Inhibiting the mTOR pathway synergistically enhances cytotoxicity in ovarian cancer cells induced by etoposide through up-regulation of c-Jun

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Translational Relevance

The mammalian target of rapamycin (mTOR) kinase is implicated in the regulation of proliferation and survival of cells. mTOR inhibitor rapamycin has been reported to enhance the effects of a variety of chemotherapeutic agents. However, the combination effects of mTOR inhibitors and cytotoxic agents have not been evaluated systematically in ovarian cancer. We tested the effects of rapamycin combined with anticancer agents to explore the mechanisms for their synergistic interactions. We showed that rapamycin and etoposide led to synergistic cytotoxic effects in ovarian cancer cells, and prolonged survival in mice with ovarian cancer xenografts. This effect was associated with up-regulation of phosphorylated c-Jun and down-regulation of Bcl-xL; suggesting that the JNK pathway had a key role in inducing apoptosis. This combination is a promising treatment for patients with ovarian cancer and should be explored in clinical trials.
Abstract

**Purpose:** The mammalian target of rapamycin (mTOR) pathway is thought to be a central regulator of proliferation and survival of cells. Rapamycin and its analogs are undergoing clinical trials in patients with epithelial ovarian cancer. The present study aimed to assess the potential to use rapamycin and anticancer agents in combination for first- and second-line chemotherapy to treat ovarian cancer.

**Experimental Design:** We used six ovarian serous adenocarcinoma cell lines (KF, KOC-2S, SHIN-3, SK-OV-3, TU-OS-3, TU-OS-4) in this study. We treated the cells with rapamycin and anticancer agents, then assessed cell viability, apoptosis, and the expression of protein in apoptotic pathways and molecules downstream of the mTOR signaling pathways. We also investigated the effect of these drug combinations on survival in nude mouse xenograft models.

**Results:** Synergistic effects were observed in five cell lines from the combination of etoposide and rapamycin. However, we observed antagonistic effects when rapamycin was combined with gemcitabine, cisplatin, or paclitaxel on more than two cell lines. Rapamycin dramatically enhanced apoptosis induced by etoposide and the expression of cleaved caspase 9. This effect was associated with up-regulation of phosphorylated c-Jun and down-regulation of Bcl-xL. The synergistic interaction of rapamycin and etoposide was lower when the c-Jun pathway was suppressed by a c-Jun N-terminal kinase inhibitor (SP600125). Finally, treating nude mice with rapamycin and etoposide significantly prolonged survival in the model mice with ovarian cancer xenografts.

**Conclusions:** Chemotherapy with rapamycin and etoposide combined is worth exploring as a treatment modality for women with epithelial ovarian cancer.
Introduction

Ovarian cancer reached 230,555 cases and accounted for 141,452 deaths worldwide in 2007, constituting 4.0% of all female cancers and 4.3% of cancer deaths in women (1). More than 70% of patients with ovarian cancer are diagnosed at the advanced stage (2). Currently, standard primary therapy for advanced disease involves combining maximal cytoreductive surgery with chemotherapy consisting of carboplatin and paclitaxel (3, 4). Though this treatment regimen initially yields a high response rate, more than 70% of patients relapse and develop resistance to platinum and taxane (2, 5). Moreover, an international study, GOG 182-ICON5, sought to improve the efficacy of standard carboplatin-paclitaxel therapy by incorporating newer cytotoxic agents (gemcitabine, pegylated liposomal doxorubicin, topotecan) in sequential doublet and triplet combinations. Unfortunately, no combination of the several agents used in standard therapy has improved overall survival (3). Alternate chemotherapeutic agents, such as pegylated liposomal doxorubicin, topotecan, gemcitabine, and etoposide, usually are used to treat recurrent ovarian cancers (6-9). However, these agents generally result in low response rates, approximately 15 to 30%, and poor survival. Thus, effective and novel treatment strategies (e.g. incorporation of molecular-targeted agents) for advanced ovarian cancer are needed urgently.

Activating the phosphatidylinositol 3'-kinase (PI3K) /Akt pathway and its downstream signaling mammalian target of rapamycin (mTOR) appears to indicate drug resistance and poor prognosis in many cancers and, therefore, may be an attractive target for therapy (10-12). In ovarian cancer amplified PI3K and activated Akt have been found in 12 to 68% of tumors and were associated closely with the up-regulation of mTOR signaling (10-12). mTOR was differentiated to two complexes: mTOR complex 1 (mTORC1) and mTORC2. mTORC1 regulates protein synthesis by directly phosphorylating the eukaryotic initiation factor
4E-binding protein 1 (4E-BP1) and also affects the activity of p70 S6 kinase (S6K1), and leads to cell growth and G1 cell cycle progression (13-15). mTORC2 has been shown to be an upstream regulator of Akt, whereas mTORC1 acts downstream of Akt, and the activity is up-regulated by compensatory response to mTORC1 down-regulation in certain circumstances (13). Therefore, inhibition of mTOR seems to be a candidate for therapy in patients with ovarian cancer.

