

Preclinical Activity of Plitidepsin Against Clear Cell Carcinoma of the Ovary

RYUTA MIYAKE, SHOICHIRO YAMANAKA, SHO MATSUBARA and SEIJI

MABUCHI

Department of Obstetrics and Gynecology, Nara Medical University, Nara, Japan

Correspondence to: Seiji Mabuchi, Department of Obstetrics and Gynecology, Nara

Medical University, 840 Shijocho, Kashihara, Nara, 634-8521 Japan. Tel: +81 744223051,

Fax: +81 744236557, e-mail: smabuchi@naramed-u.ac.jp

Running title: Plitidepsin in Ovarian Clear Cell Carcinoma

Abstract

Background/Aim: To evaluate the antitumor effects of Plitidepsin against clear cell carcinoma (CCC) of the ovary.

Materials and Methods: The expression of eEF1A2 in ovarian cancer was assessed by immunohistochemistry. Using ovarian CCC cell lines, the antitumor effect of Plitidepsin was assessed both in vitro and in vivo. By overexpressing or knocking down the eEF1A2 expression, we investigated the role of eEF1A2 in the sensitivity of CCC cells to Plitidepsin.

Results: Immunoreactivity to eEF1A2 was observed in 76.2% of CCC, which was significantly higher than other histological subtypes of ovarian cancer. Plitidepsin exhibited significant antitumor activity toward chemo-naïve and chemo-resistant CCC cells both in vitro and in vivo. Ectopic expression of eEF1A2 in CCC cells resulted in increased sensitivity to Plitidepsin. In contrast, eEF1A2 knockdown decreased sensitivity of CCC cells to plitidepsin.

Conclusion: Plitidepsin, a novel anti-cancer agent that targets eEF1A2, may be a promising agent for treating ovarian CCC.

Key Words: Plitidepsin, ovarian cancer, clear cell carcinoma, eEF1A2.

Clear cell carcinoma (CCC) of the ovary is known to be less sensitive to platinum-based frontline chemotherapy, and advanced-stage CCC is known to be associated with a worse prognosis than the more common histological subtype of serous adenocarcinoma (SAC). The lack of an effective chemotherapy for recurrent CCC is another important clinical problem (1). Therefore, novel strategies for both first-line treatment for advanced-stage CCC and salvage treatment for recurrent disease are needed.

Plitidepsin (Aplidin®) is a novel anti-cancer agent currently investigated in late phases of clinical development. In 2018, the Australian regulatory agency approved Plitidepsin in combination with dexamethasone for the treatment of multiple myeloma. Plitidepsin was originally isolated from the Mediterranean tunicate *Aplidium albicans* in 1988; currently it is chemically synthesized by Pharma Mar (Madrid, Spain) (2). According to previous preclinical studies, Plitidepsin is active against a wide range of malignancies: hematological malignancies such as multiple myeloma, lymphoma, and leukemia, and solid tumors including non-small-cell lung carcinoma, pancreas, breast, melanoma, sarcoma, gastric, and bladder cancer (3). Importantly, most reports demonstrated *in vitro* activity of Plitidepsin in a low nanomolar range. Accumulating evidence have suggested that Plitidepsin induces cell cycle arrest and apoptosis in a dose-dependent manner by inducing oxidative stress, decreasing intracellular levels of glutathione, upregulating the

JNK and p38 MAPK pathways. In addition to cytotoxic and cytostatic activities, Plitidepsin is known to inhibit tumor-angiogenesis through the inhibition of vascular endothelial growth factor (VEGF) secretion from cancer cells. In ovarian cancer, Plitidepsin exhibited anti-proliferative activity *in vitro*, and significantly inhibited the growth of a xenograft in athymic mice. However, since most ovarian cancer cell lines used in the previous preclinical studies of Plitidepsin were derived from ovarian SAC, the therapeutic potential of Plitidepsin against ovarian CCC is unknown.

The translation factor eEF1A2 is a tissue-specific variant of the eukaryotic Elongation Factor 1. The expression of eEF1A2 is normally confined to muscles and neurons (4). However, eEF1A2 is frequently over-expressed in various human malignancies and has oncogenic properties. Anand *et al.* ((5)) were the first to show that eEF1A2, while not normally expressed in ovary, is expressed in 30% of ovarian tumors. When examined according to histological subtypes, eEF1A2 was highly expressed in clear cell ovarian tumors compared to other histological subtypes of ovarian cancer (6). A recent study demonstrated that approximately 75% of ovarian CCC showed over-expression of eEF1A2 at the protein level (7).

