

## Synergistic Antitumor Effect of NVP-BEZ235 and Sunitinib on Docetaxel-resistant Human Castration-resistant Prostate Cancer Cells

HONG SEOK PARK<sup>1,2\*</sup>, SUNG KYU HONG<sup>3\*</sup>, MI MI OH<sup>1,2</sup>, CHEOL YONG YOON<sup>1,2†</sup>, SEONG JIN JEONG<sup>3</sup>, SEOK SOO BYUN<sup>3</sup>, JUN CHEON<sup>4</sup>, SANG EUN LEE<sup>3</sup> and DU GEON MOON<sup>1,2</sup>

<sup>1</sup>Department of Urology, College of Medicine, Korea University, Guro Hospital, Seoul, Republic of Korea;

<sup>2</sup>Korea University Center of Regenerative Medicine, Seoul, Republic of Korea;

<sup>3</sup>Department of Urology, Seoul National University Bundang Hospital, Gyeonggi-do, Republic of Korea;

<sup>4</sup>Department of Urology, College of Medicine, Korea University, Anam Hospital, Seoul, Republic of Korea

**Abstract.** According to recent studies, mTOR (mammalian target of rapamycin) inhibitor and tyrosine kinase inhibitor (TKI) can be used as combinational agents to enhance the antitumor effect or overcome resistance to one of the agents. In the present study, we investigated the synergistic interaction between NVP-BEZ235, a PI3K (phosphoinositide 3-kinase)/mTOR dual inhibitor, and sunitinib, a TKI, in castration-resistant prostate cancer (CRPC) cells with docetaxel resistance. Prostate cancer cells with different sensitivities to hormones and docetaxel levels were exposed to escalating doses of NVP-BEZ235 alone and in combination with sunitinib. The synergy between NVP-BEZ235 and sunitinib was determined by the combination index, three-dimensional model, and clonogenic assays. Flow cytometry and western blot analysis of proteins related to apoptosis and cell survival axis were performed. The combination of NVP-BEZ235 and sunitinib caused a significant synergistic antitumor effect over a wide range of doses in docetaxel-resistant CRPC cells. Furthermore, the IC<sub>50</sub> (half-maximal inhibitory concentration) of NVP-BEZ235 and sunitinib was reduced by 7.8-fold and 6.6-fold, respectively. The three-dimensional synergy analysis resulted in a synergy volume of 182.47  $\mu\text{M}/\text{ml}^2\%$ , indicating a strong synergistic effect of combination therapy. Combination therapy caused an

induction of caspase-dependent apoptosis in docetaxel-resistant CRPC cells. Adding sunitinib did not produce any additional effect on the NVP-BEZ235-mediated inhibition of PI3K/AKT/mTOR phosphorylation. In conclusion, combining NVP-BEZ235, a dual PI3K/mTOR inhibitor, with sunitinib can synergistically potentiate the antitumor effect in CRPC cells after docetaxel failure though induction of caspase-dependent apoptosis.

Since the FDA (Food and Drug Administration)'s approval for using docetaxel in combination with prednisone in 2004, the docetaxel-based regimen remains the mainstay of treatment for patients with castration-resistant prostate cancer (CRPC) (1, 2). However, docetaxel-based chemotherapy leads to low survival rates in patients with CRPC (*i.e.* median survival of 18 months and progression free-survival of six months) (3, 4). Therefore, there has been an increasing need for the development of new treatment modalities for this difficult to manage post-docetaxel population.

The important role of targeted therapy as first- or second-line treatment options for various disorders, including genitourinary tumors, has been established by advances in functional manipulation techniques of specific molecular targets. It has also been found that combining targeted agents is an effective method for enhancing the antitumor effect of other treatment modalities (*i.e.* conventional chemotherapy regimens and radiation therapy) (5, 6).

The phosphoinositide 3-kinase (PI3K)/AKT (protein kinase B)/mTOR (mammalian target of rapamycin) pathway is a well-known central crossroad of many signaling pathways, and is involved in the direct and indirect control of diverse molecular functions (*i.e.* cell cycle, survival, and apoptosis) (7, 8). Therefore, simultaneously targeting the PI3K/AKT/mTOR pathway in combination with interconnected signaling pathways is a promising strategy for enhancing the antitumor effect.

\*Equal study contribution as first Authors.

Correspondence to: Cheol Yong Yoon, MD, Ph.D., Department of Urology, College of Medicine, Korea University, Guro Hospital, 148 Guro-dong, Guro-gu, Seoul, 152-703, Korea. Tel: +82 226263205, Fax: +82 226261321, e-mail: yoonyong@korea.ac.kr

Key Words: Prostate, carcinoma, resistance, docetaxel, NVP-BEZ235, sunitinib.

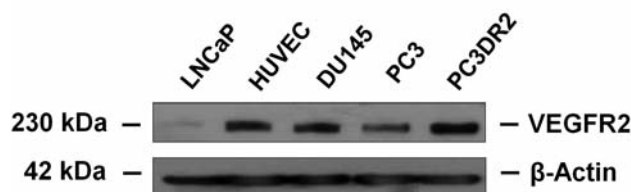


Figure 1. Expression of VEGFR2 (vascular endothelial growth factor receptor 2) in prostate cancer and endothelial cell lines. Proteins were extracted from endothelial (HUVEC, human umbilical vein endothelial cell), hormone-sensitive CaP (LNCaP), docetaxel-sensitive CRPC (castration resistant prostate cancer, DU145, PC3), and docetaxel-resistant CRPC (PC3DR2) cells and the expression of VEGFR2 was assessed by western blot.

In a recent study, Yasumizu *et al.* showed that NVP-BEZ235, a dual PI3K/mTOR inhibitor, is an orally-bioavailable imidazoquinolone derivative that has potent antitumor effects on human prostate cancer (CaP) cells and enhances the antitumor effect of docetaxel in chemotherapy-naïve, and docetaxel-resistant human CRPC cells (9). The objective of the current study was to explore the synergistic interaction between NVP-BEZ235 and sunitinib, a tyrosine kinase inhibitor (TKI), targeting VEGF (vascular endothelial growth factor) and PDGFR (platelet-derived growth factor) in docetaxel-resistant CRPC cells with focus on the activation of caspase-dependent apoptosis.

## Patients and Methods

**Cell lines and chemicals.** All cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM (Dulbecco's Modified Eagle Medium, cell lines DU145, PC3, and PC3DR2) and RPMI-1640 (cell lines LNCaP and HUVEC (human umbilical vein endothelial cell)) with 10% fetal bovine serum (Mediatech, Herndon, VA, USA) and 100 U/ml penicillin/100 mg/ml streptomycin (Gibco BRL, Grand Island, NY, USA) with 5% CO<sub>2</sub> at 37°C. The docetaxel-resistant CRPC cell line (namely PC3DR2) was generated by serial desensitization of PC3 cells.

Sunitinib, NVP-BEZ235, and docetaxel were donated by Pfizer (NY, USA), Novartis (NY, USA), and Sanofi-Aventis (Seoul, South Korea), respectively. Temsirolimus and everolimus were obtained from LC Laboratories (Woburn, MA, USA).

