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Ritonavir Interacts With Belinostat to Cause Endoplasmic Reticulum Stress and Histone Acetylation in Renal Cancer Cells

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The histone deacetylase (HDAC) inhibitor belinostat increases the amount of unfolded proteins in cells by promoting the acetylation of heat shock protein 90 (HSP90), thereby disrupting its chaperone function. The human immunodeficiency virus protease inhibitor ritonavir, on the other hand, not only increases unfolded proteins by suppressing HSP90 but also acts as a proteasome inhibitor. We thought that belinostat and ritonavir together would induce endoplasmic reticulum (ER) stress and kill renal cancer cells effectively. The combination of belinostat and ritonavir induced drastic apoptosis and inhibited the growth of renal cancer cells synergistically. Mechanistically, the combination caused ER stress (evidenced by the increased expression of the ER stress markers) and also enhanced histone acetylation by decreasing the expression of HDACs. To our knowledge, this is the first study that showed a beneficial combined effect of belinostat and ritonavir in renal cancer cells, providing a framework for testing the combination in renal cancer patients.

Key words: Belinostat; Ritonavir; Endoplasmic reticulum (ER) stress; Renal cell cancer

INTRODUCTION

Despite the wide use of tyrosine kinase inhibitors[1–4] and mammalian target of rapamycin inhibitors[5,6], there is currently no curative treatment for patients with advanced renal cancer. Development of a novel therapy is urgently needed.

Belinostat is a histone deacetylase (HDAC) inhibitor approved by the Food and Drug Administration for the treatment of relapsed or refractory peripheral T-cell lymphoma (PTCL)[7]. It is thought to increase unfolded proteins in the cell by inhibiting HDAC6, thereby disrupting the molecular chaperone function of heat shock protein 90 (HSP90)[8]. Ritonavir, on the other hand, is a human immunodeficiency virus (HIV) protease inhibitor widely used to treat HIV infection. It has recently been shown to inhibit proteasomes[9] and also increase unfolded proteins by suppressing the function of HSP90[10].

In the present study, we tried to develop a novel combination therapy against renal cancer using these two clinically available drugs. We postulated that belinostat and ritonavir would increase unfolded proteins by inhibiting HSP90 cooperatively and that ritonavir would inhibit the degradation of these unfolded proteins by suppressing the proteasome and causing the unfolded proteins to accumulate in the cell, inducing ER stress and apoptosis.

MATERIALS AND METHODS

Cell Cultures

Human renal cancer cells (769-P, 786-O, and Caki-2) were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were cultured in Roswell Park Memorial Institute medium or McCoy's 5A medium containing 10% fetal bovine serum and 0.3% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C under 5% CO_2 in a humidified incubator.

Reagents

Belinostat purchased from Selleck Chemicals (Houston, TX, USA) and ritonavir purchased from Toronto Research Chemicals (Toronto, ON, Canada) were dissolved in dimethyl sulfoxide (DMSO). The pan caspase inhibitor Z-VAD-FMK was purchased from Enzo Life Sciences (Farmingdale, NY, USA). These reagents were stored at −20°C until use.

Cell Viability Assay

Cells (5×10^3) were plated in a 96-well culture plate 1 day before being treated for 48 h with 5 μM belinostat and/or 10–50 μM ritonavir. Cell viability was evaluated by MTS assay (CellTiter 96 Aqueous kit; Promega, Madison, WI, USA) according to the manufacturer's protocol.

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Colony Formation Assay

For the colony formation assay, 100 individual cells were seeded in six-well plates 1 day before being treated for 48 h with 5 μM belinostat and/or 50 μM ritonavir. The cells were then given fresh media and cultured for 1–2 weeks. The colonies were fixed with 100% methanol, stained with Giemsa's solution, and counted.

Flow Cytometry

Flow cytometry was used to evaluate changes in the cell cycle and apoptosis. Briefly, 1.5×10^5 cells were seeded in a six-well culture plate 1 day before being cultured for 48 h in medium containing 5 μM belinostat and/or 50 μM ritonavir. They were then washed with phosphate-buffered saline and harvested by trypsinization. For cell cycle analysis, harvested cells were resuspended in citrate buffer and stained with propidium iodide. For detecting the apoptotic cells, cells were stained with annexin V according to the manufacturer's protocol (Beckman Coulter, Marseille, France) and analyzed by flow cytometry using the CellQuest Pro software (BD Biosciences, San Jose, CA, USA). To evaluate whether the apoptosis induced by the combination of belinostat and ritonavir was caspase dependent, the cells were treated with 5 μM belinostat combined with 50 μM ritonavir, with or without 40 μM Z-VAD-FMK. After 48 h, induction of apoptosis was evaluated by the annexin V assay using flow cytometry.

