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Co-application of arsenic trioxide (As_2O_3) and cisplatin (CDDP) on human SY-5Y neuroblastoma cells has differential effects on the intracellular calcium concentration ($[Ca^{2+}]_i$) and cytotoxicity

Dennis Alexander Günes [a,1], Ana-Maria Florea [a,1], Frank Splettstoesser [a], Dietrich Büsselberg [b,*]

[a] Institut für Physiologie, Universitätsklinikum Essen, Universität Duisburg Essen, Hufelandstrasse 55, 45122 Essen, Germany
[b] Texas Tech University - Health Sciences Center, Paul L. Foster School of Medicine, 5001 El Paso Drive, El Paso, TX 79905, USA

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ABSTRACT

Arsenic trioxide (As_2O_3) and cisplatin (CDDP) are clinically relevant chemotherapeutics which modulate the intracellular calcium concentration ($[Ca^{2+}]_i$) by different mechanisms: As_2O_3 depletes intracellular calcium stores while CDDP triggers an influx of Ca^{2+}. We investigate whether co-application of As_2O_3 and CDDP has an effect on $[Ca^{2+}]_i$ homeostasis, resulting in an increase of cytotoxicity/apoptosis in human SY-5Y neuroblastoma cells. Confocal imaging with Ca^{2+}-sensitive dye (fluo-4) was used for investigating $[Ca^{2+}]_i$ dynamics. The induction of cell death was assayed using Trypan blue exclusion and Hoechst 33347 staining.

Application of As_2O_3 (1 μM) or CDDP (1 μM) increased $[Ca^{2+}]_i$. The largest elevation was observed when the basic $[Ca^{2+}]_i$ concentration was low. Both, transient and sustained $[Ca^{2+}]_i$-increases were observed in response to a single application of As_2O_3 or CDDP. Sustained increase showed clear additive effects when both drugs were co-applied. The magnitude of the $[Ca^{2+}]_i$-increase depends on the order of application; the most pronounced effect occurred when the cells were preincubated with CDDP followed by a co-application with As_2O_3. The sustained $[Ca^{2+}]_i$ elevations resulted in increased cytotoxicity and apoptosis. Therefore, co-treatment with CDDP and As_2O_3 may be a more effective anti-cancer therapy then either agent alone.

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,

* Corresponding author. Tel.: +1 915 78301700x254.
E-mail address: Dietrich.Busselberg@ttuhsc.edu (D. Büsselberg).
[1] These authors share equal contribution to this work.

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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_2O_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 Büsselberg, 2005, 2006). We have also shown that an increase of $[Ca^{2+}]_i$ 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 Büsselberg, 2008) however higher concentrations of these drugs could induce cell death by necrosis (Florea, 2005; Florea and Büsselberg, 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 ($[Ca^{2+}]_i$) overload as well as a disturbance in calcium homeostasis could cause cytotoxicity and trigger either apoptotic or necrotic cell death (Orrenius et al., 2003). Interestingly, some metal-based anti-cancer drugs (e.g. As_2O_3 and CDDP) have the potential to modulate $[Ca^{2+}]_i$ (Florea and Büsselberg, 2005, 2006). Their mechanisms of action are different in that the application of As_2O_3 triggers a depletion of Ca^{2+} from intracellular stores (Florea et al., 2007; Florea and Büsselberg, 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 Büsselberg, 2007). It has been shown that independent of the calcium source, the rise of $[Ca^{2+}]_i$ is directly related to apoptosis (Splettstoesser et al., 2007; Florea et al., 2007; Florea and Büsselberg, 2008). Therefore, we hypothesize that a co-application of CDDP and arsenic will result in an additive increase of $[Ca^{2+}]_i$ 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_2O_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 Fluo4/AM (fluo-4) from Molecular Probes (Molecular Probes, Karlsruhe, Germany). Arsenic trioxide (As_2O_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 μM.

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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ were observed using a confocal laser scanning microscope (Zeiss 510) as previously described (Splettstoesser et al., 2007; Florea et al., 2007; Florea and Büsselberg, 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_2O_3 or CDDP (1 μM each). The experiment was then continued with a 60 min *co-application* of As_2O_3 and CDDP (1 μM each).

To allow offline analysis of selected regions of interest (ROI), full screen images were generated at a resolution of 1024 × 1024 points. $[Ca^{2+}]_i$ was measured using the "ion concentration" option of the META software (Zeiss). Images were background subtracted and $[Ca^{2+}]_i$ was calculated using the following equation: $[Ca^{2+}]_i = Kd \times [(F - F_{min})/(F_{max} - F)]$, where "*F*" is the variable of intensity of the used dye (Florea et al., 2007; Florea and Büsselberg, 2008). The results are illustrated in "rainbow scale", which shows $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ (with changes below 10%) were selected for further analysis. In addition, only cells that showed an increase of $[Ca^{2+}]_i \geq 10\%$ after each drug application were used for further analysis. This was done in order to clearly distinguish the effects of CDDP and/or As_2O_3 from any random fluctuations of the $[Ca^{2+}]_i$. Furthermore, cells where $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ under control conditions: (a) *low* $[Ca^{2+}]_i$ <75 nM; (b) *standard* (normal) $[Ca^{2+}]_i$ 75–125 nM; and (c) *high* $[Ca^{2+}]_i$ above 125 nM. $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ larger than 10% which returned back to baseline. The number of transient $[Ca^{2+}]_i$-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 $[Ca^{2+}]_i$ 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 MTT 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 Büsselberg, 2008). In brief, for the determination of cell viability, non-confluent (70–80% confluency) cell monolayers were exposed to As_2O_3, CDDP, and combinations of As_2O_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_2O_3 (2 h/24 h), control treated with CDDP (2 h/24 h), cells preincubated with As_2O_3 (1 h) and followed by CDDP (1 h/24 h);