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Ubiquitin-proteasome System Is a Promising Target for Killing Cisplatin-resistant Bladder Cancer Cells

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Abstract. *Background/Aim: Activation of the ubiquitin-proteasome system (UPS) has been shown to be associated with drug resistance in cancer. Using bladder cancer cells, we investigated the association between UPS activation and cisplatin resistance and also the efficacy of UPS-targeting drugs. Materials and Methods: We established cisplatin-resistant bladder cancer cells (J82-cisR, T24-cisR) and examined the activation status of the UPS and the efficacy of MLN7243, oprozomib, ixazomib, and RTS-V5. Results: The UPS in cisplatin-resistant bladder cancer cells was activated compared to that in their parental controls. All the UPS-targeting drugs induced apoptosis and inhibited growth more effectively in the cisplatin-resistant bladder cancer cells than they did in the parental controls. Furthermore, these UPS-targeting drugs induced endoplasmic reticulum stress by causing unfolded protein accumulation at lower concentrations in the cisplatin-resistant bladder cancer cells. Conclusion: Targeting the UPS could be an effective strategy for treating cisplatin-resistant bladder cancer.*

Cisplatin-based chemotherapy does not prolong the overall survival of patients with advanced bladder cancer because most of them develop cisplatin resistance (1). Immune checkpoint inhibitors have recently been approved for bladder cancer therapy (2), but their clinical benefit is limited (2, 3). An alternative approach to the existing cancer therapy is clearly needed.

In the ubiquitin-proteasome system (UPS), unfolded proteins are often repaired by molecular chaperones, but if the repair fails, they are ubiquitinated and degraded by proteasomes (4-6). Thus, the UPS is responsible for regulating the ubiquitin-dependent signaling pathways and maintaining cellular protein homeostasis (4, 5). UPS-targeting drugs have recently emerged as novel anticancer agents (7-10) because perturbation of the UPS results in the accumulation of ubiquitinated proteins, thereby causing endoplasmic reticulum (ER) stress and apoptosis in cancer cells (11, 12).

Several studies have shown that the UPS plays an important role in cancer development and chemoresistance (7, 13, 14), but the status of the UPS activity in cisplatin-resistant bladder cancer has not been fully elucidated yet. In the present study, we evaluated the expression of UPS-related proteins and the proteasome activity in cisplatin-naive and cisplatin-resistant bladder cancer cells and investigated whether cisplatin resistance influences the efficacy of UPS-targeting drugs in bladder cancer cells.

Materials and Methods

Cell cultures. Human bladder cancer cells (J82 and T24) were purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in recommended media containing 10% fetal bovine serum and 1.0% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37˚C under 5% CO_2 in a humidified incubator. Cisplatin-resistant bladder cancer cells (J82-CisR and T24-CisR) were established by culturing them in medium with continuously escalating dosages of cisplatin over several months. After acquisition of cisplatin resistance, the cells were maintained in medium containing 10 μM cisplatin.

Reagents. Cisplatin, oprozomib, and ixazomib were purchased from Selleck Chemicals (Houston, TX, USA), and MLN7243 was purchased from AOBIOUS (Gloucester, MA, USA). The dual histone deacetylase-proteasome inhibitor RTS-V5 was synthesized as previously described (15). All reagents were dissolved in dimethyl sulfoxide and stored at −80˚C until use.

Cell viability assay. Cell viability was determined by CCK-8 assay (Dojin, Kumamoto, Japan) following the manufacturer's protocol.

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Key Words: Ubiquitin-proteasome system, endoplasmic reticulum stress, cisplatin-resistant bladder cancer.