Rapamycin, one of the mTOR inhibitors, was first isolated from the soil bacterium *Streptomyces hygroscopicus* in the 1970’s (16). It belongs to the class of macrolide antibiotics and was originally developed as an antifungal and immunosuppressant agent. Then it was found to have potent antiproliferative properties in a variety of solid tumors (17). Rapamycin binds to a member of the immunophilin protein family, FK506 binding protein 12 (FKBP12), and the complex inhibits kinase activity of mTOR by directly binding mTORC1. When the downstream signaling of mTOR, such as 4E-BP and S6K1, is blocked it leads to arrest in the G1 phase cell cycle and apoptosis (18). Several clinical trials also have shown potential antitumor activities of rapamycin and its derivatives everolimus (RAD001), deforolimus (AP23573), and temsirolimus (CCI779) in solid tumors, including ovarian cancer (19).

Recently, rapamycin has been reported to enhance the effects of a variety of chemotherapeutic agents in several types of cancer (20-23). However, the effects of mTOR inhibitors combined with the range of cytotoxic agents have not been evaluated systematically in ovarian cancer. We conducted the present study to determine whether rapamycin enhances the effects of anticancer agents (cisplatin, paclitaxel, etoposide, doxorubicin, camptothecin, and gemcitabine) used as first- and second-line chemotherapy to treat ovarian cancer. We also explored the mechanisms of the synergistic interaction between rapamycin and these agents.
Materials and Methods

Cell lines and culture conditions. The six human ovarian serous adenocarcinoma cell lines (KF, KOC-2S, SHIN-3, SK-OV-3, TU-OS-3, TU-OS-4) used in this study were obtained as follows: KF, from Dr Yoshihiro Kikuchi (National Defense Medical College, Tokorozawa, Japan); KOC-2S, from Dr Toru Sugiyama (Kurume University, Kurume, Japan); and SHIN-3, from Dr Yasuhiko Kiyozuka (Nara Medical University, Kashihara, Japan). SK-OV-3 came from the American Type Culture Collection (Manassas, VA, USA). TU-OS-3 and TU-OS-4 were established by our department. These cell lines were maintained in D-MEM/Ham’s F-12 medium (Wako, Osaka, Japan) with 10% fetal bovine serum, 100 IU/mL penicillin and 50 μg/mL streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C.

Dose–response studies. The sensitivity of the cell lines to the anticancer agents was determined by a cytotoxicity assay using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), according to the specifications of the manufacturer. Briefly, cells were diluted with culture medium to a seeding density of 1 to 5 × 10⁴ cells/mL, plated on 96-well tissue culture plates at 180 μL/well (Sumitomo Bakelite, Tokyo, Japan), and incubated at 37°C overnight. The next day the cells were treated continuously with 20 μL of various concentrations of the anticancer agents to obtain a dose–response curve for each agent. Concentration for each drug were: 0.05 to 50 μM rapamycin (BIOMOL Research Laboratories Inc., PA, USA); 0.025 to 64 μM etoposide (BioVision, Inc., CA, USA); 0.015 to 3 μg/mL doxorubicin (Sigma, St. Louis, MO, USA); 7-ethyl-10-hydroxycamptothecin (SN-38) (Yakult Honsha Co., Tokyo, Japan), which is an active metabolite of CPT-11; 0.02 to 8 μM gemcitabine (LKT Laboratories, Inc., MN, USA); 1 to 30 μM cisplatin (Sigma); and 1 to 500 nM paclitaxel (Sigma). After being incubated for 72 h, 20 μL of Cell Counting Kit-8 solution was added to each well, and the plates were incubated for
another 1 to 2 h. Absorbance was measured at 450 nm with a microplate reader (iMark Microplate Absorbance Reader; Bio-Rad, Richmond, CA, USA). Inhibition of cell growth was calculated as the percentage of viable cells compared with the percentage in untreated cultures.

**Dose–effect analysis.** Rapamycin was combined with each of the different anticancer agents at a fixed ratio that spanned the individual IC$_{50}$ of each drug. The IC$_{50}$ was determined on the basis of the dose–effect curves by a cytotoxicity assay. Median-effect plot analyses and calculated combination indices (CI) were analyzed by the method of Chou and Talalay (24). CalcuSyn software (Biosoft, Ferguson, MO, USA) was used to analyze data from the cytotoxicity assays in which cells were exposed to agents alone or combined with anticancer drugs and rapamycin. CalcuSyn provides a measure of the combined agents in an additive or synergistic manner. Chou and Talalay defined CI as synergistic (CI $<$ 0.9), additive (0.9 $<$ CI $<$ 1.1) or antagonistic (CI $>$ 1.1).

