Co-application of arsenic trioxide (As$_2$O$_3$) and cisplatin (CDDP) on human SY-5Y neuroblastoma cells has differential effects on the intracellular calcium concentration ([Ca$^{2+}$]$_i$) and cytotoxicity

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1. Introduction

Cancer is still a major cause of death worldwide (Shibuya et al., 2002). Thus, cancer therapy remains one of the most important challenges of modern medicine. Classic strategies of cancer treatment aim to combat malignant cells as early and strongly as possible (Goldie and Coldman, 1979). Generally, the first attempt in cancer treatment is to remove the tumour surgically which is followed by a treatment with chemotherapeutics (e.g. platin compounds or arsenic) and/or ionizing radiation (IR). Overall, the understanding of basic cellular mechanisms of anti-cancer drugs is an important precondition for the efficient treatment of cancer.

Neuroblastoma is a frequently occurring solid tumour in children under 5 years. During the first year of life it is the most common cancer and the third most common cancer of children in the United States (Urayama et al., 2007). Neuroblastoma is a clinical heterogeneous tumour and in about half of the cases it is classified as a high-risk cancer. Despite intensive therapy of neuroblastoma, the overall survival rate is only 40% and therefore it is urgent that more effective adjuvant treatment strategies are found.

The type of neuroblastoma treatment is dependent on age, stage of disease, and biological and biochemical markers. Nuclear medicine plays an important role in the initial staging, as a prognostic indicator, for assessment of response to treatment, and also in therapy (Castel et al., 2007; Howman-Giles et al., 2007). The current treatment strategy for neuroblastoma uses an aggressive chemotherapy, consisting of a combination of cyclophosphamide, vincristine, tetrahydropyranyl [THP]-adriamycin, and cisplatin. This regimen has increased the survival rates of patients with advanced neuroblastoma (Kaneko et al., 2002). However, the general administration of chemotherapy (such as infusion with cisplatin) can lead to diverse toxic side effects such as neuro- or renal-toxicity as well as bone marrow-supression.

The response of tumours to chemo- or radio-therapy as well as to biologically active agents may depend, at least in part, on the ability of these tumours to undergo cell death. Solid tumours usually respond slowly and less effectively to treatment, with cell death characterized not only by apoptosis but also by necrosis.
or mitotic catastrophe. Resistance of tumours to treatment might be also associated with defects in, or deregulation of different steps in the apoptotic pathways (Viktorsson et al., 2005). Therefore, it is essential to identify new drugs or treatment strategies which are more efficient and result in fewer toxic side effects.

Previous studies have shown that As$_2$O$_3$ and CDDP are able to trigger apoptotic cell death that involves production of the reactive oxygen species, e.g. mitochondrial stress, release of cytochrome c and activation of caspases (for review see Florea et al., 2005; Florea and Büsselferg, 2005, 2006). We have also shown that an increase of [Ca$^{2+}$], triggered by these drugs is involved in cell death by apoptosis when low concentrations are used (nanomolar up to low micromolar concentrations) (Florea et al., 2007; Splettstoesser et al., 2007; Florea and Büsselferg, 2008) however higher concentrations of these drugs could induce cell death by necrosis (Florea, 2005; Florea and Büsselferg, 2005; Bustamante et al., 2005).

While the pharmacology of most chemotherapeutics is still not fully understood, the therapeutic goal in cancer treatment today is to trigger tumour-specific cell death. Intracellular Ca$^{2+}$-signals could have a major impact on the induction of cell death. An intracellular calcium concentration ([Ca$^{2+}$]) overload as well as a disturbance in calcium homeostasis could cause cytotoxicity and trigger either apoptotic or necrotic cell death (Orrenius et al., 2003, 2005). Interestingly, some metal-based anti-cancer drugs (e.g. As$_2$O$_3$ and CDDP) have the potential to modulate [Ca$^{2+}$], (Florea and Büsselferg, 2005, 2006). Their mechanisms of action are different in that the application of As$_2$O$_3$ triggers a depletion of Ca$^{2+}$ from intracellular stores (Florea et al., 2007; Florea and Büsselferg, 2008) whereas the application of CDDP induces a Ca$^{2+}$-influx from the extracellular space (Splettstoesser et al., 2007), while currents through voltage activated calcium channels are reduced (Tomaszewski and Büsselferg, 2007). It has been shown that independent of the calcium source, the rise of [Ca$^{2+}$] is directly related to apoptosis (Splettstoesser et al., 2007; Florea et al., 2007; Florea and Büsselferg, 2008). Therefore, we hypothesize that a co-application of CDDP and arsenic will result in an additive increase of [Ca$^{2+}$], in the cells resulting in a higher apoptotic rate in tumour cells. To test this hypothesis, we used human SY-5Y neuroblastoma cells, whose incidence is a typical indication for chemotherapy with CDDP, while As$_2$O$_3$ is regarded as a possible alternative chemotherapy.