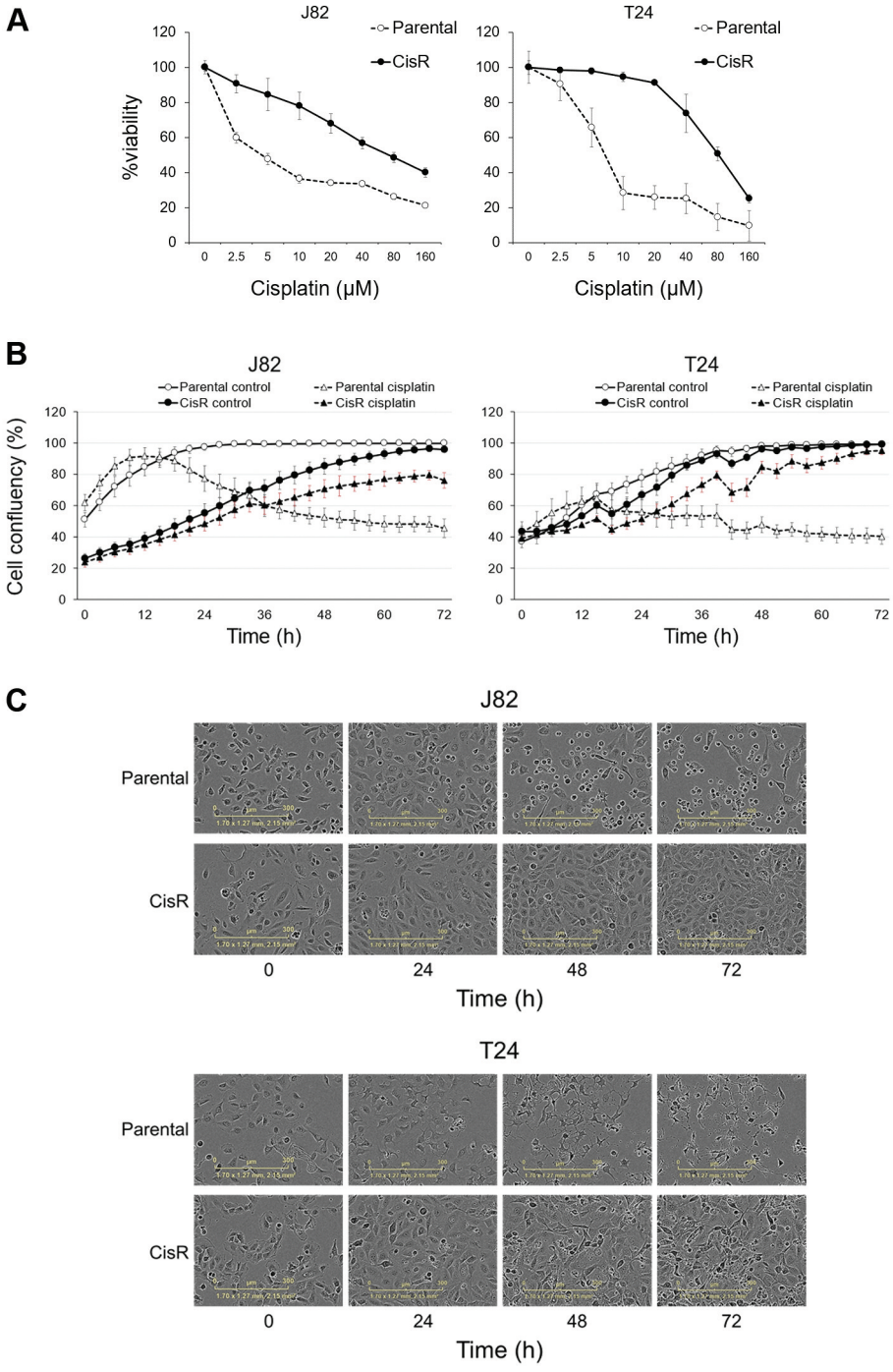


Figure 1. *Continued*

Briefly, 5×10^3 cells were plated in 96-well culture plates one day before being treated under the indicated conditions for 48 h. After treatment, the medium was replaced with 10 μl CCK-8 solution in 90 μl fresh medium and the plates were incubated for 60 min. The plates were then read in the absorbance microplate autoreader SpectraMax ABS Plus (Molecular Devices, San Jose, CA, USA).

Cell confluency assay. Five $\times10^3$ cells were seeded in a 96-well culture plate one day before being treated under indicated conditions. After treatment, confluence measurements were performed at 3-h intervals over 3 days by using the IncuCyte real-time video imaging system (Essen Instruments, Ann Arbor, MI, USA).

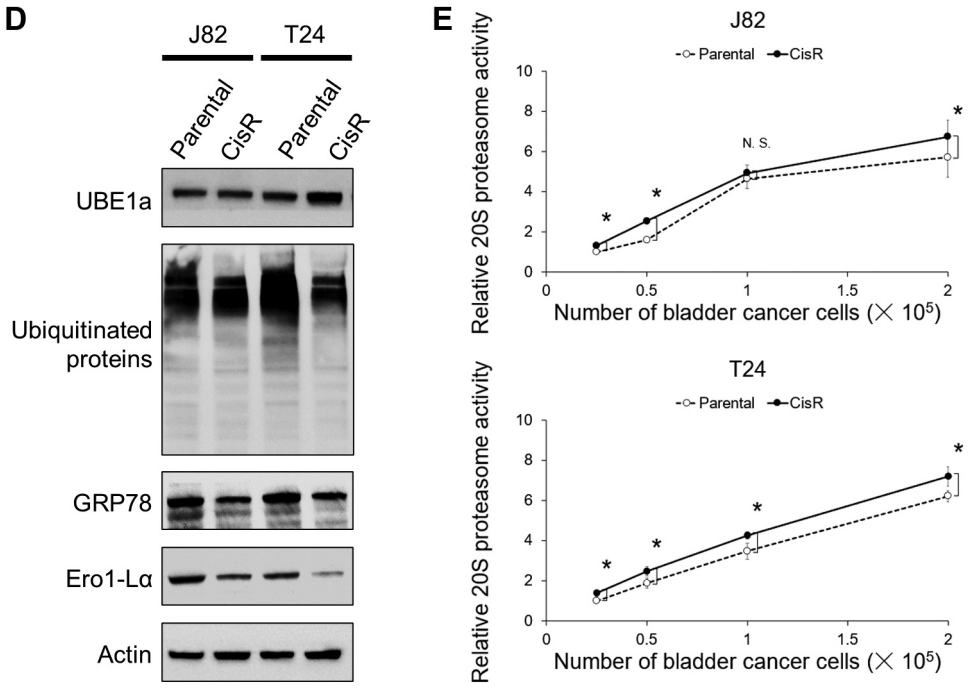


Figure 1. *Activation of the ubiquitin-proteasome system in cisplatin-resistant bladder cancer cells. A) Cells were treated for 48 h with 2.5-160 μM cisplatin, and cell viability was measured using CCK-8 assay. Mean±SD, n=6. CisR: Cisplatin-resistant. B) Cells were cultured for 72 h in medium with or without 10 μM cisplatin and confluence measurements were performed at 3-h intervals over 72 h. Mean±SD, n=6. C) Photomicrographs. Cells were cultured for 24-72 h in medium with or without 10 μM cisplatin. D) Western blotting for ubiquitin-activating enzyme (UBE1a), ubiquitinated proteins, glucose-regulated protein (GRP) 78, and endoplasmic reticulum oxidoreductin-1-like protein alpha (Ero1-Lα). Actin was used for the loading control. Representative blots are shown. E) Cells were cultured for 24 h, then the activity of purified 20S proteasome was measured. Data are expressed as mean±SD from three independent experiments. *p=0.0495; N.S.: Not significant.*

Detection of apoptosis. A total of 1 ×10^5 cells were seeded in a 12-well culture plate one day before being cultured under the indicated conditions for 48 h. Induction of apoptosis was evaluated by flow cytometry using the Annexin V FITC/7-AAD Kit (Beckman Coulter, Marseille, France) as previously described (16).

