Cancer Therapy: Preclinical

Inhibiting the mTOR Pathway Synergistically Enhances Cytotoxicity in Ovarian Cancer Cells Induced by Etoposide through Upregulation of c-Jun

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Abstract

**Purpose:** The 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. This 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, TUI-OS-3, and TUI-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 upregulation of phosphorylated c-Jun and downregulation 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, and 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 mTOR seems 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 upregulation of mTOR signaling (10–12). mTOR was differentiated to 2 complexes: mTOR complex 1 (mTORC1) and mTORC2. mTORC1 regulates protein synthesis by directly phosphorylating...
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Translational Relevance

The 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 upregulation of phosphorylated c-Jun and downregulation of Bcl-xL, suggesting that the c-Jun N-terminal kinase 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.

Materials and Methods

Cell lines and culture conditions

The 6 human ovarian serous adenocarcinoma cell lines (KF, KOC-2S, SHIN-3, SK-OV-3, TU-OS-3, and 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. TU-OS-3 and TU-OS-4 were established by our department. These cell lines were maintained in DMEM/Ham’s F-12 medium (Wako) with 10% FBS, 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), according to the specifications of the manufacturer. Briefly, cells were diluted with culture medium to a seeding density of 1 × 10⁴ to 5 × 10⁴ cells/mL, plated on 96-well tissue culture plates at 180 μL well (Sumitomo Bakelite), 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 μmol/L rapamycin (BIOMOL Research Laboratories Inc.), 0.025 to 64 μmol/L etoposide (BioVision Inc.), 0.015 to 3 μg/mL doxorubicin (Sigma), 7-ethyl-10-hydroxycamptothecin (SN38; Yakult Honsha Co.), which is an active metabolite of CPT-11, 0.02 to 8 μmol/L gemcitabine (LKT Laboratories Inc.), 1 to 30 μmol/L cisplatin (Sigma), and 1 to 500 nmol/L paclitaxel (Sigma). After being incubated for 72 hours, 20 μL of Cell Counting Kit-8 solution was added to each well, and the plates were incubated for another 1 to 2 hours. Absorbance was measured at 450 nm with a microplate reader (iMark Microplate Absorbance Reader; Bio-Rad). 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 IC₅₀ of each drug. The IC₅₀ 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) 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 3 times with PBS and then lysed in lysis buffer [(50 mmol/L Tris-HCl, 150 mmol/L NaCl, 10% glycerol, 1% Nonidet P-40, 2 mmol/L EDTA, 50 mmol/L NaF, 2 mmol/L Na3VO4, and protease inhibitors (complete Protease Inhibitor Cocktail Tablets; Roche Diagnostics)]. Protein concentrations were measured against a standardized control by using a protein assay kit (Bio-Rad Laboratories). 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). All the antibodies used came from Cell Signaling Technology, except for mouse anti-actin (Sigma) and rabbit anti-Bax (Santa Cruz Biotechnology): rabbit anti–phospho-4EBP1 (threonine 37/46, 1:1,000), rabbit anti–phospho-p70 S6 kinase (1:1,000), rabbit anti–phospho-c-Jun (serine 73, 1:500), rabbit anti–Bcl-2 (1:1,000), rabbit anti–Bcl-xl (1:1,000), rabbit anti–cleaved caspase-9 (1:500), rabbit anti–cleaved PARP (1:1,000), and mouse anti–actin (1:1,000). These were visualized with secondary anti-mouse or anti-rabbit immunoglobulin G 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) were assessed by using Cell Counting Kit-8 in KF and SK-OV-3 cells. Cells (2 × 10^5) 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 4. After incubation for 48 hours, 20 μL of Cell Counting Kit-8 solution was added to each well, and the plates were incubated for another 1 to 2 hours. 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.), 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 plate. Cells (1 × 10^5) 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 (fluorescein isothiocyanate) and 5 μL propidium iodide (PI, 50 μg/mL) for 5 minutes in the dark at room temperature. Finally, the cells were analyzed with a flow cytometer (EPICS Altra HyperSort, Beckman Coulter Inc.).

Ovarian cancer xenograft model
This 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 SK-OV-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 4 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 done with the JMP, version 7, program (SAS Institute Inc.). Data are presented as means ± 1 SD. Means for all data were compared by 1-way ANOVA with post hoc testing. Survival distributions were calculated by using the Kaplan–Meier method, and the significance of apparent differences in survival distribution between groups was tested with log-rank tests. A P < 0.05 was considered statistically significant.

