The Activity of Trabectedin As a Single Agent or in Combination with Everolimus for Clear Cell Carcinoma of the Ovary

Seiji Mabuchi¹, Takeshi Hisamatsu¹, Chiaki Kawase¹, Masami Hayashi¹, Kenjiro Sawada¹, Kazuya Mimura¹, Kazuhiro Takahashi², Toshifumi Takahashi², Hirohisa Kurachi², and Tadashi Kimura¹

Abstract

**Purpose:** The objective of this study was to evaluate the antitumor efficacy of trabectedin in clear cell carcinoma (CCC) of the ovary, which is regarded as an aggressive, chemoresistant, histologic subtype.

**Experimental Design:** Using 6 human ovarian cancer cell lines (3 CCC and 3 serous adenocarcinomas), the antitumor effects of trabectedin were examined *in vitro*, and we compared its activity according to histology. We next examined the antitumor activity of trabectedin in both cisplatin-resistant and paclitaxel-resistant CCC cells *in vitro*. Then, the *in vivo* effects of trabectedin were evaluated using mice inoculated with CCC cell lines. Using 2 pairs of trabectedin-sensitive parental and trabectedin-resistant CCC sublines, we investigated the role of mTOR in the mechanism of acquired resistance to trabectedin. Finally, we determined the effect of mTOR inhibition by everolimus on the antitumor efficacy of trabectedin *in vitro* and *in vivo*.

**Results:** Trabectedin showed significant antitumor activity toward chemosensitive and chemoresistant CCC cells *in vitro*. Mouse xenografts of CCC cells revealed that trabectedin significantly inhibits tumor growth. Greater activation of mTOR was observed in trabectedin-resistant CCC cells than in their respective parental cells. The continuous inhibition of mTOR significantly enhanced the therapeutic efficacy of trabectedin and prevented CCC cells from acquiring resistance to trabectedin.

**Conclusion:** Trabectedin is a promising agent for CCC as a first-line chemotherapy and as a second-line treatment of recurrent CCC that had previously been treated with cisplatin or paclitaxel. Moreover, trabectedin combined with everolimus may be more efficacious for the management of CCC.

Introduction

Ovarian carcinoma is the fourth most common cause of cancer death among women in the United States, with more than 21,880 new cases diagnosed and 13,850 deaths estimated to have occurred in 2010 (1). Clear cell carcinoma (CCC) of the ovary has been known to show poorer sensitivity to platinum-based frontline chemotherapy and to be associated with a worse prognosis than the more common serous adenocarcinoma (SAC; refs. 2–5). The lack of effective chemotherapy for recurrent CCC after frontline treatment is another important problem in the clinical management of CCC. Therefore, to improve the survival of patients with CCC, the development of novel treatment strategies in the setting of both first-line treatment and salvage treatment of recurrent disease are needed.

Trabectedin, which was formerly known as eceinascidin-743 (ET-743), is an antineoplastic agent that was originally derived from the Caribbean marine tunicate *Ecteinascidia turbinata*. It binds covalently to the minor groove of DNA, bending DNA toward the major groove and disrupting transcription, leading to G2-M cell-cycle arrest and ultimately apoptosis (6). Although the exact mechanism of its action is not clear, it is thought that the cytotoxic activity of trabectedin is based on the inhibition of transcription-dependent nucleotide- Excision repair (NER) via the trapping of the proteins responsible for NER, which induces cells to undergo apoptosis (7).

In a preclinical study, trabectedin showed significant antitumor activity *in vitro* and *in vivo* against a range of solid tumor cells, including soft-tissue sarcoma (STS), ovarian, breast, prostate, and renal cancers; melanoma; and non-small-cell lung cancer (8–11). On the basis of the promising results of preclinical and clinical studies

**Authors' Affiliations:** ¹Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, Osaka; and ²Department of Obstetrics and Gynecology, Yamagata University, School of Medicine, Yamagata, Japan

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**S. Mabuchi and T. Hisamatsu contributed equally to this work.**

**Corresponding Author:** Seiji Mabuchi, Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-668-793-354; Fax: 81-668-793-359; E-mail: smabuchi@gyne.med.osaka-u.ac.jp

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Translational Relevance

Clear cell carcinoma (CCC) of the ovary is a distinctive subtype of epithelial ovarian cancer associated with a poorer sensitivity to platinum-based chemotherapy and worse prognosis than the more common serous adenocarcinoma. To improve survival, the identification of a novel anticancer agent that more effectively targets CCC is necessary. Our results show that treatment with trabectedin significantly inhibited the growth of CCC in vitro and in vivo. The growth-inhibitory effect of trabectedin on CCC cells was greater than those of cisplatin, paclitaxel, and SN-38, which are clinically employed as a part of the first-line chemotherapy for this disease. This finding may be relevant for the planning of future clinical studies of first-line treatments of CCC of the ovary. Moreover, the significant antitumor activity of trabectedin against cisplatin- and paclitaxel-resistant CCC shown in the current study may represent a novel treatment option for patients with recurrent disease after first-line chemotherapy.

