Synergistic antitumor effect and mechanism of combined treatment with 17-DMAG and NVP-BEZ235 in Cisplatin-resistant human bladder cancer cells

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Directed by Professor Young Deuk Choi

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I dedicate this accomplishment to Hyun Jung, Emily, Bryan, and especially my parents, who have always been supportive.

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ABSTRACT

Synergistic antitumor effect and mechanism of combined treatment with 17-DMAG and NVP-BEZ235 in Cisplatin-resistant human bladder cancer cells

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Purpose: The current study aimed to investigate the synergistic antitumor effect and mechanism of combined treatment with 17-DMAG (HSP90 inhibitor) and NVP-BEZ235 (PI3K/mTOR dual inhibitor) in the cisplatin-resistant human bladder cancer cell.

Materials and Methods: A human bladder cancer cell line with cisplatin resistance (T24R2) was exposed to an escalating dose of 17-DMAG (2.5-20 nM) with or without NVP-BEZ236 (0.5-4 μM) in combination with cisplatin. Antitumor effects were determined by the CCK-8 analysis. Based on the dose-response study, synergistic interaction between two regimens was evaluated using a clonogenic assay and combination index. Flow cytometry and western blot were used to analyze the mechanism of synergism.

Results: Dose- and the time-dependent antitumor effect of 17-DMAG was shown in both cisplatin-sensitive (T24) and cisplatin-resistant cell lines (T24R2). However, the antitumor effect of NVP-BEZ235 was found to be self-limiting. The combination of 17-DMAG and NVP-BEZ235 in a 1:200 fixed ratio showed a significant antitumor effect in cisplatin-resistant bladder cancer cells over a

wide dose range, and clonogenic assay showed compatible results to synergy test. The three-dimensional analysis resulted in a strong synergy between two drugs with a synergy volume of 201.84 μM/ml^2%. The combination therapy resulted in a G1-phase cell cycle arrest and caspase-dependent apoptosis confirmed by the western blot.

Conclusion: HSP90 inhibitor monotherapy and in combination with PI3K/mTOR survival pathway inhibitor NVP-BEZ235 shows a synergistic antitumor effect in cisplatin-resistant bladder cancers through cell cycle arrest at the G1 phase and induction of caspase-dependent apoptotic pathway.

Key words: bladder cancer, cisplatin, NVP-BEZ235, 17-DMAG

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I.INTRODUCTION

One-third of a patient with bladder cancer (BCa) is muscle-invasive at diagnosis, and half of them develop distant metastasis to the lung, liver, and bone within two years.[1] Cisplatin-based systemic chemotherapy is the first-line treatment option for metastatic BCa. Although the initial response rate for cisplatin chemotherapy is up to 50-70%, most of them recur. Eventually, the five-year survival rate for metastatic BCa is only 6%. [2-4] In the last two decades, treatment options for these patients have not improved drastically. It could be explained by the drug resistance that often reduces the efficacy of cisplatin to cancer cells. At the same time, up to 50% of the patients with BCa are ineligible for cisplatin, mostly for decreased renal function. Over the past few decades, numerous trials have been conducted to overcome the cisplatin resistance or to reduce the effective dose for preserving renal function.

Heat shock protein 90 (HSP90) is an intracellular protein that plays a crucial role as a chaperone. It acts in post-translational maturation and activation of many oncogenic client proteins. In healthy cells, the HSP90 chaperone complex is thought to work as a facilitator of cellular response to extracellular signals. In

cancer cells, HSP90 activates many oncogenic client proteins, which intensifies the cell survival, growth, and invasiveness. As for that, HSP90 inhibitors are considered a promising therapeutic option and being investigated for several cancers preclinically.[5-8] However, HSP90 inhibitor monotherapy has shown suboptimal inhibition of target client proteins.[9] Combined approaches may be required for practical clinical use of HSP90 inhibitors.[10] Since some of the HSP90 clients have chemoprotective activity, the combination of an HSP90 inhibitor with a standard chemotherapeutic agent could markedly increase the *in vivo* efficacy of the therapeutic agent. [11] Based on the fact that HSP90 acts on ERBB2 and its down-stream (RAS-MEK-MAPK, PI3K-Akt-mTOR) signaling, targeting both HSP90 and its down-stream pathway could be a promising tactic to overcome cisplatin resistance in human BCa. [12,13]

The PI3-Akt-mTOR signaling axis is known as a significant survival pathway. It is considered an essential target in cancer therapy since it plays a critical role in development, progression, metastasis, and chemo-resistance. Several studies have shown a synergistic antitumor effect between PI3K or mammalian target of rapamycin (mTOR) inhibitors and conventional chemotherapies in chemo-naïve or resistant cancers like melanoma, nasopharyngeal, and ovarian cancers. Unfortunately, PI3K or mTOR inhibitor monotherapy has not shown significant clinical outcomes as in HSP90 inhibitor monotherapy. In BCa, however, simultaneous dual inhibition of PI3K/mTOR using NVP-BEZ235 has proven to demonstrate the synergistic antitumor effect.[3] Based on these results, we may expect a synergism when both PI3K/mTOR dual inhibitor and HSP90 inhibitor are coupled in the treatment of cisplatin-resistant bladder cancer cells.

In the present study, we investigated the synergistic effects of combining the PI3K/mTOR pathway inhibitor (NVP-BEZ235) and HSP90 inhibitor (17-DMAG) in cisplatin-based chemotherapy.

II. MATERIALS AND METHODS

1. Cell Lines and Reagents

Human bladder cancer cell lines (HTB9, J82, SW1710, T24, HTB5, UMUC14, and 253J) were maintained in MEM, DMEM or RPMI-1640 containing 10% fetal bovine serum (GE Healthcare Hyclone, UT, USA), 100 U/ml penicillin, and 100 mg/l streptomycin (Gibco BRL, Grand Island, NY, USA). Cells were incubated at 37˚C in a humidified 5% CO_2 atmosphere. UMUC14 was donated by Professor E.S. Lee (Seoul National University, Seoul, Korea), and all other cell lines were obtained from KCLB (Korean Cell Line Bank, Seoul, Korea) or ATCC (American Type Culture Collection, Manassas, VA, USA). The cisplatin-resistant T24R2 cell line was achieved by desensitizing T24 cells to cisplatin up to 2 μg/ml concentration (Figure 1).

