Inhibition of Aurora kinase A synergistically enhances cytotoxicity in ovarian clear cell carcinoma cell lines induced by cisplatin: A potential treatment strategy

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Abstract

Objective: This study aimed to clarify the incidence of Aurora kinase A (Aurora-A) protein expression and its correlation with clinical parameters in ovarian clear cell carcinoma (OCCC) tumor tissues. In addition, we assessed the efficacy of ENMD-2076, a novel selective Aurora-A inhibitor, in combination with chemotherapeutic agents for the treatment of OCCC.

Methods/ Materials: Aurora-A protein expression was determined by immunohistochemical staining of OCCC specimens from 56 patients to evaluate its correlation with clinical outcomes in OCCC. In the in vitro study, six OCCC cell lines were exposed to ENMD-2076 in combination with cisplatin, SN38, doxorubicin, or paclitaxel, and cell proliferation, cell cycle distribution, and apoptosis were assessed.

Results: The 5-year survival rates of International Federation of Gynecology and Obstetrics (FIGO) stage IC3–IV patients with intermediate or strong Aurora-A expression were significantly lower than those of patients with negative or weak Aurora-A expression. Increased Aurora-A expression was associated with significantly worse overall survival of FIGO stage IC3–IV patients (21% vs 77%). Multivariate analysis revealed that Aurora-A expression was an independent prognostic factor for stage IC3–IV OCCC patients. Furthermore, synergistic effects were observed with

ENMD-2076 in combination with cisplatin or SN-38 in four of the six tested cell lines. ENMD-2076 dramatically enhanced apoptosis and cell cycle arrest at the G2/M phase induced by cisplatin.

Conclusions: Aurora-A is a promising biomarker that is predictive of patient outcomes and a potential target for OCCC. The results suggested that chemotherapy, including ENMD-2076 in combination with cisplatin, is a potential treatment modality for patients with OCCC.

Keywords: Aurora-A, clear cell, ovarian cancer, targeted therapy, tissue microarray

Introduction

Ovarian clear cell carcinoma (OCCC) is a histopathological subtype of epithelial ovarian cancer (EOC) that accounts for approximately 4%–12% of EOC cases in Western countries and more than 20% of EOC cases in Japan [1,2]. Advanced OCCC is associated with worse clinical outcomes due to the decreased sensitivity of the tumor to standard chemotherapy, which consists of taxane and platinum [3-5]. A recent randomized phase III clinical trial comparing the efficacy of irinotecan plus cisplatin to that of paclitaxel plus carboplatin in patients with OCCC (Japanese Gynecologic Oncology Group [JGOG] 3017/Gynecologic Cancer Intergroup [GCIG] Trial) failed to observe a significant survival benefit with irinotecan plus cisplatin [6]. Therefore, effective and novel treatment strategies, such as incorporation of molecular-targeted agents to current protocols, are urgently needed to improve outcomes for patients with advanced OCCC.

Aurora kinase A (Aurora-A) is a member of the Aurora mitotic serine/threonine kinase family that plays important roles in mitosis during cell proliferation [7]. Several studies showed that Aurora-A mRNA and protein levels were increased in various tumors, including EOC [8-10]. However, to date, there are no clinical data on the potential prognostic significance of Aurora-A expression in OCCC.

ENMD-2076 is an orally available small molecule selective inhibitor of Aurora-A, which was demonstrated to reduce tumor growth in several xenograft models of various cancers such as colorectal cancer, multiple myeloma, and breast cancer [11-14]. A phase II clinical trial of ENMD-2076 for patients with platinum-resistant EOC showed that two of three patients with OCCC had longer progression-free survival (PFS) than the median PFS of the trial cohort [15]. Despite the very small number of patients with clear cell histology, a separate phase II clinical trial to examine the efficacy and safety of ENMD-2076 in OCCC (NCT01914510) is ongoing based on this result. However, a correlation between Aurora-A expression and clinicopathological features and the efficacy of Aurora-A inhibitors in combination with a range of cytotoxic agents have not been systematically evaluated in OCCC.

In this study, we assessed Aurora-A protein expression in OCCC tumor tissue specimens by immunohistochemical staining and evaluated the association of Aurora-A expression with clinical outcomes in patients with OCCC. We also aimed to determine whether ENMD-2076 enhanced the cytotoxic effects of chemotherapeutic agents currently used for OCCC.

Material and Methods

Clinical samples

Formalin-fixed and paraffin-embedded samples were collected from 56 patients with OCCC. All patients underwent surgical exploration and cytoreduction as the initial treatment at Iwate Medical University Hospital between January 2009 and December 2015. In this study, all patients were staged according to the International Federation of Gynecology and Obstetrics (FIGO) 2014 criteria. This study was approved by the institutional ethics committee of Iwate Medical University. All patients gave written informed consent before the collection of specimens according to institutional guidelines.

Tissue microarray blocks and immunohistochemistry

Tissue microarray (TMA) blocks were selected by reviewing hematoxylin/eosin-stained slides and were constructed by retrieving tissue cores from morphologically representative areas of original tumor blocks, which were then positioned in recipient paraffin blocks. TMA blocks were sectioned, deparaffinized, rehydrated, and underwent antigen retrieval using Target Retrieval Solution (High pH, K8004, Dako, Glostrup, Denmark) at 97 °C for 20 min. Staining procedures were performed in an automatic Autostainer LINK48 immunostainer (AS480, Dako) using the EnVision FLEX High pH detection system (K8000, DAKO), following the manufacturer’s instructions. Primary

antibodies used in this study were rabbit anti-Aurora-A (1:1,000; Bethyl Laboratories, Montgomery, TX, USA) and mouse anti-human Ki-67 (clone MIB-1; 1:100; DAKO).

