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Cisplatin resistance induced in germ cell tumour cells is due to reduced susceptibility towards cell death but not to altered DNA damage induction or repair

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ABSTRACT

To identify factors involved in cisplatin (CDDP) resistance of germ cell tumours (GCTs), we exposed NTERA-2 cells, and the platinum-adapted subline NTERA-2R to CDDP and compared their response. While both cell lines showed comparable proliferation, NTERA-2R cells were clearly more resistant to the drug than the parental NTERA-2 cell line. Interestingly, the two lines showed identical extent of DNA adduct formation and elimination, indicating that neither changes in CDDP uptake, nor altered drug efflux, DNA binding, or repair caused the difference in resistance. Similarly, no difference occurred in the time-course of γH2AX formation, which was not linked to 53BP1 accumulation. In contrast, NTERA-2R cells showed a more pronounced dose-dependent S phase delay, a transient G_2/M-block, and subsequent release into immediate cell death. We thus conclude that the enhanced resistance against CDDP is linked to reduced susceptibility to cell death rather than to an altered DNA adduct formation or adduct removal.

1. Introduction

Germ cell tumours (GCTs, for a recent clinical review see [1]) represent the most frequent malignancy in males between 20 and 45 years of age. Yet, using cisplatin (CDDP) based combination chemotherapy, an exceptional cure rate of 80% can be achieved even in metastatic disease. This higher-than-average chemosensitivity is also observed *in vitro* [2–6]. However, primary or secondary chemoresistance is a potentially fatal event in a minority of patients. The latter can be mimicked *in vitro* by exposing GCT cell lines to sublethal concentrations of CDDP leading to a relative CDDP resistance [7]. These cell lines provide a useful model system for investigating potential mechanisms of CDDP resistance *in vitro* [8].

CDDP is one of the most frequently used chemotherapeutic agents, displaying activity against a wide variety of solid tumours. Typically, testicular cancer regimens use 20 mg/m^2 days 1–5, every 3 weeks. This results in maximum total platinum plasma levels of 0.6–14 μM, with a median of 3.3 μM [9]. CDDP forms DNA intra- and interstrand crosslinks, DNA–protein crosslinks, and monoadducts with DNA [10]. Of these, predominantly the 1,2-d(GpG) intrastrand cross-links are considered responsible for the cytotoxic effect [11,12] as they distort the DNA structure [13] and thereby inhibit replication and transcription. There is long-standing evidence that CDDP adducts are partly processed to DNA single- and double-strand breaks (DSBs) during replication and repair [14,15] that contribute to the toxicity of the drug [16–18].

Several factors have been postulated as potential reasons for the exceptional CDDP sensitivity of GCT cells:

- They show higher adduct levels after exposure to CDDP, compared with more CDDP resistant cell lines, e.g. colon cancer cells [19].
- There is an aberrant DNA damage response following treatment with CDDP, including damage recognition and cell cycle checkpoint control [20]. This may be due to the concealment of DNA adducts by testis-specific high mobility group (HMG)-box

Abbreviations: CDDP, cisplatin; DSB, double strand break; GCT, germ cell tumour.

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proteins preventing damage detection [4,21], or p53 hypersensitivity [6], and/or other factors. In GCTs, it has been described that while the G_2/M checkpoint is functional, the G_1/S checkpoint is deregulated, leading to premature entry into S phase, a temporal S phase delay, and apoptosis upon exposure to CDDP [11,15,22–24].
- They have a defective DNA repair system, as described for some GCTs [2,7,25,26], especially for CDDP interstrand crosslinks [27].
- They are characterised by a high susceptibility to programmed cell death and a unique sensitivity to p53 activation [4,6,28].

