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Panobinostat and Nelfinavir Inhibit Renal Cancer Growth by Inducing Endoplasmic Reticulum Stress

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Abstract. *Background/Aim: There is no curative treatment for patients with advanced renal cancer. We believed that the combination of the histone deacetylase inhibitor panobinostat and the human immunodeficiency virus protease inhibitor nelfinavir would kill renal cancer cells by inducing endoplasmic reticulum (ER) stress. Materials and Methods: Using renal cancer cells (769-P, 786-O, Caki-2), the ability of this combination to induce ER stress and its mechanism of action were investigated. Results: The combination of drugs induced apoptosis and inhibited cancer growth effectively both in vitro and in vivo. Mechanistically, the combination induced ER stress and histone acetylation cooperatively. ER stress induction was shown to play a pivotal role in the anticancer effect of the combination because the protein synthesis inhibitor cycloheximide significantly attenuated combination-induced apoptosis. Nelfinavir was also found to increase the expression of the mammalian target of rapamycin (mTOR) inhibitor AMP-activated protein kinase (AMPK) and inhibited the panobinostat-activated mTOR pathway. Conclusion: Panobinostat and nelfinavir inhibit renal cancer growth by inducing ER stress.*

There is currently no curative treatment for advanced renal cancer and novel therapeutic strategies are urgently needed. Drug development is highly expensive and takes quite a long time, consequently we have been trying to develop innovative therapies against urological malignancies by repositioning drugs already clinically available.

Compounds targeting histone deacetylases (HDACs) have attracted significant attention as anticancer drugs (1, 2). Besides increasing histone acetylation, HDAC inhibitors also increase the amount of unfolded proteins in cells by inhibiting the function of molecular chaperones because inhibition of HDAC6 results in increased acetylation of molecular chaperones such as heat-shock protein (HSP) 90, suppressing their function (3). These unfolded proteins are normally degraded by the proteasome (4), therefore combining an HDAC inhibitor with drugs that inhibit proteasomes is a rational approach to causing unfolded proteins to accumulate and thereby induce endoplasmic reticulum (ER) stress in urological cancer cells (5-7).

In the present study, we investigated whether the combination of the HDAC inhibitor panobinostat and the HIV protease inhibitor nelfinavir would induce ER stress and kill renal cancer cells both *in vitro* and *in vivo*. The effect of nelfinavir on panobinostat-induced activation of the mammalian target of rapamycin (mTOR) pathway was also evaluated.

Materials and Methods

Cell lines. Renal cancer cell lines (769-P, 786-O, Caki-2) were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in Roswell Park Memorial Institute medium or McCoy's 5A medium supplemented with 10% fetal bovine serum and 1.0% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37˚C in a fully humidified atmosphere of 5% CO_2.

Reagents. Panobinostat purchased from LC Laboratories (Boston, MA, USA) and nelfinavir purchased from Tocris Bioscience (Bristol, UK) were dissolved in dimethyl sulfoxide. Cycloheximide purchased from Enzo Life Sciences (Farmingdale, NY, USA) was dissolved in distilled water. All stock solutions were kept frozen at −20˚C until use.

Cell viability assay. Cells (5×10^3) were plated in a 96-well culture plate 1 day before treatment and then cultured for 48 hours in medium containing 15-60 nM panobinostat with/without 10-20 μM nelfinavir. Cell viability was determined by MTS assay (CellTiter 96 Aqueous kit; Promega, Madison, WI, USA) following the manufacturer's instructions.

Colony formation assay. A total of 150 cells were seeded in 6-well plates 1 day before being cultured for 48 hours in medium containing 15 nM panobinostat with/without 20 μM nelfinavir. The

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Key Words: Panobinostat, nelfinavir, endoplasmic reticulum stress, histone acetylation, renal cancer.

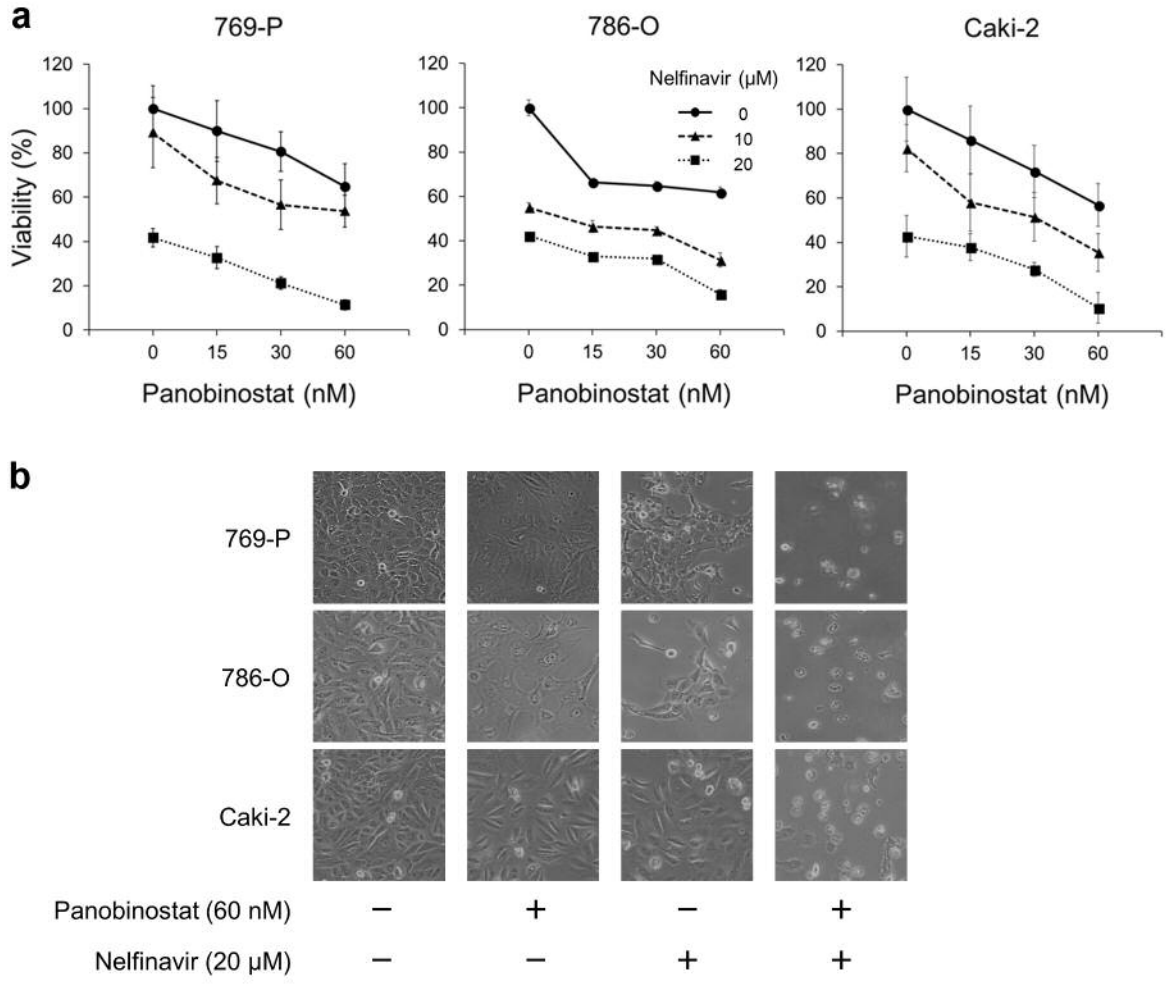

Figure 1. *Continued*

cells were then provided fresh medium and allowed to grow for 1 to 2 weeks. The colonies were then fixed with 100% methanol, stained with Giemsa's solution, and counted.

