

# Nelfinavir Induces Endoplasmic Reticulum Stress and Sensitizes Renal Cancer Cells to TRAIL

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**Abstract.** *Background/Aim: Induction of endoplasmic reticulum (ER) stress is a novel strategy for cancer treatment. The human immunodeficiency virus protease inhibitor nelfinavir was recently shown to induce ER stress, but its anti-neoplastic activity has never been investigated in renal cancer, as far as we are aware. Materials and Methods: Using renal cancer cells (769-P, 786-O, Caki-2), the ability of nelfinavir to induce ER stress and sensitize them to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was tested. Results: Nelfinavir caused apoptosis and inhibited renal cancer growth in a dose-dependent fashion. It also suppressed colony formation significantly. Nelfinavir induced ER stress and increased the expression of TRAIL death receptor (DR) 4 and DR5, sensitizing the cancer cells to TRAIL. This sensitization was blocked by human recombinant DR4/Fc and DR5/Fc chimeric protein, confirming that the sensitization was due to increased expression of both DR4 and DR5. Conclusion: Nelfinavir induces ER stress in renal cancer cells and sensitizes them to TRAIL.*

It has been estimated that in 2012 there were over 330,000 new cases of renal cancer, resulting in more than 140,000 deaths worldwide (1). Although molecular targeted drugs, such as tyrosine kinase inhibitors and mammalian target of rapamycin (mTOR) inhibitors, have a significant antitumor effect and improve the survival rates of patients with advanced renal cancer, they are not curative agents (2-7). Immune checkpoint inhibitors have recently been approved and widely used, but their therapeutic effect is also limited (8, 9). Renal cancer, thus, remains a lethal disease, and a more effective treatment modality needs to be developed.

Causing unfolded protein accumulation and endoplasmic reticulum (ER) stress is a novel approach to cancer treatment

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(10). Nelfinavir is a human immunodeficiency virus (HIV) protease inhibitor approved by the Food and Drug Administration for the treatment of patients with HIV; it increases unfolded proteins by suppressing the function of proteasomes and molecular chaperones such as heat-shock protein 90 (HSP90) (11, 12), thereby causing ER stress.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a cytokine produced by most normal tissue cells (13). It binds to the death receptors DR4 and DR5 and causes apoptosis of cancer cells (14). Interestingly, ER stress induction has recently been shown to increase the expression of these DRs (15).

In the present study, we investigated the ability of nelfinavir to induce ER stress in renal cancer cells and sensitize them to TRAIL by increasing the expression of the death receptors DR4 and DR5.

## Materials and Methods

**Cell cultures.** Human renal cancer cells (769-P, 786-O, and Caki-2) were purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured in Roswell Park Memorial Institute medium or McCoy's 5A medium containing 10% fetal bovine serum and 1.0% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C under 5% CO<sub>2</sub> in a humidified incubator.

**Reagents.** Nelfinavir purchased from Tocris Bioscience (Bristol, United Kingdom) was dissolved in dimethyl sulfoxide. Human recombinant TRAIL, DR4/Fc, and DR5/Fc chimeric protein were purchased from R&D Systems (Minneapolis, MN, USA) and dissolved in sterile phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin. These agents were stored at -80°C until use.

**Evaluation of the effect of nelfinavir on cell viability.** A total of 5×10<sup>3</sup> cells were plated in a 96-well culture plate 1 day before being treated with 2-20 μM nelfinavir. Cell viability was evaluated by MTS assay (CellTiter 96 Aqueous kit; Promega, Madison, WI, USA) according to the manufacturer's protocol. The experiment was performed using 12 wells per concentration and repeated three times.

**Colony-formation assay.** A total of 150 individual cells were seeded in 6-well plates 1 day before being treated for 48 hours with 5-20 μM nelfinavir. 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.

**Evaluation of the effect of nelfinavir on the cell cycle.** A total of  $1.5 \times 10^5$  cells were seeded in a 6-well culture plate 1 day before treatment. They were then cultured for 48 hours with 5-20  $\mu\text{M}$  nelfinavir. Changes in the cell-cycle distribution were evaluated using flow cytometry.

**Evaluation of nelfinavir-induced apoptosis.** A total of  $1.5 \times 10^5$  cells were seeded in a 6-well culture plate 1 day before being treated for 48 hours with 10 or 20  $\mu\text{M}$  nelfinavir. Apoptotic cells were detected by annexin-V assay using flow cytometry. The experiment was repeated three times.

**Aggresome detection.** Protein aggregation caused by nelfinavir was evaluated by detecting aggresomes in the cells. Briefly,  $1.5 \times 10^5$  cells were seeded in a 6-well culture plate 1 day before being cultured for 48 hours in medium containing 10 or 20  $\mu\text{M}$  nelfinavir. Cells were then fixed, permeabilized, and incubated with Hoechst 33342 and PROTEOSTAT dye (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions. Aggresomes were then qualitatively visualized by fluorescence microscopy.

**Evaluation of the combined effect of nelfinavir and TRAIL on cell viability.** A total of  $5 \times 10^3$  cells were plated in a 96-well culture plate 1 day before treatment. They were then cultured for 24 hours in medium with or without 20  $\mu\text{M}$  nelfinavir before being given 25 ng/ml TRAIL with or without 1  $\mu\text{g/ml}$  DR4/Fc or DR5/Fc chimeric protein and incubated for another 24 h. Cell viability was evaluated by MTS assay as described above. The experiment was performed using 12 wells in each condition and repeated at least twice.