Although detailed mechanisms of action have not been completely investigated, Plitidepsin has been suggested to exert its antitumor activity by directly interacting with

eEF1A2. Thus, plitidepsin might be a potential drug candidate for the treatment of CCC showing over-expression of eEF1A2.

In the current study, after investigating the expression rate of eEF1A2 in ovarian cancer specimens, we evaluated the *in vitro* and *in vivo* therapeutic efficacy of Plitidepsin as a single agent against both chemo-naïve and chemorefractory ovarian CCC cells.

Materials and Methods

Patients and clinical samples

This study was approved by the Ethics Committee of Nara Medical University, and the analysis of the patient-derived data was carried out in accordance with the Declaration of Helsinki.

The study involved screening of surgically treated patients with clinical stage III or IV ovarian cancer at Nara Medical University Hospital from April 2013 to March 2019 from our institutional tumor registry. The patient-derived formalin-fixed paraffin-embedded tissues were analyzed by immunohistochemistry. Appropriate informed consent was obtained from each patient.

Reagents and antibodies

Plitidepsin was obtained from PharmaMar (Madrid, Spain). Cisplatin was purchased from Sigma-Aldrich (St Louis, MO, USA). Antibodies recognizing β -actin were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against poly(ADP-ribose) polymerase (PARP) and cleaved PARP were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies recognizing eEF1A2 were purchased from Abcam (Cat No. Ab212171, Tokyo, Japan). Anti-rabbit and anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell culture

The human ovarian CCC cell line KOC-7C was kindly provided by Dr. H. Itamochi (Tottori University, Tottori, Japan), TOV-21G was purchased from the American Type Culture Collection (ATCC, Virginia, USA), RMG-I was purchased from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). Cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM Ham's F-12; Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin solution (Fujifilm, Tokyo, Japan). All cell lines were cultured in a humidified incubator at 37°C with 5% CO₂.

Clone selection

TOV-21G, KOC-7C and RMG-I cells were transfected in 6-well tissue culture plates with 1 µg of eEF1A2 expression vector (Sino Biological Cat No. HG14510-UT, Beijing China) and control vector (pCMV6) using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Clonal selection was performed by adding Hygromycin (Thermo Fisher Scientific, Waltham, MA, USA).

Establishment of cisplatin-resistant cell lines

Cisplatin-resistant CCC sublines (TOV-21G-CR, KOC-7C-CR and RMG-I-CR) derived from CCC cells (TOV-21G, KOC-7C and RMG-I) were developed in our laboratory by continuous exposure to Cisplatin (up to 5.0 µmol/l), as described previously (8).

Cell proliferation assay

For MTS cell growth assay, equal number of CCC cells were plated into the 96 well plates and exposed to different concentrations of Plitidepsin. After 48 h of treatment, MTS reagent was added and cell proliferation was assessed according to the manufacturer's protocol (Promega, Madison, WI, USA). The absorbance at 490 nm was used for the

evaluation of cell proliferation rate.

Western blot analysis

CCC cells treated with Plitidepsin or Control were lysed in cell lysis buffer, and resolved on a gradient gel. Protein was transferred on a nitrocellulose membrane and blocked in 5% milk powder and 0.2% Tween-20 in PBS. Membranes were probed with primary antibody, washed and incubated with horseradish peroxidase (HRP) labeled secondary antibody and developed using enhanced chemiluminescence substrate (Perkin Elmer, Massachusetts, USA). Primary antibodies used were anti-eEF1A2 (1:1,000), anti-PARP (1:1,000), anti-cleaved-PARP (1:1,000) or anti- β -actin (1:10,000) antibodies.

In vivo tumor studies

Animal studies were approved by the Animal Care and Use Committee of Nara Medical University (Nara, Japan), and were performed in accordance with institutional and National Institutes of Health guidelines under an approved protocol.

To investigate the anti-tumor activity of Plitidepsin against ovarian CCC, TOV-21G cells (5×10^6) were injected subcutaneously into nude mice (6-week-old, n=12). Cells were injected in the left flanks of mice in a volume of 200 μ l of PBS. When the tumors

reached about 50 mm³ in size, the following treatments were initiated: the first group (n=6) was intraperitoneally (i.p.) administered PBS, and the second group (n=6) was i.p. administered Plitidepsin (0.08 mg/kg) 5 days a week for 2 weeks. Caliper measurements were taken once a week and tumor volumes were calculated using the following formula:

$$V = L (\text{length}) \times W (\text{width}) \times D (\text{depth}) \times \pi / 6.$$

RNA interference

Small interfering RNA (siRNA) specifically targeting eEF1A2 and a non-targeting control siRNA were purchased from Thermo Fisher Scientific (Cat. No. AM16708). TOV-21G, KOC-7C, and RMG-I cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen).

