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Effect of cisplatin on proteasome activity☆

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ABSTRACT

Cisplatin is a widely used chemotherapy drug which exerts cytotoxic activity by affecting both nuclear and cytosolic pathways. Herewith, we report, for the first time, that cisplatin inhibits proteasome activity *in vitro*. Cisplatin induces a dose dependent inhibition of the three enzymatic activities of proteasome (*i.e.*, the chymotrypsin-like activity, the trypsin-like activity and the caspase-like activity). Moreover, cisplatin administration to neuroblastoma cells brings about a fast loss of proteasome particle activity, which is followed by a *de novo* synthesis of proteasome. Lastly, we report that the simultaneous administration of lactacystin and cisplatin enhances the cytotoxicity of cisplatin alone. The overall bulk of data opens to an intriguing scenario, concerning the biological effects of cisplatin in the control of cellular life, which goes beyond the well established genotoxic effect.

1. Introduction

Cisplatin is often part of polychemotherapy regimens in the treatment of solid tumors (*i.e.*, testicular, ovarian, head and neck, colorectal, bladder and lung cancers); however, the acquired cisplatin resistance and the overall side effects occurring during therapy, in particular cardiotoxicity, nephrotoxicity and neurotoxicity, limit its clinical use and efficiency [1–3]. The antineoplastic effects of cisplatin have been historically ascribed to the generation of nuclear DNA lesions, even though only 1% of the intracellular cisplatin reacts with DNA and a lack of correlation between cisplatin sensitivity and DNA platination has been reported [4–6]. It is worthy of note that several evidences suggest that the cytostatic and cytotoxic effects of cisplatin involve both nuclear and cytosolic pathways; it may be then reasonable to envisage an interaction of the compound with cytosolic proteins whose injury could be critical for the induction of apoptosis [6–9]. In this respect, it has been shown that cisplatin could induce endoplasmic reticulum (ER) stress and proapoptotic signaling also in enucleated cells, revealing a novel mechanism of action, at least in specific cellular models [5,9–11]. The capacity of cisplatin to activate two pathways (*i.e.*, ER stress and DNA damage), which induce apoptosis, indeed supports the idea of a multifactorial nature for molecular mechanisms underscoring cisplatin resistance [5,9]. Therefore, a series of questions, concerning the molecular mechanisms whereby cisplatin exerts its antineoplastic activity and the origin of cisplatin resistance, remain to be solved.

The unfolded proteins, which accumulate as a consequence of the ER stress (unfolded protein response), are either correctly refolded or else a degradation of the unsuccessfully refolded ones takes place by the ubiquitin–proteasome system (UPS). The UPS is the main intracellular proteolytical system whose activity and regulation are critical to the fulfillment of homeostasis and to guarantee the balancing between pro-apoptotic and anti-apoptotic factors [12,13]. Proteins targeted for proteasome degradation are selectively tagged with a poly-ubiquitin chain of variable length which is then recognized by the 26 proteasome. The 26 proteasome is a cytosolic machine composed by one or two regulatory particles (RP), called 19S, which associate with a core particle (CP), named 20S, where the three proteolytic activities reside (*i.e.* the caspase-like, the tryptic-like and the chymotryptic-like activities) [14]. The proteasome activity is essential for tumor cell proliferation and it has been reported that inhibition of the proteasome chymotrypsin-like activity is associated with induction of apoptosis in tumor cells [15–17], envisaging proteasome inhibitors as a new class of potential anti-cancer drugs. In this context, the use of a proteasome inhibitor, bortezomib, has been approved for the treatment of mantle cell lymphoma and multiple melanoma [18–22]. Moreover, proteasome inhibitors boost the action on tumor cells by widely used cancer therapeutics, including ionizing radiation and cisplatin, at least in cell experimental

Abbreviations: UPS, ubiquitin–proteasome system; ER, endoplasmic reticulum; PMSF, phenylmethylsulfonyl fluoride; DTT, di-thio-threitol; EDTA, ethylene-diamino-tetra-acetic; CP, core particle of proteasome; RP, regulatory particle of proteasome.

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models [18,23–26]. Therefore, a combined treatment of anticancer agents (such as cisplatin) with proteasome inhibitors is an attractive and novel therapeutic approach for cancer therapy [27,28]. Despite the above discussed features, no information concerning the effect of cisplatin on proteasome pathway is reported.

Herewith, for the first time, we show that cisplatin affects the activity of proteasome *in vitro* and in cellular models, suggesting that the yet un-identified cytosolic target of cisplatin could be the UPS system itself.

2. Materials and methods

2.1. Synthesis of cisplatin

Cisplatin was prepared according to the Dhara method [29].

2.2. Kinetic analysis

The characterization of the 20S proteasome activities (Boston Biochem, Boston, Ma, USA), (*i.e.* chymotryptic-like, tryptic-like and caspase-like) as a function of cisplatin concentration was carried out through a fluorimetric approach. Cisplatin was dissolved before use in PBS 1×. The 20S proteasome was diluted to a final concentration of 1 nM in the assay buffer (25 mM Tris, pH 7.7) in the absence and in the presence of the indicated concentrations of cisplatin. The reaction mixtures were incubated 25 min at 37 °C; then, the fluorogenic substrate (50 μmol/L), specific for each of the three 20S proteasome activities was added (*i.e.*, Suc-LLVY-AMC, Boc-LLRAMC and Z-LLE-AMC for the chymotryptic, tryptic and caspase-like activities, respectively). The rate of hydrolysis of the fluorogenic substrate was monitored over 45 min and the relative velocities, referred to the ratio between velocities of the reaction in the absence and in the presence of cisplatin, were extrapolated. The fluorescence was detected in an Eclipse fluorimeter (Varian Co., Palo Alto, Ca, USA) with the excitation and emission wavelengths set at 380 nm and 460 nm, respectively.