**Evaluation of the combined effect of nelfinavir and TRAIL on apoptosis induction.** A total of  $1.5 \times 10^5$  cells were seeded in a 6-well culture plate 1 day before treatment. They were cultured for 24 hours in medium with or without 20  $\mu\text{M}$  nelfinavir before being given 25 ng/ml TRAIL with or without 1  $\mu\text{g/ml}$  DR4/Fc or DR5/Fc chimeric protein and incubated for another 24 h. Apoptotic cells were detected by annexin-V assay using flow cytometry. The experiment was repeated three times.

**Flow cytometry.** Flow cytometry was used to evaluate changes in the cell-cycle distribution and apoptosis. Briefly, cells were washed with PBS and harvested by trypsinization. For cell-cycle analysis, harvested cells were resuspended in citrate buffer and stained with propidium iodide. For apoptosis analysis, cells were stained with annexin V and 7-amino-actinomycin D (7-AAD) according to the manufacturer's protocol (Beckman Coulter, Marseille, France). They were then analyzed by flow cytometry using the CellQuest Pro software (BD Biosciences, San Jose, CA, USA). A total of 10,000 cells were counted.

**Western blotting.** Cells were treated for 48 h with 5-20  $\mu\text{M}$  nelfinavir and whole-cell lysates were obtained using radioimmunoprecipitation assay buffer. Equal amount of proteins was 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 primary antibodies to cyclin D1 (sc-753, 1:500), cyclin-dependent kinase (CDK) 4 (sc-601, 1:500), survivin (sc-10811, 1:500), DR4 (sc-7863, 1:500), DR5 (sc-65314, 1:500), and glucose-regulated protein (GRP) 78 (sc-7865, 1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); cleaved poly(ADP-ribose) polymerase (PARP) (#9541, 1:1000), endoplasmic oxidoreductin-1-like protein alpha (ERO1-L $\alpha$ ) (#3264, 1:1000) and endoplasmic reticulum resident protein (ERp) 44 (#3798, 1:1000) (Cell Signaling Technology, Danvers, MA, USA); light chain (LC) 3 (PM036, 1:2000) (MBL International, Woburn, MA, USA), phorbol-12-myristate-13-acetate-induced protein 1 (NOXA/PMAIP1; ab13654, 1:500) (Abcam, Cambridge, UK); or actin (MAB1501, 1:4000) (Millipore, Billerica, MA, USA). 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 instructions.

**Statistical analysis.** The statistical significance of observed differences between samples was determined using the Mann-Whitney *U*-test (JMP pro13 software; SAS Institute, Cary, NC, USA). Differences with  $p < 0.05$  were considered to be statistically significant.

## Results

**Nelfinavir had anti-proliferative activity against renal cancer cells.** According to the cell viability assay, nelfinavir inhibited the growth of renal cancer cells in a dose-dependent fashion (Figure 1A). We then investigated whether nelfinavir affected the clonogenic survival of renal cancer cells and found that it inhibited their colony formation in a dose-dependent fashion (Figure 1B).

**Nelfinavir induced apoptosis of renal cancer cells.** The effect of nelfinavir on the cell cycle was then examined. In all the cell lines, nelfinavir increased the number of the cells in the sub-G<sub>1</sub> fraction (Figure 2A), suggesting that nelfinavir induced apoptosis. We also found that it markedly reduced the expression of cyclin D1 and CDK4 (Figure 2B), which was consistent with the observed cell-cycle changes. Nelfinavir increased the number of annexin-V-positive cells in a dose-dependent fashion (Figure 2C). It also increased the expression of cleaved PARP and NOXA and reduced the expression of survivin (Figure 2D), an anti-apoptotic protein associated with cell proliferation in renal cancer (16). Thus, nelfinavir was shown to induce apoptosis of renal cancer cells.

**Nelfinavir induced ER stress in renal cancer cells.** Next, we evaluated the changes in the expression of the ER stress markers GRP78, ERO1-L $\alpha$ , and ERp44. In all the cell lines, nelfinavir increased the expression of these markers in a dose-dependent fashion (Figure 3). Thus, nelfinavir was shown to induce ER stress. Furthermore, nelfinavir increased the expression of NOXA (Figure 2D), indicating that the ER stress induced by nelfinavir caused apoptosis.