**Western blot analyses.** Cells were washed three times with phosphate-buffered saline (PBS) and then lysed in lysis buffer [(50 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM ethylenediaminetetraacetic acid, 50 mM NaF, 2 mM Na$_3$VO$_4$, and protease inhibitors (complete Protease Inhibitor Cocktail Tablets; Roche Diagnostics, Mannheim, Germany)]. Protein concentrations were measured against a standardized control using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). A total of 50 μg protein was separated by electrophoresis on a 5 to 20% or 15% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). All the antibodies used came from Cell Signaling Technology (Beverly, MA, USA) except for mouse anti-actin (Sigma) and rabbit anti-Bax (Santa Cruz Biotechnology, CA, USA): rabbit anti-phospho-4EBP1 (threonine 37/46, 1:1000), rabbit anti-phospho-p70 S6 kinase (1:1000), rabbit anti-phospho-c-Jun (serine 73,
1:500), rabbit anti-Bax (1:1000), rabbit anti-Bcl-2 (1:500), rabbit anti-Bcl-xL (1:1000), rabbit anti-cleaved caspase-9 (1:500), rabbit anti-cleaved PARP (1:1000), and mouse anti-actin (1:1000). These were visualized with secondary anti-mouse or anti-rabbit IgG antibody coupled with horseradish peroxidase, using enhanced chemiluminescence according to the manufacturer’s recommendation.

**In vitro cytotoxicity assay.** The cytotoxicity of the various combinations of etoposide, rapamycin, and c-Jun N-terminal kinase (JNK) inhibitor SP600125 (BIOMOL International, PA, USA) were assessed using Cell Counting Kit-8 in KF and SK-OV-3 cells. Cells (2 × 10^3) were plated on 96-well tissue culture plates at 180 μL/well, and incubated at 37°C overnight. The next day the cells were treated continuously with 20 μL of rapamycin, etoposide, and SP600125 added in replicates of four. After incubation for 48 h, 20 μL of Cell Counting Kit-8 solution was added to each well, and the plates were incubated for another 1 to 2 h. Absorbance was measured at 450 nm with a microplate reader (iMark Microplate Absorbance Reader). Inhibition of cell growth was calculated as the percentage of viable cells compared with the percentage in untreated cultures.

**Assessment of colonogenic growth in soft agar.** The colony-forming capacity in KF and SK-OV-3 cells was assessed by the CytoSelect™ 96-Well In Vitro Tumor Sensitivity Assay (Soft Agar Colony Formation) Kit (Cell Biolabs, Inc, San Diego, CA, USA), according to the specifications of the manufacturer. Briefly, 50 μL of Base Agar Matrix Layer was dispensed into each well of a 96-well tissue culture plates. Cells (1 × 10^3) in 75 μL of Cell Suspension/Agar Matrix Layer were dispensed into each well, already containing the Base Agar Matrix Layer. The cells were treated with 50 μL of culture medium containing rapamycin and etoposide. After incubation for 7 days, 125 μL of the 1 × Matrix Solubilization Buffer was added to solubilize the
agar matrix completely, and then 100 µL of the mixture was transferred to a 96-well tissue culture plate. Inhibition of colony-forming capacity was assessed by Cell Counting Kit-8 (Dojindo Laboratories).

**Annexin V staining.** The annexin V–FITC Apoptosis Detection Kit (BioVision) was used to assess apoptosis as the externalization of phosphatidylserine residues, according to the specifications of the manufacturer. Briefly, cells were suspended in 500 µl of 1× binding buffer. The cells then were stained with 5 µl annexin V–FITC and 5 µl propidium iodide (PI, 50 µg/ml) for 5 min in the dark at room temperature. Finally, the cells were analyzed with a flow cytometer (Olympus, Tokyo, Japan).

**Ovarian cancer xenograft model.** The present study was carried out at the Laboratory Animal Research Center under the control of the animal research committee, in accordance with the Guidelines for Animal Experimentation in the Faculty of Medicine, Tottori University, Yonago, Japan. For these experiments, KF or SKOV-3 cells in log-phase growth were trypsinized, washed twice with PBS, and centrifuged at 250 g. Viable cells were counted, then 5 × 10^6 viable cells (in 0.5 mL PBS) were injected under aseptic conditions into the peritoneal cavities of female nude mice. The mice were assigned randomly to one of four groups (10 mice per group) and treatment was started 4 days later as follows. Group 1, intraperitoneal (i.p.) PBS weekly; group 2, i.p. rapamycin weekly (15 mg/kg per injection); group 3, i.p. etoposide weekly (20 mg/kg per injection) for 4 weeks; and group 4, i.p. etoposide with rapamycin weekly for 4 weeks. Tumors were collected and weighed (4 mice per group) on day 40.