(10, 12, 13), trabectedin is now approved in Europe for the treatment of STS after anthracycline and ifosfamide failure and for treating patients who cannot receive these agents (14).

In ovarian cancer, trabectedin has been studied in several phase I and II clinical trials, showing a favorable toxicity profile and promising activity in recurrent ovarian cancer patients (15, 16). Following the encouraging results of these studies, a phase III trial investigating the activity of trabectedin plus liposomal doxorubicin versus liposomal doxorubicin in patients with recurrent ovarian cancer has recently been conducted. As this study showed a survival benefit of trabectedin-based combination chemotherapy, trabectedin has become the focus of attention for researchers investigating the treatment of epithelial ovarian cancer (17).

As most ovarian cancer cell lines investigated in previous preclinical studies of trabectedin have been from ovarian SAC (6, 8, 9) and only a small number of patients with CCC histology were included in the previous clinical studies (15–17), the therapeutic potential of trabectedin in patients with CCC is unknown. Because of its nature as a newly developed anticancer agent, the mechanism of resistance to trabectedin is largely unknown. To further improve the prognosis of patients with ovarian cancer, a deeper understanding of the mechanism of resistance to trabectedin and the development of a novel treatment strategy to overcome this resistance are needed.

The mTOR is a serine/threonine kinase that plays a key role in cell growth, proliferation, survival, and tumor angiogenesis (18). Everolimus, an orally bioavailable mTOR inhibitor, acts as an allosteric inhibitor of mTOR. On the basis of promising preclinical and clinical findings, everolimus has been approved for the treatment of renal cell carcinoma (19). In preclinical investigations, everolimus has been shown to inhibit the proliferation of ovarian cancer cells and enhance their sensitivity to cisplatin both in vitro and in vivo (20–22), and its efficacy in ovarian cancer patients is currently being evaluated in several phase I/II trials (23). However, no reports have addressed the role of mTOR in the acquisition of resistance to trabectedin.

In the current investigation, we evaluated the therapeutic efficacy of trabectedin as a single agent in vitro and in vivo. We also examined the antitumor activity of trabectedin in both cisplatin-resistant and paclitaxel-resistant CCC cells. Finally, we investigated the role of AKT–mTOR signaling in the mechanism of acquired resistance to trabectedin and assessed the therapeutic potential of trabectedin in combination with everolimus in CCC cells.

Materials and Methods

Reagents/antibodies

Trabectedin was obtained from PharmaMar. Everolimus was obtained from Novartis Pharma AG. Cisplatin, paclitaxel, and 7-ethyl-10-hydroxycamptothecin (SN-38) were purchased from Sigma. LY294002 was purchased from Cell Signal Technology. Enhanced chemiluminescence (ECL) Western blotting detection reagents were from Perkin Elmer. Antibodies recognizing p70S6K, phospho-p70S6K (Thr389), mTOR, phospho-mTOR (Ser2448), AKT, phospho-AKT (Ser473), and β-actin were obtained from Cell Signaling Technology. The Cell Titer 96-well proliferation assay kit was obtained from Promega.

Drug preparation

Trabectedin was prepared as a 1 mg/mL stock solution in ethanol. Everolimus was diluted to the appropriate concentration in double-distilled water just before its administration by gavage in the animal studies. For the in vitro analyses, everolimus was prepared in dimethyl sulfoxide (DMSO) before being added to the cell cultures as described previously (20–22). Cisplatin was dissolved in sterilized double-distilled water to a final concentration of 1 mmol/L. SN-38 and paclitaxel were dissolved in DMSO to final concentrations of 10 and 100 mmol/L, respectively.

Cell culture

The human ovarian CCC cell lines RMG1, RMG2, and HAC2 were kindly provided by Dr. H. Itamochi (Tottori University, Tottori, Japan). These cells were cultured in phenol red–free Dulbecco’s modified Eagle’s medium (DMEM Ham’s F-12; Gibco Ltd.) with 10% FBS, as reported previously (20, 24–27). The human ovarian SAC cell lines A2780, SKOV3, and Caov-3 cells were purchased from American Type Culture Collection (ATCC). SKOV-3 cells were cultured in McCoy’s 5A with 10% FBS. A2780 and Caov-3 cells were maintained in DMEM (ATCC) with 10% FBS. All 3 cell lines were maintained in a humidified incubator at 37°C in 5% CO₂.
Determination of cell number

RMG1 and RMG2 cells were seeded into 6-well plates at a density of 10^4/well. After each incubation, the monolayers were washed once with PBS, the cells were detached with trypsin, and viable cells were counted by trypan blue dye exclusion.