20S proteasome activity assay. Cells were plated in a 96-well culture plate at indicated densities and incubated overnight. They were then processed according to the instructions of the assay kit (20S Proteasome Assay Kit; Cayman, Ann Arbor, MI, USA) and the plate was read in a fluorescence microplate autoreader SpectraMax Gemini EM (Molecular Devices) with a 360 nm excitation filter and 480 nm emission filter.

Western blotting. Cells were treated under the indicated conditions and whole cell lysates were obtained using radioimmunoprecipitation assay buffer. Equal amounts of proteins were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked using 5% skimmed milk and incubated overnight with the following primary antibodies: anti-ubiquitin, anti-ubiquitin-activating enzyme (UBE1a), and anti-endoplasmic reticulum oxidoreductin-1-like protein alpha (Ero1-Lα) (Cell Signaling Technology, Danvers, MA, USA); anti-glucose-regulated protein (GRP) 78 (Proteintech, Rosemont, IL, USA); anti-acetylated histone (Abcam, Cambridge, UK); and anti-actin (Millipore, Billerica, MA, USA). Then, the protein was detected by reaction with recommended secondary antibodies [horseradish-tagged goat anti-rabbit or goat anti-mouse antibody (GE Healthcare UK, Amersham, UK)] and staining with chemiluminescence solution (Clarity Western ECL Substrate, Bio-Rad, Hercules, CA, USA).

Detection of aggresome formation. Protein aggregation was evaluated by detecting aggresomes in cells using the PROTEOSTAT Aggresome Detection Kit (Enzo Life Sciences, Farmingdale, NY, USA) as previously described (16). Aggresomes and the nucleus were visualized using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Statistical analysis. Statistical significance of observed differences between samples was evaluated using the Mann-Whitney *U*-test (JMP Pro14 software; SAS Institute, Cary, NC, USA), and $p<0.05$ was considered statistically significant.

Results

Activation of the UPS in cisplatin-resistant bladder cancer cells. Using J82 and T24 cells, we generated cisplatin-resistant bladder cancer cells (J82-CisR and T24-CisR) by