**Cytotoxicity assay.** Cells were treated with increasing doses of docetaxel (0.5–50.0 μM), sunitinib (0.38 nM–40.0 μM), NVP-BEZ235 (0.19 nM–100.0 μM), temsirolimus (0.38 nM–100 μM), or everolimus (0.38 nM–100 μM). The antitumor effect was determined by the cell counting kit-8 (CCK-8) assay. About 4,000 cells were seeded in 96-well plates with 100 μl of media, and the cells were exposed to each drug for 24, 48, or 72 h. Afterwards, 10 μl of the CCK-8 solution (Dojindo Molecular Technologies, Gaithersburg, MD, USA) was added and the absorbance at 450 nm was measured following three hours of incubation.

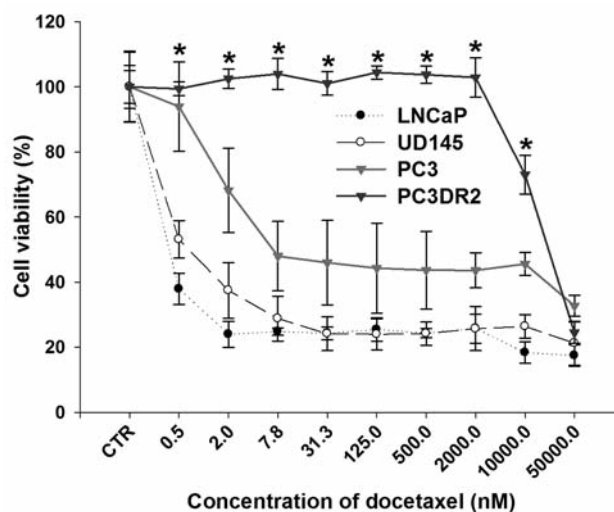


Figure 2. Antitumor effects of docetaxel in prostate cancer cell lines. Hormone-sensitive (LNCaP) and castration-resistant prostate cancer cell lines (DU145, PC3, PC3DR2) were exposed to increasing doses of docetaxel (0.5 nM–50.0 μM) for 72 h and the antitumor effect in each cell line was assessed by the CCK-8 assay. Each data point represents the mean ± standard deviation from three trials. \**p* < 0.05 for each treated group vs. untreated control.

**Determination of synergism.** The synergistic interactions between NVP-BEZ235 and sunitinib in PC3DR2 cells were assessed by combination index (CI) based on the results of CCK-8 assay in which CI values of <1.0, >1.0, and 1.0 indicates synergism, antagonism, and additivity, respectively (10). Furthermore, the degree of synergism was quantitatively determined by method of Richard and Shipman with the MacSynergy II software in which the following calculated values were obtained in either a positive (synergism) or negative (antagonism) direction: 0–25 (insignificant interaction), 25–50 (minor interaction), 50–100 (moderate interaction), and >100 μM/mL<sup>2</sup>% (strong interaction) (11–13).

**Clonogenic assay.** The PC3DR2 cells (2 × 10<sup>2</sup>) cells were plated in a 6-well culture plate and treated with NVP-BEZ235 (0.5 μM) and/or sunitinib (5.0 μM) for 72 hours with 5% CO<sub>2</sub> at 37°C. The cells were then cultured for another 10 days in drug-free media before colonies were visualized with 0.4% crystal violet staining.

**Flow cytometric analysis of cell cycle and apoptosis.** To evaluate alterations in the cell cycle following NVP-BEZ235 and sunitinib treatments, the PC3DR2 cells were exposed to NVP-BEZ235 (0.5 μM) with and without sunitinib (5.0 μM) for 72 hours and fixed in 70% ethanol before staining with a propidium iodide solution [970 μl phosphate buffered saline and 40 μl of 1 mg/ml propidium iodide (Sigma-Aldrich, St. Louis, MO, USA)] and 3 μl of RNase A (Sigma-Aldrich, St. Louis, MO, USA). A FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) was used for flow cytometry. Annexin V-FITC apoptosis detection kit (BP Pharmingen, San Jose, CA, USA) was used to assess drug-induced apoptosis.

