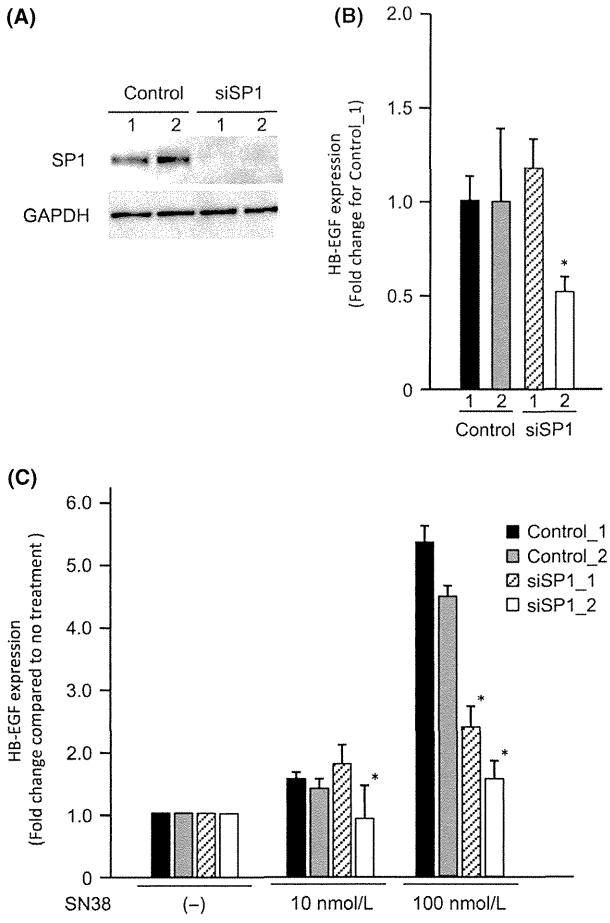

Figure 5. The contribution of SP1 to the HB-EGF induction following the treatment with SN38. (A) A Western blot analysis was performed to confirm the SP1 reduction in the transfected ES-2 cells induced by treatment with siRNAs against SP1 or a control siRNA. (B) The alterations in the HB-EGF mRNA expression in the transfected ES-2 cells induced by treatment with the siRNAs against SP1 or the control siRNA. (C) The induction of HB-EGF mRNA in the RMG-II cells transfected with siRNAs against SP1 or the control siRNA following the treatment of SN38. Each value represents the mean (n = 3) and SD. Closed bars, control_1 siRNA; gray bars, control_2 siRNA; diagonal striped bars, siSP1_1 si RNA; open bars, siSP1_2 siRNA. *P < 0.05, control_1 versus each siRNA. SP1, specific protein 1.

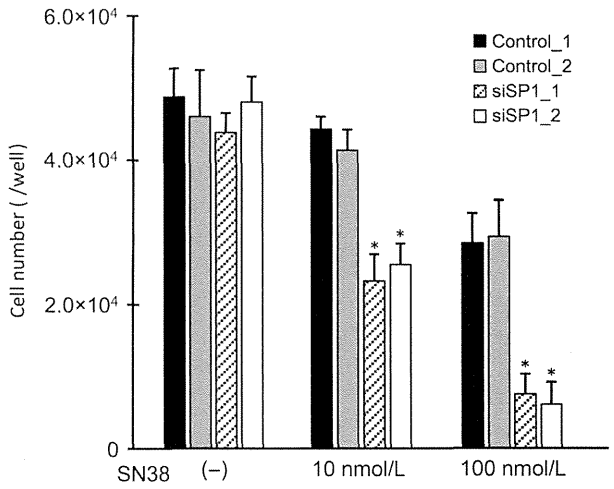

Figure 6. The contribution of SP1 to the cell viabilities following the treatment with SN38. The alterations in the numbers of RMG-II cells transfected with siRNAs against SP1 or a control siRNA after treatment with SN38. Each value represents the mean (n = 3) and SD. Closed bars, control_1 siRNA; gray bars, control_2 siRNA; diagonal striped bars, siSP1_1 si RNA; open bars, siSP1_2 siRNA. *P < 0.05, control_1 versus each siRNA. SP1, specific protein 1.

Additionally, NF-κB induced the expression of HB-EGF, and the NF-κB and SP1 binding sequence was shown to be the same GC-rich element in colon cancer cells [16, 28]. This evidence suggests that SP1 augments the expression of HB-EGF through the deacetylation of SP1 or via an interaction with NF-κB. In mice, SP1 was reported to be directly bound to the promoter regions of HB-EGF [24]. In humans, SP1 also functions as a direct regulator for the expression of HB-EGF, possibly through various posttranslational modifications of SP1 and by interactions with other transcriptional factors.

Dysregulation of SP1 is found in many types of cancer, including ovarian, breast, and gastric cancer, for which HB-EGF is a rational therapeutic target [22, 29, 30]. The hypoxia-inducible factor 2α (HIF2α)–SP1 complex activated coagulation factor VII promoter in OCCC and estrogen receptor α also form complexes with SP1 in other types of ovarian cancer [31, 32]. Additionally, previous reports showed that activation of SP1 promoted breast cancer development and the metastasis of gastric cancer [33, 34]. On the other hand, the SP1 level was highly upregulated in patients with early stage and minimally invasive lung cancer cells and in patients with stage I lung cancer compared to that in lung cancers with high invasiveness and in patients with stage IV lung cancer [35]. These previous findings show that the significance of SP1 involvement in cancer progression have been controversial. In this study, the suppression of SP1 enhanced the sensitivity of OCCC cells to SN38. Therefore, it seems to be important that the suppression of SP1 itself, as well as the inhibition of the posttranslational modifications of SP1, are critical for cancer therapy.

Several compounds that inhibit the transcriptional activity of SP1 have been developed for cancer therapy [25], for example, arsenic trioxide downregulates the expression of SP1 [36]. A phase III trial of arsenic trioxide was performed for patients with acute promyelocytic leukemia classified as having low-to-intermediate risk, and the results suggested that all-*trans*-retinoic acid plus arsenic trioxide may be superior to all-*trans*-retinoic acid

plus other chemotherapy [37]. Bortezomib has been already used for the treatment of patients with multiple myeloma [38, 39]. Bortezomib has been shown to decrease the expression of SP1 and disrupt the interaction of SP1 with NF-κB [40]. Other inhibitory compounds for SP1 will be clinically applied for various diseases, in addition to cancer [41, 42].

We have performed a clinical phase II trial using CRM197 in patients with recurrent and advanced ovarian cancer. To explore the importance of the posttranslational modifications of SP1 in the induction of HB-EGF expression and to search for compounds that can inhibit such modifications, a preclinical study should be performed using combination therapy with CRM197, irinotecan, and a compound that inhibits SP1, such as arsenic trioxide or bortezomib. Such a combination would likely improve the prognosis of patients with OCCC.

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primer sequences.

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ORIGINAL ARTICLE

Efficacy and safety of triple therapy with aprepitant, palonosetron, and dexamethasone for preventing nausea and vomiting induced by cisplatin-based chemotherapy for gynecological cancer: KCOG-G1003 phase II trial

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Abstract
Purpose Prevention of chemotherapy-induced nausea and vomiting (CINV) is crucial for maintaining the quality of life of cancer patients. Female patients have been underrepresented in previous clinical studies of aprepitant or palonosetron. We performed a prospective multicenter study to investigate the efficacy and safety of triple therapy comprising these two agents and dexamethasone in female cancer patients receiving chemotherapy that included cisplatin (≥50 mg/m^2).
Methods Aprepitant was administered at a dose of 125 mg before chemotherapy on day 1 and at 80 mg on days 2 and 3. Palonosetron (0.75 mg) was given before chemotherapy on day 1. Dexamethasone was administered at a dose of 9.9 mg before chemotherapy on day 1 and at 6.6 mg on days 2–4. The

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primary endpoint was the the proportion of patients with a complete response (CR no vomiting and no use of rescue medication) throughout the overall period (0–120 h post-chemotherapy).
Results Ninety-six women (median age 55 years) were enrolled. The overall CR rate was 54.2 %. CR was obtained during the acute phase (0–24 h post-chemotherapy) and the delayed phase (24–120 h post-chemotherapy) in 87.5 and 56.3 % of the patients, respectively. The most common adverse reactions were constipation and fatigue (reported by three patients each).
Conclusions Exhibition of a favorable overall CR rate over existing two-drug combinations suggests that the triple therapy regimen used in the present study is effective and tolerable in patients with gynecological malignancies receiving cisplatin-based chemotherapy. Female patients may have a higher risk of developing CINV.