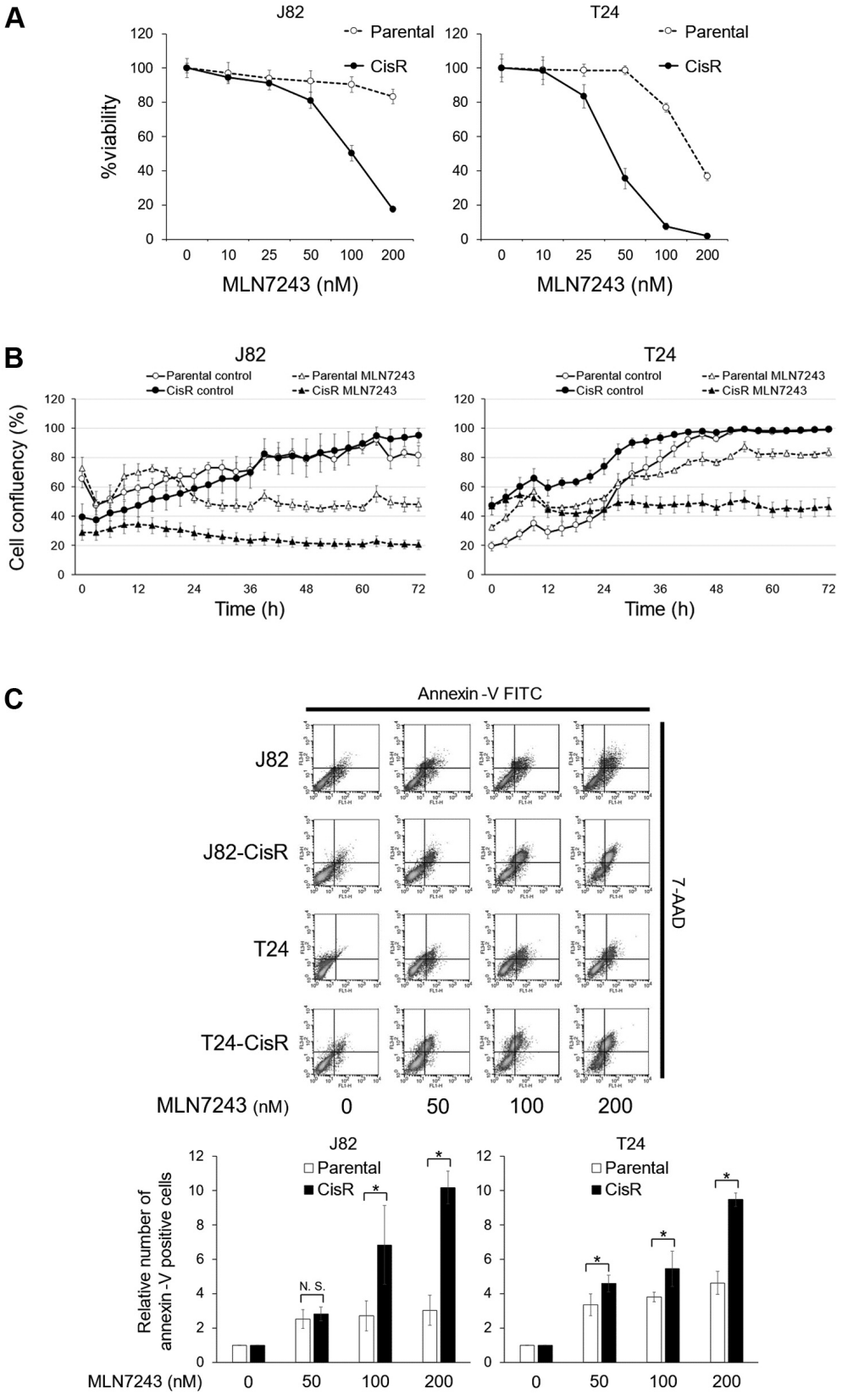


Figure 2. *Continued*

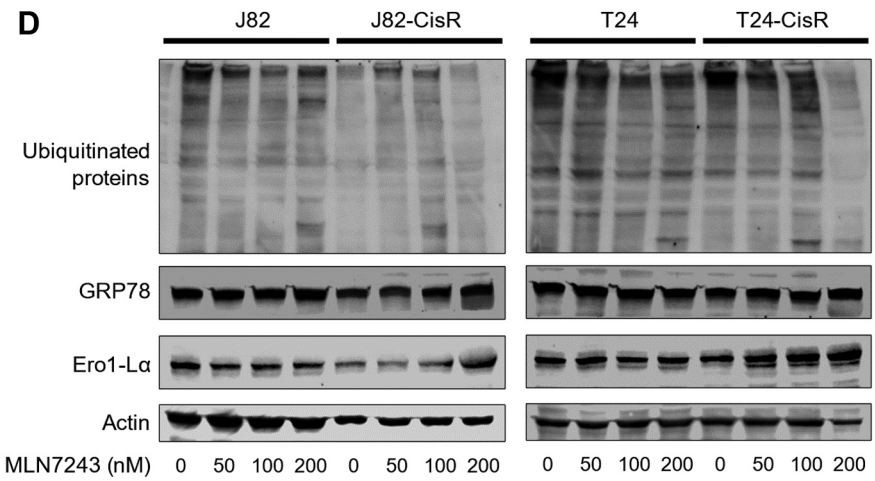


Figure 2. *The cisplatin-resistant bladder cancer cells were more susceptible to the ubiquitin-activating enzyme inhibitor MLN7243. A) Cells were treated for 48 h with 10-200 nM MLN7243 and cell viability was measured using CCK-8 assay. Mean±SD, n=6. CisR: Cisplatin-resistant. B) Cells were cultured for 72 h in medium with or without 200 nM MLN7243 and confluence measurements were performed at 3-h intervals over 72 h. Mean±SD, n=6. C: Annexin-V assay. Cells were treated for 48 h with 50-200 nM MLN7243. Apoptotic cells were detected by annexin-V assay using flow cytometry; 10,000 cells were counted. Bar graphs show the relative number of annexin-V positive cells. Data are expressed as mean±SD from three independent experiments. FITC: Fluorescein isothiocyanate; 7-AAD: 7-aminoactinomycin D. *p=0.0495; N.S.: not significant. D) Western blotting for ubiquitinated proteins, glucose-regulated protein (GRP) 78, and endoplasmic reticulum oxidoreductin-1-like protein alpha (Ero1-Lα). Cells were treated for 48 h with 50-200 nM MLN7243. Actin was used for the loading control. Representative blots are shown.*

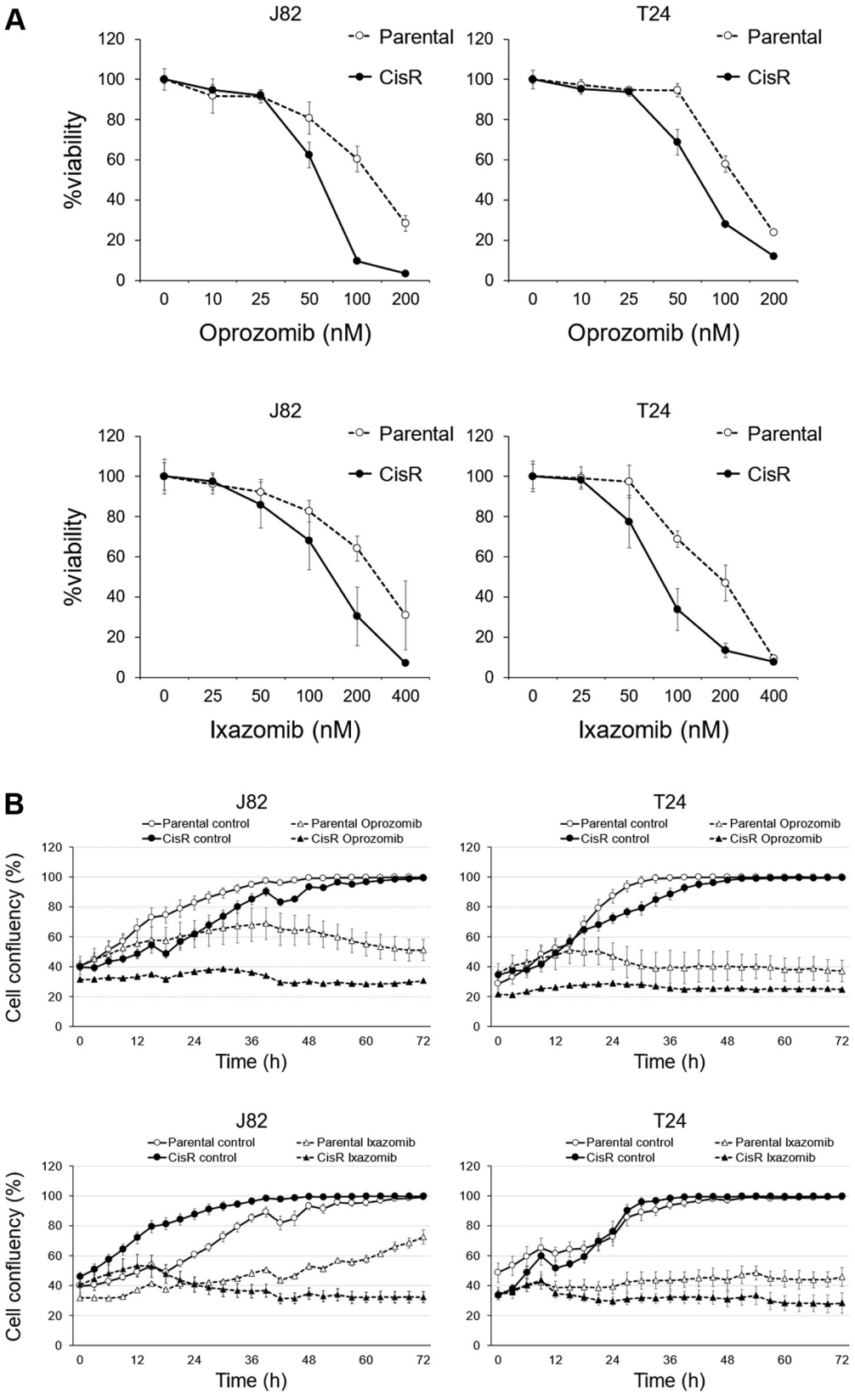
adding cisplatin after every passage at escalating doses over months. Acquisition of cisplatin resistance was confirmed by increased cisplatin IC_{50} values at 48 h compared to their parental controls (Figure 1A and Table I). Accordingly, cell confluency assay results showed that 72-h treatment with 10 μM cisplatin almost failed to inhibit the growth of J82-CisR and T24-CisR cells (Figure 1B and C).