Western Blotting

The changes in protein expression induced by the combination were evaluated using Western blotting. The cells were treated under the indicated conditions for 48 h, 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. After the membranes were blocked by 5% skimmed milk, they were incubated overnight with anti-acetylated histone (Abcam, Cambridge, UK), anti-cyclin D1, anti-glucose-regulated protein 78 (GRP78), anti-HDAC1, anti-HDAC2, anti-HDAC3, anti-HDAC6 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-cleaved poly(ADP-ribose) polymerase (PARP), anti-endoplasmic oxidoreductin-1-like protein alpha (Ero1-Lα) (Cell Signaling Technology, Danvers, MA, USA), or anti-actin (Millipore, Billerica, MA, USA) primary antibodies. They were then incubated with horseradish-tagged secondary antibodies (Bio-Rad, Hercules, CA, USA). The bands were visualized by chemiluminescence with the ECL Plus system (GE Healthcare, Wauwatosa, WI, USA) according to the manufacturer's instruction.

Statistical Analysis

CalcuSyn software (Biosoft, Cambridge, UK) was used for calculating the combination indexes (CIs). The statistical significance of observed differences between samples was determined using the Mann–Whitney *U* test (StatView software; SAS Institute, Cary, NC, USA). A value of $p < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

The Combination of Belinostat and Ritonavir Inhibited Renal Cancer Growth Synergistically

According to the cell viability assay, the combination of belinostat and ritonavir cooperatively inhibited the growth of renal cancer cells, especially when 5 μM belinostat and 50 μM ritonavir were combined (Fig. 1A). On microscopic examination, the majority of the cells treated by the combination were floating, whereas each agent alone only decreased the number of the cells (Fig. 1B). We also evaluated the combined effect using the Chou-Talalay method to calculate CI, which demonstrated that the combined effect on cell growth was additive to synergistic effect ($CI < 1$ indicates synergistic effect, whereas $CI = 1$ indicates additive effect) (Table 1). We then investigated whether the combination of belinostat and ritonavir affects the clonogenic survival of renal cancer cells. The combination inhibited colony formation by the renal cancer cells significantly ($p = 0.0369$ for 769-P cells and $p = 0.0495$ for 786-O cells) (Fig. 2). Thus, the combination of belinostat and ritonavir was shown to inhibit renal cancer cell growth effectively.

The Combination of Belinostat and Ritonavir Induced Apoptosis

Cell cycle analysis was then used to evaluate the cell cycle changes induced by the belinostat–ritonavir combination (Fig. 3A). In all the cell lines, belinostat and ritonavir each increased the number of the cells in sub-G_1 fraction, and the combination increased it. We also found that the belinostat–ritonavir combination markedly decreased the expression of cyclin D1 (Fig. 3B), which was in accordance with the cell cycle changes induced by the combination.

The combination therapy increased the expression of cleaved PARP (Fig. 4A) and annexin V-fluorescein isothiocyanate (FITC) fluorescence intensity in renal cancer cells (Fig. 4B). The combination was thus shown to induce apoptosis as well as to increase the number of cells in the sub-G_1 fraction.

We also investigated whether the combination-induced apoptosis was caspase dependent. In Caki-2 and 786-O cells,

