Arsenic Trioxide-Induced Death of Neuroblastoma Cells Involves Activation of Bax and Does Not Require p53

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ABSTRACT

Purpose: On the basis of clinical studies showing that arsenic trioxide (As₂O₃), via an apoptotic mechanism, and with minimal toxicity induces complete remission in patients with refractory acute promyelocytic leukemia and that multidrug-resistant and p53-mutated neuroblastoma cells are sensitive to As₂O₃ both in vitro and in vivo, we searched for molecular mechanisms involved in the As₂O₃-induced neuroblastoma cell death.

Experimental Design: We have studied the effect of As₂O₃ on the expression and cellular localization of proteins involved in drug-induced death in two neuroblastoma cell lines with intact p53 and two with mutated p53, the latter two displaying multidrug resistance.

Results: As₂O₃ provoked Bax expression in all tested neuroblastoma cell lines, including SK-N-BE(2) cells with mutated p53 and LA-N-1 cells, which have a deleted p53. In all cell lines exposed to As₂O₃, p21 Bax was proteolytically cleaved in a calpain-dependent way into the more proapoptotic p18 Bax, which was detected exclusively in a mitochondria-enriched subcellular fraction. As₂O₃ also caused an increase of cytoplasmic cytochrome c, translocation of antiapoptosis-inducing factor to the nuclei, and a slight activation of caspase 3. However, inhibition of caspase 3 did not prevent cell death, whereas inhibition of Bax cleavage was associated with a decreased As₂O₃-induced cell death.

Conclusions: We show that multidrug-resistant neuroblastoma cells die after exposure to As₂O₃, independent of functional p53, suggesting activation of a cytotoxic pathway different from that induced by conventional chemotherapeutic agents. We further propose that proteolytic activation of Bax is an important event in As₂O₃-induced cell death.

INTRODUCTION

Despite that children with high-risk neuroblastoma respond to induction chemotherapy with diminished tumor burden and even clearance of metastases, the majority of these patients relapse with an acquired multidrug-resistant tumor phenotype, which often includes a mutated/deleted p53 (1–4). Multimodal approaches, such as myeloablative treatment and differentiation-inducing therapy using high-dose retinoic acid against minimal residual disease, have resulted in improved overall survival (5). Nevertheless, ~60–70% of these patients still relapse and die of their neuroblastoma (6–8).

Conventional chemotherapeutic agents frequently cause damage to DNA, which triggers activation of p53, cell cycle arrest in G₁-S, and induction of apoptotic cell death (9, 10). However, in advanced tumors, mechanisms of apoptosis are frequently impaired, e.g., p53 is mutated, and/or caspases are not expressed. In addition, chemotherapy tends to enhance the accumulation of genetic aberrations, which also affects proapoptotic genes (11). In advanced neuroblastomas, effects of therapeutic agents can be bypassed because of insufficient cytotoxicity and/or through selection of cells that are resistant to multiple, unrelated cytotoxic drugs (3, 4). In the induction treatment of pediatric malignancies other than neuroblastoma, attacking the heterogeneous population of tumor cells with drugs that have different mechanisms of action has proven to be efficacious, as shown by a high rate of long-term survival. Thus, drugs that during initial treatment induce cell death through novel mechanisms are obviously of substantial clinical value.

The therapeutic effects of arsenic compounds have been exploited for centuries to remedy various medical disorders, including malignancies. In the Western world, Fowler’s solution, which contains the active substance As₂O₃ and dissolves in a solution of potassium bicarbonate, was used to treat leukemia until the early 1900s, when it was replaced by radiation therapy and later chemotherapy (12, 13). Notwithstanding, modern clinical trials have shown that As₂O₃ can induce complete remission in patients with relapsed acute promyelocytic leukemia (APL) or acute myeloid leukemia French-American-British Classification M3 (14, 15), and arsenic trioxide is now approved as the first-line treatment for these patients. It is not yet known why chemotherapy-resistant APL cells are sensitive to As₂O₃, although there is experimental evidence that the cell death involves induction of apoptotic mechanisms. The molecular events unraveled thus far in APL and nontransformed cells include direct effect(s) on the mitochondria, entailing collapse of the transmembrane potential (16–20); down-regulation of Bel-2 (21); activation of caspases 3 and 8 (15, 16, 22), and dependence of the cellular glutathione levels (Ref. 23 and reviewed in Ref. 24).
Recently, work conducted by our research group, as well as other investigators, has shown that neuroblastoma cells are sensitive to clinically tolerable concentrations of As$_2$O$_3$ (25, 26), which indicates the potential of this drug for treating neuroblastoma. Furthermore, in our previous study, we made the encouraging finding that a multidrug-resistant cell line, SK-N-BE(2), established from a patient with relapse after initial treatment, was also sensitive to As$_2$O$_3$, both in vivo and in vitro (26).

