Rapamycin-topotecan combination exhibited enhanced antitumor efficacy compared to topotecan used alone in cisplatin-resistant ovarian cancer cells

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The present study aimed to investigate the antitumor efficacy of combination of topotecan with rapamycin, a mTOR inhibitor, in cisplatin-resistant ovarian cancer cells A2780cis and COC1/DDP.

Expressions of mTOR and its target molecules p70S6K and 4E-BP1 were determined in A2780cis and COC1/DDP and the parental cells A2780 and COC1 that are sensitive to cisplatin using Western blotting. Cell proliferation was examined using MTT assay in vitro and a nude mouse model in vivo, respectively. Cell apoptosis and the relevant proteins were determined by flow cytometry and Western blotting.

We found that the levels of phosphorylated mTOR, p70S6K, and 4E-BP1 were obviously higher in A2780cis and COC1/DDP cells than that in A2780 and COC1 cells. Pretreatment with rapamycin significantly enhanced the effects of topotecan in suppressing cell proliferation and soliciting cell apoptosis in A2780cis and COC1/DDP cells. Cells that were sequentially exposed to rapamycin and topotecan had significantly higher levels of cleaved caspase-8, -3, and PARP compared to those treated with topotecan alone. Mice co-administered rapamycin and topotecan had significantly decreased terminal tumor burden without additional loss of bodyweight compared to the mice received topotecan alone.

The results suggested that rapamycin sensitized A2780cis and COC1/DDP cells to topotecan-induced apoptosis and rapamycin-topotecan combination might have a value in treatment of cisplatin-resistant ovarian cancer.

Key words: topotecan, rapamycin, mTOR, A2780cis, COC1/DDP

Ovarian cancer is the most malignant type of gynecological cancers with poor prognosis. It was estimated that there were 239,000 new cases and 152,000 deaths in the world in 2012 according to the statistics published by World Health Organization (WHO) [1]. Most patients are diagnosed at advanced stages because the disease is usually occult in onset and has no obvious symptoms at early stage. For these patients, combination of surgical reduction with cisplatin-based chemotherapy is a standard and first-line therapeutic regimen and obviously prolongs the patients’ life. However, ovarian cancer has been challenging to treat because it tends to develop resistance to platinum drugs during the primary chemotherapy. In addition, less than 15% of patients who are diagnosed with advanced ovarian cancer achieve long-term remission after first-line therapy [2]. At first relapse, approximately 25% of patients have platinum-resistant tumors and almost all patients with recurrent disease ultimately develop platinum resistance [3].

Thus, an efficient second-line treatment regimen is critical to increase the survival time of patients with platinum-resistant ovarian cancer.

Topotecan, a topoisomerase I (Topo I) inhibitor, is recently used to treat ovarian cancer. Studies demonstrated that it is also effective for recurrent or platinum-resistant ovarian cancer [4]. However, topotecan could be benefit only in a small fraction of such patients with overall response rates 20-30% and the median survival time was found to be only 59.7 weeks [5-7]. Enhancement of the efficacy of topotecan by using in combination regimen is a rational strategy. Rapamycin is a mTOR inhibitor that shows potent antitumor activity in a variety of malignances including ovarian cancers in preclinical and clinical studies [8-10]. Notably, rapamycin and its derivatives have been demonstrated to enhance the efficacy of cytotoxic chemotherapeutic drugs including topotecan, oxaliplatin, and 5-fluorouracil (5-FU) against various types of cancers such
as endometrial, gastric and colon cancer [8, 11-14]. Given that mTOR signaling pathway was shown to be hyperactivated in platinum-resistant ovarian carcinoma cells [15], we investigated the efficacy of combination of topotecan with rapamycin against cisplatin-resistant ovarian carcinoma in the present study.

Materials and methods

Agents. Rapamycin and topotecan were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rapamycin oral solution (Rapamune®) and topotecan for injection (Hycamtin®) were obtained from Wyeth Pharmaceuticals Inc. (Rouses Point, NY, USA) and GlaxoSmithKline Manufacturing S.p.A (Brentford, London, UK), respectively. For in vitro assay, rapamycin was firstly dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and further diluted in phosphate buffer solution (PBS) before use; topotecan was dissolved in PBS before use. For in vivo study, rapamycin oral solution and topotecan for injection were given by gavage and intravenous injection, respectively.