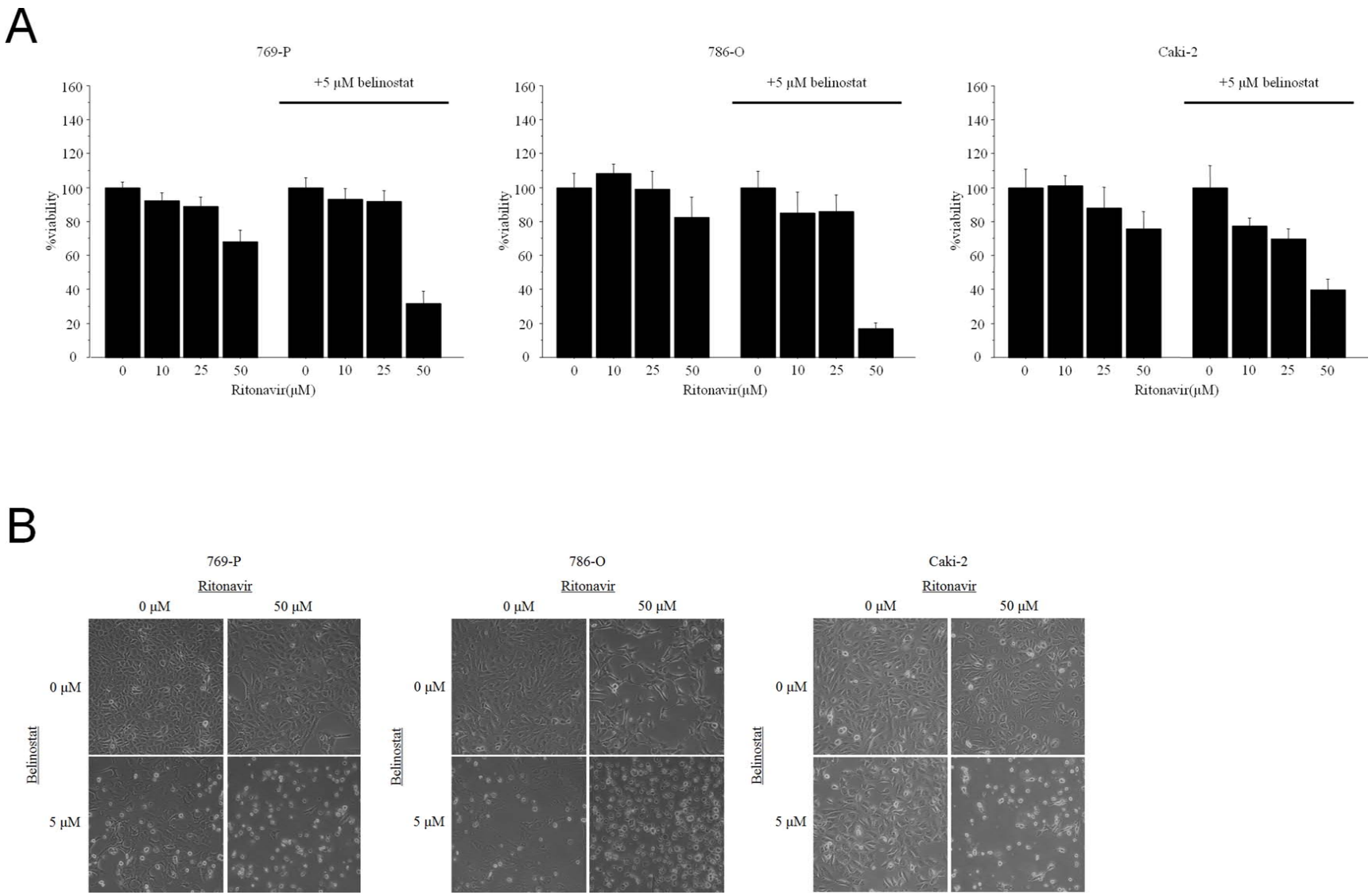

Figure 1. The combination of belinostat and ritonavir inhibited renal cancer growth effectively. (A) Cell viability assay. Cells were treated for 48 h with 5 μM belinostat and/or 10–50 μM ritonavir, and cell viability was measured using an MTS assay. Mean ± SD, $n = 6$. The viability of the control cells and that of the cells treated with 5 μM belinostat alone were both set at 100%. (B) Photomicrographs after 48-h treatment. Note that the majority of cancer cells treated with the combination were floating. Original magnification: 100×.

Table 1. Combination Indexes (CIs) for the Combination of 5 μM Belinostat and 10–50 μM Ritonavir in Renal Cancer Cells

	Ritonavir		
Cell Line	10 μM	25 μM	50 μM
769-P	0.86	0.92	0.42
786-O	1.26	1.50	0.91
Caki-2	0.83	1.01	1.08

CI < 1 indicates synergy.

coincubation with the pan caspase inhibitor Z-VAD-FMK reduced the degree to which the combination increased annexin V-FITC fluorescence intensity, whereas in 769-P cells it increased the degree to which the combination increased annexin V-FITC fluorescence intensity. This suggests that the apoptosis was at least caspase dependent.