Importantly, the first Phase I clinical trial of patients with advanced neuroblastoma and other solid tumors has been performed at the Memorial Sloan-Kettering Cancer Center, which led to the present ongoing Phase II trial,$^3$ further emphasizing the importance of studying death mechanisms induced by As$_2$O$_3$. Similar to APL cells, there is evidence for an As$_2$O$_3$-induced apoptotic death mechanism in neuroblastoma cells, as demonstrated by Bcl-2 down-regulation, caspase 3 activation, and DNA fragmentation (25, 26).

In the present study, we examined the influence of As$_2$O$_3$ on the intrinsic apoptotic pathway in four neuroblastoma cell lines by investigating this arsenic compound with regard to its impact on the expression and membrane localization of the proapoptotic Bcl-2 family member Bax and its ability to release cytochrome c to the cytoplasm. We also compared the effects of As$_2$O$_3$ with those of vincristine, etoposide, doxorubicin, and carboplatin, drugs commonly used in the induction treatment of children with advanced neuroblastoma. We found that As$_2$O$_3$ induced expression and cleavage of Bax in both drug-resistant and -sensitive cells, whereas the four mentioned cytotoxic drugs caused such changes only in the drug-sensitive cells. Therefore, we suggest that activation of Bax is a key event in As$_2$O$_3$-induced cell death, and interestingly, the activation of Bax did not depend on an intact p53 pathway.

MATERIALS AND METHODS

Cells, Drugs, and Chemicals. The neuroblastoma cell lines SH-SY5Y and SK-N-BE(2) are gifts from Dr. June Biedler (Memorial Sloan-Kettering Cancer Center, New York, NY). LA-N-1 is a gift from Dr. Robert C. Seeger (Children’s Hospital, Los Angeles, CA), and IMR-32 was from American Type Culture Collection (Manassas, VA). All but one cell line, SH-SY5Y, carry a MYCN amplification (27). SK-N-BE(2) cells are multidrug-resistant, p53-mutated (2, 3), and derived from a patient who relapsed after induction therapy (28). SH-SY5Y cells, an SK-N-SH subclone (29), were established from a patient under treatment, and IMR-32 cells were established from a tumor specimen obtained at diagnosis (30). LA-N-1 was derived from bone marrow from a patient with a chemotherapy insensitive neuroblastoma (31) and has a p53 mutation generating a stop codon (32). The four neuroblastoma cell lines were routinely grown in standard medium supplemented with 10% FCS at 37°C in a 95% air/5% CO$_2$ humidified incubator as described (26). As$_2$O$_3$ (Sigma-Aldrich Sweden AG) was dissolved in 1 M NaOH and stored as a 30 mM stock solution, which was further diluted in medium before use in culture studies.

Vincristine, etoposide, carboplatin, and doxorubicin hydrochloride were obtained from Sigma, calpeptin was purchased from Calbiochem (Merck KgaA, Darmstadt, Germany), and zVAD-fmk was from Enzyme System Products (Livermore, CA).

Viability and Cytotoxicity Assay. As$_2$O$_3$-induced cell death was confirmed by examining trypan blue-stained cells in the light microscope, which revealed that all cells died after treatment with 10–40 μM As$_2$O$_3$ for 72 h (26). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (CellTiter 96; Promega, Madison, WI) was used to determine the number of viable cells after exposure to selected drugs. Cells were harvested in log phase growth, and 5–10,000 cells were seeded per well of 96-well plates. Serial dilutions of the tested drugs were added, and the final concentration ranges investigated corresponded to those used in clinical nonmyoabative treatment (33–36) and were as follows: (a) 0.3–5 μM As$_2$O$_3$; (b) 0.1–2.5 nM vincristine; (c) 0.07–1.25 μM etoposide; (d) 0.6–10 μM carboplatin; and (e) 3–50 μM doxorubicin. Control cells received medium only. Experiments were performed at least three times, and all measurements were done in triplicates. The generated MTT product was quantified, and the results are presented as a percentage of viable cells compared with untreated controls with SDs. We also examined the capacity of zVAD-fmk and calpeptin to inhibit the cytotoxicity of As$_2$O$_3$; these compounds were added at final concentrations of 10–100 and 5–60 μM, respectively. Statistically significant effects of 100 μM zVAD-fmk on As$_2$O$_3$-induced cell death were tested according to ANOVA, followed by Duncan’s multiple range test.