Cell line and cell culture. Human epithelial ovarian cancer cells A2780 (cisplatin-sensitive) and A2780cis (cisplatin-resistant) cells were obtained from European Collection of Cell Cultures (ECACC). Human ovarian mucinous adenocarcinoma cells COC1 (cisplatin-sensitive) and COC1/DDP (cisplatin-resistant) were purchased from China Center for Type Culture Collection (CCTCC; Wuhan, China). Cells were cultured according to the ECACC and CCTCC instructions. Briefly, cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37°C in a humid atmosphere (5% CO₂–95% air). Cells were harvested by brief incubation in 0.02% (w/v) ethylenediaminetetraacetic acid (EDTA) in PBS.

Cell proliferation assay. Cells (1 × 10⁴ per well) seeded in 96-well plates were exposed to rapamycin, topotecan, or their combination for specified time. For combination therapy, cells were treated with 10 nM rapamycin and then incubated with 100 nM topotecan. Rapamycin was removed from the cultures prior to the addition of topotecan. Cells were harvested and cell lysates (30 μg of protein per lane) were fractionated by 10% SDS-PAGE. The proteins were electro-transferred onto nitrocellulose membrane and the protein levels were detected using the primary antibodies against p53 and phosphorylated mTOR, p70S6K, caspase-3 and -8, cleaved PARP, and β-actin (Santa Cruz, Dallas, TX, USA) with appropriate dilution. The bound antibodies were visualized using an enhanced chemiluminescence reagent and quantified by densitometry using ChemiDoc XR+ image analyzer (Bio-Rad, Hercules, CA, USA). Densitometric analyses of bands were adjusted with β-actin as loading control. The percentages of increase or decrease of protein were estimated by comparison to the vehicle control (100%).

In vivo inhibition of tumor growth. The in vivo efficacy of combination of rapamycin and topotecan was assessed in an A2780cis xenograft mouse model. Balb/c athymic (nu/nu) female mice, 4–6 weeks of age, were purchased from the Animal Center of China Academy of Medical Sciences (Beijing, China). The animals were housed under pathogen-free conditions. The research protocol was in accordance with the institutional ethical guideline of the Animal Care and Use Committee at Liaocheng People’s Hospital.

Tumors were generated by harvesting A2780cis cells from mid-log phase cultures. Cells were then pelleted and resuspended in PBS to a final cell count of 5 × 10⁷/mL. A volume of 0.2 mL of the cell suspension was injected s.c. in the right flank of each mouse. After 7 days, when the tumor volume had reached approximately 0.1 cm³, the mice were divided into 4 groups (n = 6) and then subjected to treatment. Group 1: mice were injected with 0.2 mL normal saline via tail vein. Group 2: rapamycin was administered p.o. at 1.5 mg/kg. Group 3: topotecan was administered i.v. at 1.0 mg/kg. Group 4: mice received 1.5 mg/kg of rapamycin (p.o.) at 7:00 AM and 1.0 mg/kg of topotecan (i.v.) at 7:00 PM. Administration of vehicle, rapamycin, topotecan, or their combination was performed daily for 14 days. Tumors were harvested at the end of experiment and tumor growth rates were defined as a percentage of the control tumor weight.

Statistical analysis. Data were expressed as mean ± S.D. for three different determinations. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple range tests. p < 0.05 was considered as statistically significant. Statistical analysis was performed using the SPSS/Win 12.0 software (SPSS, Inc, Chicago, IL, USA).
Results

Hyperactivation of mTOR signal pathway in cisplatin-resistant ovarian cancer cells. Given that mTOR signal transduction pathway has an essential role in the regulation of cell survival and suppression of this pathway may sensitize cancer cells to cytotoxic drugs [16], we first determined the levels of molecules implicated in mTOR pathway in cisplatin-resistant ovarian cancer cells A2780cis and COC1/DDP using Western blotting. As shown in Figure 1, expression of phosphorylated mTOR (p-mTOR) is obviously increased by 33.6 and 37.5% in A2780cis and COC1/DDP, respectively, compared to their parental cell lines A2780 and COC1. p70S6K and 4E-BP1 are known to be the target molecules of mTOR. Our results demonstrated that the levels of phosphorylated p70S6K and 4E-BP1 (p-p70S6K and p-4E-BP1) were increased by 35.8 and 39.3% in A2780cis, and by 68.2 and 77.6% in COC1/DDP, compared to the parental non-resistant cells (Figure 1). These results suggested that mTOR signal transduction is hyperactive in cisplatin-resistant ovarian cancer cells A2780cis and COC1/DDP.