The Combination of Belinostat and Ritonavir Induced ER Stress

Our postulation is that belinostat and ritonavir induce ER stress cooperatively. We therefore then evaluated the changes in the expression of the ER stress markers GRP78 and Ero1-Lα to see whether the combination induces ER stress (Fig. 5). In all the cell lines, 50 μM ritonavir increased the expression of GRP78, a main regulator of ER stress, showing that ritonavir alone could induce ER stress. On the other hand, 5 μM belinostat alone seemed to increase the expression of GRP78 in 769-P and 786-O cells. As we expected, in all the cell lines the expression of GRP78 was the strongest when the cells were treated with 5 μM belinostat and 50 μM ritonavir in combination. In 769-P and 786-O cells, Ero1-Lα expression was also the strongest after treatment with 5 μM belinostat and 50 μM ritonavir, consistent with the changes in the GRP78 expression. Thus, the combination was shown to induce ER stress cooperatively.

The Combination of Belinostat and Ritonavir Enhanced Histone Acetylation

Because belinostat is an HDAC inhibitor, we then examined changes in the acetylation status of histone that were caused by the combination. Belinostat alone increased histone acetylation, and, surprisingly, ritonavir enhanced this acetylation in a dose-dependent manner (Fig. 6A). To further explore the mechanism by which the combination enhanced histone acetylation, we next evaluated the changes in the expression of HDACs, which control the acetylation status of histone. We found that the combination decreased the expression of HDACs (Fig. 6B) and inferred that this decreased HDAC expression would be one mechanism of the enhanced histone acetylation caused by the combination.

DISCUSSION

Currently, there is no curative therapy against advanced renal cell carcinomas, although kinase inhibitors and mammalian target of rapamycin inhibitors are widely used[1–6]. A new treatment approach is needed. The present study shows that the combination of belinostat and ritonavir is potentially useful for implementing this approach in the treatment of advanced renal cell cancer.

The acetylation and deacetylation of histones are crucial in the modulation of chromatin structure[11]. Deacetylation of histones tightens their interaction with DNA, leading to a closed chromatin structure that inhibits gene transcription[12]. The exact mechanism of action of HDAC inhibitors is unknown, but compounds targeting HDACs are known to have multiple effects and be potent inducers of growth arrest, differentiation, and apoptosis of transformed cells in vitro and in vivo[13,14]. Furthermore, HDAC inhibitors are thought to increase the amount of unfolded proteins in cells by inhibiting the function of molecular chaperones because inhibition of HDAC6 acetylates molecular chaperones such as HSP90, suppressing their function[8]. Belinostat is a novel HDAC inhibitor that provides epigenetic control of gene expression[15]. In a phase II trial in which belinostat was given to patients with relapsed or refractory PTCL or cutaneous T-cell lymphoma (CTCL) who had failed ≥1 prior systemic therapies, an objective response was seen in 25% of the PTCL patients and 14% of the CTCL patients[16]. On the other hand, solid tumors have been proven to be refractory to monotherapy with HDAC inhibitors, and they are optimally used in combination with chemotherapies, targeted therapies, radiation, or other epigenetic modifiers rather than as single agents[17].

Ritonavir is an HIV protease inhibitor widely used against HIV infection and has recently been found to have antitumor activity. It has been shown to inhibit the Akt pathway[18], NF-κB[19], HSP90[10], and proteasomes[9]. Our group previously reported that ritonavir used in combination with 17-allylamino-17-demethoxygeldanamycin inhibited renal cancer growth by decreasing the expression of heat shock factor 1[20], an HSP90 transcription factor, showing the importance of suppressing HSP90 in the inhibition of renal cancer growth.

We thought that the combination of ritonavir and an HDAC inhibitor cooperatively caused unfolded protein accumulation in the cells and thereby induced ER stress. Our group has indeed shown that the combination of ritonavir and the HDAC inhibitor suberoylanilide hydroxamic acid is synergistically lethal against renal cancer cells[21], but in that study we did not show that it induced ER stress and the mechanism of action had remained unknown. Furthermore, to the best of our knowledge, interactions between belinostat and ritonavir have not previously been investigated in any cancer cell lines.

