RAD001 Inhibits Human Ovarian Cancer Cell Proliferation, Enhances Cisplatin-Induced Apoptosis, and Prolongs Survival in an Ovarian Cancer Model

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Abstract

Purpose: mTOR (mammalian target of rapamycin) plays a central role in regulating cell growth and cell cycle progression and is regarded as a promising therapeutic target. We examined whether mTOR inhibition by RAD001 (everolimus) is therapeutically efficacious in the treatment of ovarian cancer as a single agent and in combination with cisplatin.

Experimental Design: Using four human ovarian cancer cell lines, we determined the effect of RAD001 by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Western blot, and apoptosis assays. We evaluated the association between phospho-AKT/mTOR activity and RAD001 sensitivity. We also determined the effect of RAD001 on tumor growth and malignancy using mice inoculated with human ovarian cancer cells.

Results: RAD001 markedly inhibited cell proliferation of human ovarian carcinoma cells with high AKT activity (OVCAR10 and SKOV-3), but the effect was minimal in cells with low AKT activity (OVCAR4 and OVCAR5). Sensitivity to RAD001 was independent of p53 expression. RAD001 inhibited the phosphorylation of downstream 4E-BP1 and p70S6 kinase and attenuated the expression of Myc. RAD001 also attenuated the expression of HIF-1α and vascular endothelial growth factor, important factors in angiogenesis and tumor invasiveness. RAD001 enhanced cisplatin-induced apoptosis in cells with high AKT/mTOR activity, with minimal effect in cells with low AKT-mTOR activity. Mouse xenografts of SKOV-3 cells revealed that RAD001 inhibits tumor growth, angiogenesis, and i.p. dissemination and ascites production and prolongs survival. Moreover, treatment with RAD001 significantly enhanced the therapeutic efficacy of cisplatin in vivo.

Conclusion: These results indicate that RAD001 could have therapeutic efficacy in human ovarian cancers with hyperactivated AKT/mTOR signaling.

Ovarian carcinoma is the fourth most common cause of cancer death among women in the United States, with more than 22,000 new cases diagnosed and ~16,000 deaths each year (1). Due to the asymptomatic nature of early stages of this disease and lack of a reliable method for early detection, only 25% of ovarian cancers are diagnosed when the malignancy is confined within the ovary and when the cure rate is almost 90%. The remaining ~75% of ovarian tumors have spread beyond the ovary at the time of diagnosis, and the cure rate for these patients is <20% (2).

Currently, the standard initial treatment for advanced ovarian cancer is combination chemotherapy, using platinum-based drugs such as cisplatin coupled with paclitaxel (3). Although this treatment regimen is initially effective in a high percentage of cases, most patients will relapse due to the development of chemoresistance (4). To improve survival, a better understanding of the mechanism of drug resistance and a treatment strategy to overcome chemoresistance are needed.

One of the key molecular components that regulate sensitivity to cisplatin is p53, which is mutated in approximately one-half of all advanced ovarian cancers (5). Mutation of the TP53 gene is associated with a lack of response to cisplatin therapy in ovarian cancer patients (6), and most ovarian tumors with mutation of TP53 are incurable (4).

Inhibition of phosphoinositide-3-kinase (PI3K)/AKT signaling may be a promising strategy to enhance the efficacy of cisplatin. AKT is known to regulate various cellular pathways that promote cell survival, cell proliferation, angiogenesis, and invasion (7). We and others have previously reported that inhibition of AKT activity sensitizes human ovarian cancer cells with mutant p53 to conventional anticancer agents, including cisplatin (8) and paclitaxel (9). However, there are concerns associated with inhibiting AKT because AKT also mediates certain biologically important cell processes such as glucose...
metabolism (10). Thus, a safer approach may be to target therapeutic effectors downstream of AKT.

Among the numerous AKT substrates, mTOR (mammalian target of rapamycin) is one of the major targets of relevance to cancer therapy (11). mTOR activity, in turn, results in phosphorylation of p70S6 kinase (p70S6K) and the 4E-BP1 translational repressor (12), leading to the translation of proteins required for cell cycle progression (13). Rapamycin, a highly specific inhibitor of mTOR, has shown antitumor efficacy in certain cancers in vitro and in vivo (14, 15).

AKT activity is frequently elevated in ovarian cancer and is closely associated with the up-regulation of mTOR signaling (16). Because it is well known that high levels of AKT activity result in hypersensitivity to mTOR inhibition (17), we considered ovarian cancer to be a good candidate for therapy with an mTOR inhibitor. Currently, only in vitro data have been reported concerning the effect of rapamycin and its derivatives in ovarian cancer (16, 18).

Recently, an orally bioavailable derivative of rapamycin, RAD001 (everolimus; Novartis Pharma AG), has been developed. RAD001 has been shown to inhibit the proliferation of tumor cell growth in vitro and in vivo (19–21), and phase II clinical trials with RAD001 are currently planned or under way.

