Arsenic trioxide is highly cytotoxic to small cell lung carcinoma cells

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Abstract
Small cell lung carcinoma (SCLC) is an extremely aggressive form of cancer and current treatment protocols are insufficient. SCLC have neuroendocrine characteristics and show phenotypical similarities to the childhood tumor neuroblastoma. As multidrug-resistant neuroblastoma cells are highly sensitive to arsenic trioxide (As2O3) in vitro and in vivo, we here studied the cytotoxic effects of As2O3 on SCLC cells. As2O3 induced pronounced cell death in SCLC cells at clinically relevant concentrations, and also at hypoxia. SCLC cells were more sensitive than non–SCLC cells to As2O3. Cell death was mainly due to necrosis, although apoptotic responses were also seen. A significant in vivo effect of As2O3 on SCLC growth was shown in a nude mice-xenograft model, although a fraction of the treated tumor-bearing animals did not respond. The nonresponding SCLC tumors differed in morphology and cell organization compared with treatment-responsive tumors, which in turn, showed decreased vascularization and higher expression of neuroendocrine markers compared with control tumors. Our results suggest a potential clinical application of As2O3 in SCLC therapy. In addition to cell death induction, antiangiogenic induction of differentiation may also be part of the in vivo effect of As2O3 on SCLC growth, as suggested by an increase in neuroendocrine markers in cultured cells. [Mol Cancer Ther 2009;8(1):160–70]

Introduction
Lung cancer is the most frequent cause of cancer deaths worldwide and results in ~1 million deaths each year (1). Despite novel treatment strategies, the 5-year survival rate of lung cancer patients is only ~15%. Small cell lung carcinoma (SCLC) accounts for 15% to 20% of all lung cancers diagnosed and is a very aggressive malignancy with early metastatic spread (2). Despite an initially high rate of response to chemotherapy, which currently combines a platinum-based drug with another cytotoxic drug (3, 4), relapses occur in the absolute majority of SCLC patients. At relapse, the efficacy of further chemotherapy is poor and the need for alternative treatments is obvious.

Arsenic-containing compounds have been used in traditional Chinese medicine for thousands of years. In the last decade, clinical studies have shown that low doses of arsenic trioxide (As2O3) can induce complete remission with minimal toxicity in patients with relapsed acute promyelocytic leukemia (APL; refs. 5–8), and As2O3 is now used as first-line treatment modality in these patients. At clinically tolerable concentrations, As2O3 also induces cell death in multidrug-resistant neuroblastoma cells in vitro and in vivo (9, 10). The cytotoxic effect of As2O3 is retained in neuroblastoma cells at hypoxia (11), a property of potential clinical importance because hypoxic areas are frequent in solid tumors and have been linked to drug-resistant phenotypes (12).

The mechanisms of As2O3-induced cytotoxicity are complex and differ depending on the cell type and tumor form. In APL cells, the induction of apoptotic mechanisms, growth inhibition, as well as differentiation have been observed (13, 14). In other cells, caspase-independent pathways are also important (10, 15) and can involve the activation of the proapoptotic Bcl-2 family member Bax (16, 17), as observed in As2O3-induced death of neuroblastoma cells (10). In drug-sensitive as well as multidrug-resistant neuroblastoma cells, p21 Bax is proteolytically cleaved into the more proapoptotic p18 Bax form in a calpain-dependent process. We have further shown that p18 Bax generated upon As2O3 treatment is exclusively localized to the nucleus (18), thus indicating a novel, nuclear rather than mitochondrial death-signaling pathway induced by a Bcl-2 family member.

Neuroblastoma and SCLC have many characteristics in common, e.g., both tumors have neuronal/neuroendocrine traits and usually respond well to initial treatment with similar drugs. Furthermore, amplification of MYC, MYCN, and MYCL is observed in 20% to 30% of SCLC (19) and
amplification of MYCN occurs in ~20% of all patients with neuroblastomas (20). In both cases, amplification strongly correlates with poor clinical outcome. This, together with the observation that As2O3 effectively kills multidrug-resistant neuroblastoma cells, prompted us to investigate the in vitro and in vivo effects of As2O3 on SCLC cells. We show here that SCLC cells are highly sensitive to As2O3 in vitro, both at normoxia and hypoxia. As2O3-induced SCLC cell death is mainly necrotic but to some extent also apoptotic. Finally, As2O3 treatment results in a marked reduction in xenograft tumor growth, suggesting that As2O3-induced cytotoxicity is a potential treatment strategy for patients with relapsed SCLC.