NVP-BEZ235 was provided by Novartis Pharmaceuticals Inc. (Basel, Switzerland), and 17-DMAG was purchased from Selleckchem (Selleckchem, Boston, MA, USA). NVP-BEZ235 compound was dissolved in 100% DMSO at 85˚C to prepare a 10 mM stock solution and kept at 4˚C before use. Antibodies for p-AKT (ser473), AKT, p-mTOR (Ser2448), mTOR, p-GSK-3β (Ser9), GSK-3β, p-4E-BP1 (Thr37/46), 4E-BP1, p-MEK1/2 (Ser217/221), MEK1/2, p-ERK1/2 (Thr202/Tyr204), ERK1/2, cleaved Caspase (8 and 9), cleaved PARP, cIAP1, cIAP2, XIAP, LC3B, Beclin1, Cyclin A, Cyclin B1, Cyclin D1, Cyclin E; Cell Signaling Technology, Danvers, MA, USA; HSP90, ERBB2, and Bad; Santa Cruz Biotechnology, Santa Cruz, CA, USA; were used for immunoblotting.

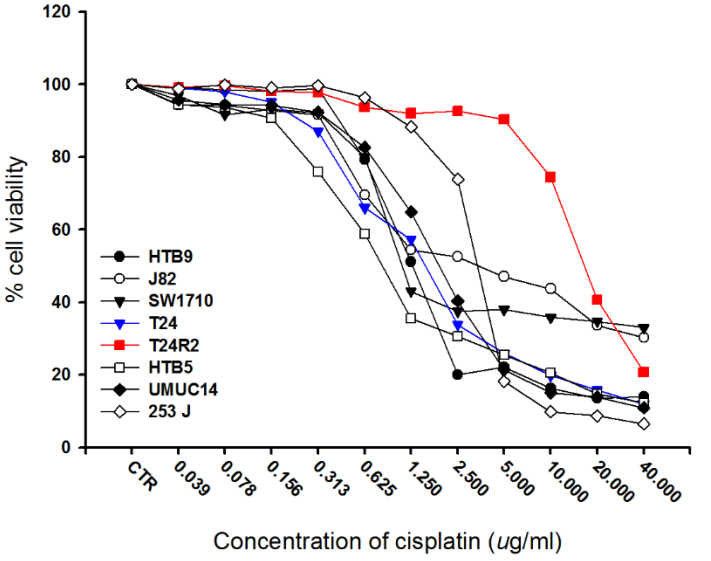

Figure 1. Dose-response studies of cisplatin in human bladder cancer cell lines with various differentiations. Human bladder cancer cells, including cisplatin-resistant T24R2, were exposed to increasing doses of cisplatin (0.039 μg/ml – 40.0 μg/ml) for 48 hours and changes in cell survival was assessed with the CCK-8 assay. Each data point stands for a mean of at least three biological replicates.

2. Dose-response and Synergy Study

Bladder cancer cells were seeded on 96-well plates and were left to be attached for 24 hours. For the dose-response study, the cells were treated with single, double combinations of NVP-BEZ235 (0.25-8.00 μM) or 17-DMAG (0.98-1000 nM) for 24, 48, or 72 hours. Cytotoxic effect was evaluated by CCK-8 assay (Cell counting kit-8; Dojindo Molecular Technologies, Gaithersburg, MD, USA) according to the manufacturer's protocol. Briefly, 10 μl of CCK-8 reagents were added to each well at the end of the incubation period, and the plates were further incubated for 2 hours. The absorbance was read at an optical density of 450 nm, and cell viability was expressed as the

surviving fraction of cells in treated versus untreated controls.

The synergistic interaction between agents was quantitatively explored based on a combination index (CI) using Calcusyn software (Biosoft, Ferguson, MO, USA) with the following interpretation: CI<1.0: synergism, CI>1.0: antagonism, and CI=1.0: additivity, respectively. The synergy between drugs was also assessed by the three-dimensional (3D) model, using the MacSynergy II software in which areas of the graph above zero indicate synergism while areas below zero indicate antagonism. The amount of synergy was expressed as surface area ($\mu M/ml^2\%$) where 0-25, 25-50, 50-100, and >100 $\mu M/ml^2\%$ means insignificant, weak, moderate, and strong interaction respectively.

3. Clonogenic assay

For the colony-forming assay, T24R2 cells were seeded on a 6-well plate with a concentration of $2x10^3$ cells/ml and treated with NVP-BEZ235 (0.5 μM) and/or 17-DMAG (2.5 nM) for 48 hours. Cells were cultured for another two weeks after removal of the drugs, and colonies formed were visualized by 0.4% crystal violet staining.

4. Flow Cytometric Cell Cycle Analysis

T24R2 cells were incubated with or without NVP-BEZ235 (2 μM) or 17-DMAG (10 nM) for 48 hours, collected and fixed in 70% ethanol and then stained with a propidium iodide solution [970 μl PBS, 40 μl of 1 mg/ml propidium iodide (Sigma) and 3 μl of RNase A (Sigma)] for 30 minutes at 37˚C. After filtering with strainer, the single-cell suspension was analyzed by the FACSCaliber flow cytometer (Becton-Dickinson, San Jose, CA, USA) for the cell cycle.

5. Western Blot Analysis.

Cells were lysed using RIPA buffer supplemented with complete proteinase inhibitors (Roche, Basel, Switzerland) and cell lysates fractionated by SDS-PAGE and transferred to PVDF membrane. Following blocking with 5% non-fat milk, the blots were incubated with the corresponding primary antibodies and secondary antibodies. The signal was detected using a chemiluminescence Western blot substrate kit (Pierce, Rockford, IL, USA).

6. Statistical Analysis

The data sets consist of at least three biological replicates, and all statistical analyses were performed using SPSS 14.0K software (SPSS Inc., Chicago, IL, USA). The data are expressed as the mean ± SD. Statistical significance was determined by a two-sample t-test, and differences were considered significant at $p < 0.05$.

III. RESULTS

1. Synergistic Antitumor Effect between 17-DMAG and NVP-BEZ235

Because HSP90 plays an import role as a chaperone in the activation and stabilization of ERBB2 and its down-stream pathway (RAS-MEK-MAPK, PI3K-Akt-mTOR) signaling, we hypothesized that concomitant targeting of HSP90 and its down-stream pathway could be a promising strategy to overcome cisplatin resistance in human BCa (Figure 2). Dual PI3K/mTOR inhibitor NVP-BEZ235 exerted dose- and time-dependent antitumor effect in both T24 and T24R2 cells (Figures 3A and 3B). However, the antitumor effect of

NVP-BEZ235 was found to be self-limiting, with all measures of antitumor impact reaching a plateau at a concentration of around 0.5 μM. When T24 and T24R2 cells were exposed to escalating doses of HSP90 inhibitor 17-DMAG, the proliferation of both cell lines was suppressed by dose- and time-dependent pattern (Figure 3C and 3D). Interestingly, cisplatin-resistant T24R2 showed a relatively higher sensitivity to 17-DMAG treatment compared with other human bladder cancer cell lines, including cisplatin-sensitive T24 (Figure 4).

