Ph.D. Dissertation of Medicine

Enhanced Efficacy of Combined Therapy with Checkpoint Kinase 1 Inhibitor and Rucaparib Via Regulation of Rad-51 Expression in BRCA Wild-Type Epithelial Ovarian Cancer Cells

– Combined therapy with chk1 inhibitor and Rucaparib in ovarian cancer–

BRCA wild-type 상피성 난소암에서 checkpoint kinase 1 과 poly ADP ribose polymerase inhibitor 의 병합 요법의 효과

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Abstract

This study aimed to evaluate anticancer effects of combination treatment with poly ADP ribose polymerase (PARP) and checkpoint kinase 1 (Chk1) inhibitors in BRCA-wild type ovarian cancer. PARP inhibitors can function as DNA-damaging agents in BRCA wild-type cancer, even if clinical activity is limited. Most epithelial ovarian cancers are characterized by a *TP53* mutation causing dysfunction at the G1/S checkpoint, which makes tumor cells highly dependent on Chk1-mediated G/M phase cell-cycle arrest for DNA repair.

We investigated the anticancer effects of combination treatment with prexasertib (LY2606368), a selective ATP competitive small molecule inhibitor of Chk1 and Chk2, and rucaparib, a PARP inhibitor, in BRCA wild-type ovarian cancer cell lines (OVCAR3 and SKOV3).

We found that combined treatment significantly decreased cell viability in all cell lines and induced greater DNA damage and apoptosis than in the control and/or using monotherapies. Moreover, we found that prexasertib significantly inhibited homologous recombination-mediated DNA repair and thus showed a marked anticancer effect in combination treatment with rucaparib. The anticancer mechanism of prexasertib and rucaparib was considered to be caused by an impaired G2/M checkpoint due to prexasertib treatment, which forced mitotic catastrophe in the presence of rucaparib.

Our results suggest a novel effective therapeutic strategy for BRCA wild-type epithelial ovarian cancer using a combination of Chk1 and PARP inhibitors.

Keyword: Chk1 inhibitor, PARP inhibitor, Rad51, Ovarian neoplasms

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Chapter 1. Introduction

1.1. Study Background

Epithelial ovarian cancer is the most lethal gynecologic malignancy, leading to more than 140,000 deaths per year worldwide [1]. In South Korea, estimated new cases of ovarian cancer are 2941 and estimated deaths from ovarian cancer are 1309 in 2020 [2]. More than 80% of patients experience recurrences and more than half show chemo-resistance. Therefore, it is critical to understand and overcome the mechanism(s) involved in chemo-resistance in order to develop better therapeutic strategies.

The DDR (DNA damage repair) pathway, an extensive network of pathways that detects and signals DNA damage for subsequent processing, is known as a key process in human cancer development [3]. ATR (Ataxia Telengiectasia and Rad3-related) and its downstream kinase, Checkpoint kinase I (Chk1), are major components of the DDR pathway [4]. Most high-grade serous ovarian cancers (HGSC) show a mutation in *TP53*, which results in the loss of G1 checkpoint control, and thus significantly rely on S and G2 checkpoints for survival [5, 6]. Therefore, targeting S and G2 checkpoints by inhibition of the ATR/Chk1 pathway in a tumor with a *TP53* mutation will prevent DNA damage-induced G2 checkpoint arrest, leading to mitotic catastrophe and tumor cell death [7].

Poly ADP ribose polymerase (PARP) inhibition, which leads to the failure of double strand break (DSB) repair in *BRCA1* and *BRCA2* defective cells, is another strategy targeting the DDR pathway. *BRCA1* and *BRCA2* are essential in homologous recombinant repair of DSBs. Approximately 15% of patients with high-grade serous ovarian cancers harbor deleterious germ-line mutations in *BRCA1* and *BRCA2* [8]. By inhibiting PARP in ovarian cancer with *BRCA* mutations, a failure of DSB repair, promotion of

genomic instability, apoptosis, and cancer cell death will occur [9]. In a clinical setting, PARP inhibitor monotherapy have shown modest activity even in *BRCA* wild-type high-grade serous ovarian cancers [10, 11]. The limited activity of a PARP inhibitor against BRCA wild-type high-grade serous ovarian cancers is partly caused by the over-expression of Rad51, which is associated with chemo-resistance [12].

Chk1 is known to play a critical role in homologous recombinant DNA repair. Chk1 facilitates the BRCA2-Rad51 interaction by phosphorylation of the BRCA2 C-terminal domain and Rad51 at T 309. This is an important step that allows the transnuclear localization of HR repair proteins in response to DSBs [13, 14].

1.2. Purpose of Research

We hypothesized that inhibiting Chk1 would sensitize BRCA wild-type HGSCs to a PARP inhibitor by preventing the formation of Rad 51 foci. In this study, we evaluated the preclinical efficacy of prexasertib (LY2606368), a selective ATP competitive small molecule inhibitor of Chk1 and Chk2, in combination with rucaparib, a PARP inhibitor, at clinically attainable concentrations in BRCA wild-type ovarian cancer.

Chapter 2. Body

2.1. Materials and Methods

2.1.1. Cell culture and drugs

SKOV-3 and OVCAR-3 (BRCA wild type) cell lines were purchased from the American Type Culture Collection (Rockville, MD). SKOV-3 cell lines were cultured in McCoy's 5A medium (Welgene, Kyungsan, Korea) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S; Invitrogen, Carlsbad, CA) in a humidified chamber with 5% CO_2 at 37° C. OVCAR-3 cells were cultured in RPMI 1640 medium (Roswell Park Memorial Institute, Buffalo, NY) supplemented with 10% FBS. Prexasertib and rucaparib were purchased from Selleckchem (Houston, TX) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MI). Final concentrations in culture medium never exceeded 0.2%.

2.1.2. Cell viability

Cell viability was measured by PrestoBlue cell viability reagent (Invitrogen). SKOV3 (1×10^5 cells/well) and OVCAR-3 cells (1×10^5 cells/well) were plated in 96-well plates in McCoy's 5A and RPMI completed media respectively. Each cell lines were treated with prexasertib, rucaparib, and both combination at 0, 1, 5, 10, 50, and 100 μM for 72 h. The treated cells were incubated with 10% PrestoBlue reagent for 30 min in room temperature. The absorbance at 540 nm was measured by ELISA microplate reader (Molecular Devices, San Jose, CA). All data are expressed as a percentage of control.

2.1.3. Cell proliferation

Cell proliferation was examined using a Cell Titer-Glo assay kit (Promega, Madison, WI). Cells were seeded in 96-well microplates in McCoy's 5A complete medium (containing 10% FBS

and 1% P/S) and treated with prexasertib, rucaparib or a combination at 0, 1, 5, 10, and 50, 100 μM for 72 h. A volume of Cell Titer-Glo reagent equal to the volume of cell culture medium was added to cells in each well. Cells were incubated at room temperature for 10 min and a luminescent signal was recorded by a luminescence microplate reader (Berthold Technologies, Bad Wildbad, Germany).

2.1.4. Rad51 siRNA transfection

Human Rad-51 siRNA was synthesized by Genolution (Seoul, South Korea), and siRNA for control was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The Rad-51 targeting-sequence was 5' - AAGCUGAAGCUAUGUUCGCCAUU -3'. The transfection was performed using Opti-MEM media and Lipofectamine RNAi MAX™ (Invitrogen) according to the manufacturer's method. Transfected cells were cultured in 96-well plates or 100-mm culture dishes. At 48 h after transfection, the cells were treated with rucaparib 50 μM for 48 h, and then cell viability and caspase-3 activity were determined or an immunoblot was undertaken.

