Gynecologic Oncology 153 (2019) 135–148

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

A novel PI3K/mTOR dual inhibitor, CMG002, overcomes the chemoresistance in ovarian cancer

Hye Joung Choi [a,b], Jin Hyung Heo [a], Ju Yeon Park [a,b], Ju Yeon Jeong [a,b], Hyeon Ju Cho [c], Kyung Soon Park [c], Se Hwa Kim [a,b], Yong Wha Moon [d], Jin Sung Kim [e], Hee Jung An [a,b,*]

[a] Department of Pathology, CHA Bundang Medical Center, CHA University, Republic of Korea
[b] Institute for Clinical Research, CHA Bundang Medical Center, CHA University, Republic of Korea
[c] Department of Biomedical Science, College of Life Science, CHA University, Republic of Korea
[d] Department of Medical Oncology, CHA Bundang Medical Center, CHA University, Republic of Korea
[e] CMG Pharmaceutical CO., LTD., Republic of Korea

HIGHLIGHTS

- A novel PI3K/mTOR dual inhibitor, CMG002, is newly developed.
- We demonstrate here for the first time that CMG002 inhibits growth of chemoresistant cancer cells both *in vitro* and *in vivo*.
- CMG002 suppresses cell proliferation and induces G1 cell cycle arrest and apoptosis in chemoresistant ovarian cancer cells.
- CMG002 re-sensitizes chemoresistant cancer cells to PTX or platinum agents.
- CMG002 is a promising therapeutic strategy for chemoresistant ovarian cancer.

ARTICLE INFO

Article history:
Received 5 October 2018
Received in revised form 7 January 2019
Accepted 9 January 2019
Available online 25 January 2019

Keywords:
PI3K/mTOR dual inhibitor
Small molecule
Chemoresistance
Ovarian cancer

ABSTRACT

Objective. Ovarian cancer is the leading cause of gynecologic-related mortality worldwide. Despite successful initial treatment, overall survival rates are very low because tumors develop resistance to chemotherapeutic drugs. The PI3K/mTOR pathway is a key signaling pathway involved in drug resistance of ovarian cancer cells. The aim of this study was to examine the effect of a newly developed PI3K/mTOR dual inhibitor, CMG002, on chemoresistant ovarian cancer cells.

Methods. We examined the effects of CMG002, and its synergistic effects when combined with paclitaxel or cisplatin, on cell viability, cell cycle arrest, and apoptosis of PTX-resistant SKpac17 or cisplatin-resistant A2780cis ovarian cancer cells *in vitro*. Western blot analysis was performed to assess expression of PI3K, p-mTOR, p-Akt, p-S6, Bim, and caspase-3. *In vivo* studies were carried out in a xenograft mouse model, followed by TUNEL and immunohistochemical staining of excised tumor tissue.

Results. CMG002 showed marked toxicity against chemoresistant ovarian cancer cells and re-sensitized these cells to chemotherapeutic agents by suppressing cell proliferation and inducing G1 cell cycle arrest and apoptosis. *In vivo* xenograft studies revealed that treatment with CMG002, either alone or in combination with paclitaxel or cisplatin, led to a marked reduction in tumor growth. CMG002 caused marked suppression of mTOR (Ser2448), Akt (Ser473), Akt (Thr308), and S6 (Ser235/236) phosphorylation, both *in vitro* and *in vivo*.

Conclusion. Taken together, CMG002, a very potent PI3K/mTOR dual inhibitor, induced cytotoxicity in chemoresistant ovarian cancer cells, suggesting that this novel inhibitor might be a new therapeutic strategy for chemoresistant ovarian cancer.

1. Introduction

Ovarian cancer is the leading cause of gynecologic-related mortality worldwide [1]. The current standard treatment comprises cytoreductive surgery followed by combination chemotherapy with platinum and paclitaxel (PTX) [2]. Unfortunately, the majority of patients treated

* Corresponding author at: Department of Pathology, CHA Bundang Medical Center, CHA University, 59 Yatapro, Sungnam, Gyonggi-do, Republic of Korea.
E-mail address: hjahn@cha.ac.kr (H.J. An).

