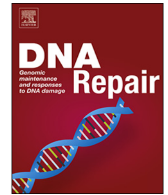
DNA Repair 73 (2019) 28–33

Contents lists available at ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair



Proteasome inhibition prevents cell death induced by the chemotherapeutic agent cisplatin downstream of DNA damage

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ARTICLE INFO

Keywords:
Cisplatin
Yeast
Proteasome
Regulated cell death
Cancer
Chemotherapy

ABSTRACT

Cisplatin is a highly effective chemotherapeutic drug acting as a DNA-damaging agent that induces apoptosis of rapidly proliferating cells. Unfortunately, cellular resistance still occurs. Mutations in p53 in a large fraction of tumor cells contribute to defects in apoptotic pathways and drug resistance. To uncover new strategies to eliminate tumors through a p53-independent pathway, we established a simplified model devoid of p53 to study cisplatin-induced regulated cell death, using the yeast *Saccharomyces cerevisiae*. We previously showed that cisplatin induces an active form of cell death accompanied by DNA condensation and fragmentation/degradation, but no significant mitochondrial dysfunction. We further demonstrated that proteasome inhibition, either with MG132 or genetically, increased resistance to cisplatin. In this study, we sought to determine how proteasome inhibition is important for cisplatin resistance by analyzing how it affects several phenotypes associated with the DNA damage response. We found MG132 does not seem to affect the activation of the DNA damage response or increase damage tolerance. Moreover, central modulators of the DNA damage response are not required for cisplatin resistance imparted by MG132. These results suggest the proteasome is involved in modulation of cisplatin toxicity downstream of DNA damage. Proteasome inhibitors can sensitize tumor cells to cisplatin, but protect others from cisplatin-induced cell death. Elucidation of this mechanism will therefore aid in the development of new strategies to increase the efficacy of chemotherapy.

1. Introduction

Cisplatin is one of the most widely used chemotherapeutic drugs, due to its effectiveness against several types of tumors such as testicular, ovarian, bladder, lung and head and neck cancers [1]. It is a platinum-based compound that binds to DNA, leading to the formation of intra- and interstrand crosslinks, which affects several cellular processes, including DNA synthesis, RNA transcription and cell cycle progression [2]. When DNA repair mechanisms are unable to repair cisplatin-induced DNA damage, cell death mechanisms are activated [3]. However, resistance to cisplatin still occurs, either intrinsic or acquired. In order to increase efficacy and reduce toxicity to normal cells, combination treatments with lower concentrations of different classes of compounds that preferentially target tumor cells are desirable. Of these, proteasome inhibitors appear as a promising strategy. Bortezomib was the first inhibitor of the 26S proteasome approved for cancer treatment, in patients with relapsed and refractory multiple myeloma, followed by carfilzomib and others under development [4]. Clinical trials have also addressed whether combining bortezomib with other chemotherapeutics would be more effective, though results varied and fell short of expectations [5]. However, there seemed to be little prior scientific evidence regarding the effectiveness of the proposed treatment regimens, since mechanistic aspects have been poorly characterized.

It has been shown that proteasome inhibitors can sensitize tumor cells such as breast, bladder, ovarian, and head and neck squamous cell carcinoma cells (HNSCC) to cisplatin, but protected cultured renal tubular epithelial cells (RTEC) from cisplatin-induced apoptosis [6–10] and attenuated cisplatin nephrotoxicity *in vivo* [11]. Proteasome inhibition also slightly sensitized breast cancer cells to 5-FU, multiple myeloma cells to curcumin and doxorubicin, among others [12–15], but protected neuroblastoma cells from taxol or vinblastine-induced apoptosis [16]. It was initially proposed that the effect of proteasome inhibitors is due to inhibition of NF-κB activation, leading to changes in the expression of pro- and anti-apoptotic genes. However, this hypothesis was later challenged, and therefore no single mechanism of action is described [17] and a systematic characterization is lacking. In