Subcellular Fractionations. Cells were treated for 72 h and then washed, harvested in ice-cold PBS, and collected by centrifugation. Cytosolic and particulate (mitochondria enriched) fractions were isolated according to a protocol described by Ahn et al. (37). The cell pellet was suspended in ice-cold extraction buffer, 50 mM PIPES (pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM MgCl$_2$, 1 mM dithiothreitol, and Complete Protease Inhibitor (Roche) containing 250 mM sucrose. The suspension was incubated on ice for 30 min, shaken, and subsequently centrifuged at 16,000 × g and 4°C for 30 min, and the supernatant was removed and used as the cytosolic fraction. The pellet was resuspended in the buffer above containing 0.1% NP40, incubated on ice for 20 min, and centrifuged at 16,000 × g (4°C) for 10 min. The supernatant was used as the particulate fraction. For nuclear-enriched fractions, the cells were suspended in 10 mM NaCl, 1.5 mM MgCl$_2$, 10 mM Tris-HCl (pH 7.5), and Complete Protease Inhibitor and incubated on ice for 10 min. The cells were homogenized 20 times using a Dounce tissue homogenizer. A solution was added giving the following final concentrations: (a) 0.25 mM sucrose; (b) 1 mM EGTA; and (c) 1 mM EDTA. The suspension was centrifuged at 500 × g for 3 min at 4°C. The remaining supernatant was removed and centrifuged again at 500 × g for 3 min at 4°C. This supernatant was used as a nuclei-depleted fraction. The pellet from the first centrifugation was washed once in PBS and resuspended in 10 mM Tris-HCl (pH 7.2), 160 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, and 1 mM EDTA in the presence of Complete Protease Inhibitor. The samples were gently shaken for 15 min and

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3 B. H. Kushner and N-K. Cheung, personal communication.
centrifuged at 15 min (4°C). The supernatant was used as a nuclei-enriched fraction.

**Western Blot and Caspase 3 Analyses.** Cells were treated for 72 h and then washed, harvested in ice-cold PBS, and lysed in 10 mM Tris-HCl (pH 7.2), 160 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, and 1 mM EDTA in the presence of Complete Protease Inhibitor. Equal amounts of protein were separated on a 12% SDS-PAGE gel and blotted onto an Immobilon-P membrane (Millipore Corp., Bedford, MA). The following primary antibodies and antisera were used at the indicated dilutions: (a) anti-Bax antisera (BD PharMingen, San Diego, CA), 1:1000; (b) anti-Bid antibody, detecting full-length Bid (Cell Signaling Technology, Inc., Beverly, MA), 1:1000; (c) anti-Bid antibody detecting tBid (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) 1:500; (d) anti-p53 antibody (DAKO, Glostrup, Denmark), 1:1000; (e) antiglyceraldehyde-3-phosphate dehydrogenase antibody (Chemicon International, Inc., Temecula, CA), 1:600; (f) antiactin antibody (ICN Biomedicals, Inc., Aurora, OH), 1:2000; (g) anticytochrome c antibody (BD PharMingen), 1:1000; (h) antivoltage-dependent anion channel antibody (Calbiochem, La Jolla, CA), 1:1000; (i) anti-poly (ADP-ribose) polymerase antibody (ProSci, Inc., Poway, CA), 1:1000; and (k) anticaspase 3 antibody (Alexis Biochemicals, San Diego, CA), 1:500. The proteins were detected using the horseradish peroxidase-conjugated secondary antibodies antimouse immunoglobulin from sheep (Amersham Pharmacia Biotech) and antirabbit immunoglobulin and antigooat immunoglobulin from rabbit (DAKO) diluted 1:5000, 1:2500, and 1:3000, respectively, and the Super Signal detection system (Pierce Chemical Co., Rockford, IL). Band intensities were quantified using the Image Gauge software (Fujifilm, Tokyo, Japan). Caspase 3 activity was determined as described (26), assaying the cleavage of the fluorogenic caspase 3 substrate DEVD-AMC (Upstate Biotechnology, Lake Placid, NY). Relative caspase 3 activity is defined as 100% activity at 6 h without As$_2$O$_3$, and the other values were calculated in relation to this.