Immunohistochemistry

Tumors were fixed in 10% neutral buffered formalin, embedded in paraffin, and sections were cut at 4- μ m thickness. Immunohistochemical (IHC) staining was performed as described previously (8). The primary antibody used was an anti-eEF1A2 antibody (1:250). Histoscore was calculated with the following formula; Histoscore = 1 \times proportion of cells with low staining + 2 \times proportion of cells with moderate staining + 3

proportion of cells with strong staining, as described previously (9).

Cell Cycle Analysis

Cells were incubated in FBS-free medium overnight, then cultured with or without 10 nM Plitidepsin in the presence of 10% FBS for 16 h. After washing with PBS, they were fixed with 70% ethanol and stained with propidium iodide (50 µg/ml) in the presence of RNase A (100 µg/ml; Cat. No.313-01461; Nippon Gene Co., Ltd, Tokyo, Japan). Cell cycle distribution was determined by analyzing 10,000 cells using a FACScan flow cytometer and Cell Quest software (Becton Dickinson, San Jose, CA, USA), as previously described (8).

Statistical analysis

Cell proliferation was analyzed by Wilcoxon's exact test. The tumor volume of the Plitidepsin-treated mice was compared with that of the PBS-treated mice and analyzed using the Wilcoxon's exact test. Histoscore was compared with Mann–Whitney-test. *p* value of less than 0.05 was considered significant.

Results

eEF1A2 expression in CCCs and other histological subtypes

Immunohistochemical analysis of eEF1A2 expression was performed using ovarian cancer tissue samples obtained from patients (21 with CCC, 19 with SAC, 10 with endometrioid adenocarcinoma (EMA), and 8 with mucinous adenocarcinoma (MAC)), and the representative photographs are shown in Figure 1A. As shown in Figure 1B, stronger immunoreactivity for eEF1A2 was observed in CCC and SAC than in the other histological subtypes. The positivity for eEF1A2 was 90.5% in CCC and 89.5% in SAC (Table 2). When CCC was compared with SAC, the frequency of strong eEF1A2 immunoreactivity was significantly higher, and the frequency of tumors with no immunoreactivity was significantly lower in CCC than in SAC. These results indicate that CCCs may be more strongly dependent on eEF1A2 expression for tumor progression than other histological subtypes.

In vitro growth-inhibitory effect of Plitidepsin in CCC cell lines

Considering the frequent eEF1A2 expression observed in human CCC tumor specimens (Figure 1), we evaluated the expression of eEF1A2 in the three human CCC cell lines KOC-7C, RMG-I, TOV-21G by western blotting. As shown in Figure 2A, eEF1A2 was expressed in all CCC cell lines, which is consistent with the immunohistochemical results

observed with tumor samples.

We next examined the efficacy of Plitidepsin on CCC cell proliferation *in vitro*. As shown in Figure 2B, treatment with Plitidepsin for 48 h inhibited the proliferation of CCC cells in a dose-dependent manner. The IC_{50} values obtained for each cell line are listed in Table I. We next investigated the mechanism by which Plitidepsin inhibits CCC cell proliferation. As shown in Figure 2C, the percentage of CCC cells in the G1 phase was significantly increased after 16 h of treatment with 10 nM Plitidepsin. Moreover, treatment of CCC cells with 10 nM Plitidepsin induced cleavage of PARP in CCC cells (Figure 2D), suggesting that Plitidepsin can induce both cell cycle arrest in the G1 phase and apoptosis of CCC cells.

Significance of eEF1A2 expression in the sensitivity of cells to Plitidepsin

To investigate the role of eEF1A2 expression in the sensitivity of CCC cells to Plitidepsin, we first established CCC cell lines stably transfected with a eEF1A2-expressing vector or empty vector (Figure 2A). When these cell lines were treated with Plitidepsin, CCC cell lines stably transfected with the eEF1A2-expressing vector showed significantly higher sensitivity compared to those transfected with the empty vector (Figure 3B, Table I). We next performed siRNA-based knockdown of the eEF1A2

expression in CCC cells (Figure 3A). As shown, knocking-down of eEF1A2 expression in CCC cells resulted in decreased sensitivity to Plitidepsin (Figure 3B). Collectively, these results indicate the significant role of eEF1A2 expression in the sensitivity of CCC cells to Plitidepsin.