On the other hand, a variety of mechanisms leading to CDDP resistance have been described in GCT cells:

- Reduced CDDP uptake and augmented detoxification mechanisms that attenuate adduct formation [29–31].
- Differentially regulated damage response pathways which may interfere with cell cycle arrest and DNA repair or enhance tolerance to CDDP-induced lesions [3,13,25,32–35].
- Deregulation of specific DNA repair pathways [36–41].
- Increased translesion synthesis that enables DNA synthesis in case of obstacles like DNA adducts [42].
- Since most studies failed to pin-point a single relevant parameter, the current hypothesis is that CDDP resistance is multifactorial [21,43].

The mechanisms linking the well described formation of CDDP DNA adducts to the sensitivity of GCT cells are not fully understood. Thus, it remains to be elucidated to which extent testicular GCT sensitivity is determined by initial DNA adduct levels and downstream mechanisms such as DNA repair or DNA damage signalling towards cell cycle arrest and cell death. Therefore, we aimed at identifying mechanisms leading to CDDP resistance in an *in vitro* model by investigating the response to the drug elicited by the GCT cell line NTERA-2 and its previously described CDDP-resistant counterpart NTERA-2R [44,45]. We assessed cytotoxicity, clonogenicity, cell cycle distribution, DNA adduct formation, and γH2AX accumulation following CDDP treatment.

2. Materials and methods

If not stated otherwise, chemicals were obtained from Merck (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), or Sigma–Aldrich (Munich, Germany). Cell culture reagents were from Invitrogen (Karlsruhe, Germany), while CDDP was a kind gift from medac GmbH (Wedel, Germany).

2.1. Cell culture

The embryonal carcinoma cell line NTERA-2 was obtained from DSMZ (Braunschweig, Germany). The CDDP resistant subline NTERA-2R was raised over a time period of 18 months by continuous exposure to increasing concentrations of CDDP, starting with the IC_{10} dose of the parental cell line. When 50% lethality was achieved, the addition of CDDP was paused and cells were allowed to recover over three passages. Genetic identity of our cells with the original NTERA-2 line was confirmed using arrayCGH (data not shown). NTERA-2 and NTERA-2R cells were cultured in DMEM + GlutamaxI® containing 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator at 37 °C in 5% CO_2. Cells were split three times a week and were used for assays in passages 3–7, exclusively. Between assays, NTERA-2R cells were cultured with 0.66 μM CDDP for 48 h to conserve resistance followed by maintenance in drug-free medium for 48 h to 96 h for recovery and re-frozen at passage 9 at the latest. The determination of the population doubling time of NTERA-2 and NTERA-2R cells (Vi-Cell TM XR cell viability analyser; Beckmann Coulter, Fullerton, CA, USA) showed equivalent growth behaviour of both cell lines within the first 48 h. Beyond this time point, NTERA-2R cells showed slightly slower growth than NTERA-2 cells. Thus, the doubling times of NTERA-2 and NTERA-2R cells were determined with 22.2 h and 23 h, respectively (data not shown).

2.2. Cytotoxicity assay (MTT)

To determine the IC_{50} of CDDP, cells were treated with different concentrations of the drug and analysed using the MTT cytotoxicity assay. This assay allows determination of the number of viable and metabolically active cells and therefore cell growth under drug treatment. Per well, 6×10^3 cells were seeded in sextuplicates in 96-well-plates, allowing exponential growth. Cells were allowed to adhere overnight prior to exposure to CDDP concentrations from 0.2 to 25.6 μM for 48 h. The medium was then replaced by MTT solution (final concentration: 0.5 mg/ml MTT; Sigma, Germany). After 2 h, MTT solution was replaced by 0.1 ml of DMSO. Solution of the formazan was enhanced by agitation of the plate for 15 min before the optical density was measured at 570 nm (Victor2 Wallac 1420 photometer, Perkin-Elmer life sciences, Wellesley, MA).