Cell proliferation assay

An MTS assay was used to analyze the effects of trabectedin, cisplatin, paclitaxel, SN-38, and everolimus on cell viability. Trabectedin is reported to show cytotoxic activity in various cancer cell lines at nmol/L concentrations. In a previous study, the IC_{50} values of trabectedin in breast cancer cell lines ranged from 0.1 to 3.7 nmol/L (28). It was also reported that the IC_{50} values of SN-38 in RMG1 cells and RMG2 cells were 37 and 34 nmol/L, respectively (29). On the basis of these findings, cell viability was assessed after the addition of trabectedin, cisplatin, paclitaxel, or SN-38 at concentrations ranging from 0 to 10 nmol/L. After 48 hours of incubation, the number of surviving cells was assessed by determination of the absorbance of the dissolved formazan product after the addition of MTS for 1 hour, as described by the manufacturer (Promega). Cell viability is expressed as follows: A_{exp \text{ group}}/A_{control} × 100.

Western blot analysis

The cells were treated with 10 nmol/L trabectedin with or without everolimus for 24 hours, washed twice with ice-cold PBS, and lysed in lysis buffer for 10 minutes at 4°C. The resultant lysates were centrifuged at 12,000 × g at 4°C for 15 minutes, and the protein concentrations of the supernatants were determined using the Bio-Rad protein assay reagent. Equal amounts of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blocking was conducted in 5% nonfat milk in 1× Tris-buffered saline. Western blot analyses were conducted with various specific primary antibodies. Immunoblots were visualized with horseradish peroxidase–coupled goat anti-rabbit or anti-mouse immunoglobulin, using the ECL Western blotting system (Perkin Elmer).

Subcutaneous xenograft model

All procedures involving animals and their care were approved by the Institutional Animal Care and Usage Committee of Osaka University, in accordance with institutional and NIH guidelines. Initial experiments were conducted to examine the effects of trabectedin on ovarian CCC (Fig. 3). Five- to 7-week-old nude mice (n = 24) were inoculated s.c. into the right flank either with 10^6 RMG1 (n = 12) or RMG2 (n = 12) cells in 200 μL of PBS. When the tumors reached about 50 mm^3, the mice inoculated with RMG1 or RMG2 were assigned to 2 treatment groups. The first group (n = 6) was i.v. administered PBS weekly for 4 weeks. The second group (n = 6) was i.v. administered trabectedin (0.2 mg/kg) weekly for 4 weeks. A second set of experiments was conducted to examine the antitumor effect of combination treatment involving trabectedin and everolimus (Fig. 6). Five- to 7-week-old nude mice (n = 48) were inoculated s.c. with 5 × 10^6 RMG1 (n = 24) or RMG2 (n = 24) into their right flank. When the resultant tumors had reached about 50 mm^3 in size, the mice were assigned to 1 of 4 treatment groups, which received PBS, trabectedin (0.2 mg/kg, weekly), everolimus (2.5 mg/kg, 3 times a week), or trabectedin (0.2 mg/kg, weekly) plus everolimus (2.5 mg/kg, 3 times a week). Caliper measurements of the longest perpendicular tumor diameter were done every week to estimate tumor volume by using the following formula: V = L × W × D × π/6, where V is the volume, L is the length, W is the width, and D is the depth as described previously (30).

Establishment of chemoresistant cell lines

Trabectedin-resistant sublines from RMG1, RMG2, and SKOV3 were developed in our laboratory by continuous exposure to trabectedin. Briefly, cells of both lines were exposed to stepwise increases in the trabectedin concentration. The initial trabectedin exposure was at a concentration of 0.1 nmol/L. After the cells had regained their exponential growth rate, the trabectedin concentration was doubled and then the procedure was repeated until selection at 10 nmol/L was attained. The resulting trabectedin-resistant sublines, called RMG1-YR, RMG2-YR, and SKOV3-YR, were subcultured weekly and treated monthly with 10 nmol/L of trabectedin to maintain a high level of chemoresistance.

Paclitaxel-resistant CCC sublines (RMG1-PR and RMG2-PR) from CCC cells (RMG1 and RMG2) were also developed by continuous exposure to paclitaxel, as previously reported (31). Cisplatin-resistant CCC sublines were developed in our laboratory as reported previously (20, 24).

Statistical analysis

Cell proliferation was analyzed by Wilcoxon’s exact test. The tumor volume of the trabectedin-treated mice was compared with that of the PBS-treated mice and analyzed using Wilcoxon’s exact test. A P value of less than 0.05 was considered significant.