Activity of Plitidepsin in cisplatin-resistant CCC cells in vitro

We first established cisplatin-resistant CCC cells by the continuous cisplatin exposure (Figure 4A). Using these cisplatin-resistant cell lines, we next investigated eEF1A2 expression by western blotting. As shown in Figure 4B, the expression of eEF1A2 in cisplatin-resistant CCC cells was equivalent to that in their respective parental cells. Consistent with this, treatment with Plitidepsin inhibited the proliferation of cisplatin-resistant CCC cells in a dose-dependent manner. The antitumor effects of Plitidepsin on cisplatin-resistant CCC cells were equivalent to those observed in their respective parental cells (Figure 4C).

In vivo growth-inhibitory effects of Plitidepsin on ovarian CCC

By using an s.c. xenograft model of TOV-21G, we next examined *in vivo* growth-inhibitory effect of Plitidepsin. Overall, drug treatment was well tolerated throughout the

study and did not cause any apparent toxicities. As shown in Figure 5, the mean TOV-21G-derived tumor burden in the mice 2 weeks after treatment with Plitidepsin for 2 weeks was 221.1 mm³, whereas it was 438.0 mm³ in the PBS-treated mice. Collectively, treatment with Plitidepsin decreased the TOV-21G-derived tumor burden by 17.3%, compared with that in PBS-treated mice.

Discussion

Accumulating evidence have suggested that Plitidepsin exerts its anti-tumor activity by directly targeting eEF1A2 (10). In an investigation using cervical, lung and gastric cancer cells, Losada A *et al.* demonstrated reduced eEF1A2 expression in Plitidepsin-resistant cancer cells, and ectopic expression of eEF1A2 in Plitidepsin-resistant cancer cells restored their sensitivity to Plitidepsin (10). These results strongly indicate that Plitidepsin has therapeutic efficacy against cancer cells that exhibit increased eEF1A2 expression.

In the current study, we observed that eEF1A2 was expressed in 90.5% of ovarian CCC. The expression rate of eEF1A2 in our study is consistent with a previous study demonstrating that eEF1A2 expression is associated with clear cell histology (7). Consistent with previous reports, the expression rate of eEF1A2 in CCC was greater than that in other histological subtypes of ovarian cancer (Table I). Previous *in vitro* and *in vivo* studies demonstrated that eEF1A2 has oncogenic properties and eEF1A2 has been regarded as a potential therapeutic target (5, 11). Thus, eEF1A2 and the process of protein elongation may play significant roles in the progression of ovarian CCC. Notably, the anti-proliferative activity of Plitidepsin was associated with the extent of eEF1A2 expression. As shown in Figure 3, ectopic expression of eEF1A2 in CCC cells resulted in

increased sensitivity to Plitidepsin, and knockdown of eEF1A2 restored sensitivity to Plitidepsin. Similar results have been reported previously: in previous *in vitro* investigations using HeLa (cervical cancer cells), NCI-H460 (NSCLC cells), and HGC27 (gastric carcinoma cells) cells and their Plitidepsin-resistant sublines, Plitidepsin-resistant cells showed significantly reduced levels of eEF1A2 protein compared to their respective parental cells (10). Moreover, in an experiment using HeLa cells, the ectopic expression of eEF1A2 increased the sensitivity to plitidepsin (10). Based on these findings, we consider that Plitidepsin is a potential drug candidate for the treatment of ovarian CCC that shows increased eEF1A2 expression.

We demonstrated that Plitidepsin exhibited significant anti-tumor activity against ovarian CCC in a low nanomolar range by inducing both cell cycle arrest in the G1 phase and apoptosis. In addition, Plitidepsin significantly inhibited the growth of CCC-derived tumors in mice without causing any apparent toxicity. The IC_{50} value of Plitidepsin against ovarian CCC observed in the current study (2.51-4.97 nM) was lower than those of other existing anticancer agents reported in previous studies: IC_{50} values of paclitaxel, 5-20 nM; cisplatin, 20-30 mM; irinotecan, 0.2-0.4 mM, and Doxorubicin 50-80 nM (12). According to previous clinical studies, the peak plasma concentration of Plitidepsin was 45 nM-72 nM (13), which is higher than that employed in the current *in vitro* and *in vivo*

experiments, indicating the rationale and clinical effectiveness of Plitidepsin in the treatment of ovarian CCC. Collectively, these findings may indicate that Plitidepsin exhibits significant clinical activity as a single agent against CCC in the setting of frontline therapy.