Materials and Methods

Cell Culture, Drugs, and Cell Viability Assays

All cell culture media were supplemented with 10% FCS (EuroClone, Ltd.), 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Life Technologies). The SCLC cell lines U-1285, grown in suspension, and U-1690, U-1906, and U-2020, all of which are semiderivative, were grown in RPMI 1640 (Sigma-Aldrich), as were the APL cell line NB4, grown in suspension, and the semiderivatized cell line U-1568, which originates from a large-cell morphologic variant of SCLC (SCLC-mv). The other lung cancer cell lines H-125 (adenocarcinoma), U-1752 (squamous cell carcinoma), and U-1810 (large cell carcinoma) were grown as monolayers in F10 medium (Life Technologies). MCF7 and T-47D breast carcinoma cells were cultured as monolayers in RPMI 1640 supplemented with 0.01 mg/mL of insulin (Actrapid Penfill; Novo Nordisk), 1.0 mmol/L of sodium pyruvate (PAA Laboratories), and 1.5 g/L of sodium bicarbonate (ICN Biomedicals). The rat cell lines PC12 and HiB5 of neuroendocrine and neural derivation, respectively, were grown as monolayers, PC12 cells in RPMI 1640 supplemented with 10% horse serum and 5% FCS, and HiB5 cells in DMEM containing 4.5 g/L of glucose (Life Technologies). All cells were cultured at 37°C in a 95% air, 5% CO2 humidified incubator, with the exception of the HiB5 cells, which were grown at 33°C. To achieve 1% O2, cells were grown in a humidified oxygen-regulated chamber (Invivo2 Hypoxia Workstation 400; Ruskinn Technology).

As2O3 (Sigma-Aldrich) was dissolved in 1.0 mol/L of NaOH, carboplatin (Sigma-Aldrich) in distilled H2O, and etoposide (Sigma-Aldrich) as well as staurosporine (Sigma-Aldrich) in DMSO.

The effects of cytotoxic drugs on cell viability were determined by the WST-1 assay (Roche Molecular Biochemicals), according to the manufacturer’s recommendation. The cells were seeded in 96-well plates at day 0 and varying concentrations of the drugs were added on day 1. Where indicated, the pan-caspase inhibitor zVAD-fmk (Enzyme System Products) was added at various concentrations on day 1. All treatments were done in triplicate and the experiments were repeated thrice. Untreated control viability was set to 100%.

To evaluate the efficacy of As2O3 on hypoxic SCLC cells, U-1690 cells were seeded on day 0 under normoxic conditions. As2O3 was added on day 1 and cells were further cultured at normoxia or hypoxia for 72 h. When indicated, cells were also preincubated at normoxia or hypoxia for 72 h before the addition of As2O3. In these experiments, the cells were re-seeded 1 day before the initiation of drug treatment. Cell viability was assessed with trypan blue (Sigma-Aldrich). Approximately 200 cells per culture were counted using a light microscope. All treatments were done in triplicate and the experiments were repeated thrice. Untreated controls were set to 100%.

Immunoblot Analyses and Flow Cytometry

Whole cell lysates were analyzed by Western blotting as described previously (10). The following primary antibodies and antisera were used: Bax antiserum (BD PharMingen) diluted 1:2,000, anti–caspase 3 antibody (Alexis Biochemicals) diluted 1:500, anti–glyceraldehyde-3-phosphate dehydrogenase antibody (Chemicon International, Inc.) diluted 1:2,000, anti–hypoxia-inducible factor-1α (HIF-1α) antibody (Abcam PLC) diluted 1:700, and anti–poly(ADP-ribose) polymerase (PARP) antibody (Biomol Research Laboratories, Inc.) diluted 1:5,000.

The fractions of apoptotic and necrotic SCLC cells were analyzed by flow cytometry using the TACS Annexin V–FITC apoptosis detection kit (R&D Systems) according to the manufacturer’s recommendation. Cells were seeded 1 day before As2O3 was added, and were collected after 24 to 72 h of As2O3 treatment, stained with Annexin V-FITC and propidium iodide (PI) and thereafter analyzed in a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems). SCLC cells treated with up to 5 μmol/L of staurosporine for 24 h were used as positive controls for the induction of apoptosis.