2.1.5. Apoptosis by annexin V-FITC by flow cytometry

For apoptotic cell death analysis, an annexin V-FITC assay was carried out using a FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA) according to the protocol provided. The cells, at a concentration of 1×10^6 cells/well, were seeded in a 6-well plate and treated with 0, 50, and 100 μM concentrations of prexasertib, rucaparib or a combination of these for 48 h. After treatment, supernatants and cells were harvested and centrifuged at 1200 rpm for 7 min. The cell pellet was resuspended in 100 μL of 1× binding buffer, and 5 μL of FITC Annexin V and propidium iodide (PI) were added. Cells were incubated for 15 min at RT (25° C) in the dark. After incubation, 1× Binding Buffer was added to each sample, and cells analyzed by flow cytometry within 1 h. Flow cytometric analysis was carried out using a FACS Calibur (BD

Biosciences) flow cytometer, by analyzing at least 10,000 cells per sample. Results are presented as a percentage of the total gated number of cells.

2.1.6. Cell-cycle arrest by flow cytometry

For cell-cycle analysis, treated cell samples were washed with ice-cold phosphate buffered saline (PBS) and fixed with 70% ethanol at −20° C overnight. Then, samples were washed with PBS and resuspended with 0.5 mL of FxCycle™ PI/RNase Staining Solution (Invitrogen) containing 50 μg/mL PI with 100 μg/mL RNase A, and incubated for 30 min at room temperature while protected from light. Samples were analyzed by FACS Calibur (BD Biosciences) flow cytometer.

2.1.7. Caspase 3/7 activity

SKOV-3 or OVCAR-3 cells (1×10^5/well) in a white-walled 96-well plate were cultured for 24 h in McCoy's 5A or RPMI 1640 complete media, and treated with prexasertib, rucaparib or a combination for 48 h. The treated cells were incubated with 100 μL of Caspase-Glo 3/7 Reagent at room temperature for 30 min. The luminescence of each sample was measured in by a luminometer (Molecular Devices, CA, USA). All data are expressed as a fold induction of control.

2.1.8. Western blot analysis

Treated cells were lysed by ice-cold cell lysis buffer (Intron Biotechnology, Seongnam, South Korea), and protein concentrations were determined with a BCA assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Equal amounts of protein (20-30 μg/lane) was separated on a 12% acrylamide gel by SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane, and blocked with 5% non-fat milk. Each membrane was incubated with anti-Chk1, anti-phospho Chk1 (Ser345), anti-phospho Chk1 (Ser296), anti-PARP, anti-cyclin B1 (Cell Signaling Technology, Danvers, MA, USA); anti-

Rad-51, anti-γ-H2AX (Ser139), anti-phospho histone H3 (Ser10), anti-cleaved caspase-3, anti-cleaved PARP (Abcam, Cambridge, UK); and alpha-tubulin (Sigma-Aldrich) antibodies. Each membrane was then incubated with horse radish peroxidase (HRP)-conjugated secondary anti-mouse or rabbit IgG antibody, and protein bands visualized using Immobilin Forte Western HRP Substrate (Millipore, Burlington, MA, USA).

2.1.9. Statistical analysis

All procedures were performed using at least 16 samples and repeated in three independent experiments. All data are represented by the mean value ± standard deviation (SD). Since, Komogorov-Smmirnov test revealed that all variables showed normal distribution, student's *t* test was used to determine the *p* value between the two different groups. A *p* value of less than 0.05 was considered significant.

2.2. Results

2.2.1. Chk1 inhibition induces apoptotic cell death compared to PARP inhibition in BRCA wild-type ovarian cancer cell lines

First, to validate the induction of cell death by Chk1 or PARP inhibition in ovarian cancer cell lines, we evaluated cell viability and proliferation in the presence of prexasertib and rucaparib using PrestoBlue and Cell Titer-Glo assays. The cell viability and proliferation of SKOV-3 and OVCAR-3 cells were reduced when cells were treated with prexasertib compared to rucaparib (Fig. 1A and B). Caspase-3 luciferase activity involved in the apoptotic signaling pathway increased in a dose-dependent manner in prexasertib-treated SKOV-3 and OVCAR-3 cells, but not in rucaparib-treated cells (Fig. 1C). Immunoblot analysis revealed that cells increased the expression of the pro-apoptotic proteins, cleaved caspase-3 and cleaved PARP, under prexasertib treatment (Fig. 1D). These results suggest that Chk1 inhibition induced apoptotic cell death in ovarian cancer cells.

2.2.2. Chk1 inhibition combined with PARP inhibition promotes apoptotic cell death in BRCA wild-type ovarian cancer cell lines

The cytotoxicity of prexasertib and rucaparib was assessed in a panel of BRCA-wild type ovarian cancer cell lines. SKOV and OVCAR cell lines were treated with different concentrations of prexasertib, rucaparib, or prexasertib with rucaparib for 3 days, and then cell viability was evaluated. Both prexasertib and rucaparib monotherapy decreased cell viability in a dose-dependent manner in both SKOV and OVCAR cell lines. Combination with prexasertib and rucaparib showed synergistic decrease of cell viability, in both SKOV (Figure 2A) and OVCAR cell lines (Figure 2B).

We next assessed the combined effect of Chk1 and PARP inhibition in ovarian cancer. Cell viability curves for combination treatments, ranging from 0-100 μM for prexasertib, and 0, 10, and 50 μM for rucaparib, were determined from PrestoBlue assays (Fig. 3A). We then selected two doses of prexasertib/rucaparib (10, 50 μM/10 μM and 10, 50 μM/50 μM) to test for a combination effect. Apoptosis and caspase-3 activity analysis demonstrated that combination therapy increased apoptotic cell death compared to prexasertib or rucaparib monotherapy in both SKOV-3 and OVCAR-3 cells (Fig. 3B and C). Western blot analysis showed that combination therapy promoted the expression of cleaved caspase-3 and cleaved PARP, compared to prexasertib or rucaparib monotherapy, in a dose-dependent manner in ovarian cancer cells (Figure 3D). This suggests that combined Chk1 and PARP inhibition, compared to the respective monotherapies, induced apoptotic cell death in ovarian cancer cells.

2.2.3. Chk1 inhibition could force mitotic entry of G2M-phase cells induced by PARP inhibitor in BRCA wild-type ovarian cancer cell lines

Cell-cycle analysis revealed that prexasertib could force mitotic entry of SubG1 phase cells induced by rucaparib in BRCA wild-type ovarian cancer cells (Fig. 4A). SubG1 phase cells, which

suggested the presence of an apoptotic population, were significantly increased after combination therapy in SKOV-3 and OVCAR-3 cell lines. Western blot analyses showed that the expression level of the mitotic marker, p-H3, increased after combination therapy, which suggested that the blockade was in fact in M phase (Fig. 4B). Moreover, prexasertib-treated cells showed a substantial reduction in the level of endogenous Chk1, whereas phosphorylation of Chk1 (Ser345) and expression of γ-H2AX was significantly increased, indicating the persistence of DSBs in the treatment group (Fig. 4B). Therefore, our results suggest that combination therapy forced mitotic entry of G2M phase cells with unrepaired DNA damage, which may have led to a synergistic anticancer effect compared to monotherapy of each drug.

2.2.4. Chk1 inhibition prevents nuclear Rad51 foci formation in response to rucaparib treatment in BRCA wild-type ovarian cancer cell lines

Gene expression data through analysis of The Cancer Genome Atlas was evaluated; this revealed Rad51 was a poor prognostic marker for patients with breast cancer. Additionally, Rad51 expression levels were much higher in African-American and Asian patients with breast cancer compared to Caucasians, suggesting Rad51 was a biomarker for racial disparities in this disease. Also, our immunoblotting data revealed that Rad51 expression was increased and regulated depending on Chk1 expression in ovarian cancer cells (Fig. 4B). Therefore, to evaluate the anti-cancer effect in response to inhibition of Rad51, we incorporated siRad51 in both cell lines and treated these with rucaparib. Cell viability reduced Rad51 knockdown and rucaparib treatment (Fig. 5A); caspase-3 activity was induced under the same conditions in both SKOV-3 and OVCAR-3 cells (Fig. 5B). Western blot analyses of BRCA wild-type ovarian cancer cell lines treated with siRad51 and rucaparib showed a similar pattern to those of cells treated with prexasertib and rucaparib, indicating that Chk1 inhibition by prexasertib prevents nuclear Rad51 foci

formation in response to rucaparib treatment (Fig. 5C). These results demonstrated that synergistic cytotoxicity in combination treatment with prexasertib and rucaparib is caused by a reduced Rad51 response. The induction of nuclear Rad51 foci by rucaparib might be abrogated when Chk1 is inhibited by prexasertib in BRCA wild-type ovarian cancer cell lines.