Results
Sensitivity to anticancer agents
We sought to determine the effect of cell proliferation for 6 first- and second-line chemotherapeutic drugs commonly used to treat ovarian cancer. Growth inhibition was measured 3 days after exposure to the anticancer agents by using Cell Counting Kit-8. The IC_{50} values of 6 ovarian cancer cell lines to the anticancer agents are shown in Table 1. IC_{50} showed varied sensitivities to these agents: 0.14 to 50 μmol/L for etoposide, 17 to 3,800 ng/mL for doxorubicin, 15 to 310 mmol/L for SN-38, 0.12 to...
28 μmol/L for gemcitabine, 3.3 to 25 μmol/L for cisplatin, and 22 to 570 nmol/L 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 by using the method of Chou and Talalay (24). Data representative of rapamycin combined with etoposide in KF cells are shown in Figure 1A. The CI value at an effective dose of 50 (effective dose means the percentage inhibition of cell growth by using the drug combinations in the actual experiment) was less than 0.9 (synergism) for 5 cell lines for etoposide, 4 cell lines for doxorubicin, and 2 cell lines for SN-38 (Fig. 1B). However, the CI value was more than 1.1 (antagonism) for 3 cell lines for gemcitabine, 5 cell lines for cisplatin, and 4 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 upregulates c-Jun and the apoptotic pathway**

Because the CI value was less than 1 for all 6 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 and B). Interestingly, 24 hours after being treated with etoposide and rapamycin, the protein expression of pc-Jun increased dramatically and Bcl-xl was downregulated, although Bcl-2 was not affected after exposure. The protein expression of cleaved caspase 9 and cleaved PARP increased when

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**Table 1. IC50 values to anticancer agents**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC50 VP-16 (μmol/L)</th>
<th>IC50 DXR (ng/mL)</th>
<th>IC50 SN-38 (nmol/L)</th>
<th>IC50 GEM (μmol/L)</th>
<th>IC50 CDDP (μmol/L)</th>
<th>IC50 PTX (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KF</td>
<td>30</td>
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<td>20</td>
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<td>3.3</td>
<td>22</td>
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<tr>
<td>KOC-2S</td>
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<td>17</td>
<td>39</td>
<td>0.12</td>
<td>2.4</td>
<td>250</td>
</tr>
<tr>
<td>SHIN-3</td>
<td>20</td>
<td>1,200</td>
<td>32</td>
<td>28</td>
<td>15</td>
<td>22</td>
</tr>
<tr>
<td>SK-OV-3</td>
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<td>77</td>
<td>47</td>
<td>0.23</td>
<td>9.8</td>
<td>110</td>
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<tr>
<td>TU-OS-4</td>
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<td>3,800</td>
<td>310</td>
<td>12</td>
<td>25</td>
<td>570</td>
</tr>
</tbody>
</table>

Abbreviation: VP-16, etoposide; DXR, doxorubicin; SN-38, 7-ethyl-10-hydroxycamptothecin; GEM, gemcitabine; CDDP, cisplatin; PTX, paclitaxel.

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Figure 1. The effects of rapamycin are synergistic or additive with those of etoposide and doxorubicine. Cells were incubated with increasing concentrations of rapamycin and etoposide (VP-16), doxorubicine (DXR), 7-ethyl-10-hydroxycamptothecin (SN-38), gemcitabine (GEM), cisplatin (CDDP), or paclitaxel (PTX) at a fixed ratio for 72 hours. Inhibition of growth was analyzed by using the Cell Counting Kit-8. A, representative data from rapamycin combined with etoposide in KF cells. Results are mean ± SD of 3 independent experiments. B, data analyzed by using CalcuSyn software to determine the CI. Chou and Talalay defined Ci < 0.9, 0.9 < Ci < 1.1, and Ci > 1.1 as synergism, additivity, and antagonism of the 2 agents, respectively.
that combining with etoposide and rapamycin upregulated blot analysis of tumor tissues from the xenografts verified treated with PBS or etoposide and/or rapamycin. Western xenograft models with ovarian cancer. Female nude mice induction of etoposide, we examined the effect of combina-
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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 combina-
tion treatment of etoposide and rapamycin on survival in xenograft models with ovarian cancer. Female nude mice were given i.p. 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 upregulated the c-Jun signaling pathways in the tumors. As expected, PC-jun, cleaved caspase 9, and cleaved PARP proteins were effectively upregulated, and the Bcl-xL protein downregulated 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 rapa-