Murine xenograft model. The efficacy of the combination of panobinostat and nelfinavir *in vivo* was assessed using a murine subcutaneous xenograft model. The experimental protocol for this *in vivo* experiment was approved by the institutional Animal Care and Use Committee of National Defense Medical College. Caki-2 cells (1×10^7) were implanted subcutaneously into male BALB/c nude mice (5 weeks old, 16-20 g) purchased from CLEA Japan (Tokyo, Japan) and treatment was initiated 4 days later (day 1), when all the mice exhibited measurable tumors. The mice were divided into control and treatment groups (n=5 per group). The treated mice received intraperitoneal injections of either panobinostat (2 mg/kg) or nelfinavir (25 mg/kg) or both, while the control mice received vehicle only. The injections were given once a day, 5 days a week, for 11 days. Tumor volumes were estimated using the following formula: volume=0.5 × length × width^2. After 11 days of treatment, the animals were euthanized and the tumors were resected, weighed, and stored at −80˚C until use.

Flow cytometry. Cells (1.5×10^5) were plated in a 6-well culture plate 1 day prior to treatment. The cells were then cultured in medium containing 60 nM panobinostat with/without 20 μM nelfinavir or in medium containing 60 nM panobinostat and 20 μM nelfinavir with/without 5 μg/ml cycloheximide for 48 hours before being 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 analysis of apoptosis by annexin-V assay, cells were resuspended in binding buffer and stained with annexin V and 7-amino-actinomycin D (7-AAD) following the instructions of the assay kit manufacturer (Beckman Coulter, Marseille, France). The cells were then analyzed using a flow cytometer and CellQuest Pro Software (BD Biosciences, San Jose, CA, USA).

Western blotting. Cell lines were treated with 15-240 nM panobinostat, or with 30-60 nM panobinostat with/without 20 μM