Rapamycin and its derivatives are generally regarded as having cytostatic effects because these drugs arrest cells in G1 phase but do not generally induce apoptosis. RAD001 has been shown to sensitize A549 lung carcinoma cells to cisplatin-induced apoptosis by inhibiting p21 expression in vitro (21). In A549 cells, RAD001-mediated sensitization was attenuated when expression of p53 was silenced using small interfering RNA.

However, other investigators have reported that rapamycin can induce apoptosis in rhabdomyosarcoma cells in a p53-independent manner (22). Rapamycin was found to sensitize multiple myeloma cells to dexamethasone-induced apoptosis in a p53-independent, AKT activity-dependent manner (23). Moreover, other in vitro work showed that rapamycin enhances the effect of cisplatin and carboplatin in ovarian and breast cancer cells with mutant p53 (24, 25). Collectively, these findings suggest that mTOR inhibition, combined with standard chemotherapies, holds promise for improved treatment of human ovarian cancers, including tumors with inactivated p53.

These considerations led us to examine whether RAD001 has efficacy in in vitro and in vivo models of ovarian cancer, and whether RAD001 accentuates the response of p53-null human ovarian cancer cells to cisplatin-induced apoptosis. Our data show that RAD001 can inhibit the progression of ovarian cancers, irrespective of p53 status, and enhances the therapeutic efficacy of cisplatin in vivo. These findings suggest that RAD001 holds promise for the treatment of women with ovarian cancer.

### Materials and Methods

#### Reagents/antibodies.

RAD001 was obtained from the Novartis Pharma AG. LY294002 was purchased from Calbiochem. Enhanced chemiluminescence Western blotting detection reagents were from Perkin-Elmer. Antibodies recognizing 4E-BP1, phospho–4E-BP1 (Thr(42)]), p70S6K, phospho-p70S6K (Thr(389)), mTOR, phospho-mTOR (Ser(2448)), AKT, and phospho-AKT (Ser(473)) were obtained from Cell Signaling Technology. Other antibodies included anti–vascular endothelial growth factor (VEGF), anti–poly(ADP ribose) polymerase (PARP), and anti–c-MYC (Santa Cruz Biotechnology), anti–β-actin antibody (Sigma), anti-HIF1α and anti-p53 (BD Bioscience), and anti-CD31/platelet/endothelial cell adhesion molecule 1 (PECAM-1) (PharMingen). The Cell Titer 96-well proliferation assay kit was obtained from Promega.

#### Drug preparation.

RAD001 was formulated at 2% (w/v) in a microemulsion vehicle (Novartis Pharma AG). For animal studies, RAD001 was diluted to the appropriate concentration in double-distilled water just before administration by gavage. For in vitro analyses, RAD001 was prepared in DMSO before addition to cell cultures.

#### Cell culture.

Human ovarian cancer cell lines SKOV-3, OVCAR4, OVCAR5, and OVCAR10 were obtained from the American Type Culture Collection. SKOV-3 cells were cultured in McCoy’s 5A with 10% fetal bovine serum (FBS). OVCAR4, OVCAR5, and OVCAR10 cells were cultured in DMEM with 10% FBS. To expose cells to hypoxia, they were placed into a modular incubator chamber (Billups) that was infused with a mixture of 1% O2, 5% CO2 and 94% N2, sealed, and incubated at 37°C.

#### Cell proliferation assay.

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to analyze the effect of RAD001 on cell viability as described (26). Cells were cultured overnight in 96-well plates (1 × 104 cells per well). Cell viability was assessed after addition of RAD001 and/or cisplatin at the indicated concentrations for 48 or 72 h. The number of surviving cells was assessed by the determination of the A490 nm of the dissolved formazan product after addition of MTT for 1 h as described by the manufacturer (Promega). Cell viability is expressed as follows: A490 group/A490 control × 100.

#### Clonogenic survival assay.

Cells (1 × 103 per well) were plated in six-well tissue culture plates in medium containing 5% serum. The cells were cultured overnight, and then cisplatin with or without RAD001 was added for 48 h, followed by two washes in medium containing 5% serum. Then, cells were incubated in medium plus 5% serum and allowed to proliferate for 6 days. Cells were fixed and stained using Diff-Quik (Dade Behring), and the number of colonies, consisting of ≥25 cells, in triplicate wells were counted for each drug combination to generate survival curves.

#### In vitro detection of apoptosis.

Apoptosis was determined as described (16). Cells were treated with 20 mmol/L RAD001 and/or 10 μmol/L cisplatin for 24 h in the presence of 5% FBS. Cells were lysed, and DNA fragmentation was detected using a Cell Death Detection ELISA Kit (Roche) per the manufacturer’s instructions.