2.3. Discussion

Currently, PARP inhibitors such as olaparib, rucaparib, and niraparib, have been approved by the US Food and Drug Administration and shown to have clinical potential in treating ovarian cancer. However, olaparib monotherapy achieved only a 30% response rate for the treatment of BRCA wild-type ovarian cancer [15].

Individual PARP inhibitors have different binding affinities for PARP1, PARP2, and PARP3 [16]. Thus, on-target effects might be different according to the types of PARP inhibitors [16]. Rucaparib inhibits PARP1, PARP2, and PARP3, whereas olaparib and niraparib inhibits only PARP 1 and PARP 2 [16]. In addition, PARP3 has been reported to activate the enzymatic activity of PARP1 in the absence of DNA. Therefore, the additional inhibition of PARP3 might potentiate the effects of rucaparib compared with olaparib or niraparib [16, 17].

A part 1 of the ARIEL2 trial revealed that rucaparib monotherapy was efficacious in women with relapsed, platinum-sensitive, BRCA mutated HGSC cell lines, as well as in those with BRCA wild-type carcinomas with high genomic loss of heterozygosity, a potential marker of homologous recombination deficiency and PARP inhibitor activity [18, 19] In our study, we applied rucaparib as a PARP inhibitor, expecting to have compatible potency with olaparib or niraparib, as well as comparable interaction with Chk1 inhibitor in BRCA wild-type ovarian cancer.

To achieve a higher complete response for the treatment of BRCA wild-type ovarian cancer, a combination strategy with PARP inhibitors and other cytotoxic agents has been attempted. From this viewpoint, the ATR/Chk1 axis can be an attractive target. Several studies reported that inhibition of the ATR/Chk1 axis caused replication catastrophe, DNA damage, and cell death [20]. Moreover, several studies have reported that Chk1 can overcome the chemo-resistance of PARP inhibitors, and synergizes cytotoxic effects in cancer cells, including in ovarian, mammary, and gastric cancer cell lines [4, 21-23].

In this study, we demonstrated that a combination of prexasertib with the PARP inhibitors, rucaparib, showed synergistic cytotoxicity against BRCA wild-type HGSC cell lines. First, we found that monotherapy of each drug, especially the Chk1 inhibitor, significantly suppressed cell proliferation. Nonetheless, combination therapy showed a synergistic effect in the suppression of cell proliferation and cytotoxicity. Our results are similar to those of several prior studies. A study exists that evaluates the *in vitro* toxicity of the PARP inhibitors, olaparib, in combination with prexasertib for the treatment of BRCA mutant and BRCA wild-type high-grade serous ovarian cancer (OVCAR3, OV90, PEO1, and PEO4) cell lines [23]. The authors suggested that combination treatment synergistically decreased cell viability in all cell lines, and induced greater DNA damage and apoptosis than monotherapy of each drug ($p < 0.05$ for all) [23]. In addition, they demonstrated that treatment with olaparib in BRCA wild-type HGSC cell lines caused the formation of Rad-51 foci, whereas combination treatment with prexasertib inhibited the transnuclear localization of Rad-51[23]. Rad-51 is a key protein in homologous recombination [23]. Therefore, they suggested that prexasertib increased the cytotoxicity of PARP inhibitor by preventing Rad-51 foci formation in BRCA wild-type HGSC cell lines [23]. In our study, we used rucaparib which are expected to have compatible potency with other PARP inhibitors, such as olaparib and niraparib. Similar to prior study using olaparib, combination of Chk1 inhibitor and rucaparib

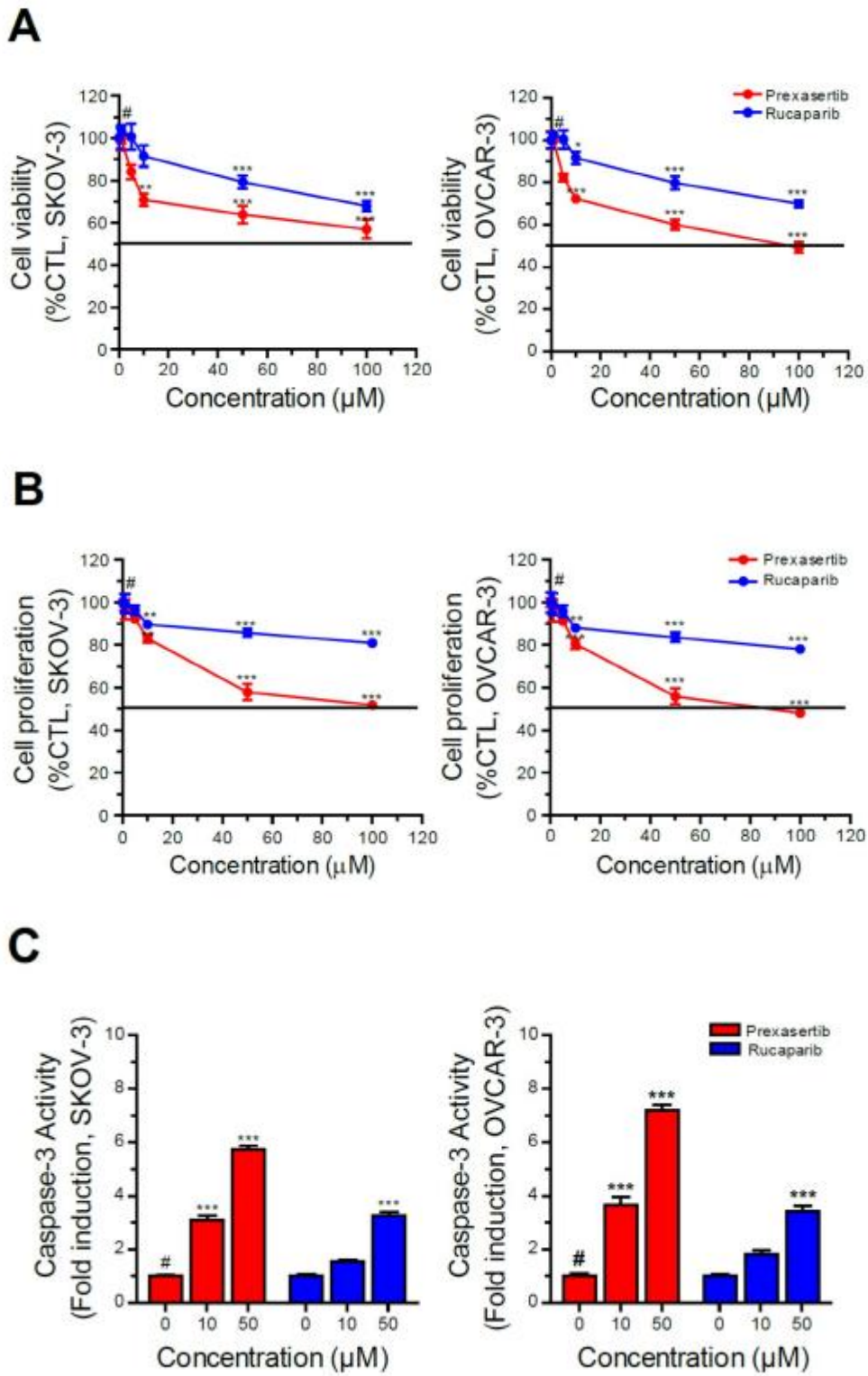
also showed synergistic anticancer effect in BRCA-wild type ovarian cancer cell lines [23]. The authors reported that olaparib treatment induced nuclear Rad foci formation in BRCA wild type HGSC cell lines, while prexasertib had no impact on nuclear Rad51 foci formation [23]. They hypothesized that the induction of nuclear Rad51 foci by olaparib was completely abrogated when Chk1 is inhibited by prexasertib in all BRCA wild type HGSC cell lines [23]. On the contrary, in our study, western blotting showed that Rad-51 expression decreased under prexasertib monotherapy and a combination of prexasertib and rucaparib, whereas it increased under rucaparib monotherapy, which might be comparable to prior study using olaparib. Furthermore, we proved that reduced levels of Rad51 expression by siRNA increases sensitivity to rucaparib in BRCA wild-type ovarian cancer cell lines. Our study confirms that Chk1 potentiates sensitivity to PARP inhibitor of BRCA wild-type ovarian cancer cells by suppression of Rad51. In addition, our data supports that the interaction mechanism of Chk1 inhibitor and PARP inhibitor was similar, regardless of types of PARP inhibitors.