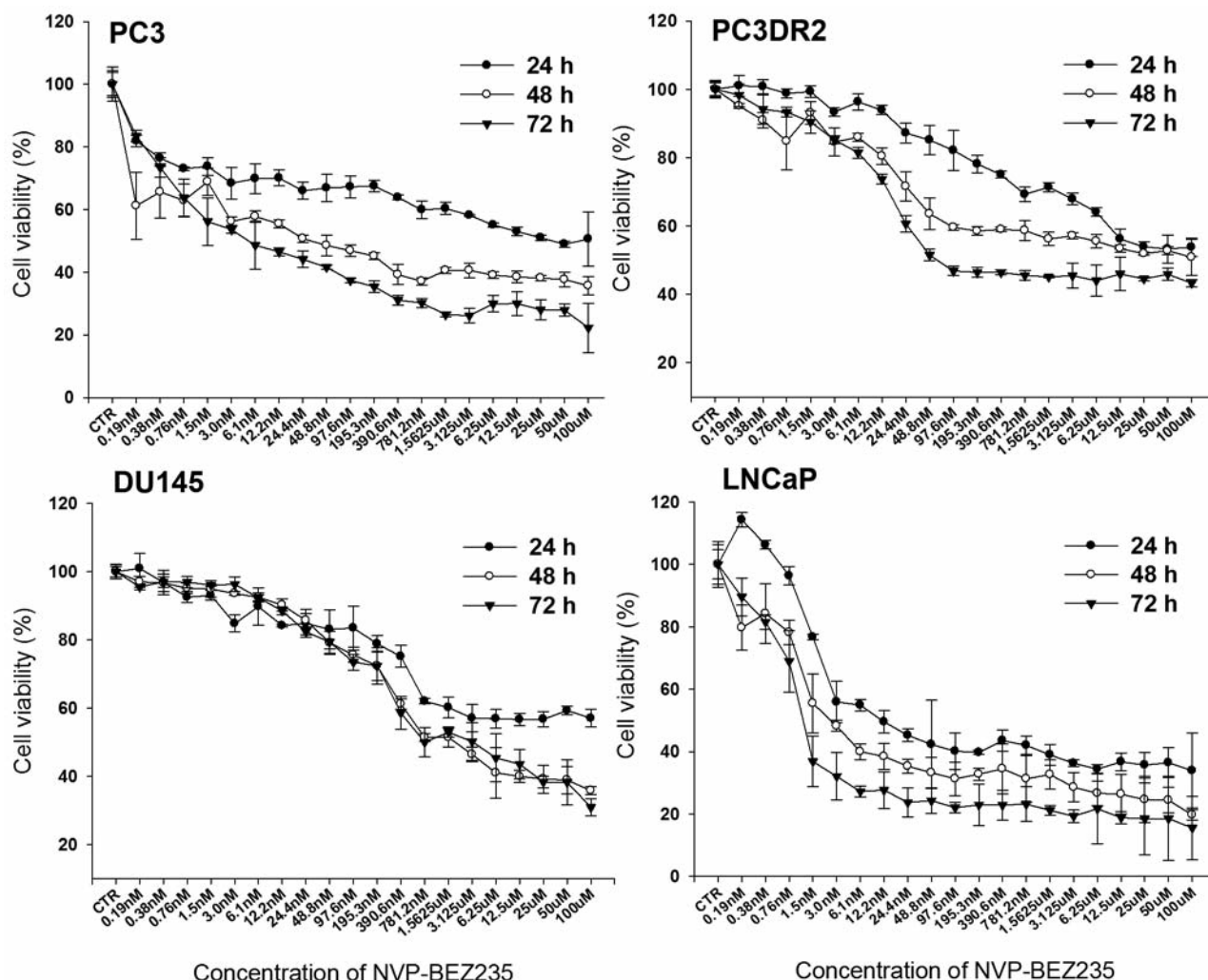


Figure 3. The antitumor effect of NVP-BEZ235 in prostate cancer cell lines. Human prostate cancer cell lines with different sensitivities to hormone and docetaxel levels were exposed to increasing doses of NVP-BEZ235 (0.19 nM–100  $\mu$ M) for 24, 48, and 72 h and the antitumor effect in each cell line was assessed by the CCK-8 assay. Each data point represents the mean  $\pm$  standard deviation from three trials. X-axis is logarithmic scale for concentration.

**Western blot analysis.** Proteins were extracted from the PC3DR2 cells using a RIPA lysis buffer 72 hours after treatment with NVP-BEZ235 (0.5  $\mu$ M) alone or combined with sunitinib (5.0  $\mu$ M). The proteins were fractionated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), and blocked with 5% milk in Tris Buffered Saline with Tween. The membranes were then incubated overnight at 4°C with the corresponding primary antibodies [namely antibodies against cleaved caspase-3, -8, and -9, cleaved PARP (Poly ADP ribose polymerase), cIAP1 (Cellular Inhibitor of Apoptosis1), cIAP2, XIAP (X-linked Inhibitor of apoptosis protein), survivin, p-IKK $\alpha$  (phospho-IkB kinase alpha, Ser176/180), IKK $\alpha$ , p-IKK $\beta$  (ser32), IKK $\beta$ , NF- $\kappa$ B (nuclear factor kappa-light-chain-enhanced of activated B cells), p-PI3K (Tyr199/458), PI3K, p-AKT (ser473), AKT, p-mTOR (Ser2448), mTOR, and VEGFR2]. Following incubation with secondary antibodies, the protein expression was

detected with an enhanced chemiluminescence western blot substrate kit (Pierce, Rockford, IL, USA).

**Statistical analysis.** Statistical analysis was conducted with SPSS 14.0K software (SPSS Inc., Chicago, IL, USA). Unless indicated otherwise, data were obtained from a minimum of three trials and were expressed as mean  $\pm$  standard deviation (SD). Statistical significance was determined by two-sample *t*-tests. A *p*-value of less than 0.05 was considered significant.

## Results

**VEGFR2 expression in CaP cells.** According to the western blot analysis, VEGFR2 was minimally expressed in hormone-sensitive LNCaP cells and significantly in DU145,

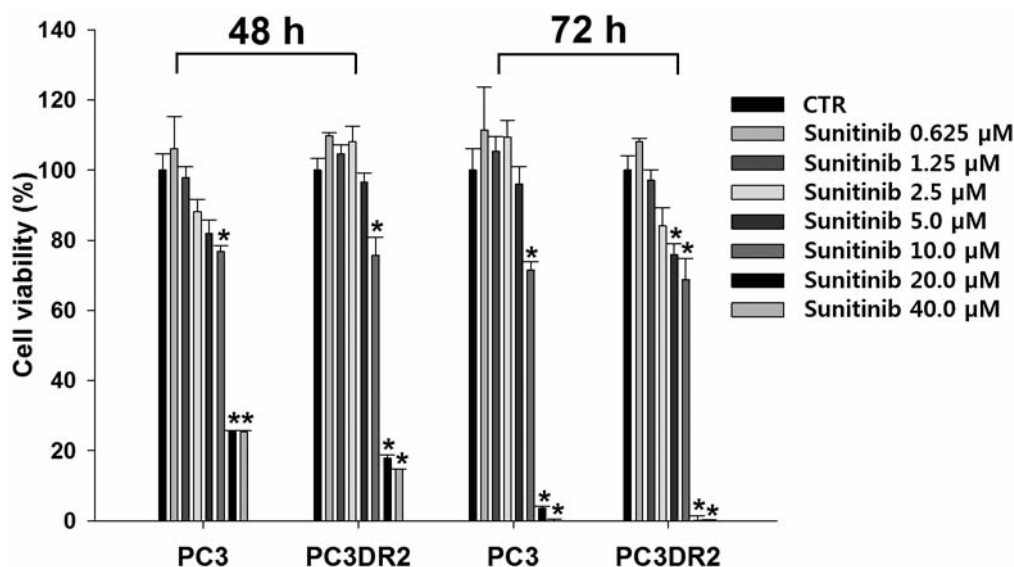


Figure 4. The antitumor effect of sunitinib in PC3 and PC3DR2 cells. Two castration resistant prostate cancer cell lines with different sensitivities to docetaxel were treated with increasing doses of sunitinib (0.625-40.0  $\mu$ M) for 48 and 72 h and the antitumor effect in each cell line was assessed by the CCK-8 assay. Each data point represents the mean  $\pm$  standard deviation from three trials. \* $p < 0.05$  for each treated group vs. untreated control.

PC3, and PC3DR2 cells, which suggests a possible direct activity of VEGFR2-targeted agents (*i.e.* sunitinib) on CRPC cells (Figure 1).

**Antitumor effect of docetaxel, NVP-BEZ235, and sunitinib.** Docetaxel showed a dose-dependent antitumor effect against LNCaP, DU145, and PC3 cells and suppressed proliferation of these cells by 75.2%, 71.2%, and 52.0% respectively, at a low concentration of 0.008  $\mu$ M after 72 h of exposure. However, the PC3DR2 cells showed no discernible response to docetaxel (up to a concentration of 2.0  $\mu$ M) after 72 h of exposure (Figure 2).

Although sunitinib showed dose- and time-dependent antitumor effects on PC3 and PC3DR2 cells, a significantly high concentration of sunitinib ( $>10$   $\mu$ M) was required for the effective suppression of growth in both cell lines (Figure 3). NVP-BEZ235 exerted dose- and time-dependent antitumor effects on all CaP cells (*i.e.* LNCaP, DU145, PC3, PC3DR2). However, docetaxel-resistant PC3DR2 cells required significantly higher amounts of NVP-BEZ235 and mTOR inhibitors (*i.e.* temsirolimus, everolimus) for inhibiting growth to a similar level as docetaxel-sensitive PC3 cells (Figures 4 and 5). At equimolar concentrations, NVP-BEZ235 exerted a more potent antitumor effect against all tested CaP cell lines compared to temsirolimus and everolimus (Figure 5).

