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Nelfinavir and Ritonavir Kill Bladder Cancer Cells Synergistically by Inducing Endoplasmic Reticulum Stress

Akinori Sato, Takako Asano, Kazuki Okubo, Makoto Isono, and Tomohiko Asano

Department of Urology, National Defense Medical College, Tokorozawa, Saitama, Japan

The human immunodeficiency virus (HIV) protease inhibitor nelfinavir acts against malignancies by inducing endoplasmic reticulum (ER) stress. The HIV protease inhibitor ritonavir, on the other hand, not only induces ER stress but also inhibits P-glycoprotein's pump activity and thereby enhances the effects of its substrate drugs. We therefore postulated that ritonavir in combination with nelfinavir would kill bladder cancer cells effectively by inducing ER stress cooperatively and also enhancing nelfinavir's effect. Nelfinavir was shown to be a P-glycoprotein substrate, and the combination of nelfinavir and ritonavir inhibited bladder cancer cell growth synergistically. It also suppressed colony formation significantly. The combination significantly increased the number of cells in the sub-G₁ fraction and also the number of annexin V⁺ cells, confirming robust apoptosis induction. The combination induced ER stress synergistically, as evidenced by the increased expression of glucose-regulated protein 78, ER-resident protein 44, and endoplasmic oxidoreductin-1-like protein. It also increased the expression of the mammalian target of rapamycin (mTOR) inhibitor AMP-activated protein kinase and caused dephosphorylation of S6 ribosomal protein, demonstrating that the combination also inhibited the mTOR pathway. We also found that the combination enhanced histone acetylation synergistically by decreasing the expression of HDACs 1, 3, and 6.

Key words: Nelfinavir; Ritonavir; Endoplasmic reticulum stress; Bladder cancer

INTRODUCTION

There is currently no curative treatment for advanced bladder cancer. Cisplatin-based chemotherapies have been the mainstay of the treatment of metastatic bladder cancer for decades, but their efficacy is quite limited¹. The anti-programmed cell death ligand 1 (PD-L1) antibody atezolizumab has recently been shown to have clinical activity in patients with metastatic cancer and approved for the treatment of bladder cancer by the US Food and Drug Administration², but the objective response rate was not satisfactory. It is evident that novel treatment approaches are urgently needed.

Drug repositioning is a powerful strategy for finding new anticancer drugs³, and our laboratory has been trying to kill urological cancer cells by combining drugs already used for other purposes. Furthermore, to develop a therapy more effective than those currently used, it is necessary to use a novel strategy for killing cancer cells. We have focused on two innovative anticancer mechanisms, inducing endoplasmic reticulum (ER) stress and acetylating histones, and reported the effective drug combinations that act by these mechanisms against renal cancer⁴⁻⁶ and prostate cancer⁷.

In the present study, we used two clinically feasible non-anticancer drugs in combination to kill bladder cancer cells by inducing ER stress. The human immunodeficiency virus (HIV) protease inhibitors have recently attracted attention as a new class of anticancer drugs with multiple effects⁸. Nelfinavir is an HIV protease inhibitor widely used to treat HIV infection and has recently been shown to induce ER stress and act against malignancies⁹. Ritonavir is another clinically available HIV protease inhibitor. It not only induces ER stress¹⁰ but also inhibits P-glycoprotein¹¹, enhancing the effects of its substrates by impeding their efflux from cells. We thought that ritonavir in combination with nelfinavir would kill bladder cancer cells effectively by inducing ER stress cooperatively and also enhancing nelfinavir's effect.

MATERIALS AND METHODS

Cell Cultures

Bladder cancer cells (5637, J82, and UMUC3) were purchased from the American Type Culture Collection (Rockville, MD, USA) and grown in RPMI or MEM

media containing 10% fetal bovine serum and 0.3% penicillin–streptomycin (Invitrogen, Carlsbad, CA, USA).

Reagents

Ritonavir (purchased from Toronto Research Chemicals, North York, ON, Canada), nelfinavir (purchased from Tocris Bioscience, Minneapolis, MN, USA), and valsopodar (purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA) were dissolved in dimethyl sulfoxide and stored at -20°C until use.

Cell Viability Assay

For the cell viability assay, 5×10^3 cells were plated in 96-well culture plates 1 day before being cultured for 48 h under the indicated conditions. Cell viability was assayed using the MTS assay (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Colony Formation Assay

For the colony formation assay, 300 cells were plated in 6-cm plates 1 day before being cultured for 48 h in