Another study evaluated the cytotoxic effect of a combination of PARP inhibitor and ATR inhibitor/Chk1 inhibitor in BRCA mutant ovarian cancer cell lines [4]. It was suggested that a combination of PARP inhibitor with ATR/CHK1 inhibitor is more effective than PARP inhibitor monotherapy in BRCA mutant ovarian cancer cell lines due to the increased reliance on ATR/CHK1 for genome stabilization under PARP inhibitor treatment [4].

Another study reported that a Chk1 inhibitor potentiated the cytotoxic effect of PARP inhibitor in gastric cancer cell lines [22]. The authors showed that a Chk1 inhibitor inhibits homologous recombination-mediated DNA repair, and thus had a marked synergistic anti-cancer effect in combination with PARP inhibitor in both *in vitro* studies and *in vivo* experiments, using a gastric cancer patient-derived xenograft model [22]. They suggested that synergy between the Chk1 inhibitor, LY2606368, and PARP inhibitor might be caused by an impaired G2M checkpoint due to LY2606368 treatment, which forced mitotic entry and cell death in

the presence of a Chk1 inhibitor [22].

2.4. Figures and Legends



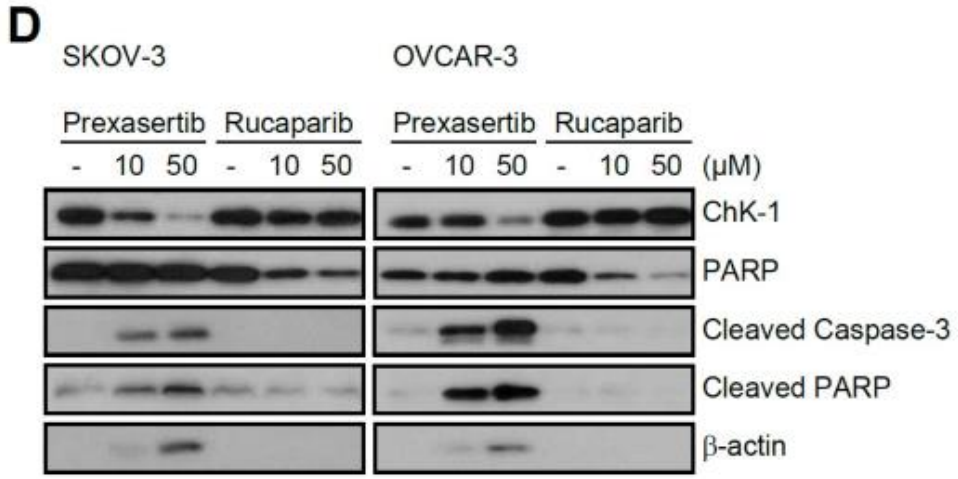


Fig. 1. Prexasertib or rucaparib reduce cell viability and proliferation and induce apoptotic cell death in BRCA wild-type ovarian cancer cell lines. The cells were treated with either prexasertib (0-100 μM) or rucaparib (0-100 μM) for 72 h after cells were seeded. (A) The cell viability and (B) proliferation of prexasertib and rucaparib was determined by PrestoBlue or Cell Titer-Glo assay in SKOV-3 and OVCAR-3 cells. (C) The activity of caspase-3 was measured by luciferase assay using Caspase-Glo 3/7 Reagent. Cell viability, proliferation, and caspase-3 activity were calculated relative to 0.01% dimethylsulfoxide (DMSO)-treated control cells. (D) Representative images of immunoblotting data for protein levels of checkpoint kinase 1 (Chk1), poly ADP ribose polymerase (PARP), cleaved caspase-3, and cleaved PARP proteins after prexasertib or rucaparib treatment. Alpha-tubulin was used as a loading control. Blue and red colors indicate prexasertib and rucaparib, respectively.

**refers to $p < 0.01$ compared to 0 μM, ***refers to $p < 0.001$ compared to 0 μM, and # refers to control group. Values are expressed as the mean ± SD (standard deviation).

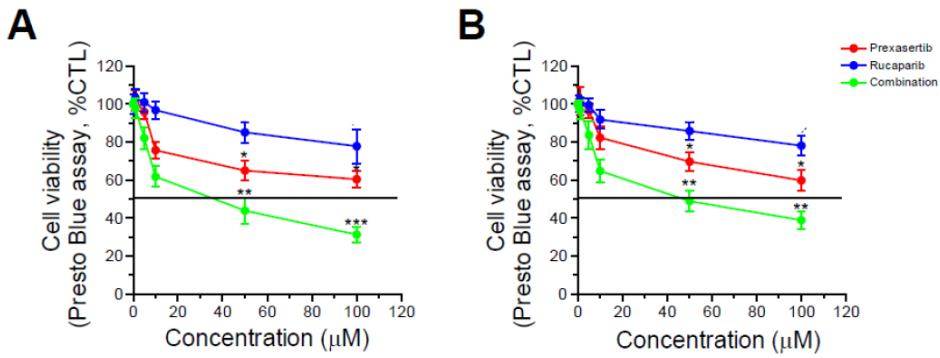
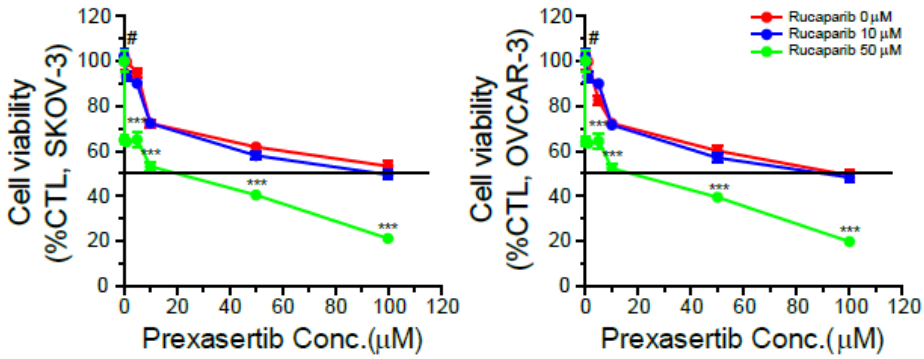


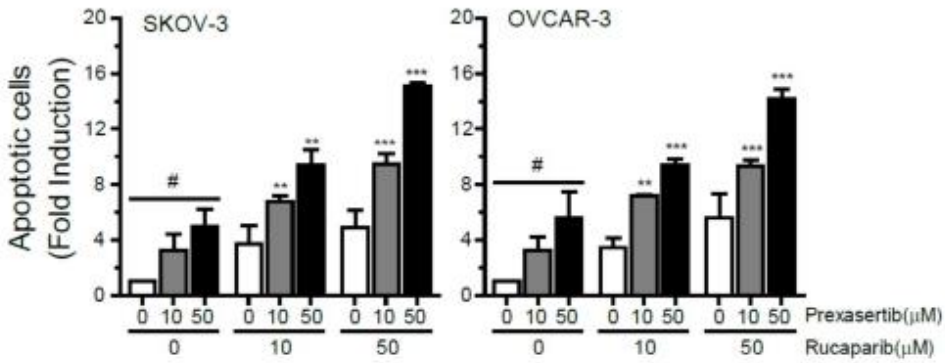
Fig. 2. Combination with prexasetib and rucaparib showed synergistic decrease of cell viability, in both SKOV (A) and OVCAR cell lines (B).