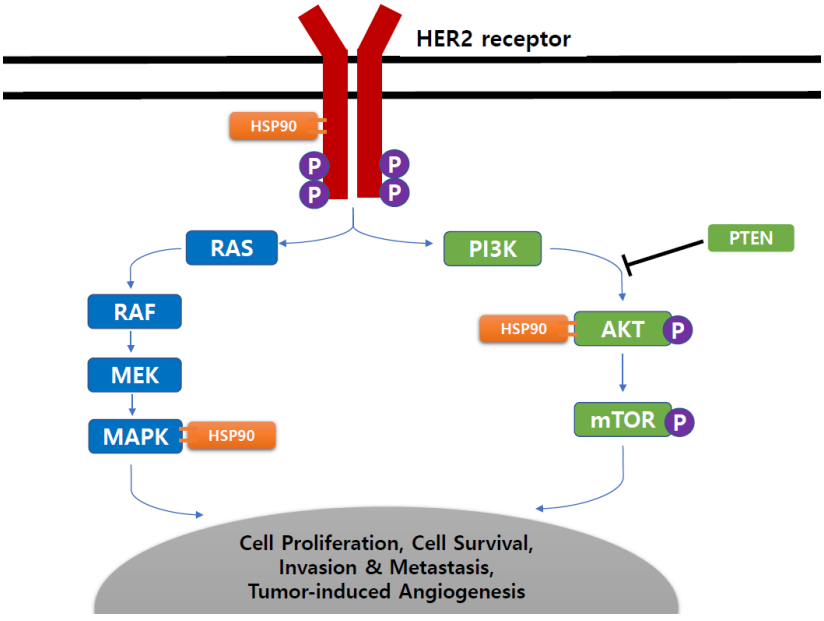

Figure 2. The schematic diagram for the crosstalk between HSP90 and PI3K-AKT-mTOR / RAS-MEK-MAPK pathways

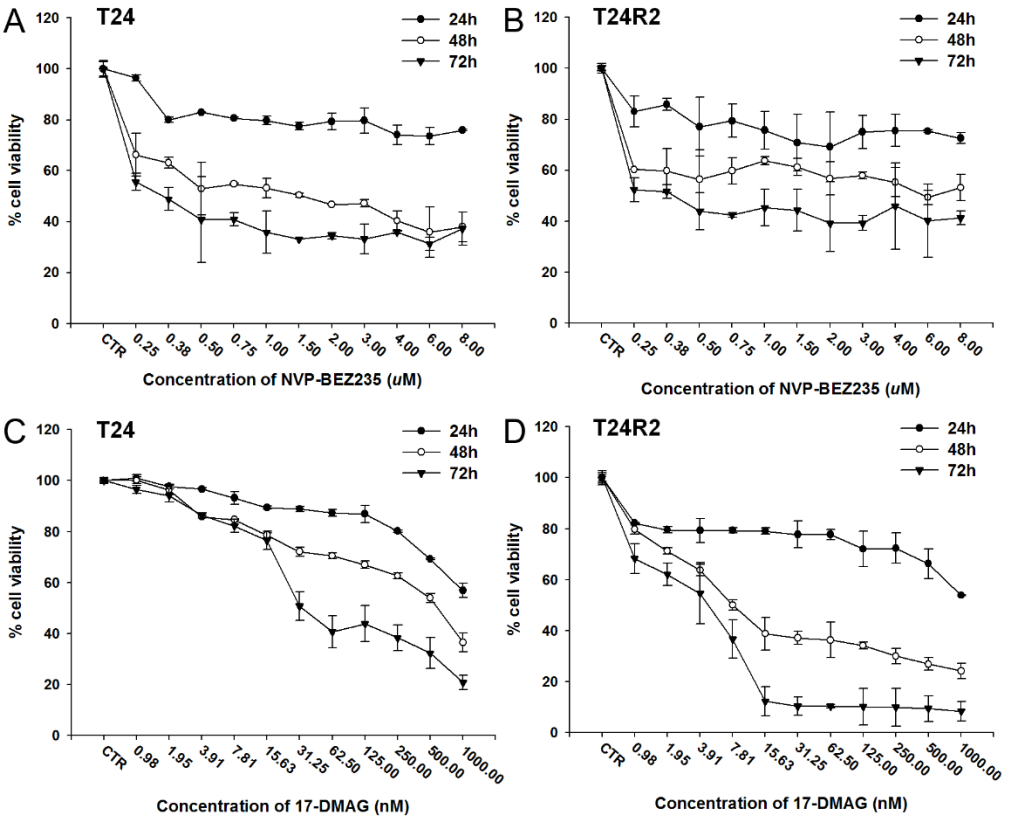

Figure 3. Dose-response studies of 17-DMAG and NVP-BEZ235 in T24 and T24R2 cells. Both cell lines were exposed to escalating doses of 17-DMAG (0.098 nM – 1 μM, upper panel) and NVP-BEZ235 (0.25 – 8.0 μM, lower panel) for 24, 48, and 72 hours and cell survival was assessed by the CCK-8 assay.

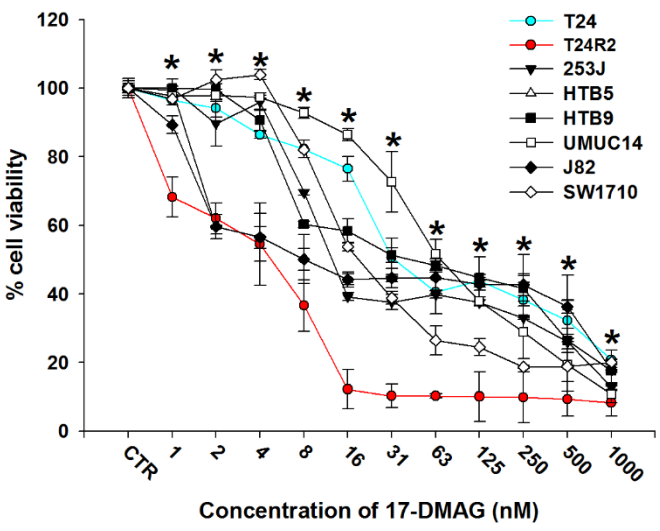

Figure 4. Varying sensitivity of human bladder cancer cell lines to HSP90 inhibitor 17-DMAG.

