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Brief communication

Cisplatin-induced cell death in *Saccharomyces cerevisiae* is programmed and rescued by proteasome inhibition

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ABSTRACT

Cisplatin is a highly effective chemotherapeutic drug used in the treatment of several tumors. It is a DNA-damaging agent that induces apoptosis of rapidly proliferating cells, an important factor underlying its therapeutic efficacy. Unfortunately, cellular resistance occurs often. A large fraction of tumor cells harbor mutations in p53, contributing to defects in apoptotic pathways and drug resistance. However, cisplatin-induced apoptosis can also occur in p53 deficient cells; thus, elucidation of the molecular mechanism involved will potentially yield new strategies to eliminate tumors that have defects in the p53 pathway. Most of the studies in this field have been conducted in cultured mammalian cells, not amenable to systematic genetic manipulation. Therefore, we aimed to establish a simplified model devoid of a p53 ortholog to study cisplatin-induced programmed cell death (PCD), using the yeast *Saccharomyces cerevisiae*.

Our results indicate cisplatin induces an active form of cell death in yeast, as this process was partially dependent on de novo protein synthesis and did not lead to loss of membrane integrity. Cisplatin also increased DNA condensation and fragmentation/degradation, but no significant mitochondrial dysfunction other than partial fragmentation. Co-incubation with the proteasome inhibitor MG132 increased resistance to cisplatin and, accordingly, yeast strains deficient in proteasome activity were more resistant to cisplatin than wild-type strains. Proteasome inhibitors can sensitize tumor cells to cisplatin, but protect others from cisplatin-induced cell death. Our results indicate inhibition of the proteasome protects budding yeast from cisplatin-induced cell death and validate yeast as a model to study the role of the proteasome in cisplatin-induced PCD. Elucidation of this mechanism will aid in the development of new strategies to increase the efficacy of chemotherapy.

1. Introduction

Cisplatin-based chemotherapy is the standard treatment for many cancers. In particular, testicular germ cells tumors are highly sensitive to cisplatin, with cure rates as high as 80% [1]. Nonetheless, prognosis is bleak for patients initially unresponsive to chemotherapy, or that later relapse [2]. Understanding the molecular basis for chemosensitivity and resistance is therefore crucial to improve treatment efficacy. Studies using cultured cell lines indicate cisplatin resistance is a multifactorial problem. Defects in mismatch repair, aneuploidy, increased cisplatin detoxification, and failure to undergo apoptosis are possible contributing causes [2,3]. However, no general mechanism of resistance has been uncovered that could account for the unresponsiveness of patients to chemotherapy. Functional inactivation of p53 may lead to impaired apoptosis and resistance to genotoxic agents commonly used in anti-cancer therapies [4]. However, endogenous p53 status or levels of Bcl-2 family proteins in testicular germ cell tumor cell lines did not seem to affect the ability of these cells to undergo cisplatin-induced apoptosis [5]. Several other reports argue against alterations in the p53-dependent mitochondrial apoptotic pathway as a common mechanism of resistance to cisplatin, as cisplatin-induced apoptosis involves both p53-dependent and independent pathways [reviewed in 2]. Elucidation of the molecular mechanisms involved in p53-independent programmed cell death (PCD) will potentially yield new strategies to eliminate tumors resistant to first-line chemotherapy.

In eukaryotic cells, the ubiquitin proteasome degradation pathway regulates many cellular processes, including cell cycle, transcription, and degradation of misfolded and/or damaged proteins, as well as apoptosis [6–8]. Proteasome inhibition using either

Abbreviations: PCD, programmed cell death; ROS, reactive oxygen species; PI, propidium iodide; DHE, dihydroethidium; MG132, Carbobenzoxy-Leu-Leu-leucinal; c.f.u., colony forming units; cDDP, cisplatin; CHX, cycloheximide; DMSO, Dimethyl sulfoxide; HNSCC, head and neck squamous cell carcinoma; RTEC, renal tubular epithelial cells; hCTR1, human copper transporter 1.

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Table 1
S. cerevisiae strains used in this work.

Strain	Genotype	Source
BF264-15D	Mat a *leu2 trp1 ade1 his3*	39
pre1-1pre4-1	BF264-15D Mat a *leu2, trp1, ade1, his3 pre1-1pre4-1*	39
W303a	Mat a *ade2 ura3 his3 trp1 leu2*	R. Rothstein
sug1-20	W303; Mat a *ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3,112 sug1-20*	40

ALLnL or lactacystin sensitized human ovarian tumor cell lines to cisplatin and other cross-linking DNA-damaging agents [9,10]. However, proteasome inhibition by MG132 can attenuate cisplatin nephrotoxicity in vivo [11]. This dual role is thought to be due to the proliferative state of the cell [12], the concentrations of inhibitory agents used, or even to other factors such as differential activation of kinases such as JNK and certain heat shock proteins [13]. Nevertheless, the mechanism underlying the involvement of proteasome in cisplatin-induced PCD in particular and apoptosis in general has not yet been uncovered.