*refers to p <0.1 to 0 μM, **refers to p < 0.01 compared to 0 μM, and ***refers to p < 0.001 compared to 0 μM. Values are expressed as the mean ± SD (standard deviation).

A


Cell viability (%CTL, SKOV-3)
Prexasertib Conc.(μM)
Cell viability (%CTL, OVCAR-3)
Prexasertib Conc.(μM)
Rucaparib 0 μM
Rucaparib 10 μM
Rucaparib 50 μM

B


SKOV-3
OVCAR-3
Apoptotic cells (Fold Induction)
Prexasertib(μM)
Rucaparib(μM)

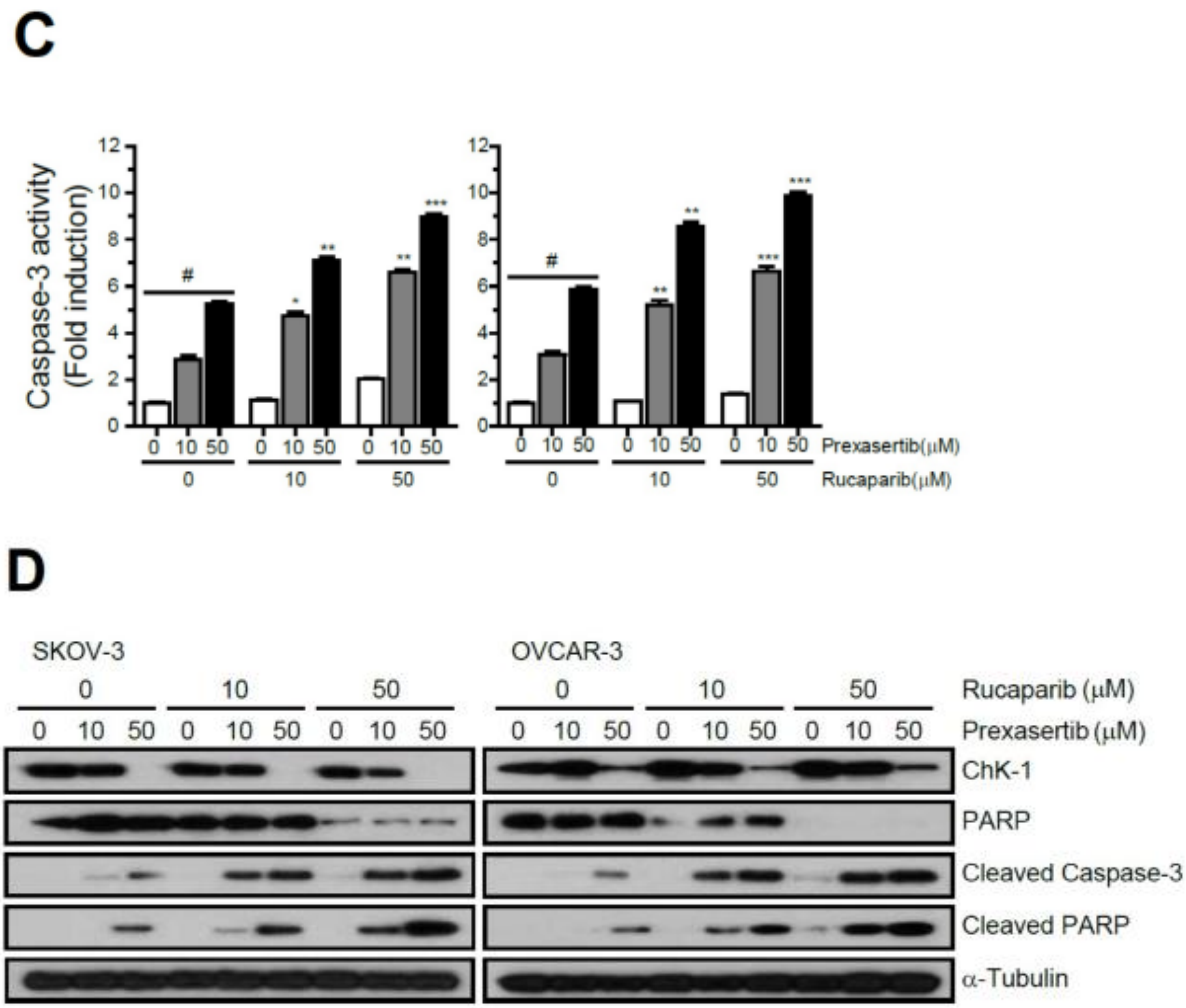


Fig. 3. Combination treatment promotes suppression of cell viability and induction of apoptotic cell death in ovarian cancer cells. SKOV-3 and OVCAR-3 cells were treated with either prexasertib (0-100 μM) or rucaparib (0, 10, 50 μM) for 72 h after cells were seeded. (A) Cell viability was determined by PrestoBlue in SKOV-3 and OVCAR-3 cells. (B) Apoptotic cell analysis was measured using an Annexin V assay by fluorescence activated cell sorting (FACS). (C) Caspase-3 activity was measured by luciferase assay using Caspase-Glo 3/7 reagent. (D) Representative images of immunoblotting data for levels of checkpoint kinase 1 (Chk1), poly ADP ribose polymerase (PARP), cleaved caspase-3, and cleaved

PARP proteins in combination treatment conditions. Alpha-tubulin was used as a loading control.
**refers to $p < 0.01$ compared to 0 μM, ***refers to $p < 0.001$ compared to 0 μM, and # refers to control group. Values are expressed as the mean ± SD (standard deviation).

