Arsenic trioxide (As$_2$O$_3$) induced calcium signals and cytotoxicity in two human cell lines: SY-5Y neuroblastoma and 293 embryonic kidney (HEK)

Ana-Maria Florea, Frank Splettstoesser, Dietrich Büsselberg *

Institut für Physiologie, Universitätsklinikum, Universität Duisburg Essen, Hufelandstrasse 55, 45122 Essen, Germany

Received 3 November 2006; revised 8 January 2007; accepted 29 January 2007

Available online 9 February 2007

Abstract

Arsenic trioxide (As$_2$O$_3$) has anticancer properties; however, its use also leads to neuro-, hepato- or nephro-toxicity, and therefore, it is important to understand the mechanism of As$_2$O$_3$ toxicity. We studied As$_2$O$_3$ influence on intracellular calcium ([Ca$^{2+}$]$_i$) homeostasis of human neuroblastoma SY-5Y and embryonic kidney cells (HEK 293). We also relate the As$_2$O$_3$ induced [Ca$^{2+}$]$_i$ modifications with cytotoxicity.

We used Ca$^{2+}$ sensitive dyes (fluo-4 and rhod-2) combined with laser scanning microscopy or fluorescence activated cell sorting to measure Ca$^{2+}$ changes during the application of As$_2$O$_3$ and we approach evaluation of cytotoxicity.

As$_2$O$_3$ (1 μM) increased [Ca$^{2+}$]$_i$ in SY-5Y and HEK 293 cells. Three forms of [Ca$^{2+}$]$_i$-elevations were found: (1) steady-state increases, (2) transient [Ca$^{2+}$]$_i$-elevations and (3) Ca$^{2+}$-spikes. [Ca$^{2+}$]$_i$ modifications were independent from extracellular Ca$^{2+}$ but dependent on internal calcium stores. The effect was not reversible. Inositol triphosphate (IP$_3$) and ryanodine (Ry) receptors are involved in regulation of signals induced by As$_2$O$_3$. 2-APB and dantrolene significantly reduced the [Ca$^{2+}$]$_i$-rise ($p<0.001$, t-test) but did not completely abolish [Ca$^{2+}$]$_i$-elevation or spiking. This indicates that other Ca$^{2+}$ regulating mechanisms are involved. In cytotoxicity tests As$_2$O$_3$ significantly reduced cell viability in both cell types. Staining with Hoechst 33342 showed occurrence of apoptosis and DNA damage.

Our data suggest that [Ca$^{2+}$]$_i$ is an important messenger in As$_2$O$_3$ induced cell death.

© 2007 Elsevier Inc. All rights reserved.

Keywords: Arsenic trioxide; Tumor cells; Neuroblastoma; HEK; Calcium homeostasis; Calcium signals; Apoptosis; Ryanodine receptors; IP$_3$ receptors; As$_2$O$_3$

Introduction

Arsenic has been medically used for over 2000 years, and is still applied in diverse treatments (e.g. leukemia, leishmaniosis, trypanosomiasis) (Shim et al., 2002; Florea, 2005; Griffin et al., 2005). Beside this, human exposure to arsenic compounds occurs by food or drinking water, being strongly associated with several illnesses (e.g. developmental abnormalities, neurological and neurobehavioral disorders, cardiovascular and hematological diseases, diabetes, hearing loss, fibrosis of the liver and lung, blackfoot disease and cancers) (Abernathy et al., 1999; Tchounwou et al., 1999; Sordo et al., 2001). Recently it was also demonstrated that arsenite impairs long-term potentiation in adult rats (Krüger et al., 2006), a indication that learning and memory formation might be impaired.

The mechanisms by which arsenic acts in living cells are not fully understood. Following intake, arsenic is biomethylated in humans to organic trivalent and pentavalent species, and this process is yet regarded as a toxification process (for review see Florea, 2005; Florea and Büsselberg, 2005, 2006; Florea et al., 2005c). Arsenic might damage cells by replacing physiological metals from their binding sites in specific molecules (Qian et al., 2003). Also, it may act as a catalyst in “redox” reactions resulting in the formation of reactive oxygen species (ROS) that further could damage DNA, lipids or proteins. Arsenic itself is triggering several signal transduction pathways leading to e.g. cell growth, proliferation and apoptosis. In addition, it promotes cell differentiation or cancer development (Qian et al., 2003, Yang and Frenkel, 2002). Currently, there is a specific scientific interest in the genotoxicity of arsenic species. Cytogenetic studies emphasized that arsenic compounds (especially trivalent forms) are clastogenic inducing micronuclei, chromosome aberrations and sister...
chromatid exchanges, even if recent research shows that relatively little arsenic is taken up in the in vitro models (Florea et al., 2005c). Arsenic compounds interact with DNA repair (e.g. enzymatic inhibition) and methylated arsenic species are promoters for urinary bladder, kidney, liver and thyroid gland carcinogenesis (for review see Florea and Büsselberg, 2006).