Human bladder cancer cell lines (T24 (grade 3), T24R2, 253J (grade 4), HTB5 (grade 4), HTB9 (grade 2), UMUC14 (grade 4), J82 (grade 3), SW1710 (grade 3)) were treated with to increasing doses of 17-DAMG (1.0 nM – 1 μM) for 72 hours and cell survival was evaluated with CCK-8 assay. The asterisks denote a significant difference between T24 and T24R2. ($p<0.05$)

T24R2 cells were treated with escalating doses of 17-DMAG alone or in a 1:200 fixed-ratio combination with NVP-BEZ235, and the antitumor effect was evaluated by CCK-8 assay (Figure 5A) to test the synergistic interaction between two drugs,. The correlation coefficients (r) were between 0.792 and 0.982, indicating fair data conforming to the median effect principle (Table I). The Dms (IC50s) of NVP-BEZ235 and 17-DMAG were 2.08175 μM and 0.01942 μM respectively, while it was 0.00174 μM in combination treatment suggesting significant synergism between two drugs. The fa (fraction affected) and CI (combination index) analysis showed a CI<1 over a wide range of fractions affected (fa, 0.45-0.85). The clonogenic assay showed results

compatible with the synergy test (Figure 5E). For a more detailed analysis of synergism, four independent full combination studies with 17-DMAG and NVP-BEZ235 was performed to generate a three-dimensional synergy plot using MacSynergy II software (Figure 5D). The three-dimensional analysis resulted in a strong synergy between two drugs with a synergy volume of 201.84 μM/mL^2%, with minimal antagonism (-10.18 μM/mL^2%). The colony-forming assay showed results compatible with the synergy test (Figure 5E).

Table 1. Dose-effect relationship parameters of 17-DMAG and NVP-BEZ235 in T24R2 cells.

Compound	m*	Dose (*u*M)†	r
17-DMAG	1.185	0.01942	0.982
NVP-BEZ235	0.135	2.08175	0.792
Combination	0.425	0.00174	0.988

* Coefficient signifying dose-effect curve shape
†IC_{50} or dose causing 50% inhibition of cell proliferation

2. 17-DMAG and NVP-BEZ235 Combined Treatment Induces G1 and sub-G1 phase Cell Cycle Arrests

Neither 17-DMAG (10 nM) nor NVP-BEZ235 (2.0 μM) single-agent treatment caused any significant alterations in cell cycle progression in T24R2 cells while combined treatment resulted in a marked increase of cells in sub-G1 (3.9±0.8%) and G1 (78.3±0.7%) population compared with untreated control (0.6±0.3% for sub-G1, 57.9±1.3% for G1 phase), 17-DMAG (1.4±0.2% for sub-G1, 59.4±1.0% for G1 phase), and NVP-BEZ235 (1.0±0.4% for sub-G1, 61.6±0.9% for G1 phase) single-agent treated cells (Figure 6).

3. Synergy Mechanisms between 17-DMAG and NVP-BEZ235

Concomitant treatment of T24R2 cell with 17-DMAG and NVP-BEZ235 suppressed cyclin A and B expression while causing increased expression of cyclin D (Figure 7). Also, the combined treatment caused increased expression of caspase 8 / 9 and PARP cleavage while suppressing the expression of cIAP1 and cIAP2 (Figure 7). There were no discernible changes in beclin 1, LC3B1, and LC3B2 expression in T24R2 cells after combined treatment with 17-DMAG and NVP-BEZ235 (Figure 7). While there was no significant change in HSP90 expression by drug treatment, combined treatment suppressed ERBB2 and its down-stream effector (p-Akt, Akt, p-mTOR, mTOR, p-4E-BP1, p-GSK-3β) expressions in T24R2 cells (Figure 8).