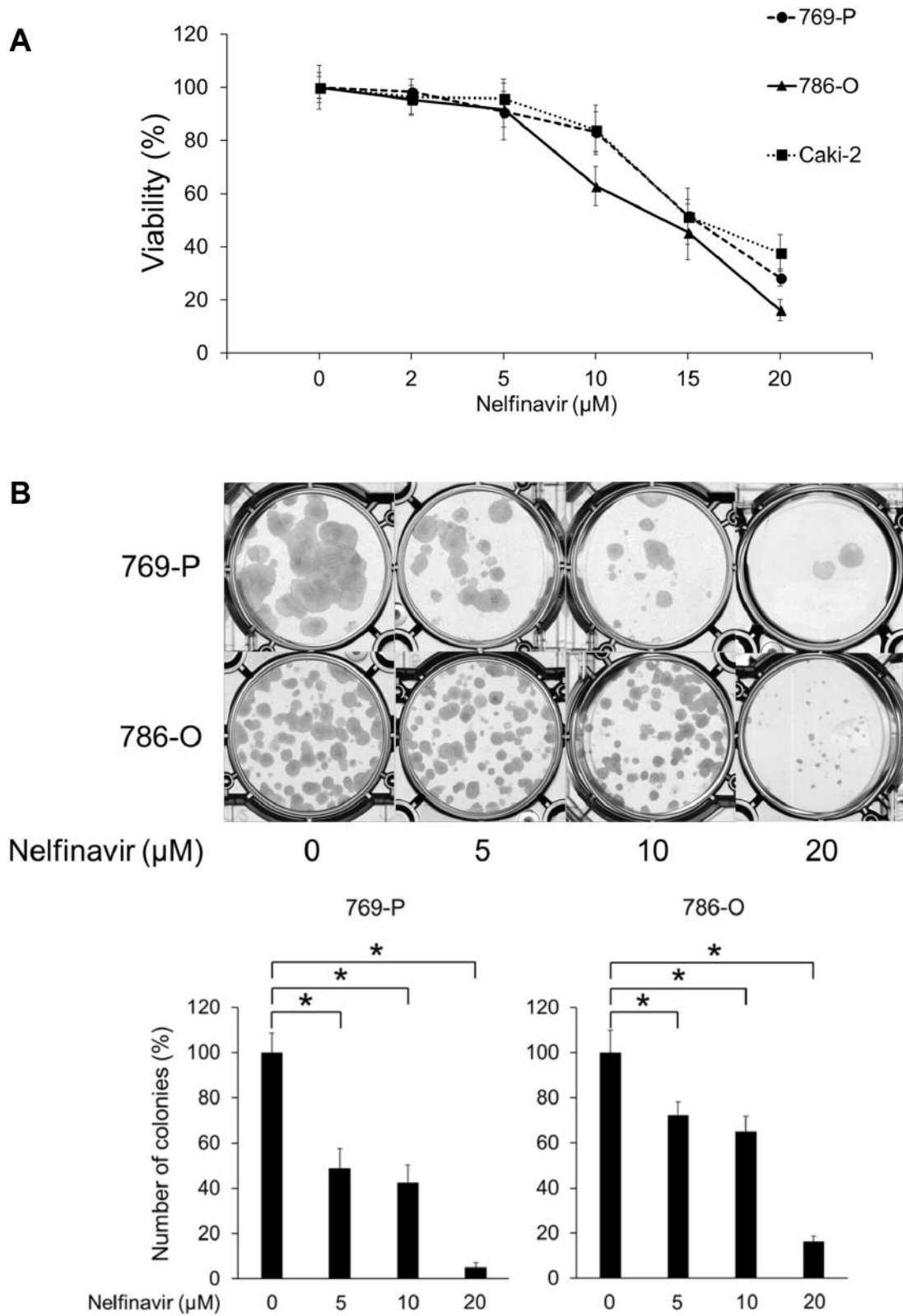


Figure 1. Nelfinavir had anti-proliferative activity against renal cancer cells. A: MTS assay. Cells were treated for 48 h with 2-20 μM nelfinavir. Bars represent mean±SD, n=12. B: Colony-formation assay. Cells were treated for 48 h with 5-20 μM nelfinavir, then given fresh media and allowed to grow for 1-2 weeks. Bars represent mean±SD, n=3. \*Relative to the untreated control.