Different possible modes of action of arsenic induced carcinogenesis have been proposed: chromosomal damage, oxidative stress, modification of gene expression, modulation of DNA repair or DNA methylation and interactions with growth factors or cell proliferation. It could also determine promotion/progression, gene amplification, suppression of p53, as well as global DNA hypomethylation or malignant transformation (Kitchin, 2001; Zhao et al., 1997).

Many of the above described physiological or pathological processes are modulated by [Ca$^{2+}$], since Ca$^{2+}$ is a major signal transducer in living cells. [Ca$^{2+}$] overloads or disturbances in local intracellular distribution determine toxicity or cell death (necrosis or apoptosis) (Orrenius et al., 2003). Therefore, we focus on determining the role of As$_2$O$_3$ induced [Ca$^{2+}$]-signals with cytotoxicity of two possible target cell models: human neuroblastoma SY-5Y and human embryonic kidney (HEK) cells. We have chosen neuroblastoma as well as HEK cells in order to evaluate the possibility of using arsenic trioxide for treatment of other forms of cancer than leukemia and also to evaluate the eventuality of kidney toxicity.

Material and methods

Material. Neuroblastoma (SY-5Y) and Human Embryonic Kidney 293 cells (HEK 293) were purchased from ATCC. Fluo-4/AM (flu-4) and rhod2/AM (rhod-2) were ordered from Molecular Probes (OR, USA). Arsenic trioxide (As$_2$O$_3$, Fluka) was solved in methanol and in phosphate saline buffer (PBS) Ca$^{2+}$ and Mg$^{2+}$ free, for 1 mM stock solution. Final solutions were made in Tyrodes (in mM: 140 NaCl, 5 KCl, 1 MgCl$_2$, 10 glucose and 10 HEPEs, pH 7.2) buffers with no Ca$^{2+}$ added or with 1.8 mM Ca$^{2+}$. 2-Aminoethoxylidiphenyl borate (2-APB; Toecris) was solved in dimethylsulphoxide (DMSO) to a stock solution of 50 mM and dantrolene (Toecris) was solved in distilled water to a stock solution of 100 mM. Final concentrations were 50 μM and 20 μM respectively. Liposomal reagents were provided by Invitrogen (Lipofectamine) and Roche (Fugen6).

Cell culture. SY-5Y neuroblastoma cells were maintained in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Cambrex Biowhiteker), 100 IU/ml penicillin and streptomycin (Gibco). HEK 293 cells were maintained in DMEM (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), 100 IU/ml penicillin and streptomycin. Cells were kept incubated at 37 °C, under an atmosphere of 5% CO$_2$. The cell suspension was aliquoted in 1.5 ml eppendorf tubes, and re-suspended in 1 ml Tyrodes /MEM (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Cambrex Bio) and for fixation, treated with cold methanol (2-APB; Toecris) was solved in dimethylsulphoxide (DMSO) to a stock solution of 50 mM and dantrolene (Toecris) was solved in distilled water to a concentration of 100 mM. Final concentrations were 50 μM and 20 μM, respectively. Liposomal reagents were provided by Invitrogen (Lipofectamine) and Roche (Fugen6).

Confocal laser scanning microscopy. To study [Ca$^{2+}$], modulations by As$_2$O$_3$, two Ca$^{2+}$ sensitive dyes were used: fluo-4/AM to observe changes of [Ca$^{2+}$] and rhod-2/AM to determine the changes of Ca$^{2+}$ within calcium stores as described before (Florea et al., 2005a,b). Fluorescence images were collected at room temperature every 30 s. To observe faster events the time interval was system at a rate of 1 ml/min. Reversibility on [Ca$^{2+}$] homeostasis was tested by applying Tyrodes buffer.

For determination of [Ca$^{2+}$] concentration the “ion concentration” option of the META software (Zeiss) was used. Images were background subtracted and the [Ca$^{2+}$]-concentration was calculated using the following equation: [Ca$^{2+}$] = K$_d$ ([F$^{-}$ − F$^{-}$/F$^{−}$/F$^{−}$]), where “K$_d$” is the dissociation constant (see Molecular Probes) and “F$^{-}$” is the fluo-4 intensity. The basal [Ca$^{2+}$]-concentration was considered 100 nM (Orrenius et al., 2003). To illustrate [Ca$^{2+}$], changes over time, the “subtraction” option from the META software was used. In the calculated images with “rainbow scale”, blue illustrates the minimum and red the maximum change of [Ca$^{2+}$].

Fluorescent activated cell sorting (FACS). Cells were grown in 125 cm$^2$ flasks and collected by trypsin treatment in culture media (with FCS), centrifuged and washed with phosphate saline buffer (PBS). The cell suspension was aliquoted in 1.5 ml eppendorf tubes, and re-suspended in each tube to a concentration of 1 ml Tyrodes buffer. Unstained and stained controls were used. After 30 min they were washed once with buffer solution and As$_2$O$_3$ was applied to the cell suspension for 0, 5, 15, 30 and 60 min. After incubation with As$_2$O$_3$, the cells were washed twice with Tyrodes buffer. Cells were fixed in 2.5% formaldehyde in PBS and measured by FACS. 10,000 events (cells) were counted for each sample. The fluorescence (x-axis) was performed with the “WinMDI” software. The modification of fluo-4 intensity was observed by a shift of the cell population to the right on the x-axis.