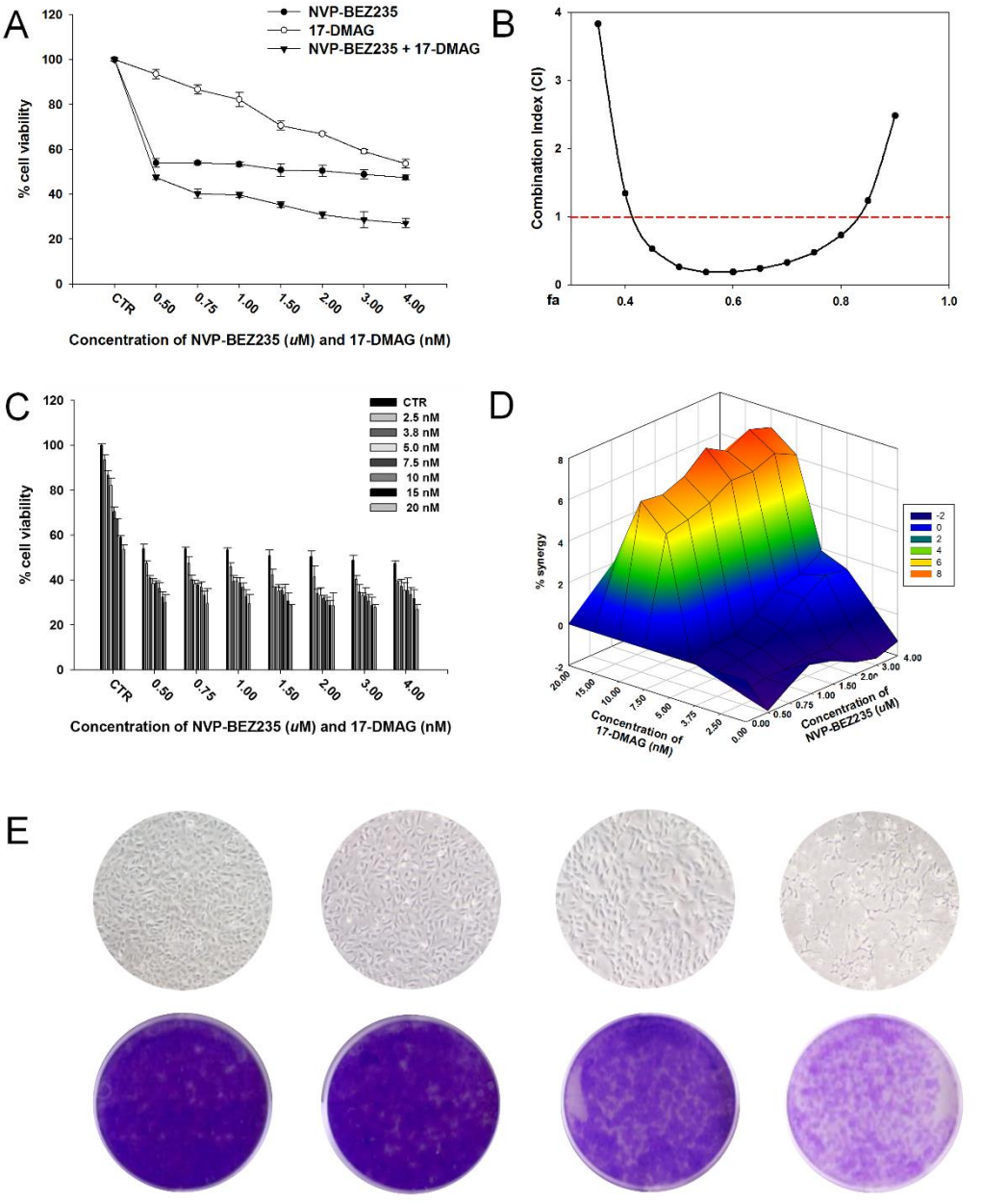

Figure 5. The synergistic antitumor effect between 17-DMAG and NVP-BEZ235 in cisplatin-resistant T24R2 cells. (A) T24R2 cells were treated with escalating doses of 17-DMAG (2.5-20 nM) alone or in combination with NVP-BEZ235 (0.5-4.00 μM) at 1:200 fixed ratio for 72 hours and cell survival was assessed using CCK-8 assay. (B)

Based on the results of the CCK-8 assay fa-CI plot was generated in which fa and CI stand for fraction affected and combination index, respectively. (C) For the more accurate test of synergism between two drugs, T24R2 cells were treated with a full combination of 17-DMAG (2.5-20 nM) and NVP-BEZ235 (0.5-4.00 μM) for 48 hours and 3D synergy plot was built using MacSynergy II data analysis program. (D) Clonogenic assay of T24R2 cells treated with 17-DMAG (2.5 nM) alone or in combination with NVP-BEZ235 (0.5 μM) for 48hours.

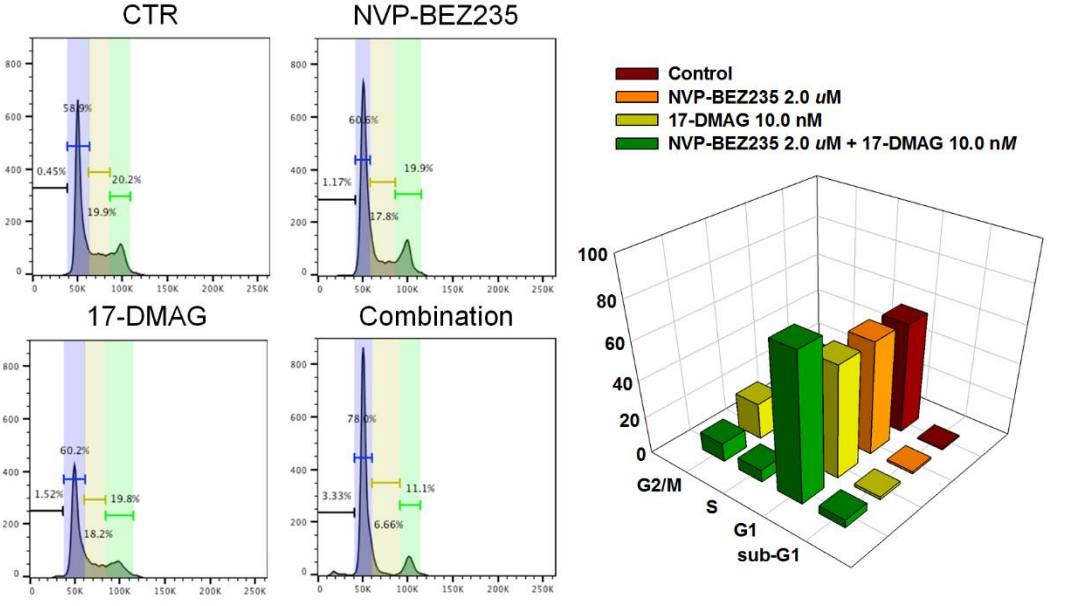

Figure 6. Alteration in the cell cycle in T24R2 cells. T24R2 cells were treated with 17-DMAG (10 nM) alone or in combination with NVP-BEZ235 (2 μM) for 48 hours, and changes in cell cycle was evaluated by flow cytometry.