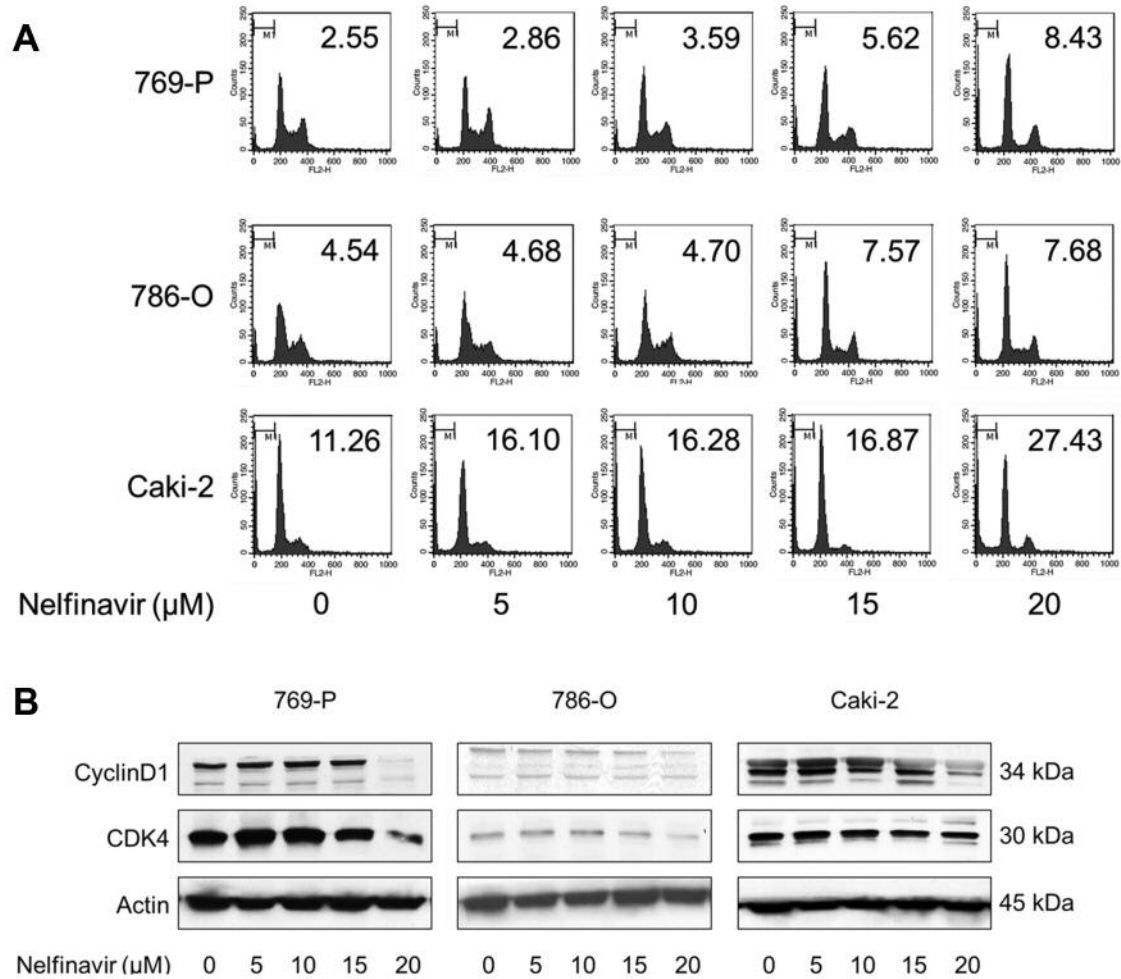


Figure 2. Continued

*Nelfinavir caused aggresome formation and induced autophagy in renal cancer cells.* Because unfolded proteins often aggregate and form aggresomes, we then evaluated nelfinavir-induced aggresome formation and autophagy. As expected, nelfinavir caused aggresome formation (Figure 4A) and induced autophagy (evidenced by the increased expression of the autophagy marker LC3-II) (Figure 4B). These results indicate that nelfinavir actually increases unfolded proteins in the cell.

*Nelfinavir increased the expression of DR4 and DR5 and thereby sensitized renal cancer cells to TRAIL.* ER stress has been reported to increase the expression of TRAIL receptors (15). We thought that nelfinavir would increase the expression of TRAIL receptors and thereby sensitize renal cancer cells to TRAIL. Treatment with nelfinavir for 48 h indeed increased the expression of the TRAIL

receptors DR4 and DR5 in a dose-dependent fashion (Figure 5A). According to the MTS assay, 25 ng/ml TRAIL alone did not kill cancer cells. When the cells were pre-treated for 24 h with 20  $\mu$ M nelfinavir, however, TRAIL exerted strong cytotoxicity (Figure 5B). Furthermore, 1  $\mu$ g/ml DR4/Fc or DR5/Fc chimeric protein attenuated nelfinavir-enhanced TRAIL cytotoxicity. The morphological change was also consistent with this sensitization: TRAIL alone had almost no effect on cell attachment, but cells treated with nelfinavir and TRAIL were floating, which was abrogated by DR4/Fc and DR5/Fc chimeric protein (Figure 5C). Annexin-V assay also confirmed this sensitization by nelfinavir: Treatment with TRAIL induced significant apoptosis only when it was preceded by nelfinavir treatment, and this apoptosis induction was significantly blocked by DR4/Fc and DR5/Fc chimeric protein (Figure 5D).