Trypan blue cytotoxicity test. For determination of cell viability, treated as well as untreated cells were used. Non-confluent cell monolayers were exposed to As$_2$O$_3$, in cell culture flasks, for 24, 48 and 72 h. All experiments were repeated twice. After treatment, the culture media were collected in a 50 ml centrifugation tube, since it might contain dead cells which will be needed for cell counts. The cell monolayer was washed with PBS and then collected in the same tube. Cells were trypsinized and trypsin and cells were also collected in the same 50 ml tube. The suspension was centrifuged (2 min, 1200 rpm) and the supernatant removed. The pellet was washed with PBS, centrifuged (2 min, 1200 rpm) and re-suspended again in complete culture media. Then, a small aliquot of the cell suspension (50 μl) was mixed with the same volume of 0.4% trypan blue (Sigma) solution and the sample was counted after 3 min of staining using a hemocytometer. The number of bright (viable) cells and blue cells (non-viable) was evaluated using a light microscope with a 20-fold magnification.

After counting, the cell viability (CV) was expressed as the percentage of surviving cells compared to the total number of cells: CV = (viable cells/total number of cells) × 100. As$_2$O$_3$ was considered to be cytotoxic, when it induced a decrease of cell viability of more than 50%. Controls and exposed samples were compared using the two-tailed Student’s t-test.

MTT-cytotoxicity test. For the MTT test we used a “Vibrant MTT Cell Proliferation Assay Kit” provided by Molecular Probes (Invitrogen, Germany) as suggested by the manufacturer’s recommendation. Briefly, 5000 cells were seeded in each well of a 96 well plate, using 100 μl culture media. They were allowed to attach over night. As$_2$O$_3$ (1 μM) was applied to non-confluent cultures, to avoid grow inhibition, for different times of exposure (2 h to 72 h). Stock solution of 12 mM MTT was prepared by adding 1 ml of sterile PBS to one 5 mg vial of MTT (component A). A stock solution of SDS was prepared by adding 10 ml of DMSO to 1 g of SDS already aliquoted as component B included in the kit. Before the exposure to As$_2$O$_3$medium was exchanged. For controls and blanks, complete culture medium was used (100 μl/well). The test cells were exposed to 1 μM As$_2$O$_3$ in a final volume of 100 μl culture medium. After exposure 10 μl of the solubilized MTT (component A) was added to the controls and exposure wells, but not to the blanks. The blanks were used to correct the microplate readings. The plate was further incubated 4 h at 37 °C. After labeling with MTT, 25 μl MTT solution was added to controls and exposure wells, and 100 μl culture media. They were allowed to attach over night. After 3 h of incubation with MTT, the cells were washed twice with PBS, and for fixation, treated with cold methanol (−20 °C) and left overnight at
−20 °C. After fixation, slides were air-dried, stained with 10 μM Hoechst 33342 (Molecular Probes) for 30 s and mounted with cover slips. Cells were analyzed using a 63× magnification with an Axiovert fluorescent microscope. The two-tailed Student’s t-test was used to compare the difference between controls and exposed samples. For the treatment with liposomes, 1 μl of 1 mM arsenic trioxide stock solution was mixed with 1.5 μl Lipofectamine or Fugene6 and further incubated with 50 μl culture media for 15 min. This mixture was given to cells cultured on chamber slides to a final volume of 1 ml per well. Cells were fixed and stained as previously described.

Results

As$_2$O$_3$ induced changes in [Ca$^{2+}$]i, in neuroblastoma and HEK cells

As$_2$O$_3$ is a potential anticancer drug; however other secondary effects can occur upon treatment such as neurotoxicity, hepatotoxicity or nephrotoxicity. To understand the cellular mechanisms of As$_2$O$_3$ interactions, we focused on [Ca$^{2+}$]-signals which are involved in cell death. The aims of the first part of this study were (1) to investigate whether micromolar concentrations of As$_2$O$_3$ influence [Ca$^{2+}$]i, (2) to define the types of [Ca$^{2+}$]-signals and (3) to investigate from which sources (extracellular or calcium release from intracellular stores) the Ca$^{2+}$ originates.

Both, LSM (Figs. 1Aa, b, c and Ca, b, c) and FACS (Figs. 1B and D) experiments demonstrated that 1 μM As$_2$O$_3$ increased [Ca$^{2+}$]i in neuroblastoma as well as in HEK cells. Figs. 1A and C show examples of LSM experiments with traces of 3 different cells (Fig. 1Aa for neuroblastoma, Fig. 1Ca for HEK). After a stable base line of [Ca$^{2+}$]i level was established, an application of As$_2$O$_3$ elevated [Ca$^{2+}$]i, with variations in time course (Figs. 1Aa and Ca). However, overall, [Ca$^{2+}$]i rose similarly in the two cell lines until a steady-state was reached. Overall [Ca$^{2+}$]i increased 170±4.84% in neuroblastoma (7 experiments, 58 cells) and 166.88±1.68% in HEK cells (3 experiments, 13 cells). In both cell lines the increase of [Ca$^{2+}$]i induced by As$_2$O$_3$ was not reversible under these experimental conditions (Figs. 1Aa and Ca).