Results

In vitro growth-inhibitory effects of trabectedin on CCC and SAC cell lines

To examine the effects of trabectedin on the proliferation of ovarian cancer cells of CCC or SAC origin, we conducted MTS assays using 6 human ovarian cancer cell lines. As shown in Figure 1A, 48 hours of treatment with trabectedin inhibited the proliferation of ovarian cancer cells in a dose-dependent manner, showing 40% to 80% growth inhibition at the highest drug concentration tested. During histology comparisons, a differential sensitivity to trabectedin was shown. CCC cells (RMG1, RMG2, and HAC2) were more sensitive to trabectedin than SAC cells (A2780, Caov-3, and SKOV-3).

Using RMG1 and RMG2 cell lines, which exhibited the greatest sensitivity to trabectedin, as shown in Figure 1A, we...
next compared the antitumor effect of trabectedin with those of cisplatin, paclitaxel, and SN-38 (Fig. 1B). As shown, both RMG1 and RMG2 cells showed poor sensitivity to cisplatin at the concentrations tested in the current study, which is consistent with the findings of a previous investigation (32). As previously reported, the activity of SN-38 was greater than those of cisplatin and paclitaxel (32). Importantly, the activity of trabectedin was significantly greater than that of SN-38. These results suggest that trabectedin is a promising agent for the treatment of CCC of the ovary.

**Effect of trabectedin on cisplatin- or paclitaxel-resistant CCC in vitro**

We next examined the growth-inhibitory effect of trabectedin on chemoresistant CCC cells by using the MTS assay. For this purpose, we employed cisplatin-resistant, paclitaxel-resistant, and their representative parental CCC cells, as described in *Materials and Methods.* As shown in Figure 2, treatment with trabectedin inhibited the proliferation of all of these CCC cells in a dose-dependent manner. The antitumor effects of trabectedin in the cisplatin- and paclitaxel-resistant CCC cells were slightly milder than those observed in their respective parental cells at lower concentrations (0.1–1 nmol/L). However, at a concentration of 10 nmol/L, the antitumor effects of trabectedin in the cisplatin- and paclitaxel-resistant CCC cells were equivalent to those observed in their respective parental cells.

**In vivo growth-inhibitory effect of trabectedin on the growth of ovarian CCC**

To examine the *in vivo* growth-inhibitory effect of trabectedin, we employed an s.c. xenograft model in which athymic mice were s.c. inoculated with RMG1 or RMG2 cells. When the tumors reached approximately 50 mm³, the mice were randomized into 2 treatment groups, receiving PBS or trabectedin, as described in *Materials and Methods.* Overall, drug treatment was well tolerated with no apparent toxicity throughout the study. Tumor volume was measured weekly after the start of treatment (Fig. 3B and D). The appearance of tumors 4 weeks from the first day of treatment is also shown in Figure 3A and C. The mean RMG1-derived tumor burden in mice treated with trabectedin was 110.1 mm³ compared with 410.2 mm³ in the PBS-treated mice, and the mean RMG2-derived tumor burden in the animals treated with trabectedin was 98.2 mm³ compared with 330.6 mm³ in the placebo-treated mice. Overall, treatment with trabectedin decreased the RMG1- and RMG2-derived tumor burden by 73% and 70%, respectively, compared with PBS. These results indicate that trabectedin has significant antitumor effects as a single agent on CCC.

**Increased AKT–mTOR activation in trabectedin-resistant CCC cell lines**

We have previously reported that AKT–mTOR signaling is involved in the resistance of ovarian CCC cells to cisplatin (20, 24). To examine whether AKT–mTOR signaling is involved in the resistance of ovarian CCC cells to cisplatin, we employed an s.c. xenograft model in which athymic mice were s.c. inoculated with RMG1 or RMG2 cells. When the tumors reached approximately 50 mm³, the mice were randomized into 2 treatment groups, receiving PBS or trabectedin, as described in *Materials and Methods.* Overall, drug treatment was well tolerated with no apparent toxicity throughout the study. Tumor volume was measured weekly after the start of treatment (Fig. 3B and D). The appearance of tumors 4 weeks from the first day of treatment is also shown in Figure 3A and C. The mean RMG1-derived tumor burden in mice treated with trabectedin was 110.1 mm³ compared with 410.2 mm³ in the PBS-treated mice, and the mean RMG2-derived tumor burden in the animals treated with trabectedin was 98.2 mm³ compared with 330.6 mm³ in the placebo-treated mice. Overall, treatment with trabectedin decreased the RMG1- and RMG2-derived tumor burden by 73% and 70%, respectively, compared with PBS. These results indicate that trabectedin has significant antitumor effects as a single agent on CCC.
signaling is involved in trabectedin resistance in CCC, we established trabectedin-resistant sublines (RMG1-YR and RMG2-YR) from RMG1 and RMG2 cells, as described in "Material and Methods." To examine whether these sublines had acquired resistance to trabectedin, we first evaluated the sensitivities of these cell lines to trabectedin by using the MTS assay. As shown in Figure 4A, clear differential sensitivity to trabectedin was observed between the trabectedin-sensitive parental and respective trabectedin-resistant sublines. We next examined trabectedin-induced apoptosis in these cell lines. Treatment with trabectedin induced cleavage of PARP in parental cells but not in trabectedin-resistant sublines (Fig. 4B), indicating that these sublines had acquired resistance to trabectedin. We next investigated the activity of AKT–mTOR in both trabectedin-resistant sublines and parental chemosensitive cells by Western blotting. As shown in Figure 4C and D (i), significantly higher phosphorylation of AKT and mTOR was observed in both trabectedin-resistant cell lines than in their respective parental cell lines. Moreover, p70S6K, a downstream effector of mTOR, was also hyperphosphorylated in both trabectedin-resistant cell lines. As shown in Figure 4C (ii) and D (ii), the increased phosphorylation of AKT, mTOR, and p70S6K was inhibited by treatment with a phosphoinositide 3-kinase (PI3K) inhibitor, LY294002. Moreover, the increased phosphorylation of p70S6K was inhibited by treatment with everolimus. These results may indicate the involvement of AKT–mTOR signaling in trabectedin resistance.