Xenograft Tumor Treatment

Six- to 8-week-old female athymic nude mice (NMRI strain nu/nu; Taconic), weighing 20 to 25 g at arrival, were housed in a controlled environment. After 1 week of acclimatization, the mice were anesthetized with halothane and injected subcutaneously on the upper back with 10 × 106 U-1690 cells suspended in PBS. When U-1690 tumors with a diameter of 5 mm were established, the animals were randomized into two groups and thereafter given daily i.v. injections of either PBS or 150 μg of As2O3 (5 mg/kg) in the tail vein. Tumor growth and animal weight were monitored on a daily basis. When the tumors reached a size of 20 mm in diameter, the mice were sacrificed with CO2 gas and the tumors were dissected and weighed. All procedures were carried out in accordance with the regional ethical committee for animal research (approval no. M66-05).

Immunohistochemistry and Quantitative Real-time PCR

Sections (4 μm) of formalin-fixed, paraffin-embedded U-1690 xenograft tumors described above were analyzed by immunohistochemistry using the following antibodies: anti-PCNA (Dako) diluted 1:4,000, anti-CD34 (Santa Cruz...
As$_2$O$_3$ is Highly Cytotoxic to SCLC Cells

Biotechnology) diluted 1:100, anti-chromogranin A (Dako) diluted 1:3,000, anti-synaptophysin (Dako) diluted 1:100, and anti–HIF-1α (Abcam PLC) diluted 1:400. After antigen retrieval in Target retrieval solution high pH (Dako), immunoreactivities were detected using the Envision system and DAKO Techmate 500 (Dako; ref. 21).

For quantitative real-time PCR experiments, RNA extraction, cDNA synthesis, and PCR reactions with SYBR Green PCR master mix (Applied Biosystems) were done as previously described (22). The comparative Ct method was used for relative quantification of expression levels, and data were normalized to the expression of three housekeeping genes, HPRT1, TBP, and UBC, which were stable during hypoxia, using geNorm (23). Each reaction was done in triplicate and the experiments were repeated four times. Primers were designed using Primer Express (Applied Biosystems), and sequences are listed in Supplementary Table S1.4

Results

As$_2$O$_3$ in Low Doses Induces Cell Death in SCLC Cells

We examined SCLC cell lines with regard to their responsiveness to As$_2$O$_3$ after 72 hours of treatment. Their sensitivities were comparable to that of the APL cell line NB4 (Fig. 1A) as well as neuroblastoma cells (10). The IC$_{50}$ values were in the 1 to 2 μmol/L range for four SCLC cell lines and the NB4 cells, i.e., concentrations well below clinically tolerable doses. By comparison, three non–small cell lung carcinoma (non-SCLC) cell lines, and two breast cancer cell lines were less sensitive to As$_2$O$_3$, with IC$_{50}$ values ranging from 2 to 5 μmol/L (Fig. 1A). The differences in sensitivity to As$_2$O$_3$ between the SCLC cells and the other cells could not be explained by differences in growth rates (data not shown). As SCLC and neuroblastoma cells have neuroendocrine and neuronal characteristics, respectively, we also tested two rat cell lines, PC-12 and HiB5, of neuroendocrine and neural derivation. These cells were not as sensitive to As$_2$O$_3$ as human SCLC or neuroblastoma cells, suggesting that As$_2$O$_3$ sensitivity is not associated with a neuroendocrine or neural phenotype per se (Fig. 1A; ref. 10).

We further examined the SCLC cells with regard to their sensitivity to etoposide and carboplatin, two drugs used in first-line treatment of SCLC (3, 4). In contrast to their As$_2$O$_3$ sensitivity, the SCLC cells differed in their responsiveness to etoposide and carboplatin (Fig. 1B). Of the tested cell lines, U-1690 was the most resistant to these two drugs, particularly to etoposide, whereas both drugs markedly reduced the viability of U-2020 and U-1285 cells after treatment for 72 hours at clinically relevant concentrations. Compared with drug-sensitive neuroblastoma cells, all four SCLC cell lines were considerably more resistant to etoposide and carboplatin (data not shown; ref. 10).