**RESULTS**

**Chemotherapy-Resistant Neuroblastoma Cells Are Sensitive to Arsenic Trioxide.** We have compared four neuroblastoma cell lines, SK-N-BE(2), LA-N-1, SH-SY5Y, and IMR-32, with regard to their sensitivity to As$_2$O$_3$ and the chemotherapeutic drugs vincristine, etoposide, doxorubicin, and carboplatin, which are currently used in the induction treatment of high-risk neuroblastoma (Fig. 1). Our results confirmed findings reported previously and showed that the SK-N-BE(2) and LA-N-1 cells, but not SH-SY5Y and IMR-32 cells, are resistant to these classical chemotherapeutic agents at concentrations corresponding to plasma levels obtained during initial therapy (33–36), although LA-N-1 cells did show a partial response to vincristine and etoposide (Fig. 1). In contrast, As$_2$O$_3$ in the micromolar range had a concentration-dependent toxic effect on both the SK-N-BE(2) and LA-N-1 cells, and these cells displayed a similar sensitivity to As$_2$O$_3$ as the nonmultidrug-resistant SH-SY5Y and IMR-32 cells (Fig. 1).

Treatment of neuroblastoma cells with As$_2$O$_3$ in combina-

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**Fig. 1** Dose-response curves for four neuroblastoma cell lines treated with As$_2$O$_3$ and cytotoxic drugs. The SK-N-BE(2) and LA-N-1 cells are drug resistant and have a mutated p53 gene, whereas SH-SY5Y and IMR-32 cells have intact p53 and are drug sensitive. The cells were exposed to the indicated concentrations of the different drugs for 72 h, after which, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data (mean ± SD; n = 2–4) are expressed as percentage of viable cells compared with untreated controls.
tion with the four cytotoxic drugs, vincristine, etoposide, doxorubicin, and carboplatin, in a wide range of concentrations resulted in an additive effect on killing of the nondrug-resistant cells SH-SY5Y and IMR-32. In the SK-N-BE(2) cells, only As$_2$O$_3$ contributed to cell death (data not shown). Thus, there was no apparent synergistic action between As$_2$O$_3$ and the four tested drugs.

**Arsenic Trioxide-Induced Cell Death Is a Late Event.**
Cell death induced by clinically relevant concentrations of As$_2$O$_3$ was massive after 3 days of exposure (Fig. 2A). Dead cells were detectable ~8 h after addition of the drug, as indicated by a slight increase in the number of floating trypan blue permeable cells (data not shown), but cell death was substantial until day 2. The late effect is exemplified with SK-N-BE(2) cells in Fig. 2B, which shows the percentage of viable cells quantified as a function of time and As$_2$O$_3$ concentration using nontreated cells at day 3 as reference. Exposing these cells to modest levels of As$_2$O$_3$ did not cause a significant change in cell number until day 2. Although As$_2$O$_3$ does kill neuroblastoma cells (Fig. 2A), the MTT assay does not discriminate between inhibited proliferation and cell death. We therefore tested the effect of As$_2$O$_3$ on cell growth by counting the number of viable and dead cells at days 2–8 after the addition of As$_2$O$_3$. SK-N-BE(2) cells did proliferate in the presence of low concentrations of As$_2$O$_3$, but at 4 μM As$_2$O$_3$ (Fig. 2C), the total number of viable cells decreased over time. At this concentration, the percentage of viable cells decreased with time showing that cell death, and not growth inhibition, is the major effect of As$_2$O$_3$ on neuroblastoma cells (Fig. 2D).

We have reported previously (26), as have others (25), that neuroblastoma cells exposed to As$_2$O$_3$ exhibit changes associated with apoptotic cell death, including a weak significant activation of caspase 3 (Fig. 3A). The activation of caspase 3 was a late event and not detectable 6 h after the addition of As$_2$O$_3$. To test whether cell death induced by the arsenic compound requires activation of caspase 3 and/or other caspases, we treated neuroblastoma cells with 10 or 20 μM the pan-caspase inhibitor zVAD-fmk for different amounts of time. Neither of these protocols blocked cell death of the three cell lines we tested, nor did repeated addition of 20 μM zVAD-fmk affect As$_2$O$_3$-induced cell death (Fig. 3B).