**NVP-BEZ235-mediated inhibition of AKT phosphorylation.** We found a higher expression and phosphorylation of AKT in hormone-sensitive LNCaP cells compared to CRPC cells (*i.e.*

DU145, PC3, PC3DR2) (Figure 6A and B). The amount of AKT phosphorylation was similar between PC3 and PC3DR2 cells. However, the level of Akt phosphorylation in both PC3 and PC3DR2 cells was lower than that of LNCaP cells, but higher than that of DU145 cells (Figure 6A and B). In both PC3 and PC3DR2 cells, NVP-BEZ235 inhibited AKT phosphorylation in a dose-dependent manner, but the suppression was more prominent in PC3 cells than PC3DR2 cells at equal NVP-BEZ235 concentrations (Figure 6C). The NVP-BEZ235-mediated antitumor effect in CaP cells was positively correlated with the level of AKT phosphorylation in each cell line. The most prominent antitumor effect was observed in LNCaP cells, which had a high level of AKT phosphorylation. The antitumor effect on DU145 cells was limited with a relatively low AKT phosphorylation (Figure 6D).

**Synergistic interaction between NVP-BEZ235 and sunitinib.** The fixed-ratio (1:10) combined treatment with NVP-BEZ235 (0.125-2.0  $\mu$ M) and sunitinib (1.25-20  $\mu$ M) for 72 hours of PC3DR2 cells had correlation coefficients ( $r$  values of median-effect plot) of 0.89649, 0.96963, and 0.98125, which indicate that our data was in accordance with the median-effect principle and had good reproducibility (Figure 7A). The  $IC_{50}$ s ( $D_m$ ) of NVP-BEZ235, sunitinib, and the combination treatment were 14.1  $\mu$ M, 11.8  $\mu$ M, and 1.8  $\mu$ M, respectively, indicating a significant synergistic interaction between the two drugs (Table I). Synergism between the two drugs over a wide range of fractions was also observed with the fa-CI (fraction affected-combination index) analysis (Figure 7B; fa=0.4-0.9).



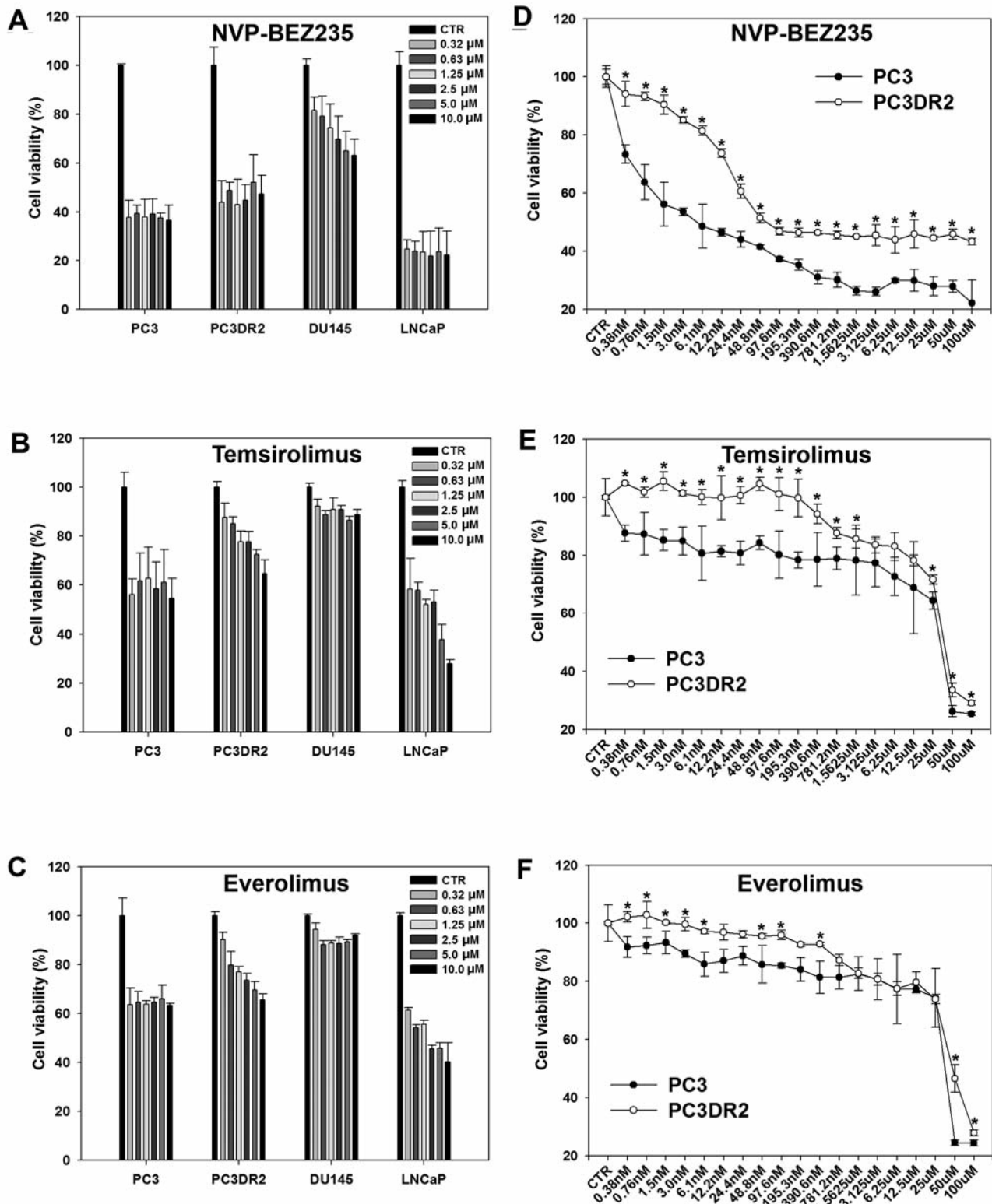


Figure 5. Comparative analysis of the antitumor effect of dual mTOR (mammalian target of rapamycin) and PI3K (phosphoinositide 3-kinase)/mTOR inhibitor in castration-resistant prostate cancer cells. PC3 and PC3DR2 cells were exposed to equimolar concentrations (0.32–10.0  $\mu$ M) of temsirolimus, everolimus, and NVP-BEZ235 for 72 h and the antitumor effect of each drug was determined by the CCK-8 assay. Each data point represents the mean  $\pm$  standard deviation from three trials. \* $p < 0.05$  for PC3 vs. PC3DR2. X-axis of D, E, and F is logarithmic scale for concentration.

