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# Delanzomib Interacts with Ritonavir Synergistically to Cause Endoplasmic Reticulum Stress in Renal Cancer Cells

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**Abstract.** *Background/Aim: To investigate the efficacy against renal cancer cells of combining the HIV protease inhibitor ritonavir with the novel proteasome inhibitor delanzomib. Materials and Methods: Renal cancer cell lines 769-P, 786-O, Caki-2 and Renca were treated with ritonavir and delanzomib in vitro and in vivo, and the efficacy of combination was evaluated. Results: The combination of ritonavir and delanzomib synergistically inhibited renal cancer growth and suppressed colony formation. It induced robust apoptosis evidenced by increased cell population in the sub-$G_1$ fraction and increased number of annexin-V-positive cells. A 13-day treatment with the combination was well tolerated in the mouse model and inhibited tumor growth significantly. Mechanistically, the combination synergistically induced endoplasmic reticulum stress and inhibited the mammalian target of rapamycin (mTOR) pathway. Conclusion: The effectiveness of combination of ritonavir and delanzomib appears to be due to the induction of ER stress and inhibition of the mTOR pathway.*

Renal cell carcinoma (RCC) accounts for approximately 3% of all malignancies in adults, its morbidity and mortality rates worldwide are rising about 2-3% per decade (1, 2), and 25-30% of patients with RCC present with metastatic disease at the time of diagnosis (1). Since 2005, targeted therapies such as inhibitors of tyrosine kinase, and of the mammalian target of rapamycin (mTOR), have been widely used against metastatic RCC (3). Although these treatment modalities have improved the prognosis of patients with RCC, the median overall survival of those with metastatic disease remains still approximately 26 months and complete responses remain the exception (4). Therefore, there remains a need for more effective regimens and agents targeting novel pathways in RCC.

The endoplasmic reticulum (ER), which mediates folding and maturation of the majority of proteins that a cell secretes or displays on its surface, is essential for diverse vital functions (5). It has been recognized as a major site of regulation of cellular homeostasis, particularly in the unfolded protein response (6). To avoid the production of an overload of unfolded proteins that cannot be properly processed, ER stress signaling mediates transient reduction of overall protein expression (5). However, a pro-apoptotic branch of the ER stress response is activated when cells cannot cope with ER stress (7). Agents inducing ER stress have, therefore, recently been used to kill cancer cells in clinical applications (8).

The HIV protease inhibitor ritonavir has been widely used to treat HIV infection and was recently shown to increase unfolded proteins by suppressing the function of heat-shock protein 90 (HSP90) (9).

Delanzomib (CEP-18770) is a novel proteasome inhibitor and clinical phase I trials have been completed in patients with multiple myeloma and solid tumors other than prostate and breast cancer (10).

We postulated that combining ritonavir with delanzomib would cause accumulation of unfolded proteins synergistically by both increasing their amount and preventing their degradation, leading to effective induction of ER stress. In the current study, the efficacy of combination of ritonavir and delanzomib to kill RCC cells *in vitro* and *in vivo* was evaluated and its underlying mechanisms were investigated.

## Materials and Methods

*Cell cultures.* Human RCC cell lines 769-P, 786-O and Caki-2, and the murine RCC cell line Renca were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were routinely maintained in RPMI or McCoy's 5A medium, depending on the cell line, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37˚C in a humidified atmosphere with 5% $CO_2$.

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*Reagents*. Ritonavir purchased from Toronto Research Chemicals (Toronto, ON, Canada) and delanzomib purchased from Selleck Chemicals (Houston, TX, USA) were dissolved in dimethyl sulfoxide (DMSO). The reagents were stored at −20˚C until use.

*Cell viability assay*. Cells were seeded at a density of $5\times10^3$ cells per well on 96-well culture plates, allowed to attach for 24 hours, then treated with different concentrations of ritonavir (25-50 μM) with/without delanzomib (25-50 nM) for an additional 48 hours. At the end of the incubation period, cell viability was measured by MTS assay (CellTiter 96 Aqueous kit; Promega, Madison, WI, USA) according to the manufacturer's protocol.