**Arsenic Trioxide Induces a p53-Independent Increase in Bax Expression and Cleavage.** Inasmuch as we found that As$_2$O$_3$-induced death of neuroblastoma cells was a late event that did not appear to require a substantial caspase activation, we looked for potential effects on Bax, because this Bcl-2 family
inhibitor 1 h before treated with As$_2$O$_3$, and thereafter, zVAD-fmk was without As$_2$O$_3$ was defined as 100%, and the other values were calculated in relation to this. Data are expressed as mean ± SD; n = 2. *p < 0.05 according to ANOVA followed by Duncan’s multiple range test. B, SK-N-BE(2) cells were exposed to increasing concentrations of As$_2$O$_3$ in combination with 20 μM the pan-caspase inhibitor zVAD-fmk. The cells were exposed to the inhibitor 1 h before treated with As$_2$O$_3$, and thereafter, zVAD-fmk was added every 24 h. The number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 3 days later. Data are expressed as mean ± SD; n = 3.

protein has been implicated in caspase-independent cell death (38–40). A striking effect of As$_2$O$_3$ on Bax in treated neuroblastoma cells was a concentration-dependent cleavage of p21 Bax to a p18 form (Fig. 4). Furthermore, As$_2$O$_3$ also caused a concentration-dependent 2–4-fold increase in the total amount of expressed Bax in the four tested cell lines, including SK-N-BE(2) and LA-N-1 cells (Fig. 4). This result was unexpected, because SK-N-BE(2) and LA-N-1 cells have a mutated and inactive p53, and Bax is a p53 target (3). p53 Western blot analysis revealed that the protein levels increased slightly with As$_2$O$_3$ treatment in both SH-SY5Y and IMR-32 but was virtually unaffected in SK-N-BE(2) cells, consistent with their p53 mutation status. LA-N-1 cells lack p53 because of a mutation causing a stop codon (32), and as expected, p53 was not detected (cf. full-length p53 in SH-SY5Y) in these cells. Thus, the increase in Bax expression in SK-N-BE(2) and LA-N-1 cells was p53 independent.

Effects of Cytotoxic Drugs on Bax Cleavage and Expression of Bax and p53. Our observation that SK-N-BE(2) and LA-N-1 cells died at low concentrations of As$_2$O$_3$ but were not severely affected by clinically relevant concentrations of vincristine, etoposide, doxorubicin, and carboplatin (Fig. 1) indicated that As$_2$O$_3$ differs from these four chemotherapeutic drugs with regard to the mechanism by which it kills neuroblastoma cells. Expression and cleavage of Bax were therefore analyzed in SK-N-BE(2), SH-SY5Y, and IMR-32 cells treated for 72 h with these four drugs or As$_2$O$_3$. In the SK-N-BE(2) cells, levels of Bax protein were virtually unchanged, and Bax was not detectably cleaved, as shown for etoposide (Fig. 5). In contrast, after exposure to doses of etoposide that did not kill all SH-SY5Y and IMR-32 cells after 72 h, Bax cleavage was substantial, and the levels of Bax protein were increased. In these, but not in SK-N-BE(2) cells, also p53 expression increased slightly (Fig. 5). In the drug-sensitive cell lines, treatment with the other three cytotoxic drugs led to increased expression and cleavage of Bax, as exemplified by the SH-SY5Y cells. We noted that the cleavage of Bax and expression of p53 induced by doxorubicin in SH-SY5Y cells were weak compared with the changes in vincristine- or carboplatin-treated cells (Fig. 5), which could suggest activation of different death mechanisms. We conclude that As$_2$O$_3$- and drug-provoked cell death of the nondrug-resistant cells we studied was generally accompanied by increased cleavage of Bax and expression of Bax and p53. However, a direct quantitative comparison between Bax cleavage and expression and cell viability measured by MTT cannot be made, because the MTT assay includes dead and floating cells, whereas only adherent cells were analyzed in the Western blotting experiments. As$_2$O$_3$-induced death of the drug-resistant, p53-mutated SK-N-BE(2) cells did not appear to involve activation of p53 but instead seemed to entail increased expression and proteolytic activation of Bax. Because the tested cytotoxic drugs did not cause either Bax cleavage or death in the SK-N-BE(2) cells, it is possible that As$_2$O$_3$ exerts its toxic effects by triggering expression and activation of Bax.

Particulate Localization of p18 Bax and Release of Cytochrome c to the Cytoplasm in Arsenic Trioxide-Treated Cells. In light of our finding that As$_2$O$_3$-induced cleavage of Bax, and the observation by others that As$_2$O$_3$ causes collapse of the mitochondrial transmembrane potential in APL cells (17), we investigated the effects of As$_2$O$_3$ on the presence of Bax in a cytoplasmic and a mitochondria-enriched particulate fraction of neuroblastoma cells. In untreated as well as treated cells, we found that unprocessed, p21 Bax was abundant in both fractions...
Examining As$_2$O$_3$-treated SK-N-BE(2), SH-SY5Y, and IMR-32 cells, the generated, low-molecular weight p18 Bax, was exclusively detected in the particulate fractions (Fig. 6A).