An additional important finding of our study is the significant antitumor activity of Plitidepsin in cisplatin-resistant CCC, because the lack of effective chemotherapy for recurrent CCC after frontline platinum-based combination chemotherapy is a major problem in the clinical management of CCC. In the current study, although the antitumor effects of Plitidepsin as a single agent in the cisplatin-resistant CCC cells were slightly milder than those observed in the respective parental cells, Plitidepsin treatment resulted in significant anti-proliferative affect in these cells. Chemotherapy-induced myelosuppression has been a clinical problem that can limit the dose intensity of chemotherapy for recurrent ovarian cancer. However, considering the facts that no bone marrow toxicity was observed in the clinical trials of Plitidepsin (although myalgia or muscle weakness have been frequently observed) and the promising anti-proliferative activity against cisplatin-resistant CCC cells *in vitro*, plitidepsin can also be an active treatment of patients with recurrent CCC developed after cisplatin treatment.

Interestingly, recent investigations have suggested that agents that target the eukaryotic

translation machinery exhibit potent antiviral activities against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (14). Since Plitidepsin, which targets eEF1A2, has demonstrated a more potent (27.5-folds) antiviral activity against SARS-CoV-2 than remdesivir (15), it has gained attention as a repurposed drug, and clinical trials of Plitidepsin are currently underway in patients suffering from coronavirus disease 2019 (COVID-19) (15). If ovarian cancer patients receiving conventional chemotherapy suffer from COVID-19, cytotoxic chemotherapy must be discontinued until recovery, due to likelihood of severe myelosuppression. However, due to its mild toxicity, there is a possibility that Plitidepsin-based anti-cancer treatment can be continued as dual antiviral and anti-cancer treatment in patients suffering from COVID-19 and CCC.

We have to recognize the limitations of the current study. First is our experimental design; *i.e.*, we used a s.c. inoculated xenograft model whereas peritoneal dissemination is the most common cause of the progression of human ovarian cancer. Second, although it has been generally accepted that combination chemotherapy exhibits greater antitumor activity than single agent chemotherapy against ovarian cancer, we did not investigate other agents that can be combined with Plitidepsin. Thus, further preclinical investigations of Plitidepsin-based combination chemotherapy or studies involving an intraperitoneal model or a genetically engineered mouse model of ovarian CCC are

required.

In conclusion, we demonstrated that Plitidepsin, a novel anti-cancer agent that targets eEF1A2, exhibits antitumor activity against chemonaive and cisplatin-resistant CCC, that frequently expresses high levels of eEF1A2. Our preclinical data provide significant scientific support for future clinical trials of Plitidepsin in patients with ovarian CCC.

Conflicts of Interest: The Authors declare that no conflicts of interests exist in relation to this study.

Authors' Contributions: Shoichiro Yamanaka revised and helped to draft the manuscript, Sho Matsubara carried out cell culture study and performed the statistical analysis, Seiji Mabuchi participated the design of the study. All authors read and approved the final manuscript.

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References

1. Mabuchi S, Sugiyama T and Kimura T: Clear cell carcinoma of the ovary:

molecular insights and future therapeutic perspectives. *J Gynecol Oncol* 27(3): e31, 2016.

PMID: 27029752. DOI: 10.3802/jgo.2016.27.e31

2. Muñoz-Alonso MJ, González-Santiago L and Martínez T, et al.: The mechanism of action of plitidepsin. *Curr Opin Investig Drugs* 10(6): 536–542, 2009. PMID: 19513942.

3. Alonso-Álvarez S, Pardal E and Sánchez-Nieto D, et al.: Plitidepsin: design, development, and potential place in therapy. *Drug Des Devel Ther* 11: 253–264, 2017. PMID: 28176904. DOI: 10.2147/DDDT.S94165

4. Knudsen SM, Frydenberg J and Clark BF et al.: Tissue-dependent variation in the expression of elongation factor-1 alpha isoforms: isolation and characterisation of a cDNA encoding a novel variant of human elongation-factor 1 alpha. *Eur J Biochem* 215(3): 549–554, 1993. PMID: 8354261. DOI: 10.1111/j.1432-1033.1993.tb18064.x

5. Anand N, Murthy S and Amann G, et al. Protein elongation factor EEF1A2 is a putative oncogene in ovarian cancer. *Nat Genet* 31(3): 301–305, 2002. PMID: 12053177. DOI: 10.1038/ng904

6. Schwartz DR, Kardia SLR and Shedden KA, et al. Gene expression in ovarian cancer reflects both morphology and biological behavior, distinguishing clear cell from other poor-prognosis ovarian carcinomas. *Cancer Res.* 62(16): 4722–4729, 2002. PMID:

12183431.