The change of [Ca$^{2+}$]i-concentration which occurred over the 2 h is illustrated in the inserts of Figs. 1Ab and Cb. In these rainbow scale images, “red” represents a maximum increase of [Ca$^{2+}$]i and “blue” those areas where [Ca$^{2+}$]i was unchanged. The confocal images shown in Figs. 1Ac and Cc illustrate the increase of fluo-4 intensity (green) over time.

Arsenic induced [Ca$^{2+}$]i-rise in both cell lines was confirmed by FACS using 10,000 counted cells. An example of FACS plots after 30 min of incubation is shown in Figs. 1B and D. The intensity of cell population shifted to the right when comparing trace (b) with trace (c) and where trace (a) is an unstained control (cells gated: unstained 0.1±0% neuroblastoma; 0.25±0.35% HEK vs. stained with fluo-4 respectively 35.15±6% neuroblastoma; 28.65±2.33% HEK vs. respectively fluo-4 fluorescence after incubation with 1 μM As$_2$O$_3$ 57.1±10% neuroblastoma; 40.1±3.39% HEK), an indication that [Ca$^{2+}$]i rose in neuroblastoma and HEK cells (data not shown for 5, 15, 60 min).

To probe whether a lower concentration of As$_2$O$_3$ influences calcium homeostasis in neuroblastoma and HEK cells, we have applied to the culture a concentration as low as 0.1 μM. Even this concentration of arsenic trioxide triggered a [Ca$^{2+}$]i-increase in both cell lines with the same patterns described for the higher concentration of 1 μM (Figs. 2A and B).

To test if the calcium increase is depending on As$_2$O$_3$ we performed experiments where no As$_2$O$_3$ was added to the bath solution. In this case the cells were maintained in the same experimental conditions as before with the difference that no As$_2$O$_3$ was present in the extracellular solution. As illustrated in Figs. 2C and D under this condition there is no relevant elevation of [Ca$^{2+}$]i over the time (up to 100 min) in either of the two cell lines tested. A fact that underlines that the [Ca$^{2+}$]i rise described before is clearly As$_2$O$_3$-dependent.

In addition to the slow increase of [Ca$^{2+}$]i, to a steady-state, two other types of [Ca$^{2+}$]i-elevations were found: (1) transient [Ca$^{2+}$]i-elevations and (2) [Ca$^{2+}$]i-spikes. Both transient [Ca$^{2+}$]i-increases were generated independently. Transient [Ca$^{2+}$]i-elevations are illustrated in Fig. 2A. To analyze the fast [Ca$^{2+}$]i-rise, images were taken at an interval of 1 s. [Ca$^{2+}$]i traces of independent cells show that the cells could express synchronized calcium rise (see the increase at ∼1500 s) and also unsynchronized transient [Ca$^{2+}$]i-increases (simultaneously with a sustained increase).

Thus, our data illustrate, that even in different compartments of the same cell localized, transient [Ca$^{2+}$]i-elevations can occur (Figs. 2B and C). Images of such localized [Ca$^{2+}$]i-events at different time points (control, 15, 30, 45, 60 and 75 min) in neuroblastoma cells are shown in Fig. 2B (see arrows). The time course of two neighboring ROIs within one neuroblastoma cell, as marked on the right side of the time series in Fig. 2B, is enlarged in Fig. 3C. The area of the cell represented by the ROI marked in “light” color (cytosol) slowly increases and decreases during the application, while [Ca$^{2+}$]i reached a higher level at the end of the experiment (after 4500 s). The near ROI placed most likely on a calcium store, shows a [Ca$^{2+}$]i-increased in three distinct steps during the presence of As$_2$O$_3$. Fig. 3D shows another example where the ROI placed in the cytosol of a neuroblastoma cell had a slight time-dependent [Ca$^{2+}$]i rise, while in the region next to this ROI, the [Ca$^{2+}$]i rose faster and higher and even a [Ca$^{2+}$]i-spike occurred. The transient [Ca$^{2+}$]i-elevations point to a (re)-uptake of the Ca$^{2+}$ by localized calcium buffer systems, which are unaffected by arsenic. When comparing the differences in [Ca$^{2+}$]i-increase at a specific time and location, the spatial distribution is even clearer (Figs. 3C and D).