Keywords Aprepitant · Palonosetron · Nausea · Vomiting · CINV · Cisplatin

Introduction

Chemotherapy-induced nausea and vomiting (CINV) occurs with a high frequency following chemotherapy for cancer and is one of the adverse reactions that causes hardship for patients receiving chemotherapy. Failure to prevent CINV may result in worsening of the physical and mental state of the patient and may even become an obstacle to the continuation of chemotherapy. Thus, prevention or alleviation of CINV is extremely important for maintenance of the quality of life of patients and for continuation of their treatment [1, 2].

Antineoplastic agents cause vomiting via two pathways. In one pathway, enterochromaffin cells of the gastrointestinal mucosa are stimulated by a chemotherapy agent and release 5-hydroxytryptamine (serotonin, 5-HT), a neurotransmitter that activates gastrointestinal 5-hydroxytryptamine type 3 (5-HT_3) receptors and transmits signals to the vomiting center in the lateral reticular formation of the medulla via vagal afferents or via the chemoreceptor trigger zone (CTZ). In the other pathway, a drug directly stimulates the CTZ, and then, signals are transmitted to the vomiting center via dopamine receptors and 5-HT_3 receptors. Furthermore, antineoplastic agents can promote the secretion of substance P in the area postrema and the nucleus solitarius of the medulla oblongata, after which substance P binds to neurokinin-1 (NK-1) receptors and induces vomiting. Attention has recently been paid to this mechanism as a new target for antiemetic therapy [3].

Aprepitant is a selective NK-1 receptor antagonist. Clinical trials of this agent with a new mechanism of action for the prophylaxis for CINV have been undertaken outside Japan, and it has been shown to be effective for both acute CINV and also delayed CINV, which responds poorly to existing medications [4–7]. In Japan, the efficacy of aprepitant was demonstrated in Japanese patients by a phase II trial [8], and authorization for manufacturing/marketing was gained in October 2009.

Palonosetron is a new second-generation 5-HT_3 receptor antagonist that differs from other 5-HT_3 receptor antagonists by showing higher receptor-binding affinity, as well as having an extended half-life of about 40 h (four to five times longer than dolasetron, granisetron, or ondansetron) and an excellent safety profile [9, 10]. In Japan, the efficacy of palonosetron was demonstrated by a randomized, parallel-group, comparative, multicenter study using granisetron hydrochloride as the comparator [11], and manufacturing/marketing authorization was obtained in January 2010.

Combined administration of NK-1 receptor antagonists, 5-HT_3 receptor antagonists, and steroids is recommended for the prevention of CINV associated with the administration of highly or moderately emetogenic antineoplastic agents in the international guidelines for antiemetic therapy issued by the American Society of Clinical Oncology (ASCO), Multinational Association of Supportive Care in Cancer (MASCC), and National Comprehensive Cancer Network (NCCN) [12–14]. In clinical studies of aprepitant or palonosetron, thus far reported in or outside Japan, there have been relatively few female patients, and the efficacy of these drugs for CINV in patients with gynecological cancer has not yet been established. In addition, no information is available in or outside Japan concerning the clinical efficacy of triple therapy with the

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combination of aprepitant, palonosetron, and dexamethasone in patients with gynecological cancer.

Therefore, we planned the present study to evaluate the efficacy and safety of combined therapy with these three agents for prevention of CINV in patients with gynecological malignancies receiving chemotherapy containing cisplatin, a highly emetogenic antineoplastic agent.

Methods

Patients

Patients aged 20 years or older were enrolled if they were scheduled to receive more than one cycle of highly emetogenic chemotherapy at any of ten facilities related to Kansai Clinical Oncology Group (KCOG) between July 1, 2010 and June 30, 2012. All patients had gynecological cancer and were scheduled to receive cisplatin at a dose of 50 mg/m^2 or more. Patients who fulfilled any of the following criteria were excluded from the study: previous cisplatin use, severe hepatic insufficiency (Child-Pugh score >9), pre-enrollment alanine aminotransferase (glutamic-pyruvic transaminase) or aspartate aminotransferase (glutamic-oxaloacetic transaminase) level >3 times the upper limit of normal, pre-enrollment total bilirubin >2 times the upper limit of normal, and pre-enrollment serum creatinine level >1.5 times the upper limit of normal.

Study treatment

Aprepitant was administered orally, with a dose of 125 mg being given at 60–90 min before chemotherapy on day 1 and 80 mg being administered once daily on days 2 and 3. Palonosetron was administered intravenously with a dose of 0.75 mg at 30–60 min before chemotherapy on day 1. Dexamethasone was administered orally or intravenously, with a dose of 9.9 mg being given at 30–60 min before chemotherapy on day 1 followed by 6.6 mg once daily on days 2–4.

Parameters assessed

The primary endpoint of the study was the proportion of patients with a complete response (CR), which was defined as no episodes of vomiting and no rescue therapy for nausea, throughout the study period from 0 to 120 h after cisplatin administration (overall CR rate). The secondary endpoints were the proportion of patients with CR in the acute phase (0–24 h after cisplatin administration) and in the delayed phase (24–120 h after cisplatin administration) of the study, as well as the proportion of patients with complete protection (CP no vomiting, no rescue therapy, and no significant nausea (visual analog scale score <25 mm)) throughout the study and in the acute and delayed phases. The proportion of patients who gave the response "Little or no effect on activities of daily living" when completing the Functional Living Index-Emesis (FLIE) questionnaire on day 6 was also determined to assess the influence on the quality of life (QOL).

Evaluation of safety

Adverse events and laboratory data were compiled according to the Common Terminology Criteria for Adverse Events (version 4).

Statistical analysis

It was calculated that 81 patients were needed to detect a difference of $P \leq 0.05$ (two-sided) with a 90 % power if the CR rate for antiemetic therapy was assumed to be 70 %. By estimating the rate of exclusion from analysis as about 20 %, the target number of subjects for enrollment was set at 100. In the main analysis, the study therapy would be judged to be effective if the proportion of patients with a CR throughout the study period (0–120 h) exceeded the proportion of patients with a CR with standard therapy (dual therapy with a first-generation 5-HT$_3$ receptor antagonists and dexamethasone) in previous reports.

Ethical considerations

The present study was conducted in accordance with ethical principles based on the Declaration of Helsinki and the "Ethical Guidelines for Clinical Studies." It was approved by an appropriate institutional review board and ethics committee at each participating center after assessment of the protocol and written information provided for the patients. All patients gave written informed consent prior to inclusion in the study. This study was registered with the University Hospital Medical Information Network (UMIN) clinical trial registry (no. UMIN000003820).

Results

Patient characteristics

A total of 96 patients were enrolled, and their characteristics are summarized in Table 1. All patients were female and their median age was 55 years (range 32–75 years). They were treated for the following gynecological malignancies: endometrial cancer in 61 patients (63.5 %), cervical cancer in 14 patients (14.6 %), and ovarian cancer in 19 patients (19.8 %). Among them, 49 patients (51.0 %) had a history of morning

Table 1 Patient characteristics

		Number	Percent
Total		96	100.0
Age (years)			
	Median	55	
	Range	32–75	
Performance status			
	0	91	94.8
	1	5	5.2
Gynecological malignancy			
	Endometrial cancer	61	63.5
	Ovarian cancer	19	19.8
	Cervical cancer	14	14.6
	Others	2	2.1
Cisplatin dose			
	≥50 and <60	57	59.4
	≥60 and <70	31	32.3
	≥70	8	8.3
	Mean	56.0	
Chemotherapy regimen			
	Cisplatin/Adriamycin	46	47.9
	Cisplatin/Irinotecan	26	27.1
	Cisplatin/Docetaxel	12	12.5
	Cisplatin/Taxol	7	7.3
	Cisplatin alone	2	2.1
	Cisplatin/5-fluorouracil	2	2.1
	Cisplatin/Doxorubicin	1	1.0
Prior chemotherapy			
	Vomiting—Yes	19	19.8
	Vomiting—No	15	15.6
No prior chemotherapy		62	64.6
Drinking alcohol			
	Yes	17	17.7
	No	79	82.3
Motion sickness			
	Yes	30	31.3
	No	66	68.8
morning sickness			
	Yes	49	51.0
	No	47	49.0

sickness during pregnancy, 30 (31.3 %) had a history of motion sickness, and 17 (17.7 %) drank alcohol. Furthermore, 34 patients (35 %) had received prior anticancer chemotherapy, and 19 patients (19.8 %) had experienced nausea. The mean dosage of cisplatin was 56 mg/cm^2, and the other drug used in combination with cisplatin was adriamycin in 46 patients (47.9 %), irinotecan in 26 patients (27.1 %), and docetaxel in 12 patients (12.5 %).