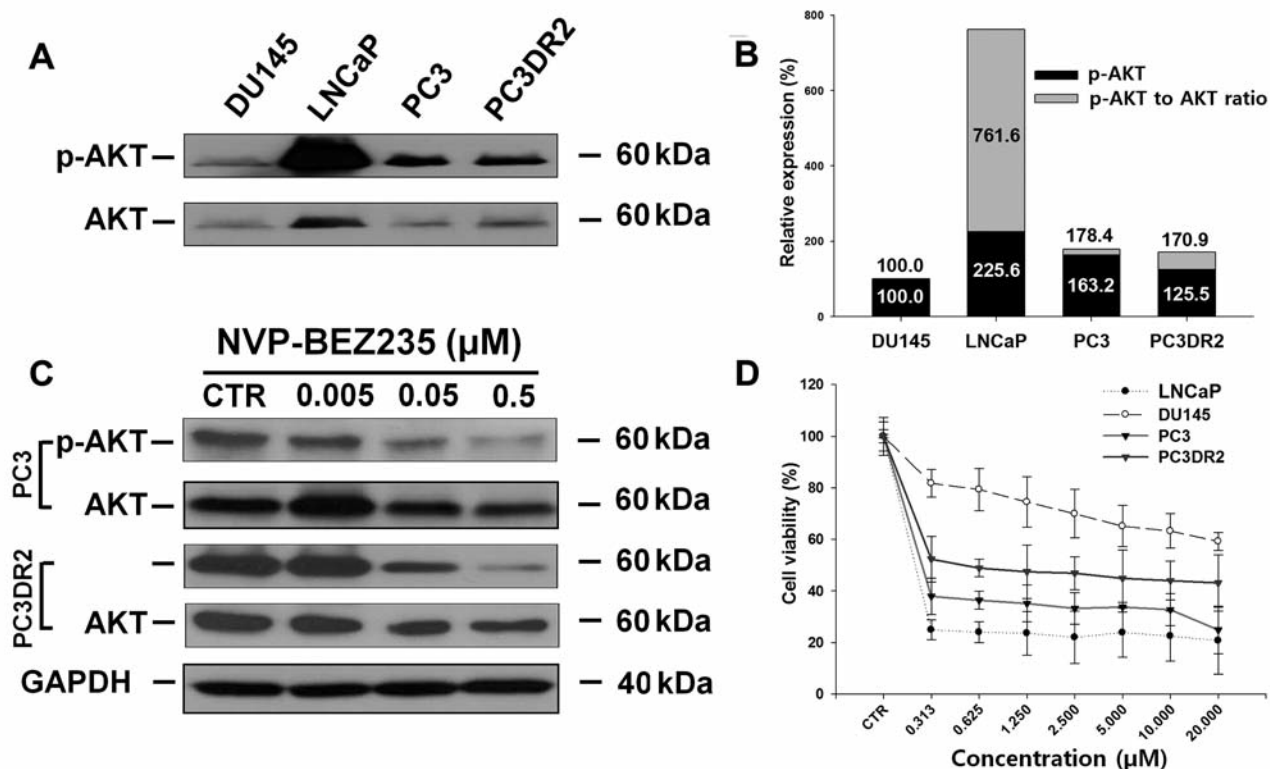


Figure 6. A: Analysis of AKT (protein kinase B)/expression and phosphorylation in prostate cancer cells. Protein was extracted from prostate cancer cell lines of differing hormone and docetaxel sensitivities (i.e. DU145, LNCaP, PC3, PC3DR2) and the AKT expression and phosphorylation status was obtained by western blot. B: Densitometric analysis of western blot results. DU145, which had the lowest AKT expression was used as standard (100%), and the relative AKT and p-AKT to AKT ratio in LNCaP, PC3, PC3DR2, and DU145 cells were expressed as percentiles. C: NVP-BEZ235-mediated suppression of AKT phosphorylation in castration resistant prostate cancer cells. PC3 (docetaxel-sensitive) and PC3DR2 (docetaxel-resistant) cells were treated with increasing doses of NVP-BEZ235 (0.005  $\mu$ M, 0.05  $\mu$ M, 0.5  $\mu$ M) for 72 h and the phosphorylation level of AKT was determined by western blot. D: Antitumor effects of NVP-BEZ235 in prostate cancer cells. prostate cancer cells were exposed to increasing doses of NVP-BEZ235 for 72 hours and the antitumor effect was assessed by the CCK-8 assay. Each data point represents the mean $\pm$ standard deviation from three trials. X-axis of D is logarithmic scale for concentration.

For a more detailed evaluation of synergy, four independent experiments of fully-combined NVP-BEZ235 (0.01-100  $\mu$ M) and sunitinib (2.5-40  $\mu$ M) treatments were performed to generate a synergy plot using the MacSynergy II data analysis program (Figure 8A). A strong synergy between NVP-BEZ235 and sunitinib in PC3DR2 cells (synergy volume of 182.47  $\mu$ M/ml<sup>2</sup>%) with moderate antagonism (89.87  $\mu$ M/ml<sup>2</sup>%) was observed (Figure 8B). The results of the clonogenic assay were also consistent with the synergy test (Figure 8C).

**Combined treatment-mediated induction of apoptosis.** Concomitant NVP-BEZ235 (0.5  $\mu$ M) and sunitinib (5.0  $\mu$ M) treatment of PC3DR2 cells induced a significant increase in the sub-G<sub>1</sub> population (14.0 $\pm$ 2.0%) compared to the untreated control (1.2 $\pm$ 0.5%) and sunitinib (2.3 $\pm$ 1.7%) and NVP-BEZ235 (2.4 $\pm$ 1.8%) single treatment groups, suggesting an important role of apoptosis in the synergistic

Table I. Dose-effect relationship parameters of NVP-BEZ235, sunitinib, and combination treatment of PC3DR2 cells in vitro.

Compound	<i>m</i>	<i>D<sub>m</sub></i>	<i>r</i>
NVP-BEZ235	0.186	14.259	0.896
Sunitinib	8.078	11.825	0.969
NVP-BEZ235 + Sunitinib	1.788	1.924	0.981

*m* is a coefficient signifying the shape of the dose-effect curve; *D<sub>m</sub>* (IC<sub>50</sub>, half-maximal inhibitory concentration) is the dose of drugs to produce 50% inhibition of cell proliferation; *r* is the correlation coefficient signifying conformity of the data to the mass action law.

interaction between the two drugs (Figure 9A-E). According to the flow cytometric analysis after annexin-V FITC/PI (Propidium Iodide) double staining, the population of annexin-V-positive and PI-negative/positive cells (early and