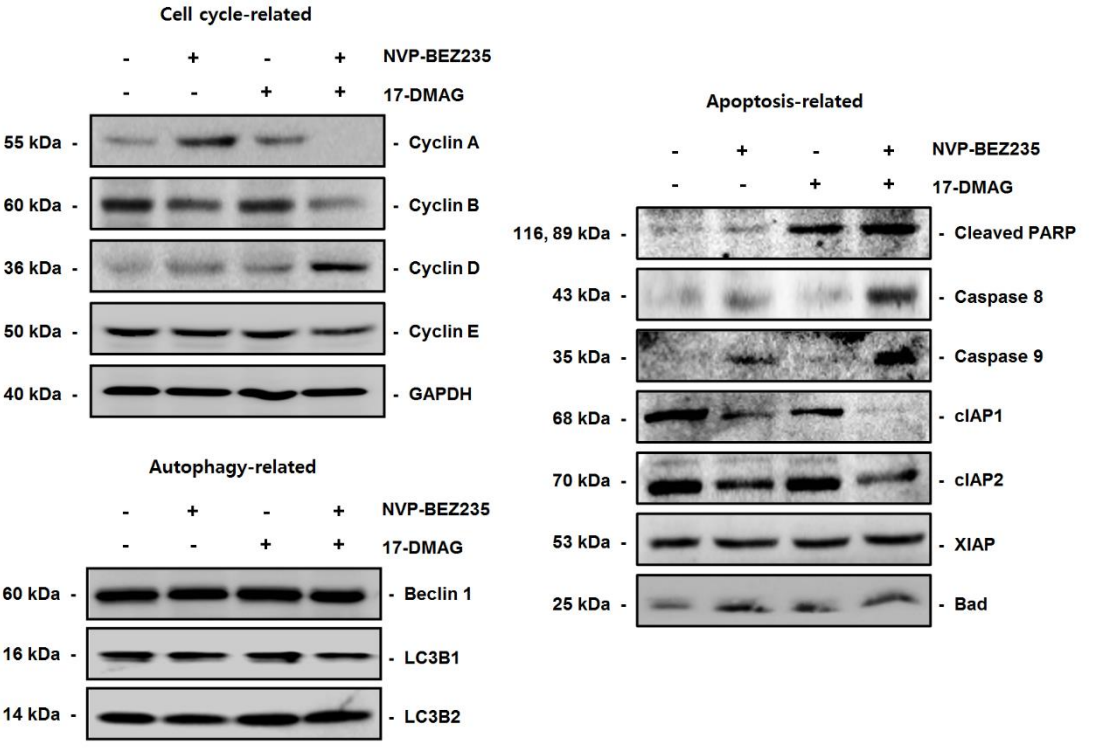

Figure 7. Western blot analysis of proteins involved in apoptosis, cell cycle, and autophagy. T24R2 cells were treated with 17-DMAG (10 nM) alone or in combination with NVP-BEZ235 (2 μM) for 48 hours and expressions of relevant proteins were evaluated by Western blot analysis

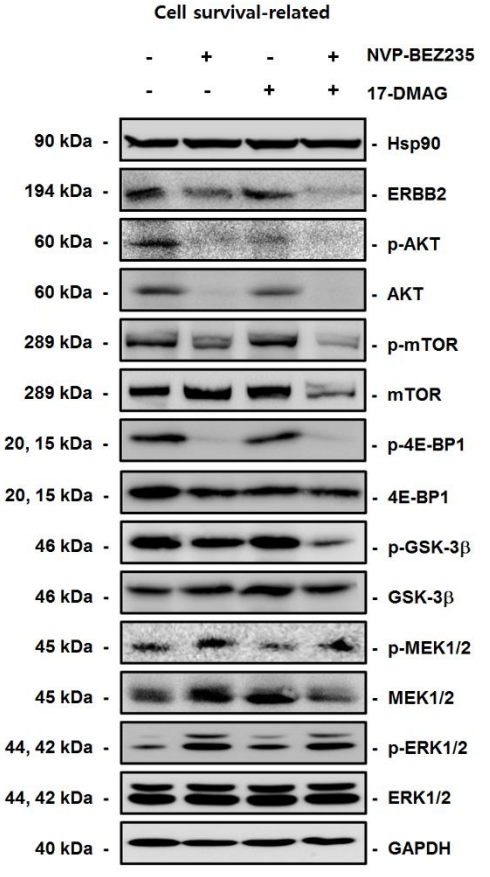

Figure 8. Western blot analysis of proteins involved in cell survival. T24R2 cells were treated with 17-DMAG (10 nM) alone or in combination with NVP-BEZ235 (2 μM) for 48 hours, and changes in cell survival-related protein expressions were explored using Western blot analysis.

IV. DISCUSSION

Cisplatin-based chemotherapy is currently the gold standard for treating metastatic BCa. However, the survival benefit of the standard of care (SOC) has not shown improvement during the last few decades. Cisplatin acts by binding to purine DNA bases, which triggers the DNA damage response. Complete response is shown in only 1 out of 4 patients. Such a high treatment failure rate could partially be explained by the development of cisplatin resistance. Reduced cisplatin binding to DNA (pre-target resistance), reduced formation of DNA-cisplatin adducts (on-target resistance), altered DNA damage response (post-target resistance), and influenced signaling pathways that reduce the DNA damage response (off-target resistance) is believed as the viable mechanisms for cisplatin-resistance.[14] Moreover, not all the patients are eligible for receiving a full therapeutic dose of cisplatin, mostly due to decreased renal function. Such profiles prompt alternative plans for sensitizing bladder cancer cells to chemotherapy and reducing the dose of cisplatin while enforcing its therapeutic efficacy. Due to a better understanding of tumor biology, sequencing after the SOC or combining with novel agents are being recognized as a promising option in treating malignancies, including genitourinary cancers.

There are several ongoing clinical trials for the treatment of advanced or metastatic urothelial carcinoma for both cisplatin-sensitive and -resistant cell lines. Diverse approaches such as inhibitors of the mTOR, tyrosine kinase inhibitors (TKIs), human epidermal growth factor receptor 2 (HER2) blockade, anti-angiogenic therapies, and immunotherapies are being evaluated. Urothelial cancers are genetically complex with various oncogenic drivers, multiple mutations within a single tumor, gene fusion transcript, copy number variations, and cytogenetic errors. Therefore, understanding the molecular mechanisms underlying BCa is of necessity in developing effective treatments for cisplatin-resistant BCa. Lee et al. revealed differential gene expression between

T24 and T24R2 cells using microarray. When the T24 cell line becomes resistant to cisplatin, gene expressions were altered among various classes, including cell signaling, transcriptional regulation, metabolism, cytoskeleton, immunity, growth factor, transporter, adhesion, and apoptosis.[14]

Recently, HER2 has emerged as a potent biomarker for many different types of cancer. HER2 belongs to the type 1 tyrosine kinase receptor family consisted of four related receptors, forming dimers with each other, which involves cell cycle regulation and cell proliferation. Amplification of the HER2 gene and overexpression of subsequent protein is associated with oncogenesis.[1,15] In a healthy environment, cells have low HER2 membrane protein content, which involves proliferation, differentiation, angiogenesis, and invasion, but it increases dramatically in cancer cells. Overexpression of HER2 protein has been presented in several malignancies, especially in gastric (20%) and breast cancers (up to 30%).[16] In BCa, the expression frequency of HER2 has been reported to range 25-100% in the primary tumor and 40-70% in corresponding metastasis, which makes HER2 an attractive target for systemic therapy.[1] Studies have shown that HER2 overexpression is associated with disease aggressiveness and more unsatisfactory outcome, suggesting HER2 targeted therapy a feasible option for locally advanced and metastatic HER2 amplified BCa.[15,16]

When HER2 proteins dimerize and bind to a ligand, the PI3K-Akt-mTOR and RAF-MEK-ERK pathways are activated.[17] Signaling via the RAF-MEK-ERK cascade leads to phosphorylation of various proteins that have multiple cellular effects, mostly involved in cell cycle progression.[18] Abnormal activation of the PI3K-Akt-mTOR signaling axis frequently induces the development, progression, metastasis, and also chemo-resistance in many tumors, including urothelial cancers.[3] These findings have drawn attention to consider PI3K or mTOR signaling as promising antitumor targets. PI3K activation is believed to be involved in cisplatin resistance mechanisms through

mutational activation of PI3K, PTEN loss, and activation of alternative receptor tyrosine kinase. Such findings suggest the possible use of PI3K inhibitors in cisplatin-resistant cancers.[6]