Table 2 Efficacy data

	Study phase	Percent	95 % confidence interval
CR	Acute	87.5	(79.2–93.4)
	Delayed	56.3	(45.7–66.4)
	Overall	54.2	(43.7–64.4)
CP	Acute	82.3	(73.2–89.3)
	Delayed	45.8	(35.6–56.3)
	Overall	44.8	(34.6–55.3)
No emesis	Acute	90.6	(82.9–95.6)
	Delayed	71.9	(61.8–80.6)
	Overall	71.9	(61.8–80.6)
No rescue therapy	Acute	95.8	(89.7–98.9)
	Delayed	65.6	(55.2–75.0)
	Overall	62.5	(52.0–72.2)
No nausea	Acute	74.0	(64.0–82.4)
	Delayed	33.3	(24.3–44.1)
	Overall	30.2	(21.5–40.8)
No significant nausea	Acute	89.6	(81.7–94.9)
	Delayed	62.5	(52.6–72.8)
	Overall	62.5	(52.6–72.8)

The results for patients with no nausea and with no significant nausea are based on data for 95 patients because there was one omission

CR complete response, *CP* complete protection

Antiemetic effect

The antiemetic effect of study therapy is summarized in Table 2. The overall CR rate, which was the primary endpoint, was 54.2 %, while the acute CR rate and delayed CR rate were 87.5 and 56.3 %, respectively. The proportion of patients with no emesis was 90.6, 71.9, and 71.9 % in the acute phase, delayed phase, and overall, respectively, while the corresponding CP rates were 82.3, 45.8, and 44.8 %. The proportion of patients with no nausea was 74.0, 33.3, and 30.2 % in the acute phase, delayed phase, and overall, respectively. The proportion of patients with no nausea on a daily basis for 5 days after administration of chemotherapy is shown in Fig. 1. Control of CINV was poorest at 4 days after the administration of chemotherapy. The proportion of patients who did not need rescue therapy was 95.8, 65.6, and 62.5 % in the acute phase, delayed phase, and overall, respectively. The proportion of patients who gave the response "Little or no effect on activities of daily living" when completing the FLIE questionnaire on day 6 was determined to assess QOL. It was 82.3 % for the vomiting domain, 43.8 % for the nausea domain, and 59.4 % for the combined nausea/vomiting domain. With regard to the nine items in the nausea domain of the FLIE questionnaire, the impact of nausea on daily activities was greatest for "Ability to enjoy a meal" (Fig. 2).

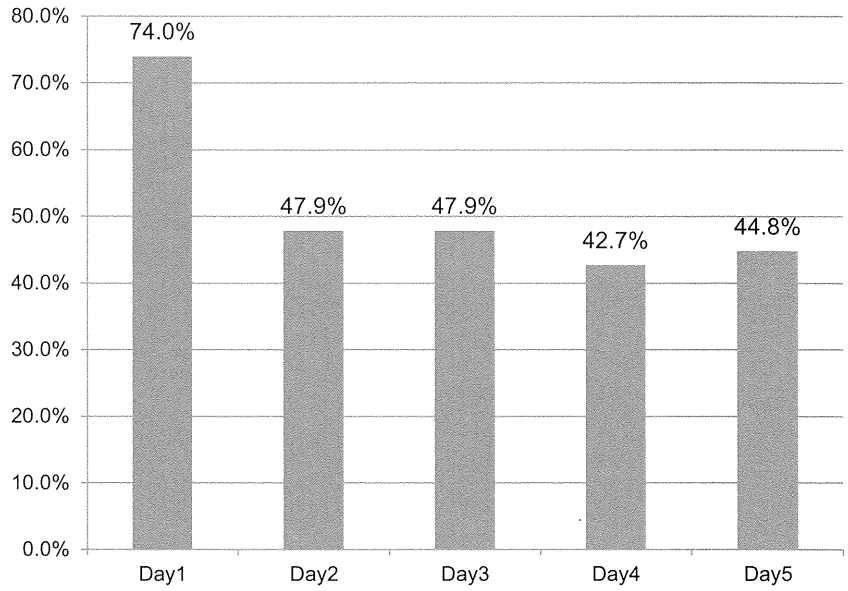

Fig. 1 Proportion of patients with no nausea

Safety

Triple therapy with aprepitant, palonosetron, and dexamethasone showed good tolerability throughout the study period. The most frequently reported adverse reactions were constipation and fatigue, each of which was noted by three patients (3 %).

Discussion

The present multicenter clinical study aimed to evaluate the effect of triple therapy with aprepitant (an NK-1 receptor antagonist), palonosetron (a second-generation 5-HT_3 receptor antagonist), and dexamethasone on CINV in patients with female malignancies who received cisplatin-based chemotherapy. Female sex is known as a risk factor for CINV. In addition, cisplatin is an antineoplastic agent that frequently causes CINV. Accordingly, the subjects of the present study may represent a population of patients in whom CINV would be difficult to control. Cisplatin-containing regimens are classified as highly emetogenic chemotherapy in the guidelines for antiemetic therapy issued by the ASCO [12], MASCC [13], NCCN [14], and Japan Society of Clinical Oncology [15].

In the present study, a CR rate of 54.2 % was achieved by triple therapy with aprepitant, palonosetron, and dexamethasone. This study demonstrated that the triple-agent strategy is

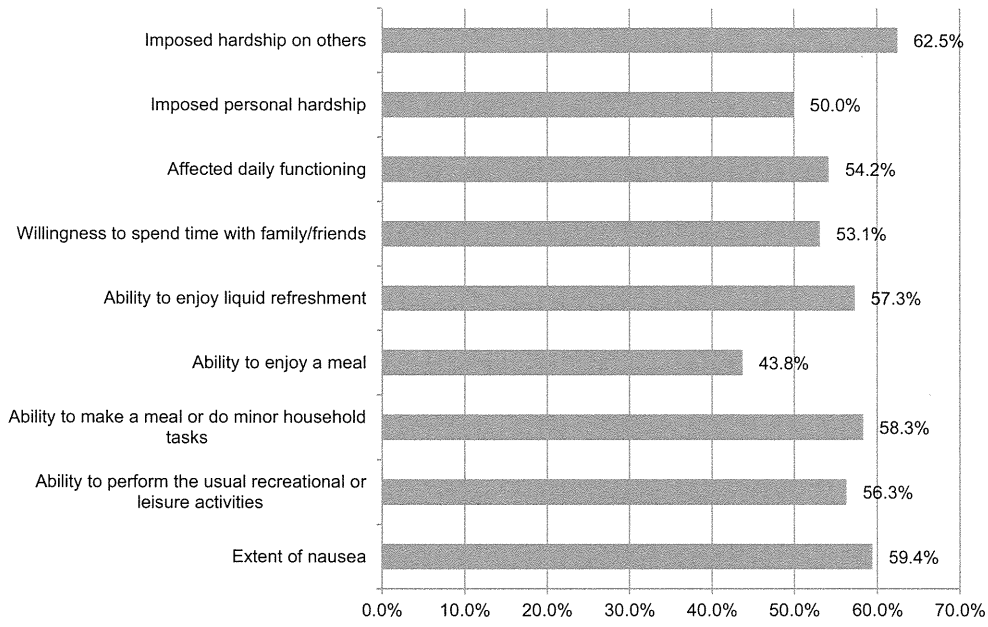

Fig. 2 Nine items in the nausea domain of the Functional Living Index-Emesis questionnaire

Table 3 Comparison of triple therapy regimens

	No. of patients	Percentage of female patients	Cisplatin ≥70 mg/m^2	CR	No emesis	No nausea	No significant nausea	5-HT$_3$ receptor antagonist
Hesketh et al. (2003)	260	48	70 %	73 %	78 %	48 %	73 %	Ondansetron
Poli-Bigelli et al. (2003)	261	37	82 %	63 %	66 %	49 %	71 %	Ondansetron
Schmoll et al. (2006)	243	39	75 %	72 %	77 %	–	73 %	Ondansetron
Takahashi et al. (2011)	149	24	100 %	71 %	77 %	34 %	69 %	Granisetron
Present study	96	100	9 %	54 %	72 %	30 %	63 %	Palonosetron