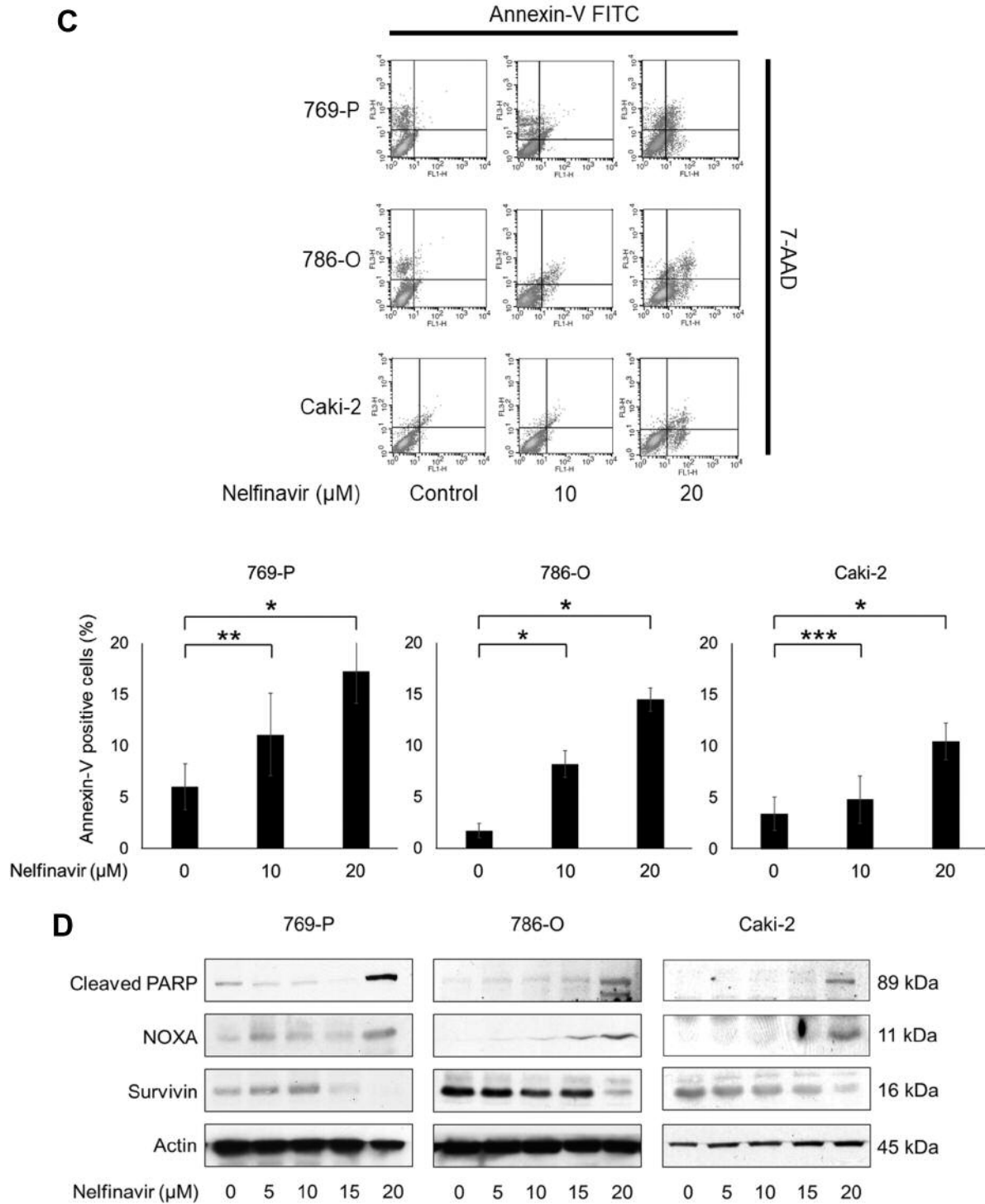


Figure 2. Nelfinavir induced apoptosis of renal cancer cells. A: Cell-cycle analysis. Cells were treated for 48 h with 5-20  $\mu$ M nelfinavir. Changes in the cell-cycle distribution were evaluated using flow cytometry; 10,000 cells were counted. The number inset in each graph shows the percentage of cells in the sub-G1 fraction. B: Western blotting for cyclin D1 and cyclin-dependent kinase 4 (CDK4). Cells were treated for 48 h with 5-20  $\mu$ M nelfinavir. Actin was used as the loading control. Representative blots are shown. C: Annexin-V assay. Cells were treated for 48 h with 10 or 20  $\mu$ M nelfinavir. Apoptotic cells were detected by annexin-V assay using flow cytometry; 10,000 cells were counted. Bar graphs show the percentages of apoptotic cells. Data are expressed as mean $\pm$ SD from three independent experiments. FITC, Fluorescein isothiocyanate; 7-AAD, 7-amino-actinomycin D. D: Western blotting for cleaved poly(ADP-ribose) polymerase (PARP), phorbol-12-myristate-13-acetate-induced protein 1 (NOXA), and survivin. Cells were treated for 48 h with 5-20  $\mu$ M nelfinavir. Actin was used for the loading control. Representative blots are shown.

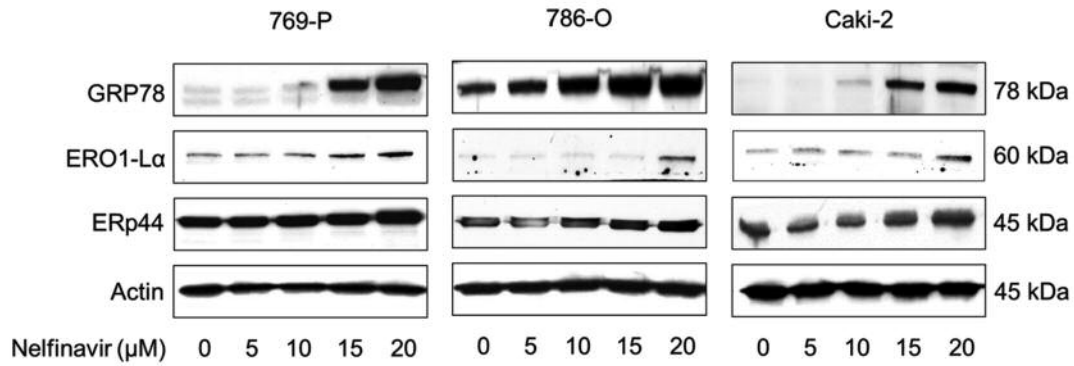


Figure 3. Nelfinavir induced endoplasmic reticulum (ER) stress in renal cancer cells. Western blotting for the ER stress markers glucose-regulated protein 78 (GRP78), endoplasmic oxidoreductin-1-like protein alpha (ERO1-Lα), and endoplasmic reticulum resident protein 44 (ERp44). Cells were treated for 48 h with 5-20 μM nelfinavir. Actin was used for the loading control. Representative blots are shown.