2. Materials and methods

Neuroblastoma SY-5Y cells were purchased from American Tissue Culture Collection (ATCC, Manassas, VA, USA) and Fluoro/AM (fluor-4) from Molecular Probes (Molecular Probes, Karlsruhe, Germany). Arsenic trioxide (As$_2$O$_3$, Fluka) was prepared as a 1 mM stock solution in methanol and phosphate buffered saline (PBS), free of Ca$^{2+}$ and Mg$^{2+}$. Cisplatin solution (CDDP) was purchased from Bristol-Meyers Squibb (Munich, Germany). Both substances were further diluted in Tyrode buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 1.8 mM CaCl$_2$, 10 mM glucose and 10 mM Hepes, pH 7.4) to a final concentration of 1 $\mu$M.

2.1. Cell culture

Non-confluent monolayers of neuroblastoma SY-5Y cells were grown in RPMI 1640 (Gibco, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal calf serum (Cambrex Biowhiteker, East Rutherford, NJ, USA), 100 IU/ml penicillin and streptomycin (Gibco). Cells were incubated at 37 °C in an atmosphere of 5% CO$_2$, 95% air. For [Ca$^{2+}$] imaging experiments the cells were trypsinized and plated onto “easy grip” culture dishes (Falcon, Franklin Lakes, NJ, USA).

2.2. Confocal laser scanning microscopy

Cells were stained with the calcium sensitive fluo-4 dye for calcium imaging. Changes in [Ca$^{2+}$], were observed using a confocal laser scanning microscope (Zeiss 510) as previously described (Splettstoesser et al., 2007; Florea et al., 2007; Florea and Büsselferg, 2008). Briefly, fluorescent images were generated at room temperature and the drugs were applied using a flow system with a flow rate of approximately 1 ml/min. Administrations of test substances were carried out using the following scheme: initially, the cells were incubated with Tyrode buffer for 20 min (control conditions), followed by a 60 min administration (preincubation) of either As$_2$O$_3$ or CDDP (1 $\mu$M each). The experiment was then continued with a 60 min co-application of As$_2$O$_3$ and CDDP (1 $\mu$M each).

To allow offline analysis of selected regions of interest (ROI), full screen images were generated at a resolution of 1024 x 1024 points. [Ca$^{2+}$], was measured using the “ion concentration” option of the META software (Zeiss). Images were background subtracted and [Ca$^{2+}$], was calculated using the following equation: 

$$[\text{Ca}^{2+}] = K_d \times \frac{(F - F_{\text{background}})}{(F_{\text{max}} - F)}$$

where “F” is the variable of intensity of the used dye (Florea et al., 2007; Florea and Büsselferg, 2008). The results are illustrated in “rainbow scale”, which shows [Ca$^{2+}$], concentration from low (blue) to high (red).

After all cells had been analyzed offline, only cells under control conditions showing a stable base line of [Ca$^{2+}$], (with changes below 10%) were selected for further analysis. In addition, only cells that showed an increase of [Ca$^{2+}$], ≥ 10% after each drug application were used for further analysis. This was done in order to clearly distinguish the effects of CDDP and/or As$_2$O$_3$ from any random fluctuations of the [Ca$^{2+}$]. Furthermore, cells where [Ca$^{2+}$], increased over 900 nM were also excluded from further analysis to eliminate errors that could result from dye saturation.

The selected cells were then subdivided into 3 categories with regard to their [Ca$^{2+}$], under control conditions: (a) low [Ca$^{2+}$], <75 nM; (b) standard (normal) [Ca$^{2+}$], 75–125 nM; and (c) high [Ca$^{2+}$], above 125 nM. [Ca$^{2+}$], obtained under control conditions were set at 100% and the following results were calculated as percent increase of control.

Transient increases were defined as changes in [Ca$^{2+}$], larger than 10% which returned back to baseline. The number of transient [Ca$^{2+}$], increases were counted for each phase of the experiment. The results are expressed as counts per cell and minute for standardization. The “duration” of the [Ca$^{2+}$], was defined as the time from beginning to the end of the transient increase.

Two-tailed, paired Student’s t-test was used for analysing changes within one experiment (calcium rise) and ANOVA for the other experiments. $p < 0.05$ was considered statistically significant.