2.3. Cell culture and MTT assay

SHSY5Y cells were seeded at a density ranging from 4×10^3 to 1×10^4 cells/well in a 96-tranwell cell plate and grown overnight in high-glucose DMEM supplemented with 10% FBS, L-glutamine (2 mM), penicillin (50 IU/mL), streptomycin (50 μg/mL), sodium pyruvate (1 mM) and ciprofloxacin (0.03 mM) at 37 °C and 5% CO_2. The following day, cisplatin and lactacystin (where indicated) were added to the medium. In details, the cells were divided into four groups as follows: (*i*) untreated cells; (*ii*) cisplatin-treated cells (at a final cisplatin concentration of 10 μM, 20 μM and 50 μM); (*iii*) lactacystin-treated cells (10 μM); (*iv*) cisplatin (at concentrations of 10 μM, 20 μM and 50 μM) and lactacystin-treated cells (10 μM). Stimulation was carried out overnight at 37 °C in a 5% CO_2 humidified atmosphere; the following day, cell viability was evaluated by MTT assay. Measurements for each experimental condition were performed in triplicate.

2.4. Western blotting analysis

SHSY5Y cells were cultivated as described above and were seeded at a density of 1.5×10^5 well in a 6-well Transwell plate and grown overnight. The following day, cells were stimulated with sub-lethal concentrations of cisplatin (*i.e.*, 0.5 μM and 1 μM) for 2 h and 24 h, respectively. After this time, cell monolayers were harvested and lysed by using a lysis buffer (50 mM Tris, 50 mM NaCl, 1% Triton X-100, pH 7.4) supplemented with phenylmethylsulfonyl fluoride (PMSF) and a protease-inhibitor mixture with broad specificity for the inhibition of serine, cysteine, aspartic, and aminopeptidase (Sigma-Aldrich, Buchs, Switzerland). After each treatment, Trypan blue exclusion viability tests demonstrated that almost 95% of cells were still alive. Total protein concentration of cell lysates was determined by Bradford assay [30] and 20 μg of whole cell lysates were separated on a 12% acrylamide gel under reducing and denaturing conditions. Thereafter, separated proteins were transferred to a HyBond-ECL nitrocellulose filters (Amersham Biosciences, Piscataway, NJ, USA) for 1 h at 4 °C and then unsatured binding sites were blocked by incubating filters in a 0.01% Tween-PBS 5% milk solution. Filters were probed overnight at 4 °C with: (*i*) anti-26S proteasome antibody and (*ii*) anti-GAPDH antibody (Covance, Princeton, NJ, USA), as internal control. The following day, membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (Abcam, UK), diluted 1:10,000 in a 0.2% Tween-PBS 1% milk solution and immunoreactive signals were detected with an ECL Advance Western Blotting Detection Kit (Amersham Biosciences, Piscatway, NJ, USA).

2.5. Proteasome assay

SHSY5Y cells were seeded at a density of 5×10^4 well in a 6-tranwell cell plate and were grown overnight; the following day, cells were stimulated with sub-lethal concentration of cisplatin (*i.e.*, 0.5 μM and 1 μM) for 2 h and 24 h respectively, as indicated above [32]. After stimulation, crude cell extracts were prepared by resuspending cells in a buffer containing 25 mM Tris, 250 mM sucrose, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM ATP, pH 7.8 followed by multiple passes through a 21G gauge. Protein concentration was normalized by the Bradford assay [30] and the proteasome assay was performed as previously described [31]. Briefly, 20 μg of crude cell extract were incubated in the 26S proteasome activity buffer (50 mM Tris, 20 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, pH 7.8,) with 100 μmol/L of chymotryptic-like activity specific substrate (*i.e.*, Suc-LLVY-AMC) (Boston Biochem, Boston, MA, USA). Each measurement was done in triplicate and the fluorimetric reports were collected every 30 min over the time interval for which linearity was observed.

2.6. Proteasome in gel-assay

The assay was performed by following the methodology described elsewhere [32]. Briefly, 150 μg of the cell lysates used for the proteasome assay described above were separated under native conditions in a 3.5% acrylamide gel. Gels were then harvested and soaked in a clean dish in a reaction buffer (50 mM Tris, 5 mM $MgCl_2$, 1 mM ATP, pH 7.5) containing 100 μM of a substrate specific for the chymotryptic-like activity of proteasome (*i.e.*, Suc-LLVY-AMC). The analysis allows to study the distribution and the activity of the three main proteasome sub-particles described in the intracellular compartment: the RP_2-CP (*i.e.*, the 30S proteasome), the RP-CP (*i.e.*, the 26S proteasome) and the CP (*i.e.*, the 20S proteasome), wherefore RP stands for Regulatory Particle (*i.e.*, the 19S proteasome) and the CP stands for the Core Particle where the proteolytic subunits are located.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine significant differences among groups. Tukey's honestly significant difference post hoc test was used for pairwise comparisons after the analysis of variance.

3. Results and discussion

3.1. Effect of cisplatin on proteasome activity in vitro

It is widely recognized that the ubiquitin proteasome system is involved in the control of each aspect of cell life. Therefore, the modulation of proteasome activity is critically linked to the cell fate [33,34]. Cisplatin is a widely used antineoplastic agent targeting nuclear DNA; however it has been recently proposed to further targets cytosolic

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