We then examined whether the acquisition of cisplatin-resistance was associated with the UPS activity. There is no established way to directly measure the UPS activity because it is a sum of the molecular chaperone function, ubiquitinating enzyme activity, and proteasomal activity. In the present study, we used the amount of undegraded ubiquitinated proteins in the cell as a surrogate indicator of the UPS activity. The cisplatin-resistant bladder cancer cells were shown to have a lower amount of undegraded ubiquitinated proteins than did their parental cells (Figure 1D). The lower expression of the ER stress markers GRP78 and Ero1-Lα was consistent with this lower amount of ubiquitinated proteins in the cisplatin-resistant cells (Figure 1D). Thus, the UPS activity was increased in the cisplatin-resistant cells. Furthermore, T24-CisR cells were shown to have the higher expression of the ubiquitin-activating enzyme UBE1a (Figure 1D) and both J82-CisR and T24-CisR cells had higher activity of the 20S proteasome (Figure 1E), both of which might contribute to the higher UPS activity.

Table I. *Mean inhibitory concentrations (IC_{50}) of cisplatin (μM). After treatment of cells with cisplatin for 48 h, CCK-8 assay was performed to assess cell viability.*

	J82	T24
Parental	4.3	10.7
CisR	71.9	85.4

The cisplatin-resistant bladder cancer cells were more susceptible to the ubiquitin-activating enzyme inhibitor MLN7243. Because the cisplatin-resistant bladder cancer cells expressed higher levels of UBE1a than the parental cells, we thought that a ubiquitin-activating enzyme inhibitor would effectively reduce the growth of cisplatin-resistant bladder cancer cells. MLN7243 is a small-molecule ubiquitin-activating enzyme inhibitor that disrupts all ubiquitin signaling and protein ubiquitination, thereby causing deubiquitinated unfolded protein accumulation and inducing ER stress (17). MLN7243 inhibited the viability and growth of the cisplatin-resistant bladder cancer cells at lower concentrations than it did in the parental cells (Figure 2A and B, Table II). It also induced apoptosis more effectively in cisplatin-resistant bladder cancer cells (Figure 2C). Mechanistically, MLN7243 decreased the amount of ubiquitinated proteins and increased the expression of the ER stress markers GRP78 and Ero1-Lα at lower