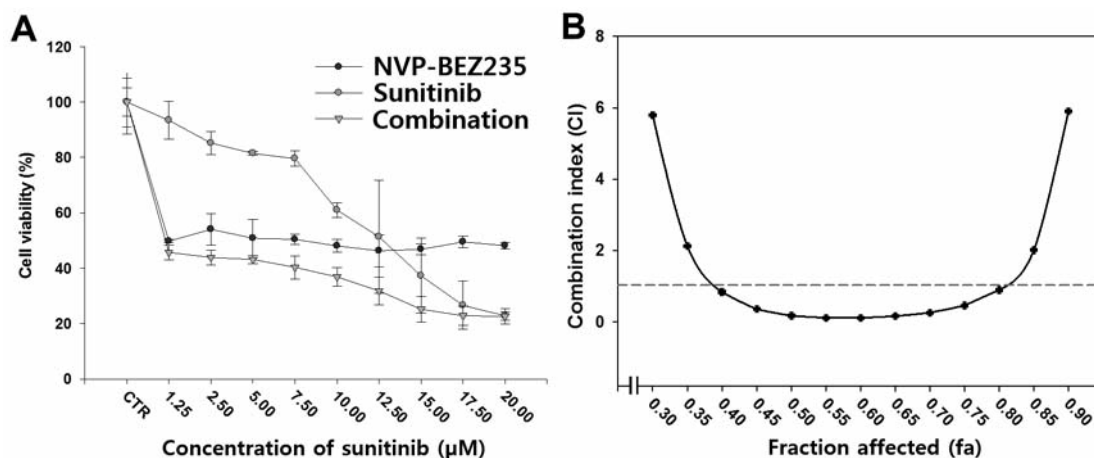


Figure 7. Analysis of the synergism of NVP-BEZ235 with sunitinib in PC3DR2 cells. A: Docetaxel-resistant PC3DR2 cells were exposed to increasing doses of NVP-BEZ235 (0.125–2.0 μM) with and without sunitinib (1.25–20.0 μM) for 72 hours, and the antitumor effect was examined by the CCK-8 assay. B: PC3DR2 cells were treated with increasing concentrations of NVP-BEZ235 alone or in combination with sunitinib at a fixed ratio (1:10) for 72 hours. The fa-CI (fraction affected-combination index) plot was generated based on the CI at the corresponding fa, which represents the fraction affected (e.g. an fa of 0.5 indicates a 50% reduction in cell proliferation). Each data point represents the mean±standard deviation from three trials.

late stage of apoptosis) significantly increased in the concomitant treatment group (30.5±4.0%) compared to the untreated control (4.4±1.2%) and groups treated with sunitinib (6.0±1.1%) or NVP-BEZ235 (4.7±0.6%) alone ( $p<0.05$ ; Figure 9F–9I).

**Alterations in apoptosis-regulatory protein expression.** Simultaneous NVP-BEZ235 and sunitinib treatment of PC3DR2 cells caused a marked increase in caspase-3, -8, -9, and PARP cleavage while suppressing the expression of anti-apoptotic proteins (*i.e.* cIAP1, cIAP2, XIAP, and survivin; Figure 10). Concomitant treatment also resulted in an increase of cytoplasmic NF-κB level and a reciprocal decrease in the level of nuclear NF-κB, which was accompanied by a decrease in IκBα and IκKα phosphorylation (Figure 10). There was no additional enhancement of NVP-BEZ235-induced inhibition of PI3K, AKT, and mTOR phosphorylation with the NVP-BEZ235 and sunitinib combined treatment in PC3DR2 cells (Figure 11).

## Discussion

The PI3K–AKT–mTOR axis has been the focus for target therapy because it plays an important role in the development, progression, metastasis, and chemoresistance of various tumors (14–18). According to recent studies, mTOR inhibitors can be also be used as combinational agents to enhance the antitumor effect of other treatment modalities, including targeted-agents (*i.e.* TKI) (19–24).

In the present study, we explored the synergistic antitumor effect between NVP-BEZ235, a PI3K/mTOR dual inhibitor,

and sunitinib, a TKI, in CRPC cells with docetaxel resistance. NVP-BEZ235 exerted superior dose- and time-dependent antitumor effects compared to first-generation mTOR inhibitors (*i.e.* temsirolimus, everolimus) in all tested CaP cell lines. However, a relatively high dose of NVP-BEZ235 was still required for the inhibition of CRPC cell growth. A dose of ≥0.5 μM of NVP-BEZ235 caused only partial effects against CRPC cells. This was comparable to a recent report by Yasumizu *et al.* in which 0.5 μM of NVP-BEZ235 was shown to only partially inhibit (~60%) CRPC cell growth (9). Moreover, CRPC cells (*i.e.* DU145, PC3, PC3DR2) had relatively lower expression and AKT phosphorylation, which were the central determinants of the antitumor effect of mTOR inhibitors compared to hormone-sensitive LNCaP cells. As expected, the antitumor effect of NVP-BEZ235 was positively correlated with the AKT phosphorylation status of each cell line, resulting in only a limited effect for CRPC cells with low level AKT phosphorylation. According to these findings, as a single agent, NVP-BEZ235 may not produce sufficient antitumor effects in patients with CRPC cells, a potential target population of novel treatments.

Mixed responses were obtained from recent studies with VEGFR-targeted TKI for docetaxel-resistant or chemotherapy-naïve CRPC cells in pre-clinical or clinical settings (25–29). For example, sunitinib (TKI targeting both PDGFR and VEGFR), which was approved by the FDA for the treatment of renal cell carcinomas and imatinib-resistant gastrointestinal stromal tumors, led to a 30% response according to the Response Evaluation Criteria In Solid Tumors (RECIST) criteria in CRPC