Figure 2. Effect of trabectedin on the growth of chemoresistant CCC cells. Cisplatin- and paclitaxel-resistant sublines were established as described in "Materials and Methods." A and B, parental (RMG1 and RMG2), cisplatin-resistant variant (RMG1-CR and RMG2-CR), and paclitaxel-resistant variant (RMG1-PR and RMG2-PR) cells were treated with the indicated concentrations of trabectedin in the presence of 5% FBS for 48 hours. Cell viability was assessed using the MTS assay. Points, mean; bars, SD (*, significantly different from RMG1-CR or RMG1-PR; **, significantly different from RMG2-CR or RMG2-PR; and ***, significantly different from RMG2-CR; P < 0.05).

Figure 3. Effect of trabectedin on the growth of CCC-derived tumor cells in vivo. Athymic nude mice were s.c. inoculated with RMG1 or RMG2 cells. When the tumors reached a mean size of about 50 mm³, the mice were i.v. administered PBS or 0.2 mg/kg trabectedin weekly for 4 weeks. A and C, appearance of s.c. tumors. B and D, graphs depicting weekly tumor volumes (mm³) for each treatment group. Points, mean; bars, SD (*, significantly different from the PBS-treated mice; P < 0.05).
Figure 4. The increased activation of AKT–mTOR signaling in trabectedin-resistant CCC cells. A and B, establishment of trabectedin-resistant variant cell lines. Trabectedin-resistant sublines were established as described in "Materials and Methods." A, trabectedin-sensitive parental (RMG1 and RMG2) and trabectedin-resistant variant (RMG1-YR and RMG2-YR) cells were treated with the indicated concentrations of trabectedin in the presence of 5% FBS for 48 hours. Cell viability was assessed using the MTS assay. Points, mean; bars, SD (*, P < 0.05). B, effect of trabectedin on the cleavage of PARP in trabectedin-sensitive parental and trabectedin-resistant variant cell lines. RMG1, RMG1-YR, RMG2, and RMG2-YR treated with 10 nmol/L trabectedin for 24 hours. The cells were harvested and then the lysates were subjected to Western blotting by using anti-PARP or anti-β-actin antibody. C and D, AKT/mTOR signaling activation in trabectedin-sensitive parental and trabectedin-resistant variant cells in vitro. RMG1, RMG1-YR, RMG2, and RMG2-YR cells were serum starved overnight in the presence or absence of 20 μmol/L LY294002 or 20 nmol/L everolimus. The 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, or anti-β-actin antibodies.
Trabectedin induces the activation of mTOR in an AKT-dependent manner

To further examine whether AKT–mTOR signaling is involved in the mechanism of resistance to trabectedin, we next examined whether AKT–mTOR signaling is activated by trabectedin treatment in CCC cells. As shown in Figure 5A and B (i), treatment with trabectedin-induced prolonged phosphorylation of AKT, mTOR, and p70S6K in

Figure 5. Treatment with trabectedin induces the prolonged activation of mTOR in CCC cells. A and B (i and iii), trabectedin induces the activation of mTOR signaling in an AKT-dependent manner. Trabectedin-sensitive parental (RMG1 and RMG2) and trabectedin-resistant variant (RMG1-YR and RMG2-YR) cells were treated with 10 nmol/L trabectedin in the presence or absence of 20 nmol/L of everolimus for 24 hours. Then, the medium was removed and replaced with medium with or without 10 nmol/L of everolimus. After an additional 0, 24, 48, or 72 hours of incubation, the cells were harvested. A and B (ii), RMG1 and RMG2 cells were treated with 1 nmol/L trabectedin in the presence or absence of 20 μmol/L LY294002 or 20 nmol/L of everolimus for 24 hours. Then, cells were harvested. 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, or anti-β-actin antibodies.