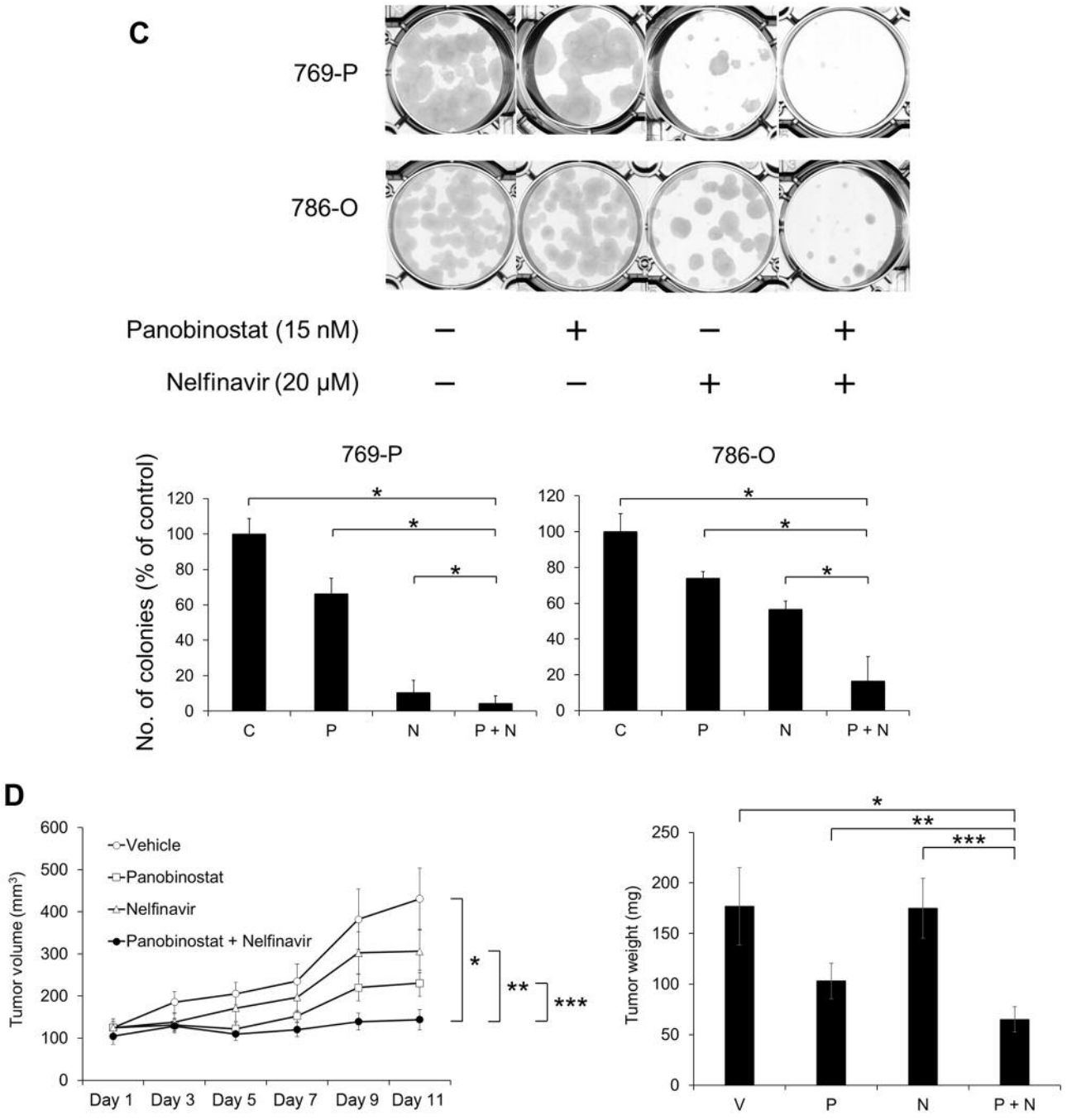

Figure 1. *The combination of panobinostat and nelfinavir inhibited renal cancer growth effectively (data are the mean±SD). A: Cell viability assay. Cells were treated for 48 h with 15-60 nM panobinostat with and without 10-20 μM nelfinavir, and cell viability was measured using the MTS assay (n=6). B: Photomicrographs after 48-h treatment. Note that the majority of cancer cells treated with the combination were floating. Original magnification, ×100. C: Colony formation assay. A total of 150 cells were treated for 48 h with 15 nM panobinostat with and without 20 μM nelfinavir. The cells were then given fresh medium and allowed to grow for 1-2 weeks then colonies were counted (n=3). C: Control; P: 15 nM panobinostat; N: 20 μM nelfinavir; P + N: 15 nM panobinostat and 20 μM nelfinavir. *Significantly different at p=0.0495. D: In vivo study. A murine xenograft model was established using Caki-2 cells. The vehicle-treated group received intraperitoneal injections of dimethyl sulfoxide and the treatment groups received 2 mg/kg panobinostat or 25 mg/kg nelfinavir or both. The injections were given once a day, 5 days a week for 11 days (n=5). Left panel: Tumor volume. *Significantly different at day 11 from: vehicle-treated (p=0.001), nelfinavir-treated (p=0.0046) and panobinostat-treated (p=0.0211) groups. Right panel, Tumor weight. V: Vehicle-treated group; P: panobinostat-treated group; N: nelfinavir-treated group; P + N: combination-treated group. Significantly different at *p=0.011, **p=0.043, and ***p=0.027 at day 11.*

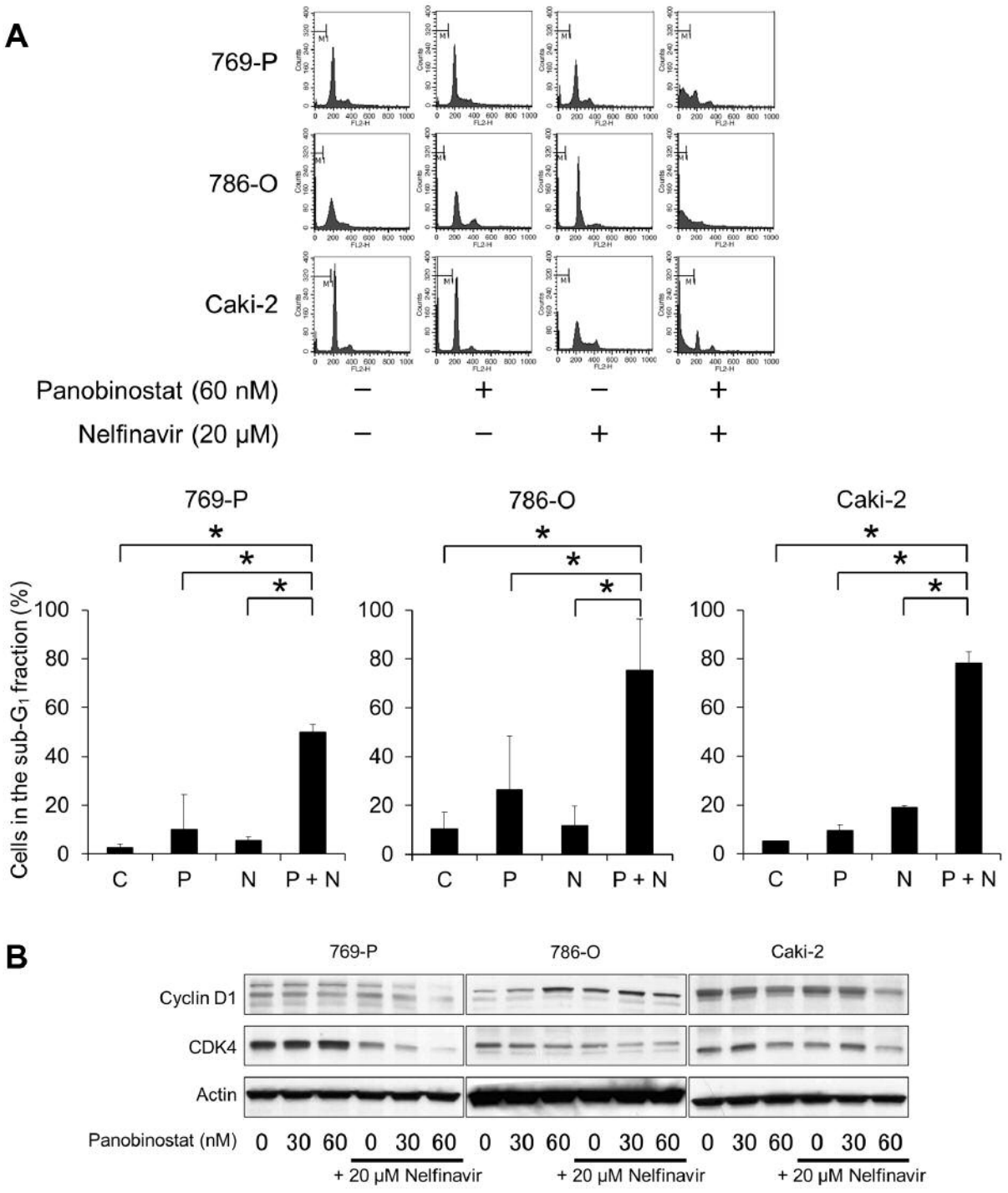

Figure 2. *Continued*

nelfinavir, or with 60 nM panobinostat and 20 μM nelfinavir with/without 5 μg/ml cycloheximide for 48 h and whole-cell lysates were obtained using radioimmunoprecipitation assay (RIPA) buffer. Tumor specimens obtained from mice used in the *in vivo* study were also homogenized using RIPA buffer and whole-cell lysates were obtained. Samples were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes and blocked in 5% non-fat skimmed milk. The following primary antibodies were used for overnight incubation: anti-AMP-activated protein kinase (AMPK) from Proteintech