2.3. Trypan Blue cytotoxicity test

We have previously tested the sensibility of cytotoxicity test by using Trypan blue exclusion method as well as the MITT assay where we show that for our experimental set up the results are similar and therefore for this study we used the Trypan Blue cytotoxicity test (Florea et al., 2007). The cytotoxicity tests were carried out as previously published (Florea et al., 2007; Florea and Büsselferg, 2008). In brief, for the determination of cell viability, non-confluent (70–80% confluency) cell monolayers were exposed to As$_2$O$_3$, CDDP, and combinations of As$_2$O$_3$/CDDP in 6-well plates. Two time points were analyzed: 2 h exposure, similar to the duration of the intracellular calcium measurements, and 24 h. Each experiment included an untreated control, control treated with As$_2$O$_3$ (2 h/24 h), control treated with CDDP (2 h/24 h), cells preincubated with As$_2$O$_3$ (1 h) and followed by CDDP (1 h/24 h);
Following drug treatment, the culture medium was collected and centrifuged to collect the non-adherent cells. The remaining cells were trypsinized and combined with the cells harvested from the culture medium. The suspension was centrifuged (2 min, 1200 rpm) and the supernatant was discarded. The pellet was washed with PBS, centrifuged (2 min, 1200 rpm) and re-suspended again in complete culture media. Then, a small aliquot (20 μl) of the cell suspension was mixed with the same volume of 0.4% Trypan blue (Sigma, Taufkirchen, Germany) solution and the cells were counted after 3 min of staining, using a hemocytometer (Neubauer).

The number of bright (viable) cells and blue cells (non-viable) was evaluated using a light microscope at 20× magnification. 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. Control cells and treated cells were compared using the two-tailed Student’s t-test (Florea et al., 2007; Florea and Büsselferg, 2008).

2.4. Hoechst staining to assess apoptotic cells

Sterile cover slips were placed in each well of 6-well plates (Greiner), seeded with 100,000 cells in 1 ml culture media and incubated overnight. A similar treatment scheme as described above for the cytotoxicity test was applied. After the incubation period, the cells were washed twice in PBS, fixed 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, Karlsruhe, Germany) for 30 s and mounted on cover slips. Cells were analyzed using 63× magnification with an Axiovert fluorescent microscope (×2000 cells were counted per cover slip). The results are presented as number of apoptotic cells (pyknotic morphology and condensed chromatin)/500 cells. The two-tailed Student’s t-test was used to compare the difference of the apoptotic rate between controls and treated cells (Florea et al., 2007; Florea and Büsselferg, 2008).

3. Results

As previously demonstrated in tumour models, As2O3 as well as CDDP triggers a concentration-dependent increase of [Ca2+]i, that derives from two independent sources: calcium stores and the extracellular space. Thus, the aim of this study was to analyze whether a combination of these drugs would additively/synergistically increase the [Ca2+]i level and enhance toxicity in tumour cells. Neuroblastoma cells were chosen as the model for the study. Several independent experiments were performed in order to check the influence on intracellular calcium levels of As2O3 or CDDP as well as of the two drugs combined. In 12 independent experiments, 449 cells were analyzed for the case where As2O3 was used in preincubation that was followed by co-application of CDDP and in 8 independent experiments, 274 cells for CDDP preincubation that was followed by co-application with As2O3. Cells that moved out of the microscopic field or became detached during experimentation were excluded from further analysis since they do not provide relevant information about the physiological processes.

3.1. Effects of As2O3 and CDDP on sustained [Ca2+]i-increase

Our results confirm previous studies showing that both As2O3 and CDDP increases [Ca2+]i in a concentration-dependent manner to a steady state (sustained increase) level and/or trigger transient [Ca2+]i elevations. Fig. 1 illustrates the sustained elevation of [Ca2+]i over time for three selected cells. After a stable baseline of [Ca2+]i was recorded under control conditions either As2O3 or CDDP was applied followed by a simultaneous administration of both substances.

In the preincubation phase, either As2O3 or CDDP (each 1 μM) induced a sustained [Ca2+]i-increase within approximately 20 min for As2O3 and within 11 min for CDDP. The [Ca2+]i-increase reached a steady state level after 43 min for As2O3 (27.6 ± 13.6%, mean ± S.D.; Figs. 1C and 2B) and 50 min for CDDP (72.6 ± 47.7%; Figs. 1D and 2B). These [Ca2+]i elevations were significantly higher than controls for both substances. An additional application of 1 μM CDDP to the cells that were previously exposed to As2O3 (Fig. 1C) or of 1 μM As2O3 to cells that were preincubated with CDDP (Fig. 1D) elicited a secondary increase of [Ca2+]i, after 18 min and 22 min. A steady state [Ca2+]i was reached after 50 min with As2O3, and 46 min with CDDP of 74.8 ± 46.5% (p = 6.8 × 10−8) and 119.1 ± 60.4% (p = 4.1 × 10−14), above controls (Fig. 2B). Therefore, our results indicated that the co-application of As2O3 after pre-application of CDDP resulted in an additive increase of [Ca2+]i, while there is no clear additive effect observable by co-application of CDDP.

3.2. The elevation of [Ca2+]i depends on the [Ca2+]i before application of either of the two anti-cancer drugs

The effects of As2O3 and CDDP on neuroblastoma cells, when administered alone and in combinations, are influenced by the initial [Ca2+]i (Fig. 1A and B). Generally, the cells having an initially low [Ca2+]i are most sensitive to the application of the anti-cancer drugs when applied alone or in combination. However, here the variation is large (see Figs. 1A, 1B and 2). In cells that initially had [Ca2+]i, higher than 125 nM under control conditions, treatment resulted in a [Ca2+]i-increase which was less pronounced. In these cells, also the variance was smaller (Fig. 2).