**Statistical Analyses.** Statistical analyses were performed with the JMP, version 7, program (SAS Institute Inc., Cary, NC). Data are presented as means ± 1 standard deviation. Means for all data were compared by one-way analysis of variance with post hoc testing. Survival distributions
were calculated using the Kaplan–Meier method, and the significance of apparent differences in survival distribution between groups was tested with log-rank tests. A P value < 0.05 was considered statistically significant.

**Results**

**Sensitivity to anticancer agents.** We sought to determine the effect of cell proliferation for five first- and second-line chemotherapeutic drugs commonly used to treat ovarian cancer. Growth inhibition was measured three days after exposure to the anticancer agents using Cell Counting Kit-8. The IC$_{50}$ values of six ovarian cancer cell lines to the anticancer agents are shown in Table I. IC$_{50}$ showed varied sensitivities to these agents: 0.14 to 50 μM for etoposide, 17 to 3800 ng/mL for doxorubicin, 15 to 310 nM for SN-38, 0.12 to 28 μM for gemcitabine, 3.3 to 25 μM for cisplatin, and 22 to 570 nM for paclitaxel.

**Combination effects of rapamycin and anticancer agents.** We next analyzed the synergistic activity of combining rapamycin with each anticancer agent by calculating CI values using the method of Chou and Talalay (24). Data representative of rapamycin combined with etoposide in KF cells is shown in figure 1A. The CI value at an effective dose of 50 (effective dose means the percentage inhibition of cell growth using the drug combinations in the actual experiment) was less than 0.9 (synergism) for five cell lines for etoposide, four cell lines for doxorubicin, and two cell lines for SN-38 (Fig. 1B). However, the CI value was more than 1.1 (antagonism) for three cell lines for gemcitabine, five cell lines for cisplatin, and four cell lines for paclitaxel. Rapamycin combined with etoposide had a synergistic effect in the greatest number of cell lines.

We then assessed the colony-forming capacity by a soft agar assay to determine whether the
clonogenic growth of ovarian cancer cells also was reduced by rapamycin combined with etoposide. At 7 days after exposure the colony-forming capacity was reduced significantly by this combination in KF and SK-OV-3 cells (Fig. 2).

**Rapamycin combined with etoposide up-regurates c-Jun and the apoptotic pathway.** Because the CI value was less than 1 for all six cell lines when etoposide was combined with rapamycin we examined whether the synergism arose from an increase in apoptosis induced by etoposide. The level of PTEN, phosphorylated (p) Akt, pmTOR, p4E-BP1, and pS6K1 proteins expressed were confirmed in all cell lines (data not shown). Bax and pc-Jun proteins increased after treatment with etoposide in KF and SK-OV-3 cells (Fig. 3A, B). Interestingly, 24 h after being treated with etoposide and rapamycin the protein expression of pc-Jun increased dramatically and Bcl-xL was down-regulated, although Bcl-2 was not affected after exposure. The protein expression of cleaved caspase 9 and cleaved PARP increased when etoposide was combined with rapamycin. Furthermore, activation of the apoptotic pathway induced by combination treatment of rapamycin and etoposide was lower when the c-Jun pathway was suppressed by a JNK inhibitor (SP600125). Similar results were obtained in the other four cell lines (data not shown).

Finally, to determine whether the synergistic effect of combining rapamycin with etoposide was caused by up-regulation of the c-Jun pathway, we examined cell viability after treatments with etoposide and with or without both rapamycin and SP600125 or each individually. At two days after exposure inhibition of cell growth and apoptosis were measured using the in vitro cytotoxicity assay and annexin V staining, respectively. Etoposide combined with rapamycin dramatically suppressed cell growth ($P < 0.05$, Fig. 4A) and increased the number of apoptotic cells in KF and SK-OV-3 cell cultures ($P < 0.05$, Fig. 4B, 4C). However, cells in which the c-Jun pathway was suppressed by SP600125 showed less inhibition of cell growth and apoptosis. The effect of combination treatment was not as pronounced as when both rapamycin and etoposide were used individually (Fig. 4A, 4B, 4C).
pathway was suppressed by SP600125 showed a lower synergistic effect. Similar results were obtained in the other four cell lines (data not shown). These findings suggest that apoptosis induced with etoposide in ovarian cancer cells may be enhanced by up-regulation of the c-Jun pathway by adding rapamycin.