Aurora-A expression was determined by semiquantitative scoring of the TMA blocks according to previously published criteria; samples with <5%, 6%–25%, 26%–50%, 51%–75%, and >75% positive cells were scored as 0, 1, 2, 3, and 4, respectively [16]. Staining intensity was scored as negative (0), weak (1), moderate (2), or strong (3). Scores for the percentage of stained cells and the intensity of positive cells were added; overall scores for Aurora-A expression were categorized into four groups: negative (0–1), weak (2–3), intermediate (4–5), and strong (6–7). The Ki-67 LI was determined by the following formula: LI (%) = 100 × labeled cells/labeled and unlabeled cells. All samples were reviewed by a gynecologic oncologist in a blinded manner.

Cell lines and culture conditions

Six human OCCC cell lines used in this study were obtained from the following sources: KK from Prof. Yoshihiro Kikuchi (National Defense Medical College, Tokorozawa, Japan), KOC-7C from Dr. Toru Sugiyama (Kurume University, Kurume, Japan), OVAS and OVMANA from Prof. Hiroshi Minaguchi (Yokohama City University, Yokohama, Japan), and RMG-I from Prof. Shiro Nozawa (Keio University,

Tokyo, Japan). We previously established the TU-OC-1 cell line [17]. These cell lines were maintained in DMEM/Ham's F-12 medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum, penicillin, and streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

Dose–response studies

Chemosensitivity of cell lines was determined using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's specifications. Briefly, cells were plated into 96-well tissue culture plates (Corning, NY, USA) at 100 µL per well. The next day, the cells were incubated with various concentrations of ENMD-2076 (0.1–10 µM; Chemscene, NJ, USA), cisplatin (0.3–30 µM; Yakult Pharmaceutical Industry, Tokyo, Japan), 7-ethyl-10-hydroxycamptothecin (SN-38, an active metabolite of irinotecan; 1.0–4,000 nM; Toronto Research Chemicals, Toronto, Canada), paclitaxel (10–1,000 nM; Wako Pure Chemical Industries), and doxorubicin (30–600 µg/mL; Wako Pure Chemical Industries). At 72 h after addition of the drugs, 100 µL of Cell Counting Kit-8 10% solution was added to each well and the plates were incubated for another 20–40 min at 37 °C. Absorbance at 450 nm was measured with a microplate reader (iMark™ microplate absorbance reader; Bio-Rad

Laboratories, Richmond, CA, USA). Inhibition of cell growth was calculated as the percentage of viable cells compared to untreated cultures.

Dose–effect analysis

The effect of ENMD-2076 in combination with each chemotherapeutic agent was tested using a fixed ratio that spanned individual 50% inhibitory concentration of a substance (IC_{50}) value of each drug. Median effective dose (ED_{50}) for each drug combination was determined and median–effect plot analyses and combination index (CI) calculations were achieved by the method described by Chou and Talalay [18]. CI for each combination was defined as synergistic ($CI < 0.9$), additive ($0.9 \leq CI < 1.1$), or antagonistic ($CI \geq 1.1$). CompuSyn software (ComboSyn, Paramus, NJ, USA) was used to analyze data obtained by the WST-8 assay.

Western blotting

After indicated treatments, cells were lysed in radio-immunoprecipitation assay lysis buffer (20 mM HEPES, 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.1% *sodium dodecyl sulfate*, 0.5% sodium deoxycholate pH 7.5, supplemented with protease and phosphatase inhibitors; ATTO, Tokyo, Japan). A total of 20–30 μg protein was separated by electrophoresis on 4%–20% polyacrylamide gels (Mini-PROTEAN®TGX™ gels; Bio-Rad Laboratories) and transferred to blotting membranes using the Trans-Blot®

Turbo™ transfer system (Bio-Rad Laboratories). Membranes were next incubated with rabbit anti-phospho-Aurora-A (Thr288; 1:500; Abcam, Boston, USA) and mouse anti-actin (1:1,000; Sigma-Aldrich, St. Louis, MO, USA) primary antibodies. Bands were visualized with secondary anti-rabbit or anti-mouse IgG antibodies coupled with horseradish peroxidase using an enhanced chemiluminescence kit according to the manufacturer's recommendations.

Cell cycle distribution analysis

Cell cycle distribution was analyzed by flow cytometry. Cells were seeded in 6-well plates at a density of 1×10^5 cells/well in culture medium. The next day, cells were treated with ENMD-2076, cisplatin, or ENMD-2076 plus cisplatin for 24–72 h. At the end of treatment, adherent and suspension cells were fixed and stored in −20 °C for at least two hours. Cells were then incubated with propidium iodide (PI) and RNase for 30 minutes in the dark at room temperature. DNA content was measured with a FACSCalibur flow cytometer and CellQuest Pro software (Becton-Dickinson, Franklin Lakes, NJ, USA).

Annexin V staining

Cells, which were treated with chemotherapeutic agents as indicated above, were collected and suspended in binding buffer, followed by staining with Annexin V-

fluorescein isothiocyanate (FITC) and PI using ApoScreen® Annexin V apoptosis kit-FITC (Southern Biotech, Birmingham, AL, USA), according to the manufacturer's specifications. In each experiment, a total of 3×10^5 cells were analyzed with a FACSCalibur flow cytometer.

Statistical analysis

Statistical analyses were performed using JMP statistical software version 12 (SAS Institute, Cary, NC, USA). The chi-squared test was used for comparisons of categorical data. Means were compared by one-way analysis of variance with post-hoc testing. Survival distributions were calculated using the Kaplan–Meier method and the significance of apparent differences in survival distribution between groups was tested with the log-rank test. Additionally, multivariable analysis was performed to fit the Cox proportional hazards model. A $P < 0.05$ was considered statistically significant.

Results

Altered Aurora-A and Ki-67 expression in ovarian clear cell carcinoma specimens.

Representative images of specimens stained for Aurora-A and Ki-67 are shown in Fig. 1A. OCCC specimens exhibited a predominantly cytoplasmic Aurora-A staining, with limited nuclear staining. Among a total of 56 OCCC specimens examined, 42

(75%) showed an intermediate or strong Aurora-A expression. There were no significant associations between Aurora-A expression and patient age, disease stage, residual tumor size, or first-line chemotherapy regimen.