https://doi.org/10.1016/j.ygyno.2019.01.012
0090-8258/

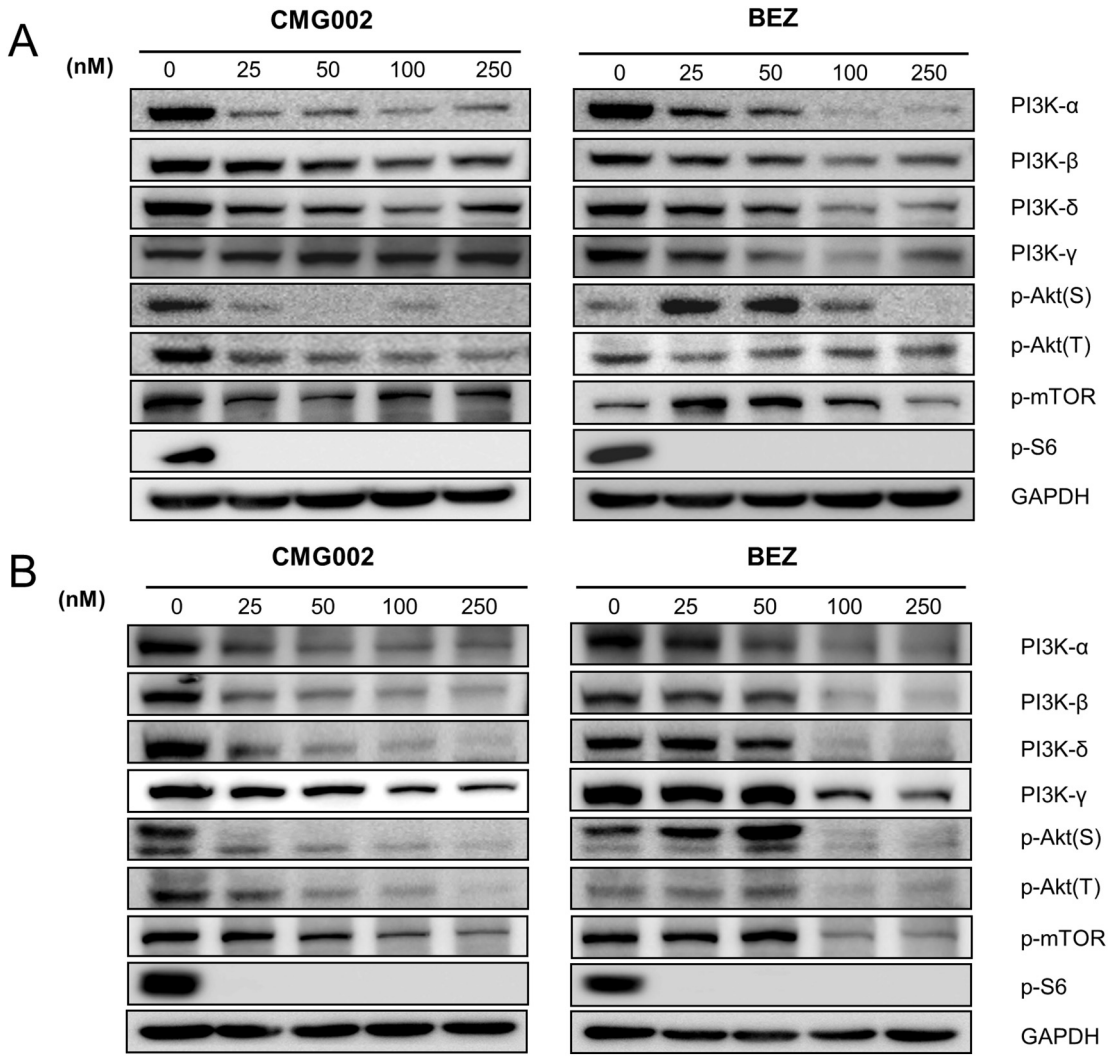


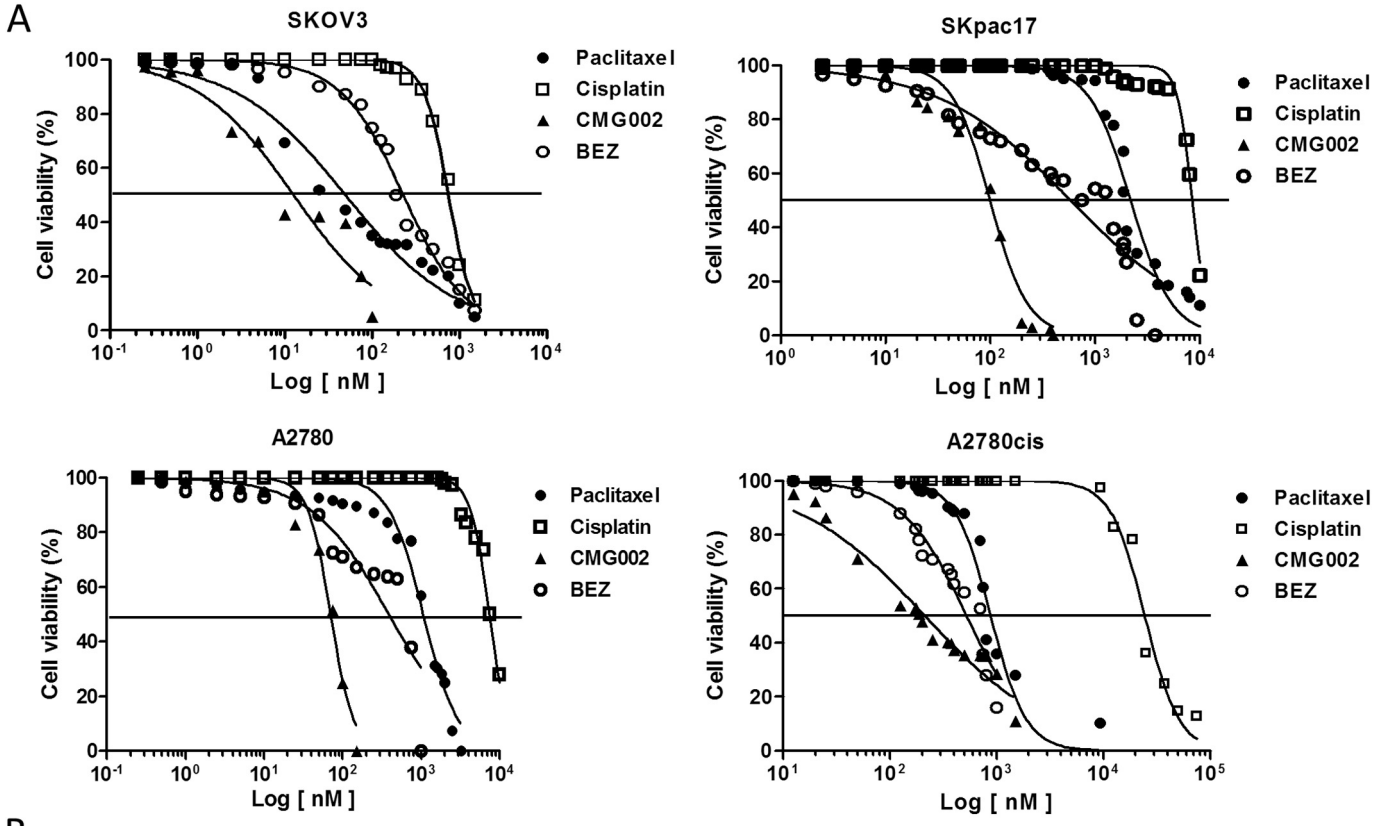
Fig. 1. Decrease of PI3K/mTOR signaling proteins induced by CMG002 treatment. Western blotting showed significant reduction of PI3K, p-AKT, p-mTOR, and p-S6 with CMG002 treatment at low dose in ovarian cancer cell line SKOV3 (A) or OVCAR3 (B). The reduction of these proteins was comparable with previously developed PI3K/mTOR inhibitor, NVP-BEZ235.