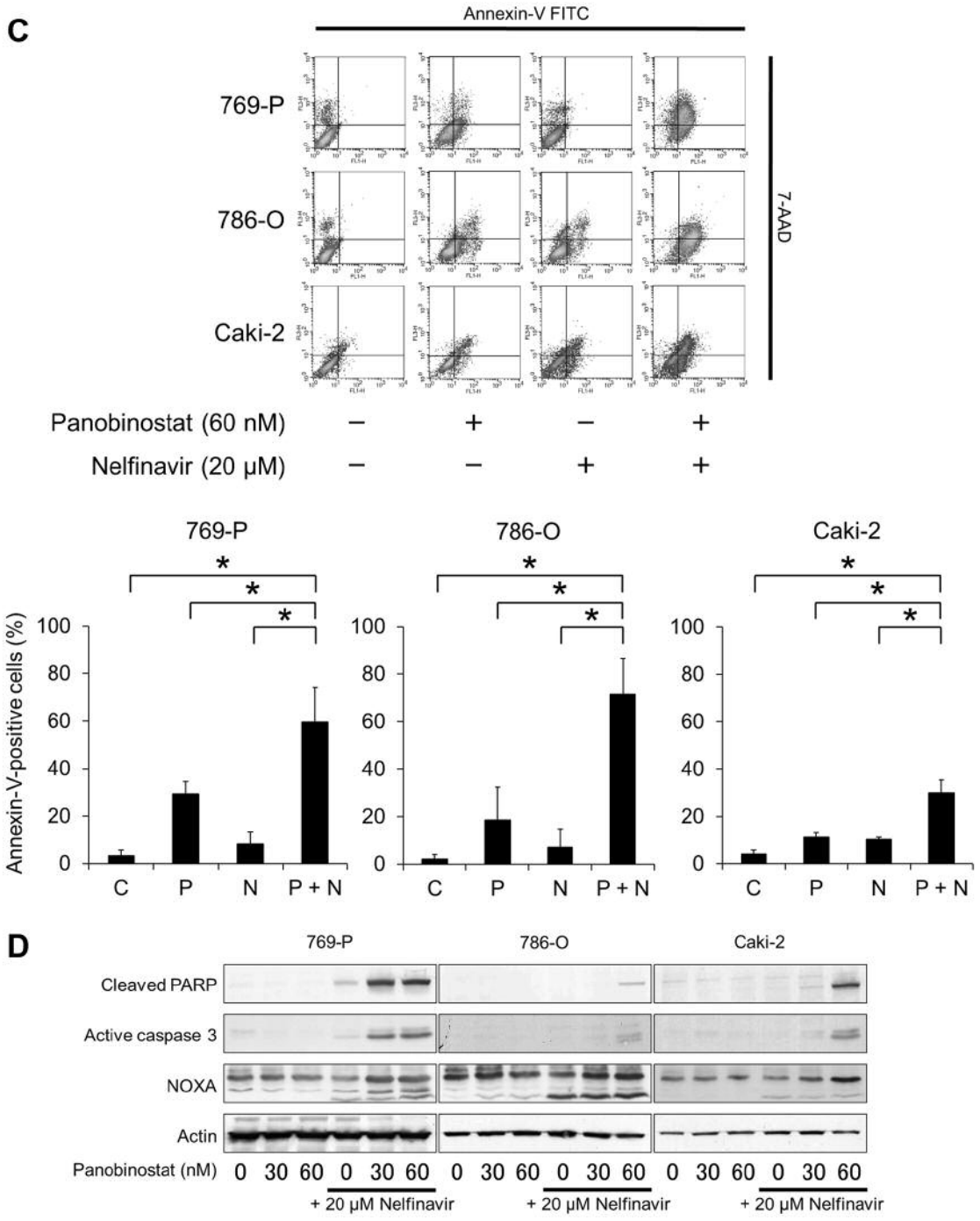

Figure 2. *The combination of panobinostat and nelfinavir induced apoptosis. A: Cell-cycle analysis. Cells were treated for 48 h with 60 nM panobinostat with and without 20 μM nelfinavir. Changes in the cell cycle were evaluated using flow cytometry; 10^4 cells were counted. Bar graphs show the percentages of cells in the sub-G1 fraction. Data are expressed as mean±SD from three independent experiments. C: Control; P: 60 nM panobinostat; N: 20 μM nelfinavir; P+N: 60 nM panobinostat and 20 μM nelfinavir. *Significantly different at p=0.0495. B: Western blotting for cyclin D1 and cyclin-dependent kinase (CDK) 4. Cells were treated for 48 h with 30-60 nM panobinostat with and without 20 μM nelfinavir. Actin was used for the loading control. Representative blots are shown. C: Annexin-V assay. Cells were treated for 48 h with 60 nM panobinostat with and without 20 μM nelfinavir. Apoptotic cells were detected by annexin-V assay using flow cytometry; 10^4 cells were counted. Bar graphs show the percentages of annexin-V-positive cells. Data are expressed as mean±SD from three independent experiments. FITC: Fluorescein isothiocyanate; 7-AAD: 7-amino-actinomycin D; C: control; P: 60 nM panobinostat; N: 20 μM nelfinavir; P+N: 60 nM panobinostat and 20 μM nelfinavir. *Significantly different at p=0.0495. D: Western blotting for cleaved poly(ADP-ribose) polymerase (PARP), active caspase 3, and phorbol-12-myristate-13-acetate-induced protein 1 (NOXA). Cells were treated for 48 h with 30-60 nM panobinostat with and without 20 μM nelfinavir. Actin was used for the loading control. Representative blots are shown.*

(Rosemont, IL, USA); anti-cyclin D1, anti-cyclin-dependent kinase (CDK) 4, anti-glucose-regulated protein 78 (GRP78), anti-HDAC1, anti-HDAC3, and anti-HDAC6 from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-cleaved poly(ADP-ribose) polymerase (PARP), anti-ribosomal protein S6 (S6), and anti-phosphorylated S6 from Cell Signaling Technology (Danvers, MA, USA); anti-active caspase 3, anti-phorbol-12-myristate-13-acetate-induced protein 1 (NOXA), and anti-acetylated histone from Abcam (Cambridge, UK); and pan-actin antibody from Millipore (Billerica, MA, USA). The target proteins were then detected using horseradish-tagged goat anti-rabbit or goat anti-mouse antibody (Bio-Rad, Hercules, CA, USA) and staining with a chemiluminescence solution with the ECL Plus system (GE Healthcare, Wauwatosa, WI, USA).

For western blotting of the resected tumor specimens, five tumor lysates from each treated group were separated on the same gel with two specific tumor lysates from the control group. The densities of the bands were semiquantified using public domain ImageJ software and normalized to the expression of actin. The inter-membrane difference of band density was calibrated using the two specific bands from the control group on each membrane.

Statistical analysis. CalcuSyn software (Biosoft, Cambridge, UK) was used for calculating the combination indices according to the Chou-Talalay method (a combination index<1, =1, and >1 indicate synergism, additive effect, and antagonism) (8). Statistical significance of observed differences between samples was evaluated using the Mann-Whitney *U*-test (JMP Pro14 software; SAS Institute, Cary, NC, USA), and values of $p<0.05$ were considered to indicate statistically significant differences.