In contrast, Ki-67 LI, which is known as a representative cell proliferative marker, has been used widely for various malignancies including ovarian cancer to evaluate the proliferative activity or the prognosis of the cancer [19], ranged widely from 0.2% to 53.8%. In this study, the cut-off value of Ki-67 LI was set at 12.5% (median value for OCCC). There was no relationship between Aurora-A expression and Ki-67 LI (Table 1).

Prognosis of patients with ovarian clear cell carcinoma.

Univariate analysis demonstrated that both FIGO stage IC3–IV ($P < 0.001$) and residual tumor >1 cm ($P < 0.0001$) were significantly correlated with shorter overall survival (OS) in patients with OCCC. Patients with intermediate or strong Aurora-A expression tended to have worse clinical outcomes than those with negative or weak tumor Aurora-A expression (Fig. 1B). The OS rate was significantly lower in FIGO stage IC3–IV patients with intermediate or strong tumor Aurora-A expression than in those with negative or weak tumor Aurora-A expression, and the 5-year survival rates were 21% and 77%, respectively (Fig. 1C). Interestingly, the OS rate was significantly

lower in FIGO stage IC3 or II patients with intermediate or strong Aurora-A expression (n=10) compared with those with negative or weak Aurora-A expression (n=8) (the 5-year survival rate: 21.6% vs 85.7%, $P = 0.019$). However, Aurora-A expression was not associated with OS in patients with OCCC having FIGO stage IA or IC1 disease, or patients with complete tumor resection (data not shown). Among 34 cases of FIGO stage IC3-IV, 30 cases (88.2%) received taxane and platinum-based chemotherapy, 3 (8.8%) received irinotecan plus cisplatin therapy, and the remaining one case with poor performance status (3%) did not receive any first-line chemotherapy. Multivariable analysis revealed that Aurora-A expression and residual tumor were independent prognostic factors for OCCC (Table 2).

Combination effects of ENMD-2076 and chemotherapeutic agents

Given our novel clinical observations that Aurora-A expression is an independent prognostic factor for advanced OCCC, inhibition of Aurora-A could be an attractive approach. We first confirmed the presence of phosphorylated Aurora-A at equivalent levels across all six OCCC cell lines by Western blot analysis (data not shown). We next sought to determine the effect of four chemotherapeutic agents commonly used to treat ovarian cancer, as well as ENMD-2076, on proliferation of six OCCC cell lines. The sensitivity to individual chemotherapeutic agents and ENMD-2076 varied among six

OCCC cell lines and ranged from 1.1 to 2.8 μmol/L for ENMD-2076, 3.6 to 8.9 μmol/L for cisplatin, 9.5 to 42.2 nmol/L for SN-38, 77 to 380 ng/mL for doxorubicin, and 29.9 to 3,730 nmol/L for paclitaxel. We then determined the potential synergistic effects of ENMD-2076 in combination with other chemotherapeutic agents on cytotoxicity by determining CI values. Representative data of ENMD-2076 combination with cisplatin in RMG-I cells are shown in Figure 2A. The CI values at ED_{50} was less than 0.9 (synergism) for four cell lines for cisplatin and SN-38 and three cell lines for doxorubicin (Fig. 2B). Conversely, the CI value was more than 1.1 (antagonism) in four cell lines for paclitaxel. These results showed that ENMD-2076 in combination with cisplatin or SN-38 had a synergistic effect in more cell lines. Cisplatin is one of the key chemotherapeutic agents used in EOC. Therefore, we focused on determining the mechanism underlying the cytotoxic effects of cisplatin in combination with ENMD-2076 in OCCC.

ENMD-2076 in combination with cisplatin induces G2/M phase arrest and apoptosis in ovarian clear cell carcinoma cell lines

We first examined cell cycle distribution in RMG-I and TU-OC-1 cells by flow cytometry (Fig. 3A, B). In both cell lines, after treatment with ENMD-2076 alone, the proportion of cells in G2/M phase was markedly increased. Conversely, the proportion

of cells in S phase and those accumulating in G2/M were significantly increased 24 h after treatment with cisplatin alone, whereas the proportion of cells in S phase was decreased 48 h after treatment. In contrast, the proportion of cells in S phase after 24 h was lower in cultures treated with ENMD-2076 plus cisplatin than in those exposed to cisplatin alone. Moreover, 48 h after treatment, the proportion of sub-G1 cells was significantly higher in cultures treated with ENMD-2076/cisplatin combination than in those exposed to cisplatin alone. These results suggested that the addition of ENMD-2076 to cisplatin abrogated S-phase arrest, which led to subsequent death of OCCC cells.

ENMD-2076 leads to a rapid reduction in phospho-Aurora-A expression and subsequent apoptosis in ovarian clear cell carcinoma cell lines

Western blot analysis revealed that phospho-Aurora-A protein levels were decreased at 0.5–12 h after treatment with 1 μM ENMD-2076 in RMG-I cells (Fig. 4A); similar findings were observed in the remaining five OCCC cell lines (data not shown). Next, we used the Annexin V-FITC apoptosis detection kit to detect and discriminate between early apoptotic and secondary necrotic cells. Treatment of TU-OC-1 cells with 2 μmol/L cisplatin and/or 1.5 μmol/L ENMD-2076 for 24–72 h led to a significantly increase in the number of Annexin V–FITC-positive cells (Fig. 4B, C).

Discussion

In the current study, we demonstrated that an intermediate/strong Aurora-A expression was independently associated with poor OS in patients with OCCC. We also found that the Aurora-A kinase inhibitor ENMD-2076 in combination with cisplatin had a significant cytotoxic effect in OCCC cell lines. To our knowledge, this is the first study to show Aurora-A as a novel prognostic biomarker in OCCC and Aurora-A inhibition as an effective strategy to treat OCCC.