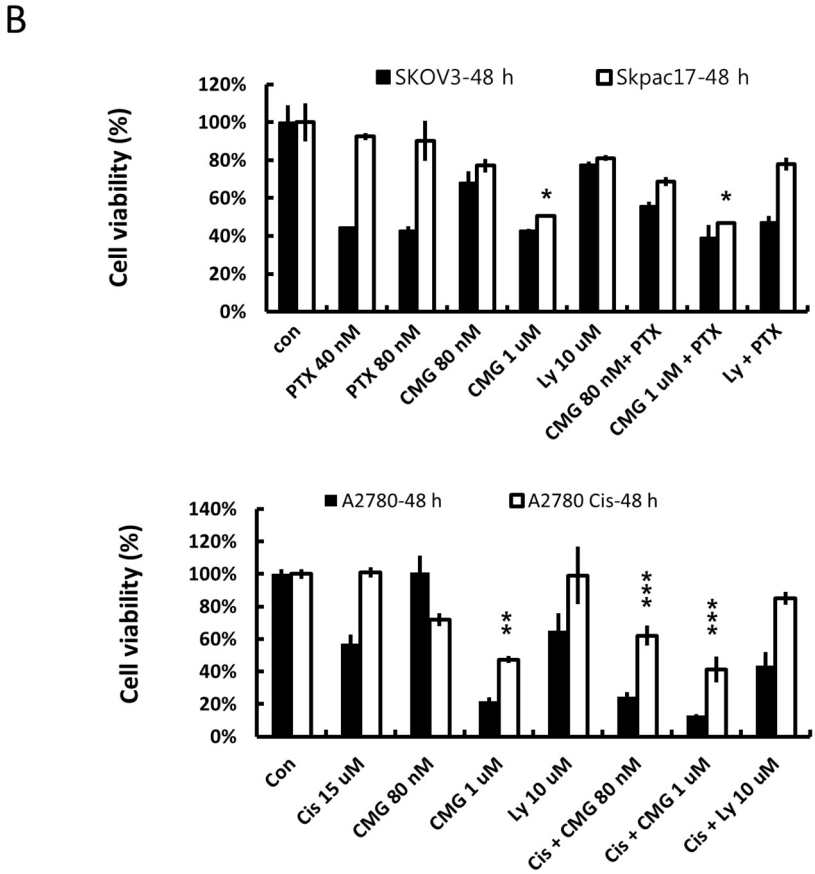
with PTX eventually develop resistance, resulting in recurrence and low survival rates [3]. Therefore, there is an urgent need to improve outcomes for patients with relapsed and refractory ovarian cancer [4] by developing new therapeutics that target chemoresistant cancer cells.

The phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway is constitutively activated in several human cancers. This pathway plays an important role in many biological processes in cancer cells, including proliferation, growth, survival, and metabolism [5–8]. PI3Ks are heterodimers comprising a regulatory and a catalytic subunit [9] and consists of three different classes (I, II, and III) that differ in terms of structure and mechanism of activation. Class I PI3K is the most well-characterized and includes Class IA (p110α, p110β, and p110δ) and class IB (p110γ) enzymes. Aberrant activation of this pathway occurs in many human cancers, including ovarian cancer [9,10]. PI3K regulates G1 cell cycle progression in ovarian cancer cells *via* activation of the Akt/mTOR/p70S6K1 signaling pathway [11,12]. Hence, inhibiting the PI3K/Akt pathway can suppress cell proliferation and trigger cell death. In addition, inhibiting this pathway increases the efficacy of chemotherapeutic agents and re-sensitizes drug-resistant cancer cell populations within malignant human tumors to chemotherapeutic agents [13]. Thus, the components of this pathway are attractive targets for cancer therapeutics.