As2O3 (1 μM) applied to cells having a control [Ca2+]i below 75 nM induced a [Ca2+]i-rise of 78.4 ± 69.2%, on average 76.5 nM (p = 0.004). Furthermore, in the cells with an initial [Ca2+]i, of 75–125 nM the [Ca2+]i-rise was 27.6 ± 13.6% or on average 133.2 nM (p = 9.4 × 10−10). In cells with [Ca2+]i levels higher than 125 nM under control conditions the rise was 29.5 ± 16.1% or on average 371.5 nM (p = 1.5 × 10−14). An additional application of 1 μM CDDP to the cells induced a further increase in [Ca2+]i, to 265.9 ± 276.9% or 157.3 nM (p = 1.6 × 10−15) in cells with a low initial [Ca2+]i, while in the cells with an initial medium level of calcium the rise was 74.8 ± 50.2% or 182.5 nM (p = 2.2 × 10−18) and 73.2 ± 37.1% or 497.4 nM (p = 2.0 × 10−28) in cells with the highest initial [Ca2+]i levels (summarized in Table 1 and Fig. 2A).

Similar effects were observed when CDDP (1 μM) was applied first to neuroblastoma cells. The initial increase of [Ca2+]i, after application of CDDP was: 112.8 ± 80.1% or 106.6 nM (p = 1.2 × 10−12) in cells with low initial [Ca2+]i, level: 72.6 ± 47.7% or 156.8 nM (p = 6.8 × 10−8) in cells with medium initial [Ca2+]i; and
Fig. 1. Time course of $[\text{Ca}^{2+}]_i$ during single application and co-application of 1 μM As$_2$O$_3$ (A and C) and/or 1 μM CDDP (B and D). Each experiment started with an initial control where the $[\text{Ca}^{2+}]_i$ level was monitored without drug addition in order to show a stable baseline which was followed by application of drugs as described above. The increase of $[\text{Ca}^{2+}]_i$ depends on the level of $[\text{Ca}^{2+}]_i$ at the beginning of the experiment (A and B) as shown in every cell with a low (blue), normal (green) and high (red) baseline concentration. There is a sustained increase of $[\text{Ca}^{2+}]_i$ after single- and co-application of anti-cancer drugs (C and D) demonstrated by cells with an initial normal calcium concentration. The images in the right upper corner illustrate in rainbow scale (blue indicates a low calcium concentration while red indicates a high concentration) the cultured cells and the regions of interest (ROI) where the measurements were taken. The original pictures in the lower part of the figure were taken at control (1 min), single application (40 min), co-application (100 min), and end of the experiment (140 min). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 2. Increase of $[\text{Ca}^{2+}]_i$ after application of As$_2$O$_3$ (white) or CDDP (black) and additional application of the other substance (grey) respectively represented as mean with standard deviation. The graph is divided by the $[\text{Ca}^{2+}]_i$-concentration before application (A). The increase of $[\text{Ca}^{2+}]_i$ induced by either of the substances highly depends on the initial $[\text{Ca}^{2+}]_i$-concentration (t-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). In (B) the results are depicted when the $[\text{Ca}^{2+}]_i$-concentration was between 75 and 125 nM before application. Furthermore, the first substance applied largely influences the $[\text{Ca}^{2+}]_i$-increase when the second substance is applied. For significance analysis the ANOVA test was applied in order to compare the different conditions (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).
41.6 ± 32.3% or 328.4 nM ($p = 1.2 \times 10^{-13}$) in cells with high initial $[Ca^{2+}]_i$. Additional application of As$_2$O$_3$ resulted in an increase of 298.1 ± 165.9% or 199.4 nM ($p = 1.5 \times 10^{-18}$) in cells with a low initial $[Ca^{2+}]_i$, 119.1 ± 61.9% or 199.1 nM ($p = 4.1 \times 10^{-14}$) in cells with a medium initial $[Ca^{2+}]_i$, and 105.1 ± 53.2% or 475.9 nM ($p = 8.7 \times 10^{-22}$) in the cells with a high initial $[Ca^{2+}]_i$ (summarized in Table 1 and Fig. 2).

### 3.3. Effects of As$_2$O$_3$ and CDDP on transient increases of $[Ca^{2+}]_i$

In addition to the effects of As$_2$O$_3$ and CDDP on the sustained increase of $[Ca^{2+}]_i$, we also analyzed the frequency and duration of transient $[Ca^{2+}]_i$-increases. Both anti-cancer drugs modulated the number of transient $[Ca^{2+}]_i$ elevations. While As$_2$O$_3$ administration followed by CDDP co-application (Fig. 3A) reduced the incidence of transient elevations compared to controls by 25.6 ± 25.8% ($p = 0.287$) and 30.6 ± 55.0% ($p = 0.281$) (Fig. 3C), CDDP followed by As$_2$O$_3$ co-application (Fig. 3B) enhanced the number of transients by 61.3 ± 55.4% ($p = 0.280$) and 58.4 ± 44.8% ($p = 0.284$; Fig. 3C).