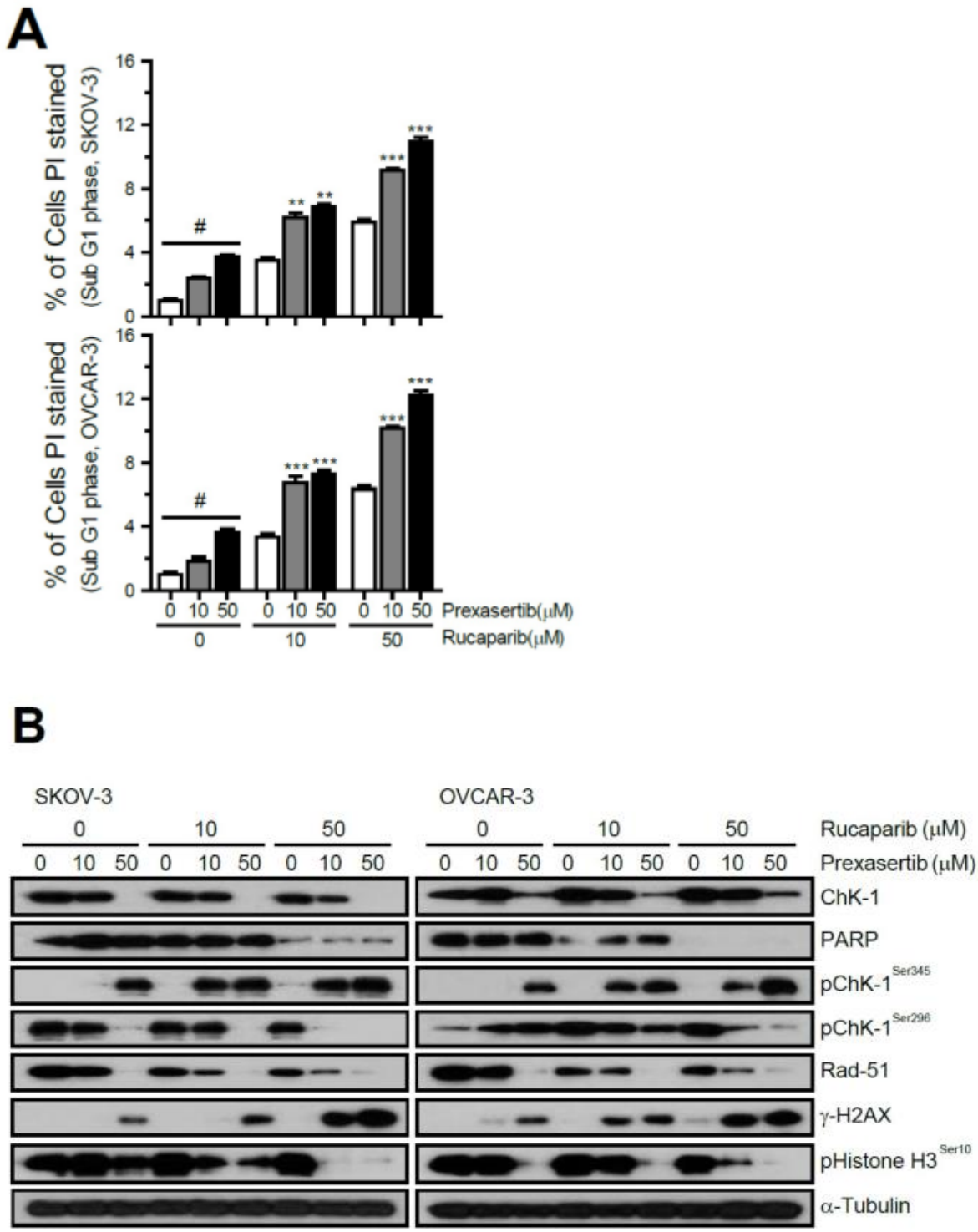
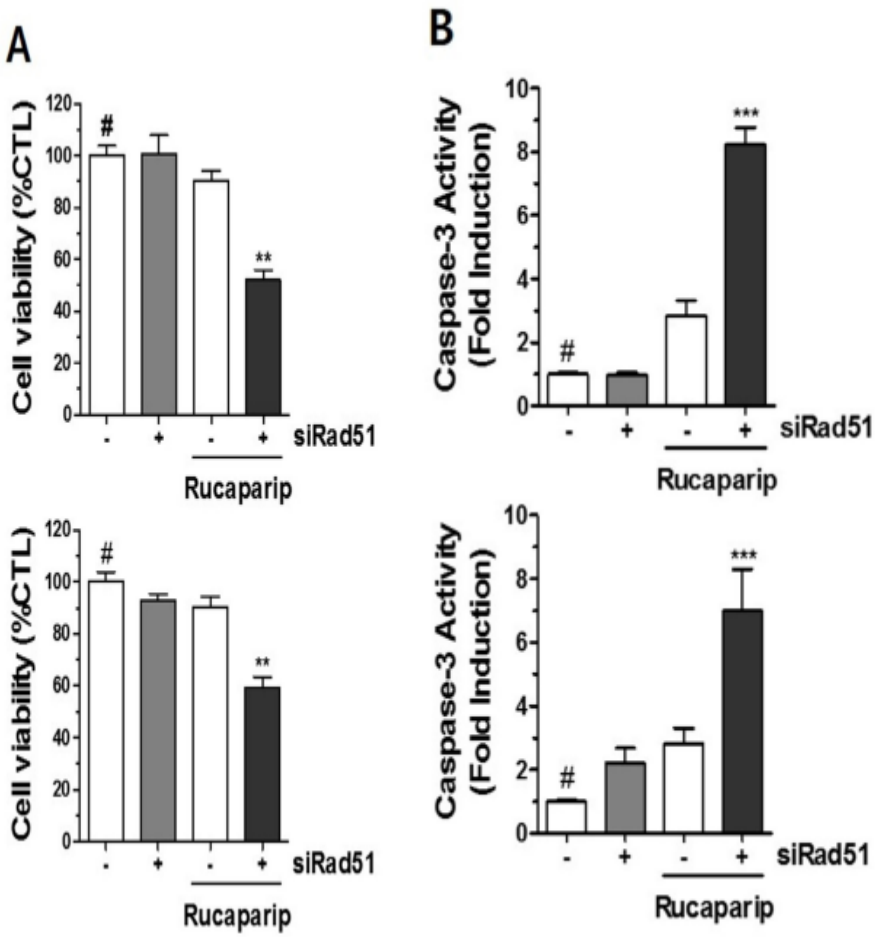


Fig. 4. Combination treatment increases mitotic entry of Sub G1 phase cells and activates the DNA damage signaling pathway. (A)

Cell-cycle analysis was determine using FxCycle™ propidium iodide (PI)/RNase Staining Solution by fluorescence activated cell sorting (FACS) in both SKOV-3 and OVCAR-3 cell lines. (B) Representative images of immunoblotting data for levels of phospho-Ser345 checkpoint kinase 1 (Chk1), phospho-Ser296 Chk1, Rad-51, γH2AX, and phosphor-Ser10 Histone H3 proteins in combination treatment conditions. Alpha-tubulin was used as a loading control. **refers to $p < 0.01$ compared to 0 μM, ***refers to $p < 0.001$ compared to 0 μM, and # refers to control group. Values are expressed as the mean ± SD (standard deviation).


A
B
Cell viability (%CTL)
Caspase-3 Activity (Fold Induction)
siRad51
Rucaparip
#
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***