CR complete response

useful, because it achieved similar CR rates to standard therapy (with a first-generation 5-HT$_3$ receptor antagonist plus dexamethasone) that was used as the comparator in previous clinical trials of aprepitant or palonosetron: Hesketh et al. [4] and Poli-Bigelli et al. [5], respectively, reported a CR rate of 52.3 and 43.3 % with the standard ondansetron-dexamethasone combination. In addition, a CR rate of 40.3 % was reported with the combination of palonosetron and dexamethasone [11]. However, the triple therapy used in the present study achieved a lower CR rate than other triplet regimens, since the CR rate was 72.7, 62.7, and 72.0 % with triple therapy using ondansetron (a first-generation 5-HT$_3$ receptor antagonist) as reported by Hesketh et al. in 2003 [4], Poli-Bigelli et al. in 2003 [5], and Schmoll et al. in 2006 [16], respectively. In addition, a CR rate of 70.5 % for a triplet regimen using granisetron (another first-generation 5-HT$_3$ receptor antagonist) was reported by Takahashi et al. in 2011 (Table 3) [8]. One reason for this difference from other studies may be that our subjects were all female patients. Women are known to be susceptible to CINV, and stratified analysis has shown that dual therapy with a first-generation 5-HT$_3$ receptor antagonist and dexamethasone achieves a lower CR rate in women than in men [17]. In other clinical studies with a lower proportion of women than the present study, the CR rate for triple therapy that included palonosetron as the first-generation 5-HT$_3$ receptor antagonist was reported to be 70.3 % (percentage of women in the study population 23.4 %) by Longo et al. [18] and 81.0 % (percentage of women 23 %) by Miura et al. [19]. Since patients with previous chemotherapy were also enrolled in the present study, an analysis was conducted to compare the subgroups with and without prior chemotherapy, but similar results were obtained (Table 4). Accordingly, although the fact that the subjects of the present study were all women should have an influence, the possibility of other factors cannot be ruled out because it has been reported that the addition of aprepitant to dual therapy with a first-generation 5-HT$_3$ receptor antagonist and dexamethasone can overcome the increased risk of CINV associated with the female gender [17].

In the present study, only 30 % of patients had no nausea throughout the observation period, and adequate control of nausea was not achieved. Rescue medication was administered at the discretion of the attending physician at each participating center because the present study was a multicenter investigation. Accordingly, one reason for the low CR rate may be that rescue therapy was provided without careful consideration of patient's complaints about nausea at some centers. Therefore, different measures should be taken for the control of nausea in future studies.

In another study of patients undergoing highly emetogenic chemotherapy (TRIPLE), comparison was performed between granisetron (a first-generation 5-HT$_3$ receptor antagonist) and palonosetron (a second-generation 5-HT$_3$ receptor antagonist) with basal antiemetic therapy using aprepitant and dexamethasone [20]. The overall CR rate was the primary endpoint, and this showed no statistically significant difference between the two groups. Palonosetron group was significantly superior to the granisetron group with regard to the nausea domain rates for complete control and total control. In view of our present finding that the control of nausea was even poor with

Table 4 Results for patients with and without prior chemotherapy

	No. of patients	Percentage of female patients	Cisplatin ≥70 mg/m^2	CR	No emesis	No nausea	No significant nausea	Prior chemotherapy
Longo et al. (2010)	222	23	98 %	70 %	93 %	60 %	91 %	Chemo-naïve
Miura et al. (2013)	64	23	95 %	81 %	–	54 %	67 %	Chemo-naïve
Present study	96	100	9 %	54 %	72 %	30 %	63 %	Cisplatin-naïve
	62*	100	8 %	55 %	68 %	31 %	63 %	Chemo-naïve

CR complete response

palonosetron, the influence of female gender is considered to be strong.

Recently, the efficacy of olanzapine for controlling nausea has been reported. This agent is one of the multi-acting receptor antipsychotics (MARTA) used for the treatment of schizophrenia, which can block dopamine receptors, serotonin receptors, histamine receptors, adrenergic receptors, and other receptors associated with CINV. Olanzapine was recently reported to be effective for preventing CINV based on its mechanism of action [21]. Therefore, concomitant use of olanzapine is another option that is available.

In order to achieve further improvement of the control of CINV, several additional treatments are likely to be introduced. Since nausea often reaches a peak at 4 days after the administration of anticancer agents (Fig. 2), treatment with aprepitant for 5 days is also likely to be a useful option. Furthermore, administration of dexamethasone for a period of up to 5 days is recommended by guidelines established in Japan, and this regimen is also available.

The present study was the first to investigate the usefulness of triple therapy with aprepitant, palonosetron, and dexamethasone for prevention of CINV in Japanese patients with gynecological cancer receiving cisplatin-containing chemotherapy. This triple therapy was ascertained to be effective compared with the current standard therapy using first-generation 5-HT_3 receptor antagonists and dexamethasone. Nevertheless, the preventive effect of triple therapy tended to be weaker in the present study than that reported previously. All of the subjects were female and therefore may have been predisposed to develop CINV, but the reason is unclear. Control of delayed nausea is an important measure against CINV in the gynecology field. Thus, it may be necessary for medical and co-medical staff, including pharmacists and nurses, to conduct further follow-up of patients.

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Conflict of interest The authors have no conflicts of interest to declare.

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The PI3K/mTOR dual inhibitor NVP-BEZ235 reduces the growth of ovarian clear cell carcinoma

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Abstract. Patients with clear cell carcinoma of the ovary (OCCC) have poor survival due to resistance to standard chemotherapy. OCCC has frequent activating mutations of the *PIK3CA* gene. The present study was conducted to clarify the efficacy of the inhibition of the PI3K-AKT-mTOR pathway in OCCC. We used 8 OCCC cell lines and 5 ovarian serous adenocarcinoma (OSAC) cell lines. The mutation status of the *PIK3CA* and *KRAS* genes was examined by direct sequencing. The IC_{50} values of NVP-BEZ235 (BEZ235) and temsirolimus were determined by WST-8 assay. Protein expression levels of PI3K-AKT-mTOR pathway molecules were examined by western blotting. Cell cycle distribution was analyzed by flow cytometry. Annexin V staining was used for detecting apoptosis. We also investigated the effects of BEZ235 on OCCC tumor growth in a nude mouse xenograft model. Four of the 8 OCCC cell lines showed a *PIK3CA* mutation while none of the 5 OSAC cell lines showed a mutation. The IC_{50} values of BEZ235 for the OCCC cell lines were lower than these values for the OSAC cell lines. The IC_{50} value of temsirolimus was higher than BEZ235 in the OCCC cell lines. The *PIK3CA* mutation was more frequently noted in OCCC than OSAC cells, but the sensitivity of these cell lines to BEZ235 or temsirolimus was not related to the mutation status. pHER3 and pAkt proteins were expressed more frequently in OCCC compared with OSAC. However, protein expression levels were distributed widely, and were not related to the sensitivity. Treatment with BEZ235 suppressed expression of pAkt, although treatment with temsirolimus did not. OCCC cells exhibited G_1 phase arrest after treatment with BEZ235 and apoptosis with a higher concentration of the agent. BEZ235 significantly inhibited tumor growth in mice bearing OVISE and TU-OC-1 cell tumors. The present study indicated that the PI3K-AKT-mTOR pathway is a potential target for OCCC, and that BEZ235 warrants investigation as a therapeutic agent.

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Abbreviations: PI3K, phosphatidylinositol 3-kinase; PDK1, phosphatidylinositol-dependent kinase 1; mTOR, mammalian target of rapamycin; OCCC, ovarian clear cell carcinoma; OSAC, ovarian serous adenocarcinoma

Key words: clear cell carcinoma, PI3K, mTOR, ovarian carcinoma

Introduction

Clear cell carcinoma of the ovary (OCCC) is recognized in the World Health Organization classification of ovarian tumors as a distinct histological entity. Its clinical behavior is distinctly different from other epithelial ovarian cancers (1). OCCC accounts for 3.7-12.1% of epithelial ovarian cancers (2,3). We found that response rates for platinum-based chemotherapy were 11.1% for OCCC and 72.5% for serous adenocarcinoma (SAC), suggesting that OCCC resists conventional platinum-based chemotherapy (4). A novel therapeutic strategy is needed to improve the prognosis of patients with OCCC.