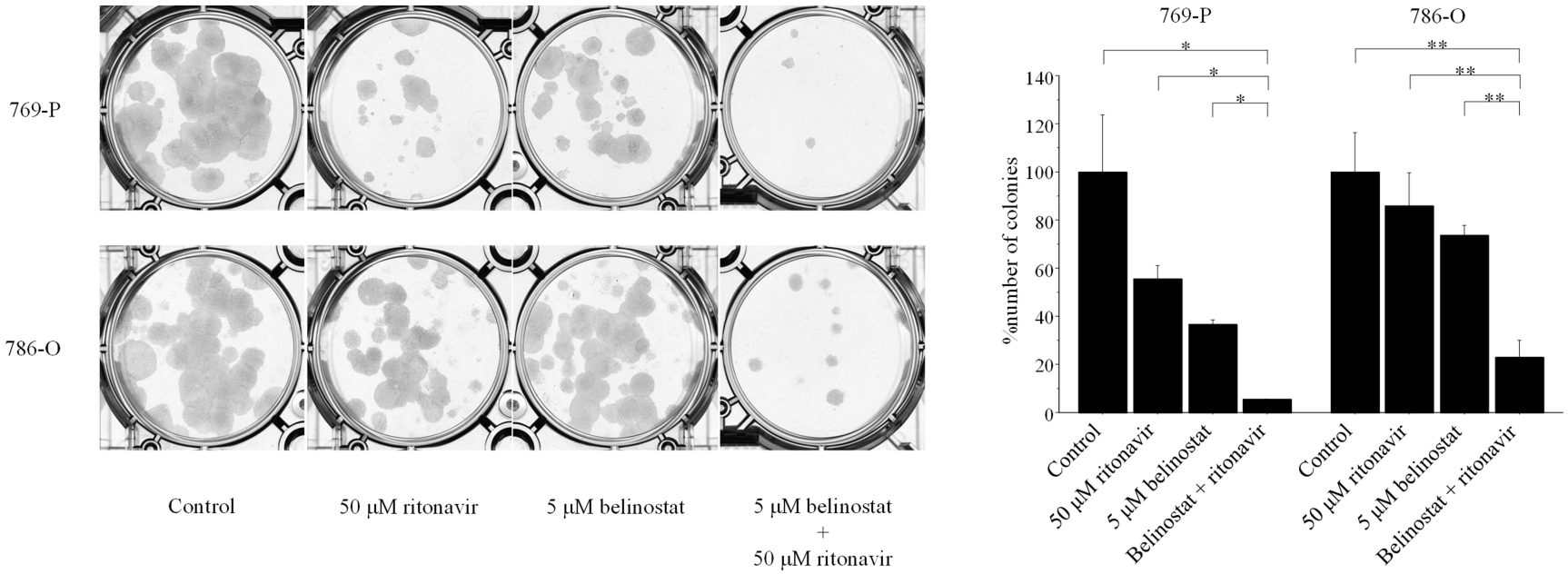

Figure 2. Colony formation assay. One hundred cells were treated for 48 h with 5 μM belinostat and/or 50 μM ritonavir. The cells were then given fresh media and allowed to grow for 1–2 weeks. Mean±SD, $n=3$. *$p=0.0369$ for 769-P cells, **$p=0.0495$ for 786-O cells.