In regard to the durations of the transient $[Ca^{2+}]_i$ elevations, As$_2$O$_3$ extended this duration by 30.7 ± 68.2%, which was not significantly different from controls (Fig. 3D) while the additional CDDP co-application (Fig. 3A) further enhanced the transient duration only by 3.4 ± 43.1%, which was clearly not significantly different from controls (Fig. 3D). CDDP increased the transient extension by 17.9 ± 23.0% (not significant; $p = 0.200$) while when followed by As$_2$O$_3$ co-application (Fig. 3B) the transient duration was enhanced by 25.6 ± 24.2% (not significant; $p = 0.071$; Fig. 3D). In summary, As$_2$O$_3$ followed by CDDP co-application (Fig. 3A) reduced the incidence of transient increases compared to controls by 25.6 ± 25.8% ($p = 0.287$) and 30.6 ± 55.0% ($p = 0.281$) (Fig. 3C), CDDP followed by As$_2$O$_3$ co-application (Fig. 3B) enhanced the number of transients by 61.3 ± 55.4% ($p = 0.280$) and 58.4 ± 44.8% ($p = 0.284$; Fig. 3C).

### Table 1

The elevation of $[Ca^{2+}]_i$ depends on the $[Ca^{2+}]_i$ before application of either of the two anti-cancer drugs.

<table>
<thead>
<tr>
<th>$[Ca^{2+}]_i$, level in the control (nM)</th>
<th>$[Ca^{2+}]_i$-rise after initial application of 1 μM As$_2$O$_3$</th>
<th>Average (nM)</th>
<th>t-test</th>
<th>$[Ca^{2+}]_i$-rise after second application of 1 μM CDDP</th>
<th>Average (nM)</th>
<th>t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;75</td>
<td>78.4 ± 69.2%</td>
<td>76.5</td>
<td>$p = 0.004$</td>
<td>265.9 ± 276.9%</td>
<td>157.3</td>
<td>$p = 1.6 \times 10^{-15}$</td>
</tr>
<tr>
<td>75–125</td>
<td>27.6 ± 13.6%</td>
<td>133.2</td>
<td>$p = 9.4 \times 10^{-10}$</td>
<td>74.8 ± 50.2%</td>
<td>182.5</td>
<td>$p = 2.2 \times 10^{-18}$</td>
</tr>
<tr>
<td>&gt;125</td>
<td>29.5 ± 16.1%</td>
<td>371.5</td>
<td>$p = 1.5 \times 10^{-14}$</td>
<td>73.2 ± 37.1%</td>
<td>497.4</td>
<td>$p = 2.0 \times 10^{-28}$</td>
</tr>
</tbody>
</table>

Fig. 3. CDDP as well as As$_2$O$_3$ also induce transient $[Ca^{2+}]_i$ elevations. Illustrated is the time course of $[Ca^{2+}]_i$ during application of As$_2$O$_3$ and additional application of CDDP (A) and inverse application order (B). In addition to the sustained increase there are numerous transient increases of $[Ca^{2+}]_i$. Mean modulation (presented as mean ± standard deviations) of transients-incidence (C) and duration (D) by application of substances show no significant differences except for the application of CDDP as a first application which was stronger as compared with the other conditions.
followed by CDDP application reduces the incidence of calcium transients of As$_2$O$_3$, while CDDP followed by As$_2$O$_3$ co-application, increased the transient frequency. Both anti-cancer drugs slightly elongate the duration of transient elevations but none of these observations was statistically significant.

3.4. Co-application of As$_2$O$_3$ and CDDP causes a synergistic increase in cytotoxicity of SY-5Y neuroblastoma cells

In cytotoxicity tests, CDDP had a more pronounced effect than As$_2$O$_3$. While the application of As$_2$O$_3$ (1 $\mu$M) for 2 h decreased the cell viability by 16.9 \pm 6.4%, the application of CDDP (1 $\mu$M) decreased the cell viability by 25.0 \pm 2.8%, indicating that CDDP is more cytotoxic in neuroblastoma cells than As$_2$O$_3$ (Fig. 4A). The sequential administration of As$_2$O$_3$ (1 h alone), followed by CDDP administration and additional 1 h incubation, resulted in a similar cytotoxicity as when CDDP was administered alone (25.4 \pm 7.2%).