7. Tomlinson V a. L, Newbery HJ and Bergmann JH, et al. Expression of eEF1A2 is associated with clear cell histology in ovarian carcinomas: overexpression of the gene is not dependent on modifications at the EEF1A2 locus. *Br J Cancer*. 96(10):1613–1620, 2007. PMID: 17437010. DOI: 10.1038/sj.bjc.6603748
8. Mabuchi S, Kawase C and Altomare DA, et al. mTOR is a promising therapeutic target both in cisplatin-sensitive and cisplatin-resistant clear cell carcinoma of the ovary. *Clin Cancer Res*. 15(17): 5404–5413, 2009. PMID: 19690197. DOI: 10.1158/1078-0432.CCR-09-0365
9. Alotaibi FT, Peng B and Klausen C, et al. Plasminogen activator inhibitor-1 (PAI-1) expression in endometriosis. *PLoS One*. 14(7): e0219064, 2019. PMID: 31315131. DOI: 10.1371/journal.pone.0219064
10. Losada A, Muñoz-Alonso MJ and García C, et al. Translation Elongation Factor eEF1A2 is a Novel Anticancer Target for the Marine Natural Product Plitidepsin. *Sci Rep*. 6: 35100, 2016. PMID: 27713531. DOI: 10.1038/srep35100.
11. Lee M-H, Choi BY and Kundu JK, et al.. Resveratrol suppresses growth of human ovarian cancer cells in culture and in a murine xenograft model: eukaryotic elongation factor 1A2 as a potential target. *Cancer Res*. 69(18): 7449–7458, 2009. PMID:

19738051. DOI: 10.1158/0008-5472.CAN-09-1266.

12. Yokoi E, Mabuchi S and Shimura K, et al. Lurbinectedin (PM01183), a selective inhibitor of active transcription, effectively eliminates both cancer cells and cancer stem cells in preclinical models of uterine cervical cancer. *Invest New Drugs*. 37(5): 818–827, 2019. PMID: 30374654. DOI: 10.1007/s10637-018-0686-6.

13. Izquierdo MA, Bowman A and García M, et al. Phase I clinical and pharmacokinetic study of plitidepsin as a 1-hour weekly intravenous infusion in patients with advanced solid tumors. *Clin Cancer Res*. 14(10): 3105–3112, 2008. PMID: 18483378. DOI: 10.1158/1078-0432.CCR-07-1652.

14. Gordon DE, Jang GM and Bouhaddou M, et al. A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. *Nature*. 583(7816): 459–468, 2020. PMID: 32353859. DOI: 10.1038/s41586-020-2286-9.

15. White KM, Rosales R and Yildiz S, et al. Plitidepsin has potent preclinical efficacy against SARS-CoV-2 by targeting the host protein eEF1A. *Science*. 371(6532): 926–931, 2021. PMID: 33495306. DOI: 10.1126/science.abf4058.

Figure Legends

Figure 1. Expression of eEF1A2 in ovarian cancer assessed by immunohistochemical staining. A, representative photographs showing eEF1A2 expression in ovarian tissue specimens of clear cell carcinoma (CCC), serous adenocarcinoma (SAC), endometrioid adenocarcinoma (EMA), and mucinous adenocarcinoma (MAC). Magnifications: $\times 400$. B, histogram indicating immunoreactivity profiles of the four histological subtypes, with the immunoreactivity for eEF1A2 being the strongest in CCC.

Figure 2. eEF1A2 expression in ovarian cancer cells. A, eEF1A2 expression levels in three ovarian CCC cell lines. Three CCC cells and their sublines stably transfected with a eEF1A2 vector or control vector were harvested, and the expression of eEF1A2 was determined by western blot analysis. Actin expression was used as a loading control. B, sensitivity of CCC cells to Plitidepsin. CCC (KOC-7C, RMG-I, and TOV-21G) cells were treated with the indicated concentrations of Plitidepsin in the presence of 10% FBS for 48 h. Cell viability was assessed by the MTS assay. * mean $p < 0.05$. C, effect of Plitidepsin on cell cycle distribution of CCC cells. TOV-21G, RMG-I and KOC-7C cells were cultured in FBS-free medium overnight and then treated with or without 10 nM Plitidepsin for 16 h. Cell cycle analysis was performed by flow cytometry as described in

the “Materials and Methods”. Experiments were repeated three to four times with similar results, and representative results are shown. D, effect of Plitidepsin on induction of apoptosis. KOC-7C, RMG-I, and TOV-21G cells were treated with 10 nM Plitidepsin for 48 h. Cells were harvested, and apoptosis was examined by western blotting using anti-PARP and anti-cleaved-PARP antibodies.