Pretreatment of rapamycin enhanced the anti-proliferation effect of topotecan. The efficacy of topotecan monotherapy was first tested in the cisplatin-resistant cells A2780cis and COC1/DDP. The two cell lines were cultured in medium for 24 h and then treated with increasing doses of the drug (10, 50, 100, 500, and 1,000 nM) for 48 h and cell proliferation was determined using MTT assay. These concentrations of topotecan represent a range that typically bracket therapeutic dose levels achieved in the circulation [17]. Results showed that topotecan at designed concentrations inhibited cell proliferation by 5.8, 11.6, 22.5, 33.8, and 45.3%, respectively, in A2780cis cells; by 7.1, 16.3, 25.9, 40.1, and 52.1%, respectively, in COC1/DDP cells (Figure 2).

To determine whether rapamycin could sensitize cells to topotecan-induced growth inhibition, A2780cis and COC1/DDP were pretreated with rapamycin (10 nM) for 24 h followed by treatment with increasing doses of topotecan for 48 h. The concentration of rapamycin used in the combination therapy was selected at 10 nM based on that rapamycin at this concentration significantly enhanced the anti-proliferation effect of topotecan in cisplatin-resistant cells A2780cis and COC1/DDP.
dose for 24 h exposure did not significantly affect the proliferation of A2780cis and COC1/DDP in a pilot experiment. Results showed that pretreatment of rapamycin enhanced the inhibitory effect of topotecan on the proliferation of two cell lines (Figure 2). Inhibitory rates of rapamycin-topotecan combination (topotecan at 10, 50, 100, 500, and 1,000 nM) were determined to be 15.3, 22.8, 35.6, 50.1, and 75.3%, respectively, in A2780cis cells, and 18.7, 32.5, 48.6, 60.5, and 85.3%, respectively, in COC1/DDP cells. There is a significant difference in inhibition rate at each concentration between topotecan monotherapy and combined therapy ($p < 0.05$; Figure 2).

**Pretreatment of rapamycin enhanced cell apoptosis induced by topotecan.** We next determined topotecan-induced cell apoptosis with or without rapamycin pretreatment using flow cytometry. Topotecan alone at doses of 10, 100, and 1,000 nM for 48 h exposure induced apoptotic rates of 7.5, 15.6, and 23.6%, respectively, in A2780cis cells; 8.6, 17.5, and 26.9%, respectively, in COC1/DDP cells. Rapamycin at 10 nM for 24 h exposure had no apparent effect on cell apoptosis in A2780cis and COC1/DDP cells (Figure 3). When pretreatment with 10 nM rapamycin for 24 h followed by treatment with 10, 100, and 1,000 nM topotecan for 48 h, percentages of

![Figure 3](image_url)
apoptotic cells were determined to be 13.4, 27.2, and 39.7%, respectively, in A2780cis cells, and 15.6, 30.2, and 43.4%, respectively, in COC1/DDP cells (Figure 3). These results suggested that pretreatment of rapamycin increased the activity of topotecan in soliciting apoptosis in cisplatin-resistant ovarian cancer cells.