As$_2$O$_3$ Induces Proteolytic Activation of Caspase 3, PARP, and Bax in SCLC Cells

Depending on the cell system investigated, As$_2$O$_3$-induced cell death has been associated with caspase-dependent apoptosis as well as caspase-independent death pathways (10, 14, 15). To characterize the mechanisms by which As$_2$O$_3$ kills SCLC cells, we studied the cleavage of caspase 3 and PARP, two classical apoptosis markers. Caspase 3 was proteolytically activated in all tested SCLC cell lines in an As$_2$O$_3$ dose-dependent manner (Fig. 1C); however, the cleavage seemed modest. PARP is a target protein for caspasess during apoptosis and As$_2$O$_3$ treatment induced cleavage of PARP to an 89 kDa form (Fig. 1C). PARP can also be processed during necrosis, resulting in multiple cleavage products, one of which is of a similar molecular size as the apoptosis-induced fragment, i.e., 89 kDa. In previous reports, the antibody used in this study (C-2-10) detected main fragments of markedly lower molecular weights (50 and 62 kDa) under necrotic conditions (24). Here, only weak bands corresponding to such fragments were detected in As$_2$O$_3$-treated U-1690 cells (data not shown).

In neuroblastoma cells, As$_2$O$_3$ induces a caspase-independent cell death involving proteolytic activation of the proapoptotic Bcl-2 family member Bax (10, 18). In the SCLC cell lines tested, As$_2$O$_3$ similarly induced dose-dependent cleavage of full-length p21 Bax to a truncated p18 form (Fig. 1C). However, compared with As$_2$O$_3$-treated neuroblastoma cells, the induction was weak (Fig. 1C). A slight increase in total Bax levels was also noted in two of the cell lines (Fig. 1C).

Calpains are a group of proteases known to be involved in the degradation of proteins in dying cells (25) and also in proteolytic activation of the proapoptotic proteins Bax and Bid (10, 18, 26, 27). To investigate the involvement of caspasess and calpains in As$_2$O$_3$-induced SCLC cell death, cells were treated with zVAD-fmk, a pan-caspase inhibitor which also inhibits calpains at high concentrations (28, 29). The viability of As$_2$O$_3$-treated U-2020 cells, i.e., the SCLC cells with the most pronounced As$_2$O$_3$-induced caspase 3 activation and Bax cleavage (Fig. 1C), was rescued to a limited extent by high concentrations (100 μmol/L) of zVAD-fmk (Fig. 1D). The effect of zVAD-fmk was only observed upon treatment with 2 μmol/L of As$_2$O$_3$, a suboptimal drug concentration upon which the involvement of caspasess and calpains in As$_2$O$_3$-induced SCLC cell death may be the most pronounced. By contrast, the same concentration of zVAD-fmk clearly prevented a classical apoptotic cell death induced by staurosporin (Fig. 1D).

As$_2$O$_3$ Induces a Mixed Necrotic and Apoptotic Cell Death in SCLC Cells

We further studied the relative contributions of apoptosis and necrosis to As$_2$O$_3$-induced SCLC cell death by flow cytometry using Annexin V-FITC and PI. In U-1690, U-1906, and U-2020 cells, this assay revealed different well-defined cell populations (Fig. 2A; data not shown). The dot plots in Fig. 2 show viable cells (bottom left quadrants, Annexin V−/PI−), necrotic cells (top left quadrants, Annexin
V⁺/PI⁻), and early apoptotic cells (bottom right quadrants, Annexin V⁺/PI⁻). In all our experiments, the top right quadrant comprised two well-defined cell populations which were both Annexin V⁺/PI⁺: PIhigh (gate G1) and PILow cells (gate G2). However, this assay does not distinguish, per se, between cells that are in late apoptosis and those that have died/lysed as a result of necrosis because in either case, dead cells will be Annexin V⁺/PI⁺. Here, we have defined cells within the top left quadrant plus within gate G1 as lytic-necrotic cells, and cells within the bottom right quadrant plus within gate G2 as apoptotic and late apoptotic. These assumptions are based on the following facts: in our experiments, the necrotic cells in the top left quadrant together with the PIhigh cells in gate G1