Several PI3K pathway inhibitors have been developed and are being evaluated in preclinical studies and early clinical trials; such inhibitors include PI3K isoform inhibitors, AKT inhibitors, mTOR catalytic site inhibitors, and PI3K-mTOR dual inhibitors. One possible advantage of a PI3K/mTOR dual inhibitor is suppression of mTORC1 and mTORC2 as well as all catalytic isoforms of PI3K. These inhibitors might effectively turn off this pathway completely, thereby overcoming feedback

Fig. 2. Effect of CMG002 on the viability of chemosensitive and chemoresistant ovarian cancer cell lines. A. Dose-response curve and IC50 values of CMG002, BEZ, paclitaxel and cisplatin for chemosensitive and chemoresistant ovarian cancer cell lines. SKOV3, SKpac17, A2780, and A2780cis cells were seeded at 5×10^3 cells/well and treated for 72 h with various concentrations (0–125 μM) of paclitaxel, cisplatin, CMG002, and BEZ. Cell viability was then measured by a CCK-8 assay. Data were normalized to the values for untreated control cells. The IC_{50} values and best-fit lines were generated during the logarithmic growth phase. The IC_{50} values against SKOV3 (paclitaxel, 49.93 nM; cis, 752.7 nM; CMG002, 12.74 nM; and BEZ, 228.8 nM), SKpac17 (paclitaxel, 2.39 μM; cis 8.49 μM, CMG002, 99.52 nM; and BEZ, 586.6 nM), A2780 (paclitaxel, 1.06 μM; cis, 7.39 μM; CMG002, 70.47 nM; and BEZ, 388.9 nM), and A2780cis (paclitaxel, 879 nM; cis, 24.5 μM: CMG002, 213.1 nM: and BEZ, 522.3 nM) cells. B. Viability of cells treated with CMG002 alone or in combination with PTX or cisplatin. SKOV3 and SKpac17 cells were treated with PTX (40 nM or 80 nM), CMG002 alone (80 nM or and 1 μM), CMG002 + PTX (40 nM), Ly (a pan PI3K inhibitor, 10 μM), or Ly (10 μM) + PTX (40 nM), for 48 h. A2780 and A2780cis cells were treated with cis (15 μM), CMG002 alone (80 nM and 1 μM), CMG002 + cis (15 μM), Ly (10 μM), or Ly (10 μM) + cis (15 μM), for 24 and 48 h. Each bar represents the mean of at least three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, *versus* the control (con) group.





inhibition normally observed with mTORC1 inhibitors (*i.e.*, rapamycin analogs). Recently, a dual PI3k/mTOR inhibitor, CMG002, was newly developed.

Here, we performed *in vitro* and *in vivo* studies to examine whether CMG002 inhibits the growth of chemosensitive and chemoresistant human ovarian cancer cells. We found that CMG002 inhibited

proliferation of chemosensitive and chemoresistant ovarian cancer cells by inducing G1 cell cycle arrest and apoptosis. We also investigated whether combined treatment with CMG002 plus conventional chemotherapeutic agents, PTX and cisplatin (cis), had a synergistic antitumor effect. The results showed that CMG002 re-sensitized cancer cells to these conventional chemotherapeutic agents and acted synergistically to inhibit proliferation of chemoresistant ovarian cancer cells both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Compounds

A new product of PI3K/mTOR dual inhibitor, CMG002, was developed (Supplementary Fig. 1, Fig. 1) by CMG Pharmaceutical Co., Ltd. (Seoul, South Korea). NVP-BEZ235 (BEZ) was kindly provided by CMG Co., Ltd. The PI3K inhibitor Ly294002 (Ly) was purchased from Cell Signaling Technology (Beverly, MA).

2.2. Cell lines

The human ovarian cancer cell line SKOV3 was obtained from American Type Culture Collection (Manassas, VA). SKpac17, a PTX-resistant subline, was established by continuous exposing SKOV3 cells to a stepwise increase in PTX concentration for >8 months [14]. A2780 (cis-sensitive) and A2780cis (cis-resistant) cells were obtained from European Type Culture Collection (Manassas, VA). SKOV3 and SKpac17 cells were maintained in McCoy's 5A medium. A2780 and A2780cis cells were maintained in RPMI 1640. The mutation profiles of these cell lines are presented in Supplementary Table S1.

2.3. Cell viability assay

Cell viability was measured using the CCK-8 assay. Briefly, cells (5×10^3/well) were seeded into a 96-well plate and incubated overnight. The cells were then treated with various concentrations (0–125 μM) of paclitaxel, cis, CMG002, or BEZ for 72 h. Next, 10 μL of the CCK-8 reagent (Dojindo, Kumamoto, Japan) was added and the cells incubated for 3 h. The optical density at 450 nm was measured using a microplate reader (Bio-TeK Instruments, Winooski, VT, USA). Cell viability was calculated and expressed as a percentage of the untreated control cells. To compare the synergistic effects of CMG002 plus PTX or cis with the effects of other PI3K inhibitors, cells were treated for 48 h with PTX (40 nM), cis (10 μM), CMG002 (80 nM or 1 M), or Ly (10 μM).

2.4. Western blot analysis

Cells were washed first with phosphate-buffered saline (PBS, pH 7.4) and then suspended in 100 μL lysis buffer (Pro-Prep, iNtRON Biotechnology, South Korea). Equal amounts of protein were loaded into each well of a 8–12% SDS-polyacrylamide gel, separated by electrophoresis, and electrically transferred to a polyvinylidene difluoride membrane (GE Health Care, Piscataway, NJ). After blocking the membrane with 5% skim milk, target proteins were detected using specific antibodies (Supplementary Table S2). Thereafter, blots were incubated with secondary antibodies, and positive bands detected using a luminol-based chemiluminescence detection kit (Bio-Rad).