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https://doi.org/10.1016/j.dnarep.2018.10.006
Received 14 February 2018; Received in revised form 23 October 2018; Accepted 23 October 2018
Available online 25 October 2018
1568-7864/ © 2018 Published by Elsevier B.V.

many cases, it appears that a functional p53 is fundamental for cisplatin-induced cell death [18,19]. Yet, other studies found no correlation between p53 status and cell death mediated by this drug under different cellular contexts [20,21]. Since mutations in p53 are highly frequent in most human cancers [22], we previously used yeast cells as a model to elucidate p53-independent regulation of cell death induced by cisplatin. We showed that cisplatin induces a mitochondria-independent regulated cell death (RCD) in budding yeast and that proteasome inhibition, either genetically or pharmacologically, led to resistance to this drug [23]. Since the mechanisms proposed to explain the involvement of the proteasome in cisplatin-induced cell death in human cell lines are either not present in yeast or would only explain synergistic increased sensitivity, we concluded an unknown mechanism underlies the observed phenotype. In the present study, we therefore assessed the involvement of DNA damage and DNA damage repair in the increased resistance of yeast cells to cisplatin imparted by proteasome inhibition, in order to shed light into a conserved pathway modulating cisplatin sensitivity that can be exploited in novel therapies.

2. Materials and methods

2.1. Growth conditions and treatments

Saccharomyces cerevisiae strains (Table 1) were grown overnight in Synthetic complete medium (SC; 1.37 g/L Drop-Out mix, 1 g/L proline, with 2% (w/v) glucose) at 30 °C and 200 rpm, diluted to OD 0.3 in the same medium containing 0.003% SDS, and grown for an additional 3 h. Afterwards, cells were treated with 118 μM MG132 (Sigma) or the equivalent volume of DMSO for 30 min. Then, cisplatin (30–300 μg/mL, Sigma) resuspended in DMSO or 0.1% methyl methanesulfonate (MMS, Fluka) was added to the cell cultures. In specific cases, after 3 h of treatment, cells were washed, resuspended in fresh medium and incubated at 30 °C, 200 rpm for an additional period of 24 h. Treatment with 75 μg/mL of cycloheximide (CHX, Sigma) was used when indicated. Though cisplatin was stored in aliquots in the dark and resuspended immediately prior to use, to minimize exposure to air, some variability in cytotoxicity between independent experiments was unavoidable.

2.2. Viability assays

For semi-quantitative spot assays, cells were collected at specific time points, and 5 μL of the cell suspensions and serial dilutions (10^{-1} to 10^{-4}) were spotted on YPDA plates (1% yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose, 2% (w/v) agar), followed by incubation for 2 days at 30 °C. Plates were then photographed using an appropriate equipment.

Table 1
S. cerevisiae strains used in this work.

Strain	Genotype	Source
BF264-15D	*MAT*a, *leu2, trp1, ade1, his3*	S. Reed
RDKY3615	*MAT*a, *ura3-52, leu2Δ1, trpΔ63, his3-200, lys2-Bgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3*	R.Kolodner
mlh1Δ	RDKY3615 *mlh1::URA3*	R.Kolodner
sml1Δ	RDKY3615 *sml1::KANmx4*	R.Kolodner
sml1Δmec1Δ	RDKY3615 *sml1::KANmx4, mec1::HIS3*	R.Kolodner
RDKY3023	*MAT*a, *ura3-52, leu2Δ1, trp1Δ63, his3-200, lys2-Bgl, hom3-10, ade2Δ1, ade8*	R.Kolodner
rad4Δ	RDKY3023 *rad4::URA3*	R.Kolodner
W303	*MAT*a, *ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1, RAD5+*	X. Zhao
sgs1Δ	W303 *MAT*a, *sgs1::HIS3*	X. Zhao
rad51Δ	W303 *MAT*a, *rad51::LEU2*	X. Zhao
apn1Δ	W303 *MAT*alpha, *apn1::KANmx4*	X. Zhao