The combination of a 1 h administration of CDDP followed by the addition of As$_2$O$_3$ and additional 1 h incubation resulted in an approximately threefold higher cytotoxicity (80.3 \pm 8.5%) compared to controls (Fig. 4A). After 24 h incubation with As$_2$O$_3$ the cytotoxicity was slightly reduced compared to the 2 h incubation (13.5 \pm 12.7%). Incubation for 24 h with CDDP caused a slight increase in cytotoxicity to 36.6 \pm 15.0% (Fig. 4B). The combined administration of As$_2$O$_3$ and CDDP for 24 h was similarly effective as CDDP administered alone for 24 h (31.9 \pm 0.0% over control). The combination of CDDP and the additional application of As$_2$O$_3$ generated the largest cytotoxicity at 24 h (59.8 \pm 1.6%), which is surprisingly lower than in the short term application of 1 h CDDP and 1 h As$_2$O$_3$ (Fig. 4B).

4. Discussion

4.1. [Ca$^{2+}$]$_i$-increase induced by As$_2$O$_3$ and CDDP is additive

Calcium homeostasis is an important mechanism for maintenance of all living cells. Thus, modulation of [Ca$^{2+}$]$_i$-signaling in tumour cells could represent a modality of activating/deactivating pathways that lead to cell death specifically in tumour cells. Therefore, we have investigated the effects of anti-cancer drug combinations in cultured neuroblastoma (SY-5Y) cells. We have chosen As$_2$O$_3$ and CDDP as test substances because they induce [Ca$^{2+}$]$_i$ elevations by two different mechanisms, namely As$_2$O$_3$ releases Ca$^{2+}$ from intracellular stores (Florea et al., 2007) while CDDP triggers a Ca$^{2+}$-influx from the extracellular space by an IP$_3$-dependent mechanism (Splettstoesser et al., 2007).

Fig. 4. Average increase (mean \pm standard deviations) of cytotoxicity expressed as cell viability (%) as described in Section 2 (A and B) and apoptotic counts expressed as number of apoptotic nuclei/500 cells (C and D) induced by single- (each 2 h) and pretreatment/co-application (1 h plus 1 h (A and C) or 24 h (B and D)) of As$_2$O$_3$ and/or CDDP (A and C). There is significant difference in induced cytotoxicity and apoptosis between a single application of As$_2$O$_3$ or CDDP, and co-application following a previous application of As$_2$O$_3$ or CDDP. For significance analysis the ANOVA test was applied in order to compare the different conditions (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).
In this study we demonstrate that the mechanisms of \([\text{Ca}^{2+}]_i\)-increase caused by \(\text{As}_2\text{O}_3\) and \(\text{CDDP}\) are indeed independent. Thus, co-application of both anti-cancer drugs in neuroblastoma cells have additive effects in the sustained increase of \([\text{Ca}^{2+}]_i\), while additive effects on transient \([\text{Ca}^{2+}]_i\) elevations are not very clear. In addition, a low baseline \([\text{Ca}^{2+}]_i\), level led to a larger increase of \([\text{Ca}^{2+}]_i\), when the anti-cancer drugs were applied (this is also true for their co-application), but a high variation in the \([\text{Ca}^{2+}]_i\)-rise induced in each cell was observed. In addition, the \([\text{Ca}^{2+}]_i\)-rise triggered by \(\text{As}_2\text{O}_3\) and \(\text{CDDP}\) in cells with a medium or high \([\text{Ca}^{2+}]_i\)-base level under control conditions was similar; however, a smaller variance was observed in the cells with a low \([\text{Ca}^{2+}]_i\), before the application.

The fact that cells have different initial \([\text{Ca}^{2+}]_i\), levels under control conditions could reflect different stages of the cell cycle, such as exit from quiescence in early G1 phase, G1/S transition or, control conditions could reflect different stages of the cell cycle, position in the cell cycle. Furthermore, some studies have reflected a different filling status of the stores; e.g. based on the position of the cell cycle. Furthermore, some studies have suggested that chemotherapy resistant cells have a modified \([\text{Ca}^{2+}]_i\)_homeostasis, although contradictory observations have also been reported. Liang and Huang (2000) carried out experiments using CDDP-sensitive as well as CDDP-resistant human lung adenocarcinoma cells. They observed a \([\text{Ca}^{2+}]_i\)-elevation after administration of CDDP. Their results show that CDDP-resistant cells have a smaller \([\text{Ca}^{2+}]_i\)-increase compared to CDDP-sensitive cells. Contrary to these results, Nygren et al. (1991) as well as Tsuruo et al. (1984) observed an elevated \([\text{Ca}^{2+}]_i\)-increase in resistant cells compared to the cells that were sensitive to CDDP. Clearly, further research is needed to elucidate what causes the tumour cell resistance to anti-cancer drugs.