*Colony-formation assay*. Cells were seeded in 6-well plates at a density of 100 cells per well 1 day before being treated with 50 μM ritonavir with/without 25 nM delanzomib for 48 hours. Ten days later, the cells were washed with phosphate-buffered saline (PBS), fixed in 100% methanol, and stained with Giemsa's solution (Muto, Tokyo, Japan). The numbers of colonies were then counted.

*Flow cytometry*. For flow cytometry to evaluate changes in the cell cycle distribution and induction of apoptosis, $1.5\times10^5$ cells were seeded in a 6-well culture plate 1 day prior to treatment with different concentrations of ritonavir with/without delanzomib for 48 h. They were then washed with PBS and harvested by trypsinization. For cell-cycle analysis, harvested cells were resuspended in citrate buffer and stained with propidium iodide. For annexin V assay, cells were stained with annexin V and 7-amino-actinomycin D (7-AAD) according to the manufacturer's protocol (Beckman Coulter, Marseille, France) and analyzed by flow cytometry using CellQuest Pro software (BD Biosciences, San Jose, CA, USA).

*Murine renal cancer model*. The animal protocol for this experiment was approved by the institutional Animal Care and Use Committee of National Defense Medical College (approval number 14068). Five-week-old male nude mice (strain BALB/c Slc-*nu*/*nu*) were obtained from CLEA (Tokyo, Japan). Renca cells ($1\times10^7$ cells) were implanted subcutaneously into the flank of each mouse and treatments were initiated 5 days after implantation (day 1). The mice were then randomly assigned to four groups, each consisting of five animals. The control group received intraperitoneal injections of dimethylsulfoxide solution (vehicle), and the other groups received ritonavir (15 mg/kg) two delanzomib (30 μg/kg). Injections were given once a day, 5 days a week, for 2 weeks. The mice were carefully monitored and tumor size was measured every 2 days. Tumor volume was calculated as one half of the product of the tumor length and the square of the width (volume=$0.5 \times \text{length} \times \text{width}^2$).

*Western blotting*. Changes in protein expression caused by the combination were evaluated using western blotting. Cells were treated with 50 μM ritonavir with/without 50 nM delanzomib for 12, 24, or 48 h and total protein lysates were prepared with RIPA-buffer containing 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 50 mM Tris (pH 7.6), and 10 μl/ml protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA) for 30 min on ice. Equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% (w/v) skimmed milk, washed, and incubated with primary antibodies at room temperature for 1 h or at 4˚C overnight. The primary antibodies used were against acetylated histone (Abcam, Cambridge, UK), histone deacetylase (HDAC)1, HDAC2, HDAC3, HDAC6, cyclin-dependent kinase (CDK) 4, cyclin D1, glucose-regulated protein 78 (GRP78), ubiquitinated proteins, sestrin-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), ER-resident protein (ERp) 44, cleaved poly(ADP-ribose) polymerase (PARP), endoplasmic oxidoreductin-1-like protein (ERO1L), mammalian target of rapamycin (mTOR), phosphorylated mTOR (p-mTOR), S6 ribosomal protein (S6) (Cell Signaling Technology, Danvers, MA, USA), adenosine monophosphate-activated protein kinase (AMPK) (ProteinTech Group, Chicago, IL, USA), light chain 3 (LC3) (Cosmo Bio Co, Tokyo, Japan), and actin (Millipore, Billerica, MA, USA). They were then incubated with horseradish peroxidase (HRP)-tagged secondary antibodies (Bio-Rad, Hercules, CA, USA) for 1 h. The bands were visualized by chemiluminescence with the ECL Plus system (GE Healthcare, Wauwatosa, WI, USA) according to the manufacturer's instructions.

*Statistical analysis*. The Chou-Talalay method was used for calculating combination indices using CalcuSyn software (Biosoft, Cambridge, UK) (11). The statistical significance of the observed differences between differently treated groups was determined using the Mann-Whitney *U*-test (StatView software; SAS Institute, Cary, NC, USA). Values of $p<0.05$ were considered to indicate a statistically significant difference.