2.5. Flow cytometry analysis

Cells were harvested and then resuspended in 1 mL of 0.9% NaCl, followed by addition of ice-cold 90% ethanol (2.5 mL). After incubation at room temperature for 30 min, cells were centrifuged and resuspended in 1 mL PBS containing 50 μg/mL propidium iodide and 100 μg/mL ribonuclease A, and then incubated at 37 °C for 30 min. Flow cytometry analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

2.6. TUNEL analysis of apoptosis

The *In Situ* Cell Death Detection Kit (Roche, Mannheim, Germany) was used to detect and quantify apoptosis by flow cytometry, according to the manufacturer's instructions. In brief, SKpac17 and A2780cis cells (1×10^6 cells/mL) were seeded in 100-mm dishes and then treated with PTX (40 nM), cis (10 μM), CMG002 (80 nM or 1 μM), Ly (10 μM), PTX (40 nM) + CMG002 (80 nM or 1 μM), or cis (10 μM) + CMG002 (80 nM or 1 μM) for 48 h. Cells (2×10^7) were fixed with 75% ethanol for 2 h at −20 °C, and incubated with 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. The cells were then incubated with a TUNEL labeling mixture for 1 h at 37 °C in the dark and analyzed by flow cytometry.

2.7. Immunohistochemistry (IHC) and TUNEL staining

IHC analysis of paraffin-embedded tumor sections was performed using anti-pAkt (Thr308), anti-pAkt (Ser473), anti-pmTOR (Ser2448), and anti-pS6 (Ser235/236) antibodies. Slides were deparaffinized, and then underwent antigen retrieval in citrate buffer. Slides were incubated overnight at 4 °C with an appropriate primary antibody diluted in PBS. Staining was completed using ABC kits (VECTOR Laboratories, Burlingame, CA, USA) and enzyme substrates from VETOR, according to the manufacturer's instructions. Finally, sections were counterstained with hematoxylin. Deparaffinized slides were used for TUNEL stain using an ApoTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (EMD Millipore Corp. Temecula, CA, USA), according to the manufacturer's instructions.