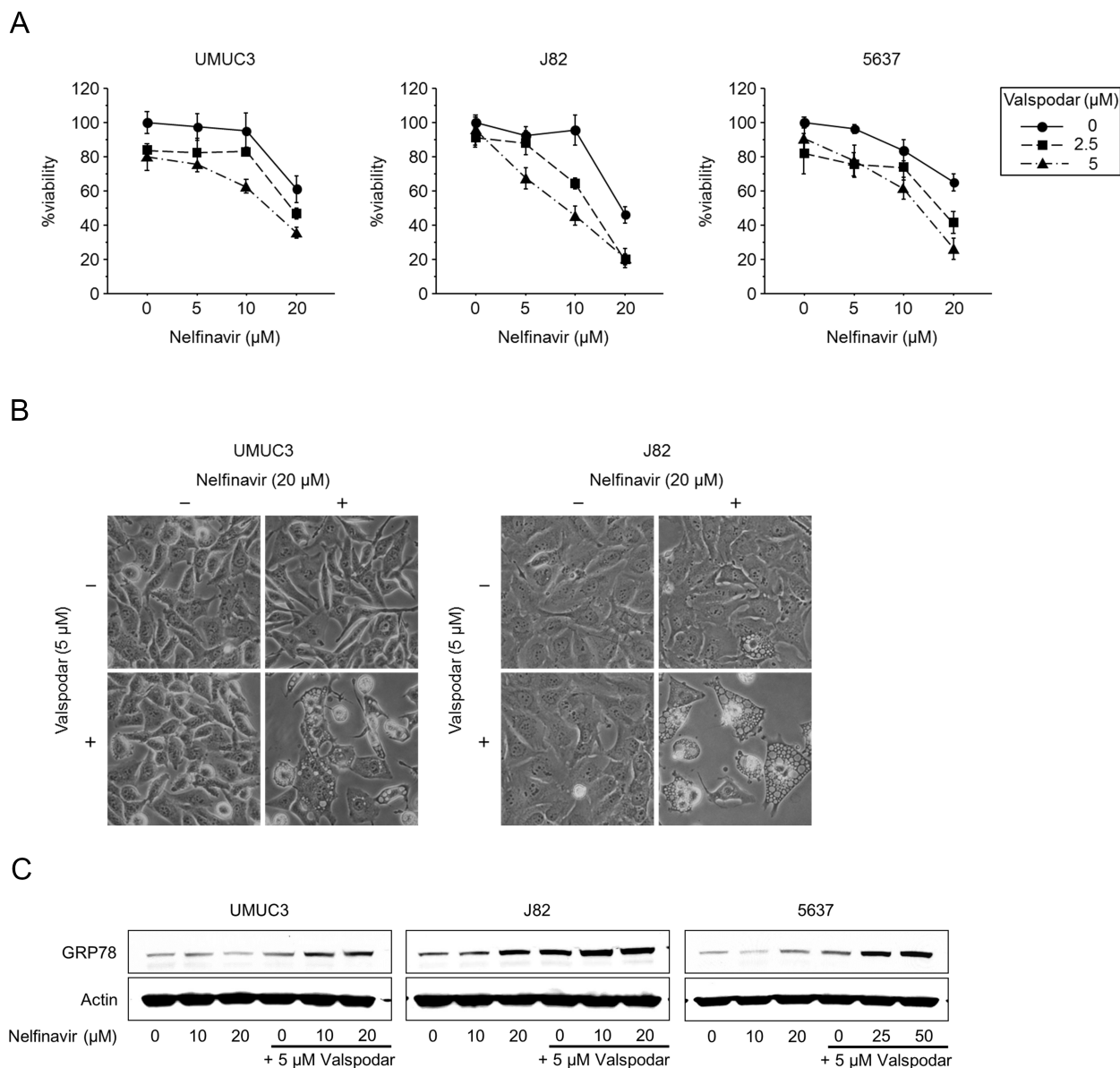


Figure 1. Nelfinavir was a P-glycoprotein substrate. (A) MTS assay. Cells were treated for 48 h with 5–20 μM nelfinavir with or without 2.5–5 μM valsopodar. Bars represent mean \pm SD, $n=6$. (B) Photomicrographs after 48 h of treatment with 20 μM nelfinavir and/or 5 μM valsopodar. Note marked cytoplasmic vacuolization after treatment with nelfinavir and valsopodar. Original magnification: 200 \times . (C) Western blotting for glucose-regulated protein 78 (GRP78) after 48 h of treatment with 10–20 μM nelfinavir and/or 5 μM valsopodar. Actin was used for the loading control. Representative blots are shown.

media containing 20 μM nelfinavir and/or 25 or 50 μM ritonavir. They were then given fresh media and allowed to grow for 1–2 weeks before being fixed with 100% methanol, stained with Giemsa's solution, and counted.

Flow Cytometry

Induction of apoptosis was detected by measuring the number of cells in the sub- G_1 fraction on cell cycle analysis and by annexin V assay. Cells (1.5×10^5) were plated in six-well culture plates 1 day before being cultured for 48 h in media containing 20 μM nelfinavir and/or 50 μM ritonavir. They were then washed with phosphate-buffered saline and harvested by trypsinization. For the cell cycle analysis, the harvested cells were resuspended in citrate buffer, stained with propidium iodide, and analyzed by flow cytometry. For the annexin V assay, the harvested cells were stained with annexin V and 7-amino-actinomycin D according to the manufacturer's instruction (Beckman Coulter, Marseille, France) and then analyzed by flow cytometry. Data were analyzed using CellQuest Pro Software (BD Biosciences, San Jose, CA, USA).