#### Western blot analysis.

Cells were treated with either DMSO (vehicle) or 20 mmol/L RAD001 for 0, 6, or 16 h. Cells were washed twice with ice-cold PBS and lysed in lysis buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L Na3VO4, 1 mmol/L l-glycerophosphate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L L-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 10 μg/mL aprotinin, 1 μg/mL leupeptin, and 1% Triton X-100) for 10 min at 4°C. Lysates were centrifuged at 12,000 × g at 4°C for 15 min, and protein concentrations of the supernatants were determined using Bio-Rad protein assay reagent. Equal amounts of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blocking was done in 5% nonfat milk in 1× TBS. Western blot analyses were done with various specific primary antibodies. Immunoblots were visualized with horseradish peroxidase–coupled goat anti-rabbit or anti-mouse immunoglobulin by using the enhanced chemiluminescence Western blotting system (Perkin-Elmer).

#### Subcutaneous xenograft model.

Nude mice 5 to 7 weeks old were inoculated s.c. into the right flank with 5 × 106 SKOV-3 cells in 200 μL of PBS (n = 24). When tumors reached about 100 mm³, mice were assigned into four treatment groups, each with six mice. The first group was treated with placebo twice a week. The second group was treated with RAD001 (5 mg/kg) twice a week. The third group was treated with cisplatin (3 mg/kg) every 2 weeks. The fourth group was treated with RAD001 (5 mg/kg) twice a week plus cisplatin (3 mg/kg) every 2 weeks. RAD001 was given intragastrically using an animal-feeding device.
needle. Cisplatin was given i.p. Body weight was measured weekly. Caliper measurements of the longest perpendicular tumor diameters were done every week to estimate tumor volume using the following formula: \( V = \frac{L \times W \times D \times \pi}{6} \), where \( V \) is the volume, \( L \) is the length, \( W \) is the width, and \( D \) is the depth (27).

**Intraperitoneal xenograft model.** All procedures involving animals and their care were approved by the Institutional Animal Care and Usage Committee of Fox Chase Cancer Center in accordance with institutional and NIH guidelines. SKOV-3 cells were suspended in PBS at 10^6 cells/500 μL. Then, 1 × 10^6 cells were injected i.p. into each of 48 female 5- to 7-week-old severe combined immunodeficiency mice. Two weeks after inoculation, mice were assigned into four treatment groups, each consisting of 12 mice. The first group of mice was treated with placebo twice a week. The second group was treated with RAD001 (5 mg/kg) twice a week. The third group was treated with cisplatin (3 mg/kg) every 2 weeks. The fourth group was treated with RAD001 (5 mg/kg) twice a week plus cisplatin (3 mg/kg) every 2 weeks. RAD001 was given intragastrically using an animal-feeding needle purchased from Popper & Sons, Inc. Cisplatin was given i.p. Body weight was measured weekly. One-half of the animals (\( n = 24 \)) were sacrificed 5 weeks after starting treatment to assess antitumor efficacy of the drug treatment by measuring volume of ascites, size of tumors, and extent of dissemination. One mouse treated with both cisplatin and RAD001 and one mouse in the placebo-treated group were excluded from the study due to complications independent of tumor formation. In the remaining 24 animals, survival was evaluated from the first day of treatment until death.

**Magnetic resonance microimaging.** Mice inoculated i.p. were scanned before sacrifice by magnetic resonance microimaging (MRM), an extension of clinical magnetic resonance imaging. Mice were imaged in a 7-Tesla vertical wide-bore magnet, using a Bruker DRX 300 spectrometer with a microimaging accessory, which included a micro 2.5 gradient set, a 30-cm birdcage coil, and Paravision software. Mice were anesthetized by exposure to a mixture of oxygen and isofluorane (2%) for 10 min, after which the isofluorane concentration was reduced to 1%. The mice received a Gd-DTPA injection consisting of 0.2 mL of 10:1 diluted Magnevist (Berlex Labs) contrast agent into the shoulder muscle immediately preceding the scan. Mice were positioned such