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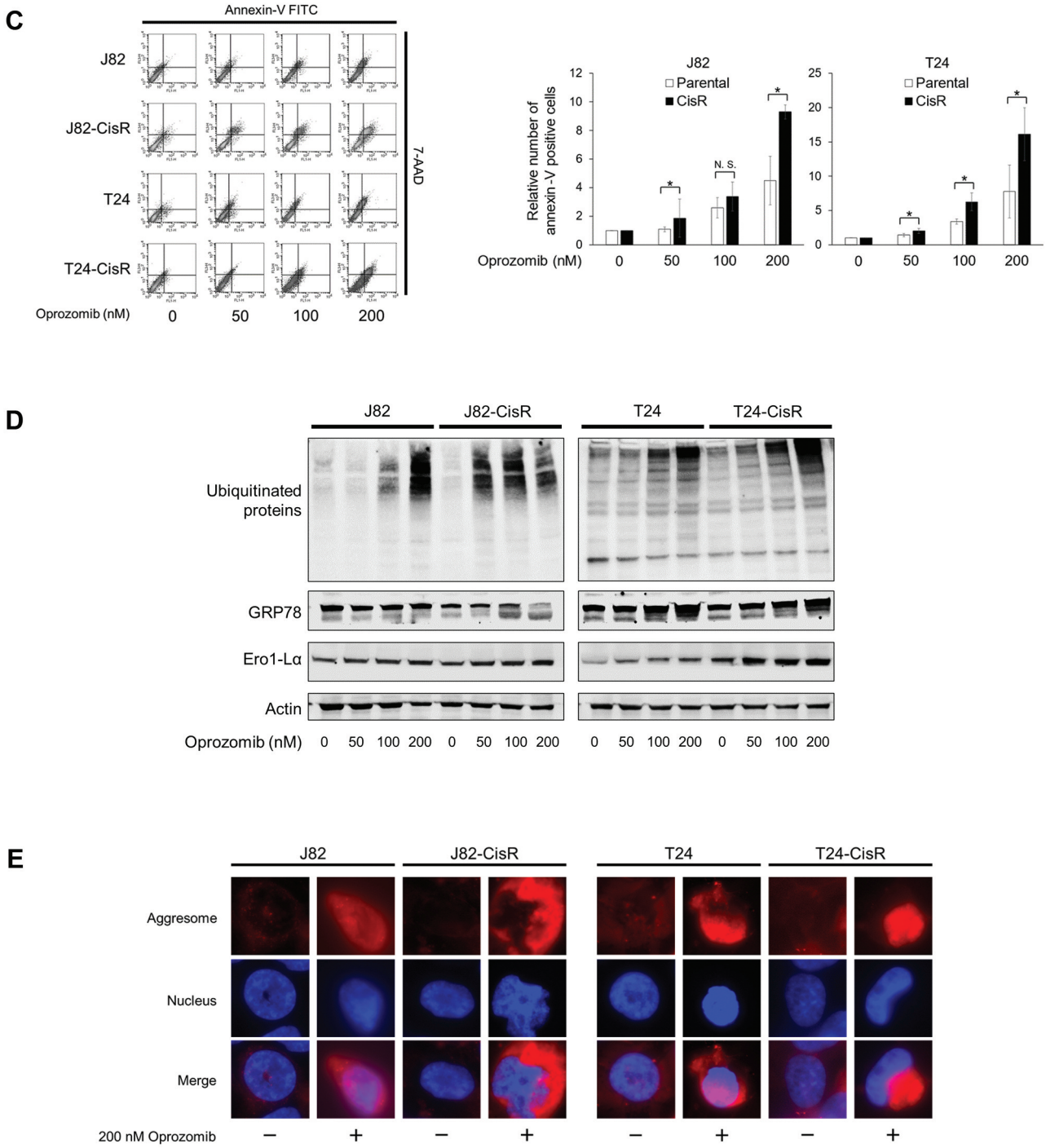
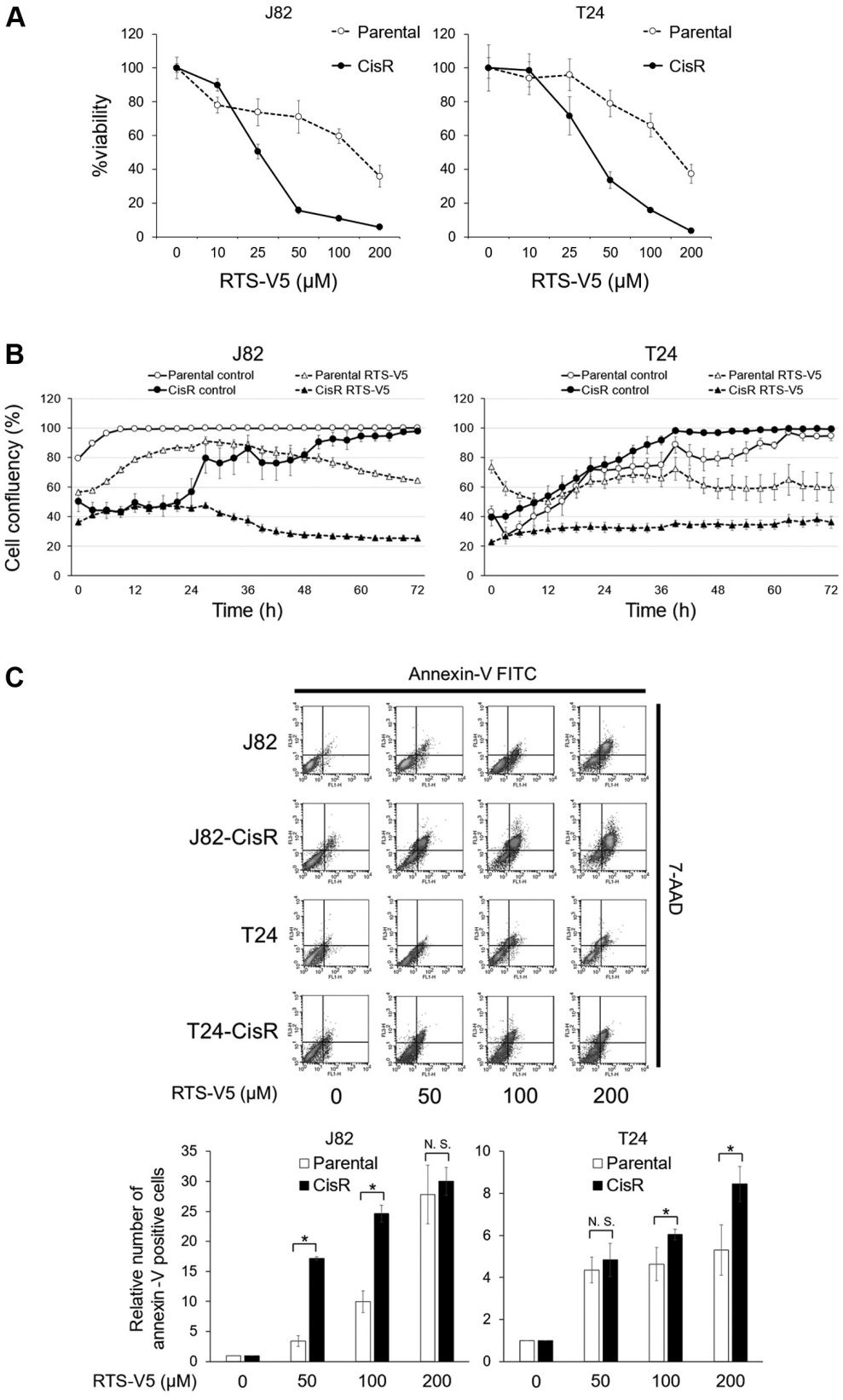