Trabectedin induces the activation of mTOR in an AKT-dependent manner

To further examine whether AKT–mTOR signaling is involved in the mechanism of resistance to trabectedin,
Figure 6. Inhibition of mTOR activity by everolimus prevents CCC cells from acquiring resistance to trabectedin. A–C, in vitro experiments. A, treatment schedule. Trabectedin-sensitive parental cells (RMG1 or RMG2) were seeded into 6-well plates at a density of $10^4$/well. Then, the cells were treated with 1 nmol/L trabectedin and/or 20 nmol/L everolimus in the presence of 5% FBS in accordance with the treatment schedule. The first group was treated with PBS (control). Then, the medium was removed and replaced with normal growing medium. The second group was treated with 20 nmol/L everolimus for 24 hours. Then, the medium was removed and replaced with normal growing medium. The third group was treated with 1 nmol/L trabectedin for 24 hours. Then, the medium was removed and replaced with normal growing medium. The fourth group was treated with 1 nmol/L trabectedin plus 20 nmol/L everolimus for 24 hours. Then, the medium was removed and replaced with normal growing medium. The fifth group was treated with 1 nmol/L trabectedin plus 20 nmol/L everolimus for 24 hours. Then, the medium was removed and replaced with normal growing medium. After 72 hours of incubation, more everolimus was added. All treatments were repeated every 7 days for 7 weeks. B and C (i), cell growth was monitored by weekly cell counting, as described in “Materials and Methods,” and the results are shown. Points, mean; bars, SD (*, significantly different from group 2; **, significantly different from group 3, 4, or 5). (ii), the magnified graphs for groups 3 to 5 are shown. Points, mean; bars, SD (*, significantly different from group 3; **, significantly different from group 4). D, in vivo experiments. Athymic nude mice were s.c. inoculated with RMG1 or RMG2 cells. When the resultant tumors reached a mean size of about 50 mm³, the mice were treated with PBS, 0.2 mg/kg trabectedin once a week, 2.5 mg/kg everolimus 3 times a week, or weekly 0.2 mg/kg trabectedin plus 2.5 mg/kg everolimus 3 times a week, for 4 weeks. i and ii, graphs depicting weekly tumor volumes (mm³) for each treatment group. Points, mean; bars, SD (*, significantly different from the PBS-treated mice; **, significantly different from the trabectedin-treated mice; $P<0.05$).
both RMG1 and RMG2 cells (lanes 1–5). As shown in Figure 5A and B (ii), trabectedin-induced p70S6K phosphorylation in RMG1 and RMG2 cells was inhibited by cotreatment with everolimus or LY294002 (lane 3). Moreover, trabectedin-induced p70S6K phosphorylation in RMG1 and RMG2 cells was inhibited by cotreatment with everolimus and trabectedin [Fig. 5A and B (i), lanes 6–10, and Fig. 5A and B (ii), lane 4], suggesting that trabectedin induces p70S6K phosphorylation via the AKT–mTOR signaling cascade. In contrast, in trabectedin-resistant CCC cells (RMG1-YR and RMG2-YR), treatment with trabectedin did not induce the phosphorylation of AKT, mTOR, or p70S6K [Fig. 5A and B (iii)]. These results indicate that the AKT–mTOR activation induced by trabectedin treatment in trabectedin-sensitive parental cells plays an important role during the acquisition of trabectedin resistance in CCC cells.

We also investigated whether the activation of AKT–mTOR signaling is involved in the mechanism of resistance to trabectedin in SAC cells. As shown, treatment with trabectedin induced prolonged activation of the AKT–mTOR signaling pathway in SKOV3 cells (Supplementary Fig. S1A). Moreover, hyperactivation of AKT–mTOR signaling was also observed in the trabectedin-resistant SKOV3 cells (SKOV3-YR; Supplementary Fig. S1B). These results indicated that the effect of trabectedin on AKT–mTOR signaling is not unique to CCC cells.

Continuous inhibition of mTOR by everolimus prevents CCC cells from acquiring resistance to trabectedin

Given the elevated basal activation of AKT–mTOR signaling found in trabectedin-resistant CCC cell lines (Fig. 4C and D) as well as the prolonged activation of AKT–mTOR signaling induced by trabectedin treatment in trabectedin-sensitive parental cells (Fig. 5A and B), we considered that the inhibition of AKT–mTOR signaling in CCC cells hold promise as a method for overcoming the resistance to trabectedin. Thus, we next examined the effect of mTOR inhibition by everolimus during the acquisition of trabectedin resistance.