4.2. Relevance of transient \([\text{Ca}^{2+}]_i\), elevations

Transient \([\text{Ca}^{2+}]_i\), elevations are generated by an interaction of the drugs with the IP_3- or ryanodine-receptors resulting in a depletion of intracellular calcium stores to induce or modulate intracellular processes; e.g. contribution to resting level, or activation of ion channels (Berridge, 1997). The ability of \(\text{As}_2\text{O}_3\) to deplete intracellular calcium stores and to increase the frequency and duration of transient \([\text{Ca}^{2+}]_i\), elevations could correlate with each other. While CDDP possibly interacts with an IP_3-receptor at the cell membrane (Dellis et al., 2006; Splietstoesser et al., 2007) the increase of the \([\text{Ca}^{2+}]_i\)-frequency could also be the result of a possible interaction of CDDP with mitochondrial IP_3-receptors. This could generate or modulate transient \([\text{Ca}^{2+}]_i\),-increases. Interestingly, the duration of \([\text{Ca}^{2+}]_i\), elevation was nearly unchanged after treatments with the two drugs which could be an indication that the re-uptake into the stores is not influenced by either of the two anti-cancer drugs used in this study.

These agonist-evoked \(\text{Ca}^{2+}\)-signals have a complex spatial-temporal arrangement and are able to spread through the cell as a repetitive wave (Bootman and Berridge, 1996). Moreover there are diverse transient \([\text{Ca}^{2+}]_i\)-signals such as puffs, spikes, sparks or spikes, which appear in different cell-types. These signals are characterized by different attributes like amplitude (50–600 nM), spatial spread (1–10 \(\mu\)M) and duration (~1 s) (Bootman et al., 2001). Taking all these details into consideration, especially since such transient elevations have a limited spatial spread and a short duration, our observations are based on a limited time and spatial resolution. Specific concerns are: (1) scans were taken every 60 s, while some signals have a duration of ~1 s; (2) the 2D-imaging are used for processes that in fact occur within a 3D volume with a minimal spatial spread; (3) the size of ROI makes it difficult to analyze minimal spatial processes (for details see: Florea et al., 2007). For that reason, in this study, we are only able to monitor relatively prolonged changes of \([\text{Ca}^{2+}]_i\) previously it was shown that transient intracellular calcium signals are involved in several cellular processes, including the induction of apoptosis (Szalai et al., 1999). In our study we did not find any evidence in support of this hypothesis because the transient signals triggered by the anti-cancer drugs did not significantly correlate with an increased induction of cytotoxicity, while the sustained increase of \([\text{Ca}^{2+}]_i\) triggered by \(\text{As}_2\text{O}_3\) and/or CDDP did correlate with the induction of apoptosis.

4.3. Additive effects of co- and pre-application on \([\text{Ca}^{2+}]_i\) and cytotoxicity

\(\text{As}_2\text{O}_3\) and \(\text{CDDP}\) induced a sustained increase of \([\text{Ca}^{2+}]_i\), while \(\text{As}_2\text{O}_3\) was less effective than \(\text{CDDP}\) at a concentration of 1 \(\mu\)M. Interestingly, pre-application of \(\text{As}_2\text{O}_3\) followed by a co-application of \(\text{CDDP}\) increased the \([\text{Ca}^{2+}]_i\), similarly to the increase seen when \(\text{CDDP}\) was given as a single application. Hence, pre-application of \(\text{CDDP}\) followed by a co-application of \(\text{As}_2\text{O}_3\) induces a \([\text{Ca}^{2+}]_i\)-increase that was higher than the sum of both single drug applications. Therefore, we conclude that co-application of \(\text{As}_2\text{O}_3\) after pre-application with \(\text{CDDP}\) induces a “synergistic” sustained increase of \([\text{Ca}^{2+}]_i\). A possible explanation for this effect involves the different mechanisms of \([\text{Ca}^{2+}]_i\), elevation induced by these two drugs. While \(\text{CDDP}\) induces an influx of extracellular calcium ions (Splietstoesser et al., 2007) that could lead to secondary filling of the calcium stores, a further application of \(\text{As}_2\text{O}_3\) induces a depletion of intracellular calcium stores (Florea et al., 2007) and consequently elevates \([\text{Ca}^{2+}]_i\), to a higher level than when given without the pre-application of \(\text{CDDP}\). The observation that co-application of \(\text{CDDP}\) with \(\text{As}_2\text{O}_3\) results in an increase similar to the single application by \(\text{CDDP}\) converges with this assumption that the effect induced of \(\text{CDDP}\) seems to be more dominant than \(\text{As}_2\text{O}_3\)-induced modulation of \([\text{Ca}^{2+}]_i\).