Figure 3. *Proteasome inhibitors kill the cisplatin-resistant cancer cells effectively. A) Cells were treated for 48 h with 10-200 nM oprozomib or 25-400 nM ixazomib and cell viability was measured using CCK-8 assay. Mean±SD, n=6. B) Cells were cultured for 72 h in medium with or without 200 nM oprozomib or 400 nM ixazomib and confluence measurements were performed at 3-h intervals over 72 h. Mean±SD, n=6. C: Annexin-V assay. Cells were treated for 48 h with 50-200 nM oprozomib. Apoptotic cells were detected by annexin-V assay using flow cytometry; 10,000 cells were counted. Bar graphs show the relative number of annexin-V positive cells. Data are expressed as mean±SD from three independent experiments. FITC: Fluorescein isothiocyanate; 7-AAD: 7-aminoactinomycin D. *p=0.0495; N.S.: not significant. D) Western blotting for ubiquitinated proteins, glucose-regulated protein (GRP) 78, and endoplasmic reticulum oxidoreductin-1-like protein alpha (Ero1-Lα). Cells were treated for 48 h with 50-200 nM oprozomib. Actin was used for the loading control. Representative blots are shown. E: Aggresome detection after 48-h treatment with 200 nM oprozomib. Red: aggresome; blue: nucleus. Original magnification, 1,000×.*



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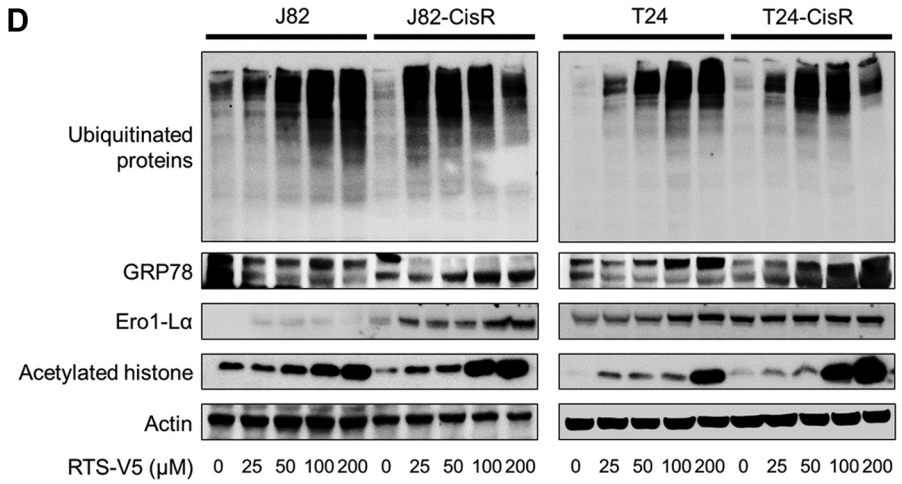

Figure 4. *Efficacy of the dual histone deacetylase-proteasome inhibitor RTS-V5 in cisplatin-resistant bladder cancer cells. A) Cells were treated for 48 h with 10-200 μM RTS-V5 and cell viability was measured using CCK-8 assay. Mean±SD, n=6. CisR: Cisplatin-resistant. B: Cells were cultured for 72 h in medium with or without 200 μM RTS-V5 and confluence measurements were performed at 3-h intervals over 72 h. Mean±SD, n=6. C) Annexin-V assay. Cells were treated for 48 h with 50-200 μM RTS-V5. Apoptotic cells were detected by annexin-V assay using flow cytometry; 10,000 cells were counted. Bar graphs show the relative number of annexin-V positive cells. Data are expressed as mean±SD from three independent experiments. FITC: Fluorescein isothiocyanate; 7-AAD: 7- aminoactinomycin D. *p=0.0495; N.S.: not significant. D) Western blotting for ubiquitinated proteins, glucose-regulated protein (GRP) 78, endoplasmic reticulum oxidoreductin-1-like protein alpha (Ero1-Lα), and acetylated histone. Cells were treated for 48 h with 25-200 μM RTS-V5. Actin was used for the loading control.*

concentrations in the cisplatin-resistant bladder cancer cells than in their parental cells (Figure 2D). Taken together, this is evidence that the cisplatin-resistant bladder cancer cells were more susceptible to MLN7243.

Proteasome inhibitors kill the cisplatin-resistant cancer cells effectively. Because the cisplatin-resistant bladder cancer cells exhibited higher 20S proteasome activity, we suggested that proteasome inhibitors would kill the cisplatin-resistant bladder cancer cells effectively. The proteasome inhibitors oprozomib and ixazomib inhibited the viability and growth of the cisplatin-resistant bladder cancer cells at lower concentrations than it did in their parental cells (Figure 3A and B, Table III). Because oprozomib had lower IC_{50} values than ixazomib, we used oprozomib for further analysis. In consistence with the result of the cell viability assay, oprozomib induced apoptosis at lower concentrations in cisplatin-resistant bladder cancer cells (Figure 3C). Mechanistically, oprozomib increased the amount of ubiquitinated proteins and induced ER stress at lower concentrations (Figure 3D). Next, we evaluated aggresome formation by oprozomib. An aggresome is an inclusion body produced by UPS inhibition and the resultant accumulation and aggregation of unfolded proteins (18, 19). Two hundred nM oprozomib caused massive aggresome formation in cisplatin-resistant bladder cancer cells, whereas it caused only slight aggresome formation in parental cells (Figure 3E).