2.3. Mutator assay

Fluctuation analysis [24] was used to determine frequencies of *CAN1* inactivation and *hom3-10* reversion. Individual BF264-15D or RDKY3615 *mlh1Δ* colonies that survived 100 μg/mL cisplatin treatment (without or with MG132), as well as DMSO-treated control were grown overnight in YPD, after which cells were washed, diluted in water, and appropriate dilutions plated on complete media, SC-Glucose medium lacking arginine containing 60 μg/mL canavanine (Sigma) and/or SC-Glucose medium lacking threonine. After 2 days at 30 °C, colonies were counted and mutation frequencies calculated as described [25].

2.4. Western blot

Protein samples from total cell extracts of wild type BF264-15D cells treated with cisplatin or MMS were separated by SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) on a 12.5% polyacrylamide gel and transferred to a PVDF (polyvinylidene difluoride) membrane. Then, membranes were incubated in 5% non-fat dry milk in PBS-T (PBS solution with 0.1% Tween-20) for 1 h. For phosphorylated H2A and Pgk1p detection, membranes were incubated overnight at 4 °C with the primary antibodies anti-γH2AX (1:5000, Abcam) and anti-PGK1 (1:5000, Molecular Probes), respectively. Incubations with anti-rabbit (for γH2AX) and anti-mouse (for Pgk1p) secondary antibodies (Jackson Laboratories) were performed at RT for 1 h. Chemiluminescence detection of the bands was achieved using the ECL system (GE Healthcare) and a Chemi-Doc XRS (BioRad).

2.5. Flow cytometry

For cell cycle analysis, cells were collected by centrifugation, washed with dH_2O and the pellet was resuspended in 70% (v/v) ethanol. After washing and resuspending the pellet in 50 mM sodium citrate, pH 7.5, samples were incubated with 0.25 mg/mL RNase A (Sigma) for 1 h at 50 °C. Afterwards, 1 mg/mL Proteinase K (NZYtech) was added and samples incubated 1 h at 50 °C. Sytox Green (Molecular Probes) diluted in Tris-EDTA was added to a final concentration of 1 μM and samples incubated overnight at 4 °C in the dark. Samples were sonicated (3 pulses, 1–3 s each) to avoid aggregates and analyzed in an Epics® XL™ flow cytometer (Beckman Coulter), with excitation and emission wavelengths of 490 nm and 520 nm, respectively (FL-1 channel). Data was analyzed using FlowJo 7.6 software (Tree Star, Inc).

3. Results and discussion

3.1. The proteasome does not affect cisplatin-induced DNA damage response

We have previously shown that inhibition of the proteasome results in increased resistance of yeast cells to cisplatin [23] (also, see Fig. 4A). Resistance to DNA damaging agents is usually attributed to decreased intracellular availability of the drug, increased DNA repair, tolerance to DNA lesions, or defective cell death induction downstream of DNA damage, with the common result of failure to undergo cell death [26]. We first addressed whether inhibition of the proteasome could affect DNA damage by monitoring phosphorylation of H2A. Like the H2AX histone variant, H2A is phosphorylated in the presence of DNA double strand breaks (DSBs) in response to several DNA damaging agents, one of the first events signaling for the recruitment of DNA damage response (DDR) factors and the repair of the DNA damage [27,28]. It is important to highlight that cisplatin does not induce DSBs directly, but these lesions may result from the processing of interstrand crosslinks. We observed a clear increase in H2A phosphorylation after 3 h of treatment with cisplatin or with the positive control MMS, an alkylating agent known to induce DNA damage [29] (Fig. 1). These results are in agreement with other studies, where an increase in phosphorylation