## Results

*Renal cancer growth in vitro and in vivo*. The efficacy of the combination treatment against RCC cells was first evaluated using MTS assay. The combination of ritonavir and delanzomib inhibited renal cancer growth effectively (Figure 1A). Microscopic examination also revealed this marked combined effect in that the majority of the cells treated with the combination were floating (Figure 1B). Isobologram analysis using the Chou-Talalay method revealed that the combined effect on cell growth was in fact synergistic (combination indices <0.9) under most of the treatment conditions (Table I). It was then investigated whether the combination of ritonavir and delanzomib affected the clonogenic survival of renal cancer cells. The combination treatment inhibited colony formation of 769-P and 786-O cells almost completely, whereas ritonavir or delanzomib alone did so only moderately (Figure 1C). Thus, the combination of ritonavir with delanzomib efficiently inhibited long-term growth of renal cancer cells *in vitro*. In murine subcutaneous models, a 13-day treatment with the combination of ritonavir and delanzomib was well tolerated and suppressed tumor growth significantly (Figure 1D).

*Analysis of cell-cycle distribution and apoptosis*. To follow the induction of growth arrest in renal cancer cells induced by the ritonavir–delanzomib combination, cell-cycle analysis was performed. In all cell lines, 48-h treatment with ritonavir and delanzomib increased the number of cells in the sub-$G_1$ fraction