Trabectedin-sensitive parental cells (RMG1 or RMG2) were treated with 1 nmol/L trabectedin and/or 20 nmol/L everolimus in accordance with the treatment schedule shown in Figure 6A. On the basis of our in vitro experiment, which showed that the inhibition of mTOR activity by everolimus lasted for 72 hours [Fig. 5A and B (i), lanes 6–10], in the fifth group, everolimus was administered every 3 days for 1 month to continuously inhibit mTOR activity. As shown in Figure 6B, 24 hours of treatment with trabectedin or everolimus significantly decreased the number of viable CCC cells (group 1 vs. group 2 and group 1 vs. group 3); however, after roughly 3 weeks of exposure, the surviving cells started to proliferate again as a result of acquired resistance to trabectedin. When trabectedin was administered in combination with everolimus for 24 hours (group 4), the antitumor activity of trabectedin was significantly enhanced (group 3 vs. group 4); however, this combination chemotherapy failed to prevent the development of trabectedin-resistant cells. However, importantly, when everolimus was administered every 3 days to continuously inhibit the activity of mTOR (group 5), the combination of trabectedin and everolimus resulted in the complete disappearance of CCC cells. These results indicate that the continuous inhibition of mTOR during trabectedin treatment is required to prevent CCC cells from acquiring resistance to trabectedin. We further examined the in vivo growth-inhibitory effect of trabectedin in the setting of combination therapy with everolimus (Fig. 6C). In mice inoculated with RMG1 cells and then treated with PBS, trabectedin, and everolimus, the mean tumor burden was 382.2, 240.7, and 141.5 mm³, respectively. The tumors that developed in the mice treated with a combination of trabectedin and everolimus were very small (mean tumor burden = 48.1 mm³). Similarly, in the mice inoculated with RMG2 cells and then treated with PBS, trabectedin, everolimus, and trabectedin and everolimus, the mean tumor volume was 276.2, 184.0, 87.2, and 32.2 mm³, respectively. Collectively, these results indicate that everolimus enhanced the growth-inhibitory effect of trabectedin in this model.

Discussion

CCC of the ovary was recognized as a distinct histologic subtype of epithelial ovarian tumors by the World Health Organization in 1973 (33). The precise incidence of CCC is unknown, but CCC is the second most frequent histologic subtype in Japan, with an incidence of more than 20%, which is more frequent than the 5% incidence observed in Western countries (34, 35).

In the setting of frontline chemotherapy, the response rate of CCC to conventional platinum-based chemotherapy involving a platinum agent alone or in combination with cyclophosphamide and doxorubicin was reported to be as low as 11%. In contrast, SAC patients displayed a response rate of 72% (3). The efficacy of carboplatin–paclitaxel, the current standard regimen, against CCC has not been fully investigated. A retrospective review of 6 randomized phase III clinical trials showed that patients with stage III CCC who were treated with carboplatin–paclitaxel displayed shorter survival than those with other histologic subtypes of epithelial ovarian cancer (2). However, a recent retrospective analysis suggested that clear cell histology is not associated with inferior survival (4). In the setting of salvage treatment of recurrent CCC after frontline treatment, the response rates of various regimens were extremely low (5). Collectively, these previous findings suggested that CCC is associated with a diminished sensitivity to chemotherapy and a worse prognosis than other epithelial ovarian cancers. To improve survival, the development of new treatment strategies that target CCC more effectively is necessary.

On the basis of a previous preclinical study suggesting that SN-38, an active metabolite of irinotecan, is more effective in CCC cells than any other anticancer agent including cisplatin (32), the activity of irinotecan-based combination chemotherapy had been investigated in

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we found that the percentage of cells in the G1 phase was longer doubling time than SAC cells. Moreover, when the phase (44). As reported previously (45), in the current study, treatment with irinotecan plus cisplatin failed to show its superiority over carboplatin plus paclitaxel with regard to progression-free survival, indicating the urgent need for the development of new antineoplastic agents for patients with CCC of the ovary.

In the current study, we have shown that trabectedin possesses significantly greater antitumor activity than SN38, which was reported to be the most effective agent for the treatment of CCC of the ovary. The in vitro growth-inhibitory effect of trabectedin was greater than those of other agents tested in the current studies (Fig. 1B). We also evaluated the efficacy of trabectedin in vivo by using s.c. xenograft models (Fig. 3). As previously reported, i.v. treatment with trabectedin was well tolerated with no apparent toxicity. In mice that were s.c. inoculated with RMG1 or RMG2 cells, treatment with trabectedin significantly inhibited tumor growth. Collectively, these findings indicate that trabectedin has significant clinical activity as a single agent for CCC in a setting of frontline therapy.