**Etoposide combined with rapamycin prolongs the survival of mice injected with ovarian cancer cells.** After confirming that rapamycin enhanced cytotoxicity induced by etoposide, we examined the effect of combination treatment of etoposide and rapamycin on survival in xenograft models with ovarian cancer. Female nude mice were given intra-peritoneal injections of KF or SK-OV-3 cells and then treated with PBS or etoposide and/or rapamycin. Western blot analysis of tumor tissues from the xenografts verified that combining with etoposide and rapamycin up-regulated the c-Jun signaling pathways in the tumors. As expected, pc-Jun, cleaved caspase 9, and cleaved PARP proteins were effectively up-regulated, and the Bcl-xL protein down-regulated in tumors from mice treated with both etoposide and rapamycin (Fig. 5A). There were no signs of overt toxicity (weight loss or gross clinical signs) in any group (Fig. 5B). In nude mice bearing KF the mean tumor weight of tumors disseminated peritoneally in the group treated with rapamycin combined with etoposide (0.12 ± 0.04 g) was significantly lower than that of the group treated with PBS (1.42 ± 0.45 g), rapamycin alone (0.68 ± 0.08 g), or etoposide alone (0.62 ± 0.15 g) (P < 0.05). Similarly, in nude mice bearing SK-OV-3 the mean tumor weight of tumors disseminated in the peritoneum in the group treated with rapamycin combined with etoposide (0.24 ± 0.11 g) was significantly lower than that of the group treated with PBS (1.96 ± 0.59 g), rapamycin alone (1.10 ± 0.37 g), or etoposide alone (0.98 ± 0.18 g) (P < 0.05). In nude mice bearing KF the median survival times were 61 days for rapamycin treatment, 64 days for etoposide, 98 days for rapamycin with etoposide, and 50 days for PBS.
Mice treated with etoposide combined with rapamycin survived significantly longer than those treated with PBS, rapamycin, or etoposide alone ($P < 0.001$, Fig. 5D). Similarly, in nude mice bearing SK-OV-3 the median survival times were 95 days for rapamycin treatment, 98 days for etoposide, 174 days for rapamycin with etoposide, and 77 days for PBS. Mice treated with etoposide combined with rapamycin survived significantly longer than those treated with PBS, rapamycin, or etoposide alone ($P < 0.001$, Fig. 5E). These findings indicate that combining etoposide and rapamycin prolonged survival in nude mice bearing KF or SK-OV-3 cells.
Discussion

Several molecular targeted agents have been developed and have already entered clinical practice. These agents are attractive treatment options either alone or in combination with traditional cytotoxic drugs (19, 25). The present study aimed to determine the best therapy to combine the mTOR inhibitor rapamycin with six cytotoxic agents (etoposide, doxorubicin, camptothecin, gemcitabine, cisplatin, paclitaxel) used commonly to treat ovarian cancer. We found rapamycin and etoposide had the strongest cytotoxic effect. The effectiveness of the combination was confirmed in ovarian cancer xenograft models. Combining rapamycin and etoposide prolonged the survival of these mice compared with those treated by rapamycin or etoposide alone. These data provide clear evidence that this combination may be effective for ovarian cancer. To our knowledge, this was the first study to show that rapamycin combined with etoposide was effective against ovarian cancer, both in vitro and in vivo, and explored the mechanisms of synergistic interaction between these drugs.

The Akt/mTOR signaling pathway plays a central role in cell growth, proliferation, and apoptosis. The activity of this pathway frequently is elevated in several cancers, including ovarian cancer (10-12). Loss of phosphatase and tensin homolog deleted from chromosome 10 (PTEN) and activation of Akt have been associated closely with the up-regulation of mTOR signaling and result in hypersensitivity to mTOR inhibitors (26, 27). Recently, Mondesire, et al., (20) reported that rapamycin synergistically enhanced apoptosis induced by paclitaxel and carboplatin only in cells sensitive to rapamycin. Mabuchi, et al., (28) also reported that the rapamycin analog RAD001 enhanced cisplatin-induced apoptosis in ovarian cancer cells (SK-OV-3 and OVCAR10) with high Akt/mTOR activity, whereas a minimal effect was seen in cells with low Akt/mTOR activity. Similarly, we observed the synergistic effect of rapamycin and
cisplatin on cell growth inhibition in only one cell line (KF) with high AKT/mTOR activity. Furthermore, no synergistic effects were seen when rapamycin was combined with paclitaxel in all six cell lines tested. We found previously that simultaneous inhibition of the mitogen-activated protein kinase kinase and Akt pathways was necessary to enhance sensitivity to paclitaxel in ovarian cancer cells (29). These results suggested that the combination effects of rapamycin with cisplatin or paclitaxel, both of which are used for first-line chemotherapy, might be limited for treatment of patients with ovarian cancer.