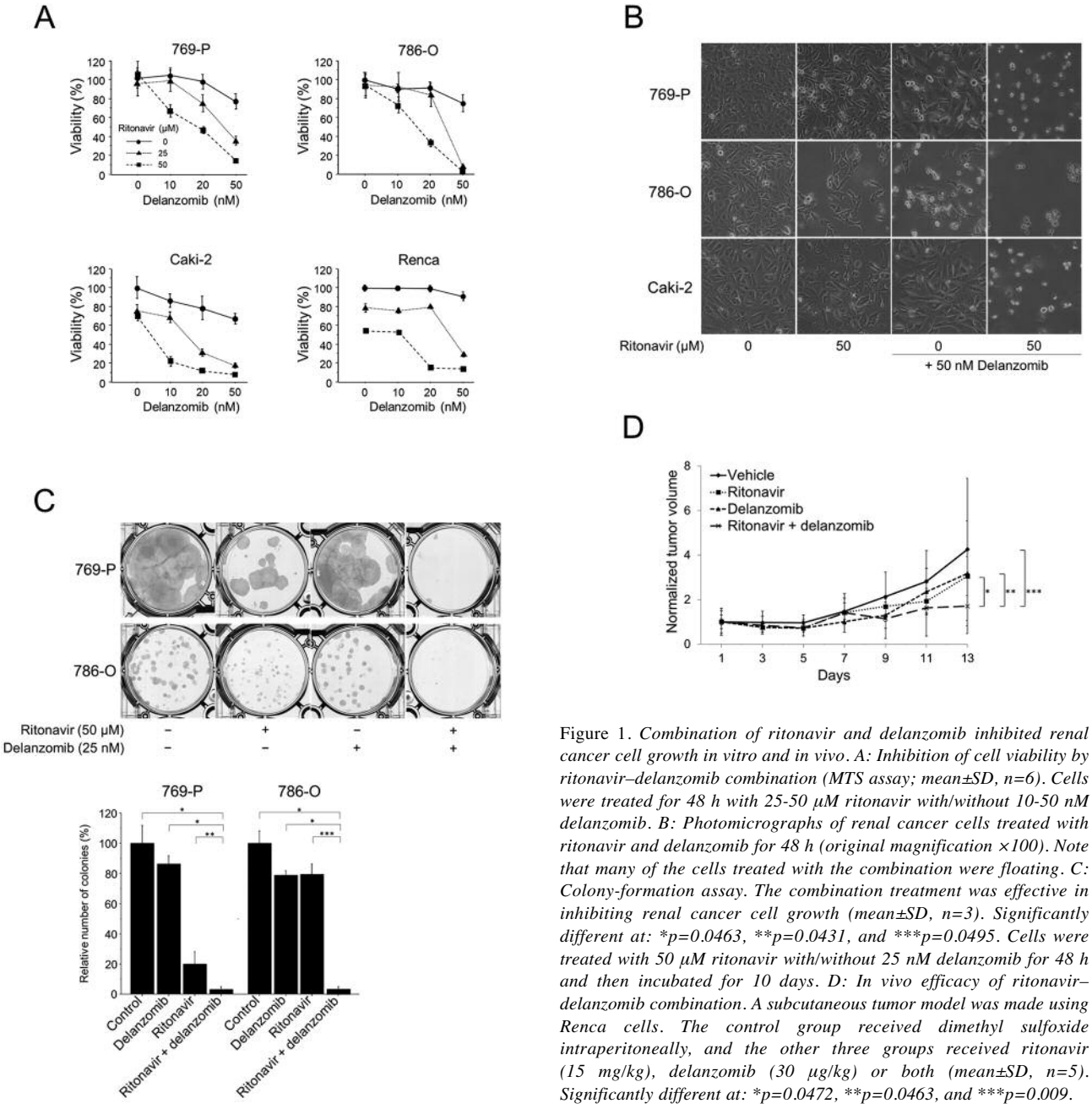


Figure 1. *Combination of ritonavir and delanzomib inhibited renal cancer cell growth in vitro and in vivo. A: Inhibition of cell viability by ritonavir–delanzomib combination (MTS assay; mean±SD, n=6). Cells were treated for 48 h with 25-50 μM ritonavir with/without 10-50 nM delanzomib. B: Photomicrographs of renal cancer cells treated with ritonavir and delanzomib for 48 h (original magnification ×100). Note that many of the cells treated with the combination were floating. C: Colony-formation assay. The combination treatment was effective in inhibiting renal cancer cell growth (mean±SD, n=3). Significantly different at: *p=0.0463, **p=0.0431, and ***p=0.0495. Cells were treated with 50 μM ritonavir with/without 25 nM delanzomib for 48 h and then incubated for 10 days. D: In vivo efficacy of ritonavir–delanzomib combination. A subcutaneous tumor model was made using Renca cells. The control group received dimethyl sulfoxide intraperitoneally, and the other three groups received ritonavir (15 mg/kg), delanzomib (30 μg/kg) or both (mean±SD, n=5). Significantly different at: *p=0.0472, **p=0.0463, and ***p=0.009.*

(Figure 2A). The combination correspondingly reduced the expression of CDK4 and cyclin D1 as shown by western blot analysis (Figure 2B). To confirm that the combination enhanced the induction of apoptosis, annexin V assay was conducted. Treatment with 50 μM ritonavir or 50 nM delanzomib alone increased the number of annexin-V-positive cells, while the combination increased it markedly (Figure 2C). Accordingly, increased levels of cleaved PARP were detected following the combination treatment (Figure 2D). Thus, the combination was shown to induce apoptosis of renal cancer cells.

*Induction of ER stress*. As anticipated, a 48-h treatment with the combination of ritonavir and delanzomib synergistically increased the expression of the ER stress markers GRP78, ERp44, and ERO1L in all the cell lines studied here (Figure 3A). Our hypothesis was that delanzomib inhibited the