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**Fig. 1.** AKT activity is a predictor of RAD001 sensitivity. A, analysis of phospho-AKT status in four ovarian cancer cell lines. Ovarian cancer cells were serum-starved overnight, after which the expression of phospho-AKT was determined by Western blot analysis. Actin expression was used as a loading control. **B**, expression of p53 in ovarian cancer cell lines. Ovarian cancer cells were incubated in the presence of 5% FBS, and then the expression of p53 was determined by Western blot analysis. **C**, differential sensitivity of ovarian cancer cells to RAD001. Ovarian cancer cells were treated with the indicated concentrations of RAD001 in the presence of 5% FBS for 72 h. Cell viability was assessed byMT assay. Points, mean; bars, SD. **D** and **E**, SKOV-3 and OVCAR5 cells were treated with the indicated concentrations of RAD001 for 6 h in the presence of 5% FBS. Cells were harvested, and equivalent amounts (30 μg) of protein were subjected to SDS-PAGE and blotted with anti- phospho-mTOR (Ser2448), anti-mTOR, anti-phospho-AKT (Ser473), anti-AKT, anti-phospho-p70S6K (Thr389), anti-p70S6K, anti-phospho-4E-BP1 (Thr65), anti-4E-BP1, or anti-α-actin antibodies. **F**, SKOV-3 and OVCAR5 cells were treated with or without the indicated concentrations of RAD001 for 48 h in the presence of 5% FBS. Cells were harvested, and then the expression of MYC was determined by Western blot analysis. Actin expression was used as the loading control.
that the abdominal cavity was in the center of the MRM radiofrequency coil. Images were made in an axial orientation with a slice thickness of 1 mm, field of view of 2.56 mm, and in-plane resolution of 0.1 mm, with four signal averages and 20–28 slices to cover the entire abdomen. Volumetric measurements were done offline on a Microsoft Windows-based computer. Images acquired in Paravision were converted to Analyze format using the Bruker2analyze program. Tumor volumes were estimated by manually outlining the ovaries using MRICRO program. Measured tumor volume was plotted against actual tumor volume obtained at sacrifice. Linear regression analysis gave a correlation coefficient of 0.94 and P value of <0.01.

**Immunohistochemistry.** Tumor tissues were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned, and processed for either H&E staining or immunohistochemical staining. For immunohistochemistry, sections were incubated with primary antibodies, followed by peroxidase-conjugated secondary antibodies. Primary antibodies were anti-CD31/PECAM-1 at a 1:50 dilution. Microvessel areas (MVA) of individual tumors were analyzed by anti-CD31 immunostaining as reported (28).

**Statistical analysis.** Cell proliferation and apoptosis were analyzed by Wilcoxon exact test. Body weight, tumor volume, ascites formation, dissemination, MVA, cell proliferation, and survival were compared among placebo-, RAD001-, cisplatin-, and cisplatin-plus-RAD001–treated mice. Incidence of ascites formation and dissemination were analyzed by Fisher’s exact test. Tumor burden, volume of ascites, body weight, and MVA were analyzed by Wilcoxon exact test. Survival was analyzed by log-rank test. A P value of <0.05 was considered significant.

**Results**

**Phospho-AKT in association with RAD001 sensitivity.** To examine the effect of mTOR pathway inhibition by RAD001 on proliferation of ovarian cancer in vitro, we did a MTT assay using four human ovarian cancer cell lines with different phospho-AKT and p53 expression levels. As shown in Fig. 1A, under serum-starvation conditions, phospho-AKT levels were low in OVCAR4 and OVCAR5 cells. In contrast, AKT was strongly phosphorylated in OVCAR10 and SKOV-3 cells. The activation state of AKT under conditions of 5% serum was similar to that observed in serum-deprived cells (data not shown). Figure 1B shows the expression of p53 in these lines. Although OVCAR4 and OVCAR10 cells express p53, OVCAR5

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**Fig. 2.** RAD001 enhances cisplatin-induced apoptosis. A, MTT assay (top) and clonogenic survival (bottom) of OVCAR5 and SKOV-3 cells treated with various concentrations of cisplatin with or without RAD001 in the presence of 5% FBS. MTT and clonogenic survival assays were done as described in Materials and Methods. Experiments were repeated three to four times with similar results, and representative results are shown. Points, mean; bars, SD. B, effect of RAD001 on cisplatin-induced cleavage of PARP. SKOV-3 cells were treated with 10 μmol/L cisplatin with or without 20 nmol/L RAD001 for 24 h. Cells were harvested, and then lysates were subjected to Western blotting using anti-PARP or anti–β-actin antibody. C, effect of RAD001 on cisplatin-induced DNA fragmentation. SKOV-3 cells were treated with 10 μmol/L cisplatin with or without 20 nmol/L RAD001 for 24 h. The cells were lysed, and DNA fragmentation was examined as described in Materials and Methods. Columns, mean; bars, SD. **, P < 0.05.
and SKOV-3 do not, as reported previously (29, 30). As shown in Fig. 1C, a differential sensitivity to RAD001 was shown in association with AKT activity. OVCAR10 and SKOV-3 were more sensitive to RAD001 than OVCAR4 and OVCAR5. The in vitro growth inhibition by RAD001 was not striking, with almost 100 mm³, mice were treated with placebo, RAD001, cisplatin, or cisplatin plus RAD001 for 5 wks as described in Materials and Methods. Graph depicting weekly tumor volumes for each treatment group. Points, mean; bars, SD. * P < 0.05, significantly different from placebo-treated mice. ** P < 0.05, significantly different from RAD001- or cisplatin-treated mice.