We subsequently reprobed the filters used for Bax analysis to determine whether As$_2$O$_3$ causes a release of cytochrome c to the cytoplasm. In all three neuroblastoma cell lines, we detected an As$_2$O$_3$ concentration-dependent increase in cytochrome c in the cytoplasm (Fig. 6A), as also observed in As$_2$O$_3$-treated APL cells (41). Again, the weakest signals were obtained with the IMR-32 cell fractions. We reprobed the filters with an antibody directed against the mitochondrial voltage-dependent anion channel (VDAC). All particulate fractions gave a strong VDAC signal, but no VDAC was detected in the cytoplasmic fractions (Fig. 6A). Therefore, we conclude that the cytochrome c detected in the cytoplasm of cells treated with As$_2$O$_3$ was not the result of mitochondrial contamination.

The release of cytochrome c is often accompanied by the translocation of the mitochondrial protein AIF to the nucleus. In agreement with the small increase in cytoplasmic cytochrome c, a weak AIF signal was detected in the nuclei-enriched fractions of As$_2$O$_3$-treated cells (Fig. 6B). In these fractions, a small increase of cleaved poly (ADP-ribose) polymerase (PARP) was also detected, suggesting that a fraction of the As$_2$O$_3$-treated neuroblastoma cells was dying in a classical apoptotic process via caspase activation. The low levels of PARP in the nuclei-depleted and low glyceraldehyde-3-phosphate dehydrogenase levels in the nuclei-enriched fractions suggest that these fractions were comparatively clean (Fig. 6B).

Bid is another proapoptotic member of the Bcl-2 family.

Fig. 4 As$_2$O$_3$ induces cleavage of Bax in neuroblastoma cells. SK-N-BE(2), SH-SY5Y, IMR-32, and LA-N-1 cells were treated with 0.5–4 μM As$_2$O$_3$ for 72 h. Thereafter, the cells were lysed, and 100 μg of total protein from each sample were subjected to SDS-PAGE and Western blotting. The same filter was consecutively incubated with antibodies against Bax, p53, actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH); actin and GAPDH were used as loading controls. The asterisks indicate the M$_r$ 21,000 (**) and 18,000 (*) product of Bax cleavage. In the LA-N-1 SDS-PAGE analysis, a SH-SY5Y (SY) control cell lysate was used as a positive control for wt p53. Positions of the molecular weight markers are given to the right. Total amounts of Bax (p21 and p18) and p53 were estimated densitometrically, and values were corrected for the corresponding GAPDH protein levels. Values are expressed as fold induction relative to nontreated controls and are the means of three separate experiments.

Fig. 5 Effects of cytotoxic drugs on Bax and p53 expression and Bax cleavage in neuroblastoma cells. The cells were treated for 3 days with etoposide, doxorubicin, vincristine, and carboplatin. Thereafter, cell lysates were analyzed by Western blotting, and the filters were probed with anti-Bax and -p53 antibodies. Lysates from As$_2$O$_3$-treated cells were used as positive Bax cleavage controls. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a loading control. Positions of the molecular weight marker proteins are indicated to the right.
that can be proteolytically activated (tBid) by caspases during activation of the extrinsic apoptotic pathway (42), but advanced neuroblastomas frequently lack expression of caspase 8 because of gene methylation (43), like the SK-N-BE(2), SH-SY5Y, and IMR-32 cells used in this study (Ref. 44 and data not shown). Bid can also be cleaved by calpain (45), and we asked whether As$_2$O$_3$ can interfere with the extrinsic pathway and trigger expression and cleavage of Bax in neuroblastoma cells and thereby activate a mitochondria-dependent apoptotic cell death. As shown in Fig. 7, A and B, As$_2$O$_3$ did not consistently change the Bid protein levels; we noted a slight increase in As$_2$O$_3$-treated SK-N-BE(2) and decrease in treated IMR-32 cells. Although tBid levels decreased in treated IMR-32 cells, there was a moderate As$_2$O$_3$-induced increase in tBid in the two other tested cell lines (Fig. 7A). Our results suggest that cleavage of Bid is not a major and consistent event in As$_2$O$_3$-induced neuroblastoma cell death but could be involved in triggering the small release of cytochrome c and AIF and weak activation of caspase 3.