PIK3CA is located at the 3q26.3 locus and encodes the catalytic subunit of the phosphatidylinositol 3-kinase (PI3K), p110α (5). In response to an extracellular signal, the activated p110α phosphorylates PIP2 to generate PIP3. The PIP3 recruits AKT to the plasma membrane, where it is phosphorylated and activated by phosphatidylinositol-dependent kinase 1 (PDK1) and PDK2. Activated AKT can directly activate the mammalian target of rapamycin (mTOR) by phosphorylation at Ser2448. mTOR is a serine/threonine kinase that acts as an effector in the PI3K/Akt pathway. Aberrations of the PI3K pathway are frequently present in many different types of cancer. A number of studies have shown amplification or mutations of the *PIK3CA* gene in ovarian cancers (6-8). AKT and mTOR are also hyperactivated in ovarian cancer (9,10). Additionally, a high frequency of activating mutations of *PIK3CA* has been observed in OCCC (11).

NVP-BEZ235 is an imidazoquinoline derivative that potently and reversibly inhibits class 1 PI3K and mTOR catalytic activity by competing at its ATP-binding site (12). It has been demonstrated to reduce tumor growth in several xenograft models and is currently in clinical trials (12-14). The present study was conducted to clarify the efficacy of NVP-BEZ235 treatment on OCCC.

Materials and methods

Cell lines and cell cultures. Eight human OCCC cell lines (OVISE, SMOV-2, KK, TU-OC-1, OVTOKO, KOC-7c, RMG-I and OVMANA) and five OSAC cell lines (KF, KOC-2s, TU-OS-3, TU-OS-4 and SHIN-3) were used. Cells were obtained as follows: OVISE and OVTOKO from Dr Hiroshi Minaguchi (Yokohama City University, Yokohama, Japan); SMOV-2 from Dr Tomohiro Iida (St. Marianna University, Kawasaki, Japan); KK and KF from Dr Yoshihiro Kikuchi (National Defense Medical College, Tokorozawa, Japan); KOC-7c and KOC-2s from Dr Toru Sugiyama (Kurume University, Kurume, Japan); RMG-I from Dr Shiro Nozawa (Keio University, Tokyo, Japan); and SHIN-3 from Dr Yasuhiko Kiyozuka (Nara Medical University, Kashihara, Japan). TU-OC-1, TU-OS-3, and TU-OS-4 cells were established by our department (15,16). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 at 37˚C.

Mutation screening. Screening for mutations was performed as previously described (17). Genomic DNA was purified from all cell lines using a DNeasy Tissue kit (Qiagen, Valencia, CA, USA). PCR primers used to amplify the sequence of interest (exons 9 and 20 of the *PIK3CA* gene, exons 2 and 3 of the *KRAS* gene) were the same as reported in the literature (18,19). DNA was amplified in reactions of 30 sec at 94˚C; 30 sec at 55˚C; followed by 90 sec at 72˚C for 30 cycles. Then, PCR products were subjected to sequencing using BigDye Terminator v3.1 Cycle Sequencing kit and an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems Foster City, CA, USA).

Reagents. NVP-BEZ235 and temsirolimus were purchased from LC Laboratories (Woburn, MA, USA). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at -20˚C for the *in vitro* experiments. The drugs were diluted in fresh medium immediately before each experiment. In all the experiments, the final DMSO concentration was <0.1%.

Dose-response studies. The cytotoxicities of NVP-BEZ235 and temsirolimus were assessed by the WST-8 assay using Cell Counting Kit-8 (Dojindo Laboratories, Tabaru, Japan) as previously described (17). Cells ($2\text{-}4x10^3$ cells/80 μl) were seeded into each well of a 96-well tissue culture plate, cultured overnight, and then treated with 20 μl of NVP-BEZ235 or temsirolimus solution at a final concentration of 0.001, 0.01, 0.1, 1 or 10 μM for 72 h. After that, 20 μl of Cell Counting Kit-8 solution was added to each well, and the plates were incubated for another 1-2 h. Absorbance was measured at 450 nm with a microplate reader (iMark Microplate Absorbance Reader). Cell viability was calculated as the percentage of cells killed by the treatment. All experiments were conducted in triplicate. Median inhibitory concentrations were determined from these calculations.

Western blot analysis. Cells were washed twice with ice-cold PBS. Cell pellets were then lysed in a buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4 and protease inhibitors (Complete Protease Inhibitor Cocktail Tablets; Roche Diagnostics)] as previously described (17). Protein concentrations were measured against a standardized control using a protein assay kit (Bio-Rad Laboratories). A total of 50 mg protein was separated by electrophoresis on a 5-20% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore). The antibodies were as follows: rabbit anti-erbB3 antibody (C17) (diluted 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-β-actin (AC-40) antibody (1:1,000; Sigma-Aldrich, St. Louis, MO, USA); and anti-phospho-erbB3 (Tyr1289) (21D3) antibody (1:1,000), rabbit anti-AKT antibody (1:1,000), rabbit anti-phospho-AKT (Ser473) antibody (1:500), rabbit anti-mTOR antibody (1:500), rabbit anti-phospho-mTOR (Ser2448) antibody (1:500), rabbit anti-p70S6K antibody (1:500), rabbit anti-phospho-p70S6K (Thr389) antibody (1:500), rabbit anti-4E-BP1 antibody (1:1,000) and rabbit anti-phospho-4E-BP1 (Thr37/49) antibody (1:1,000) (all from Cell Signaling Technology (Danvers, MA, USA). Signals were detected with secondary anti-mouse or anti-rabbit immunoglobulin G antibody coupled with horseradish peroxidase, using an Ez-Capture II chemiluminescent imaging system (ATTO, Tokyo, Japan).

Cell cycle distribution analysis. Cell cycle distribution was analyzed by flow cytometry. Briefly, cells were plated in a 6-well plate, cultured overnight, and then treated with NVP-BEZ235 or left untreated for 48 or 72 h (final concentration of 10 or 100 nM). Floating and adherent cells were fixed overnight in ice-cold 70% ethanol. The cells were then resuspended in PBS containing propidium iodide (PI, 25 μg/ml) supplemented with 0.1% RNase A and incubated at 37˚C for 30 min. DNA content was measured with a FACSCalibur flow cytometer with CellQuest software (Becton-Dickinson, Franklin Lakes, NJ, USA). Cell fit analysis determined the percentage of the cell count in a specific phase of the cell cycle.

Annexin V staining. The Annexin V-FITC Apoptosis Detection kit (BioVision, Mountain View, CA, USA) was used to assess apoptosis as the externalization of phosphatidylserine residues, according to the specifications of the manufacturer. Briefly, cells were suspended in 500 ml of 1X binding buffer. The cells then were stained with 5 ml Annexin V-FITC (fluorescein isothiocyanate) and 5 ml PI (50 mg/ml) for 5 min in the dark at room temperature. Finally, the cells were analyzed with a flow cytometer (FACSCalibur; Becton-Dickinson).

Ovarian cancer xenograft model. OVISE or TU-OC-1 cells in log-phase growth were trypsinized, washed twice with PBS and centrifuged at 250 x g. For subcutaneous tumor development, $4x10^6$ viable cells (in 0.1 ml of PBS) were injected subcutaneously under aseptic conditions into female athymic mice. Seven days after the injection, we confirmed the development of measurable tumors, and then treatment was initiated with NVP-BEZ235 at doses of 25 or 50 mg/kg/day, and continued for 3 weeks. Mice treated with vehicle (10% 1-methyl-2-pyrrolidone-90% polyethylene glycol 300) were used as the control group. All agents were administered by oral gavage. Ten mice were used in each experimental group. The tumor volume was measured with a caliper twice weekly. The body weight of mice was also measured twice weekly.

Table I. Characteristics of the OCCC and OSAC cell lines.

Cell line	Original tumor	*KRAS*		*PIK3CA*		IC_{50} of BEZ235 (nM)
		Exon 2	Exon 3	Exon 9	Exon 20	
OVISE	Clear cell carcinoma	wt	wt	wt	wt	44
SMOV-2	Clear cell carcinoma	wt	wt		3141 A>A/T	65
KK	Clear cell carcinoma	wt	wt	1634 A>A/C	wt	74
TU-OC-1	Clear cell carcinoma	wt	wt	1624 G>G/A	wt	131
OVTOKO	Clear cell carcinoma	wt	wt	wt	wt	534
KOC-7c	Clear cell carcinoma	wt	wt	wt	wt	600
OVMANA	Clear cell carcinoma	wt	wt	1634 A>T	wt	641
RMG-I	Clear cell carcinoma	wt	wt	wt	wt	777
KF	Serous adenocarcinoma	wt	wt	wt	wt	779
KOC-2s	Serous adenocarcinoma	wt	wt	wt	wt	989
TU-OS-3	Serous adenocarcinoma	wt	wt	wt	wt	1,004
TU-OS-4	Serous adenocarcinoma	wt	wt	wt	wt	3,951
SHIN-3	Serous adenocarcinoma	34 G>A	wt	wt	wt	25,400

OCCC, ovarian clear cell carcinoma; OSAC, ovarian serous adenocarcinoma; wt, wild-type.