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 PBS (VP-16), or 8 or 1.5 μmol/L 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-forming capacity was significantly reduced in both cell lines. Points represent mean ± SD from 4 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 μmol/L etoposide (VP-16) and with PBS (control), or 8 or 1.5 μmol/L rapamycin (Rap), and/or 7 μmol/L c-Jun N-terminal kinase inhibitor (SP600125), respectively. The cells were then collected and the protein expression of phosphorylated (p) S6K1, 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 effectively upregulated, and the Bcl-xL protein downregulated 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 rapa-

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 4 cell lines (data not shown).

Finally, to determine whether the synergistic effect of combining rapamycin with etoposide was caused by upregulation 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 2 days after exposure, inhibition of cell growth and apoptosis were measured by using the in vitro cytotoxicity assay and annexin V staining, respectively. Etoposide combined with rapamycin dramatically suppressed cell growth (Fig. 4A) and increased the number of apoptotic cells in KF and SK-OV-3 cell cultures (Fig. 4B and C). However, cells in which the c-Jun pathway was suppressed by a JNK inhibitor (SP600125). Similar results were obtained in the other 4 cell lines (data not shown). These findings suggest that apoptosis induced with etoposide in ovarian cancer cells may be enhanced by upregulation of the c-Jun pathway by adding rapamycin.
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). This study aimed to determine the best therapy to combine the mTOR inhibitor rapamycin with 6 cytotoxic agents (etoposide, doxorubicin, camptothecin, gemcitabine, cisplatin, and 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 upregulation of mTOR signaling and result in hypersensitivity to mTOR inhibitors (26, 27). Recently, Mondesire and colleagues (20) reported that rapamycin synergistically enhanced apoptosis induced by paclitaxel and carboplatin only in cells sensitive to rapamycin. Mabuchi and colleagues (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 6 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.

Figure 5. Combination treatments with etoposide and rapamycin prolonged survival in mice with implanted KF or SK-OV-3 cells. A, the levels of phosphorylated (p)jc-Jun, Bcl-xL, cleaved caspase 9, and cleaved PARP proteins were determined by Western blotting 24 hours after i.p. treatment with PBS (control), rapamycin (Rap), etoposide (VP-16), or a combination of Rap and VP-16. pc-Jun, cleaved caspase 9, and cleaved PARP. Proteins were upregulated, and Bcl-xL proteins downregulated, in tumors from mice treated with Rap and VP-16. The results shown represent duplicate experiments. B to E, female nude mice (6 per group) were given an i.p. injection of 5 × 10⁶ 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 and 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.
Patients who relapse within 6 months after first-line chemotherapy end 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 pyrimidine 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 6 cell lines tested. The combination of rapamycin and etoposide led to especially synergistic effects on 5 of 6 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 has also 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 antiapoptotic proteins, such as Bcl-2 and Bcl-xL (35–37). These evidences support our findings from Western blot analysis that upregulation of pc-Jun, cleaved caspase 9 and downregulation of Bcl-xL were observed after rapamycin was combined with etoposide to treat ovarian cancer cells. Furthermore, 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, in which 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 vivo 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 i.v. 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 i.v. injection and oral administration reaches 25.8 and 5.8 μmol/L (at day 1) to 7.8 μmol/L (at day 21) every treatment day, respectively (38–40). The IC50 value of KF cell line for etoposide was 30 μmol/L, but a significant synergistic effect was seen in combination with rapamycin and 4 or 8 μmol/L etoposide. Furthermore, it is reported that the Cmax of the mice after 10 mg/kg i.v. treatment is 54.5 μmol/L, though the Cmax after i.p. 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 upregulation 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

mTOR Inhibition Enhanced Chemotherapy-Induced Cytotoxicity


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Inhibiting the mTOR Pathway Synergistically Enhances Cytotoxicity in Ovarian Cancer Cells Induced by Etoposide through Upregulation of c-Jun

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

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