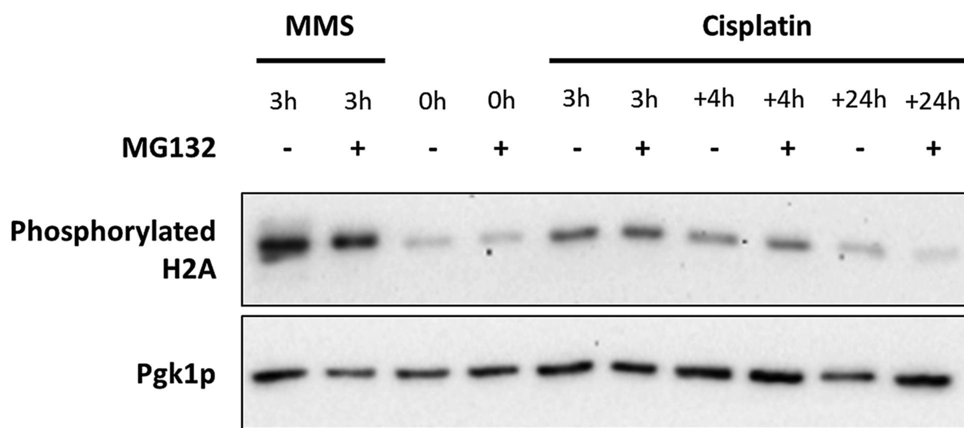

Fig. 1. The effect of proteasome inhibition on H2A phosphorylation in response to cisplatin treatment. Wild type BF264-15D cells were treated with MG132 or DMSO for 30 min followed by incubation with cisplatin (100 μg/mL) or MMS (0.1%) for 3 h. Afterwards, cells were washed, ressupended in new medium and cells collected after an additional 4 h and 24 h. H2A phosphorylation levels were assessed by Western Blot. Pgk1p levels were used as a loading control.

Fig. 2. Resistance of DNA damage response-deficient mutants to cisplatin in the absence or presence of proteasome inhibition. The indicated mutant strains and respective wild type controls were pre-incubated for 30 min with MG132 or DMSO as indicated, followed by treatment with cisplatin for 3 h at 30 °C (2 h for *rad4Δ* and respective wild type control). After exposure, cell viability was evaluated by spot assay on YPD plates. Spots of mutants and corresponding controls shown pairwise for experiments performed in parallel.

levels of this protein was detected in yeast in response to DNA damage, including exposure to MMS [30,31]. However, inhibition of the proteolytic activity of the 26S proteasome complex with the peptide aldehyde MG132 (carbobenzoxy-Leu-Leu-leucinal) did not influence H2A phosphorylation, since the phosphorylation levels were not significantly different in the absence and presence of MG132. Finally, 24 h after the removal of cisplatin from the medium, the phosphorylation returned to basal levels, indicating recovery from damage of surviving

Table 2
Mutator phenotype of wild type 15D cells.

Treatments	Can^r ($\times 10^{-7}$)[a]
DMSO	7.87
Cisplatin	7.44
Cisplatin + MG132	8.27

[a] Median frequencies of nine cultures. Values not statistically significant (Mann–Whitney test).

Table 3
Mutator phenotype of *mlh1Δ* cells.

Treatments	*hom3-10* ($\times 10^{-6}$)[a]	Can^r ($\times 10^{-6}$)[a]
DMSO	2.18	2.59
Cisplatin	2.44	2.93
Cisplatin + MG132	2.98	2.16

[a] Median frequencies of nine cultures. Values not statistically significant (Mann–Whitney test).