In previous clinical studies of ovarian cancer, trabectedin has usually been administered at concentrations ranging from 1 to 1.5 mg/m² every 3 to 4 weeks (15–17). The maximum serum trabectedin concentration in these patients was reported to be 1.8 ng/mL, which is equivalent or higher than that observed in mice when trabectedin was administered at a dose of 0.2 mg/kg (42, 43). The concentration of trabectedin used in the present in vitro experiments (1–10 nmol/L) has also been achieved in patients in phase 1 clinical trials (43). On the basis of these previous findings, we consider that our treatment schedule is reasonable and clinically achievable.

The reason why CCC cells showed greater sensitivity to trabectedin than SAC cells in the current study remains unknown (Fig. 1A). In a preclinical study, experiments with synchronized cells showed that cancer cells in the G1 phase are more sensitive to trabectedin than cells in the G2 or S phase (44). As reported previously (45), in the current study, the CCC cells used in the current study showed a longer doubling time than SAC cells. Moreover, when the cell-cycle distribution was determined by flow cytometry, we found that the percentage of cells in the G1 phase was significantly higher in the CCC cell lines than in the SAC cells (data not shown). This may explain why CCC cells showed greater sensitivity to trabectedin than SAC cells.

An additional important finding of our study is the significant antitumor activity of trabectedin in cisplatin- and paclitaxel-resistant CCCs (Fig. 2) because the lack of effective chemotherapy for recurrent CCC after frontline platinum-based combination chemotherapy is a major clinical problem in the management of CCC. In the current study, although at lower concentrations (0.1–1 nmol/L), the antitumor effects of trabectedin in the cisplatin- and paclitaxel-resistant CCC cells were slightly milder than those observed in the respective parental cells, at the higher concentration (10 nmol/L), the antitumor effects of trabectedin in the cisplatin- or paclitaxel-resistant CCC cells were similar to those observed in their respective parental cells. Collectively, these results suggest that cisplatin- and paclitaxel-refractory CCCs are also candidates for trabectedin treatment.

In this study, we investigated the role of the AKT–mTOR signaling pathway in trabectedin resistance and found that trabectedin-resistant CCC cell lines exhibit enhanced activation of AKT–mTOR signaling compared with the corresponding trabectedin-sensitive parental cell lines (Fig. 4C and D). Moreover, treatment with trabectedin induced prolonged activation of AKT–mTOR signaling in CCC cells (Fig. 5). To the best of our knowledge, this is the first report to show the involvement of AKT in the mechanism of trabectedin resistance. AKT is known to be involved in chemoresistance to various chemotherapeutic agents (46, 47). As it has been previously reported that the inhibition of AKT activity sensitizes human ovarian cancer cells to conventional anticancer agents (46, 48), we consider that the inhibition of AKT–mTOR signaling holds promise for overcoming trabectedin resistance. As AKT inhibitors have not been convincingly proven to be clinically efficacious, we employed a more clinically practical approach—the use of mTOR inhibitor, which is now approved for the clinical treatment of renal cell carcinoma, 90% of which shows a clear cell histology. In the current study, we employed everolimus as an mTOR inhibitor, as it showed significant in vitro and in vivo antitumor activity toward CCC cells (20). Importantly, treatment with everolimus in combination with trabectedin enhanced the efficacy of trabectedin in our experimental model. Moreover, the continuous inhibition of mTOR by everolimus prevented CCC cells from acquiring resistance to trabectedin (Fig. 6). Because the RMG1-YR and RMG2-YR cells used in this study mimic the clinical situation of resistance development in trabectedin-treated patients, our results suggest that administering trabectedin in combination with mTOR inhibitor is a promising treatment of patients with CCC of the ovary. In addition, the finding that the effect of trabectedin on AKT–mTOR signaling was also observed in SAC cells indicates that treatment with trabectedin in combination with everolimus might be efficacious for epithelial ovarian cancer in general (Supplementary Fig. S1).

We have to recognize the potential weakness of our experimental design; that is, we used a s.c. xenograft model. As peritoneal dissemination is the main process responsible for the progression of human ovarian cancer, the intraperitoneal injection of cancer cells might more accurately model advanced disease. Therefore, further investigation using an intraperitoneal model or a genetically engineered mouse model of ovarian cancer would be useful.

In conclusion, our findings indicate that trabectedin is a promising agent for treating CCC of the ovary both as a frontline treatment and as a salvage treatment of recurrence after platinum- or paclitaxel-based chemotherapy. We believe that our preclinical data provide significant evidence that the selective targeting of the AKT–mTOR pathway with trabectedin in combination with everolimus might be a promising strategy for the treatment of drug-resistant CCC of the ovary.
support for future clinical trials of trabectedin in this patient population.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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The Activity of Trabectedin As a Single Agent or in Combination with Everolimus for Clear Cell Carcinoma of the Ovary

Seiji Mabuchi, Takeshi Hisamatsu, Chiaki Kawase, et al.

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