RAD001 attenuates phosphorylation of p70S6K and 4E-BP1 in vitro. To determine whether the antiproliferative effects of RAD001 result from inhibition of mTOR signaling, we first examined the phosphorylation status of AKT, mTOR, p70S6K, and 4E-BP1 using OVCAR5 and SKOV-3 cells. As shown in Fig. 1D and E, elevated phosphorylation of AKT, mTOR, p70S6K, and 4E-BP1 were much lower in OVCA5 cells. We next examined the effect of RAD001 on the phosphorylation of p70S6K and 4E-BP1, and we found that phosphorylation of both of these downstream targets of mTOR was significantly decreased in SKOV-3 cells by the treatment with RAD001. We next evaluated whether RAD001 effectively inhibits protein translation, which is required for cell cycle progression. For this purpose, we determined the effect of RAD001 on the expression of MYC, whose translation initiation is known to be regulated by the mTOR pathway (32). As shown in Fig. 1F, Western blot analysis showed that the expression of MYC was attenuated in response to RAD001 in SKOV-3 cells. In contrast, the effect was negligible in OVCAR5 cells. Collectively, these results show that RAD001 effectively inhibits the mTOR signaling pathway in ovarian cancer cells with hyperactivated AKT/mTOR signaling.

RAD001 enhances efficacy of cisplatin in vitro. Using OVCAR5 and SKOV-3 cells, which do not express p53 and have been reported to be resistant to cisplatin (33, 34), we next determined whether RAD001 enhances the efficacy of cisplatin. As shown in Fig. 2A, in the presence of 20 nmol/L of RAD001, the ability of cisplatin to inhibit cell proliferation and cell survival was significantly enhanced in SKOV-3 cells, which have elevated AKT/mTOR signaling. In contrast, the effect was minimal in OVCAR5 cells, which have low AKT/mTOR activities. The same association with the AKT/mTOR pathway was also observed in ovarian cancer cells that do express p53. The ability of cisplatin to inhibit cell proliferation was significantly enhanced by RAD001 in OVCAR10 cells, which also have elevated AKT/mTOR signaling, whereas the effect was minimal in OVCAR4 cells, which have low AKT/mTOR activities (data not shown). These data suggest that, irrespective of p53 expression, enhanced response to cisplatin was caused by RAD001 is associated with AKT/mTOR signaling in the ovarian cancer cells. We next determined whether RAD001 can enhance cisplatin-induced apoptosis. Although 20 nmol/L of RAD001 alone did not induce apoptosis in RAD001-sensitive SKOV-3 cells, RAD001 significantly enhanced cisplatin-induced cleavage of PARP (Fig. 2B) and DNA fragmentation (Fig. 2C), suggesting that RAD001 can enhance cisplatin-induced apoptosis in ovarian cancer cells lacking expression of p53.

RAD001 inhibits tumor growth in a s.c. xenograft model. We examined the growth-inhibitory effect of RAD001 on ovarian cancer cells in vivo. For this purpose, we employed a s.c. xenograft model in which athymic mice were inoculated s.c. with RAD001-sensitive SKOV-3 cells. When tumors reached ~100 mm³, mice were randomized into four treatment groups receiving placebo, RAD001, cisplatin, or RAD001 plus cisplatin as described in Materials and Methods. Treatment with cisplatin was well tolerated, with no apparent toxicity throughout the study. Tumor volume was measured weekly after the start of treatments (Fig. 3). Treatment with RAD001 or cisplatin alone decreased tumor burden by 83% and 68%, respectively, compared with placebo, indicating that use of RAD001 as a single agent has a marked antitumor activity. Moreover, treatment with cisplatin plus RAD001 decreased the tumor burden by 92%, suggesting that treatment with RAD001 can enhance the growth-inhibitory effect of cisplatin.

RAD001 inhibits intra-abdominal dissemination of ovarian cancer cells and enhances efficacy of cisplatin in an i.p. model. Although the results from the RAD001 treatment of s.c. ovarian tumors cells were encouraging, peritoneal dissemination is the primary course of progression in human ovarian cancer, and the amount of ascites and size of the disseminated tumor are correlated with patient prognosis (35). Therefore, athymic mice were inoculated i.p. with SKOV-3 cells to examine the effect of RAD001 on intra-abdominal dissemination of ovarian cancer, ascites formation, and tumor growth. Two weeks after inoculation, mice were randomized into four groups receiving placebo, RAD001, cisplatin, or RAD001 plus cisplatin. Overall, cisplatin-treated mice showed a ~7% decrease in body weight compared with placebo-treated mice, although the difference did not reach statistical significance (data not shown).

Although a disadvantage of the i.p. xenograft model is the difficulty in monitoring disease progression, it is possible to visualize tumor burden by MRM (36). To examine the antitumor efficacy, we randomly selected five mice from each group for scanning with MRM, as described in Materials and Methods. As shown in Fig. 4A and B, 5 weeks after the start of...
treatment, large abdominal tumors were frequently detected by MRM in placebo-treated mice, whereas the tumors in cisplatin- or RAD001-treated mice were smaller. Furthermore, tumors in mice treated with RAD001 together with cisplatin were collectively much smaller than tumors found in mice treated with cisplatin or RAD001 alone.