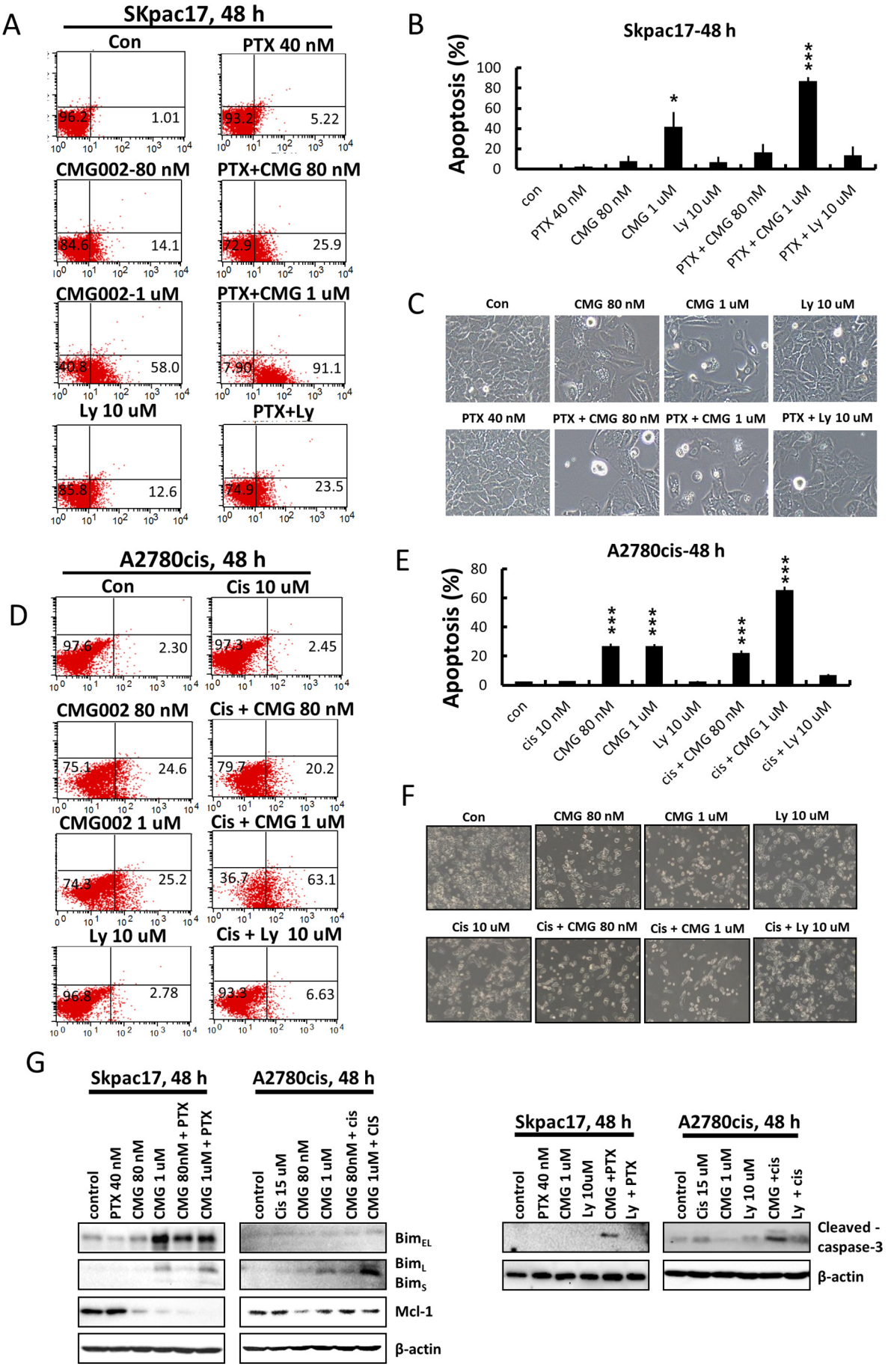
2.8. Tumor xenograft model

SKpac17 (5×10^6) and A2780cis (1×10^7) cells were inoculated subcutaneously into the right and left dorsal flanks of 5-week-old BALB/C nude mice (OrientBio, Sungnam, Korea). When the subcutaneous tumors reached approximately 30 mm^3, mice were assigned randomly to one of eight groups (n = 6–8 per group). Mice bearing tumors were orally administered CMG002 or BEZ (10 mg/kg or 20 mg/kg dissolved in 40% PEG-400/16% 2-hydroxypropyl-*b*-cyclodextrin) for five consecutive days per week. Mice with SKpac17 xenografts were injected intraperitoneally once per week with PTX (10 mg/kg) or cisplatin (2 mg/kg). Tumor volume was measured three times a week and calculated using the following formula: $length^2 \times width^2 \times 0.5$ (length = longest diameter across the tumor, width = corresponding perpendicular diameter).

2.9. Statistical analysis

Statistical analysis was performed using SPSS software version 21.0 (SPSS, Chicago, IL). Quantitative data were obtained from

Fig. 3. Synergistic effect of combined treatment with CMG002 plus PTX or cisplatin on apoptosis of chemoresistant ovarian cancer cells. SKpac17 and A2780cis cells were seeded (5×10^4 cells/mL) in plates and treated with PTX, cis, CMG002 alone, or CMG002 + PTX or cis, for 48 h (con: control). A and D. Apoptosis was assessed by flow cytometry after TUNEL staining. B and E. Histograms show the percentage of apoptotic cells. C and F. Representative images show morphological changes. All images were obtained under a phase-contrast microscope (original magnification, ×200). All experiments were repeated three times, with similar results. G. Western blot analysis for apoptosis related proteins. SKpac17 and A2780cis cells were treated with CMG002 for 48 h. Total cell lysates were prepared and equal amounts of protein electrophoretically separated on 8–12% SDS-polyacrylamide gels. The proteins were then transferred to a nitrocellulose membrane for Western blotting with antibodies specific for Bim, Mcl-1 (G, left panels), cleaved caspase-3 (G, right panels), and β-actin (loading control). Experiments were repeated three times with similar results.


A
SKpac17, 48 h
Con
PTX 40 nM
CMG002-80 nM
PTX+CMG 80 nM
CMG002-1 uM
PTX+CMG 1 uM
Ly 10 uM
PTX+Ly
96.2
1.01
93.2
5.22
84.6
14.1
72.9
25.9
40.8
58.0
7.90
91.1
85.8
12.6
74.9
23.5
B
Skpac17-48 h
Apoptosis (%)
con
PTX 40 nM
CMG 80 nM
CMG 1 uM
Ly 10 uM
PTX + CMG 80 nM
PTX + CMG 1 uM
PTX + Ly 10 uM
C
Con
CMG 80 nM
CMG 1 uM
Ly 10 uM
PTX 40 nM
PTX + CMG 80 nM
PTX + CMG 1 uM
PTX + Ly 10 uM
D
A2780cis, 48 h
Con
Cis 10 uM
CMG002 80 nM
Cis + CMG 80 nM
CMG002 1 uM
Cis + CMG 1 uM
Ly 10 uM
Cis + Ly 10 uM
97.6
2.30
97.3
2.45
75.1
24.6
79.7
20.2
74.3
25.2
36.7
63.1
96.8
2.78
93.3
6.63
E
A2780cis-48 h
Apoptosis (%)
con
cis 10 nM
CMG 80 nM
CMG 1 uM
Ly 10 uM
cis + CMG 80 nM
cis + CMG 1 uM
cis + Ly 10 uM
F
Con
CMG 80 nM
CMG 1 uM
Ly 10 uM
Cis 10 uM
Cis + CMG 80 nM
Cis + CMG 1 uM
Cis + Ly 10 uM
G
Skpac17, 48 h
A2780cis, 48 h
control
PTX 40 nM
CMG 80 nM
CMG 1 uM
CMG 80nM + PTX
CMG 1uM + PTX
control
Cis 15 uM
CMG 80 nM
CMG 1 uM
CMG 80nM + cis
CMG 1uM + CIS
BimEL
BimL
BimS
Mcl-1
β-actin
Skpac17, 48 h
A2780cis, 48 h
control
PTX 40 nM
CMG 1 uM
Ly 10uM
CMG +PTX
Ly + PTX
control
Cis 15 uM
CMG 1 uM
Ly 10 uM
CMG +cis
Ly + cis
Cleaved - caspase-3
β-actin

three or more experiments and expressed as the mean ± S.D. Comparisons between two different treatment groups were analyzed using analysis of variance, followed by a multiple comparison test using a Bonferroni adjustment. A p value < 0.05 was considered statistically significant.