Statistical analysis. Statistical analyses were performed with Prism version 5 (GraphPad Software Inc., San Diego, CA, USA). Data are presented as means ± 1 standard error. Means for all data were compared by one-way analysis of variance with *post hoc* testing or by unpaired t-test. A P-value of <0.05 was considered to indicate a statistically significant result.

Results

Identification of PIK3CA and KRAS mutations in OCCC and OSAC cell lines. We first screened the mutation status of *PIK3CA* (exons 9 and 20) and *KRAS* (exons 2 and 3) in the 8 OCCC and 5 OSAC cell lines. Four out of the 8 OCCC cell lines showed a *PIK3CA* mutation while none of the 5 OSAC cell lines showed the mutation (Table I). One of the 5 OSAC cell lines showed a *KRAS* mutation (34G>A) while none of the 8 OCCC cell lines showed this mutation.

Sensitivity to NVP-BEZ235 or temsirolimus. The IC_{50} values of NVP-BEZ235 in the OCCC cell lines were lower than these values in the OSAC cell lines (Table I). In the OCCC cell lines, the IC_{50} of temsirolimus was higher than that of BEZ235 (Table II). Although the *PIK3CA* mutation was more frequently noted in OCCC than OSAC, the sensitivity of these cell lines to NVP-BEZ235 or temsirolimus was not related to the mutation status.

Expression levels of PI3K-Akt-mTOR pathway molecules in the OCCC and OSAC cell lines. Comparison of the OCCC and OSAC cell lines showed that pHER3 and pAkt expression was more frequent in OCCC than OSAC (Fig. 1A). That is, 7 of the 8 OCCC cell lines expressed pHER3 whereas 2 of the 5 OSAC cells lines exhibited expression. Similarly, 6 of the 8 OCCC cell lines expressed pAkt while 2 of the 5 OSAC cell lines did. The protein expression levels were distributed widely, and did not relate to the sensitivity to NVP-BEZ235 or temsirolimus.

Table II. IC_{50} of temsirolimus in the OCCC cell lines.

Cell line	IC_{50} (nM)	
	BEZ235	Temsirolimus
OVISE	44	9,122
SMOV-2	64	8,924
KK	74	5,929
TU-OC-1	131	7,224
OVTOKO	534	12,776
KOC-7c	600	9,779
OVMANA	641	17,650
RMG-I	777	4,045

When OVISE cells were treated with NVP-BEZ235, expression levels of p-p70S6K and p4E-BP1 were suppressed in a dose-dependent manner (Fig. 1B). Treatment with temsirolimus incompletely suppressed p-p70S6K and p4E-BP1 expression in the OVISE cells. Moreover, treatment with NVP-BEZ235 suppressed pAKT expression, while treatment with temsirolimus did not. Similar results were observed in the KK cells (Fig. 1C).

NVP-BEZ235 induces G_1 phase arrest and apoptosis in OCCC cells. OVISE cells were arrested at the G_1 phase, but did not exhibit apoptosis (denoted by an increased proportion of cells in sub-G_1), after 72 h of treatment with 10 and 100 nM NVP-BEZ235 (Fig. 2A). We observed similar results of G_1 arrest in the KK cells (Fig. 2A). Although the same conditions as those in the cell cycle analysis did not induce apoptosis,

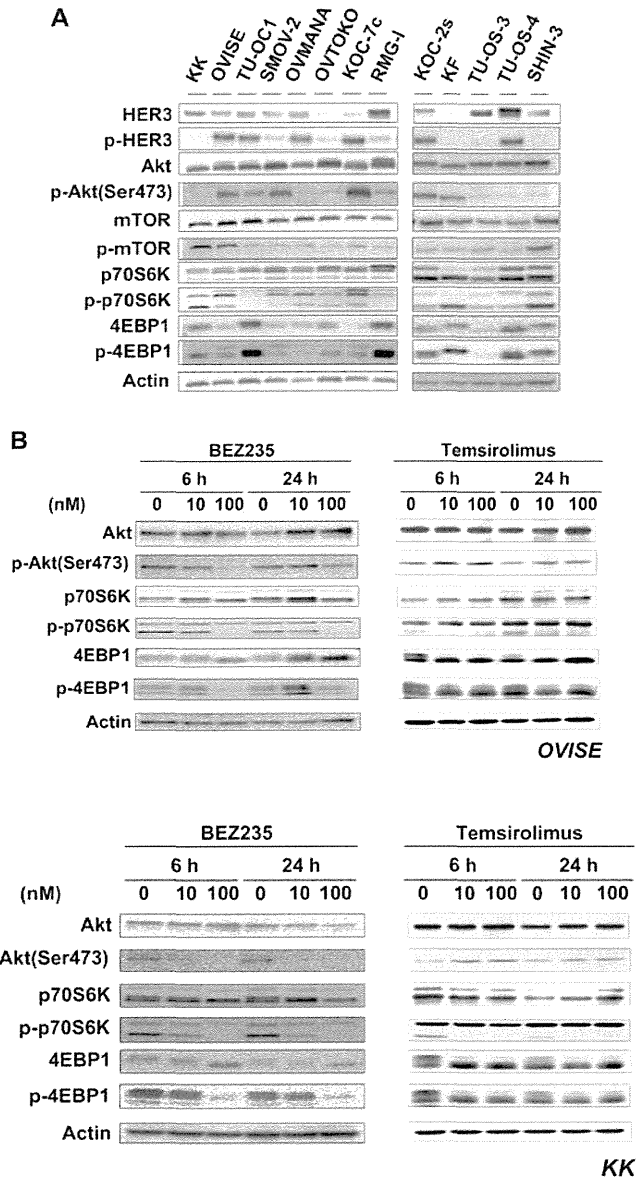

Figure 1. (A) Baseline expression of PI3K-Akt-mTOR pathway molecules in the OCCC and OSAC cell lines. Eight OCCC cell lines (KK, OVISE, TU-OC-1, SMOV-2, OVMANA, OVTOKO, KOC-7c and RMG-I) and 5 OSAC cell lines (KOC-2s, KF, TU-OS-3, TU-OS-4 and SHIN-3) were cultured in DMEM/F12 medium with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37°C. Western blot analysis was performed to detect the expression levels of HER3, p-HER3, Akt, p-Akt, mTOR, p-mTOR, p70S6K, p-p70S6K, 4E-BP1 and p-4E-BP1. β-actin was used as a loading control. Each experiment was repeated 3 times independently. (B and C) NVP-BEZ235 suppressed pAkt expression in OCCC cells. Two OCCC cell lines (OVISE and KK) were plated in 6-well plates. The protein samples were collected after treatment with 10 and 100 nM NVP-BEZ235 or temsirolimus for 6 or 24 h. Western blot analysis was performed to detect Akt, p-Akt, p70S6K, p-p70S6K, 4E-BP1 and p-4E-BP1 expression. β-actin was used as a loading control.