Western Blotting

Cells were treated under the indicated conditions, and whole-cell lysates were obtained using radioimmuno-precipitation buffer. The lysates were then subjected to Western blot analysis as reported previously⁶. The primary antibodies used were anti-cyclin D1, anti-cyclin-dependent kinase 4 (CDK4), anti-glucose-regulated protein 78 (GRP78), anti-ubiquitinated protein, and anti-histone deacetylases (HDACs) 1, 3, and 6 (Santa Cruz Biotechnology); anti-ER-resident protein 44 (ERP44), anti-endoplasmic oxidoreductin-1-like protein (Ero1L), anti-S6 ribosomal protein, and anti-phosphorylated S6 ribosomal protein (Cell Signaling Technology, Danvers, MA, USA); anti-AMP-activated protein kinase (AMPK; Proteintech, Rosemont, IL, USA); anti-acetylated histone (Abcam, Cambridge, UK); and anti-actin (Millipore, Billerica, MA, USA).

Statistical Analysis

Combination indexes were calculated using CalcuSyn software (Biosoft, Cambridge, UK). The differences observed in the colony formation assay, cell cycle analysis, and annexin V assay were tested for statistical significance using the Mann–Whitney *U*-test (StatView software; SAS Institute, Cary NC, USA). Differences were considered significant with a value of $p < 0.05$.

RESULTS

Nelfinavir Was a P-glycoprotein Substrate

We examined whether nelfinavir was a substrate of P-glycoprotein using the specific P-glycoprotein inhibitor valsopodar. The cancer cells were treated for 48 h with

5–20 μM nelfinavir in combination with 2.5–5 μM valsopodar. MTS assay results showed that valsopodar enhanced the cytotoxic effect of nelfinavir, whereas valsopodar itself had only slight cytotoxicity (Fig. 1A). According to the combination indexes, the combined effect was synergistic (combination index < 1) under many of the treatment conditions (Table 1). Interestingly, the cells treated with nelfinavir and valsopodar displayed massive cytoplasmic vacuolization (Fig. 1B), a morphological change associated with ER stress¹². Valsopodar also enhanced nelfinavir-induced ER stress as evidenced by the increased expression of GRP78, a central regulator of ER function¹³ (Fig. 1C). From these results, we inferred that nelfinavir was a P-glycoprotein substrate.

The Combination of Nelfinavir and Ritonavir Inhibited Bladder Cancer Growth Synergistically

We treated the cells with 5–20 μM nelfinavir for 48 h with or without 25–50 μM ritonavir. MTS assay results showed that the combination effectively inhibited the viability of bladder cancer cells (Fig. 2A). This combined effect was also evident on microscopic examination (Fig. 2B): each agent alone had only a moderate effect on the morphology of the cancer cells, whereas most of the combination-treated cells were floating. We also calculated combination indexes and found the combined effect to be synergistic under many of the treatment conditions (Table 2). Furthermore, the combination-treated cells displayed massive cytoplasmic vacuolization (Fig. 2C), indicating the induction of ER stress. The combination also significantly inhibited colony formation by the bladder cancer cells (Fig. 2D). Thus, the combination of nelfinavir and ritonavir was shown to effectively inhibit bladder cancer growth.

The Combination of Nelfinavir and Ritonavir Perturbed the Cell Cycle

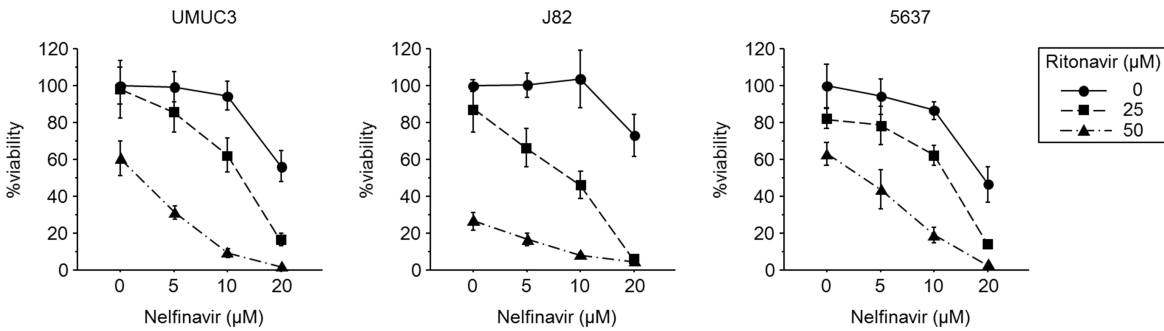
Cells were cultured for 48 h in medium containing 20 μM nelfinavir and/or 50 μM ritonavir. Each agent

Table 1. Combination Indexes (CIs) for the Combination of Nelfinavir and Valsopodar in Bladder Cancer Cells

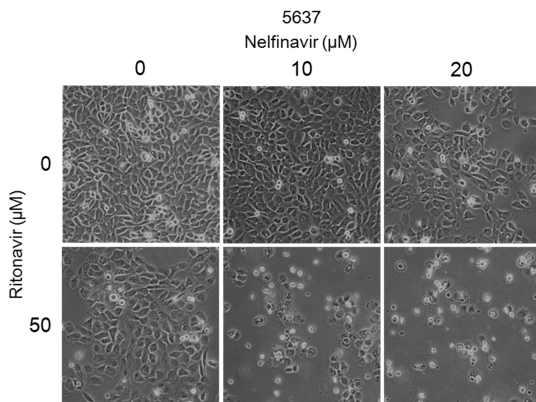
Bladder Cancer Cells	Nelfinavir		
	5 μM	10 μM	20 μM
UMUC3			
2.5 μM valsopodar	1.176	1.632	0.692
5 μM valsopodar	0.84	0.559	0.562
J82			
2.5 μM valsopodar	1.047	0.607	0.423
5 μM valsopodar	0.354	0.403	0.434
5637			
2.5 μM valsopodar	0.353	0.664	0.637
5 μM valsopodar	0.395	0.487	0.444

CI < 1 indicates synergy.