Patients who relapse within 6 months after first-line chemotherapy ends have a form of the disease that is likely to resist platinum agents and taxane compounds. Therefore, a topoisomerase I inhibitor (topotecan), a topoisomerase II inhibitor (etoposide, pegylated liposomal doxorubicin), and a pyrimidin analogue (gemcitabine) are used commonly for second-line chemotherapy in these patients (6-9). Among these agents we found additive or synergistic effects of topoisomerase I or II inhibitors combined with rapamycin in all six cell lines tested. The combination of rapamycin and etoposide led to especially synergistic effects on five out of six cell lines regardless of the levels at which the pAkt and pmTOR proteins were expressed. This set of results suggested that rapamycin enhances the cytotoxicity induced by etoposide independently of AKT/mTOR activity and that this combination may be an effective treatment for ovarian cancer.

Several studies have reported on the synergic interaction between rapalogues and etoposide in hematologic tumors, but the mechanisms of this interaction have not been shown (30, 31). Etoposide has been shown to activate JNK, and this is thought to correlate with induced cell death (32, 33). Indeed, apoptosis induced by etoposide was lower in combination with the JNK inhibitor, SP600125, though this effect was not statistically significant. Rapamycin also has been
shown to induce rapid and sustained activation of apoptosis signal-regulating kinase 1, JNK; and to elevate pc-Jun, resulting in apoptosis (34). Therefore, the combination of rapamycin with etoposide may induce further activation of the c-Jun signaling pathway. The mechanism by which JNK promotes apoptosis is thought to involve potentiation of release of mitochondrial cytochrome c, which cleaves caspase 9 or inactivates anti-apoptotic proteins, such as Bcl-2 and Bcl-xL (35-37). These evidences support our findings from western blot analysis that up-regulation of pc-Jun, cleaved caspase 9 and down-regulation of Bcl-xL were observed after rapamycin was combined with etoposide to treat ovarian cancer cells. Further, inhibition of JNK by SP600125 attenuated apoptosis induced by this drug combination. Our findings suggested that the JNK pathway has a key role in the synergistic induction of apoptosis by the treatment of ovarian cancer cells with rapamycin and etoposide together.

Sustained activation of the JNK cascade and apoptosis by rapamycin are suppressed by wild-type p53, where the cells are arrested in the G1 phase (34). However, the effect of rapamycin on cytotoxicity induced as chemotherapy was observed in both p53 mutant and p53 wild-type breast cancer cells (20). We also found a synergistic interaction in both p53 wild-type (KF, SHIN-3) and p53 mutant or deleted ovarian cancer cells (KOC-2S, SK-OV-3). Furthermore, we confirmed that the c-Jun apoptosis pathway was activated with rapamycin and etoposide in ovarian cancer xenograft models (KF, SK-OV-3). The combination prolonged survival of these mice compared with those treated with rapamycin or etoposide alone. Thus, rapamycin may potentiate the cytotoxic effect of etoposide on ovarian cancer cells having both wild-type p53 and mutant p53.

Etoposide is used mainly for patients with recurrent ovarian cancer. The doses of etoposide are 100 mg/m²/day by intra-venous injection for 5 days, every 28 days or 50 mg/m²/day orally.
for 21 days, every 28 days. The maximum serum etoposide concentration (Cmax) after intra-venous injection and oral administration reaches 25.8 μM and 5.8 μM (at Day 1) to 7.8 μM (at Day 21) every treatment day, respectively (38-40). The IC\textsubscript{50} value of KF cell line for etoposide was 30 μM, but a significant synergistic effect was seen in combination with rapamycin and 4 or 8 μM etoposide. Furthermore, it is reported that the Cmax of the mice after 10 mg/kg intra-venous treatment is 54.5 μM, though the Cmax after intra-peritoneal injection is not available (41). Thus, the dose of etoposide in our experiment is roughly equivalent to the standard clinical dose used in patients.

In summary, our study showed that the mTOR inhibitor rapamycin enhanced the cytotoxicity of some chemotherapeutic agents, especially etoposide, in ovarian cancer cells. We also found that the synergistic interaction of rapamycin and etoposide may be related to up-regulation of the pc-Jun protein that results in induction of apoptosis. Furthermore, this combined treatment prolonged the survival of nude mice injected with ovarian cancer cells. Therefore, we concluded that combining rapamycin with etoposide is worth exploring as a treatment for ovarian cancer. We hope that this combination therapy will improve the survival of patients with advanced ovarian cancer.
References


Figure Legends

**Figure 1** The effects of rapamycin are synergistic or additive with those of etoposide (VP-16) and doxorubicine (DXR). Cells were incubated with increasing concentrations of rapamycin and VP-16, DXR, SN-38, gemcitabine (GEM), cisplatin (CDDP), or paclitaxel (PTX) at a fixed ratio for 72 h. Inhibition of growth was analyzed using the Cell Counting Kit-8. A) representative data from rapamycin combined with etoposide in KF cells. Results are mean ± standard deviation of three independent experiments. B) data analyzed using CalcuSyn software to determine the combination index (CI). Chou and Talalay defined CI < 0.9, 0.9 < CI < 1.1, and CI > 1.1 as synergism, additivity, and antagonism of the two agents, respectively.