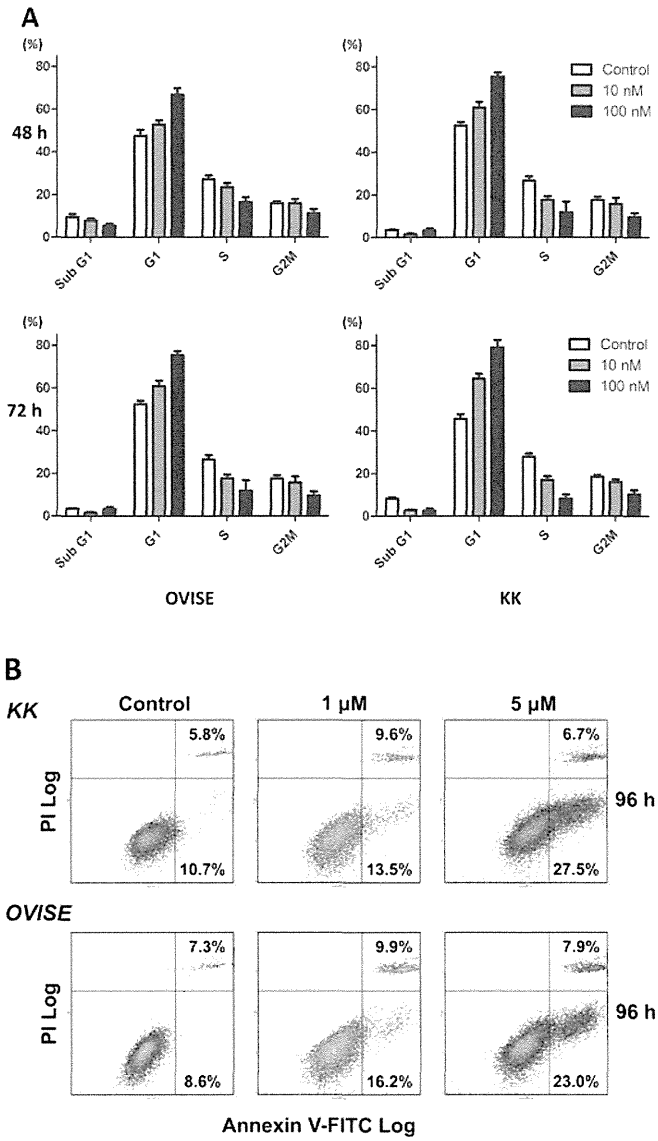

Figure 2. (A) NVP-BEZ235 induced G_1 phase arrest in the OCCC cells. OVISE or KK cells were treated with 10 or 100 nM NVP-BEZ235 for 48 or 72 h. FACScan analysis was performed to detect the cell cycle distribution. (B) NVP-BEZ235 induced apoptosis in the OCCC cells. OVISE and KK cells were treated with 1 or 5 μM NVP-BEZ235 for 96 h. Apoptosis was determined by the Annexin V-FITC Apoptosis Detection kit. Early apoptotic cells were scored as Annexin V-FITC-positive and propidium iodide (PI)-negative to exclude necrotic cells.

treatment of OVISE cells with 1 or 5 μM of NVP-BEZ235 for 96 h increased the number of Annexin V-positive and PI-negative cells (Fig. 2B). Similar results were observed in the KK cells (Fig. 2B).

NVP-BEZ235 suppresses tumor growth in an OCCC xenograft model. To assess short-term systemic toxicity of the agent, we recorded body weight changes of mice in addition to visual observation. After treatment, no mice had detectable changes in body weight, implying that there was no severe toxicity (Fig. 3A). At doses of 25 or 50 mg/kg/day, NVP-BEZ235 significantly inhibited subcutaneous tumor growth in mice bearing OVISE cells ($P<0.05$ for 25 mg/kg/day, $P<0.01$ for 50 mg/kg/day) (Fig. 3B). TU-OC-1 tumor volume in the 50 mg/kg/day group was significantly lower than that of the vehicle control although that in the 25 mg/kg/day group was not ($P<0.01$ for 50 mg/kg/day) (Fig. 3C).

Discussion

Many authors have reported poorer prognoses for patients with advanced stage OCCC (4,20,21). Low survival rates in OCCC

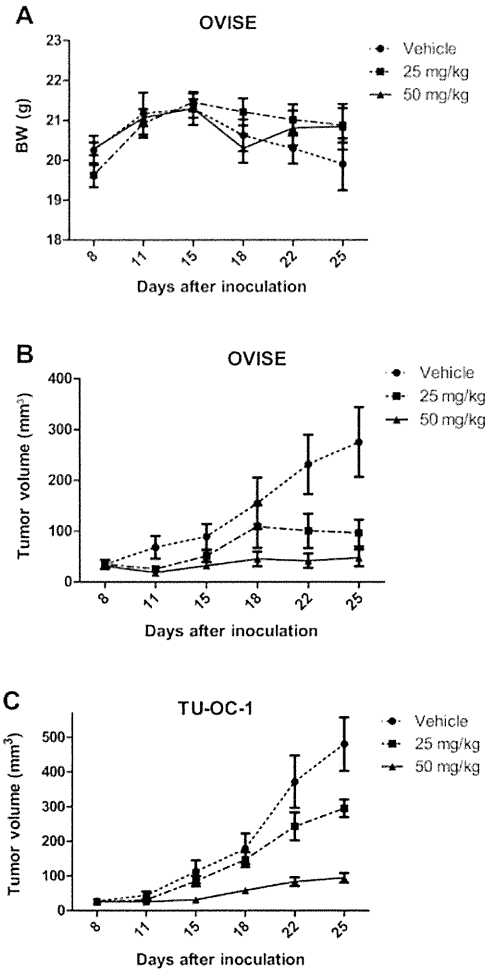

Figure 3. NVP-BEZ235 inhibits tumorigenicity in an OCCC xenograft model without producing toxic effects. Female athymic nude mice were injected subcutaneously with $4x10^6$ OVISE or TU-OC-1 cells. The experimental groups were treated with vehicle control (10% 1-methyl-2-pyrrolidone-90% polyethylene glycol 300), or 25 or 50 mg/kg/day NVP-BEZ235 for 3 weeks beginning 7 days after cell injection. (A) Mean body weight in each treatment group. (B and C) The tumor volume in OVISE or TU-OC-1 xenografts was measured with a caliper twice weekly. Error bars represent the standard error.

may, in part, reflect its lack of sensitivity to platinum-based chemotherapy. There are no antineoplastic agents definitely active and effective to treat OCCC. Therefore, novel therapeutic strategies, including targeted therapy, are needed to improve the prognosis of patients with OCCC.

It is known that mutations of *PIK3CA* are common molecular genetic alterations identified in OCCC (11). Expression of phospho-mTOR was found to be more prominent in OCCC than in OSAC (22). mTOR exists in two distinct complexes, mTOR complex 1 (mTORC1) and mTORC2. The downstream targets of mTORC1 are p70 ribosomal S6 kinase 1 (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), both of which are crucial to the regulation of protein synthesis.

In practice, inhibitors of mTORC1, such as temsirolimus and everolimus, have been used for renal cell carcinoma. A phase II study (GOG268) combining temsirolimus with carboplatin and paclitaxel following temsirolimus consolidation as first-line therapy is underway in patients with OCCC (23). However, the efficacy of mTORC1 blockade may be attenuated due to the loss of an mTORC1-dependent negative feedback loop on PI3K signaling and the mTORC2-mediated activation of Akt (24). p70S6K inhibits insulin receptor substrate 1 (IRS-1) by phosphorylating it, by inducing it to degrade and by altering its localization (25,26). The inhibition of IRS by p70S6K attenuates PI3K-AKT activation. Rapamycin (and its analogs temsirolimus and everolimus) stops this negative feedback loop from the p70S6K to the PI3K signaling pathway, resulting in activation of proliferative and prosurvival effectors such as AKT.

NVP-BEZ235 is a dual pan-class I PI3K and an mTOR kinase inhibitor that has the possible advantage of inhibiting PI3K, mTORC1 and mTORC2. Therefore, it should turn off this pathway completely and overcome feedback inhibition that is normally observed with mTORC1 inhibitors (e.g. rapamycin analogs). It is known that NVP-BEZ235 displays significant antitumor activities in glioblastoma, lung, breast, renal cell and uterine endometrial carcinomas (12,14,13,27).

In the present study, IC_{50} of temsirolimus was markedly higher than NVP-BEZ235 in all OCCC cell lines. In contrast, NVP-BEZ235 effectively suppressed proliferation of OCCC cells. Additionally, treatment with temsirolimus increased expression of pAKT while p-p70S6K and p4E-BP1 were suppressed. Treatment with NVP-BEZ235 suppressed pAkt, p-p70S6K and p4E-BP1. Accordingly, NVP-BEZ235 may be the more effective agent.

We found that NVP-BEZ235 suppressed tumor growth in an OCCC xenograft model. A few authors have reported on the antitumor activity of this compound in ovarian carcinoma. Montero *et al* (28) showed that NVP-BEZ235 effectively suppressed proliferation of 4 ovarian carcinoma cell lines which were not derived from OCCC. Santiskulvong *et al* (29), investigated the *in vivo* effects of NVP-BEZ235 on an immunocompetent transgenic murine ovarian endometrioid adenocarcinoma model. They also examined *in vitro* activity of the compound in 17 human ovarian carcinoma cell lines including 2 OCCC cell lines (ES-2 and OV207). Unfortunately, these studies did not focus on OCCC. Recently, Rahman *et al* (30) investigated the frequency of *PIK3CA* mutations in patients with OCCC and the relationship between the mutations and clinicopathological or biological variables. They also examined the *in vitro* sensitivity of 9 OCCC cell lines to LY294002, temsirolimus and NVP-BEZ235. No relationship was observed between the mutation status and sensitivity to these inhibitors. We also examined the mutation status of *PIK3CA* and *KRAS* genes and baseline protein expression levels of the PI3K/Akt/mTOR pathway molecules. Although the *PIK3CA* mutation was more common in OCCC than in OSAC in our series, there were no relationships between the mutation status or protein expression levels and sensitivity to NVP-BEZ235. These findings supported those of a previous report (30).