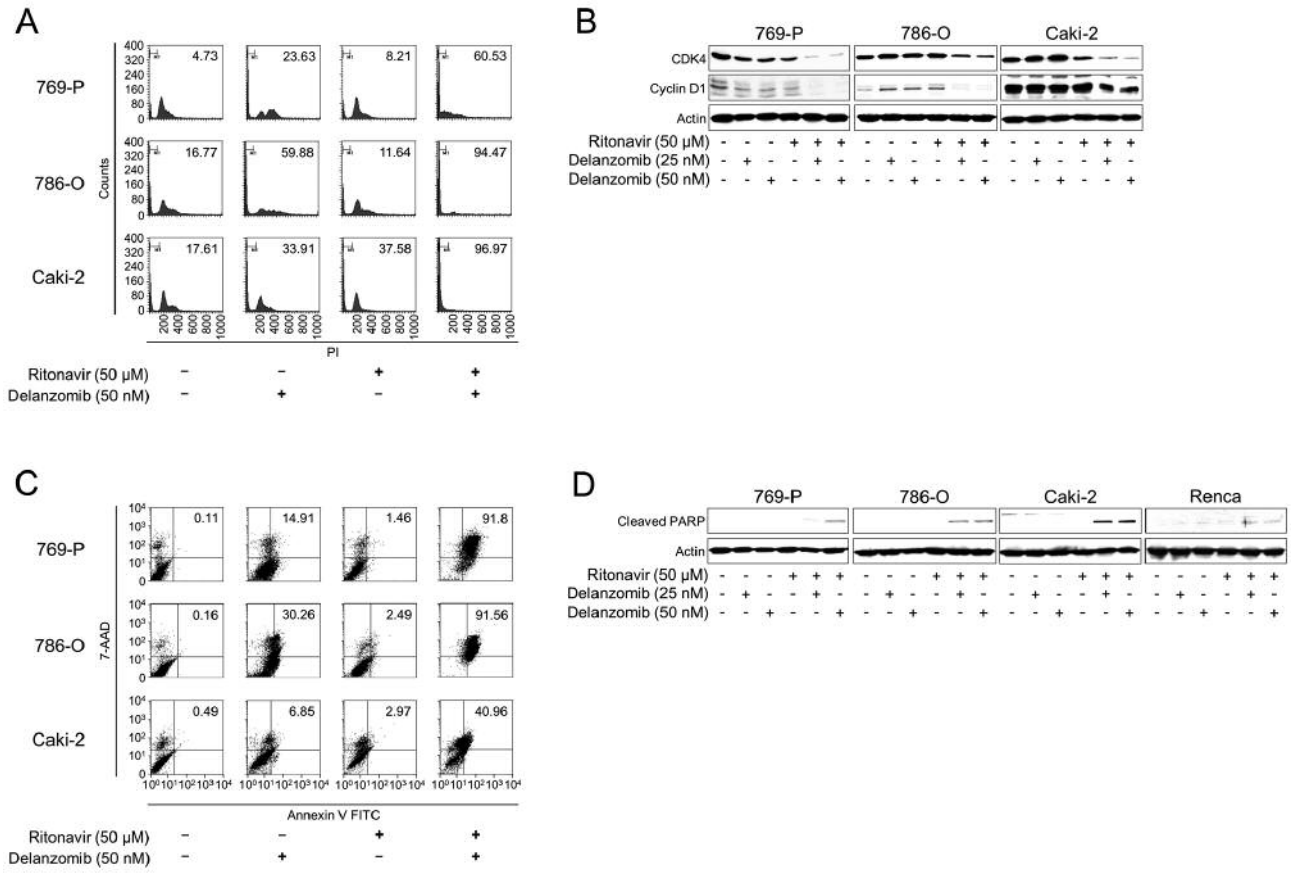


Figure 2. *Combination of ritonavir and delanzomib perturbed the cell cycle and induced apoptosis of renal cancer cells. A: Cell-cycle analysis. Cells were treated for 48 h with 50 μM ritonavir with/without 50 nM delanzomib. Ten thousand cells were counted and changes in the cell cycle were evaluated using flow cytometry. The inset number in each graph shows the percentage of cells in the sub-G1 fraction. Representative results of flow cytometry are shown. B: Western blot analysis for cyclin D1 and cyclin-dependent kinase 4 (CDK4). Cells were treated for 48 h with 25 or 50 nM delanzomib with or without 50 μM ritonavir. Actin was used as a loading control. Representative blots are shown. C: Annexin V assay. Cells were treated for 48 h with 50 μM ritonavir with/without 50 nM delanzomib. Ten thousand cells were counted and apoptotic cells were detected by annexin V assay using flow cytometry. The inset number in each graph is the percentage of annexin-V-positive cells. Representative results of flow cytometry are shown. D: Western blot analysis for cleaved poly(ADP-ribose) polymerase (PARP). Cells were treated for 48 h with 25 or 50 nM delanzomib with or without 50 μM ritonavir. Actin was used as a loading control. Representative blots are shown.*

degradation of ubiquitinated unfolded proteins which were indeed by ritonavir and thereby caused their accumulation in RCC cells.Therefore the changes in the expression of ubiquitinated proteins were examined (Figure 3A). In Renca cells, 50 μM ritonavir combined with 25 or 50 nM delanzomib caused accumulation of ubiquitinated proteins as expected. On the other hand, in other cell lines, the combination of 50 μM ritonavir and 25 or 50 nM delanzomib reduced their ubiquitination, that is seemingly incompatible with our hypothesis. However, the combination did increase the expression of the ER stress markers in all the cell lines, indicating that the unfolded protein response was induced. To further explore the mechanism of decreased expression of ubiquitinated proteins, RCC cells were treated with 50 μM