The Arsenic Trioxide-Induced Cleavage of Bax Is Calpain Dependent. Besides an As$_2$O$_3$-induced cleavage of Bax, we also observed limited spontaneous cleavage under standard growth conditions. Bax is known to be cleaved and activated by calpain in a calcium-dependent process (46–48); hence, we tested the effects of the calpain inhibitor calpeptin on As$_2$O$_3$-induced Bax cleavage. Ten µM calpeptin partially inhibited the spontaneous (data not shown) and As$_2$O$_3$-induced Bax cleavage, as shown for SK-N-BE(2) cells in Fig. 8A. Already at this concentration, calpeptin was toxic to the neuroblastoma cells, and 20 µM still inhibited the Bax cleavage, although a complete inhibition was not obtained. Even higher concentrations were too toxic to give informative results regarding inhibition of Bax cleavage. We tried to study the impact of calpeptin on As$_2$O$_3$-induced neuroblastoma cell death. However, as calpeptin was toxic to the cells, the results were inconclusive (data not shown).

Concentrations of zVAD-fmk ≤ 20 µM are considered to be specific for caspases (49, 50). Higher concentrations have been reported to also inhibit calpains (49, 50), and because the neuroblastoma cells tolerated zVAD-fmk concentrations ≤ 100 µM (Fig. 8), we tested the effect of zVAD-fmk on proteolytic activation of caspase 3 and calpain, respectively. Twenty µM zVAD-fmk blocked all As$_2$O$_3$-induced proteolytic cleavage of caspase 3, and most of the residual caspase 3 activation was blocked by 50 µM zVAD-fmk (Fig. 8B). In contrast, 20 µM zVAD-fmk had only a limited effect on the cleavage of p21 Bax to the p18 form, whereas 100 µM zVAD-fmk almost completely inhibited the As$_2$O$_3$-induced proteolytic activation of Bax (Fig. 8C). We next tested whether the inhibition of the formation of p18 Bax by the high concentration of zVAD-fmk resulted in increased survival of As$_2$O$_3$-treated SK-N-BE(2) cells. As dem-
has been shown to enhance the cell death-inducing potency of Bax (46, 47), which could imply that cleavage of this protein in neuroblastoma cells is an important step in death caused by As$_2$O$_3$.

It is well established that Bax is an important proapoptotic molecule and that enforced expression of Bax renders cells more susceptible to death signals (52). A central role of Bax in drug-induced death of epithelial cancer cells has further been demonstrated in Fig. 8D. 100 μM, but not 20 μM zVAD-fmk, did significantly inhibit As$_2$O$_3$-induced neuroblastoma cell death.

DISCUSSION

Neuroblastoma cells treated with As$_2$O$_3$ exhibit activated caspase 3 and fragmented DNA (25, 26). In the present study, we further demonstrate the release of cytochrome c to the cytoplasm and cleavage of PARP, two distinctive features of apoptotic cell death. However, these changes, including the activation of caspase 3, were modest, and using the pan-caspase inhibitor zVAD-fmk at concentrations reported to be caspase specific (49, 50), support that the activation of caspases in general is not a central component of the molecular mechanisms underlying As$_2$O$_3$-effected death of neuroblastoma cells. In this context, the As$_2$O$_3$-provoked translocation of AIF to the nucleus is interesting, because AIF is a major factor involved in caspase-independent neuronal cell death (51). In addition, As$_2$O$_3$ did not seem to activate death receptor downstream events in an extrinsic apoptotic pathway because As$_2$O$_3$ could kill caspase-8-negative neuroblastoma cells, and it did not affect the downstream target Bid to any large extent. It has been proposed that this arsenic compound influences the intrinsic apoptotic pathway in hematopoietic cells by affecting the mitochondrial membrane potential (17), possibly by altering the expression and activation of members of the Bcl-2 family (20). We have found previously that Bcl-2 is down-regulated in neuroblastoma cells exposed to As$_2$O$_3$ (26), and in the current study, we observed that such treatment increased the levels of the proapoptotic protein Bax, two changes that will cooperate in promoting cell death. In addition, we discovered that Bax was cleaved into a p18 form, all of which was found in a mitochondria-enriched particulate fraction. In other cell systems, generation of p18 Bax