Our results revealed that NVP-BEZ235 effectively suppressed not only p-p70S6K and p4E-BP1, but also pAKT expression in OCCC cell lines and suppressed tumor growth in an OCCC xenograft model. This is the first report to demonstrate the efficacy of NVP-BEZ235 in OCCC.

We conclude that the PI3K-AKT-mTOR pathway is a potential therapeutic target for OCCC and that NVP-BEZ235 warrants investigation as a therapeutic agent.

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Dual Inhibition of Phosphatidylinositol 3′-Kinase and Mammalian Target of Rapamycin Using NVP-BEZ235 as a Novel Therapeutic Approach for Mucinous Adenocarcinoma of the Ovary

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Abstract: Ovarian mucinous adenocarcinoma (MAC) resists standard chemotherapy and is associated with poor prognosis. A more effective treatment is needed urgently. The present study assessed the possibility of molecular-targeted therapy with a novel dual inhibitor of phosphatidylinositol 3′-kinase (PI3K) and mammalian target of rapamycin (mTOR), NVP-BEZ235 (BEZ235) to treat of MAC. Seven human MAC cell lines were used in this study. The sensitivity of the cells to BEZ235, temsirolimus, and anticancer agents was determined with the WST-8 assay. Cell cycle distribution was assessed by flow cytometry, and the expression of proteins in apoptotic pathways and molecules of the PI3K/Akt/mTOR signaling pathways was determined by Western blot analysis. We also examined the effects of BEZ235 on tumor growth in nude mice xenograft models. The cell lines showed half-maximal inhibitory concentration values of BEZ235 from 13 to 328 nmol/L. Low half-maximal inhibitory concentration values to BEZ235 were observed in MCAS and OMC-1 cells; these 2 lines have an activating mutation in the *PIK3CA* gene. NVP-BEZ235 down-regulated the protein expression of phosphorylated (p-) Akt, p-p70S6K, and p-4E-BP1, suppressed cell cycle progression, up-regulated the expression of cleaved PARP and cleaved caspase 9, and increased apoptotic cells. Synergistic effects were observed on more than 5 cell lines when BEZ235 was combined with paclitaxel or cisplatin. The treatment of mice bearing OMC-1 or RMUG-S with BEZ235 significantly suppressed tumor growth in MAC xenograft models without severe weight loss. We conclude that the PI3K/Akt/mTOR pathway is a potential therapeutic target and that BEZ235 should be explored as a therapeutic agent for MAC.

Key Words: PI3K, mTOR, NVP-BEZ235, Ovarian mucinous adenocarcinoma, Molecular-targeted therapy

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Mucinous adenocarcinoma of the ovary (MAC) is the third most common type of epithelial ovarian cancer (EOC), comprising 10% to 12% of EOC.[1–3] Mucinous adenocarcinoma of the ovary seems to have a clinical behavior distinct from other EOC. Several studies have shown that MAC often is diagnosed at an early stage, and therefore, confers a relatively good prognosis. However, advanced MAC has a poorer prognosis than other histopathologic subgroups.[4–6] Poor response (13% to 42%) to conventional platinum- or taxane-based chemotherapy is associated with poor prognosis because chemosensitivity is one of the main prognostic factors for patients with advanced EOC.[4–9] Thus, novel treatment strategies (eg, incorporation of molecular-targeted agents) for advanced MAC are needed.

The phosphatidylinositol 3′-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway plays a key role in cellular metabolism, proliferation, survival, and motility.[10] This pathway is hyperactivated by several mechanisms, such as deletion or decreased function of phosphatase and tensin homolog (PTEN), activating mutations of PI3K or Akt, and activating receptor tyrosine kinases (eg, epidermal growth factor receptor, human epidermal growth factor receptor 2).[10,11] Activating this cascade is indicated to relate drug resistance and poor prognosis in many cancers including EOC.[10,12] The frequent mutation of *PIK3CA* has been reported in clear cell carcinoma (33%) and endometrioid adenocarcinoma (20%) of the ovary, and this cascade is thought to be a potential therapeutic target.[13,14] Several studies have shown that inhibiting the Akt/mTOR signaling pathway is one of these promising therapeutic targets in clear cell carcinoma of the ovary.[15,16] Therefore, we focused on this cascade to treat MAC. In MAC, overexpressed proteins of the epidermal growth factor receptor family and activated downstream signaling of Akt/mTOR have been found in 48% of tumors and were associated with poor patient outcomes.[17,18] This pathway could be an attractive target for therapy for MAC.

Recently, a novel imidazoquinoline derivative, NVP-BEZ235 (BEZ235), was developed and has entered clinical trials.[19–21] NVP-BEZ235 is an orally bioavailable agent that targets the ATP-binding clefts of the class 1 PI3K and mTOR kinases, thereby inhibiting the activity of PI3K, mTOR complex (mTORC) 1, and mTORC2. Several studies have shown potential antitumor activities by BEZ235 in vitro and in vivo in a variety of cancers, including EOC.[19,22–25] However, the effects of a dual inhibitor of PI3K and mTOR have not been evaluated systematically in MAC. We therefore conducted the present study to assess the possibility of molecular-targeted therapy with BEZ235 to treat MAC.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The 7 human MAC cell lines used in this study were obtained as follows: JHOM-1 and JHOM-2B from Riken BioResource Center cell bank (Tsukuba City, Ibaraki, Japan); MCAS from Health Science Research Resources Bank (Chiyoda-ku, Tokyo, Japan); OMC-1 from Dr Tsuyoshi Saito (Sapporo Medical University, Sapporo, Japan); and RMUG-L and RMUG-S from Dr Daisuke Aoki (Keio University, Tokyo, Japan). TU-OM-1 was established by our department. These cell lines were maintained in DMEM/Ham's F-12 medium (Wako Pure Chemical Industries, Ltd) with 10% fetal bovine serum, 100 IU/mL penicillin, and 50 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C.

Dose-Response Studies

The sensitivity of the cell lines to BEZ235 (LC Laboratories) and temsirolimus (LC Laboratories) was determined by a cytotoxicity assay using Cell Counting Kit-8 (Dojindo Laboratories). Briefly, cells were diluted with culture medium to a seeding density of 3 to 5 × 10^4/mL, plated on 96-well tissue culture plates at 180 μL per well, and incubated at 37°C for 24 hours. The next day, the cells were treated continuously with 20 μL of various concentrations of the agents to obtain a dose-response curve for each agent. The concentration for each agent was 1 to 10,000 nmol/L. After being incubated for 72 hours, 20 μL of Cell Counting Kit-8 solution was added to each well, and the plates were incubated for another 1 to 2 hours. Absorbance was measured at 450 nm with a microplate reader (iMark Microplate Absorbance Reader; Bio-Rad). Inhibition of cell growth was calculated as the percentage of viable cells compared with the percentage in untreated cultures.

Dose-Effect Analysis

NVP-BEZ235 was combined with each of the different anticancer agents at a fixed ratio that spanned the individual half-maximal inhibitory concentrations (IC_{50}) of each drug. The IC_{50} was determined from dose-effect curves created by a cytotoxicity assay. Median effect plot analyses and calculated combination indices (CI) were analyzed by the method of Chou and Talalay.[26] CalcuSyn software (Biosoft) was used to analyze data from the cytotoxicity assays in which cells were exposed to agents alone or combined with the anticancer drugs and BEZ235. CalcuSyn provided a measure of the combined agents in an additive or synergistic manner. We used the Chou and Talalay[26] definition of CI as synergistic (CI < 0.9), additive (0.9 < CI < 1.1), or antagonistic (CI > 1.1).

Mutational Analysis

Mutation screening for *PIK3CA* was performed at exons 9 and 20 and *K-Ras* at exons 2 and 3, covering the mutational hot spots in human cancers. These exons of *PIK3CA* and *K-Ras* were amplified using polymerase chain reaction (PCR) for genomic DNA. The primers for PCR and sequencing were prepared according to previous reports.[27,28]

The PCR conditions were as follows: 1 cycle at 94°C for 5 minutes, 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90 seconds, followed by 1 cycle at 72°C for 7 minutes. The PCR products subsequently were subjected to direct sequencing PCR with BigDye terminator v3.1 cycle sequencing reagents (Applied Biosystems). The samples were finally analyzed on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems) with Sequence Scanner Software v1.0 (Applied Biosystems).