Table II. *Mean inhibitory concentrations (IC_{50}) of MLN7243 (nM). After treatment of cells with MLN7243 for 48 h, CCK-8 assay was performed to assess cell viability.*

	J82	T24
Parental	3,561.2	172.2
CisR	94.8	43.5

Table III. *Mean inhibitory concentrations (IC_{50}) of oprozomib (nM) and ixazomib (nM). After treatment of cells with oprozomib or ixazomib for 48 h, CCK-8 assay was performed to assess cell viability.*

	Oprozomib		Ixazomib	
	J82	T24	J82	T24
Parental	129.8	137.7	266.6	165.2
CisR	48.8	73.4	129.6	102.5

Efficacy of the dual histone deacetylase-proteasome inhibitor RTS-V5 in cisplatin-resistant bladder cancer cells. RTS-V5 is the world's first dual histone deacetylase (HDAC)-proteasome inhibitor and can simultaneously inhibit the two relevant parts

Table IV. *Mean inhibitory concentrations (IC_{50}) of RTS-V5 (μM). After treatment of cells with RTS-V5 for 48 h, CCK-8 assay was performed to assess cell viability.*

	J82	T24
Parental	135.4	163.6
CisR	28.3	45.8

of the UPS, *i.e.*, HDACs and the proteasome, causing ubiquitinated proteins to accumulate and inducing ER stress (15). We postulated that the cisplatin-resistant bladder cancer cells would be more sensitive to RTS-V5 than their parental cells. As expected, RTS-V5 inhibited the viability and cell proliferation of the cisplatin-resistant bladder cancer cells more effectively than those of their parental cells (Figure 4A and B, Table IV). Accordingly, apoptosis was induced at a lower concentration of RTS-V5 in the cisplatin-resistant bladder cancer cells (Figure 4C). Mechanistically, RTS-V5 increased the amount of ubiquitinated proteins and the expression of GRP78 and Ero1-Lα (only in J82-CiR cells) at lower concentrations in the cisplatin-resistant bladder cancer cells (Figure 4D). Of note, 50-200 μM RTS-V5 in J82-CisR cells and 200 μM RTS-V5 in T24-CisR cells apparently decreased the expression of ubiquitinated proteins (Figure 4D). Because the expression of the ER stress markers was increased, this decrease means that excessively accumulated ubiquitinated proteins aggregated and shifted to the detergent-insoluble fraction (20, 21). Thus, RTS-V5 was shown to cause excessive ubiquitinated protein accumulation in cisplatin-resistant bladder cancer cells but not in their parental cells. Interestingly, RTS-V5 also induced histone acetylation more effectively in cisplatin-resistant bladder cancer cells (Figure 4D).

Discussion

The UPS plays a pivotal role in protein homeostasis and regulates DNA repair, gene expression, cell survival, and apoptosis (4-6). Because its activation correlates with oncogenesis, cancer development, and chemoresistance (22-25), targeting the UPS is a novel strategy for cancer treatment (7-10).

There have been several studies that demonstrated the association of the UPS activity and drug resistance (7, 13, 14); however, to our knowledge, little is known about the association of the UPS activity and cisplatin resistance. In the present study, we found that the expression of UBE1a and the 20S proteasome activity were increased in the cisplatin-resistant bladder cancer cells, *i.e.*, the UPS activity was increased by activating the two major steps of the protein degradation process: ubiquitylation and proteasomal degradation. The activated UPS would enable the cisplatin-resistant bladder cancer cells a faster recovery from cisplatin treatment by repairing unfolded proteins, such as antiapoptotic proteins and transcription factors, and restoring their function. Identifying such associated proteins, however, would require further investigation.

Since the UPS activity was increased in cisplatin-resistant bladder cancer cells, we presumed that they would be more susceptible to drugs inhibiting the UPS than their parental cells. UPS-targeting drugs undergo multiple mechanisms of action as anticancer agents (22-25) and one of the crucial mechanisms is inducing ER stress (26). ER stress is caused by accumulation and aggregation of unfolded or misfolded proteins (6), and excessive ER stress leads to apoptosis in cancer cells (11, 12). Our results demonstrate that the ubiquitin-activating enzyme inhibitor MLN7243, the proteasome inhibitors oprozomib and ixazomib, and the dual HDAC-proteasome inhibitor RTS-V5 induced ER stress more effectively in the cisplatin-resistant bladder cancer cells than in their parental cells. MLN7243 acts as a mechanism-based inhibitor of ubiquitin-activating enzymes, all subsequent ubiquitin signaling, and protein ubiquitination, thereby causing accumulation of deubiquitinated unfolded proteins and inducing ER stress (17, 27). On the other hand, proteasome inhibitors block the degradation of multi-ubiquitinated target proteins, thereby causing ubiquitinated unfolded proteins to accumulate and induce ER stress (28). RTS-V5 increases the amount of unfolded proteins by inhibiting the molecular chaperone function *via* HDAC inhibition and inhibiting the proteasome, leading to ER stress induction (15). We also found that RTS-V5 induced histone acetylation more effectively in cisplatin-resistant bladder cancer cells, suggesting that the cisplatin-resistant bladder cancer cells displayed higher HDAC activity than their parental cells. Another explanation for this phenomenon is that histone acetylation occurs as a consequence of ER stress induction (16, 21). Although further studies are needed to clarify the actual mechanism, RTS-V5 is an attractive agent for the treatment of cisplatin-resistant bladder cancer by causing both ER stress and histone acetylation.

Thus, UPS-targeting drugs could be novel drug candidates against cisplatin-resistant bladder cancer. Furthermore, our previous studies revealed that the clinically feasible drug combinations ritonavir and ixazomib (20), nelfinavir and ritonavir (29), and ritonavir and lopinavir (30) killed bladder cancer cells synergistically by inhibiting the UPS and thereby inducing ER stress. The present study provides a theoretical basis for using these UPS-targeting combinations to treat cisplatin-resistant bladder cancer in patients, for whom there have been no curative treatment modalities.

In conclusion, the present study demonstrates that the cisplatin-resistant bladder cancer cells activated the UPS by increasing the expression of UBE1a and the 20S proteasome activity and sensitized themselves to UPS-targeting drugs.

Targeting the UPS would be a novel strategy for treating cisplatin-resistant bladder cancer.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

K.O., M.I., and A.S. designed the study. K.O. carried out all the experiments. N.R. and F.K.H. synthesized RTS-V5. K.O., M.I., T.A., N.R., W.A.S., F.K.H., and A.S. contributed to the interpretation of the results. K.O. wrote the manuscript. N.R., W.A.S., F.K.H., and A.S. edited the manuscript. A.S. supervised the study. All Authors read and approved the final manuscript.

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