The association of Aurora-A overexpression with aggressive tumor behavior such as invasiveness, poor differentiation, and nodal metastasis was shown in esophageal, breast, and hepatocellular carcinoma [20-22]. In ovarian cancer, Aurora-A overexpression was reported to represent early changes and was suggested to play an important role in the development of malignancy [23]. Furthermore, Landen *et al.* [16] found that 58 of 70 tumor specimens (83%) from patients with stage III or IV high-grade serous carcinoma of the ovary overexpressed Aurora-A compared with normal ovarian surface epithelium. They also reported that Aurora-A overexpression was correlated with poor survival in these patients and suggested that Aurora-A might be a useful prognostic marker and therapeutic target in EOC. Indeed, we observed that 96% of the OCCC tumor specimens

expressed Aurora-A, with 75% of the samples exhibiting intermediate or strong Aurora-A expression. Furthermore, we found that Aurora-A expression in tumor specimens was an independent prognostic factor for FIGO stage IC3–IV patients with OCCC. Altogether, these novel findings suggest that Aurora-A expression should be considered as a valuable predictor for prognosis and survival in patents with OCCC, especially those with advanced-stage disease.

Aurora-A regulates cell cycle events from the late S phase to the M phase and plays a crucial role in the G2/M checkpoint of cell cycle in cells undergoing mitosis. Aurora-A is activated during the G2-to-M transition, with peak kinase activity in pro-metaphase [24]. Several studies recently showed that inhibiting Aurora-A activity increased chemosensitivity in various cancers such as mantle cell lymphoma, hepatocellular carcinoma, and non-small-cell lung cancer [25-27]. However, the potential synergic interaction of Aurora-A inhibitors with chemotherapeutic agents have not been evaluated in OCCC. We therefore conducted the present study to determine whether ENMD-2076 enhanced the effects of chemotherapeutic agents and used cisplatin, SN-38, doxorubicin, and paclitaxel as first- and second-line agents currently in use for EOC. We determined that ENMD-2076 in combination with cisplatin showed a synergistic effect on cell growth inhibition and cell death in four of the six cell lines

included in the current study. Similarly, Yao *et al*. [27] reported that another Aurora-A inhibitor, VX680, enhanced the chemosensitivity of HepG2 hepatocellular carcinoma cells to cisplatin. They also showed that VX680/cisplatin combination significantly increased the number of apoptotic cells and p53 expression and led to a reduction in Bcl-2 expression. Aurora-A has been reported as a key regulator in the p53 pathway and its overexpression was shown to result in p53 degradation [28]. Given that *p53* mutation is infrequent in OCCC and that p53 activation was shown to induce apoptosis in OCCC cells [29], our findings suggest that ENMD-2076 may augment cisplatin-induced apoptosis via activation of the p53 pathway.

In summary, we showed that FIGO stage IC3–IV patients with intermediate or strong tumor expression of Aurora-A might have a worse prognosis. We also found that the Aurora-A inhibitor ENMD-2076 augmented the cytotoxicity of certain chemotherapeutic agents, especially that of cisplatin, in OCCC cells *in vitro*. Therefore, Aurora-A might be a promising prognostic marker in patients with FIGO stage IC3–IV OCCC, and combination therapies incorporating Aurora-A inhibitors such as ENMD-2076 might improve survival of patients with OCCC.

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Figure legends

Figure 1. Expression of Aurora-A and overall survival (OS) in patients with ovarian clear cell carcinoma (OCCC). (**A**) Immunohistochemical staining for Aurora-A and Ki-67 in OCCC tumor specimens. Staining intensity was scored as negative (0–1), weak (2–3), intermediate (4–5), or strong (6–7); × 20. (**B**) The Kaplan–Meier survival curve for OS for all patients included in this study (n = 56). The difference in OS for patients with high Aurora-A expression was not statistically significant (P = 0.18). (**C**) The estimated 5-year survival rate for stage IC3–IV patients with intermediate or strong Aurora-A expression was significantly worse than that for patients with negative or weak Aurora-A expression (21% vs 77%, P = 0.02).

Figure 2. Cisplatin in combination with ENMD-2076 shows a synergistic or additive effect in ovarian clear cell carcinoma cell lines. (**A**) Representative data showing growth inhibition elicited by ENMD-2076 in combination with cisplatin in RMG-I cells. Results are presented as means ± standard deviation from three biological replicates. (**B**) Combination index (CI) of ENMD-2076 in combination with cisplatin (CDDP), 7-ethyl-

10-hydroxycamptothecin (SN-38), doxorubicin (DXR), or paclitaxel (PTX) added to cell cultures at a fixed ratio for 72 h. Combination, ENMD-2076 + cisplatin.

Figure 3. Effects of ENMD-2076 on the cell cycle distribution in response to cisplatin (CDDP). Two ovarian clear cell carcinoma cell lines, RMG-I and TU-OC-1, were treated with PBS (control), CDDP (5.4 or 2.0 μmol/L), and/or ENMD-2076 (ENMD, 0.8 or 1.6 μmol/L), respectively. (**A** and **B**) Representative flow cytometry histograms show cell cycle distribution in RMG-I and TU-OC-1 cells at 24 to 72 h after treatment. Cell cycle distribution is displayed as the intensity of propidium iodide (x axis) versus cell number (y axis). CDDP in combination with ENMD-2076 led to a reduction in G1- and S-phase fractions and a shift in the cell cycle distribution to the sub-G1 phase at 48 and 72 h in RMG-I and TU-OC-1 cells. Combination, ENMD-2076 + cisplatin.

Figure 4. Enhanced cytotoxicity in ovarian clear cell carcinoma cells treated with ENMD-2076 plus cisplatin. (**A**) Western blot analysis showing that phospho-Aurora-A protein levels were decreased at 0.5–12 h after treatment with ENMD-2076. (**B**) Representative scatter plots from a fluorescence-activated cell sorting experiment is shown. Apoptosis was determined by ApoScreen® Annexin V Apoptosis Kit-FITC.