Table I. *Combination indices (CI) for the effect of the combination of ritonavir and delanzomib on renal cancer cell lines. The CI represents a quantitative definition for synergistic (CI<1), additive (CI=1), and antagonistic (CI>1) effects.*

| | | Delanzomib (nM) | | |
|---|---|---|---|---|
| Cell line | Ritonavir (μM) | 10 | 25 | 50 |
| 769-P | 25 | 0.195 | 0.137 | 0.26 |
| | 50 | 0.049 | 0.049 | 0.102 |
| 786-O | 25 | 1.157 | 0.781 | 0.003 |
| | 50 | 0.121 | 0.024 | 0 |
| Caki-2 | 25 | 0.695 | 0.068 | 0.038 |
| | 50 | 0.02 | 0.011 | 0.009 |

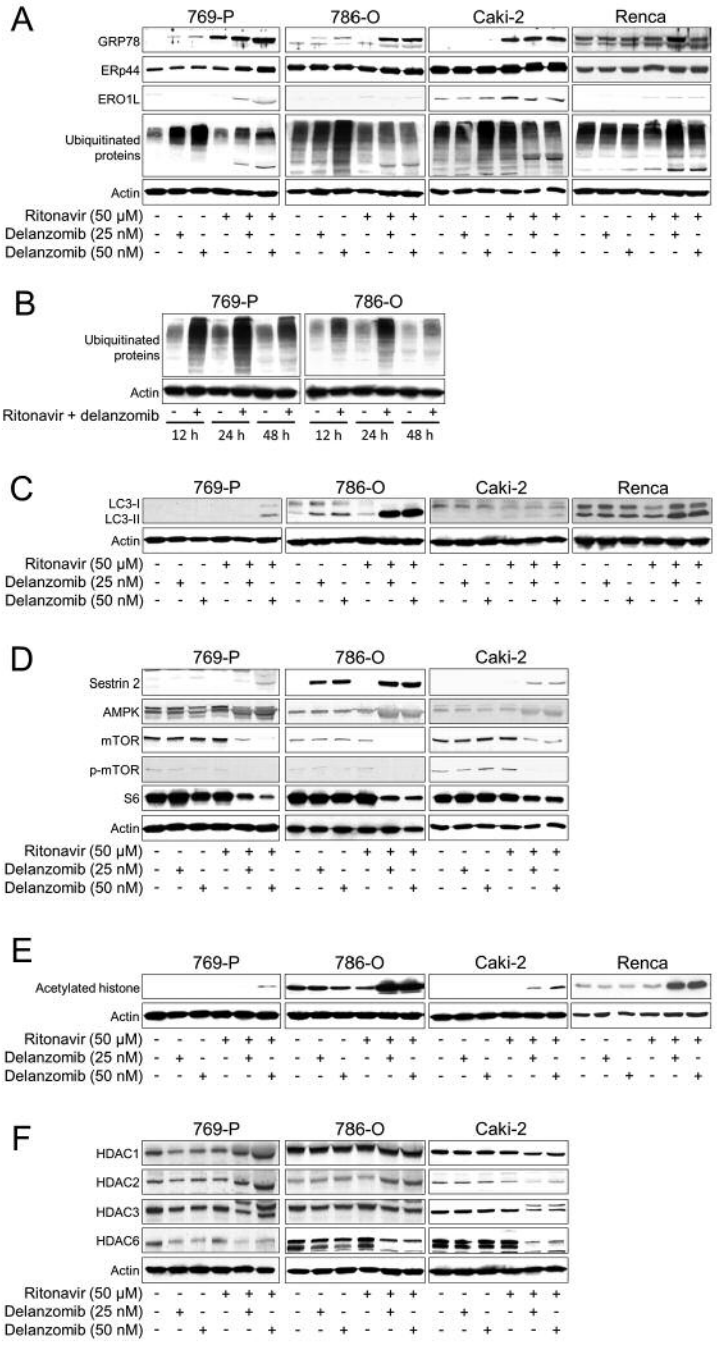


Figure 3. *Combination of ritonavir and delanzomib induced endoplasmic reticulum (ER) stress and autophagy, inhibited mammalian target of rapamycin (mTOR) pathway, and caused histone acetylation in renal cancer cells. A: Western blot analysis for the ER stress markers glucose-regulated protein 78 (GRP78), endoplasmic reticulum resident protein 44 (ERp44), and endoplasmic oxidoreductin-1-like protein (ERO1-L), and for ubiquitinated proteins. Cells were treated for 48 h with 25 or 50 nM delanzomib with or without 50 μM ritonavir. Actin was used as a loading control. Representative blots are shown. B: Western blot analysis for ubiquitinated proteins. Cells were treated for 12, 24, and 48 h with 50 μM ritonavir and 50 nM delanzomib. Actin was used for the loading control. Representative blots are shown. C: Western blot analysis for the autophagy marker light chain 3 (LC3). Cells were treated for 48 h with 25 or 50 nM delanzomib with or without 50 μM ritonavir. Actin was used for the loading control. Representative blots are shown. D: Western blot analysis for sestrin 2, AMP-activated protein kinase (AMPK), mTOR, and S6 ribosomal protein (S6) after 48-h treatment with 25 or 50 nM delanzomib with or without 50 μM ritonavir. Actin was used as a loading control. Representative blots are shown. E: Western blot analysis for acetylated histone after 48-h treatment with 25 or 50 nM delanzomib with or without 50 μM ritonavir. Actin was used as a loading control. Representative blots are shown. F: Western blotting for histone deacetylase (HDAC) 1,-2,-3, and-6 after 48-h treatment with 25 or 50 nM delanzomib with or without 50 μM ritonavir. Actin was used as a loading control. Representative blots are shown.*