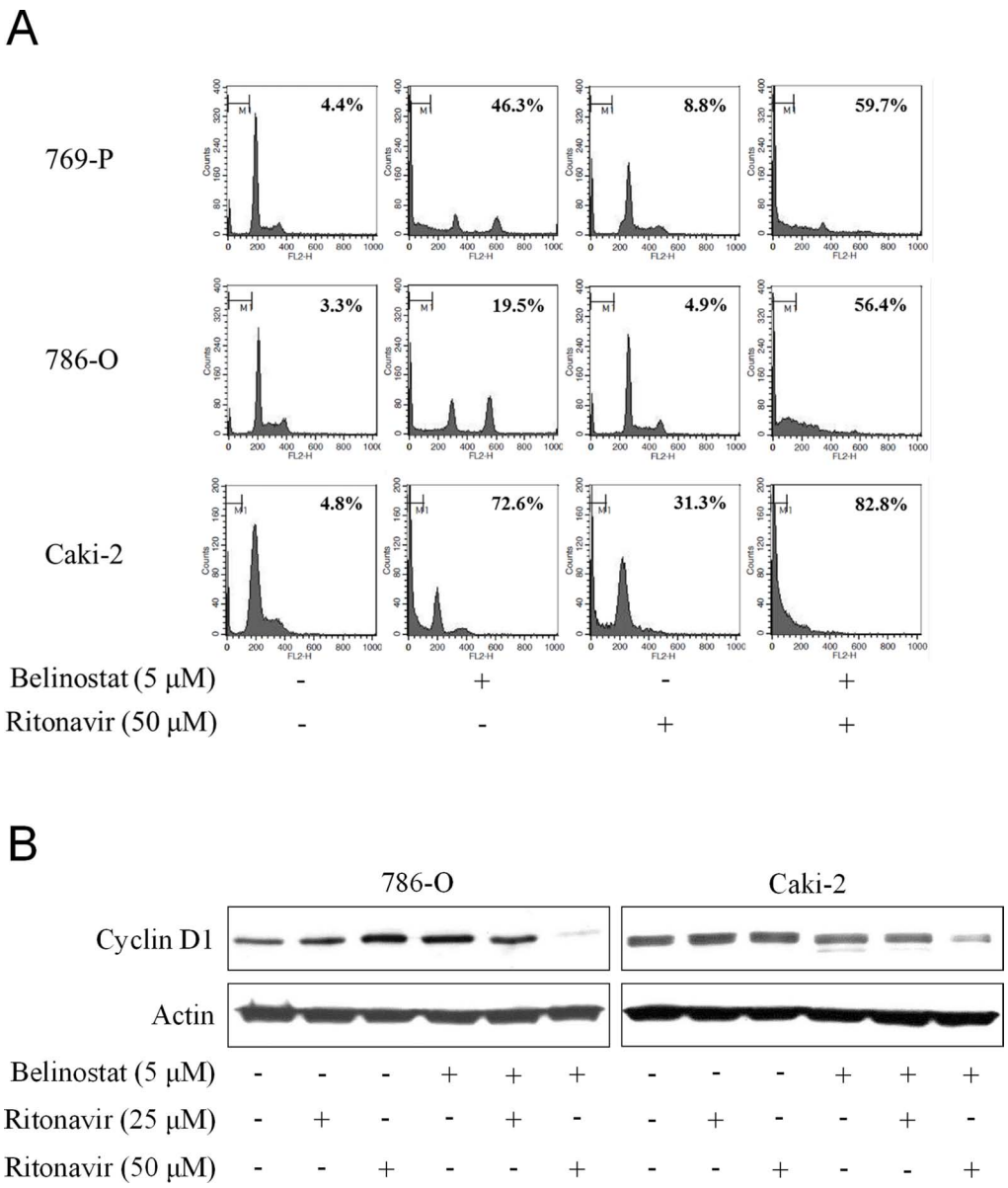

Figure 3. The combination of belinostat and ritonavir perturbed the cell cycle and decreased the expression of cyclin D1 in renal cancer cells. (A) Cell cycle analysis. Cells were treated for 48 h with 5 μM belinostat and/or 50 μM ritonavir; 10,000 cells were counted, and changes in the cell cycle were evaluated using flow cytometry. The number inset in each graph is the percentage of cells in the sub-G_1 fraction. (B) Western blotting for cyclin D1. Cells were treated for 48 h with 5 μM belinostat and/or 25 or 50 μM ritonavir. Actin was used for the loading control. Representative blots are shown.

The belinostat–ritonavir combination induced apoptosis and synergistically inhibited the growth of renal cancer cells in many of the treatment conditions. As we postulated, the combination induced ER stress cooperatively as evidenced by the increased expression of ER stress markers such as GRP78 and Ero1-Lα. Ritonavir itself was shown to induce ER stress to lesser extent than did the combination. This is compatible with ritonavir's mechanism of action, namely, inhibition of both proteasomes[9] and HSP90[10]. The addition of belinostat was thought to further inhibit HSP90[8] and therefore further increase unfolded proteins, thus enhancing the ER stress. This induction of ER stress is an important mechanism of action by the combination because profound ER stress due to the accumulation of unfolded proteins has reportedly been toxic to tumor cells[22]. The present result is also consistent with our previous studies showing the importance of ER stress induction in inhibiting renal cancer growth[23,24].