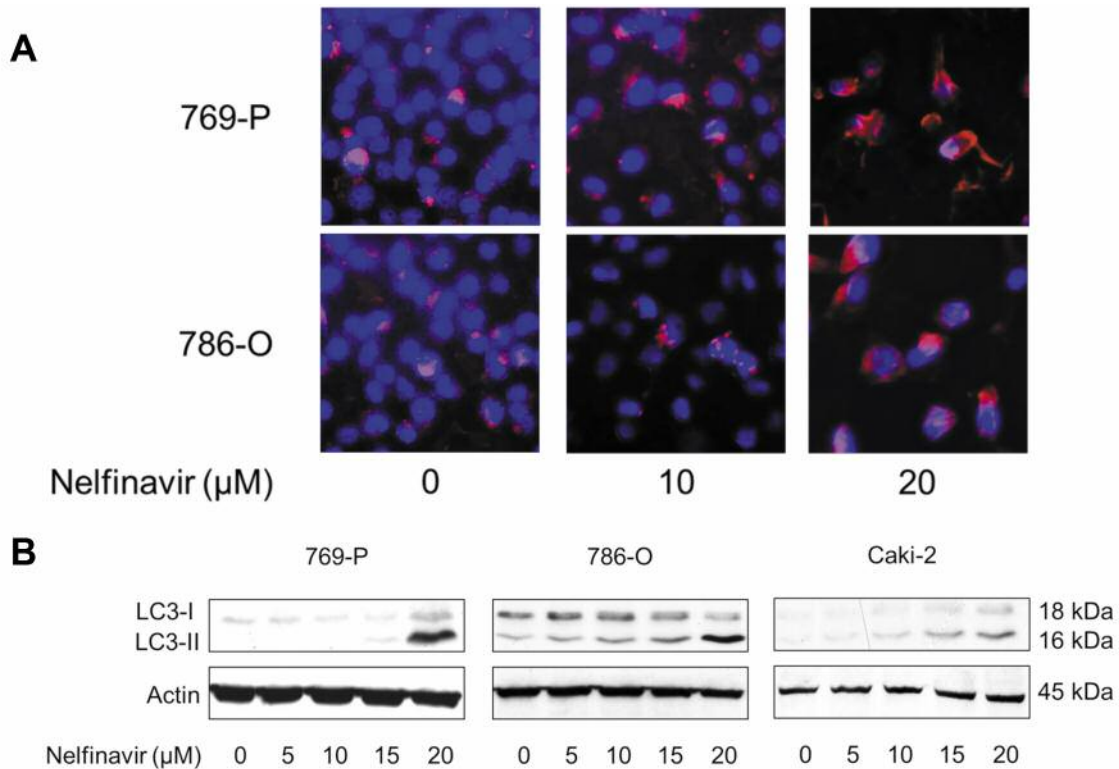


Figure 4. Nelfinavir caused aggresome formation and induced autophagy in renal cancer cells. A: Aggresome detection after 48 h treatment with 10 or 20 μM nelfinavir. Red, aggresome; blue, nucleus. Original magnification, 100×. B: Western blotting for the autophagy marker light chain 3 (LC3). Cells were treated for 48 h with 5-20 μM nelfinavir. The emergence of LC3-II marks the occurrence of autophagy. Actin was used for the loading control. Representative blots are shown.

## Discussion

A significant number of patients diagnosed with renal cancer present with locally invasive or metastatic disease (17), and novel therapeutic approaches are urgently needed because

although tyrosine kinase inhibitors, mTOR inhibitors, and immune checkpoint inhibitors are widely used against advanced renal cancer (2-9), there is no curative therapy for it.

Induction of ER stress reportedly killed cancer cells (18, 19) and has been recognized as a novel strategy for treating cancer