ritonavir and 50 nM delanzomib for 12, 24, and 48 h and the changes in the expression of ubiquitinated proteins were examined (Figure 3B). In this experiment, the expression of ubiquitinated proteins was increased by the combination in a time-dependent manner up to 24 h and then decreased at 48 h. Excessive ubiquitinated proteins are accumulated and aggregate, and are shifted into the detergent-insoluble fraction (12). Therefore, the decrease in the amount of ubiquitinated proteins at 48 h was thought to reflect excessive accumulation, not an actual decrease.

*Induction of autophagy, inhibition of mTOR pathway, and induction of histone acetylation*. Studies showed that HIV protease inhibitors induced ER stress and autophagy, and inhibited mTOR activity in cancer cells (13, 14). We believed that delanzomib, in combination, might enhance these effects of ritonavir. As anticipated, the combination of ritonavir and delanzomib induced autophagy in all the cell lines, as evidenced by the increased expression of the autophagy marker LC3-II (Figure 3C). This autophagy induction is consistent with the observed decreased in ubiquitinated protein expression because aggregated proteins are degraded by autophagy (15). As induction of ER stress reportedly inhibits the mTOR pathway (14), the combination's effect on the mTOR pathway were evaluated. The combination of ritonavir and delanzomib increased the expression of the endogenous mTOR inhibitors sestrin-2 and AMPK and reduced that of mTOR, phosphorylated mTOR, and S6 ribosomal protein (Figure 3D). Thus, the combination was thought to inhibit the mTOR pathway by suppressing both the expression and the function of mTOR.

Because we previously showed that inducing ER stress caused histone acetylation in RCC cells (16), we believed that the combination of ritonavir and delanzomib would also cause histone acetylation. As anticipated, the combination increased the expression of acetylated histone (Figure 3E). To investigate the mechanism of this histone acetylation, the expression of HDACs was evaluated. The ritonavir–delanzomib combination reduced the expression of HDACs 1,-2,-3, and-6 in Caki-2 cells and the expression of HDAC6 in 769-P and 786-O cells (Figure 3F). We attributed this reduction of HDACs to be one mechanism of histone acetylation caused by the combination.