Ca$^{2+}$ stores and Ca$^{2+}$ receptors are involved in the As$_2$O$_3$ mediated [Ca$^{2+}$]i changes

To analyze from which sources the increase of [Ca$^{2+}$]i originates, we performed experiments where: (1) Ca$^{2+}$ in the stores of neuroblastoma and HEK cells was labeled with rhod-2 before As$_2$O$_3$ was applied, and (2) [Ca$^{2+}$]i was stained with fluo-4 under two experimental conditions; (a) no calcium added to the external solution (to exclude a Ca$^{2+}$ entry from the extracellular space) and (b) these results were compared with
the data obtained in extracellular solution which contained 1.8 mM Ca^{2+}. Exposure of neuroblastoma and HEK cells to As_{2}O_{3} showed a Ca^{2+} release from the internal calcium stores. An application of 1 \mu M As_{2}O_{3} decreased over time the rhod-2 intensity (Figs. 4A for neuroblastoma and B for HEK cells). As we have shown earlier in this study, the rise of [Ca^{2+}]_i clearly depends on the application of As_{2}O_{3}. The concentration of [Ca^{2+}]_i was basically not changed when As_{2}O_{3} was not present in the Tyrodes solution, respectively the [Ca^{2+}]_i rise was 100.4±0.7% in neuroblastoma and 100.5±0.5% in HEK cells (Figs. 5A and B) which was not statistically significant (compare also Figs. 2A–C). Inserts Aa (for SY5Y neuroblastoma cells) and Ca (for HEK293 cells) illustrate images taken from the cell culture tested, where the [Ca^{2+}]_i-level is indicated by false colors (blue=background=no calcium; green to yellow=intermediate calcium concentration; red=high calcium concentration). The colored circles indicate which cells have been taken for the time course of the main part of the figure. Parts Ab and Cb illustrate the difference of [Ca^{2+}]_i; after the application of As_{2}O_{3}. The image series in Ac and Cc show the modification in fluo-4 intensity that is in direct relationship with [Ca^{2+}]_i, at different time points of the experiment. The increase of [Ca^{2+}]_i was confirmed also by FACS experiments using a large population of cells (10,000 counted cells). Figs. 1B and D represent such FACS plots of neuroblastoma (B) and HEK cells (D). The cells were stained with fluo-4 and incubated with As_{2}O_{3} (1 \mu M). The traces show the autofluorescence level of unstained cells (a), the FITC fluorescence after staining with fluo-4 for 30 min (b) and fluo-4 fluorescence after incubation with 1 \mu M As_{2}O_{3} (30 min) (c). Notice the shift of the intensity to the right that is in concordance with increased [Ca^{2+}]_i, as the cells were arbitrary gated as: unstained 0.1±0% neuroblastoma; 0.25±0.35% HEK vs. stained with fluo-4 respectively 35.15±6% neuroblastoma; 28.65±2.33% HEK vs. respectively fluo-4 fluorescence after incubation with 1 \mu M As_{2}O_{3} (30 min) 57.1±10% neuroblastoma; 40.1±3.39% HEK. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To determine whether inositol-1,4,5-triphosphate (IP_{3}) and/or ryanodine (Ry) receptors, which are found at the endoplasmatic reticulum (ER) and/or the Golgi apparatus (Pinton et al., 1998), are involved in the regulation of [Ca^{2+}]_i, we used specific blockers for these receptors (2-APB and dantrolene). A preincubation (20 min) with 2-APB (50 \mu M) and/or dantrolene (20 \mu M) reduced significantly the [Ca^{2+}]_i-rise (in 1.8 mM calcium buffer, Figs. 5A and B). After preincubation with 2-APB, As_{2}O_{3} determined a [Ca^{2+}]_i-increase of: 121±0.17% (3 experiments, 23 cells) in neuroblastoma and 113±0.91% in HEK cells (3 experiments, 28 cells). Similar effects were observed with dantrolene: 137±1% (3 experiments, 28 cells) increase of [Ca^{2+}]_i in neuroblastoma and 125±2.45% in HEK cells (3 experiments, 18 cells). The rise was highly significantly reduced (\rho<0.001) compared with the [Ca^{2+}]_i-rise induced by As_{2}O_{3} (1 \mu M) in normal containing calcium buffer. These
results drive the conclusion that $[\text{Ca}^{2+}]_{\text{i}}$-rise induced by As$_2$O$_3$ is modulated by IP$_3$- and Ry-receptors.

We conclude that As$_2$O$_3$ interacts with $[\text{Ca}^{2+}]_{\text{i}}$ in neuroblastoma as well as in HEK cells as a result of $\text{Ca}^{2+}$-release from the calcium stores.

As$_2$O$_3$ determines cell death and damages DNA of neuroblastoma and HEK cells

With Trypan blue staining and with MTT cytotoxicity test, we tested whether As$_2$O$_3$ (1 $\mu$M) decreases cell viability after 2, 24, 48 and 72 h and whether the used concentration is cytotoxic, expressed as less than 50% cell survival, in both cell lines. As$_2$O$_3$ (1 $\mu$M) had an increased toxic effect on both cell lines. In Trypan blue exclusion assay, after exposure to 1 $\mu$M As$_2$O$_3$, the cell viability was decreased significantly ($t$-test, $p<0.05$), reaching an approximate 80% survival after 72 h of exposure. However, it did not drop under 50% cell survival. The effect was similar for both cell types (Fig. 6A). When applying MTT test, the neuroblastoma cells had higher sensitivity to 1 $\mu$M As$_2$O$_3$ than HEK cells (70% vs. 85%), but the cell survival did not drop under 50% (Fig. 6A).