Results

The combination of panobinostat and nelfinavir inhibited renal cancer growth effectively. According to the cell viability assay, the combination of panobinostat and nelfinavir cooperatively inhibited the growth of renal cancer cells (Figure 1A). On microscopic examination, the majority of the cells treated with the combination of 60 nM panobinostat and 20 μM nelfinavir were floating but each agent alone only reduced the number of the cells (Figure 1B). The combined effect was also evaluated using the Chou-Talalay method to calculate combination indices, which demonstrated that the combined effect on cell growth was synergistic under many of the combinations (Table I). Combination of agents also significantly inhibited the clonogenicity of renal cancer cells (Figure 1C). In the murine subcutaneous tumor model using Caki-2 cells, an 11-day treatment using the combination of panobinostat and nelfinavir was well tolerated and suppressed tumor growth significantly (Figure 1D).

The combination of panobinostat and nelfinavir induced apoptosis. We then evaluated apoptosis induction by the combination. On cell-cycle analysis, 48-hour treatment with the combination significantly increased the number of cells in the sub-G1 fraction (Figure 2A). We also found that the panobinostat–nelfinavir combination markedly reduced the expression of the cell-cycle regulators cyclin D1 in 769-P and Caki-2 cells and CDK4 in all cell lines (Figure 2B), which was in accordance with the cell-cycle changes induced by the combination. Annexin-V assay was then performed and changes in the expression of apoptosis-related proteins were also evaluated to confirm apoptosis induction by the combination. The combination significantly increased the number of annexin-V-positive cells (Figure 2C) and increased the expression of apoptosis-related proteins: cleaved PARP, active caspase 3, and NOXA (Figure 2D). In 786-O and Caki-2 cells, a higher concentration of panobinostat (60 nM) was required to increase the expression of cleaved PARP and active caspase 3 in combination with 20 μM nelfinavir. Thus the combination of panobinostat and nelfinavir was shown to induce apoptosis.

Table I. *Combination indices for the combination of 15-60 nM panobinostat and 10-20 μM nelfinavir against renal cancer cells. A combination index of <1, 1, and >1 indicates synergism, additive effect, and antagonism.*

		Panobinostat (nM)		
Cell line	Nelfinavir (μM)	15	30	60
769-P	10	0.953	0.96	1.234
	20	0.973	0.849	0.71
786-O	10	0.629	0.576	0.262
	20	0.583	0.546	0.159
Caki-2	10	0.899	0.994	0.917
	20	1.054	0.95	0.616

The combination of panobinostat and nelfinavir induced ER stress and histone acetylation cooperatively both in vitro and in vivo. Our postulation was that panobinostat and nelfinavir induce ER stress cooperatively. We therefore evaluated the changes in the expression of the ER stress marker GRP78 to see whether the combination induced ER stress (Figure 3A). In all the cell lines, GRP78 expression was prominently enhanced by 20 μM nelfinavir in combination with 30 or 60 nM panobinostat, whereas panobinostat alone increased it only slightly. Thus the combination was shown to induce ER stress cooperatively.

Panobinostat is an HDAC inhibitor and induces histone acetylation. We therefore examined how nelfinavir influenced the acetylation status of histone when given in combination with panobinostat. As expected, panobinostat alone increased histone acetylation in a dose-dependent manner and, interestingly, nelfinavir enhanced this acetylation (Figure 3B). To investigate the mechanism of this enhanced histone acetylation, we then evaluated changes in the expression of HDACs, enzymes which control the acetylation status of histone. Finding that the