We further examined cell apoptosis by measuring the levels of apoptosis-related proteins including cleaved caspase-8, -3, and PARP in A2780cis and COC1/DDP cells. Western blotting showed that sequential treatment of rapamycin and topotecan had more potent effect than topotecan monotherapy in regulating the expressions of apoptotic proteins. Topotecan at 100 nM for 48 h exposure, the levels of cleaved caspase-8, -3, and PARP were increased by 132.6, 95.6, and 84.2%, respectively, in A2780cis cells, and by 74.1, 95.9, and 102.3%, respectively, in COC1/DDP cells (Figure 4). When pretreatment with 10 nM rapamycin for 24 h exposure did not obviously affect the levels of cleaved caspase-8, -3, and PARP in both A2780cis and COC1/DDP cells (Figure 4). Rapamycin at 10 nM for 24 h exposure increased the levels of cleaved caspase-8, -3, and PARP by 165.8, 127.3, and 100.5%, respectively, in A2780cis cells; and by 115.6, 127.6, and 131.3%, respectively, in COC1/DDP cells. Significant differences existed between combined therapy and topotecan monotherapy \( (p < 0.05) \) (Figure 4).

**p53 expression was decreased by rapamycin and increased by topotecan.** p53 expression was determined in A2780cis and COC1/DDP cells subjected to rapamycin, topotecan, and their sequential combination treatment, respectively, using Western blotting. Results showed that rapamycin at 10 nM for 24 h exposure decreased p53 expression by 67.3% in A2780cis cells \((p < 0.01 \text{ vs. control})\) (Figure 5). On the other hand, topotecan at 100 nM for 48 h exposure increased the levels of p53 by 32.5% \((p < 0.05 \text{ vs. control})\) in A2780cis cells (Figure 5). The expression of p53 in sequential treatment group was demonstrated to be lower than that in control and topotecan alone treatment group \((p > 0.05 \text{ vs. control}; p < 0.05 \text{ vs. topotecan alone})\). Similar with A2780cis cell line, p53 expression in COC1/DDP cells was significantly reduced by rapamycin and increased by topotecan (Figure 5). In addition, p53 level in sequential treatment group was less than that in control and topotecan alone treatment group \((p > 0.05 \text{ vs. control}; p < 0.05 \text{ vs. topotecan alone})\). Since high expression of p53 could cause resistance of cancer cells to topotecan-induced apoptosis [18], downregulation of p53 by rapamycin might be the possible mechanism underlying the effect of rapamycin in augmenting topotecan-induced cell apoptosis in A2780cis and COC1/DDP.

**Suppression of tumor growth in vivo.** To assess the efficacy of the rapamycin-topotecan combination in vivo, an A2780cis human xenograft model was established and animals were randomly assigned to control group, rapamycin group, topotecan group, and drug combination group. Rapamycin dosed orally at 1.5 mg/kg slightly inhibited tumor growth by 5.6% compared to control (Figure 6A). Topotecan significantly reduced terminal tumor burden by 20.5% when dosed at 1.0 mg/kg (Figure 6A). The rapamycin-topotecan combination (1.5 and 1.0 mg/kg, respectively) significantly reduced terminal tumor
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burden by 44.1% which is obviously higher than that of the corresponding monotherapy controls (Figure 6A). These data suggested that rapamycin and topotecan dosed in combination act synergistically in reducing overall tumor burden using the A2780cis ovarian cancer tumor model.

Oral rapamycin was generally well tolerated by mice with no significant loss of body weight ($p > 0.05$ vs. vehicle) (Figure 6B). Topotecan injection resulted in a significant reduction in body weight ($p < 0.05$ vs. vehicle). A significant loss of body weight was also observed in mice receiving the rapamycin-topotecan combination (Figure 6B). However, there was no significant difference in body weight between combined treatment group and topotecan alone treatment group ($p > 0.05$).

Discussion

Topotecan is usually used to treat ovarian cancers that develop platinum-resistance. To enhance the efficacy of this drug, rapamycin, a mTOR specific inhibitor, was combined in the present study. We showed that pretreatment of rapamycin obviously enhanced the efficacy of topotecan in suppressing cell proliferation and soliciting cell apoptosis in two cisplatin-resistant ovarian cancer cell lines A2780cis and COC1/DDP in vitro. The expressions of caspase-8, -3, and PARP were increased to higher levels in cells exposure to rapamycin-topotecan sequential treatment compared to topotecan treatment alone. Importantly, our results showed that combination of rapamycin and topotecan significantly reduced tumor burden in ovarian cancer mouse xenograft model. These results suggested that rapamycin might be capable of sensitizing cisplatin-resistant ovarian cancer cells A2780cis and COC1/DDP to topotecan chemotherapy.