Figure 1. A and B, SCLC cells are as sensitive to As₂O₃ as the APL cells NB4, which is in contrast with non-SCLC, breast carcinoma, and neurally derived cells. Cells were treated with cytotoxic drugs for 72 h and the relative amounts of viable cells were evaluated using the WST-1 assay. Points, mean from three experiments presented as a percentage of viable cells as compared with untreated controls; bars, SD. A, the indicated cell lines treated with various concentrations of As₂O₃. B, dose-response curves of SCLC cells upon treatment with either etoposide or carboplatin. C and D, analysis of As₂O₃-induced cell death pathways in SCLC cells. C, immunoblot analyses of PARP, caspase 3, Bax, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in SCLC cells treated with the indicated concentrations of As₂O₃ for 72 h. SK-N-BE2(c) neuroblastoma cells (NB) treated in the same way were included for comparison. D, U-2020 cells exposed to the indicated concentrations of ZVAD-fmk together with increasing concentrations of As₂O₃ or staurosporine for 72 and 24 h, respectively. After treatment, the fraction of viable cells was determined using the WST-1 assay. Columns, mean from three experiments presented as a percentage of viable cells as compared with untreated controls; bars, SD. **, P < 0.01; ***, P < 0.001, statistically significant differences determined by ANOVA followed by Duncan’s multiple range test.
comprised a continuous horizontal cell population ranging over the upper two quadrants (Fig. 2A). Their high uptake of PI, as compared with gate G2 cells, reflects a higher membrane permeability, i.e., a more lytic condition. In addition, it is known that when apoptosis is measured over time, cells can often proceed from Annexin V⁻/PI⁻ (viable, or no measurable apoptosis) to Annexin V⁺/PI⁻ (early apoptosis), and finally to Annexin V⁺/PI⁺ (end stage apoptosis and death). The movement of cells through these three stages, or quadrants, reflects cell death dynamics. In our experiments, the apoptotic cell population shifted from the bottom right quadrant toward gate G2 when cell death in As₂O₃-treated U-1690 cells was assessed over time (Fig. 2A and B). Exposure to As₂O₃ gave rise to a concentration-dependent and time-dependent increase in necrotic as well as apoptotic cells. After 24 hours of treatment, the majority of the U-1690 cells was viable, and the fraction of necrotic as well as apoptotic cells was ~20% irrespective of the As₂O₃ concentration used. Cell death was not substantial unless the cells were treated with 3 μmol/L of As₂O₃ for 48 h, resulting in necrosis and apoptosis levels of 35 and 30%, respectively. Cells undergoing apoptosis accounted for no more than 30% of the total U-1690 cell population at all time points and treatment concentrations tested (Fig. 2B). In contrast, the frequency of necrotic cells was as high as 70% in cells treated with 3 μmol/L of As₂O₃ for 72 hours.

As₂O₃ treatment gave rise to a concentration-dependent increase of necrosis as well as apoptosis in U-1906 and U-2020 cells (Fig. 2C). However, in these cells, the differences in levels of necrosis and apoptosis were much less pronounced, and the frequency of necrotic cells never exceeded 35% in either cell line after 72 hours of treatment. Altogether, these results indicate that As₂O₃ induces both necrosis and apoptosis in SCLC cells, and necrosis seems to be the main cell death mode.

**SCLC Cells Are Sensitive to As₂O₃ at Hypoxia**

The oxygen tension in solid tumors is generally low compared with normal adjacent tissues (~1% versus 5–6% O₂) and tumor cells have adapted to growth under hypoxic conditions. The phenotypical changes induced by hypoxia are frequently associated with induced drug resistance (reviewed in ref. 12), and in *vitro* testing of drugs under normoxic (21% O₂) culture conditions might, for this simple reason, generate data of reduced clinical relevance. The most drug-resistant as well as the most drug-sensitive of the SCLC cell lines tested, i.e., U-1690 and U-2020 cells (Fig. 1B), were therefore grown and treated at 21% and 1% O₂, respectively. As shown in Fig. 3A and B, SCLC cells grown under either normoxia or hypoxia were equally sensitive to As₂O₃. Furthermore, U-1690 cells pre-exposed to hypoxic conditions for 72 hours before treatment at hypoxia or normoxia retained their sensitivity to As₂O₃ (Fig. 3C).

To ensure that U-1690 cells cultured under hypoxic conditions had adapted to hypoxia, we investigated the accumulation of the hypoxia-induced transcription factor HIF-1α (Fig. 3D). As expected, after a 4-hour incubation under normoxia or hypoxia, no HIF-1α protein could be detected in normoxic cells (Fig. 3D, lane 1), whereas a...
marked accumulation was observed in hypoxic cells (Fig. 3D, lane 2). A robust HIF-1α protein stabilization was also detected in cells treated with As2O3 under hypoxia (Fig. 3D, lane 3). Altogether, this indicates that the cells adapted to the hypoxic environment.