4.4. The \(\text{As}_2\text{O}_3\) and/or \(\text{CDDP}\)-induced sustained elevation of \([\text{Ca}^{2+}]_i\), correlates with increased cytotoxicity in neuroblastoma cells

An excessive sustained increase of \([\text{Ca}^{2+}]_i\) (Trump and Berezovsky, 1995; McConkey and Orrenius, 1996) as well as the occurrence of transient \([\text{Ca}^{2+}]_i\), elevations (Chang et al., 2000; Mills et al., 1995) can result in cell death. This work demonstrates that there is a strong correlation between the effects of \(\text{As}_2\text{O}_3\) and \(\text{CDDP}\) on neuroblastoma cells regarding the sustained increase of \([\text{Ca}^{2+}]_i\) and the induction of cytotoxicity. However, in this study the effects of \(\text{As}_2\text{O}_3\) and \(\text{CDDP}\) on transient \([\text{Ca}^{2+}]_i\),-increases seems to be independent in regard to the cytotoxicity. While there are limitations in the observation of transient \([\text{Ca}^{2+}]_i\), elevations (for details see above and Florea et al., 2007), our results do not support that they have a major influence on modulation of cell death induced by \(\text{As}_2\text{O}_3\) and \(\text{CDDP}\).

The results presented here indicate not only that the effects of \(\text{As}_2\text{O}_3\) and \(\text{CDDP}\) on the sustained \([\text{Ca}^{2+}]_i\)-increase are additive but also that the cytotoxicity is synergistically elevated by co-application of the two drugs, especially when a preincubation of \(\text{CDDP}\) is followed by an application of \(\text{As}_2\text{O}_3\). This observation
could be important in regard to the use of these two substances in anti-cancer therapy. Thus, the stronger effects observed after 2 h incubation compared to a treatment of 24 h could be the reason of an acute effect that could result in recovery upon a longer time of incubation. In addition the longer time of incubation with drugs could stimulate the expression of drug resistance proteins that will increase resistance of cells to drugs (see Florea et al., 2007; Florea and Büsselberg, 2008).

In conclusion, at a concentration of 1 µM, CDDP was not only more effective in increasing the [Ca2+]i, but also in triggering cell death in neuroblastoma cells compared to As2O3. However, the most intriguing observation of our study is that the order of pre-application of the drugs is not only most important for the increase of [Ca2+]i, but also for the induction of cytotoxicity.

In regard to the mechanism inducing cell toxicity previous studies have shown that in neuroblastoma cells As2O3 induces apoptosis. This was identified by morphological changes and nucleosomal DNA fragmentation at a low concentration of 1 µM via activation of caspase 3 and caspase 8 (Akao et al., 2000). As2O3 also stimulated a concentration-dependent release of cytochrome c via induction of mitochondrial permeability transition and subsequent swelling of mitochondria. Mitochondrial GSH does not seem to be a target for As2O3. In mouse embryonic fibroblasts, 10 µM As2O3 stimulated cytochrome c release and apoptosis via a Bax/Bak-dependent mechanism (Bustamante et al., 2000). Similarly, CDDP triggered chromatin fragmentation characteristic for apoptosis, strong cytoplasmatic accumulation of cytochrome c an activation of caspase-9 (Schloffer et al., 2003). Caspases increase [Ca2+]i concentration-dependent which completely depended on extra-cellular Ca2+ and was reduced by the IP3 receptor blocker, 2-APB. This effect was not due to a Ca2+ release triggered by Ca2+-entry. The increase of [Ca2+]i was related to the activation of calpain but not caspase-8 and triggered apoptosis (Splettstoesser et al., 2007).

4.5. Clinical relevance

As2O3 is used in therapy of acute promyelocytic leukaemia. Nevertheless, there is evidence that As2O3 could be a potent option in therapy of other malignancies (Wang, 2001). Studies have shown effects on myeloid leukemic cells (Chen et al., 1996), megakaryocytic leukemic cell lines (Lu et al., 1999), esophageal carcinoma cells (Shen et al., 1999), gastric carcinoma cell lines (Zhang et al., 1999), neuroblastoma cell lines (Akao et al., 1999; Florea et al., 2007), prostate carcinoma cell lines (Uslu et al., 2000), ovarian cancer cell lines (Kong et al., 2005), and breast cancer cell lines (Ling et al., 2002), while its use in other malignancies is limited by cytotoxicity to non-malignant cells (Diaz et al., 2005). While CDDP is a frequently used anti-cancer drug being co-applied with other anti-cancer drugs in chemotherapy (Gebbia et al., 1993; Findlay et al., 1994; Du and Ho, 2001) there is a potential overlap in the use of As2O3 and CDDP in order to increase the effectiveness of treatment of promyelocytic leukaemia (Previati et al., 2006) or to increase the sensitivity of CDDP-resistant cells to apoptosis (Du and Ho, 2001; Kong et al., 2005). Our study strongly supports that a combination-therapy with different drugs could be more effective. Furthermore, the order of application might be an important factor when using different drugs for therapy since this study indicates that cytotoxicity and apoptosis also depend on the order of application of anti-cancer drugs. This should be considered when different anti-cancer drugs are co-applied. Our study could led to a better understanding of the mechanisms involved.

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Conflict of interest

The authors state no conflict of interest.

References

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