After exposure to As$_2$O$_3$ neuroblastoma and HEK cells were scored for DNA damage (micronucleated cells), apoptotic cells (condensed nuclei) and cells in mitosis (Fig. 6Ba–c: neuroblastoma; d–f: HEK). The number of cells with damaged DNA as well as apoptotic cells was significantly increased ($p<0.05$, $t$-test) in neuroblastoma (Fig. 6C) and HEK cells (Fig. 6D) comparing with the conditions where cells were incubated without As$_2$O$_3$. The number of mitotic cells was not significantly affected in any of the cell lines (compare Table 1).

Since liposomal therapy is an option in anticancer drug delivery, we tried liposomes containing As$_2$O$_3$ in order to see consolidated effects. Fugene6 and Lipofectamine did not modify the background level of DNA damage, apoptosis or mitosis (Fig. 6E, F). In Figs. 6E (neuroblastoma) and F (HEK) it is illustrated that Fugene6 and Lipofectamine had similar effects. The cells with damaged DNA and the number of cells in apoptosis were significantly increased ($p<0.05$) while the number of cells in mitosis was significantly decreased ($p<0.05$). However, the DNA damaged and apoptotic level of cells was not significantly different compared to the experiments without usage of liposomes, an indication that intracellular concentration of As$_2$O$_3$ is possibly regulated (Table 2).

Discussion

As$_2$O$_3$ influences $[\text{Ca}^{2+}]_{\text{i}}$, homeostasis

Metal ions and compounds affect $[\text{Ca}^{2+}]_{\text{i}}$, homeostasis of living cells (Florea and Büsselberg, 2005). $[\text{Ca}^{2+}]_{\text{i}}$, is highly
controlled and deregulation of \([\text{Ca}^{2+}]\), affects the plasma membrane, mitochondria or ER (Orrenius et al., 2003) and could result in cytotoxicity (reviewed by Kass and Orrenius, 1999). Although \(\text{As}_2\text{O}_3\) is an effective drug for therapy in acute promyelocytic leukemia, its use in other malignancies is limited by the dose-toxicity, required to induce apoptosis in non-APL tumor cells (Diaz et al., 2005). It was shown that an \(\text{As}_2\text{O}_3\) triggered increase of \([\text{Ca}^{2+}]\) inhibits cell growth and induces apoptosis in human malignant cell lines, accompanied by an increase of cellular \(\text{H}_2\text{O}_2\), a decrease of mitochondrial membrane potential and an activation of caspase-3 (Zhang et al., 1999; Kajiguchi et al., 2003; Miller et al., 2002). While it is documented that \([\text{Ca}^{2+}]\) overloads could trigger apoptosis (Orrenius et al., 2003), there is no detailed work describing how \(\text{As}_2\text{O}_3\) induced \([\text{Ca}^{2+}]\), modulations are involved in programmed cell death. Therefore we investigated the mechanisms of \([\text{Ca}^{2+}]\)-elevation triggered by \(\text{As}_2\text{O}_3\) using two different target models: neuroblastoma and embryonic kidney cells.

Generally, \([\text{Ca}^{2+}]\), could be increased in living cells by (1) facilitated \(\text{Ca}^{2+}\)-entry from the extracellular space by activation (or reduced inactivation) of \(\text{Ca}^{2+}\) selective pores, (2) \(\text{Ca}^{2+}\) release from the stores (mitochondria and/or endoplasmic reticulum; Florea et al., 2005a,b) and (3) impairment of \(\text{Ca}^{2+}\) selective transport proteins which pump \(\text{Ca}^{2+}\) in the extracellular space and/or in the calcium stores (e.g. nanomolar concentrations of lead; Ferguson et al., 2000). \([\text{Ca}^{2+}]\), could be decreased by a reduction of \(\text{Ca}^{2+}\)-entry by blocking calcium selective pores (Büsselberg, 1995, 2004; Büsselberg et al., 1994) or by an enhancement of the efficiency of calcium transport proteins.

In this study we have demonstrated that low concentrations of \(\text{As}_2\text{O}_3\) (1 and 0.1 \(\mu\text{M}\)) triggered an irreversible \([\text{Ca}^{2+}]\)-increase to a steady-state or/and (fast and slower) calcium transients while experiments performed with no \(\text{As}_2\text{O}_3\) added show no modification in intracellular calcium concentration. These effects were similar in the two cell lines, suggesting that...
As$_2$O$_3$ targets these cells in a similar manner. While the extracellular calcium level had no significant influence in As$_2$O$_3$ derived [Ca$^{2+}$]$_i$-elevation, the Ca$^{2+}$-release from intracellular calcium stores was most important. On the first view, this qualifies the influence of mechanisms at the cellular membrane, like an enhanced Ca$^{2+}$ entry from the extracellular space, but a Ca$^{2+}$ uptake from the extracellular space is not totally excluded by our experiments. Ma and colleagues (2006) showed that in a low extracellular Ca$^{2+}$ concentration As$_2$O$_3$ (10 $\mu$M) did not induce the opening of the mitochondrial transition pore (PTP) and cytochrome $c$ release from mitochondria, while the same concentration of As$_2$O$_3$ with high extracellular Ca$^{2+}$ concentration induced PTP opening and cytochrome $c$ release. A possible explanation could be the different cell systems, as well as the 10-fold lower concentration of As$_2$O$_3$ used in our study.