Fig. 8 Inhibition of As$_2$O$_3$-induced Bax cleavage and cell death of SK-N-BE(2) cells. In A, cells were grown in the presence of 1 μM As$_2$O$_3$ with or without 10 or 20 μM calpeptin, a calpain inhibitor. After 72 h, the cells were analyzed by Western blotting for proteolytic cleavage of Bax. In B and C, the cells were exposed for 72 h to 4 μM As$_2$O$_3$, and/or indicated concentrations of daily added zVAD-fmk. The cell lysates were subjected to Western blot analyses, and the filters were probed with antibodies directed against caspase 3 in B and Bax in C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The percentage of cleaved Bax in relation to the total Bax (A and C), as well as cleavage (activation) of caspase 3 in relation to untreated cells after correlation to GAPDH levels (B), were estimated densitometrically and are the means of three separate experiments. In D, the cells were exposed to increasing concentrations of As$_2$O$_3$ and the indicated concentrations of zVAD-fmk, which was added every 24 h. The number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are expressed as mean ± SD; n = 6. *Statistically significant differences (P < 0.05) according to ANOVA followed by Duncan’s multiple range test.
demonstrated, because absence of Bax completely abolished the death response to some cytotoxic drugs (53, 54). Therefore, it seems likely that the increase in Bax expression is an important event in the neuroblastoma cell death process induced by As₂O₃ and the other tested cytotoxic drugs. However, unlike drug-induced, Bax-dependent death of cells of epithelial origin (54), it seems that caspase activity is not required for As₂O₃-induced neuroblastoma cell death. Thus, our results suggest a Bax-dependent, but caspase-independent, cell death mechanism caused by As₂O₃, which also has been reported to occur with other drugs in other cell systems (55, 56).

Cleavage of Bax was partially inhibited by calpeptin, which demonstrates that calpain is involved in the induced proteolytic activation of Bax, but we could not block As₂O₃-induced cell death by calpeptin. Because calpeptin was toxic in itself to neuroblastoma cells, a concentration range in which this inhibitor functions in a nontoxic fashion, but still inhibits Bax cleavage, might not exist. Data obtained using the broad caspase inhibitor zVAD-fmk suggested that caspase-dependent, As₂O₃-cleavage, might not exist. Data obtained using the broad caspase inhibitor functions in a nontoxic fashion, but still inhibits Bax itself in neuroblastoma cells, a concentration range in which this drug kills neuroblastoma cells. However, by using the nonspecific action of high concentrations of zVAD-fmk, i.e., that zVAD-fmk at these concentrations can inhibit calpains, we could demonstrate that 100 μM zVAD-fmk block Bax cleavage as well as As₂O₃-induced neuroblastoma cell death. These results suggest that not only an increase in total Bax level will affect cell death in As₂O₃-treated neuroblastoma cells, but the cell death-inducing effect of As₂O₃ is enhanced by the proteolytic cleavage of Bax. In this context, it is noteworthy that cell death induced via overexpression of p21 Bax in human embryonic kidney cells can be completely blocked by 50 μM zVAD-fmk, whereas death induced by overexpression of p18 Bax in the same cells was only partially blocked by zVAD-fmk (47).

We observed that increased expression of Bax and cleavage of this protein also occur in drug-sensitive neuroblastoma cells treated with the four other tested cytotoxic drugs. Expression of Bax can be driven by p53 (57), and in our experiments with drug sensitive cells, p53 was induced by all protocols that resulted in cell death. Although activation of p53 and Bax is probably important during drug-induced death of nondrug-resistant neuroblastoma cells, neither As₂O₃ nor the four tested cytotoxic drugs induced expression of p53 in SK-N-BE(2) cells. Thus, increased expression and proteolytic activation of Bax appear to be common characteristics of neuroblastoma cells that are dying because of treatment with As₂O₃ or the tested cytotoxic drugs. Importantly, our data demonstrate that p53 was not required for the As₂O₃-induced increase in Bax expression, as demonstrated in the p53-negative LA-N-1 cells (Fig. 4). In summary, our data suggest that activation of Bax and subsequent destabilization of the mitochondria are key events in As₂O₃-induced cell death. We further conclude that Bax activation and cell death by As₂O₃ are independent of p53 and that this could be a general death mechanism, which might be possible to activate in any tumor cell with nonfunctioning p53. A future aim is to further clarify the death mechanism and screen for additional drugs working by the same, p53-independent death mechanism.

The ongoing Phase II clinical study of patients with refractory neuroblastoma, together with our results showing that multidrug-resistant, p53-mutated neuroblastoma cells respond to clinically relevant doses of As₂O₃, give hope that this drug as an adjuvant to existing treatment regimens can improve the outcome in children with tumors that are presently untreatable. If the side effects of As₂O₃ prove to be acceptably low in a larger patient material, this compound might even be included during induction therapy for high-risk neuroblastoma patients.

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REFERENCES

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