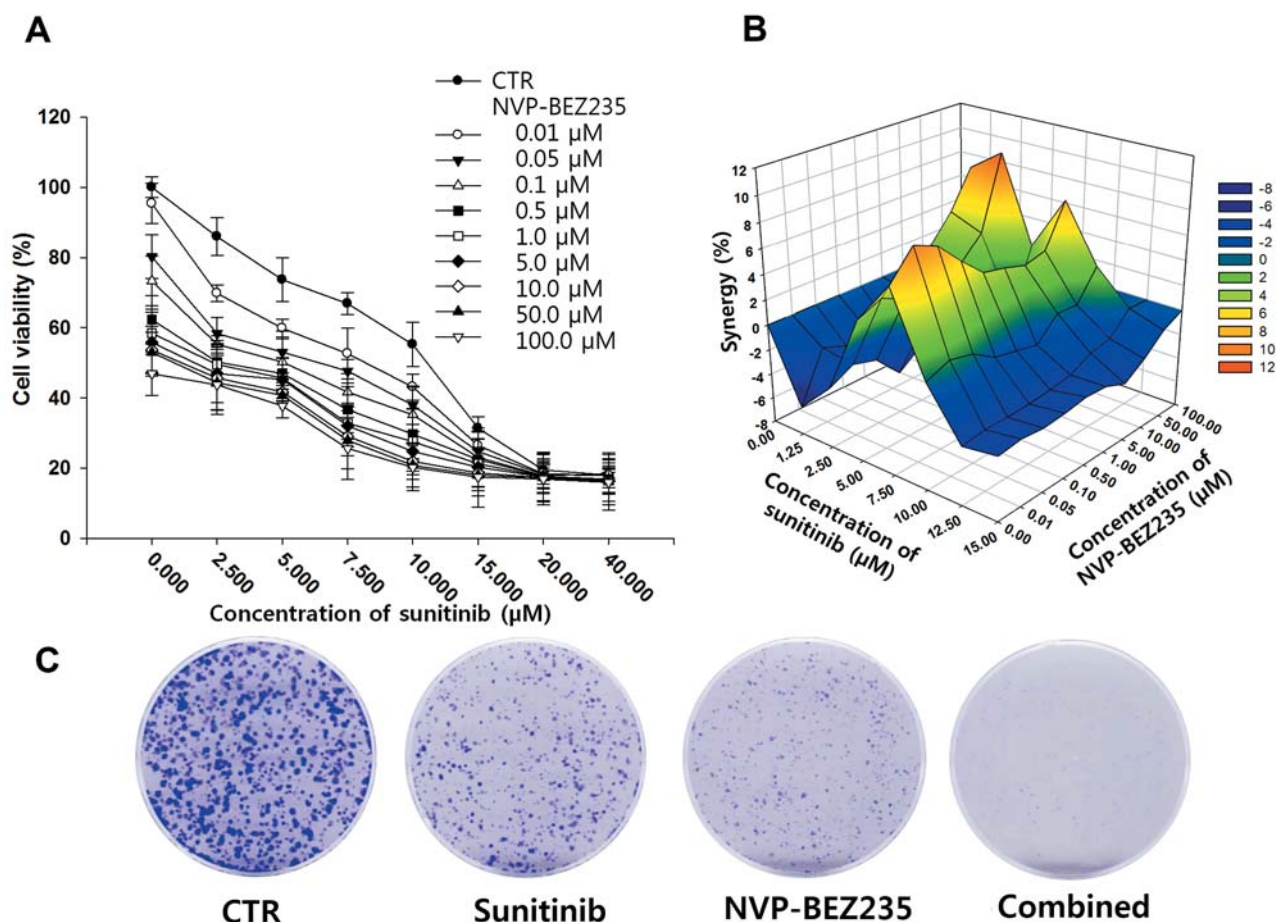


Figure 8. Analysis of the synergism of NVP-BEZ235 with sunitinib in PC3DR2 cells. A: For more detailed analysis of synergism, four independent combination experiments of NVP-BEZ235 (0.01  $\mu\text{M}$ -100.0  $\mu\text{M}$ ) and sunitinib (2.5  $\mu\text{M}$ -40  $\mu\text{M}$ ) were performed and the results were three-dimensionally reconstructed using the MacSynergy II data analysis program. The peak, depression, and horizontal planes indicate synergistic, antagonistic, and additive interactions, respectively (B). C: The clonogenic assay of PC3DR2 cells exposed to NVP-BEZ235 (0.5  $\mu\text{M}$ ) with and without sunitinib (5.0  $\mu\text{M}$ ). CTR: Untreated control.

patients with full (11%) and limited (44.4%) response (29). An improvement in the overall survival rates in patients with CRPC was not observed in a recent phase III clinical trial for sunitinib treatment (30). In the current study, we found that unlike hormone-sensitive LNCaP cells, VEGFR2 expression in CRPC cells (*i.e.*, DU145, PC3, PC3DR2) was markedly increased. These results were similar to previous findings of tumor cell expression of VEGF and VEGFR during neoplastic transformation, which enabled autocrine and paracrine activities of VEGF in some tumors (31-36). Comparable to these results, sunitinib caused a dose- and time-dependent antitumor effect in both PC3 and PC3DR2 cells. However, proper inhibition of CRPC cell growth (at least >50% of control) could only be obtained at the relatively high concentration

ranges of sunitinib. Therefore, although CRPC cells have relatively high VEGFR expression, the antitumor effect of sunitinib by its direct action on VEGFR in CRPC cells is not strong enough for the proper suppression of CRPC tumor cell growth.

We hypothesized that a combined regimen of NVP-BEZ235 and sunitinib can be an option for enhancing the relatively poor antitumor effect of each agent in patients with CRPC, especially those with docetaxel-resistant tumors. Our hypothesis was based on promising results from recent studies about the combined use of mTOR inhibitors and TKI to enhance the antitumor effect or overcome the resistance to each other (19-24).

A significant synergistic interaction between NVP-BEZ235 and sunitinib in PC3DR2 cells was detected by CI and



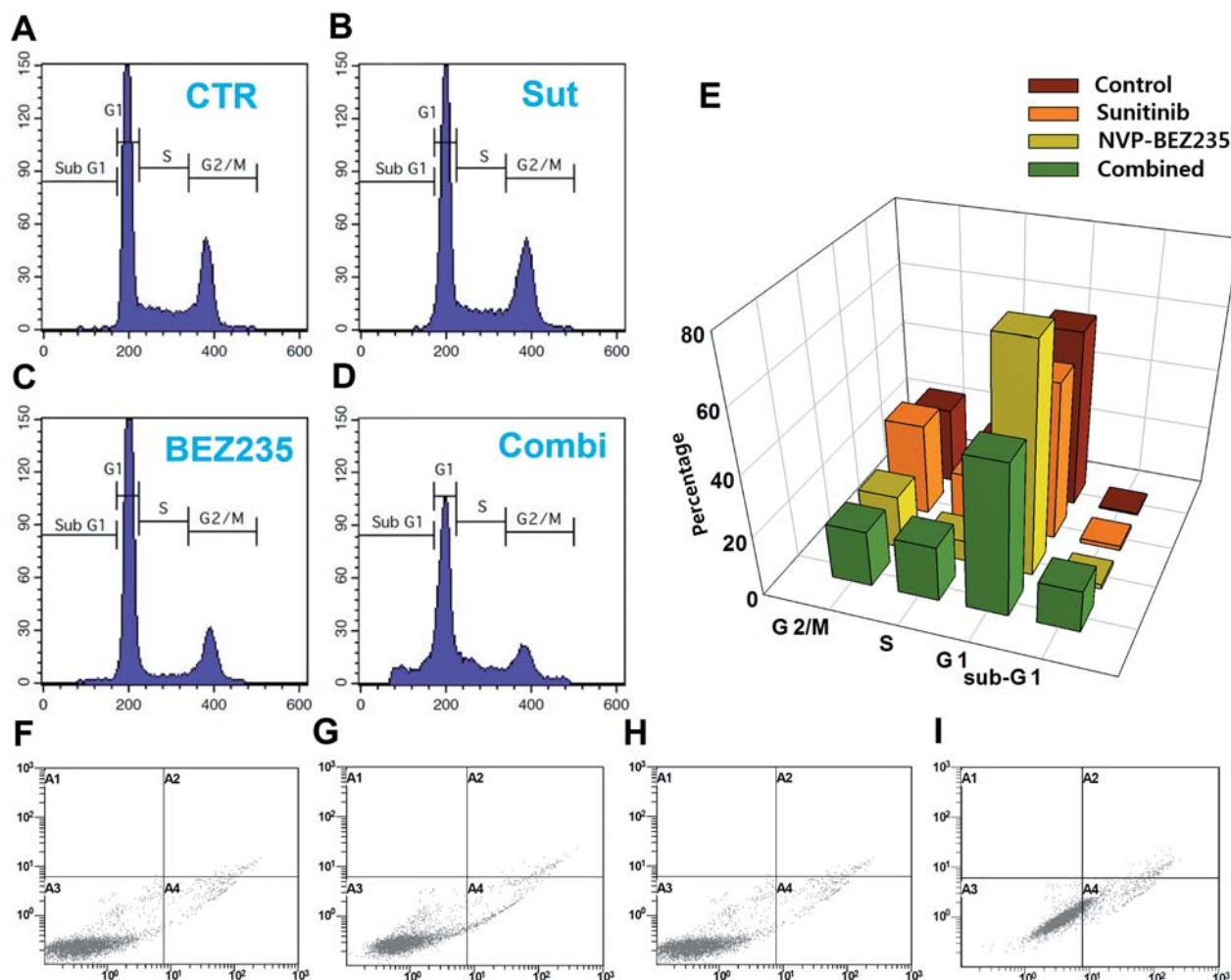


Figure 9. A-D: Representative flow cytometric DNA content histogram of PC3DR2. PC3DR2 cells exposed to sunitinib (5.0  $\mu$ M, B) with and without NVP-BEZ235 (0.5  $\mu$ M, C) for 72 h and alteration in the cell cycle was assessed by FACScalibur flow cytometer after propidium iodide (PI) staining. E: The quantitative analysis of duplicate flow cytometric studies. F-I: PC3DR2 cells were treated with NVP-BEZ235 (0.5  $\mu$ M) with and without sunitinib (5  $\mu$ M) for 72 h and the degree of apoptosis was examined by annexin-V FITC/PI flow cytometry. CTR: Untreated control.