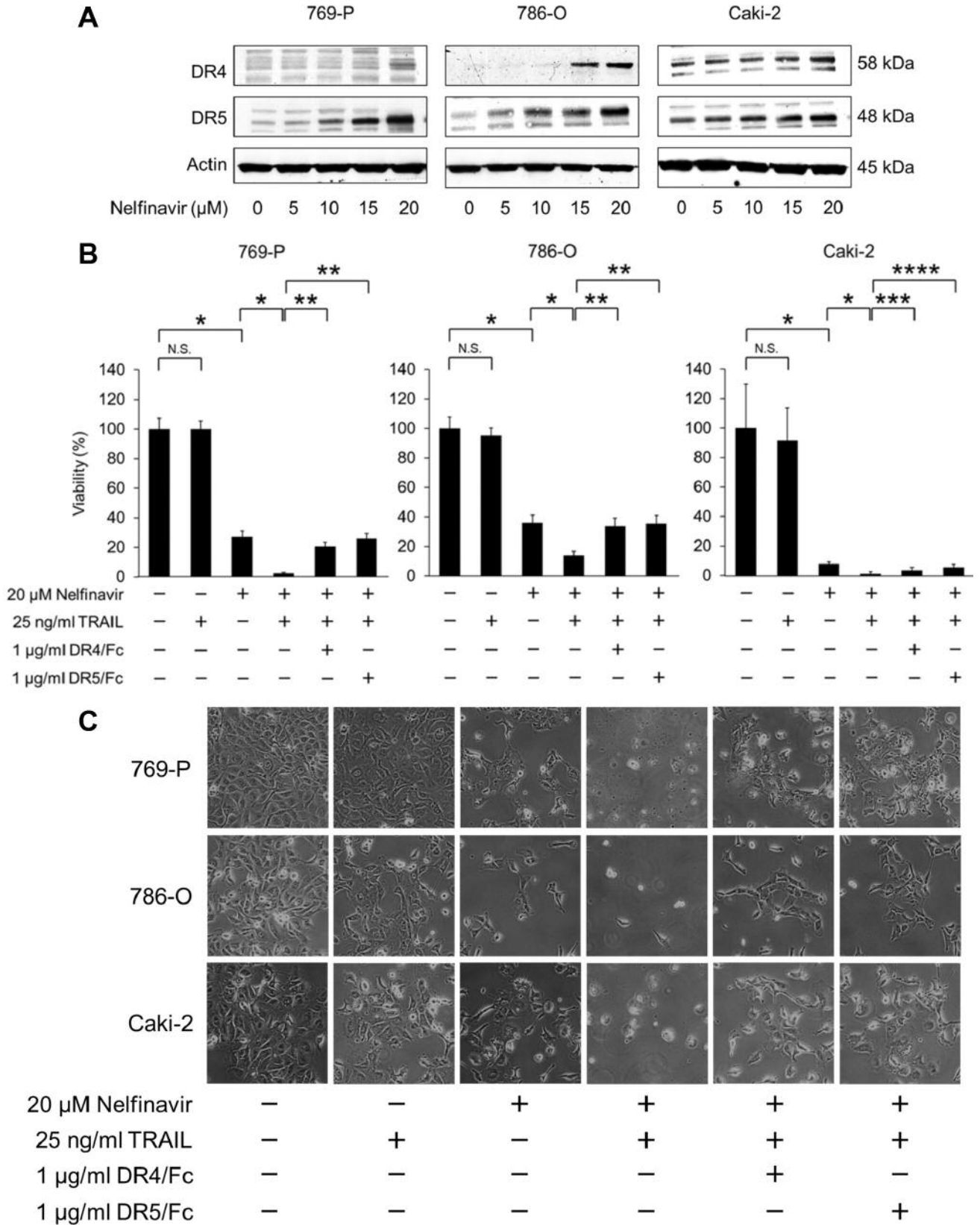


Figure 5. Continued

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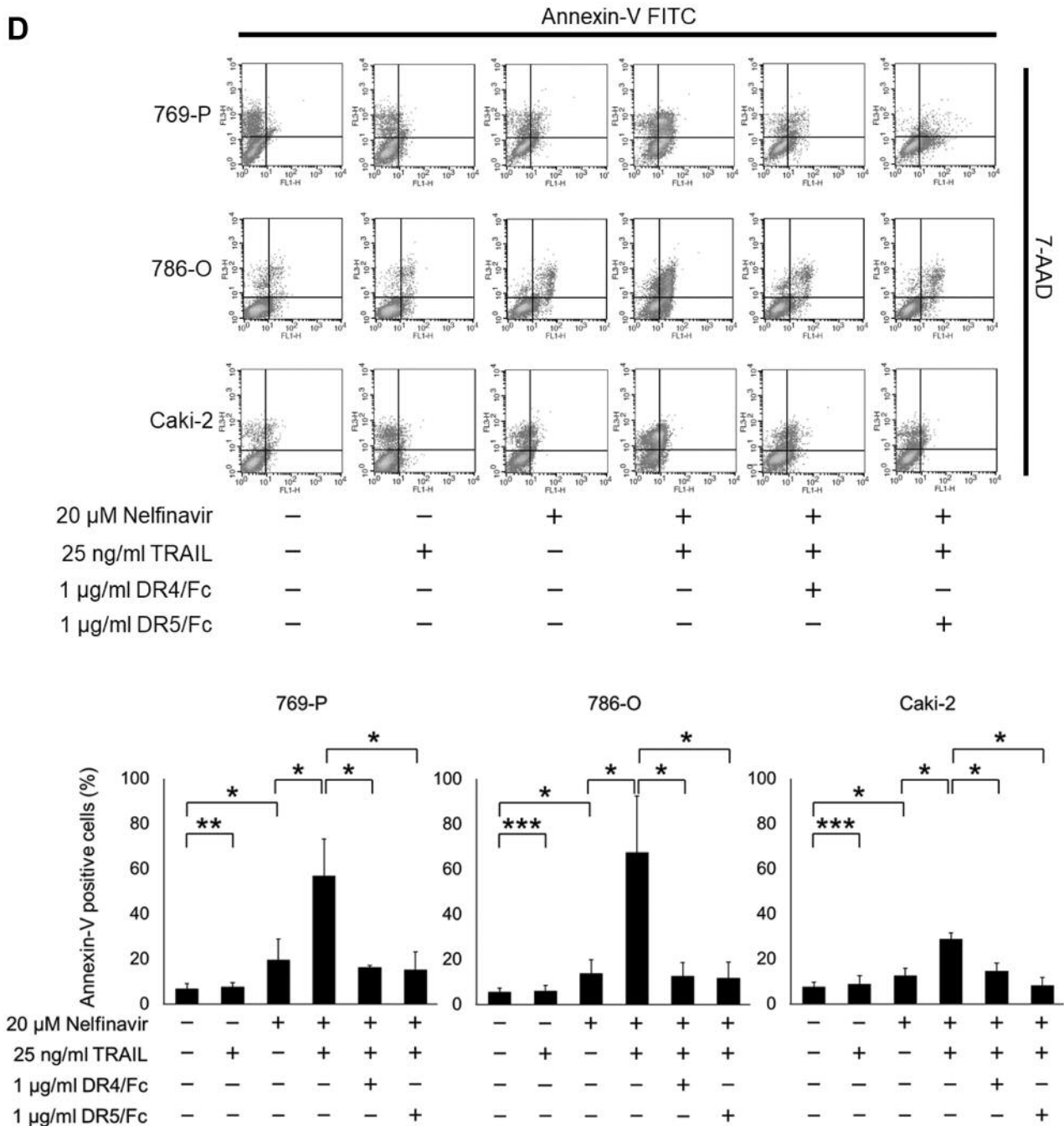