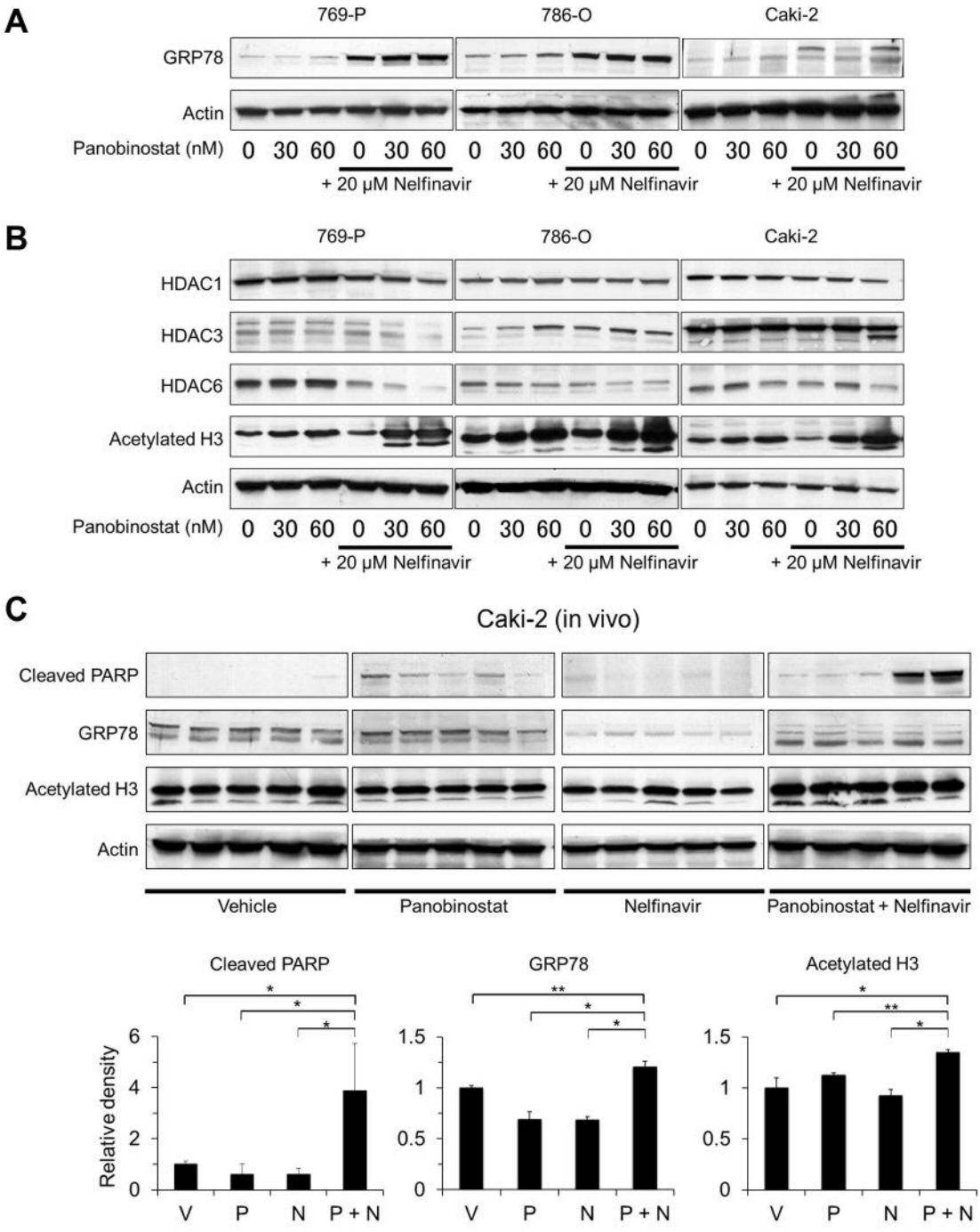

Figure 3. *The combination of panobinostat and nelfinavir induced endoplasmic reticulum (ER) stress and histone acetylation cooperatively both in vitro and in vivo. A: Western blotting for the ER stress marker glucose-regulated protein 78 (GRP78). Cells were treated for 48 h with 30-60 nM panobinostat with and without 20 μM nelfinavir. Actin was used for the loading control. Representative blots are shown. B: Western blotting for acetylated histone (H3) and histone deacetylases (HDACs). Cells were treated for 48 h with 30-60 nM panobinostat with and without 20 μM nelfinavir. Actin was used for the loading control. Representative blots are shown. C: Western blotting of resected tumor specimens for cleaved poly(ADP-ribose) polymerase (PARP), GRP78, and acetylated H3. Mice bearing Caki-2 tumors were killed at day 11 and the tumors were resected, homogenized in radioimmunoprecipitation assay buffer, and subjected to western blotting. Five tumor lysates from each treated group were separated on the same gel with two specific tumor lysates from the control group (not shown). The densities of the bands were semiquantified using ImageJ and normalized to actin expression. The inter-membrane difference of band density was calibrated using the two specific bands from the control group on each membrane. Relative density (vehicle group=1) is shown. Data are the mean±SD, n=5. V: Vehicle group; P: panobinostat-treated group; N: nelfinavir-treated group; P+N: combination-treated group. Significantly different at *p=0.0367 and **p=0.0216.*

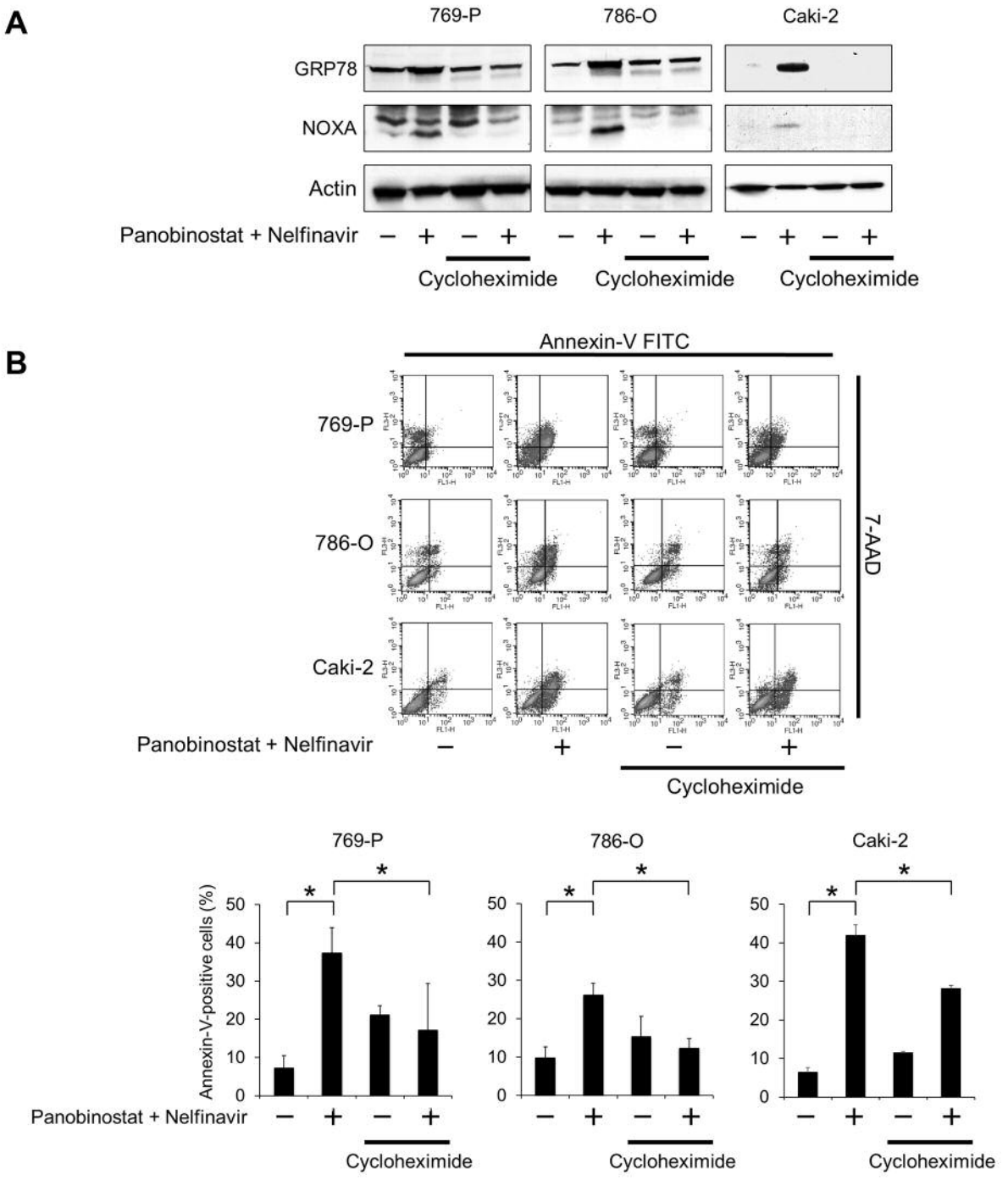

Figure 4. *Continued*

combination reduced the expression of HDACs (HDAC1, 3, and 6 in 769-P cells, HDAC6 in 786-O cells, and HDAC1 and 6 in Caki-2 cells), we inferred that reduction of HDAC expression would be one mechanism of the enhanced histone acetylation caused by the combination.