MacSynergy II analysis. A significant increase in the apoptosis of PC3DR2 cells exposed to concomitant treatment was observed by flow cytometric analyses of cell cycle and apoptosis. Combination treatment suppressed the expression of anti-apoptotic cIAP1, cIAP2, XIAP, and survivin, while enhancing the cleavage of caspase-3, -8 and -9, and PARP. Furthermore, the exposure of PC3DR2 cells to concomitant treatment suppressed the phosphorylation of p-IKK $\alpha$  and IKB $\alpha$  accompanied by an increase in cytoplasmic NF- $\kappa$ B levels and a reciprocal decrease of nucleic NF- $\kappa$ B levels, indicating the suppression of NF- $\kappa$ B signaling by combined treatments. According to these findings, a synergistic effect through the induction of caspase-dependent apoptosis is exerted by NVP-BEZ235 and sunitinib in docetaxel-resistant PC3DR2 cells.

Treatment of PC3DR2 cells with sunitinib did not alter PI3K/AKT/mTOR phosphorylation. Furthermore, the addition of sunitinib to NVP-BEZ235 did not change the NPV-BEZ235-mediated PI3K-AKT-mTOR phosphorylation status in PC3DR2 cells. Therefore, the synergistic interaction between NVP-BEZ235 and sunitinib is not caused by the synergistic inhibition of PI3K-AKT-mTOR axis signaling in docetaxel-resistant PC3DR2 cells.

Preliminary data about the synergistic induction of caspase-dependent apoptosis by concomitant NVP-BEZ235 and sunitinib treatment in docetaxel-resistant CRPC cells was obtained in this current study, but it is not without flaws and a few limitations. The limitations of the current study include the *in vitro* nature of the design and the small sample

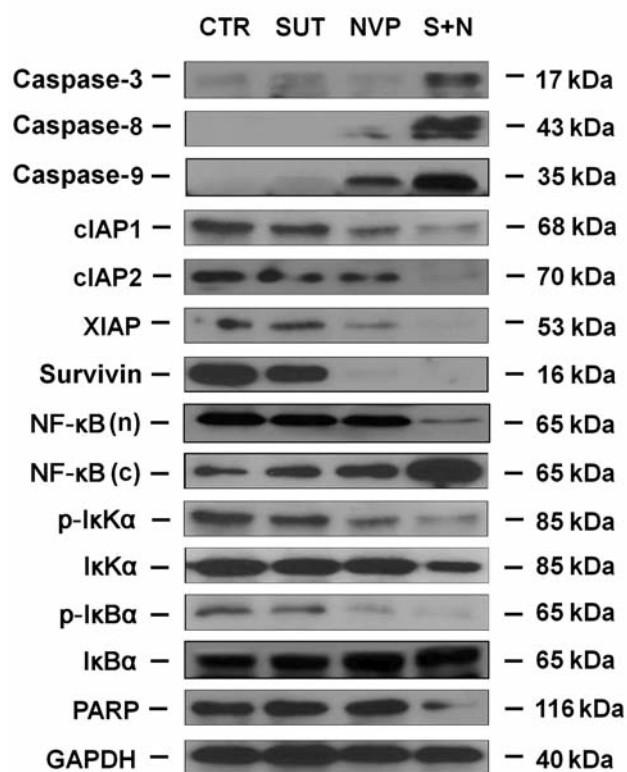


Figure 10. Analysis of apoptosis regulator expression in PC3DR2 cells. PC3DR2 cells were exposed to NVP-BEZ235 (0.5  $\mu$ M) alone (NVP) or in combination with sunitinib (SUT) (5.0  $\mu$ M) (S+N) for 72 h. Apoptosis-related protein expression was analyzed by western blot.

size. We explored the antitumor effect of NVP-BEZ235 and sunitinib in only four human CaP cell lines with different sensitivities to hormonal and docetaxel levels. Furthermore, we tested the antitumor effect of sunitinib *in vitro*. Although there are reports about the autocrine or paracrine activities of VEGF and VEGFR, the main target of sunitinib is VEGFR in endothelial cells, which means that sunitinib as a single or combinational agent may have different antitumor potentials under *in vivo* conditions compared to the current *in vitro* study. Therefore, we must be careful when directly extrapolating the current *in vitro* data to the clinical field, in which the *in vivo* tumor microenvironment, including tumor vasculature, can influence the antitumor effect of sunitinib as a single or combined agent with NVP-BEZ235. In the current study, we primarily focused on apoptosis, one of the most potent antitumor phenomena, as an underlying mechanism of synergy between NVP-BEZ235 and sunitinib. Furthermore, we tested whether a sunitinib combination can enhance the NVP-BEZ235-mediated inhibition of PI3K-AKT-mTOR signaling, but we obtained negative results. However, considering the possible role of the

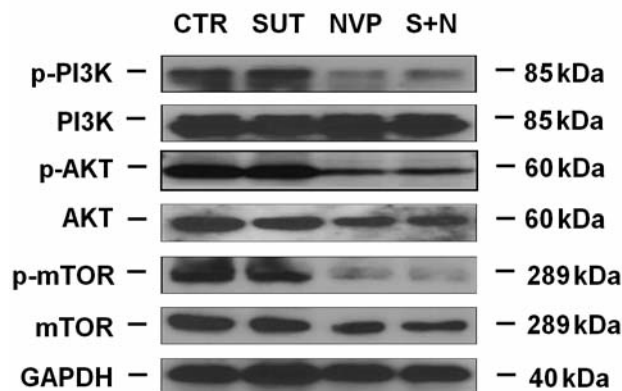


Figure 11. Analysis of AKT (protein kinase B) and mTOR (mammalian target of rapamycin) phosphorylation in PC3DR2 cells. PC3DR2 cells were exposed to NVP-BEZ235 (0.5  $\mu$ M) (NVP) with (S+N) and without sunitinib (SUT) (5.0  $\mu$ M) for 72 h, and the AKT and mTOR expression and phosphorylation status were examined by western blot.

synergistic interaction between NVP-BEZ235 and sunitinib in numerous other signaling pathways, the induction of apoptosis may only be only a small part of a multifactorial synergy mechanism.

Further comprehensive molecular studies, including *in vivo* studies, to validate our results and to elucidate the interactions between NVP-BEZ235 and sunitinib are required before clinical application of the presented findings.

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