Figure 3. *In vitro* growth-inhibitory effects of Plitidepsin as a single agent on chemo-naïve CCC cells. A, effect of siRNA-based knockdown of eEF1A2 in CCC cells. Three CCC cells were transfected with siRNA targeting eEF1A2 or control siRNA, as described in “Materials and Methods”. Twenty-four hours after transfection, the expression of eEF1A2 was determined by western blot analysis. Actin expression was used as a loading control. B, role of eEF1A2 in the sensitivity of CCC cells to Plitidepsin. CCC cells transfected with eEF1A2-vector, empty vector, or siRNA targeting eEF1A2 were treated with the indicated concentrations of Plitidepsin in the presence of 10% FBS for 48 h. Cell viability was assessed by the MTS assay. Points mean median value, bars mean SD, * mean $p < 0.05$.

Figure 4. *In vitro* growth-inhibitory effect of Plitidepsin as a single agent on

Cisplatin-resistant CCC cells. A, establishment of cisplatin-resistant CCC cell lines.

Cisplatin-resistant sublines were established as described in “Materials and Methods.”

Cisplatin-sensitive parental (KOC-7C, RMG-I, and TOV-21G) and cisplatin-resistant

(KOC-7C-CR, RMG-I-CR, and TOV-21G-CR) cells were treated with the indicated

concentrations of cisplatin in the presence of 10% FBS for 48 h. Cell viability was

assessed by the MTS assay. Points mean median value, bars mean SD, * mean $p < 0.05$. B,

eEF1A2 expression levels in cisplatin-resistant CCC cell lines. Cisplatin-resistant

sublines incubated in the presence of 10% FBS were harvested, and then the expression

of eEF1A2 was determined by western blot analysis. C, effect of Plitidepsin on the growth

of cisplatin-resistant CCC cells. Cisplatin-resistant sublines were established as described

in “Materials and Methods”. Parental (KOC-7C, RMG-I and TOV-21G) and Cisplatin-

resistant (KOC-7C-CR, RMG-I-CR and TOV-21G-CR) cells were treated with the

indicated concentrations of Plitidepsin in the presence of 10% FBS for 48 h. Cell viability

was assessed by the MTS assay. Points mean median value, bars mean SD, * mean

$p < 0.05$. Experiments were repeated three to four times with similar results, and

representative results are shown.

Figure 5. Effect of Plitidepsin on the growth of CCC-derived tumors *in vivo*. Athymic nude mice were s.c. inoculated with TOV-21G cells. When the tumors reached a mean size of about 50 mm³, the mice were i.p. administered PBS or 0.2 mg/kg Plitidepsin 5 days a week for 2 weeks. A, a graph depicting weekly tumor volumes (mm³) for each treatment group. Points mean median value, bars mean SD, * mean p<0.05.

Table legends

Table 1: Half-maximal inhibition concentration (IC50) of plitidepsin in proliferation of clear cell carcinoma cells.

Table 2: Immunohistostaining with eEF1A2 in each histological subtype.

Figure 1

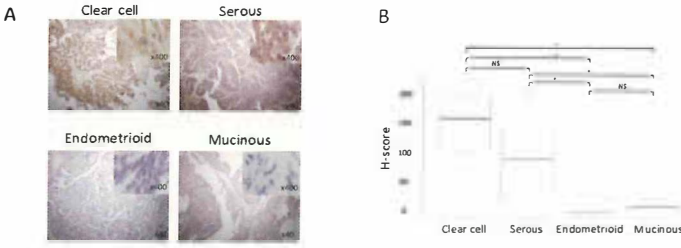


Figure 2 A

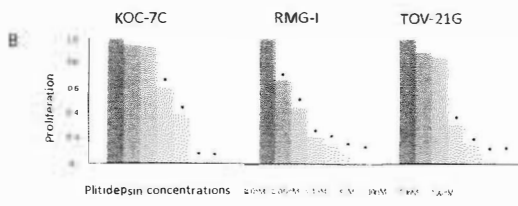
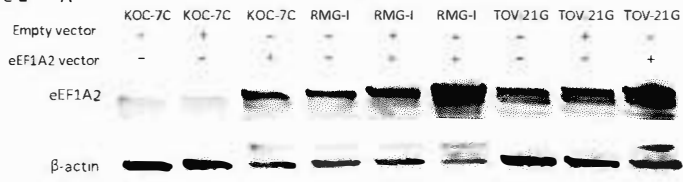