**Figure 2** Rapamycin combined with etoposide enhanced the inhibition of clonogenic growth in ovarian cancer cells. KF (A) and SK-OV-3 cells (B) were treated with etoposide (VP-16) at the indicated concentrations and with phosphate-buffered saline (VP-16), or 8 or 1.5 μM rapamycin (VP-16 + Rap), respectively. Colony forming capacity was assessed by the CytoSelect™ 96-Well In Vitro Tumor Sensitivity Assay (Soft Agar Colony Formation) Kit. At 7 days after treatment with VP-16 and Rap, the colony-formation capacity was significantly reduced in both cell lines. Points represent mean ± standard deviation from four duplicate experiments.

**Figure 3** Rapamycin enhances the c-Jun signaling and apoptotic pathways induced by etoposide in ovarian cancer cells. KF (A) and SK-OV-3 cells (B) were treated at the indicated times with 30 or 1.9 μM etoposide (VP-16) and with phosphate-buffered saline (Control), 8 or 1.5 μM rapamycin (Rap), and/or 7 μM c-Jun N-terminal kinase inhibitor (SP600125), respectively. The
cells were then collected and the protein expression of phosphorylated (p) p70 S6 kinase (S6K1), p4E-binding protein 1 (p4E-BP1), pc-Jun, Bax, Bcl-2, Bcl-xL, cleaved caspase 9, and cleaved PARP were tested by western blotting. After treatment with VP-16 combined with Rap the expression of pc-Jun was up-regulated and cleaved caspase 9 and cleaved PARP were seen. SP60125 down-regulated the expression of pc-Jun, cleaved caspase 9, and cleaved PARP induced by VP-16 and Rap. The results shown represent duplicate experiments.

**Figure 4** Rapamycin enhanced cytotoxicity induced by etoposide by up-regulating the c-Jun signaling pathway in ovarian cancer cells. Each of the cell lines was treated with 30 or 1.9 μM etoposide (VP-16) and with phosphate-buffered saline or 8 or 1.5 μM rapamycin (Rap), and/or 7 μM c-Jun N-terminal kinase inhibitor (SP600125) for 48 h. The cytotoxicity of the various combinations of etoposide, rapamycin, and c-Jun N-terminal kinase (JNK) inhibitor SP600125 was assessed by Cell Counting Kit-8. Apoptosis was determined by the Annexin V-FITC Apoptosis Detection Kit. Early apoptotic cells were scored as annexin-V-FITC positive and propidium iodide (PI) negative to exclude necrotic cells. A) Cell proliferation was significantly suppressed by VP-16 combined with rapamycin in KF and SK-OV-3 cells compared with other treatment conditions. B) Fluorescence-activated cell sorting data from a representative sample. C) SP60125 reduced apoptosis induced by VP-16 and rapamycin in KF and SK-OV-3 cells. Points represent mean ± standard deviation from four duplicate experiments. NS: not significant.

**Figure 5** Combination treatments with etoposide (VP-16) and rapamycin (Rap) prolonged survival in mice with implanted KF or SK- OV-3 cells. A) the levels of pc-Jun, Bcl-xL, cleaved caspase 9, and cleaved PARP proteins were determined by western blotting 24 h after
intra-peritoneal treatment with phosphate-buffered saline (Control), Rap, VP-16, or a combination of Rap and VP-16. pc-Jun, cleaved caspase 9, and cleaved PARP. Proteins were up-regulated, and Bcl-xL proteins down-regulated, in tumors from mice treated with Rap and VP-16. The results shown represent duplicate experiments. B-E) female nude mice (six per group) were given an intra-peritoneal (i.p.) injection of $5 \times 10^6$ KF cells or SK-OV-3 cells followed by weekly i.p. injections of 15 mg/kg Rap and/or 20 mg/kg VP-16 for 4 weeks. B) Mean body weight of each treatment group. Error bars represent standard error. C) Tumors were collected and weighed on day 40. In mice inoculated with KF and SK-OV-3 cells the weight of the peritoneally disseminated tumors was significantly lower in the mice treated with Rap combined with VP-16 than with the other treatments ($P < 0.05$). D, E) Treatment with VP-16 and Rap prolonged survival of mice inoculated with KF or SK-OV-3 cells relative to treatment with PBS, Rap, or VP-16 ($P < 0.001$). NS: not significant.
Fig. 1A

Growth Inhibition (%)

Concentration (μM)

Rap, VP-16, and VP-16 + Rap concentrations are shown.