We also demonstrate that As$_2$O$_3$ triggered different kinds of Ca$^{2+}$ signals: slow (sustained), transient elevations and calcium spikes. During a slow increase of [Ca$^{2+}$], the Ca$^{2+}$ concentration in the stores was reduced over time; this could be explained by an enhanced calcium release probably by opening IP$_3$ and/or ryanodine mediated pores or by an impaired re-uptake into the stores such as a reduced transport performance of calcium pumps. Therefore, calcium stores play a major role in the As$_2$O$_3$ induced changes of [Ca$^{2+}$]. The calcium stores could also take up Ca$^{2+}$ from the cytosol by calcium transport proteins. While we cannot exclude an impaired re-uptake in the stores for the slower transient [Ca$^{2+}$], the fast calcium spikes

![Fig. 4: As$_2$O$_3$ releases Ca$^{2+}$ from the calcium stores.](image-url)
are contradictory to this conclusion, since they depend on potent extrusion mechanisms for the cytosolic Ca$^{2+}$, which are obviously not impaired. The transient increases of [Ca$^{2+}$]$_{i}$ occur fast and are localized and appeared independently, in different regions of the same cell. Also, cells have individual changes of [Ca$^{2+}$]$_{i}$ after the application of arsenic. This could be explained by: (1) differential protein (receptor) expression at the cell membrane, (2) different activity levels of specific
pathways or (3) extrusion mechanisms with a variable efficiency.

More difficult to explain are the fast calcium spikes. Due to the limitations of our recording parameters such as (1) taking an image generally every 30 s, to avoid bleaching; (2) setting the ROIs to a specific seize and therefore very small and localized events could hardly be analyzed; and (3) searching for events which happened in the 3D-structure of the cell while analyzing them in a 2D confocal image; those spikes (comp. Fig. 2D) are difficult to judge because they could occur any time in the interval when no images were taken.

Yet, we have not addressed the question whether As2O3 needs to enter the cytosol to accomplish the rise of \([\text{Ca}^{2+}]\). Our data do not allow to determine to which extent As2O3 enters the cells, but previously it was documented that inorganic trivalent arsenic (arsenite) has a very low uptake in vitro (below 5% of the substrate, see detailed results in Florea, 2005). In addition, our experiments using liposomes, to increase the amount of As2O3 in the cell, did not result in a higher cytotoxicity. Therefore we speculate that either As2O3 binds to receptors at the extracellular membrane, which trigger intracellular pathways resulting in an elevated \([\text{Ca}^{2+}]\)-level, or the relatively small amount of As2O3 which enters the cytosol is sufficient to release \(\text{Ca}^{2+}\) from the stores. In other work the involvement of mitochondria on the rise of \([\text{Ca}^{2+}]\) was demonstrated (Ma et al., 2001; Shen et al., 2002) thus, little or no investigations have been done in analyzing the involvement of ER in As2O3 induced \([\text{Ca}^{2+}]\) rise and cell death. In this work we show that As2O3, we faced the challenge that the blockers themselves induced a high rate of micronuclei and apoptotic cells after 24 h of incubation, and therefore it was impossible to obtain dependable results (data not shown). Thus, micromolar concentrations of As2O3 with liposomal association rose \([\text{Ca}^{2+}]\), triggered apoptosis, damaged DNA and stopped the cells to enter mitosis. \([\text{Ca}^{2+}]\) rise from mitochondria or ER could induce mitochondrial and ER stress, formation of ROS that could further damage DNA. Thus, localized \(\text{Ca}^{2+}\) accumulation could be a result of a \(\text{Ca}^{2+}\) re-uptake, \(\text{Ca}^{2+}\) overload, PTP opening and apoptosis. The non-reversibility indicates that \([\text{Ca}^{2+}]\), disturbance could also lead to “a point of no return” and cell death. The different types of \([\text{Ca}^{2+}]\)-signals induced by As2O3 could also be interpreted as modulators for specific processes such as resistance, transport, gene expression.

Induction of enhanced DNA damage by As2O3 is a supplementary risk in the case of non-tumor cells, since clastogenesis increases the possibility of secondary malignancies. In addition, the number of cells in mitosis was significantly decreased only in the co-treatment of As2O3 with liposomes, a fact that underlines that the cells do not divide anymore. Unfortunately, this effect was not strictly related to tumor cells but also affected non-tumor cells, a finding that emphasizes a therapeutic strategy that protects non-tumor cells.