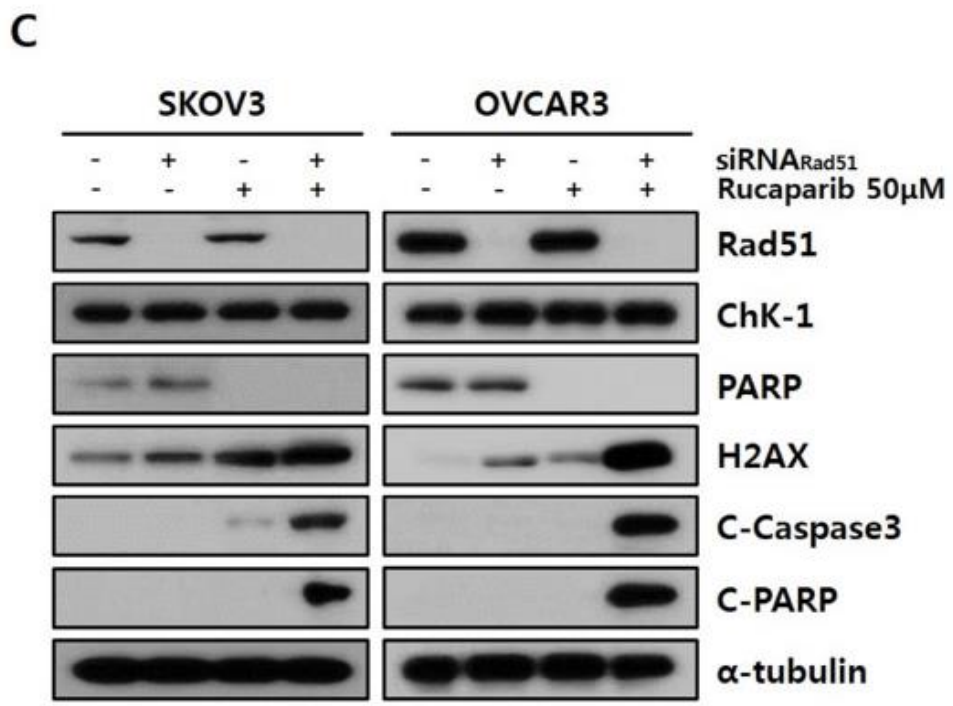
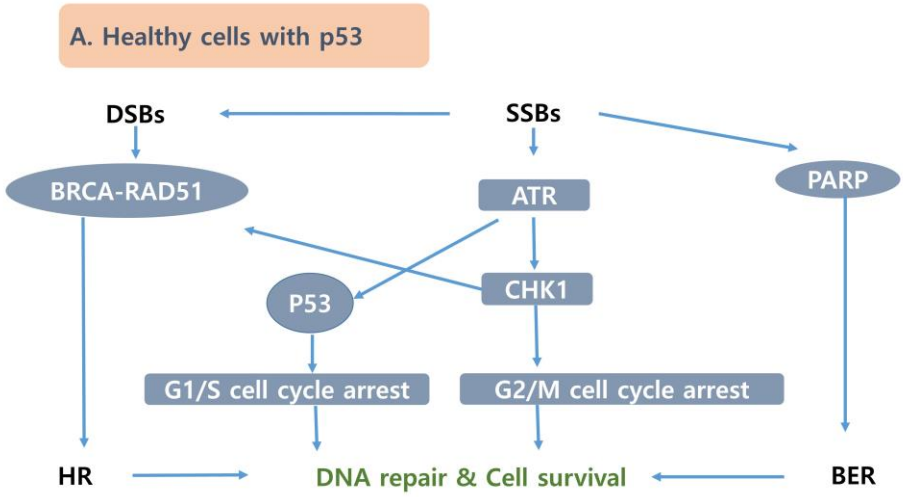
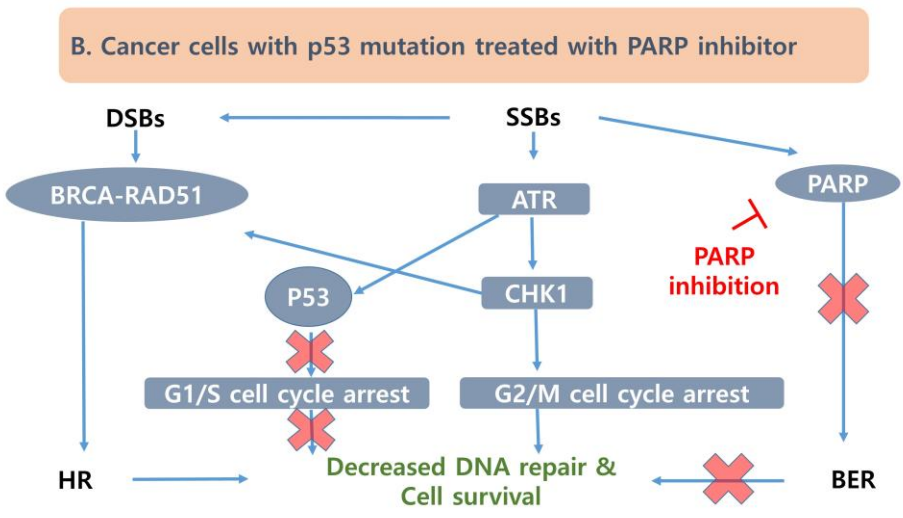


Fig. 5. Regulation of Rad51 expression affects anti-cancer effect by PARP inhibition. (A, B) Knockdown of Rad51 expression was performed by Lipofectamine in ovarian cancer cells. After inhibition of Rad-51, SKOV-3 and OVCAR-3 cells were treated with 50 μM rucaparib for 48 h. Cell viability and caspase-3 activity were measured by PrestoBlue and Cell Titer-Glo assays, respectively. (C) Representative images of immunoblotting data for protein levels of Rad51, checkpoint kinase 1 (Chk1), poly ADP ribose polymerase (PARP), γH2AX, cleaved caspase-3, and cleaved PARP under specific conditions. Alpha-tubulin was used as a loading control. **refers to $p < 0.01$ compared to 0 μM, ***refers to $p < 0.001$ compared to 0 μM, and # refers to control group. Values are expressed as the mean ± SD (standard deviation).


A. Healthy cells with p53
DSBs
SSBs
BRCA-RAD51
ATR
PARP
CHK1
P53
G1/S cell cycle arrest
G2/M cell cycle arrest
HR
DNA repair & Cell survival
BER


B. Cancer cells with p53 mutation treated with PARP inhibitor
DSBs
SSBs
BRCA-RAD51
ATR
PARP
PARP
inhibition
CHK1
P53
G1/S cell cycle arrest
G2/M cell cycle arrest
HR
Decreased DNA repair &
Cell survival
BER

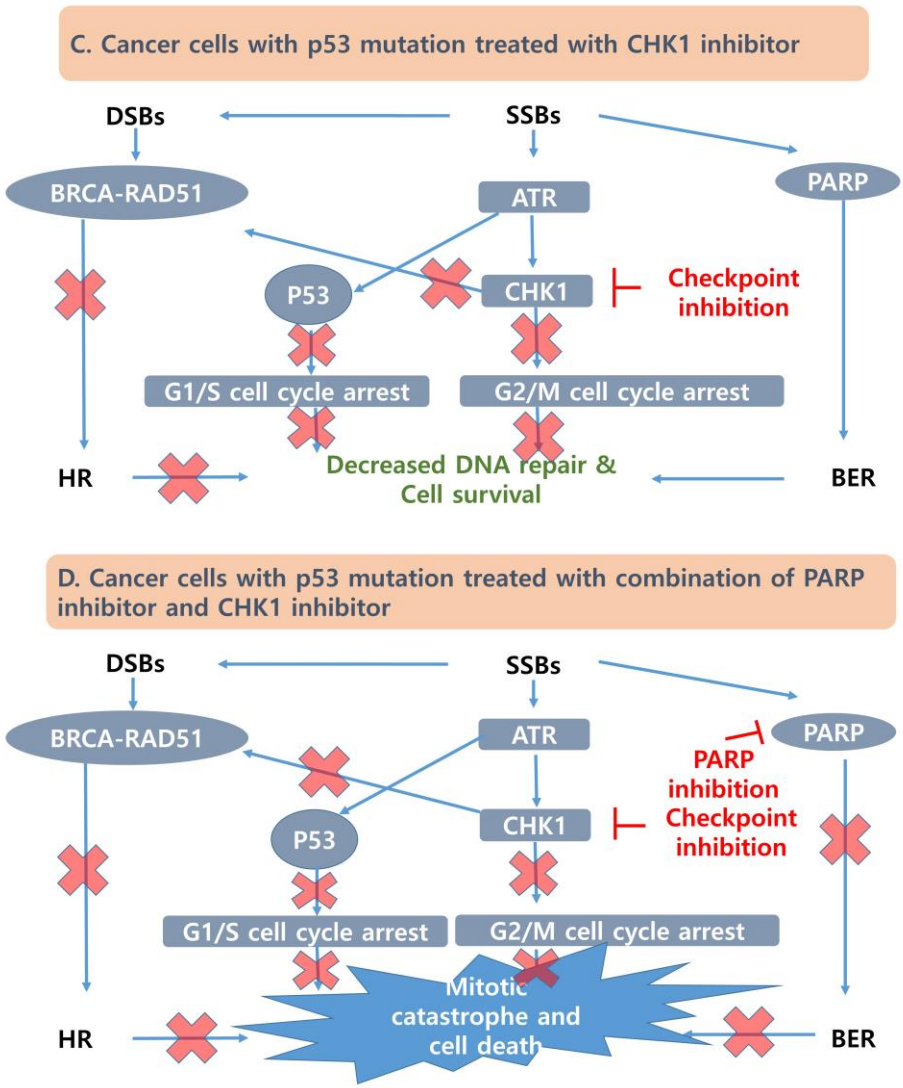


Fig. 6. Sensitizing cancer cells to PARP inhibitor with checkpoint inhibitor. Replication stress, ultraviolet, and radiation induces DNA damages. Single strand breaks (SSBs) activates BER and ATR/CHK1 pathway. Double strand breaks (DSBs) activates HR pathway. In healthy cells with p53, G1/S checkpoint cell cycle arrest and G2/M checkpoint cell cycle arrest normally operate and cell survives. (A) Cancer cells with p53 mutation are highly dependent on G2/M checkpoint. Even under the PARP inhibition, G2/M checkpoint normally operates and cell damage is minimal. (B) Checkpoint inhibitor suppresses HR pathway via inhibition of RAD51 formation, as well as G2/M checkpoint cell cycle arrest. In