Early apoptotic cells were scored as Annexin V–FITC-positive and propidium iodide (PI)-negative to exclude necrotic cells. (**C**) Bar graphs showing the percentage of apoptotic and necrotic cells in response to the indicated treatments. Data represent means ± standard deviation from at least three independent experiments (*$P < 0.01$). Combination, ENMD-2076 + cisplatin.

Table 1. Association of Aurora-A expression with clinical characteristics in patients with ovarian clear cell carcinoma

Characteristics	Aurora-A expression		*P* value
	intermediate/strong (*n* = 42)	negative/weak (*n* = 14)	
Age(years)			0.16
Range	30-78	42-69	
Median	50	54	
FIGO stage			0.24
IA	12	2	
IC1	6	2	
IC3	8	6	
II	2	2	
III	10	2	
IV	4	0	
Node status			0.28
Positive	9	1	
Negative	31	11	
Unknown	2	2	
Residual tumor			0.43
No residual	31	8	
< 1cm	6	4	
≥ 1cm	5	2	
First-line chemotherapy			0.32
Paclitaxel+Carboplatin (TC)	36*	10	
Irinotecan+Cisplatin (CPT-P)	3	3	
No treatment	3	1	
Ki-67 expression			1.00

High (≥ 12.5)	21	7
Low (< 12.5)	21	7

FIGO, International Federation of Gynecology and Obstetrics

* 1 received intraperitoneal therapy; 3 received TC plus Temsirolims; 1 received TC plus Velipalib

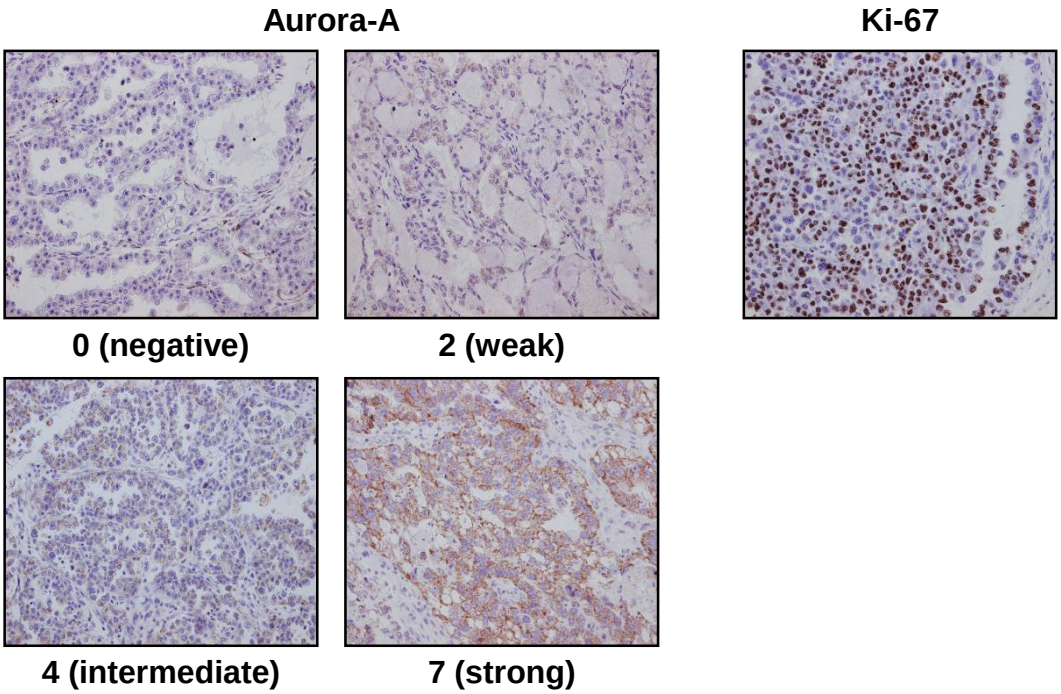
Table 2. Multivariable Cox proportional hazards model for overall survival in clear cell carcinoma of the ovary

	Relative risk (95% CI)	*P* value
Age(years)		0.0639
< 52 vs ≥ 52	3.2582 (0.9347 to 12.8980)	
FIGO stage		0.9787
IC3/II vs III/IV	0.9824 (0.2593 to 3.7023)	
Residual tumor		0.0058
< 1cm vs ≥ 1 cm	7.7746 (1.8126 to 38.3010)	
Aurora-A expression		0.0141
negative/weak vs intermediate/strong	7.0381 (1.4539 to 52.0524)	

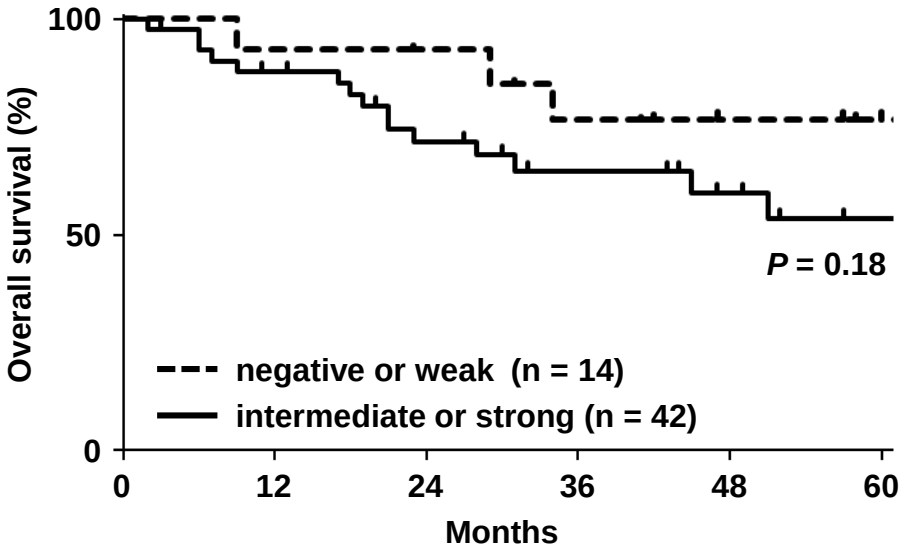
CI, confidence interval

Fig. 1

A


Aurora-A
Ki-67
0 (negative)
2 (weak)
4 (intermediate)
7 (strong)