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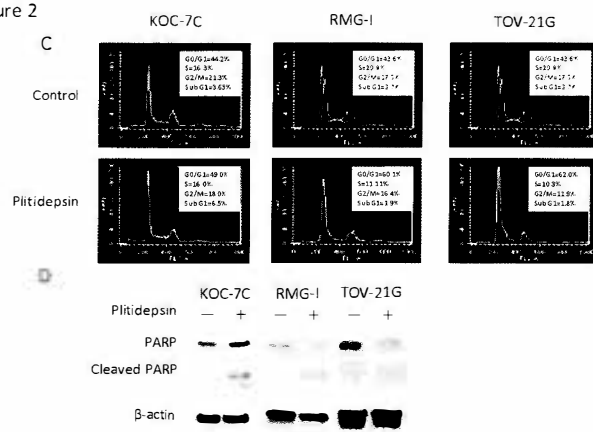


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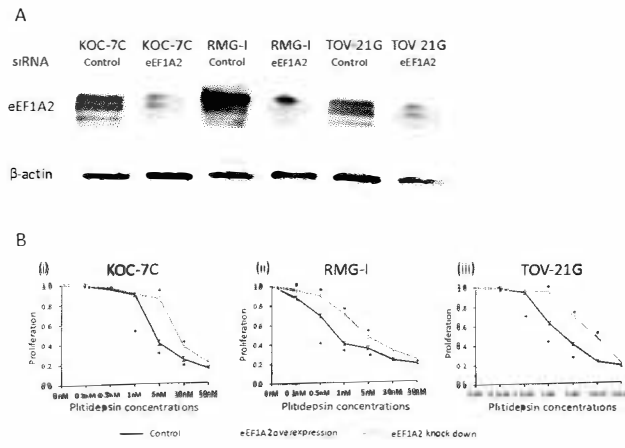


Figure 4

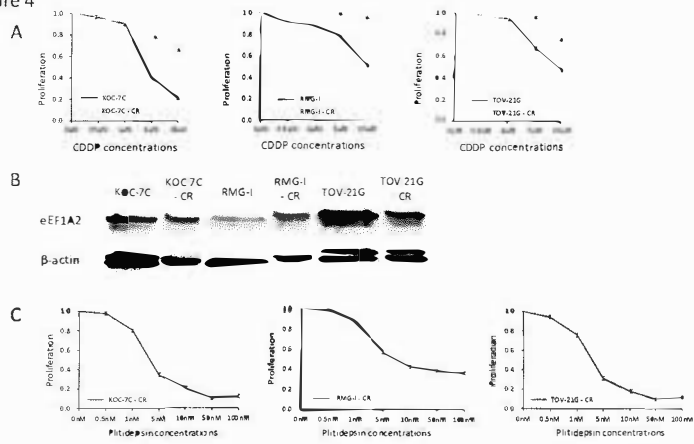


Figure 5

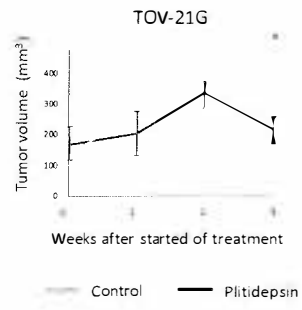


Table I. Concentration of Plitidepsin causing half-maximal inhibition of cell proliferation (IC₅₀).

		IC ₅₀ of Plitidepsin (nM)
KOC-7C	Control / Parent	3.61
	Over-expression of eEF1α2	0.86
	Knock down of eEF1α2	8.21
RMG-1	Control / Parent	4.97
	Over-expression of eEF1α2	1.69
	Knock down of eEF1α2	21.68
TOV-21G	Control / Parent	2.51
	Over-expression of eEF1α2	0.75
	Knock down of eEF1α2	7.60

CDDP: Cisplatin; eEF1α2: eukaryotic translation elongation factor 1 alpha 2.

Table 2. Immunoreactivity of ovarian cancer for eEF1A2 according to histological subtypes.

Histology	Number	Average H-score	No. of strong (H-score>200)	No. of moderate (100<H-score<200)	No. of weak (1<H-score<100)	Negative (H-score=0)	% of positive
Clear Cell	21	138.8	6(28.6%)	10(47.6%)	3(14.3%)	2(9.5%)	19(90.5%)
Serous	19	86.1	1(5.3%)	7(36.8%)	9(47.4%)	2(10.5%)	17(89.5%)
Endometrioid	10	3	0(0%)	0(0%)	1(10%)	9(100%)	1(10%)
Mucinous	8	8.1	0(0%)	0(0%)	5(62.5%)	3(37.5%)	5(62.5%)