## Discussion

In the present study, we provided evidence that ritonavir, a widely used HIV protease inhibitor, in combination with delanzomib, a novel proteasome inhibitor, effectively suppresses the growth of RCC cells. The effect of the ritonavir-delanzomib combination is associated with induction of ER stress, as indicated by our findings that the combination increased the expression of the ER stress markers GRP78, ERp44, and ERO1L. Profound ER stress has been reported to be toxic to tumor cells (12). Malignant cells are characterized by increased basal ER stress (compared with that of nonmalignant cells), so increasing ER stress may selectively eliminate malignant cells (17).

The HIV protease inhibitor ritonavir has proven its clinical potential in treating cancer (18). There are several likely reasons for ritonavir's antitumor activity, including its inhibitory effects on the AKT8 virus oncogene cellular homolog (AKT) pathway (19), nuclear factor-kappa B (20), and HSP90 (9). Our group previously showed that ritonavir inhibited renal cancer growth by inhibiting heat-shock factor 1, a transcription factor of HSP90, when used in combination with 17-allylamono-17-demethoxygeldanamycin (21), showing the importance of the suppression of HSP90 in inhibiting renal cancer growth. Unfortunately, ritonavir exhibits limited efficacy as a single agent. Ritonavir alone is thought to fail to induce enough ER stress to kill cancer cells because although it increases the amount of unfolded proteins, these are degraded by the proteasome if it functions normally. Delanzomib suppresses proteasome function and blocks the degradation of unfolded protein (22). We, therefore, thought that the combination of ritonavir and delanzomib would lead to accumulation of unfolded proteins, causing enough ER stress to kill cancer cells effectively. Indeed, the combination of ritonavir with delanzomib inhibited renal cancer growth synergistically both *in vitro* and *in vivo* and induced ER stress, as postulated.

Autophagy is thought to be another cellular process associated with enhanced ER stress (23). It seems to be induced in response to cellular stress, such as nutrient deprivation, hypoxia, and toxin accumulation (24). Although its precise mechanisms remain unclear, autophagy may be associated with excessive ER stress in the current combination treatment. It may help degrade unfolded proteins and engulf overloaded parts of the ER. Treatment of cancer cells with ER stress-inducing drugs also results in the expression of endogenous mTOR inhibitor sestrin-2, indicating that ER stress inhibits mTOR activity (14). The inhibition of mTOR favors the concomitant induction of autophagy, which is often associated with programmed cell death in tumor cells (25). Profound autophagy affects cancer cells in various ways, in some cases causing growth arrest and triggering apoptosis (26). Although it is unclear whether apoptosis and autophagy are interconnected in this setting, induction of autophagy may be another important mechanism of action of the combination of ritonavir with delanzomib.

Induction of histone acetylation is thought to be another important mechanism of action because histone acetylation is an epigenetic phenomenon that causes increased transcriptional activity and inhibits cancer cell growth (27, 28). The exact mechanism of this enhanced histone acetylation is unknown, but the current study showed that the ritonavir–delanzomib combination reduced the expression of

HDACs. This reduction in HDAC expression was also reported in our previous study using the combination of ritonavir and other proteasome inhibitors (29) and might in part explain the enhanced histone acetylation. Among HDACs, inhibition of HDAC6 has been reported to induce hyperacetylation of HSP90, leading to suppression of its chaperone function (30). Decreased expression of HDAC6 by combination of ritonavir with delanzomib might further suppress HSP90 function, thus leading to enhancement of accumulation of ubiquitinated proteins.

To our knowledge, this is the first study that showed a beneficial combined effect of ritonavir and delanzomib on cancer cells, and it may help exploit the therapeutic potential of novel ER stress-based treatments against renal cancer. The combination may be tested in patients with advanced RCC that is refractory to current treatment modalities because the combination acts by completely different mechanisms of action: by inducing ER stress and histone acetylation. However, a phase-I trial with careful monitoring of drug concentration will be needed because ritonavir is also a potent cytochrome P450 inhibitor (31) and therefore could increase serum concentration of delanzomib by inhibiting its degradation by the liver.

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