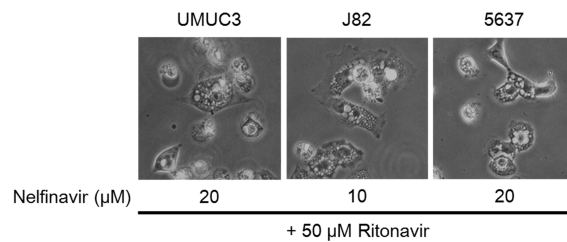
A



B



C



D

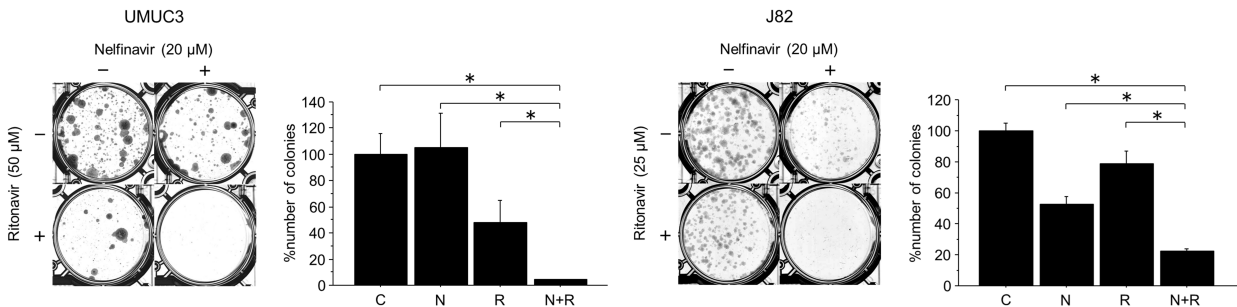


Figure 2. The combination of nelfinavir and ritonavir inhibited bladder cancer growth synergistically. (A) MTS assay. Cells were treated for 48 h with 5–20 μM nelfinavir with or without 25–50 μM ritonavir. Bars represent mean \pm SD, $n=6$. (B) Photomicrographs after 48 h of treatment with 10–20 μM nelfinavir and/or 50 μM ritonavir. Note that most of the cells treated with the combinations are floating. Original magnification: 100 \times . (C) High-power field image of the cells treated by the combination, showing marked cytoplasmic vacuolization. Original magnification: 200 \times . (D) Colony formation assay. A total of 300 cells were cultured for 48 h in media containing 20 μM nelfinavir and/or 25 or 50 μM ritonavir. They were then given fresh media and allowed to grow for 1–2 weeks. Bars represent mean \pm SE, $n=3$. C, control; N, nelfinavir; R, ritonavir. $*p=0.0495$.

alone caused a slight to moderate increase in the number of the cells in the sub- G_1 fraction, but in combination increased it significantly (Fig. 3A). These changes were in accordance with the decreased expression of the cell cycle regulators cyclin D1 and CDK4 (Fig. 3B).

The Combination of Nelfinavir and Ritonavir Induced Apoptosis

Cells were treated for 48 h with 20 μM nelfinavir and/or 50 μM ritonavir. Nelfinavir or ritonavir alone increased the number of annexin V^+ cells only moderately, but

Table 2. Combination Indexes (CIs) for the Combination of Nelfinavir and Ritonavir in Bladder Cancer Cells

Bladder Cancer Cells	Nelfinavir		
	5 μ M	10 μ M	20 μ M
UMUC3			
25 μ M ritonavir	1.033	1.04	0.936
50 μ M ritonavir	0.977	0.838	0.737
J82			
25 μ M ritonavir	0.936	0.955	0.813
50 μ M ritonavir	1.009	0.967	1.081
5637			
25 μ M ritonavir	1.284	1.107	0.595
50 μ M ritonavir	0.796	0.532	0.306

CI < 1 indicates synergy.

together increased it significantly (Fig. 3C). This, along with the increased number of cells in the sub-G₁ fraction, showed that the combination of nelfinavir and ritonavir induced apoptosis in bladder cancer cells.

The Combination of Nelfinavir and Ritonavir Induced ER stress

Ritonavir or nelfinavir alone induced ER stress, and the combination enhanced this ER stress, as indicated by the increased expression of the ER stress markers GRP78, ERP44, and Ero1L (Fig. 4). Our hypothesis is that the combination increases the amount of unfolded proteins in the cell and induces ER stress, so we also examined the change in the amount of ubiquitinated unfolded proteins. Surprisingly, the expression of ubiquitinated proteins was seemingly decreased by the combination.

The Combination of Nelfinavir and Ritonavir Inhibited the Mammalian Target of Rapamycin (mTOR) Pathway

Because induction of ER stress has been reported to inhibit the mTOR pathway¹⁴, a relevant pathway for cancer proliferation, we thought that the combination could also inhibit it. The combination increased the expression of the mTOR inhibitor AMPK and caused dephosphorylation of the S6 ribosomal protein (Fig. 5), demonstrating that the combination indeed inhibited the mTOR pathway.