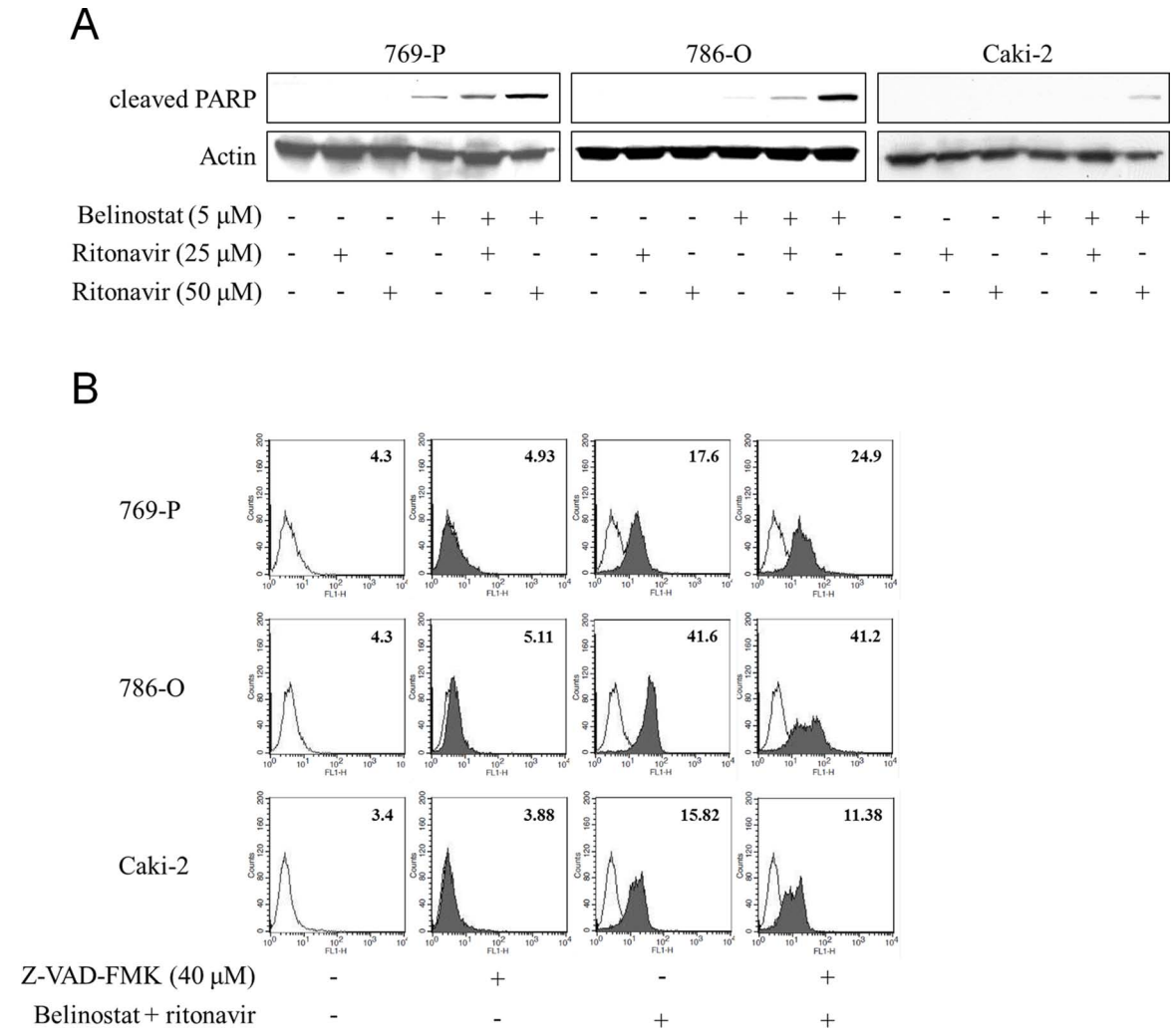

Figure 4. The combination of belinostat and ritonavir induced apoptosis in renal cancer cells. (A) Western blotting for cleaved PARP. Cells were treated for 48 h with 5 μM belinostat and/or 25 or 50 μM ritonavir. Actin was used for the loading control. Representative blots are shown. (B) Annexin V assay. Cells were treated for 48 h with the combination of 5 μM belinostat and 50 μM ritonavir with or without 40 μM pan caspase inhibitor Z-VAD-FMK; 10,000 cells were counted, and apoptotic cells were detected by annexin V assay using flow cytometry. The number inset in each graph shows annexin V fluorescein intensity. Gray, control; white, treated.

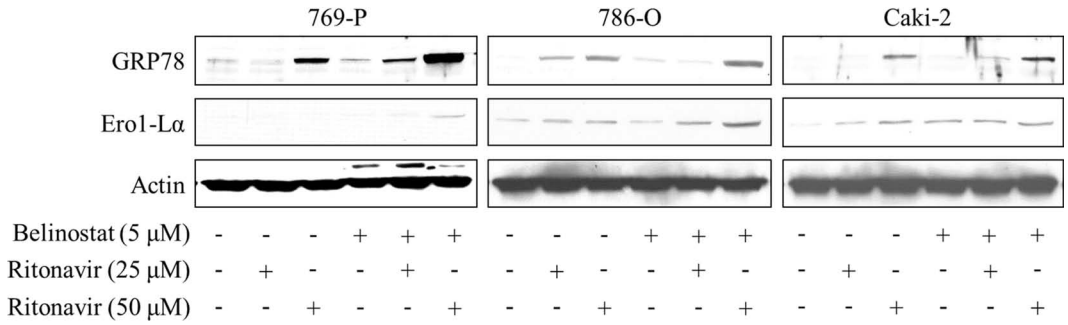

Figure 5. The combination of belinostat and ritonavir induced endoplasmic reticulum (ER) stress. Western blotting for ER stress markers. Cells were treated for 48 h with 5 μM belinostat and/or 25 or 50 μM ritonavir. Actin was used for the loading control. Representative blots are shown.