B


Overall survival (%)
100
50
0
P = 0.18
negative or weak (n = 14)
intermediate or strong (n = 42)
0
12
24
36
48
60
Months

C

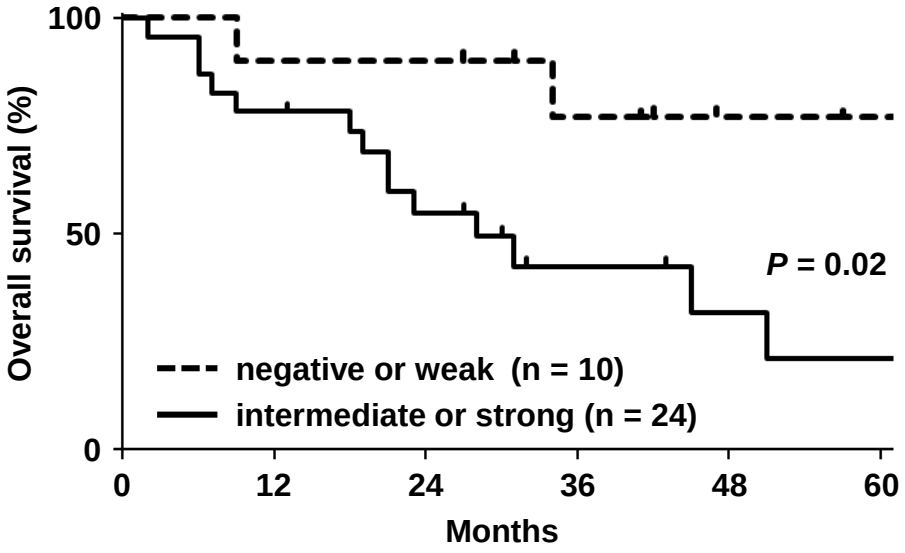
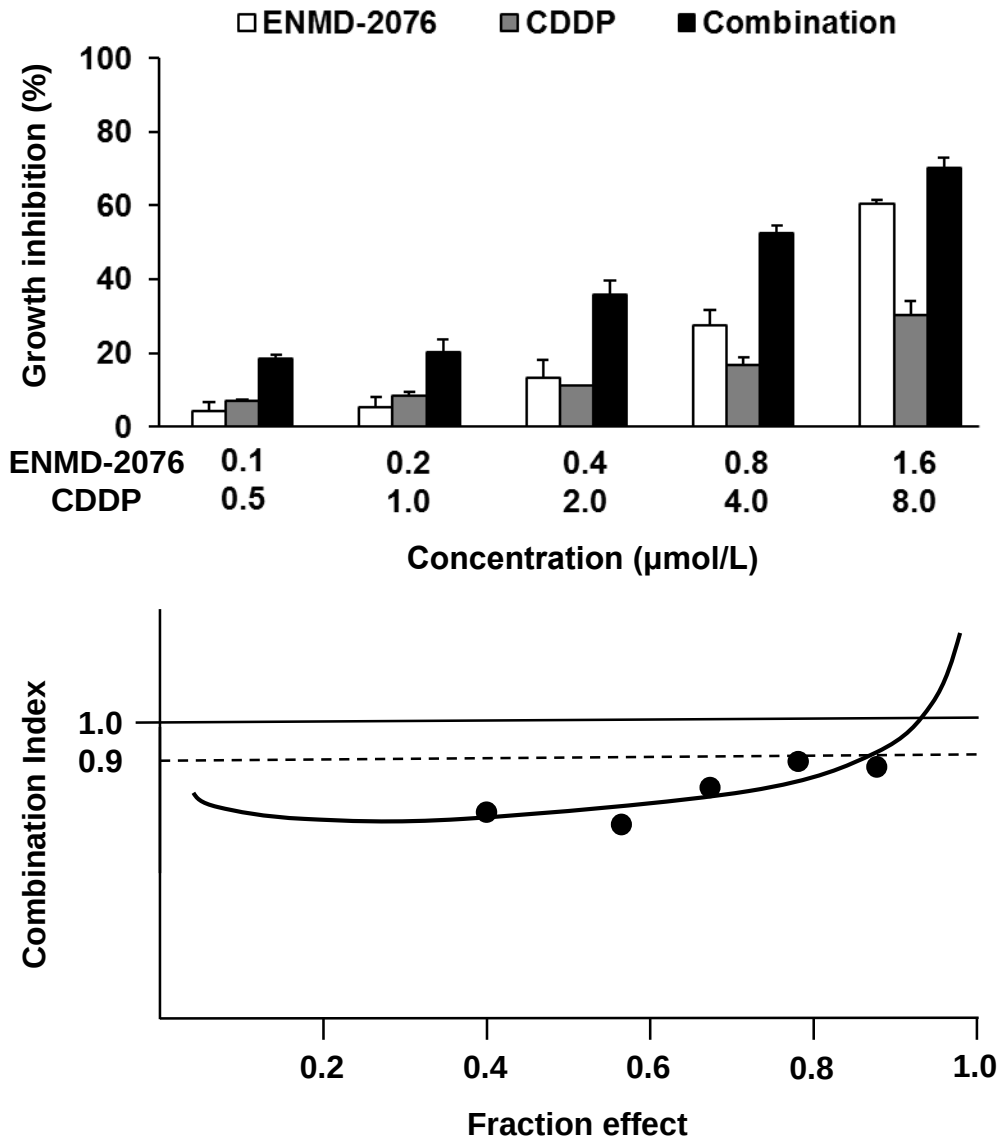