Figure 5. Nelfinavir increased the expression of death receptor 4 (DR4) and DR5 and sensitized renal cancer cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). A: Western blotting for DR4 and DR5. Cells were treated for 48 h with 5-20  $\mu$ M nelfinavir. Actin was used as the loading control. Representative blots are shown. B: MTS assay. Cells were cultured for 24 h in medium with or without 20  $\mu$ M nelfinavir. Then they were given 25 ng/ml TRAIL with or without 1  $\mu$ g/ml DR4/Fc or DR5/Fc chimeric protein and incubated for another 24 h. Bars represent mean $\pm$ SD, n=12. Significantly different at \*p=0.0001, \*\*p=0.0007, \*\*\*p=0.0033, and \*\*\*\*p=0.0021; N.S., not significantly different. C: Photomicrographs after 48-h treatment. Cells were cultured for 24 h in medium with or without 20  $\mu$ M nelfinavir. Then they were given 25 ng/ml TRAIL with or without 1  $\mu$ g/ml DR4/Fc or DR5/Fc chimeric protein and incubated for another 24 h. Note that TRAIL exerted very strong cytotoxicity only when it was preceded by nelfinavir treatment, and this cytotoxicity was blocked by DR4/Fc and DR5/Fc chimeric protein. Original magnification, 100 $\times$ . D: Annexin-V assay. Cells were cultured for 24 h in medium with or without 20  $\mu$ M nelfinavir. Then they were given 25 ng/ml TRAIL with or without 1  $\mu$ g/ml DR4/Fc or DR5/Fc chimeric protein and incubated for another 24 h. Apoptotic cells were detected by annexin-V assay using flow cytometry; 10,000 cells were counted. Bar graphs show the percentages of apoptotic cells. Data are expressed as mean $\pm$ SD from three independent experiments. FITC, Fluorescein isothiocyanate; 7-AAD, 7-amino-actinomycin D. \*Significantly different at p=0.0495; N.S., not significantly different.

(10). The present study showed that the HIV protease inhibitor nelfinavir induced ER stress and killed renal cancer cells.

Nelfinavir is widely used against HIV infection and was recently found to exert antitumor activity by inhibiting proteasomes (11), HSP90 (12), the AKT serine/threonine kinase pathway (20), and nuclear factor-kappa B (NF- $\kappa$ B) (21). We believed that nelfinavir would inhibit both proteasomes and molecular chaperones and thereby cause unfolded proteins to accumulate in the cells. When unfolded protein accumulation is persistent, it triggers ER stress, resulting in cell death (22).

We found that nelfinavir induced apoptosis and inhibited the growth of renal cancer cells in a dose-dependent fashion. As postulated, nelfinavir induced ER stress, evidenced by the increased expression of the ER stress markers GRP78, ERO1- $\alpha$  and ERp44. We previously showed the importance of ER stress induction in inhibiting renal cancer growth (23-25), and the induction of ER stress is thought to be one important mechanism of action of nelfinavir. In addition, nelfinavir caused aggresome formation and induced autophagy. An aggresome is a cytoplasmic inclusion body containing unfolded protein aggregates (26), and it facilitates elimination of unfolded proteins from cells by autophagy (27, 28). The role of autophagy in cancer cells remains controversial, but excessive autophagy reportedly killed cancer cells (29). Although further study would be needed to clarify the exact role of nelfinavir-induced aggresome formation and autophagy, these phenomena well support our postulation that nelfinavir causes unfolded protein to accumulate in the cell.

TRAIL, as a member of the TNF family, can bind TRAIL receptors such as DR4 and DR5 and induce apoptosis in many kinds of tumor cells *in vitro* and *in vivo* (13). After binding its receptors, TRAIL activates caspase cascades (14), which accelerate apoptosis (30). ER stress was recently shown to regulate TRAIL receptors in malignant diseases (31-33), and we postulated nelfinavir might also increase the expression of TRAIL receptors in renal cancer cells. As expected, nelfinavir did induce ER stress and increased the expression of DR4 and DR5. Because TRAIL killed cancer cells only when they had been pretreated with nelfinavir and blockade of each of the receptors with the corresponding chimeras (DR4/Fc and DR5/Fc) attenuated TRAIL-induced cytotoxicity, the sensitization of renal cancer cells to TRAIL was shown to be due to the increased expression of both DR4 and DR5 caused by nelfinavir. In gastric cancer, overexpression of TRAIL- $\gamma$  in gastric carcinoma tissue samples was reported to be associated with a significantly higher survival rate (34). Anees *et al.* showed that elevated TRAIL expression in the tumor microenvironment was significantly associated with increased recurrence-free survival in patients with prostate cancer (35). Thus, intrinsic TRAIL is thought to have significant anticancer activity. Furthermore, patients with bladder cancer with high expression of DR4 or DR5 were shown to have significantly

longer postoperative recurrence-free periods (36). Increasing the sensitivity of cancer cells to intrinsic TRAIL by using nelfinavir to increase the expression of TRAIL receptors is thus thought to be a reasonable approach to killing cancer cells through activation of the TRAIL pathway.

To our knowledge, this is the first study showing that nelfinavir induced ER stress in renal cancer cells and sensitized them to TRAIL. The present study provides a basis for clinical studies using nelfinavir in patients with renal cancer.

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