**SCLC Cells Are Responsive to As2O3 In vivo**

To test the in vivo cytotoxic efficacy of As2O3 on SCLC cells, we used a xenograft model of subcutaneously grown U-1690–derived tumors in nude mice. We chose the U-1690 cells because they gave a high tumor take (~80%; 16 of 20 injected animals in the experiment shown in Fig. 4), and of the tested SCLC cell lines, U-1690 showed the highest resistance to etoposide and carboplatin (Fig. 1B). Animals were treated daily with i.v. injections of either PBS (control) or 150 μg of As2O3 (5 mg/kg), a concentration chosen to mimic clinically relevant plasma concentrations and comparable to those used in in vivo models to treat APL and neuroblastoma (9, 30). In the experiment presented in Fig. 4, tumors in the PBS control animals seemed to follow one fast and one slower growth pattern (Fig. 4A). In the As2O3-treated cohort of animals, the picture was slightly more complex. First, in three out of seven animals, As2O3 almost completely prevented tumor growth, and in another two out of seven As2O3-treated animals, significant initial inhibition of tumor growth was observed. In contrast, the remaining two animals developed tumor burdens comparable to those seen in the PBS control animals. Despite the lack of response to As2O3 in these two animals, the mean tumor growth in the treated cohort was significantly different from that of PBS control animals (P ≤ 0.05; Fig. 4B). An eighth As2O3-treated animal in this experiment had to be sacrificed early during the experiment due to a skin lesion in the tumor area and was excluded from the study. The growth-inhibiting effect of As2O3 was not related to a general intoxication of treated animals as they appeared healthy and did not lose weight during treatment (Fig. 4C).

**Distinct Morphology of As2O3 Nonresponding SCLC Tumors**

In an attempt to understand the varying degrees of As2O3-induced growth inhibition of SCLC xenograft tumors, we did immunohistochemical investigations of tumor morphology, fractions of proliferating cells, differentiation status, and levels of vascularization. H&E-stained tumor sections revealed striking morphologic intertumor differences as exemplified by tumors from animal C4, As1, and As4 (Fig. 5). The PBS-treated tumors had large areas of necrosis and a large fraction of dividing cells (PCNA

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Figure 3. Hypoxic SCLC cells are sensitive to As2O3. After As2O3 treatment at hypoxia or normoxia for 72 h, the numbers of viable (trypan blue excluding) and dead cells were counted. Points, mean percentage of dead cells or viable cells as compared with untreated controls from three experiments; bars, SD. U-1690 cells (A) and U-2020 cells (B) exposed to the indicated concentrations of As2O3 at 1% and 21% oxygen, respectively, for 72 h. C, U-1690 cells cultured at normoxia or hypoxia for 72 h before addition of the indicated concentrations of As2O3. After another 72 h of incubation under normoxic or hypoxic conditions in the presence of As2O3, the cells were collected and counted. D, immunoblot analyses of HIF-1α and actin in untreated or As2O3-treated SCLC cells grown at normoxia or hypoxia for 72 h. SK-N-BE2(c) neuroblastoma cells (NB) cultured under normoxic or hypoxic conditions for 4 h were included for comparison.
positivity; Fig. 5). As expected, the drug-responding tumors had extensive areas of necrotic cells and a smaller fraction of dividing cells (Fig. 5). Interestingly, the cells of the As$_2$O$_3$ nonresponding SCLC tumor As1 showed a distinctly different morphology in that they were larger, the nuclei varied in size, and they had disintegrated chromatin and prominent nucleoli. Furthermore, these variant tumor cells showed a more organized growth pattern with a high number of PCNA-positive cells (Fig. 5), suggesting selection for a different, nonresponding cell type in this tumor. Regarding tumor vascularization, the number of blood vessel endothelial (CD34 positive) cells was low in As$_2$O$_3$-responding tumors and considerably higher in nonresponding and PBS-treated tumors (Fig. 5).

To test whether the variant, nonresponding tumor type developed as a result of As$_2$O$_3$ selection, the morphologies of tumors from 15 nontreated animals were analyzed. Two of these showed the variant morphologic pattern (Fig. 6A), clearly demonstrating that the development of this particular morphology did not require As$_2$O$_3$ treatment, and suggesting that the U-1690 cell line contains different subpopulations of cells, which both can give rise to tumors in nude mice. Of interest, one large cell morphologic variant of SCLC (SCLC-mv) cell line showed markedly reduced sensitivity to As$_2$O$_3$ (Fig. 6B) as compared with the classic SCLC cell lines presented in Fig. 1A.