3. Results

3.1. CMG002 reduces the viability of chemoresistant ovarian cancer cells more potently than other PI3K/mTOR inhibitors

We examined the effects of CMG002 on the viability of SKOV3, SKpac17 (PTX-resistant), A2780, and A2780cis (cis-resistant) cells using a CCK-8 assay and compared the results with those for another dual PI3K/mTOR inhibitor (BEZ). Exposure to 0–125 μM CMG002, BEZ, PTX, or cis for 72 h reduced the viability of chemosensitive (SKOV3 and A2780) and chemoresistant (SKpac17 and A2780cis) ovarian cancer cells in a dose-dependent manner (Fig. 2A). CMG002 was able to reduce the viability of chemoresistant SKpac and A2780cis cells with IC_{50} values of 99.52 nM and 213.1 nM, respectively. The values were much lower than those for BEZ (IC_{50} = 586.6 nM and 522.3 nM, respectively).

We then examined whether combination treatment with CMG002 plus primary chemotherapeutic agents (PTX or cis) increased cytotoxicity against chemoresistant ovarian cancer cells (Fig. 2B). We found that CMG002 caused a significant reduction in the viability of Skpac17 cells (by 77% at 80 nM and 50% at 1 μM) as well as SKOV3 cells (by 68% at 80 nM and 42% at 1 μM). By contrast, there was no significant reduction in the viability of SKpac17 cells treated with PTX. However, the combined treatment of Skpac17 cells with CMG002 and PTX showed stronger cytotoxic effects (cell viability was reduced by 67% [PTX + 80 nM CMG002] and 46% [PTX + 1 μM CMG002]).

In addition, treatment of A2780cis cells with CMG002 reduced viability by 62% [80 nM] and 47% [1 μM], whereas no significant reduction was found in the viability of A2780cis cells treated with cis alone. However, the combination treatment with CMG002 plus 15 μM cis reduced viability by 67% [80 nM CMG002] or 41% [1 μM CMG002]. Treatment of SKpac17 with Ly [10 μM] alone (81%) or with Ly + PTX [40 nM] (77%) had little effect. Similar results were observed for A2780cis cells after treatment with Ly alone (99%) and after treatment with Ly plus cis (85%).

These results indicate that CMG002 reduces the viability of chemoresistant as well as chemosensitive ovarian cancer cells, and that it has a synergistic effect against chemoresistant cancer cells when used alongside primary chemotherapeutic agents.

3.2. Combined treatment of chemoresistant ovarian cancer cells with CMG002 plus PTX or cis induces apoptosis

To assess the apoptotic effects of CMG002 and its ability to resensitize cells to PTX or cis, we exposed chemoresistant SKpac17 and A2780cis cells to either CMG002 alone or to CMG002 plus PTX or cis for 48 h. Flow cytometry analysis of TUNEL-positive cells revealed that CMG002 induced apoptosis in Skpac17 (Fig. 3A–C) and A2780cis (Fig. 3D–F) cells. The percentage of apoptotic Skpac17 cells was much higher after CMG002 treatment (80 nM, 7.8%; 1 μM, 42%; $p < 0.05$ and $p < 0.01$, respectively) than after treatment with PTX (40 nM, 2.5%) or Ly (10 μM; 6.7%). Moreover, the number of apoptotic cells detected after combined treatment with CMG002 and PTX was higher (16.7% in CMG002 [80 nM] + PTX and 87% in CMG002 [1 μM] + PTX, $p = 0.06$ and $p < 0.001$, respectively) than that with PTX + Ly (13.8%). Similar results were obtained for A2780cis cells (CMG002 [1 μM] *vs.* CMG [1 μM] + cis = 26% *vs.* 65%). In comparison, treatment with Ly only or Ly + cis had no significant effect on apoptosis in chemoresistant cells. Taken together, CMG002 was consistently a more potent inducer of apoptosis in chemoresistant ovarian cancer cells than Ly (a pan PI3K inhibitor), and showed a synergistic effect when used along with PTX or cis.

Next, we explored the effect of CMG002 on expression of anti- and pro-apoptotic proteins (Fig. 3G). We found a significant increase in expression of Bim upon treatment with CMG002, either alone or in combination with PTX or cis. Bim_{EL} (23 kDa) was main form identified in SKpac cells whereas Bim_L (19 kDa) was the main form in A2780cis cells. Among the anti-apoptotic proteins tested, treatment with CMG002 led to a significant reduction in expression of Mcl-1. However, expression of other pro-apoptotic proteins (Bax and Bak), or of anti-apoptotic proteins (Bcl-2 and Bcl-xL) was unchanged by CMG002 treatment (data not shown). Expression of cleaved caspase-3 increased significantly in SKpac and A2780cis cells upon combined treatment with CMG002 + PTX or cis.

3.3. CMG002 induces cell cycle arrest at G1 phase

On cell cycle analysis in chemosensitive and chemoresistant ovarian cancer cells treated with CMG002, either alone or in combination with PTX, for 24 h (Fig. 4A–C) and 48 h (Supplementary Fig. S2), the results showed that 64% of control SKOV3 and 55% of control SKpac17 cells were in G0/1 phase; however, CMG002 arrested both cell lines at G0/G1 (SKOV3, 88%; SKpac17, 80%, $p < 0.05$). We found it interesting that PTX induced G2/M arrest in SKOV3 cells; however, the percentage of G2/M arrested cells fell from 94% to 56% when PTX was combined with CMG002 (Fig. 4A). By contrast, PTX had no effect on the cell cycle of PTX-resistant SKpac17 cells (Fig. 4B). However, G1 arrest was observed even in SKpac17 cells treated with CMG002 or Ly, either alone (80% *vs.* 66%, respectively) or in combination with PTX (PTX + CMG002 = 78%; PTX + Ly = 63%).