Inhibition of mTOR has shown antitumor activity both in monotherapy and in combination with chemotherapy. In urothelial carcinoma cells, mTOR inhibition enhanced chemosensitivity by inducing cell cycle arrest at the sub-G1 and G1 growth phase and inhibiting VEGF production. The 1st generation mTOR inhibitors like rapamycin, temsirolimus, and everolimus showed synergistic activity with cisplatin but yielded inadequate treatment responses.[5] These 1st generation mTOR inhibitors act by allosteric inhibition of mTORC1 but not mTORC2, which induces a paradoxical activation of survival axis signaling by mTORC2-mediated Akt phosphorylation.[19,20] Therefore, the dual inhibition of PI3K/mTOR or Akt/mTOR may be an answer to these feedback loops, providing a better strategy for overcoming the development of resistance of cancer cells to targeted therapy. Moon et al. have demonstrated the dose-dependent antitumor effect of PI3K/mTOR dual inhibitor NVP-BEZ235 in bladder cell lines.[3] NVP-BEZ235 blocked the negative feedback activation of Akt and showed a more significant antitumor effect compared to the 1st generation rapalogues. However, NVP-BEZ235 monotherapy had an only partial inhibitory effect even at a relatively high dose (0.5 μM or higher) and showed a transient antitumor effect lasting no more than three days. These findings were more pronounced in cisplatin-resistant T24R2 cells compared to cisplatin-sensitive T24 cells, suggesting possible cross-resistance between NVP-BEZ235 and cisplatin in human BCa. NVP-BEZ235 and cisplatin in combination showed the significant synergistic antitumor effect by cell cycle arrest and caspase-dependent apoptosis in cisplatin-resistant bladder cancer cells compared to either agent in monotherapy.

The activity of HER2 is affected and partly controlled by “molecular chaperones,” which assures accurate folding of proteins, maintaining cellular

integrity and homeostasis. In normal conditions, Heat shock proteins (HSPs) act as classical chaperones. Among the several classes of HSPs, HSP90 is most abundant, and its loss of expression is related to a poorer prognosis.[7] Given that therapeutic resistance is attributed to diverse accumulating mutations in cancer cells, it is ideal for targeting multiple anti-apoptotic pathways. There are HSP90 client proteins other than the Akt and ERK that act as vital components of various oncogenic pathways related to growth and survival, such as hypoxia-inducible factor-1a, erbB2 and inhibitor of κB kinase β, which mediates NF-κB activation.[21] Since these molecules are deeply involved in significant cancer pathways and play essential roles as oncogenes, ubiquitous molecular chaperone HSP90 is a promising target to enhance treatment response. In several malignancies such as gastric and breast cancer, HSP90 expression was significantly associated with tumor aggressiveness and poor prognosis. Anti-HSP90 treatment for these cancers has shown potent benefits in numerous preclinical and clinical studies.[9,12,15,17,22,23] In bladder cancer cells, 17-AAG clearly showed a dose-dependent and cell type-specific inhibition of cell proliferation, survival, and motility in previous studies.[24] Yoshida et al. suggested that HSP90 inhibitors, even at a low concentration (17-DMAG or 17-AAG at 100 nM), would be potent enough to improve chemoradiation therapy response by inactivating erbB2, Akt, and NF-κB without increasing toxicities.[6,25] Tatokoro et al. demonstrated that administration of HSP90 inhibitors at noncytocidal concentrations synergistically potentiated the cytotoxic effects of cisplatin on BCa-initiating cells *in vitro* and successfully sensitized cisplatin-resistant, BCa-initiating, cell-derived tumor xenografts to cisplatin.[21] These data suggest that it may be feasible to utilize HSP90 inhibitors to improve the therapeutic outcomes of cisplatin-based combination chemotherapy for advanced BCa.[21]

Not only applying HSP90 inhibitor but combining it with other targeted therapies in conjunction with SOC may block the compensatory signaling

pathway and convey a clinical benefit.[5] An optimal strategy for the combination is to target the same pathway vertically.[26] Since Akt is one of the client proteins of HSP90, we hypothesized that a combined regimen of HSP90 inhibitor (17-DMAG) and a PI3K/mTOR dual inhibitor (NVP-BEZ235) could be an optimal option to augment the relatively low antitumor effect of each agent in a patient with cisplatin-resistant bladder cancer cells. In line with previous results, the antitumor effect of NVP-BEZ235 alone is self-limiting with an antitumor effect reaching a plateau at a concentration around 0.5 μM. However, the proliferation of both T24 and T24R2 cell lines were suppressed by dose- and time-dependent pattern when exposed to escalating doses of HSP90 inhibitor 17-DMAG. Synergy test for a combination of NVP-BEZ235 and 17-DMAG with cisplatin in cisplatin resistant bladder cancer also significantly enhanced the antitumor effect compared to that of either drug in monotherapy. The fa-CI plot exposed that these two agents exert synergistic effect over a wide range of dose combination in T24R2 cell lines. Dose reduction index analysis presented that when this combination is applied to treat T24R2 cells, the IC_{50} of NVP-BEZ235 and 17-DMAG can be reduced by 1,196.0- and 11.2- fold, respectively, indicating strong synergistic interactions within two drugs. 3D synergy test demonstrated in synergy volume 201.84 $\mu M/ml^2$ with minimal antagonism. Such an extent of synergy volume suggests the importance of this effect *in vivo*.