cancer cells with p53 mutation treated with Chk1 inhibitor, G1/S checkpoint, G2/M checkpoint, and HR pathway do not normally operate and cell damage is increased. (C) In cancer cells with p53 mutation treated with combination of PARP inhibitor and Chk1 inhibitor, G1/S checkpoint, G2/M checkpoint, HR pathway, and BER pathway do not normally operate, then, cells with unrepaired DNA damage advances to mitotic catastrophe and cell deaths. (D)

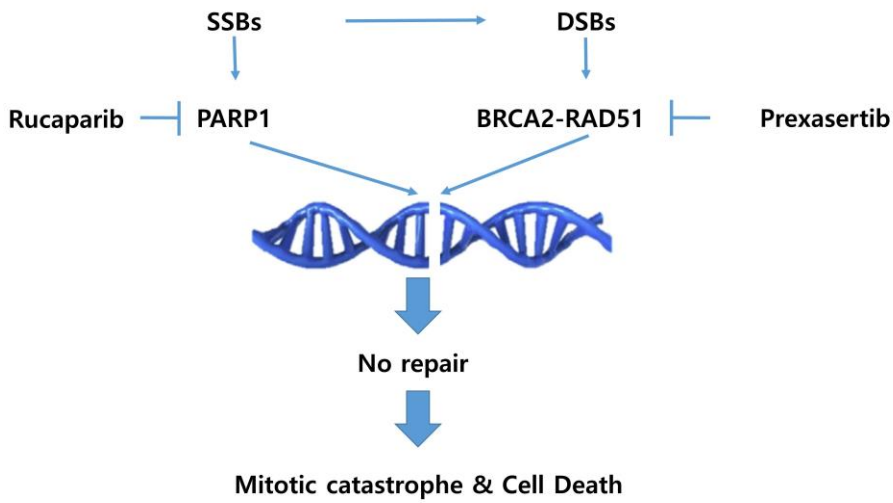


Fig. 7. Mechanism of cell death triggered by combination of Prexasertib and Rucaparib. Prexasertib downregulates HR pathway via disruption of BRCA2-RAD51 interaction, which makes BRCA-wild type cancer cells more sensitive to PARP inhibitor, rucaparib.

Chapter 3. Conclusion

In conclusion, we demonstrate that a Chk1 inhibitor suppresses Rad51, which affects a decrease in homologous recombinant repair. Moreover, we found that Chk1 inhibitor and PARP inhibitor combination therapy forced mitotic catastrophe and cell death in p53-mutated ovarian cancer cell lines, which are highly dependent on G2/M phase cell-cycle arrest (Fig.6, 7).

Importantly, we found that suppression of Rad51 sensitized cells to the anticancer effect of the PARP inhibitor, which might be applied to the treatment of various human cancer cells. This provides a potentially new therapeutic strategy for the treatment of BRCA wild-type HGSC, which is the most common type of epithelial ovarian cancer.

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국문 초록

PARP 억제제는 BRCA 유전자 변이가 있는 상피성 난소암에서 효능이 입증되어 임상적으로 널리 적용되고 있다. 그러나 BRCA 유전자 변이가 없는 상피성 난소암에서는 PARP 억제제의 임상적인 효능이 BRCA 유전자 변이가 있는 상피성 난소암과 비교하여 낮은 것으로 보고되고 있다. TP53은 손상된 DNA의 복구과정에 중요한 역할을 하는데, 특히 세포주기 확인점 중에서도 G1/S 확인점의 조절과정에 중요한 역할을 한다. 대부분의 상피성 난소암은 TP53 변이를 갖는데, TP53 변이를 가진 상피성 난소암은 G1/S 확인점의 조절과정에 의한 DNA의 복구가 불가능하므로, G2/M 확인점의 조절과정에 관여하는 ATR/Chk1 등을 통하여 DNA 복구를 하게 된다.

따라서 이론적으로 BRCA 유전자 변이가 없는 상피성 난소암에서 PARP 억제제와 Chk1 억제제를 병합하였을 때 각각을 단독으로 투여하였을 때 보다 더 높은 항암효과를 기대할 수 있다. 이에 본 연구에서는 BRCA 유전자 변이가 없는 상피성 난소암 세포주에서 PARP 억제제인 rucaparib과 Chk1 억제제인 prexasertib (LY2606368)을 단독 혹은 병합하여 항암효과를 비교하였다.

본 연구의 목적은 다음과 같다. 첫째, Chk1 억제제가 BRCA 유전자 변이가 없는 상피성 난소암 세포의 성장을 억제하는지 확인하고자 하였다. 둘째, Chk1 억제제가 BRCA 유전자 변이가 없는 상피성 난소암 세포에서 G2/M 확인점의 조절과정에 미치는 영향을 확인하고자 하였다. 셋째, Chk1 억제제와 PARP 억제제의 병합투여가 BRCA 유전자 변이가 없는 상피성 난소암 세포의 성장을 억제하는 데 있어서 상승효과가 있는지 확인하고자 하였다.

주요 결과는 다음과 같다. 첫째, rucaparib과 비교하여 prexasertib이 유의하게 더 강한 세포독성을 보였다. 둘째, rucaparib과 prexasertib 각각의 단독투여와 비교하여 병합투여가 유의하게 세포독성을 증가시키는 것으로 나타났다. 셋째, rucaparib과 prexasertib을 병합투여했을 때 rucaparib의 효과가 미치지 않는 G2/M 세포주기 정지 세포에서도 prexasertib에 의하여 Chk1이 억제됨으로써 세포독성이

유도되었다. 넷째, BRCA 유전자 변이가 없는 상피성 난소암에서 Rad51의 과발현은 rucaparib의 효과를 감소시키는 기전 중의 하나인데, prexasertib이 Rad51 발현을 차단함으로써 rucaparib의 세포독성을 증가시켰다.

rucaparib과 prexasertib 각각의 단독투여와 비교하여 병합투여가 유의하게 세포독성을 증가시키는 기전은 다음과 같다. 첫째, rucaparib을 단독으로 투여했을 때에는 영향을 받지 않는 G2/M 확인점이 Chk1 억제제에 의하여 작동하지 않게 되면 난소암 세포의 유사분열 파국이 유도된다. 둘째, Chk1은 Rad51을 발현시키고 Rad51과 BRCA2의 결합을 증가시켜서 상동 재조합을 유도하는데, Chk1을 억제하면 Rad51의 발현과 BRCA2와의 결합이 감소하여 상동 재조합이 억제된다.

결론적으로, Chk1 억제제와 PARP 억제제의 병합투여를 BRCA 유전자 변이가 없는 상피성 난소암에서 새로운 효과적 치료 전략으로 제시할 수 있을 것이다. 또한, 다른 암종에서도 PARP 억제제의 저항성 기전을 Rad 51의 과발현으로 설명하고 있는데, Chk1 억제제에 의하여 Rad51의 발현을 감소시킴으로써 PARP 억제제의 저항성을 극복할 수 있을 것이다.

Keyword: Chk1 억제제, PARP 억제제, Rad51, 상피성 난소암