To determine whether the combination indeed acted against tumors by the expected mechanism of action *in vivo*, we then evaluated the changes in the status of apoptosis, ER stress, and histone acetylation using the *in vivo* tumor specimens. The expression of cleaved PARP, GRP78, and

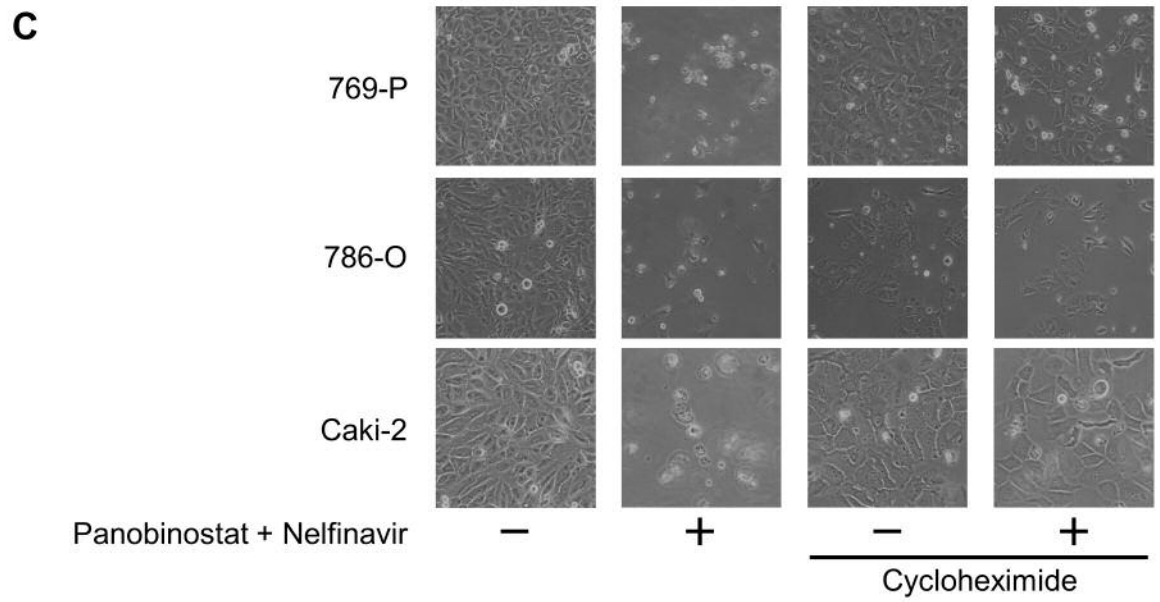

Figure 4. *Induction endoplasmic reticulum stress played a pivotal role in the anticancer effect of panobinostat-nelfinavir combination. A: Western blotting for glucose-regulated protein 78 (GRP78) and phorbol-12-myristate-13-acetate-induced protein 1 (NOXA). Cells were treated for 48 h with the combination of 60 nM panobinostat and 20 μM nelfinavir with and without 5 μg/ml of the protein synthesis inhibitor cycloheximide. Actin was used as a loading control. Representative blots are shown. B: Annexin-V assay. Cells were treated for 48 h with the combination of 60 nM panobinostat and 20 μM nelfinavir with and without 5 μg/mI cycloheximide. Apoptotic cells were detected by annexin-V assay using flow cytometry; 10^4 cells were counted. Bar graphs show the percentages of annexin-V-positive cells. Data are expressed as mean±SD from three independent experiments. FITC: Fluorescein isothiocyanate; 7-AAD: 7-amino-actinomycin D. *Significantly different at p=0.0495. C: Photomicrographs of cells after 48-h treatment with the combination of 60 nM panobinostat and 20 μM nelfinavir with and without 5 μg/ml cycloheximide. Original magnification, ×100.*

acetylated histone was significantly higher in the tumors of the combination-treated group than it was in the tumors of the control group and the single-agent-treated groups (Figure 3C). Thus the combination of panobinostat and nelfinavir was shown to act against renal cancer cells by inducing ER stress and histone acetylation both *in vitro* and *in vivo*.

Induction of ER stress played a pivotal role in the anticancer effect of the panobinostat-nelfinavir combination. We then evaluated the role of ER stress induction in the combination's action. Cycloheximide reportedly inhibits protein synthesis and thereby suppresses induction of ER stress (9). In our study, 5 μg/ml cycloheximide inhibited the combination-increased expression of GRP78 and NOXA, showing that it actually suppressed the ER stress induction by the combination (Figure 4A). Furthermore, cycloheximide attenuated the ability of the combination to induce apoptosis (Figure 4B). This marked attenuation of the combination's anticancer effect was also evident on microscopic examination (Figure 4C). Thus, ER stress induction was shown to play a pivotal role in the anticancer effect of the combination.

Panobinostat induced histone acetylation and ER stress, but also activated the mTOR pathway. Panobinostat has been reported to cause not only histone acetylation but also ER stress because it increases unfolded proteins in the cell by inhibiting the molecular chaperone function (10). In the present study, panobinostat itself also induced ER stress as well as histone acetylation in a dose-dependent manner (Figure 5). Furthermore, we found that it also activated the mTOR pathway as evidenced by phosphorylation of S6. Because mTOR activation generally promotes cell proliferation, its activation is thought to attenuate the anticancer effect of panobinostat.

Nelfinavir inhibited mTOR pathway activation by panobinostat. We believed that nelfinavir in combination might affect panobinostat-induced mTOR activation. Interestingly, nelfinavir inhibited the phosphorylation of S6 caused by panobinostat (Figure 6), showing that it inhibited activation of the mTOR pathway by panobinostat. Furthermore, we also found that the combination increased the expression of the mTOR inhibitor AMPK, which would be one of the mechanisms of this inhibition of mTOR. We

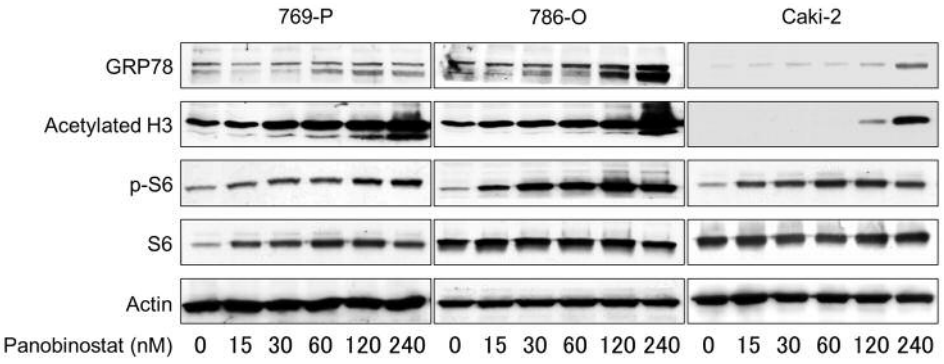

Figure 5. *Panobinostat induced endoplasmic reticulum stress and histone acetylation but also activated the mammalian target of rapamycin pathway. Western blotting for glucose-regulated protein 78 (GRP78), acetylated histone (H3), phosphorylated ribosomal protein S6 (p-S6), and S6. Cells were treated for 48 h with 15-240 nM panobinostat. Actin was used as a loading control. Representative blots are shown.*