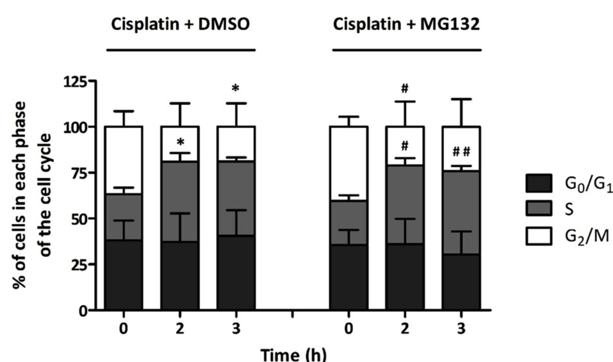

Fig. 3. The effect of proteasome inhibition on cell cycle progression of cisplatin-treated cells. Wild type BF264-15D cells were treated with MG132 or DMSO for 30 min, followed by exposure to cisplatin (100 μg/mL) for 3 h. Samples were collected and, after staining with Sytox Green, fluorescence was measured by flow cytometry. The percentage of cells in each phase of the cell cycle is represented: G_0/G_1 (black), S (grey) and G_2/M (light grey). Values represent means and standard deviations of 3 independent experiments. * $P < 0.05$ cells treated with cisplatin compared with the corresponding T0 (0 h); $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ cells treated with MG132 and cisplatin compared with the corresponding T0 (0 h).

cells. Taken together, these results indicate that MG132 does not reduce cisplatin-induced DNA damage in yeast cells.

Next, we assessed if genes involved in DDR were required for the observed phenotype. We chose genes coding for proteins involved in cell cycle checkpoint (*MEC1*) and nucleotide excision repair, the major pathway repairing cisplatin lesions (*RAD4*), as well as double strand break repair (*RAD51*, *SGS1*); indeed mutants in these pathways were shown to be sensitive to cisplatin [32]. In addition, since cisplatin can also cause abasic sites to form [33], and the mismatch repair system is involved in the processing of cisplatin adducts in mammalian cells [34], we also chose the *APN1* and *MLH1* genes, respectively. Wild type strains and mutants defective in cell cycle checkpoint (*sml1Δmec1Δ* and respective *sml1Δ* control) and in the different DNA repair mechanisms (*rad4Δ*, *mlh1Δ*, *rad51Δ*, *sgs1Δ*, *apn1Δ*) were pre-incubated for 30 min with MG132 or DMSO, followed by exposure to cisplatin for 3 h. Different cisplatin concentrations were tested according to the strain sensitivity to the chemotherapeutic agent. Although the differential sensitivities to cisplatin hamper a direct comparison of the extent of protection afforded by MG132 in the different mutants, increased viability in cells exposed to cisplatin in the presence of MG132 was always observed, indicating that these particular genes are not required for this process (Fig. 2).

3.2. The proteasome does not affect mutation frequencies of cisplatin-treated cells

Since proteasome inhibition does not seem to affect the DNA damage response following cisplatin exposure, we hypothesized that it could lead to increased DNA damage tolerance (DDT). Through DDT, DNA replication can bypass the lesions, allowing for later repair, although it can be associated with increased mutagenesis (reviewed in [35]). If this was the case, cells surviving exposure to cisplatin in the presence of MG132 should have increased mutations over cells exposed only to cisplatin. To test this hypothesis, we assessed the *CAN1* inactivation frequencies of wild type cells surviving exposure to 100 μg/mL of cisplatin for 3 h, pre-treated with DMSO or MG132, and compared it with those of cells treated with DMSO alone. As seen in Table 2, there were no significant differences between the 3 conditions. We also used *mlh1Δ* cells surviving the different treatments, in order better assess whether MG132 affects mutation frequencies of cisplatin-treated cells in a strain prone to mutations. For this purpose, we assessed *CAN1* forward mutations as well as *hom3-10* frameshifts in the RDKY3615 *mlh1Δ* reporter strain (Table 3). In accordance with a previous report [36], exposure to cisplatin did not increase *CAN1* inactivation frequencies in *mlh1Δ* cells, and we found that it did not increase *hom3-10* frameshifts. Moreover, exposure to MG132 had no effect on the frequency of these mutations. Taken together, our results indicate that increased resistance to cisplatin imparted by MG132 is not related with a change in mutation frequency.