Combination Index vs. Fractional Effect

The combination index (CI) is calculated to determine the synergistic or antagonistic interaction between Rap and VP-16.

CI = (Fractional Effect of Rap + Fractional Effect of VP-16) / (Fractional Effect of Rap + VP-16)

A CI < 1 indicates synergy, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

CI vs. Fractional Effect

The graph shows the relationship between CI and fractional effect, illustrating the synergy and interaction between the drugs at different concentrations.
Fig. 1B

Combination Index

- KF
- KOC-2s
- SHIN-3
- SK-OV-3
- TU-OS-3
- TU-OS-4

Chemotherapeutic agents:
- VP-16
- DXR
- SN-38
- GEM
- CDDP
- PTX
**Fig. 2**

**KF**

![Graph showing colony formation in KF cells](image)

**SK-OV-3**

![Graph showing colony formation in SK-OV-3 cells](image)

* $P < 0.01$ vs. VP-16

** $P < 0.001$ vs. VP-16
Fig. 3A

<table>
<thead>
<tr>
<th>KF</th>
<th>6 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Rap</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>VP-16</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>SP600125</td>
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</tr>
<tr>
<td>pc-Jun</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bax</td>
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<tr>
<td>Cleaved PARP</td>
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Hours after treatment
Fig. 3B

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<td>+ - + + +</td>
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<tr>
<td></td>
<td>- - + + +</td>
<td>- + + + +</td>
</tr>
<tr>
<td></td>
<td>- - - - -</td>
<td>- - - - +</td>
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<tr>
<td>Rap VP-16 SP600125</td>
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<tr>
<td>Bax</td>
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<td>Bcl-2</td>
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<td>actin</td>
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Hours after treatment
Fig. 4A

KF

SK-OV-3

Cell Viability (%)

0 25 50 75 100

Cell Viability (%)

Rap - - + + - - + +
VP-16 - - - - + + + +
SP600125 - + - + - + - +

Rap - - + + - - + +
VP-16 - - - - + + + +
SP600125 - + - + - + - +

* P < 0.05 vs. other treatment conditions

NS

* P < 0.05 vs. other treatment conditions
Fig. 4B

Annexin V

PI

SP600125
Rap
Rap + SP600125

VP-16
Fig. 4C

**Annexin V-positive Cells (%)**

- **KF SK-OV-3**
  - VP-16
  - SP600125
  - Rap

- **Annexin V-negative Cells (%)**
  - **PI-negative cells**
  - **PI-positive cells**

- *P < 0.05 vs. other treatment conditions

- *P < 0.05 vs. untreated controls

- **Research.**
  - **Author Manuscript** Published OnlineFirst on May 24, 2011; DOI: 10.1158/1078-0432.CCR-11-0190
Fig. 5A

<table>
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<th>Rap</th>
<th>VP-16</th>
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</tbody>
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KF SK-OV-3
Fig. 5B

KF

SK-OV-3

Days after Inoculation

Mean Body Weight (g)

Days after Inoculation

Mean Body Weight (g)

- control
- Rap
- VP-16
- VP-16 + Rap

Treatment

0 4 11 18 25 32

15 20 25 30

15 20 25 30
Fig. 5C

KF

SK-OV-3

Tumor Burden (g)

control
Rap
VP-16
VP-16 + Rap

*, **, $P < 0.05$ vs. other treatment conditions
**Fig. 5D**

KF

**Treatment**

- Control
- Rap
- VP-16
- VP-16 + Rap

* $P < 0.001$ vs. other treatment conditions
Fig. 5E

SK-OV-3

Treatment

- Control
- Rap
- VP-16
- VP-16 + Rap

Survival Rate (%)

0 30 60 90 120
Survival Time (Days)

* P < 0.001 vs. other treatment conditions

* P < 0.001 vs. other treatment conditions
<table>
<thead>
<tr>
<th>Cell lines</th>
<th>VP-16 (μM)</th>
<th>DXR (ng/mL)</th>
<th>SN-38 (nM)</th>
<th>GEM (μM)</th>
<th>CDDP (μM)</th>
<th>PTX (nM)</th>
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<td>SHIN-3</td>
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</table>

VP-16, etoposide; DXR, doxorubicin; SN-38, 7-ethyl-10-hydroxycamptothecin; GEM, gemcitabine; CDDP, cisplatin; PTX, paclitaxel.
Inhibiting the mTOR pathway synergistically enhances cytotoxicity in ovarian cancer cells induced by etoposide through up-regulation of c-Jun

Hiroaki Itamochi, Tetsuro Oishi, Muneaki Shimada, et al.

Clin Cancer Res  Published OnlineFirst May 24, 2011.