After MRM scanning, mice were sacrificed, and volume of ascites, size of disseminated tumors, and extent of dissemination were measured. Figure 4 illustrates the effect of RAD001 on tumor growth, dissemination, and ascites formation. Representative images are shown for placebo, RAD001, cisplatin, or cisplatin plus RAD001. Tumor volumes were determined by manually outlining the ovaries using the MRCRO program. Columns, mean; bars, SD. **, P < 0.05. C, mice were sacrificed 5 weeks after the first day of treatment. I, physical appearance of representative mice. II and III, magnified view of intra-abdominal dissemination of the same mice. Metastasis to liver (II) and diaphragm (III). Arrows and bracket, metastatic sites. IV, Intra-abdominal ovarian tumors. Q, histologic features of ovarian tumors. Tissues sections were stained with H&E, and representative fields are shown. I, dissemination to diaphragm. Magnification, ×100. II, dissemination to intestine. Magnification, ×100. III, poorly differentiated adenocarcinoma of the ovary. Magnification, ×200. **, P < 0.05. E, effect of RAD001 on survival. Athymic mice were inoculated i.p. with SKOV-3 cells. Two weeks after inoculation, the mice were randomized into four treatment groups, i.e., placebo, RAD001, cisplatin, or cisplatin plus RAD001. Survival of the mice was evaluated from the first day of treatment until death.
were examined at autopsy. Large abdominal tumors, bloody ascites, and peritoneal dissemination were commonly observed in placebo-treated mice (Fig. 4C, I-IV, and D, I-II). Histologically, these abdominal tumors were poorly differentiated adenocarcinomas (Fig. 4D, III). Mean tumor burden in mice treated with RAD001 or cisplatin was 107.4 and 341.5 mm$^3$, respectively, compared with 698.2 mm$^3$ in placebo-treated mice (Table 1). It is noteworthy that the ability of RAD001 to inhibit abdominal tumor growth was significantly greater than that of cisplatin, which is consistent with the findings of the s.c. xenograft study (Fig. 3). Surprisingly, tumors developing in mice treated with combined cisplatin and RAD001 were very small (mean size, 5.6 mm; Table 1), indicating that RAD001 dramatically enhanced the growth-inhibitory effect of cisplatin in this model. Striking effects were also seen with regard to tumor dissemination and ascites formation. As shown in Table 1, abundant ascites was detected in placebo-treated mice. Treatment with either RAD001 or cisplatin significantly attenuated the volume of ascites. Abdominal effusions were not detected in mice treated with cisplatin plus RAD001 (Table 1). Tumor cell dissemination to the liver or diaphragm was commonly observed in placebo-treated mice (Fig. 4C, II and III; Table 1), whereas dissemination of tumor cells was significantly inhibited by treatment with RAD001, cisplatin, or RAD001 plus cisplatin.

We also examined the effect of RAD001 on survival of mice inoculated i.p. As shown in Fig. 4E, median overall survival was 41 days in the placebo group versus 54.5, 61, and 71 days in groups treated with either RAD001, cisplatin, or RAD001 plus cisplatin, respectively. All mice treated with cisplatin plus RAD001 survived until the end of the study. Thus, although RAD001 alone significantly prolonged survival, RAD001 also enhanced cisplatin-mediated maintenance of survival in this preclinical model of human ovarian cancer.

Taken together, these findings indicate that RAD001 has significant antitumor effects as a single agent, with the ability not only to inhibit ovarian tumor growth, but also to inhibit dissemination of tumor cells and production of ascites. Moreover, RAD001 enhanced the antitumor effects of cisplatin in vivo, especially in tumors with elevated AKT/mTOR activity.

**RAD001 inhibits the expression of HIF-1α and VEGF in vitro and angiogenesis in vivo.** We next investigated the mechanism by which RAD001 inhibits ascites formation and peritoneal dissemination in vivo. Tumor growth, metastasis, and dissemination have been shown to be dependent on angiogenesis (37). Hypoxia commonly develops within tumors, and under hypoxic conditions, HIF-1α regulates the expression of VEGF and promotes angiogenesis to meet the metabolic requirements for sustained tumor growth (38). In ovarian cancer, VEGF has been reported to be a major mediator of ascites formation (39), invasiveness (40), metastasis (41), and dissemination (42) and is thereby associated with poor patient prognosis (43). Because AKT-mTOR signaling has been shown to regulate the expression of HIF-1α and VEGF (19), we tested the effect of RAD001 on the expression of HIF-1α and VEGF in SKOV-3 cells in vitro.