Several authors describe the cytotoxicity of As2O3. For example, As2O3 induced cytotoxicity in vitro with an IC50 of 10 μM after 24 h of exposure (Shim et al., 2002). The same authors described DNA fragments, morphological changes and chromatin condensation of the cells undergoing apoptosis after incubation with a two times higher As2O3 concentration. Zhang et al. (1999) showed that 1 μM As2O3 had cytotoxic effects in malignant cells but not in human embryonic pulmonary cells. We show that As2O3 (1 μM) exhibits cytotoxic effects by significantly decreasing cell viability, enhances cell death by apoptosis and induces DNA damage in neuroblastoma as well as in HEK cells while absence of As2O3 did not. These different results could be explained by the use of different in vitro models that could have different sensibility, since kidney cells represent a target for As2O3.

Arsenic induces apoptosis in several tumor and non-tumor cell lines. Several \([\text{Ca}^{2+}]\)-dependent apoptotic pathways were emphasized: (1) an intrinsic mitochondrial pathway, (2) an activation/repression MAPK and/or of (3) other protein kinases (Chen et al., 2001; Fernandez et al., 2004; Davison et al., 2004; Verma et al., 2002; Maeda et al., 2001; Zhou et al., 2004; Jarvis et al., 1994). Trying to determine the involvement of IP3 and Ry receptors in DNA damage, apoptosis and mitosis induced by As2O3, we faced the challenge that the blockers themselves induced a high rate of micronuclei and apoptotic cells after 24 h of incubation, and therefore it was impossible to obtain dependable results (data not shown). Thus, micromolar concentrations of As2O3 with liposomal association rose \([\text{Ca}^{2+}]\), triggered apoptosis, damaged DNA and stopped the cells to enter mitosis. \([\text{Ca}^{2+}]\) rise from mitochondria or ER could induce mitochondrial and ER stress, formation of ROS that could further damage DNA. Thus, localized \(\text{Ca}^{2+}\) accumulation could be a result of a \(\text{Ca}^{2+}\) re-uptake, \(\text{Ca}^{2+}\) overload, PTP opening and apoptosis. The non-reversibility indicates that \([\text{Ca}^{2+}]\), disturbance could also lead to “a point of no return” and cell death. The different types of \([\text{Ca}^{2+}]\)-signals induced by As2O3 could also be interpreted as modulators for specific processes such as resistance, transport, gene expression.

Induction of enhanced DNA damage by As2O3 is a supplementary risk in the case of non-tumor cells, since clastogenesis increases the possibility of secondary malignancies. In addition, the number of cells in mitosis was significantly decreased only in the co-treatment of As2O3 with liposomes, a fact that underlines that the cells do not divide anymore. Unfortunately, this effect was not strictly related to tumor cells but also affected non-tumor cells, a finding that emphasizes a therapeutic strategy that protects non-tumor cells.

---

**Table 1**
The level of DNA damage (MN) and apoptosis (AP) compared with control levels, induced by 1 μM As2O3 in neuroblastoma and HEK cells

<table>
<thead>
<tr>
<th></th>
<th>MN</th>
<th>AP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neuroblastoma</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>370%</td>
<td>260%</td>
</tr>
<tr>
<td>48 h</td>
<td>514%</td>
<td>152%</td>
</tr>
<tr>
<td>72 h</td>
<td>400%</td>
<td>214%</td>
</tr>
<tr>
<td><strong>HEK</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>335%</td>
<td>150%</td>
</tr>
<tr>
<td>48 h</td>
<td>481%</td>
<td>192%</td>
</tr>
<tr>
<td>72 h</td>
<td>316%</td>
<td>200%</td>
</tr>
</tbody>
</table>

**Table 2**
The level of DNA damage (MN) and apoptosis (AP) compared with control levels, induced by liposomal treatment with As2O3 in neuroblastoma and HEK cells

<table>
<thead>
<tr>
<th></th>
<th>MN</th>
<th>AP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neuroblastoma</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>As2O3 and Fugene6 (24 h)</td>
<td>231%</td>
<td>246%</td>
</tr>
<tr>
<td>As2O3 and Lipofectamine (24 h)</td>
<td>264%</td>
<td>240%</td>
</tr>
<tr>
<td><strong>HEK</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>As2O3 and Fugene6 (24 h)</td>
<td>348%</td>
<td>252%</td>
</tr>
<tr>
<td>As2O3 and Lipofectamine (24 h)</td>
<td>356%</td>
<td>240%</td>
</tr>
</tbody>
</table>
Conclusion

Our study could open new perspective in improving the therapy with \( \text{As}_2\text{O}_3 \) because understanding of the arsenic induced, IP\(_3\) and Ry-receptor mediated rise of [Ca\(^{2+}\)]\(_i\), might help to increase the efficiency of the anti-cancer treatment and diminish secondary effects such as kidney toxicity.

References


Finton, P., Pozzan, T., Rizzuto, R., 1998. The golgi apparatus is an inositol 1,4,5-trisphosphate-sensitive Ca\(^{2+}\) store, with functional properties distinct from those of the endoplasmic reticulum. EMBO 17, 5298–5308.