2.3. Colony formation assay

Clonogenicity and therefore stem cell survival was assessed by the colony formation assay as described by Zhu et al. [46]. Briefly, one hundred treated or control cells were seeded in 6-well plates and maintained in drug-free medium for 14 days. Once a week, medium was changed. For analysis, cells were fixed with methanol and stained according to Giemsa. All colonies visible to the naked eye (usually containing several hundred cells) were counted. Colony formation of treated cells is expressed as percentage of the colony number arising from untreated control cells. The absolute colony number of untreated NTERA-2 cells was approximately 20% lower than in NTERA-2R cells. The results are given as means and standard deviations of at least three independent experiments with triplicate to quadruplicate samples for every treatment condition.

2.4. Cell cycle analysis and Annexin V/PI staining

For the analysis of the cell cycle, DNA content was measured in at least 5.000 cells by flow cytometry (FACScan, BD-Biosciences, Franklin Lakes, NJ, USA) in treated and control cells stained with propidium iodide as described earlier [47]. The results are given as means and standard deviations (SDs) of at least three independent experiments for every treatment condition.

To discriminate between apoptotic and necrotic cells, analysis of phosphatidyl serine presentation and membrane integrity was performed by flow cytometric analysis (BD-Biosciences, Franklin Lakes, NJ, USA) of 30,000 treated or control cells per sample, using simultaneous staining with Annexin V-FITC and propidium iodide as described earlier [47]. Cellular debris was excluded from the analysis by gating based on the FSC/SSC plot and quadrants were set as exemplified in the insert in Fig. 5. Cells in the lower left quadrant (negative for both Annexin V and propidium iodide) were counted as viable, cells in the lower right quadrant (positive for Annexin V, indicating phosphatidyl serine externalisation, and negative for propidium iodide, indicating an intact cell membrane) as apoptotic, and cells in the upper quadrants (positive for propidium iodide, indicating a disrupted membrane) as necrotic.

2.5. Pt-adduct formation

Plated cells were grown in 6-well plates, treated with 2.5 μM CDDP for 24 h and subsequently maintained in drug-free medium for up to 24 h. At defined time points, cells were trypsinised, washed in PBS, resuspended in starch-solution (25% HAES-steril Fresenius Kabi, Bad Homburg, Germany, in PBS) and placed on precoated microscopic slides (Squarix ImmunoSelect® Adhesion Slides, Squarix, Marl, Germany). Pt-1,2d(GpG) intrastrand cross-links in the nuclear DNA of single cells were visualised and measured by an immunocytological assay (ICA) using the Pt-1,2d(GpG) adduct-specific antibody 'R-C18', as described previously [48]. Briefly, cells were fixed in methanol followed by (partial) alkaline denaturation and sequential proteolytic digestion with pepsin and proteinase K (both Roche, Grenzach, Germany). After blocking, Pt-(GpG)-adducts were visualised by indirect immunostaining with primary antibody R-C18 (generated at the Institute of Cell Biology, Essen, Germany) and Cy3-rabbit-anti-rat secondary antibody (Dianova, Hamburg, Germany). Nuclear DNA was counterstained with DAPI (200 μg/ml). Cy3 and DAPI fluorescence within single cells was measured by digital image analysis (ACAS 6.0 Cytometry Analysis System, ACAS II, Ahrens Electronics, Bargteheide, Germany) for at least 70 nuclei per sample. As the DNA content did not vary systematically in the samples, Pt adduct fluorescence was not corrected for DNA content. Mean values and standard deviations of at least 3 independent experiments were calculated and the values of untreated cells (background) were subtracted from those of all samples.

2.6. Accumulation of γH2AX

Cells treated with 2.5 μM CDDP for different time periods and subsequently maintained in drug-free medium for up to 24 h were centrifuged onto slides, fixed and double-stained with DAPI and anti-phospho(Ser 139)-Histone H2A.X antibody, (clone JBW301, Millipore, Schwalbach, Germany) as described previously [49]. For the concomitant analysis of 53BP1 co-localisation with γH2AX, cells were addition-