The Combination of Nelfinavir and Ritonavir Induced Histone Acetylation

We have previously shown that a combination causing ER stress in renal cancer cells also induced histone acetylation in them⁵. Therefore, we thought that the combination of nelfinavir and ritonavir might also induce histone acetylation. As expected, the combination caused histone acetylation synergistically (Fig. 6). In J82 cells, either 10 or 20 μ M nelfinavir or 50 μ M ritonavir caused

slight histone acetylation, and in 5637 cells 20 μ M nelfinavir did, whereas in UMUC3 cells neither agent alone caused histone acetylation. However, given in combination, they caused drastic histone acetylation in all the cell lines.

To explore the mechanism of this histone acetylation, we then evaluated the expression of HDACs and found that the combination decreased the expression of HDACs 1, 3, and 6 in all the cell lines.

DISCUSSION

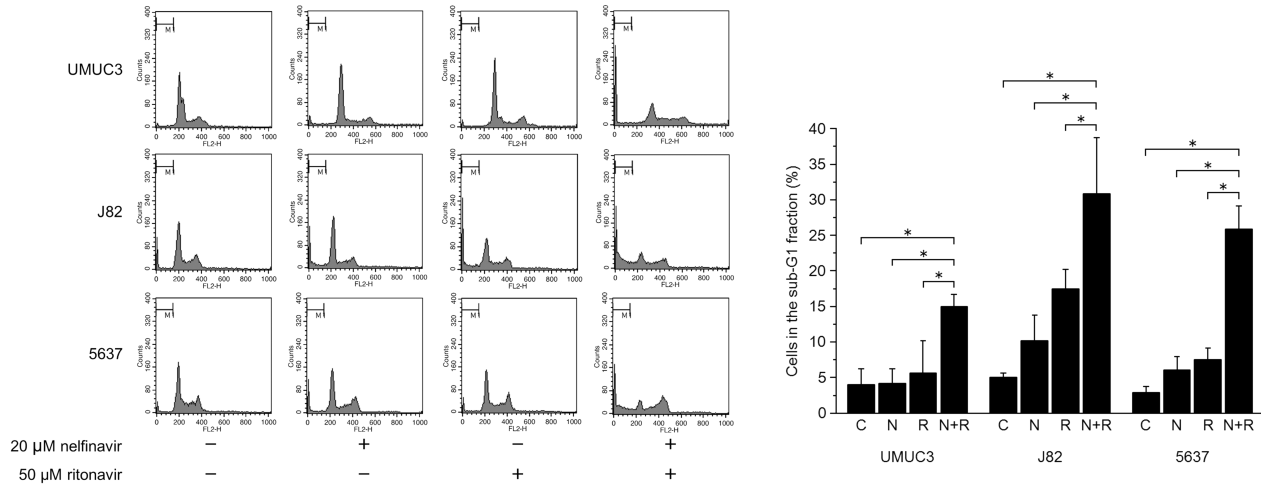
Cisplatin-based chemotherapies have been the mainstay of the treatment of advanced bladder cancer for decades, but their efficacy is quite limited¹. Recently, the PD-L1 antibody atezolizumab was approved for the treatment of bladder cancer by the US Food and Drug Administration, but the objective response rate was only 26%². Development of a novel treatment is clearly needed.

Inducing ER stress is an emerging strategy to treat malignancies¹⁵. Starting with the combination of ritonavir and suberoylanilide hydroxamic acid in renal cancer¹⁶, we have investigated ways to kill urological cancer cells by inducing ER stress⁴⁻⁷. In the present study, we tried to introduce the concept of killing cancer cells by inducing ER stress to bladder cancer treatment. The most important thing was effectively inducing this ER stress.

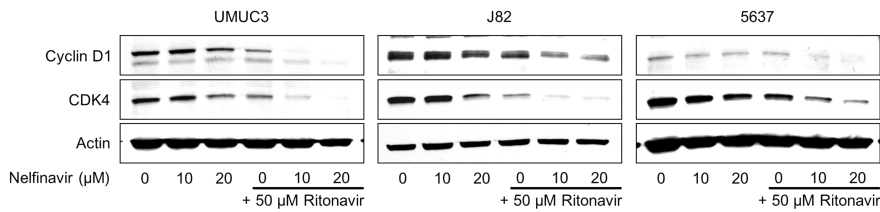
Developing a new agent costs much, and therefore recent innovative anticancer agents such as kinase inhibitors, mTOR inhibitors, anti-PD-1 antibodies, and anti-PD-L1 antibodies are all very expensive. Furthermore, because development processes are long and approval rates are low, it takes a long time for a new agent to be applied clinically. Therefore, drug repositioning has emerged as an alternative approach to finding novel anticancer agents, an approach better in terms of both economic cost and time efficiency¹⁷. In the present study, using the concept of drug repositioning, we combined clinically feasible common non-anticancer agents to induce ER stress effectively.