Expression of Neuroendocrine Differentiation Markers in As$_2$O$_3$-Treated SCLC Tumors

As As$_2$O$_3$ has been reported to induce differentiation in APL cells (14), we investigated neuroendocrine differentiation status in the SCLC tumors based on the expression of chromogranin A and synaptophysin, two neuroendocrine

Figure 4. SCLC in vivo growth is reduced by As$_2$O$_3$ treatment. Human U-1690 cells (10 x 10$^6$) were xenotransplanted in nude mice. When tumors with a diameter of 5 mm were established, treatment with daily i.v. injections of either PBS or 150 µg of As$_2$O$_3$ (corresponding to 5 mg/kg) was initiated. A, tumor burden in all animals (top) after the indicated number of days of daily treatment. PBS-treated animals are labeled C1–C8 (middle), As$_2$O$_3$-treated animals As1–As7 (bottom). B, mean tumor burden in PBS-treated and As$_2$O$_3$-treated animals from A. Statistically significant differences were determined by Mann-Whitney’s one-tailed U test (P < 0.05). Bars, SE. C, total weight of PBS-treated and As$_2$O$_3$-treated animals from A.
Figure 5. Immunohistochemical characterization of xenotransplanted U-1690 tumors from the experiment presented in Fig. 4. Sections of formalin-fixed paraffin-embedded tumors treated with PBS (C4) or As$_2$O$_3$ (nonresponding As1 and As$_2$O$_3$-responding As4) were stained with H&E and analyzed immunohistochemically for PCNA, CD34, chromogranin A, synaptophysin, and HIF-1α, respectively. Note the unspecific staining for PCNA, chromogranin A, and HIF-1α in the necrotic parts. Magnifications are shown as insets in the respective panels. Bars, 100 μm (bars in insets, 50 μm).
marker genes known to be expressed in SCLC cells. As exemplified in Fig. 5, these two differentiation markers were high in the As2O3-responding tumors, fairly high in the PBS controls, but virtually absent in the nonresponding As1 tumor.

To further evaluate the potential influence of As2O3 on neuroendocrine differentiation, we studied the expression of chromogranin A and synaptophysin in As2O3-treated U-1690 cells. After 6 days of treatment, both genes were slightly down-regulated compared with untreated controls (Fig. 6C). As vascularization seemed to be lower in the As2O3 responding versus nonresponding tumors, expression of differentiation marker genes was investigated in cells grown at hypoxia in the presence or absence of As2O3. Hypoxic cells up-regulated both chromogranin A and synaptophysin significantly, whereas As2O3 in combination with hypoxia did not induce any further increase in marker gene expression levels (Fig. 6C).

Immunohistochemical analysis of HIF-1α protein in xenograft tumor sections revealed that treatment-responsive tumors accumulated HIF-1α, whereas less HIF-1α immunoreactivity was seen in PBS-treated or nonresponding tumors (Fig. 5).

**Discussion**

We show here that As2O3, at clinically tolerable concentrations, is highly cytotoxic to SCLC cells *in vitro*. Interestingly, non–SCLC cells were considerably more resistant to As2O3. The sensitivity of SCLC cells to As2O3 was comparable to that of APL cells as the toxic effect is achieved within the same concentration range for both cell types. In addition, As2O3 treatment significantly impaired SCLC tumor growth in a mouse xenograft model. Our findings that As2O3 retains its cytotoxicity at *in vitro* hypoxia as well as under *in vivo* conditions provide proof of principle regarding the potential use of As2O3 for treatment of patients with SCLC.

As2O3-induced cytotoxicity is complex and has been shown to involve several different cell death pathways. In APL cells, As2O3 treatment results in apoptosis, growth inhibition and differentiation (13, 14). Moreover, caspase-independent necrotic cell death has also been shown upon As2O3 treatment (31). The precise mechanisms of As2O3-induced SCLC cell death are yet to be determined. We observe what seemed to be a modest induction of caspase 3, which only marginally contributed to As2O3-induced death.

**Figure 6.** A, morphologic characterization of subcutaneously grown U-1690–derived tumors in nude mice. Human U-1690 cells (10^6) were xenotransplanted and tumors were collected after 15 d of *in vivo* growth. Sections of the formalin-fixed, paraffin-embedded tumors were stained with H&E. B, cell viability was determined by the WST-1 assay in a large-cell morphologic variant of SCLC (SCLC-mv, U-1568) cell line and in U-1690 cells after 72 h of As2O3 treatment. Points, mean from three experiments presented as a percentage of viable cells as compared with untreated controls; bars, SD. C, quantitative real-time PCR analyses of expression levels of neuroendocrine differentiation genes in U-1690 cells after As2O3 treatment for 3 and 6 d at hypoxia and normoxia, respectively. Columns, mean from four experiments; bars, SD. *, P < 0.05; **, P < 0.01, statistically significant differences determined by ANOVA followed by Duncan’s multiple range test.
because treatment with the pan-caspase inhibitor zVAD-fmk did not prevent cell death. In line with these results, fluorescence-activated cell sorting analyses showed low levels of apoptosis compared with the high frequency of necrotic cells.