3.3. Proteasome inhibition does not affect cell cycle progression of cisplatin-treated cells

After DNA damage induction, cell cycle arrest allows the cells to repair these lesions and, afterwards, cell cycle progresses normally [37]. Since the proteasome has a well-established role in the cell cycle [38], we next evaluated whether MG132 affected cell cycle progression of cisplatin-treated cells by flow cytometry. However, as seen in Fig. 3 proteasome inhibition had no effect on the cell cycle progression of cisplatin-treated cells. Again, these results point to a role of the proteasome in cisplatin-induced cell death independent of DNA damage.

3.4. MG132 does not protect cells from universal DNA damage-induced cell death

In the previous sections, we show that no significant differences are observed in DNA damage-associated phenotypes of cisplatin-treated cells in the presence or absence of MG132, which indicates that the proteasome is likely involved in the cell death mechanism. To assess the specificity of this mechanism, we assessed whether MG132 also protected cells from death induced by a different DNA damaging agent. Under our experimental conditions, we observed that MG132 does not protect cells from death induced by MMS, unlike the clear increased resistance to cisplatin (Fig. 4A). This is in agreement with another report showing that proteasome inhibition only clearly protected cells from chronic exposure to 4-Nitroquinoline 1-oxide (4-NQO), and had minimal effects on cell death induced by 4-NQO or MMS [39]. Also, differently from the case of cisplatin, MMS exposure did not lead to an increased number of cells with sub G_0/G_1 DNA content (Fig. 4B), indicative of DNA degradation during the cisplatin-induced cell death process [40]. We also previously showed that inhibition of protein biosynthesis increased the viability of yeast cells treated with cisplatin, indicating that it induces an active form of cell death [23] (Fig. 4C). However, we found that cycloheximide did not revert cell death induced by MMS (Fig. 4C). Taken together, these results indicate that the cell death processes induced by MMS and cisplatin are fundamentally different and propose that MG132 functions by inhibiting an active form of cell death induced by cisplatin, downstream of DNA damage.