Because ritonavir not only induces ER stress¹⁰ but also inhibits P-glycoprotein¹¹, we thought that ritonavir in combination with nelfinavir kills bladder cancer cells effectively by inducing ER stress cooperatively and also enhancing nelfinavir's effect. Ritonavir or nelfinavir alone induced ER stress and in combination enhanced this stress, which was in accordance with our hypothesis. Nelfinavir alone inhibited cell viability in a dose-dependent fashion, and the specific P-glycoprotein inhibitor valspodar enhanced this inhibition. Furthermore, enhanced expression of GRP78 confirmed that valspodar enhanced the nelfinavir-induced ER stress. Nelfinavir was thus shown to be one of the substrates of P-glycoprotein, and its inhibition is thought to be one mechanism of the

A



B



C

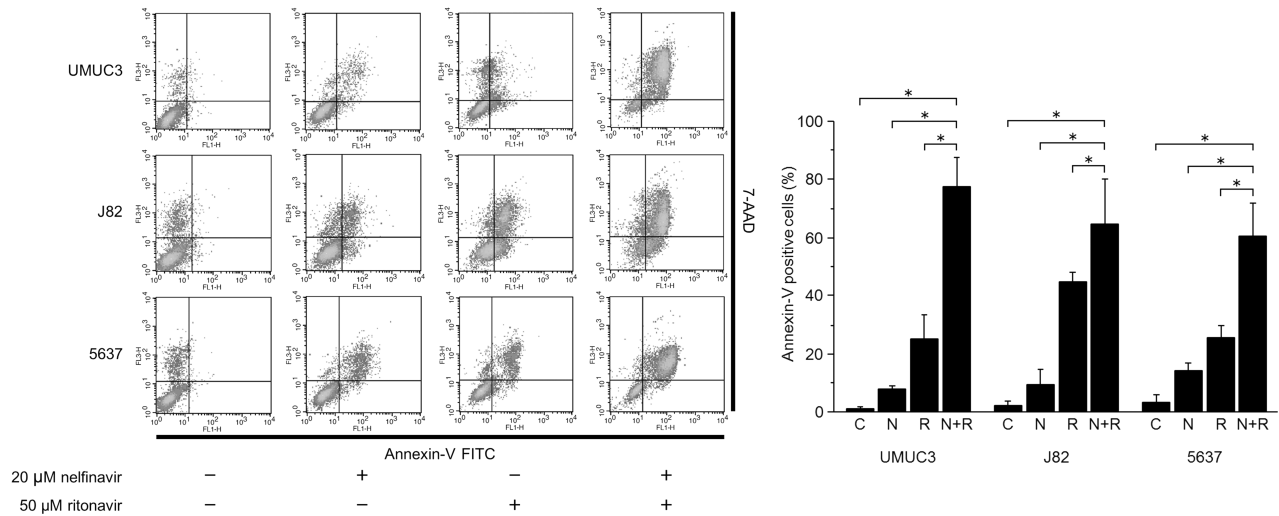


Figure 3. The combination of nelfinavir and ritonavir perturbed the cell cycle and caused apoptosis. (A) Cell cycle analysis. Cells were treated for 48 h with 20 μM nelfinavir and/or 50 μM ritonavir. A total of 10,000 cells were counted, and changes in cell cycle were analyzed using flow cytometry. Bar graphs show cells in the sub-G₁ fraction. Data are expressed as mean ± SD from three independent experiments. **p* = 0.0495. (B) Western blotting for cyclin D1 and cyclin-dependent kinase 4 (CDK4) after 48 h of treatment with 10–20 μM nelfinavir and/or 50 μM ritonavir. Actin was used for the loading control. Representative blots are shown. (C) Annexin V assay. Cells were treated for 48 h with 20 μM nelfinavir and/or 50 μM ritonavir. A total of 10,000 cells were counted, and induction of apoptosis was evaluated by annexin V assay using flow cytometry. Bar graphs show percentage of annexin V⁺ cells. Data are expressed as mean ± SD from three independent experiments. FITC, fluorescein isothiocyanate; 7-AAD, 7- amino-actinomycin D. **p* = 0.0495.