Caspase-independent cell death has, in some cell systems, been associated with increased expression and/or proteolytic activation of the proapoptotic Bcl-2 family member Bax (16, 17). Although the proteolytic cleavage of p21 Bax to a p18 form by calpains could be linked to the cell death in As2O3-treated neuroblastoma cells (10, 18), proteolytic activation of Bax did not seem to be of major importance for As2O3-induced SCLC cell death. However, the combined effect of the observed increase in total Bax and the formation of low levels of the more proapoptotic form, p18 Bax, might contribute substantially to As2O3-induced SCLC cell death. Altogether, our results indicate that As2O3-induced SCLC cell death is mainly due to caspase-independent necrotic cell death, whereas the involvement of apoptosis is more cell line–dependent.

We have previously shown that As2O3 induces neuroblastoma cell death independently of p53 status (10), a feature of great interest because loss of p53 is commonly seen in SCLC. The fact that As2O3 can activate several death pathways is likely important for its clinical efficacy and may explain why As2O3 is able to kill SCLC cells such as U-1690, which is less sensitive to etoposide and carboplatin but is as sensitive to As2O3 as the other tested SCLC cells. Thus, similar to neuroblastoma cells (9, 10), SCLC cells retain sensitivity to As2O3 despite acquired resistance to other drugs. The significant As2O3-induced reduction of SCLC tumor growth observed in vivo is therefore promising.

The varying degree of drug response in the different tumors arising from the same cell line may relate to subpopulation(s) of cells, as suggested by the distinct cellular morphology observed in As2O3 nonresponding SCLC tumors. This is an important issue that needs to be further evaluated. As tumors containing cells with this morphology were also observed with some frequency in untreated animals, we propose that this particular phenotype does not develop as a result of As2O3-induced selection. Previous reports have described SCLC cell lines with different morphologies and treatment responses (32, 33). Thus, it is possible that there exist subpopulations of tumor cells in established SCLC cell lines that are resistant to As2O3. A highly relevant question is whether corresponding cell populations can also be found in tumor specimens from patients with SCLC. Patients with mixed histology small/large cell carcinoma exist and this group of patients shows lower response rates and dies earlier than patients with archetypical small cell cancer (34). The fact that cells of As2O3 nonresponding SCLC tumors were larger and that the SCLC-mv cell line (derived from a large cell variant of SCLC; ref. 35) was less sensitive to As2O3 treatment may indicate that the highest efficacy of As2O3 is to be expected in patients with classic small cell type SCLC.

We observed high expression levels of neuroendocrine differentiation markers in SCLC tumors that responded well to As2O3. As As2O3 alone did not induce a similar up-regulation of differentiation markers in vitro at normoxia, the in vivo result is most likely not a direct effect of As2O3 on the tumor cells. Instead, it could be indirect, e.g., via an antiangiogenic effect of As2O3, leading to tumor hypoxia. This hypothesis is supported by our observations that hypoxia induced the expression of chromogranin A and synaptophysin in cultured SCLC cells and that treatment-responsive tumors had the highest HIF-1α protein levels (Fig. 5). As the level of tumor cell differentiation can influence clinical outcome, an indirect, hypoxia-driven differentiating effect of As2O3 could be clinically relevant and might contribute to the effects of As2O3 seen in vivo.

As2O3 has shown substantial efficacy in the treatment of patients with relapsed and refractory APL (5–8), and in recent years, also in patients with untreated, newly diagnosed APL (36, 37). In addition, As2O3 induces cell death at clinically relevant doses in a variety of cancer cells in vitro (38, 39), and a number of phase I and II clinical trials have been initiated to evaluate the potential effects of As2O3 in various cancer types (40). The observed As2O3-induced cytotoxicity under hypoxic conditions is clinically important because low tumor oxygenation has been linked to aggressive behavior in several tumor forms and hypoxic areas are frequent in solid tumors. In conclusion, the findings presented in this study suggest a potential clinical application of As2O3 in SCLC therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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