mTOR belongs to the phosphatidylinositide-3OH kinase (PI3K) related family of protein kinases and plays a central role in the regulation of cell growth and survival by integrating nutrient/energy signaling with that of growth factor signaling. Given tumor cells are more sensitive than normal cells to nu-
trients and energy supply, mTOR has been an important target for cancer treatment. Since the PI3K/AKT/mTOR is activated in approximately 70% of ovarian cancers [19], mTOR targeting agents including rapamycin and its closely related derivatives are currently undergoing phase I or II clinical studies either as single agent or in combination with chemotherapeutic agents in the treatment of ovarian cancers [19, 20]. In the present study, we found that the levels of p-mTOR and its down-stream molecules including p-p70S6K and p-4E-BP1 were obviously higher in cisplatin-resistant cells A2780cis and COC1/DDP than that in the parental cisplatin-sensitive cells. It is possible that hyperactive mTOR survival cascade is involved in the sensitivity of ovarian cancer cells A2780cis and COC1/DDP to cisplatin or other chemotherapeutic drugs. These results provide a clue for testing the efficacy of an mTOR inhibitor rapamycin in combination of topotecan in treatment of platinum-resistant ovarian cancer in the current study.

We found p53 expression was significantly decreased in A2780cis and COC1/DDP cells exposure to rapamycin for 24 h, which might contribute to sensitizing the cells to topotecan-induced apoptosis. Topotecan is an inhibitor of Topo I, an enzyme that can cleave the DNA and covalently bound to the nicked DNA, forming the so-called Topo I-DNA cleavage complex [21]. Under physiological condition, Topo I-DNA complexes are reversible covalent intermediates catalyzing the cleavage-religation reaction of the enzyme. In the presence of topotecan, the cleavable complexes are stabilized and relegation of Topo I mediated DNA single-strand breaks are blocked, leading to accumulation of DNA strand breaks that may interfere with DNA replication [21]. Tomicic and co-workers showed that p53 mediated the repair of Topo I-cleavable complex [18]. They showed that p53-deficient mouse embryonic fibroblasts (MEF) and p53 mutated glioblastoma cells U138 were significantly more sensitive to the apoptotic activity of topotecan than the p53-proficient and p53 wild-type counterparts. The authors suggested that tumor response to topotecan was dependent on the p53 level in cancer cells [18]. In the present study, we observed reduced p53 expression in A2780cis and COC1/DDP cells after rapamycin treatment, which may sensitize the cells to subsequent topotecan-induced apoptosis. Although exposure to topotecan increased p53 expression, the p53 level in combined treatment group was still lower than that in topotecan alone treatment group. Thus, down-regulation of p53 by rapamycin may be responsible for the enhanced antitumor effect of rapamycin-topotecan combined therapy.

Topotecan produces DNA damage that induces autophagy in cancer cells [22]. Previous studies revealed that p53 status affected cell response to topotecan via modulation of autophagy. Li et al. reported that DNA damage induced by topotecan treatment resulted in cytoprotective autophagy in colon cancer cells with wild-type p53 [23]. However, in cells with mutant p53 or p53 knockout, treatment with topotecan induced autophagy-associated cell death [23]. These results imply that p53-dependent induction of cytoprotective autophagy is a cell response that reduces cellular sensitivity to the DNA-damaging drug topotecan. We observed that rapamycin down-regulated p53 levels in A2780cis and COC1/DDP cells, which might alleviate the cytoprotective autophagy and thus enhance topotecan-induced apoptosis. The effects of rapamycin and topotecan on autophagy in cisplatin-resistant ovarian cancer cells warrants further studies.

In conclusion, suppression of mTOR by rapamycin reduced p53 expression and sensitized platinum-resistant ovarian cancer cells A2780cis and COC1/DDP to topotecan chemotherapy both in vitro and in vivo. This study provided evidence for possible usage of mTOR inhibitors such as rapamycin in combination of topotecan to treat platinum-resistant ovarian cancers. The efficacy and tolerance of this combined therapy in treating chemoresistant or recurrent ovarian cancers warrant further preclinical and clinical research in the future.

References


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