Overall survival (%)
100
50
0
P = 0.02
negative or weak (n = 10)
intermediate or strong (n = 24)
0
12
24
36
48
60
Months

Fig. 2

A


ENMD-2076
CDDP
Combination
Growth inhibition (%)
100
80
60
40
20
0
ENMD-2076 0.1 0.2 0.4 0.8 1.6
CDDP 0.5 1.0 2.0 4.0 8.0
Concentration (μmol/L)

Combination Index
1.0
0.9
0.2
0.4
0.6
0.8
1.0
Fraction effect

B

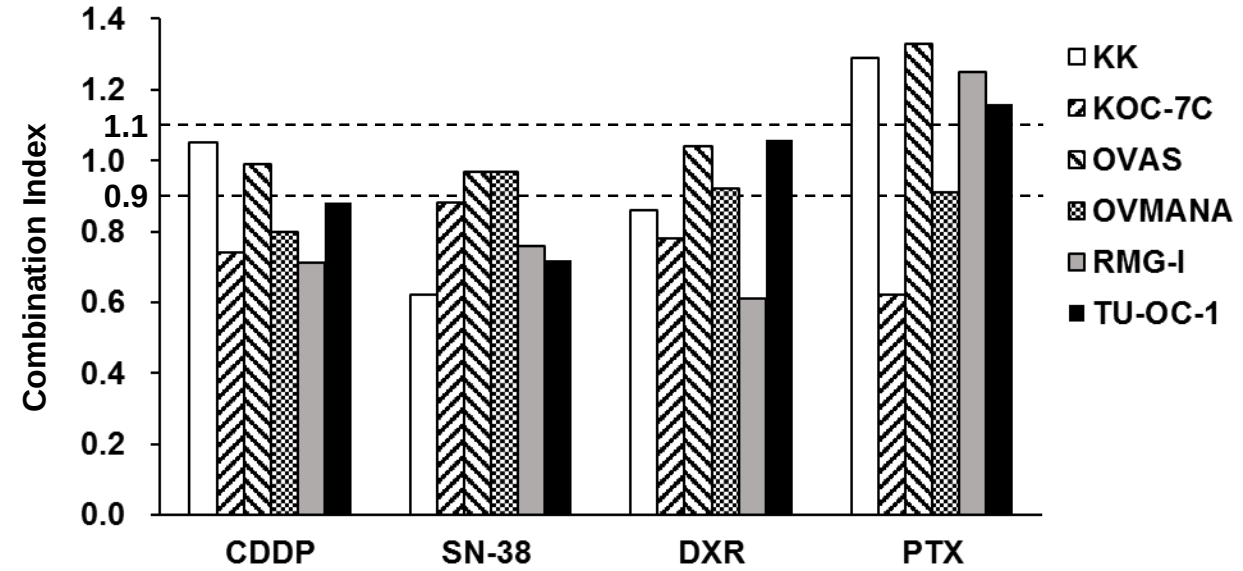

Combination Index
1.4
1.2
1.1
1.0
0.9
0.8
0.6
0.4
0.2
0.0
CDDP
SN-38
DXR
PTX
KK
KOC-7C
OVAS
OVMANA
RMG-I
TU-OC-1