In human urinary bladder cancer cells, the diverse pattern of cell cycle arrest in response to 17-DMAG administration associated with the malignancy grade and cellular environment. Low malignancy p53wt cells such as RT4 present with a prominent G1/S block, while p53 mutant T24 cells develop a various pattern of cell cycle arrest. In the previous study, the combination of NVP-BEZ235 and cisplatin showed a prominent cytostatic effect rather than the apoptogenic effect confirmed by a mild increase in sub-G1 population fraction while inducing a marked increase in S phase fraction. In the present study, however, co-treatment

of 17-DMAG and NVP-BEZ235 produced a mild rise in sub-G1 fraction while a marked increase in the G1 phase population in flow cytometry suggesting prominent apoptotic effect. Western blot analysis demonstrated that combined treatment resulted in a marked decrease in cyclin A and cyclin B while causing increased expression of cyclin D. These findings indicate that the antitumor mechanism of combined therapy is at least partly through G2/M phase cell cycle arrest. 17-DMAG and NVP-BEZ235 combination depressed the expression of anti-apoptotic cIAP1, cIAP2 while enhancing the cleavage of caspase 8 / 9 and PARP, indicating induction of both extrinsic and intrinsic caspase-dependent apoptotic pathway. Akt, which is a crucial protein kinase of the complex cell signaling network and an essential member of the HSP90 client, is downregulated upon 17-DMAG administration. The exposure of T24R2 cells to 17-DMAG in combination with NVP-BEZ235 suppressed the phosphorylation of PI3K, mTOR, and its immediate client GSK-3β and 4E-BP1. Simultaneous suppression of PI3K and mTOR with the deactivation of Akt and its downstream signaling evidence antiproliferative and proapoptotic activities of 17-DMAG and NVP-BEZ235 in combination with cisplatin in cisplatin-resistant bladder cancer cells. The result of our study corresponds with Chen et al., where the combination of PI3K/mTOR dual inhibitor (NVP-BEZ235) and HSP90 inhibitor (NVP-AUY922) showed significant synergistic proapoptotic and antiproliferative effect in cholangiocellular carcinoma cell line *in vitro*. Furthermore, they have demonstrated that HSP inhibitor NVP-AUY922 induced ER stress and mitochondrial damage fueled by oxidative stress when combined with NVP-BEZ235.[6] There were no apparent changes in the expression of the autophagy-related genes, beclin 1, LC3B1, and LC3B2 in T24R2 cells after combined treatment with 17-DMAG and NVP-BEZ235.

Previous studies have shown that 17-DMAG simultaneously inactivates both Akt and ERK signaling at non-cytocidal concentration when combined with

cisplatin in the human bladder cancer cell.[21] In our study, however, the combination of NVP-BEZ235 and 17-DMAG decreased the expression of PI3K related proteins (Akt, mTOR) while increasing the MERK-ERK associated proteins. Activation of MAPK/ERK pathway signaling is known as a resistance mechanism in mTOR inhibitor-based therapy. Roccaro et al. have demonstrated that NVP-BEZ235 upregulated ERK phosphorylation in Waldenstrom macroglobulinemia cell line.[27] NVP-BEZ235 mono- and combination therapy with cisplatin in bladder cancer cell line upregulates MEK1/2, and ERK1/2.[3] Thus, our findings could be interpreted that 17-DMAG did not reverse the effect of NVP-BEZ235 regarding the ERK signaling. Such crosstalk indicates the MAPK/ERK pathway as another potential concomitant target to enhance the antitumor effect of combination therapy in cisplatin-resistant bladder cancer.

The limitations of the current study include in vitro study design and small number sample size. However, it outlined the synergistic interaction between 17-DMAG and NVP-BEZ235 in cisplatin-resistant BCa. Further comprehensive molecular studies, including in vivo studies, would be required to validate our findings and to elucidate the interactions between 17-DMAG and NVP-BEZ235 in cisplatin-resistant BCa to be clinically applied.

V. CONCLUSION

Not only 17-DMAG in combination with cisplatin showed an antitumor effect in bladder cancer cells, but also the HSP90 inhibitor in combination with PI3K/mTOR survival pathway inhibitor NVP-BEZ235 shows a synergistic antitumor effect in cisplatin-resistant BCa through cell cycle arrest at the G1 phase and induction of caspase-dependent apoptotic pathway.

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ABSTRACT(IN KOREAN)

Cisplatin에 내성을 가지는 방광암 세포에서의 17-DMAG와 NVP-BEZ235의 항암효과 상승 작용

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목적: 분자 샤페론 HSP90은 암세포 내에서 과도하게 발현되거나, 변이가 생긴 단백질을 보호하고 안정화시키는 기능을 가진다. 특히 종양유전자 단백질 (oncoprotein)을 비롯해 세포의 다양한 신호분자들을 조절하는 HSP90은 최근 여러 암종에서 이상적인 연구 타겟으로 지목되어 왔다. 이에 본 연구에서는 HSP90 차단제 17-DMAG와 PI3K/mTOR survival pathway 차단제인 NVP-BEZ235를 이용하여 cisplatin 내성 방광암 세포에서의 항암효과 상승 작용에 대하여 분석하였다.

대상 및 방법: Cisplatin에 내성을 가지는 세포주인 T24R2에 17-DMAG (2.5-20 nM)와 NVP-BEZ235 (0.5-4 uM)를 용량을 증가시키며, 단독, 혹은 복합 투여한 후 CCK-8 분석 및 clonogenic assay를 통해 두 약제간의 항암효과 상승 여부를 분석하였다. 또한 flow cytometry와 Western blot를 이용하여 두 약제간의 항암효과 상승 작용의 관련 기전에 대하여 분석하

였다.

결과: Cisplatin에 높은 감수성을 가지는 T24와 내성 세포주인 T24R2 모두에서 17-DMAG의 우수한 dose- 및 time-dependent한 항암효과를 볼 수 있었다. NVP-BEZ235 역시 두 세포 모두에서 dose- 및 time-dependent이긴 하나 제한적인 항암효과를 보였다. 두 약제를 1:200 fixed ratio로 복합 투여한 후 combination index를 이용하여, 분석한 결과 wide dose range에서 두 약제 간에 항암효과 상승작용이 존재함을 확인하였으며, clonogenic assay에서도 동일한 항암효과 상승작용이 확인되었다. 17-DMAG와 NVP-BEZ235의 항암효과 상승작용에 대한 3D 분석 결과 synergy volume은 201.84 $uM/ml^2\%$로서 매우 우수한 상승 효과가 확인되었다. Flow cytometry 결과 T24R2 세포에서 두 약제의 복합 투여는 apoptosis 및 G1-phase cell cycle arrest를 유발하는 것으로 나타났으며 Western blot에서도 이와 동일한 caspases 활성의 증가 및 cyclin의 활성 억제가 확인되었다.

결론: 본 연구를 통하여 cisplatin에 내성을 가지는 방광암 세포주에서 17-DMAG의 단독 제제로서의 항암효과를 확인하였으며, 또한 PI3K/mTOR 차단제를 함께 처리하였을 때 apoptosis 및 G1-phase cell cycle arrest를 통해 우수한 항암효과 상승작용을 나타내는 것을 확인하였다.

핵심되는 말 : 방광암, cisplatin, NVP-BEZ235, 17-DMAG