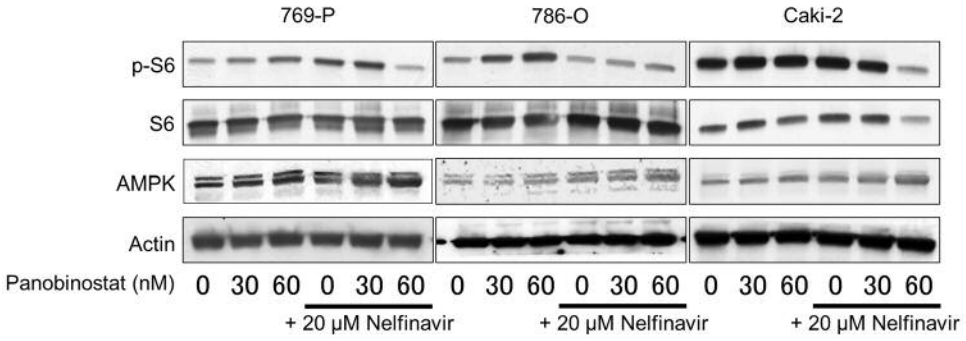

Figure 6. *Nelfinavir inhibited the activation of mammalian target of rapamycin pathway by panobinostat. Western blotting for phosphorylated ribosomal protein S6 (p-S6), S6, and AMP-activated protein kinase (AMPK). Cells were treated for 48 h with 30-60 nM panobinostat with and without 20 μM nelfinavir. Actin was used for the loading control. Representative blots are shown.*

inferred that inhibition of the mTOR pathway activated by panobinostat is another anticancer mechanism of the panobinostat–nelfinavir combination.

Discussion

Because of the lack of curative treatment, there is an urgent need for new therapeutic strategies for patients with advanced renal cancer. Inducing ER stress is a novel strategy for cancer treatment (11). ER stress is initiated when the amount of unfolded protein exceeds the capacity of the ER to properly refold these proteins, and severe ER stress kills cancer cells (12). Using combinations of clinically available drugs, our laboratory has been trying to develop novel therapies that kill urological cancer cells by inducing ER stress. We reported that ER stress-inducing combinations exert strong cytotoxicity against renal cancer (5, 7, 13-15), prostate cancer (6), and bladder cancer cells (16, 17). In the present study, we investigated the effects of the combination of the HDAC inhibitor panobinostat and the HIV protease inhibitor nelfinavir and tried to clarify the mechanism of the combination's action both *in vitro* and *in vivo*.

Panobinostat is a pan-HDAC inhibitor approved for the treatment of relapsed and refractory multiple myeloma (18). It has been shown to inhibit the chaperone function of HSP90 (3). Nelfinavir is an HIV protease inhibitor clinically used to treat HIV infection and has been reported to inhibit both proteasomes (19) and HSP90 (20). Furthermore, nelfinavir was recently shown to induce ER stress and sensitize renal cancer cells to tumor necrosis factor-related apoptosis-inducing ligand (21). We believed that the combination of panobinostat and nelfinavir would cause ER stress effectively because nelfinavir would inhibit the degradation of unfolded proteins produced by the effect of panobinostat and nelfinavir, causing them to accumulate in cells.

The combination of panobinostat and nelfinavir induced significant apoptosis and inhibited renal cancer growth effectively. The combination increased the expression of

GRP78 cooperatively, confirming that the combination induced ER stress. GRP78 acts as a master regulator of the unfolded protein response (22) and is widely used as a marker of ER stress (23). Furthermore, the combination increased the expression of NOXA, a BH3-only protein that is induced by ER stress and activates the mitochondrial apoptotic pathway (24). Thus apoptosis induced by the combination was shown to be caused by ER stress. We also found that cycloheximide attenuated combination-induced ER stress. This is consistent with our hypothesis that panobinostat and nelfinavir induce ER stress cooperatively by causing unfolded proteins to accumulate because cycloheximide is an inhibitor of protein synthesis (9) and should therefore reduce the amount of unfolded protein in cells. Furthermore, cycloheximide inhibited combination-induced apoptosis significantly. These results show that induction of ER stress plays a pivotal role in the cytotoxicity of this combination.

The combination of panobinostat and nelfinavir induced histone acetylation cooperatively. We inferred that this enhanced histone acetylation was due to reduced expression of HDACs. Although the exact cause of this combination-enhanced histone acetylation is unknown, one possible mechanism would be induction of ER stress. The proteasome inhibitor bortezomib, also an ER stressor, was reported to increase histone acetylation in renal cancer (14) and prostate cancer (6) cells. Furthermore, ER stress-inducing drug combinations were shown to reduce the expression of HDACs and induce histone acetylation in renal cancer (7, 13) and bladder cancer cells (16, 17), even though some of the combinations did not include an HDAC inhibitor (13, 16, 17). It is also notable that reduction of HDAC expression can enhance ER stress because inhibition of HDAC6 increases the acetylation of molecular chaperones and suppresses their function (3). Thus the combination may cause a 'vicious cycle' of ER stress–histone acetylation in cancer cells.

We showed, for the first time, that panobinostat activates the mTOR pathway in renal cancer cells, which is consistent with the results of a previous study using Hodgkin lymphoma cell lines (25). The mTOR pathway is one of the pathways activated in renal cancer, regulating survival and cell growth (26). A clinical study found panobinostat not to be effective in patients with metastatic renal cancer (27), and we believed that its activation of the mTOR pathway might attenuate its anticancer activity. We found in the present study that nelfinavir in the combination increased the expression of AMPK and inhibited mTOR pathway activation by panobinostat, as shown by the mediator of S6 phosphorylation. AMPK is a serine-threonine kinase that is activated by cellular stresses which deplete ATP and inhibits the mTOR pathway (28-30). It was recently implied to be a therapeutic target for controlling cancer cell growth through the suppression of mTOR function (31, 32). Increased AMPK expression as a result of the combination may be associated with the induction of ER stress because the combination with HIV protease inhibitors was reported not only to induce ER stress, but also to increase AMPK expression (17) and proteasome inhibitors, which are known to act as ER stressors, were found to activate AMPK (33, 34). Clarifying the mechanism by which the combination increases the expression of AMPK needs further study, but increasing AMPK expression would be an important mechanism of the anticancer action of the combination.

Because both these drugs have already been used clinically and the effectiveness and mechanisms of action of this combination *in vivo* we also demonstrated, the next step is to translate our results into clinical use. However, there is also a limitation. Many HIV protease inhibitors, including nelfinavir, are known to inhibit liver enzymes (*e.g.* cytochrome *P450* enzymes), and their clinical use may be complicated by drug–drug interactions (35). An optimal safe therapeutic dose should be determined by a careful monitoring of serum drug concentration on clinical application.

In summary, the combination of panobinostat and nelfinavir induced ER stress and inhibited renal cancer growth. Increasing histone acetylation and inhibiting panobinostat-induced mTOR activation would also be important mechanisms of action. To the best of our knowledge, this is the first study demonstrating the beneficial combined effect of panobinostat and nelfinavir in renal cancer cells both *in vitro* and *in vivo*.

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