As shown in Fig. 5A, the expression of HIF-1α under non-hypoxic conditions is very low in SKOV-3 cells (Fig. 5A, lane 1), and the expression of HIF-1α was significantly up-regulated in response to exposure to hypoxia (Fig. 5A, lane 2). Up-regulation of HIF-1α expression was decreased following treatment with RAD001 or the PI3K inhibitor LY294002 (Fig. 5A, lanes 3-5), consistent with expression of HIF-1α being regulated through PI3K-AKT signaling.

We also examined whether RAD001 inhibits the expression of VEGF in SKOV-3 cells (Fig. 5B). The basal expression of VEGF in SKOV-3 cells was significantly higher in cells exposed to hypoxia than in those under nonhypoxic conditions. Treatment of cells with RAD001 for 16 h attenuated the expression of VEGF in hypoxic SKOV-3 cells. These results suggest that RAD001 not only inhibits cell proliferation, but may also attenuate angiogenesis. To test this hypothesis, we next examined whether RAD001 inhibits angiogenesis in vivo. We examined the distribution of the endothelial marker CD31 in tumors by immunohistochemistry and evaluated tumor MVA in the same samples. As shown in Fig. 5C and D, large CD31-immunopositive vessels were observed in peritoneal tumors from placebo-treated mice, but the CD31-immunopositive vessels were smaller and fewer in peritoneal tumors from RAD001-treated mice. These results indicate that the antitumor effect of RAD001 is associated, at least in part, with the inhibition of tumor angiogenesis. RAD001-mediated inhibition of VEGF, HIF-1α, and angiogenesis is consistent with the fact that RAD001 inhibits tumor dissemination and ascites production in mice.

**Discussion**

Mutation in the p53 gene (TP53) is a common occurrence in ovarian cancer (5) and presents a challenging therapeutic problem because it is associated with a resistance to cisplatin therapy in ovarian cancer patients (6). Previous in vitro studies have shown that a higher dose of cisplatin is required to induce

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**Table 1. Effect of RAD001 on tumor development, ascites formation, and dissemination**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of mice</th>
<th>Number of mice with tumors (%)</th>
<th>Mean tumor volume (mm$^3$)</th>
<th>Volume of ascites (mL)</th>
<th>Number of mice with dissemination to liver (%)</th>
<th>Number of mice with dissemination to diaphragm (%)</th>
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<td>Placebo</td>
<td>5</td>
<td>5 (100)</td>
<td>698.2</td>
<td>1.86</td>
<td>4 (80)</td>
<td>5 (100)</td>
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<td>6</td>
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<td>107.4*</td>
<td>0.2*</td>
<td>0 (0)*</td>
<td>1 (0)*</td>
</tr>
<tr>
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<td>6 (100)</td>
<td>341.5*</td>
<td>0.1*</td>
<td>0 (0)*</td>
<td>0 (0)*</td>
</tr>
<tr>
<td>Cisplatin + RAD001</td>
<td>5</td>
<td>2 (40)</td>
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<td>0*</td>
<td>0 (0)*</td>
<td>0 (0)*</td>
</tr>
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*Significantly different from placebo group (P < 0.01).
†Significantly different from cisplatin group (P < 0.01).
‡Significantly different from RAD001 group (P < 0.01).
apoptosis in ovarian cancer cells with p53 mutations than in ovarian cancer cells with wild-type p53 (44). Clinical use of high-dose cisplatin is limited because of its toxicity, with high doses leading to multiorgan toxicities, including kidney damage, bone marrow failure, or neuropathy (45). Therefore, development of a new treatment strategy to circumvent p53-induced cisplatin resistance with minimal side effects is needed.

The sensitivity of cancer cells to chemotherapeutic drug-induced apoptosis depends on the balance between pro-apoptotic and antiapoptotic signals. Therefore, inhibition of antiapoptotic signals, such as those mediated by the AKT pathway, has been proposed as a promising strategy to enhance the efficacy of conventional chemotherapeutic agents (7). Among the numerous AKT substrates, mTOR is thought to be one of the major targets of relevance to cancer therapy (19–21). Thus, mTOR inhibition by RAD001 may have potential not only to inhibit tumor growth, but also to enhance the effect of cisplatin without increasing multiorgan toxicity.

Our data show that treatment with RAD001 attenuates phosphorylation of both 4E-BP1 and p70S6K and expression of MYC, and RAD001 markedly inhibits the proliferation of ovarian cancer cells in vitro. We also showed that elevation of both phospho-AKT/mTOR activity can predict sensitivity to RAD001 in ovarian cancer cells. Although RAD001 alone did not induce apoptosis, RAD001 significantly enhanced cisplatin-induced apoptosis in vitro. The increased sensitivity to cisplatin caused by RAD001 was correlated with hyperactivated AKT/mTOR signaling (OVCAR10 and SKOV-3 cells), whereas the effect was minimal in OVCAR5 and OVCAR4, which exhibit low levels of phospho-AKT/mTOR activity. Interestingly, increased sensitivity to cisplatin in combination with RAD001 was observed in SKOV-3 cells, even when a relatively low concentration (1 μmol/L) of cisplatin was used (Fig. 2A).