Similarly to metazoans, yeast cells can exhibit several characteristics of apoptosis, including chromatin condensation, DNA breakage, flipping of phosphatidylserine to the outer membrane, accumulation of reactive oxygen species (ROS), and cytochrome *c* release from the mitochondria [14]. However, several regulators of this response in mammalian cells (such as the p53 family of transcription factors and most members of the Bcl-2 family of proteins) are absent from this system [15]. The yeast apoptotic-like program might therefore be a conserved core mechanism that is expanded in higher eukaryotic cells to include the p53 or Bcl-2 families.

2. -Materials and methods

2.1. Growth conditions and cisplatin treatments

Saccharomyces cerevisiae strains (listed in Table 1) were grown overnight in Synthetic Complete medium (SC) (1.37 g/L Drop-Out mix; 6.7 g/L Yeast Nitrogen Base, with 2% (w/v) glucose) at 30 °C, 200 rpm, collected at OD (A_{600}) 0.5∼0.8, and transferred to SC medium with or without cisplatin (200–400 μg/mL) (Sigma), at 30 °C for up to 300 min. Cisplatin was stored in aliquots in the dark and resuspended in medium immediately prior to use, to minimize exposure to air, though some variability regarding cisplatin cytotoxicity in independent experiments was inevitable. For specific assays, cells were then washed and resuspended in fresh SC medium to a final OD 0.5 and incubated for 24 h at 30 °C. 100 μg/mL of Cycloheximide (CHX) (Merck) or the equivalent volume of DMSO was used where indicated. Proteasome inhibition with MG132 (Sigma) was performed as described [16].

2.2. Cell viability assays

At specific time intervals, cells were collected by centrifugation, resuspended in water or medium, diluted and plated on YPDA plates (1% yeast extract, 2% (w/v) BactoPeptone, 2% (w/v) glucose 2% (w/v) agar) at least in triplicate, and incubated 2 days at 30 °C, prior to colony forming units (c.f.u.) counting. c.f.u. means and standard deviations were normalized to the OD (A_{600}) values at each time and viability calculated relative to time 0 (corresponding to 100% viability). Statistical analysis was performed with GraphPad Prism 5. For semi-quantitative assays, 4 μL of cell suspensions and of ten-fold serial dilutions (10^{-1} to 10^{-4}) were spotted onto YPDA plates, incubated 2 days at 30 °C, and photographed with ChemiDoc XRS (BioRad) using Quantity One software (BioRad).

2.3. Chromatin condensation

Cells were fixed in 50% ethanol in PBS (80 mM Na_2HPO_4, 20 mM NaH_2PO_4,100 mM NaCl), stained with 2 μg/mL 4,6-diamido-2-phenyl-indole (DAPI, Sigma) for 5 min, washed twice with PBS and visualized by fluorescence microscopy (Leica DM 5000B with a Leica DFC350 FX camera) and analyzed with LAS AF (Leica).

2.4. Flow cytometry

2.4.1. Plasma membrane integrity and ROS accumulation

Cells were diluted 1:10 in PBS and incubated with 5 μg/mL PI (Propidium Iodide, Sigma–Aldrich) to assess plasma membrane integrity or with 5 μg/mL of DHE (dihydroethidium, Molecular Probes) to detect ROS, for 10 or 30 min in the dark, respectively. Fluorescence was measured in an Epics® XL™ flow cytometer (Beckman Coulter). Cells with red fluorescence [FL-3 channel (488/620 nm)] were considered to display plasma membrane disruption or ROS accumulation, respectively.

2.4.2. Cell cycle analysis

Cell cycle analysis was performed as described [17], but using 1 μM of Sytox Green (Molecular Probes). Cells were analyzed in an Epics® XL™ flow cytometer (Beckman Coulter), with excitation and emission wavelengths of 488 nm and 520 nm, respectively (FL1 channel). Results were analyzed with FlowJo 7.6 software and Flowing Software.

3. Results

3.1. Cisplatin induces an active form of cell death in yeast

Loss of plasma membrane integrity is considered a marker of necrotic cell death. Therefore, we tested if cisplatin-induced cell death in *S. cerevisiae* is accompanied by loss of membrane integrity by staining cells with propidium iodide (PI) and evaluating the fluorescence by flow cytometry. Cells remained impermeable to PI even after 180 min of exposure to cisplatin, although there was a significant loss of cell viability under the same conditions (Fig. 1A and B). This indicates cisplatin-induced yeast cell death occurs without loss of plasma membrane integrity, supporting that it is a regulated process. We therefore determined the effect of the protein biosynthesis inhibitor cycloheximide (CHX) on the viability of cells treated with and without cisplatin (Fig. 2A and B). Indeed, cycloheximide increased the viability of yeast cells exposed to cisplatin, indicating cisplatin-induced death in *S. cerevisiae* requires de novo protein synthesis and is, therefore, an active form of cell death.

3.2. Cisplatin does not induce major mitochondrial alterations

ROS are crucial cell death regulators and have been connected to many of the known apoptotic pathways [14,18,19]. We therefore assessed ROS levels in cisplatin-treated cells by flow cytometry using DHE, which preferentially detects the accumulation of intracellular superoxide anion. We could not detect a significant increase in the percentage of cells stained with DHE after