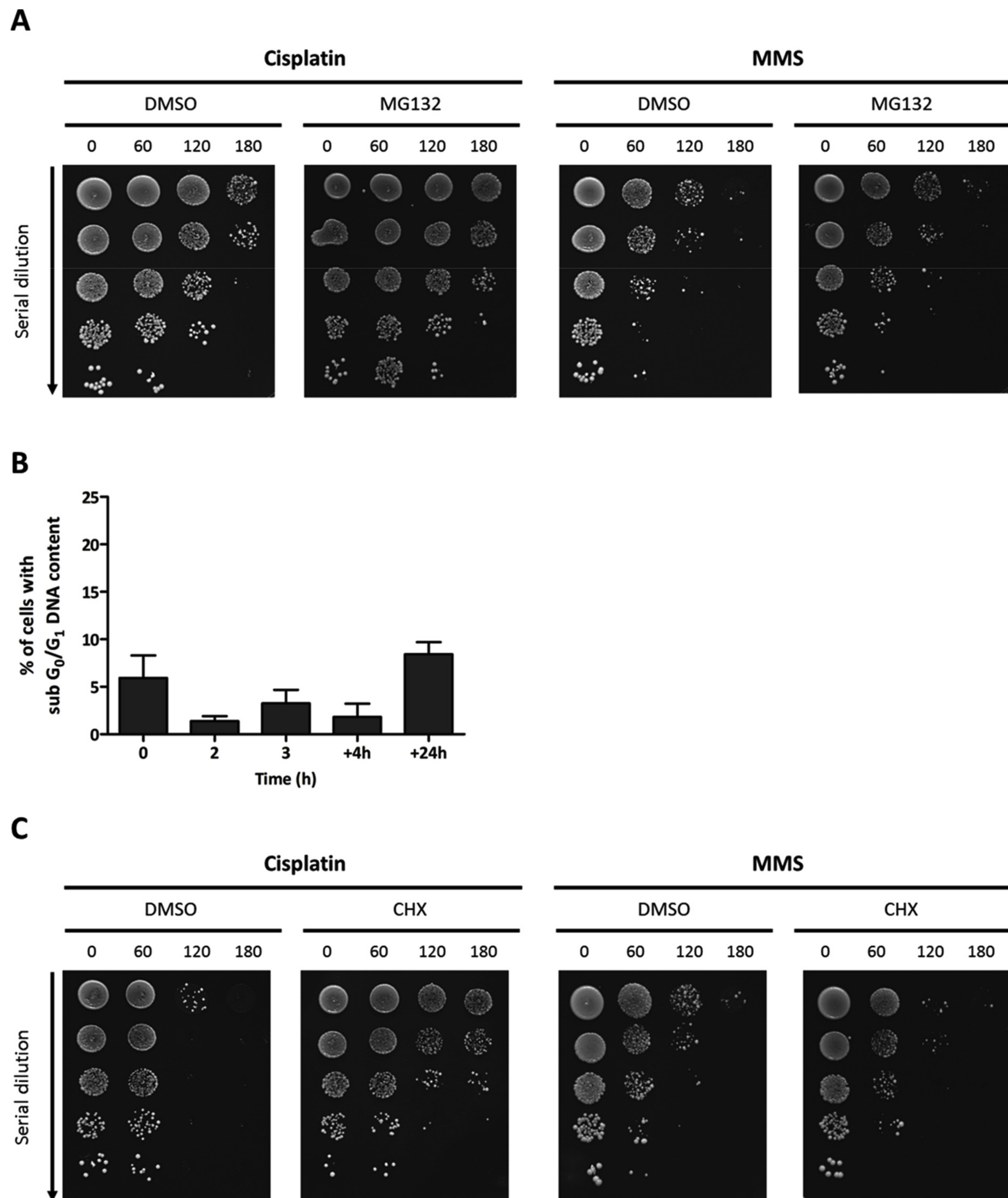

Fig. 4. The effect of proteasome inhibition on cisplatin- or MMS-induced cell death. BF264-15D cells were grown in SC-Glu medium containing proline as nitrogen source and 0.003% SDS for cell permeabilization. After 3 h, 118 μM of MG132 or the equivalent volume of DMSO was added to the medium for an additional period of 30 min. (A) Spot assay of cells treated with 100 μg/mL of cisplatin or 0.1% MMS for 180 min at 30 °C. (B) Sub G_0/G_1 DNA content of cells treated with 0.1% MMS. Samples were collected at 0 h, 2 h, 3 h and after 4 h/24 h of MMS removal. After processing and staining with Sytox Green, fluorescence was measured by flow cytometry. Values represent means and standard deviations of 3 independent experiments. (C) Spot assay of cells treated with 0.1% MMS or 100 μg/mL cDDP and 75 μg/mL of cycloheximide (CHX) for up to 180 min at 30 °C.

4. Conclusions

Combination of cisplatin with other anticancer drugs is a promising strategy to overcome cisplatin resistance. In particular, combinational therapy with cisplatin and proteasome inhibitors proved to be effective against several cancer cells lines. In contrast, proteasome inhibition seemed to protect tubular renal cells and yeast cells from cisplatin-induced cell death. The present study indicates the mechanisms underlying evasion from cisplatin-induced cell death imparted by proteasome inhibitors occur downstream of DNA damage, and are amenable to pharmacological modulation. Further elucidation of these pathways will contribute towards strategies to preferentially sensitize tumor cells

to chemotherapy, both by increasing its efficacy and reducing its toxicity and side effects.

Conflicts of interest

None.

Acknowledgements

We thank Dr. Richard Kolodner, Dr. Steven Reed, and Dr. Xiaolan Zhao for strains, as well as Dr. Vincent Pennaneach for help with the mutation frequencies protocol. This work was supported by FCT I.P. through the strategic funding UID/BIA/04050/2013 and FCT-ANR/BEX-BCM/0175/2012, as well as a Post-doc fellowship to S. Chaves (SFRH/BPD/89980/2012) and a PhD fellowship to A. Rego (SFRH/BD/79523/2011).

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