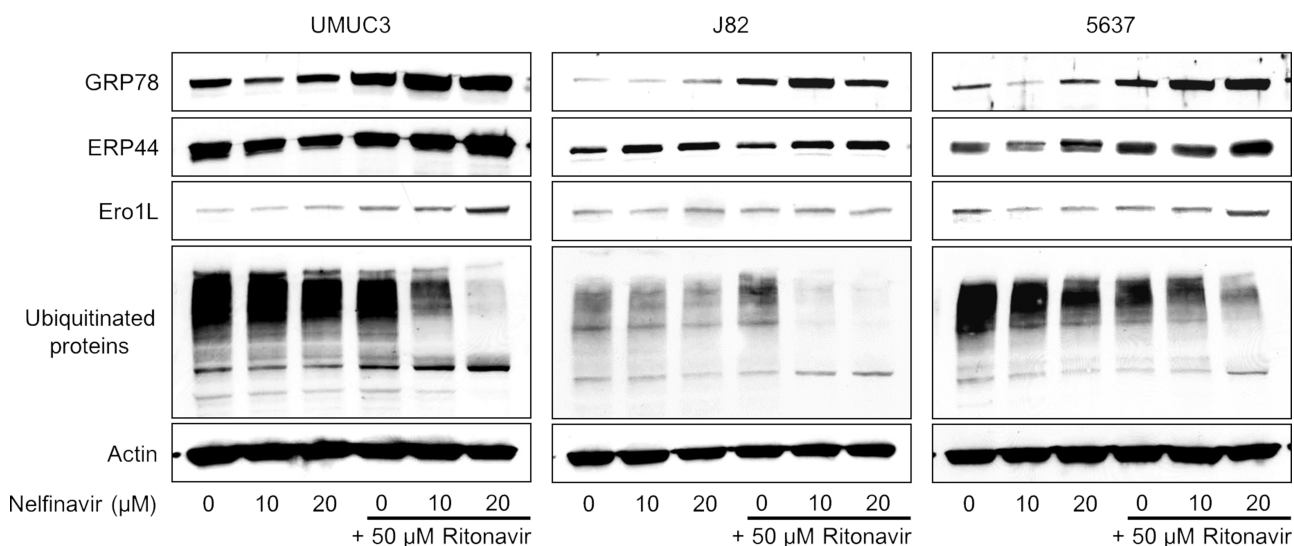


Figure 4. The combination of nelfinavir and ritonavir induced endoplasmic reticulum (ER) stress. Western blotting for ER stress markers and ubiquitinated proteins after 48 h of treatment with 10–20 μM nelfinavir and/or 50 μM ritonavir. Actin was used for the loading control. Representative blots are shown. ERP44, endoplasmic reticulum-resident protein 44; Ero1L, endoplasmic oxidoreductin-1-like protein.

combination's action. On the other hand, ritonavir itself caused ER stress and inhibited bladder cancer viability. Ritonavir reportedly inhibits the proteasome¹⁸ and molecular chaperones¹⁹, inducing ER stress¹⁰. Therefore, cooperative ER stress induction would be another important mechanism of the combination's action.

The combination of nelfinavir and ritonavir decreased the expression of ubiquitinated proteins. This result seems to be inconsistent with our hypothesis. Because the combination actually induced ER stress or unfolded protein response, unfolded ubiquitinated proteins were thought to accumulate. Mimnaugh et al.²⁰ reported that the ER stress-inducing combination of the HSP90 inhibitor geldanamycin and the proteasome inhibitor bortezomib decreased the expression of ubiquitinated proteins by causing their

aggregation and shift into the detergent-insoluble fraction, which may explain the seeming decrease in the expression of ubiquitinated proteins in the present study. Another possible mechanism of the decreased ubiquitinated protein expression would be protein synthesis inhibition due to the extensive ER stress induction²¹, but further study will be needed to clarify the exact mechanism of this phenomenon.

Inhibition of the mTOR pathway is another important mechanism by the combination. Genetic variations in the phosphoinositide-3 kinase–AKT–mTOR pathway were reported to increase bladder cancer risk²². The expression of phosphorylated-S6 ribosomal protein, a downstream target of mTOR, was shown to be an independent predictor of disease-specific survival in bladder cancer

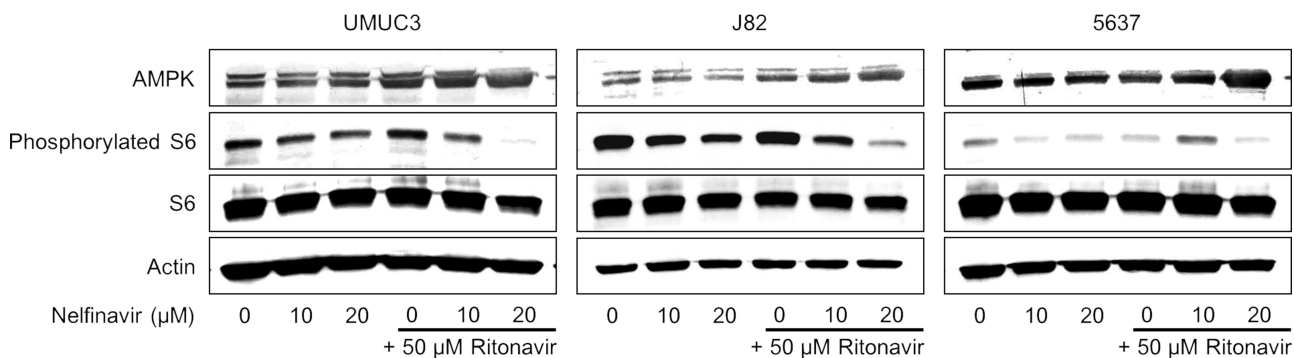


Figure 5. The combination of nelfinavir and ritonavir inhibited the mammalian target of rapamycin pathway. Western blotting for AMP-activated protein kinase (AMPK), phosphorylated S6 ribosomal protein, and total S6 ribosomal protein after 48 h of treatment with 10–20 μM nelfinavir and/or 50 μM ritonavir. Actin was used for the loading control. Representative blots are shown.