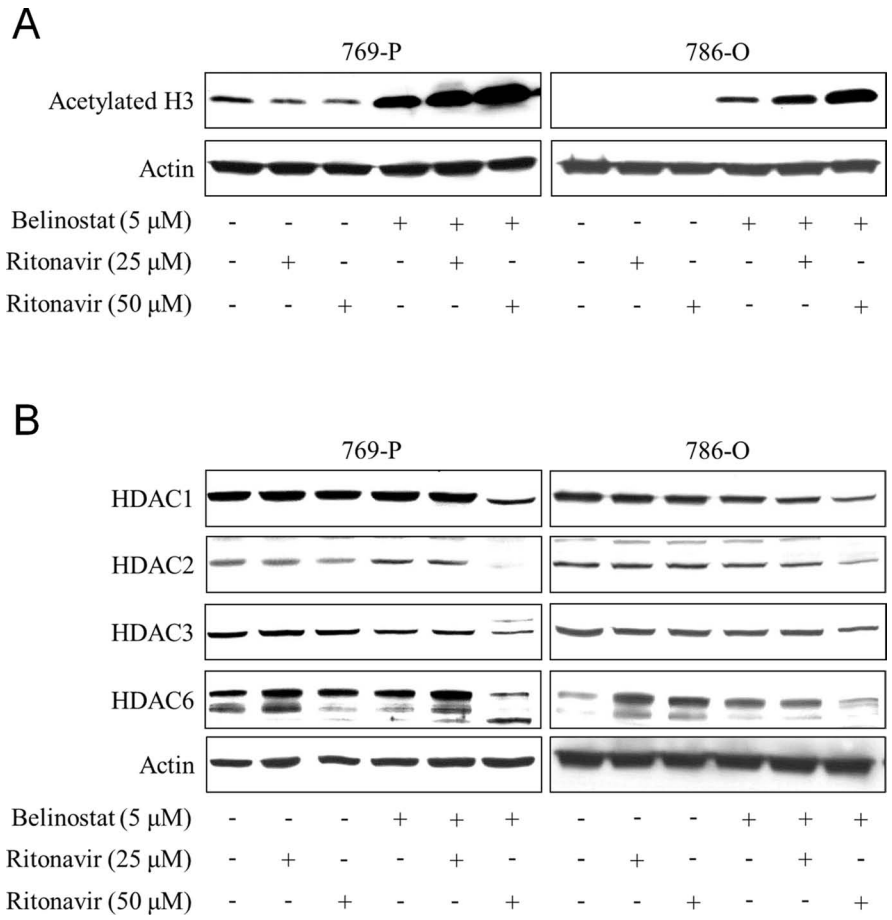

Figure 6. The combination of belinostat and ritonavir enhanced histone acetylation. Western blotting for acetylated histone (A) and histone deacetylases (B). Cells were treated for 48 h with 5 μM belinostat and/or 25 or 50 μM ritonavir. Actin was used for the loading control. Representative blots are shown.

Ritonavir also enhanced the histone acetylation caused by belinostat, and this enhanced acetylation is thought to be another important mechanism of the combination's action therapy because histone acetylation is an epigenetic approach that causes increased transcriptional activity and inhibits cancer growth[25]. Furthermore, we found that the combination decreased the expression of HDACs, which in part explained the enhanced histone acetylation. However, the exact mechanism of this decreased HDAC expression has not been revealed by the present study.

To our knowledge, this is the first study that showed a beneficial combined effect of belinostat and ritonavir in cancer cells. In addition, it provides a basis for testing the combination in patients with advanced renal cell carcinoma that is refractory to current treatment modalities because the combination acts by completely different mechanisms: inducing ER stress and histone acetylation. A phase I trial with careful monitoring of drug concentration will be needed, however, because ritonavir is also a CYP3A4 inhibitor[26] and therefore could increase the serum concentration of belinostat by inhibiting its degradation by the liver.

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