Fig. 3

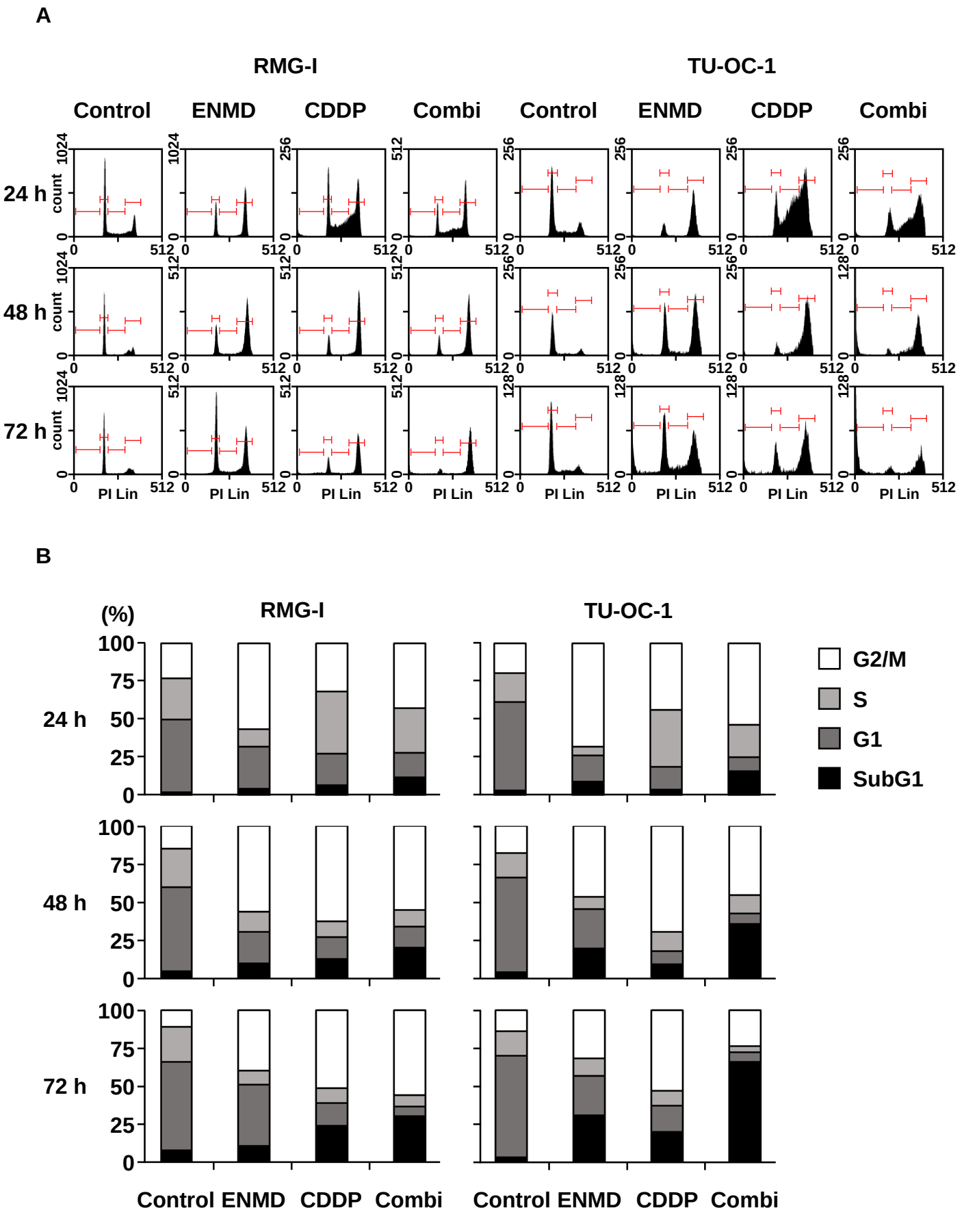
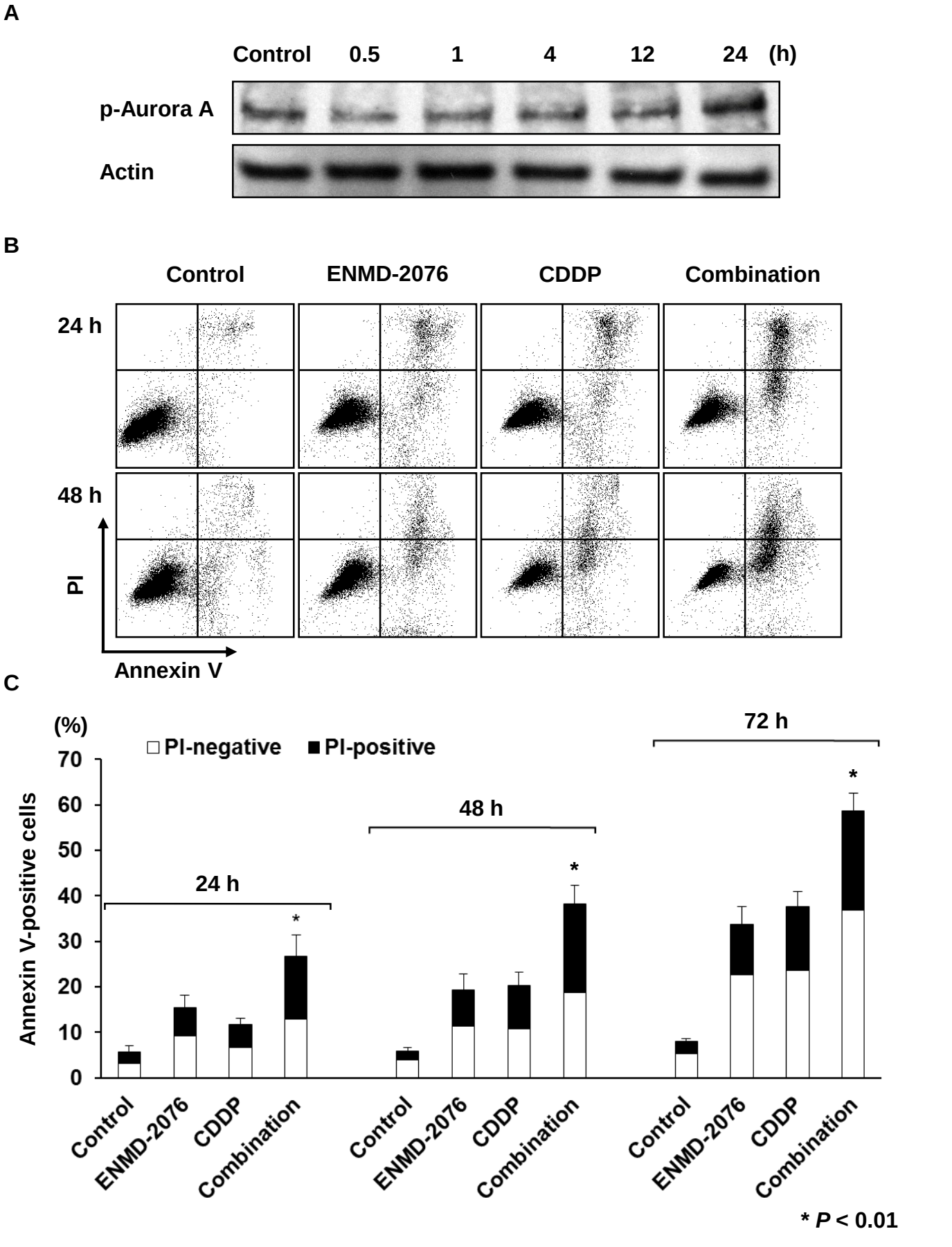

A
RMG-I
TU-OC-1
Control
ENMD
CDDP
Combi
Control
ENMD
CDDP
Combi
24 h
48 h
72 h
count
PI Lin
B
(%)
RMG-I
TU-OC-1
100
75
50
25
0
24 h
48 h
72 h
G2/M
S
G1
SubG1
Control
ENMD
CDDP
Combi
Control
ENMD
CDDP
Combi

Fig. 4


A
Control
0.5
1
4
12
24
(h)
p-Aurora A
Actin
B
Control
ENMD-2076
CDDP
Combination
24 h
48 h
PI
Annexin V
C
(%)
PI-negative
PI-positive
72 h
48 h
24 h
Annexin V-positive cells
70
60
50
40
30
20
10
0
*
Control
ENMD-2076
CDDP
Combination
* P < 0.01