A similar correlation has been reported between AKT activation and rapamycin sensitivity in breast cancer cell lines (31). However, AKT kinase activity is not the sole determinant of sensitivity to mTOR inhibition because other pathways can signal to mTOR, and certain factors, such as amplification of S6K1, can result in sensitivity to rapamycin in the absence of AKT activation (31). In ovarian cancers, we have found a
strong correlation between phosphorylation of AKT and phosphorylation of mTOR and p70S6K in ovarian carcinomas (16, 46).

Although in vitro treatment of cell lines with RAD001 induced only ~30% growth inhibition with OVCAR10 and SKOV-3 at the highest drug concentration tested, a more effective response to mTOR inhibition was observed in vivo than in vitro. Similarly, treatment of MCF-7 cells with rapamycin resulted in a modest inhibition of cell proliferation after 2 to 5 days of treatment in vitro, but treatment of MCF-7 xenografts for 19 days resulted in a more pronounced decrease in tumor volume (>50%) compared with untreated mice (47). This increased efficacy in vivo may result, in part, from the longer duration and repeated administration of drug. Moreover, in addition to inhibiting cell proliferation, we show that RAD001 inhibits angiogenesis, which might further explain the greater efficacy seen in our in vivo studies.

In mice inoculated s.c. or i.p. with SKOV-3 cells, treatment with RAD001 alone significantly inhibited tumor growth. The i.p. xenograft model more closely reflects disease progression in humans because tumor dissemination and ascites are observed in human ovarian cancers, which frequently metastasize and disseminate throughout the peritoneum (48). Treatment with RAD001 inhibited intra-abdominal dissemination and production of ascites and resulted in prolonged survival of these mice. Although the effect of RAD001 alone on tumor growth was more profound than cisplatin alone (Figs. 3 and 4B; Table 1), cisplatin may inhibit ascites formation and dissemination more effectively than RAD001 (Table 1). Moreover, mice treated with cisplatin alone survived somewhat longer than those treated with RAD001 alone, consistent with the clinical importance of controlling ascites production and dissemination in ovarian cancer.

It has been reported that the maximum serum cisplatin concentration (C_max) after i.p. treatment of 1.33 and 6.67 mg/kg cisplatin in nude mice are 2.9 and 8.76 μg/mL, respectively (49). Because the maximum serum cisplatin concentration (C_max) after i.v. treatment with 75 mg cisplatin in humans is known to be ~6 μg/mL, the dose of cisplatin employed in our experiment (3 mg/kg) is roughly equivalent to the standard clinical dose (50-75 mg/mm^2) used in patients.

The molecular mechanism by which RAD001 enhanced the efficacy of cisplatin in our experimental model is not known. Although immunostaining for caspase-3 did not show any significant additive effect of RAD001 on the induction of apoptosis by cisplatin, RAD001 may enhance the efficacy of cisplatin in vivo through other mechanisms such as inhibition of cell proliferation and angiogenesis. These findings are consistent with our recent work with a transgenic mouse model of ovarian cancer, in which RAD001 treatment resulted in a marked decrease in Ki-67–positive cells but only a small increase in the number with immunoreactivity for cleaved caspase-3 (46).

In a recent study, RAD001 was found to sensitize A549 cells with wild-type TP53 to cisplatin-induced apoptosis by inhibiting p21 translation in vivo (21). Unlike A549 cells, treatment of p53-deficient SKOV-3 cells with cisplatin and RAD001 resulted in induction of p21 (data not shown; ref. 16). Thus, in our study, RAD001 enhanced the sensitivity of ovarian cancer cells to cisplatin in a p53-independent, AKT/mTOR-dependent manner. A similar finding was reported for myeloma cells (23). The reason for the discrepancy between these studies is not known. However, there are numerous alterations in cancer that may impact response to combination therapy, and different tumor types may have different responses to mTOR inhibition when combined with DNA damage-inducing drugs.

Cytotoxic agents, such as cisplatin, are usually administered with intervals of several weeks to allow the recovery of normal tissue. However, surviving cancer cells also proliferate during this interval, and this process of repopulation is thought to be an important cause of treatment failure (50). In our in vivo studies, cisplatin was given every 2 weeks to avoid multigran toxicity, and mice were treated with RAD001 twice a week between courses of cisplatin treatment. This regimen may have inhibited the repopulation of RAD001-sensitive SKOV-3 cells and improved the efficacy of cisplatin in vivo.

Collectively, these results indicate that RAD001 is a promising agent for the treatment of aggressive ovarian cancers with hyperactivation of the AKT/mTOR pathway, including tumors lacking expression of p53. These results provide further rationale for future clinical trials with RAD001 in combination with cisplatin in ovarian cancer patients.

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