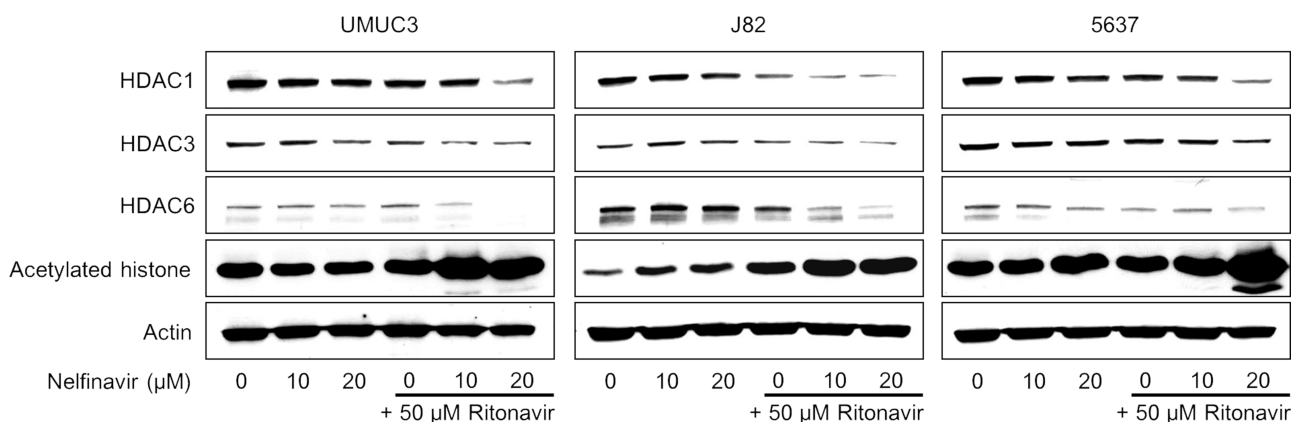


Figure 6. The combination of nelfinavir and ritonavir induced histone acetylation. Western blotting for histone deacetylases (HDACs) 1, 3, and 6, and acetylated histone after 48 h of treatment with 10–20 μM nelfinavir and/or 50 μM ritonavir. Actin was used for the loading control. Representative blots are shown.

patients²³. Furthermore, mTOR inhibition by rapamycin reportedly reduced cancer cell proliferation in RT4, T24, J82, and UMUC3 cells, corresponding to reduced phosphorylated-S6 ribosomal protein levels²⁴. Thus, the mTOR pathway is thought to play an important role in bladder cancer proliferation. In the present study, the combination of nelfinavir and ritonavir caused dephosphorylation of S6 ribosomal protein, showing that the combination inhibited the mTOR pathway. As reported by Brüning et al.¹⁴, this mTOR inhibition would be secondary to the ER stress. The increased expression of the mTOR inhibitor AMPK is one important mechanism of the mTOR inhibition in the present study. AMPK is activated under conditions that deplete cellular ATP and elevate AMP levels such as glucose deprivation, hypoxia, ischemia, and heat shock²⁵ and inhibits the mTOR pathway^{26,27}. The increased expression of AMPK is thought to also be due to the ER stress because the ER stressor bortezomib was reported to increase the expression of AMPK²⁸. This mTOR inhibition is an attractive important mechanism of the combination's action because targeting AMPK–mTOR has recently emerged as novel cancer therapy²⁹.

Surprisingly, the combination of nelfinavir and ritonavir caused histone acetylation, although we did not use any HDAC inhibitor in the present study. To our knowledge, there has been no report that an HIV protease inhibitor itself caused histone acetylation. The acetylation and deacetylation of histones play important roles in the regulation of gene transcription and in the modulation of chromatin structure³⁰, and the acetylation status is determined by the activities of histone acetyltransferases and HDACs³¹. Because aberrant HDAC activity is associated with tumorigenicity and survival of cancer cells, compounds targeting HDACs (i.e., HDAC inhibitors) have generated great interest as anticancer drugs^{32,33}. Because nelfinavir and ritonavir are not so-called HDAC

inhibitors, we examined the expression of HDACs themselves in pursuit of a mechanism of the histone acetylation and found that the combination actually decreased the expression of HDACs. Another possible mechanism of the histone acetylation would be the ER stress induction, which has been shown to induce histone acetylation in other cancer cells^{5,7}. Clarifying the exact mechanism will require further study, but inhibition of HDACs might play an important role in the combination's antineoplastic activity because it not only causes histone acetylation but also causes acetylation of non-histone proteins such as transcription factors, signal transduction mediators, and DNA repair enzymes, modulating their function³⁴.

There are also limitations in the present study. In the treatment of HIV infection, ritonavir is normally administered with other HIV protease inhibitors such as saquinavir, indinavir, lopinavir, and amprenavir³⁵, but the combination of nelfinavir and ritonavir has not been used routinely. However, the results of some clinical studies^{36–38} indicate that the combination of nelfinavir and ritonavir could be administered safely. Careful drug monitoring would be needed, however, because ritonavir inhibits CYP3A4 in the liver³⁵ and may increase the serum concentration of nelfinavir.

In summary, the combination of nelfinavir and ritonavir killed bladder cancer cells by inducing ER stress and histone acetylation. Inhibition of the mTOR pathway was also an important mechanism of action. Because both agents are common HIV protease inhibitors and have already been used clinically, a clinical trial using the combination would be more cost effective and feasible than one using novel agents even though careful drug monitoring would be needed.

ACKNOWLEDGMENT: This work was supported by JSPS KAKENHI Grant No. JP26462434.

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