Exposure of SKOV3 cells to CMG002 alone reduced expression of cyclins D3, B1, and E; there was no change in expression after treatment with PTX or Ly alone (Fig. 4C). However, expression of cyclin B1 increased after treatment with PTX; this was not reversed after combined treatment with CMG002. Even in PTX-resistant SKpac17 cells, CMG002 led to a significant reduction in expression of all three cyclins, whereas PTX or Ly alone, or PTX + Ly, had no effect.

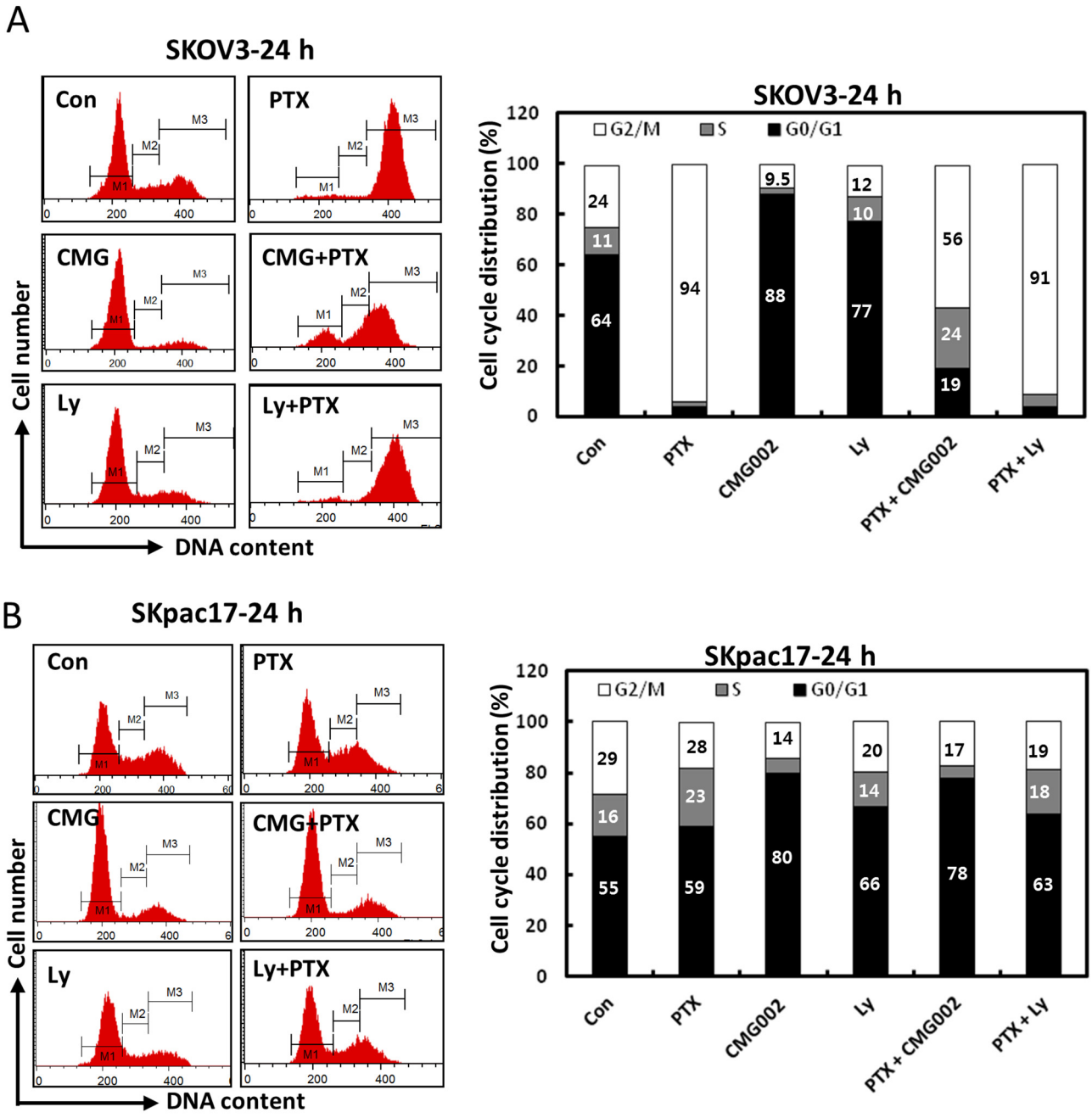
Treatment of A2780 and A2780cis cells with CMG002 (Fig. 4D–F, Supplementary Fig. S3), either alone or in combination with cis, induced cell cycle arrest at G0/G1 phase (A2780: 88% and 81% by CMG002 alone or CMG002 + cis, respectively; A2780cis: 88% and 83%, by CMG002 alone or CMG002 + cis, respectively). By contrast 65% of A2780 and 68% of A2780cis ($p < 0.05$) control cells were at G0/G1. Western blot analysis revealed that CMG002 caused a significant reduction in expression of cyclin D3 in A2780 and A2780cis cells.

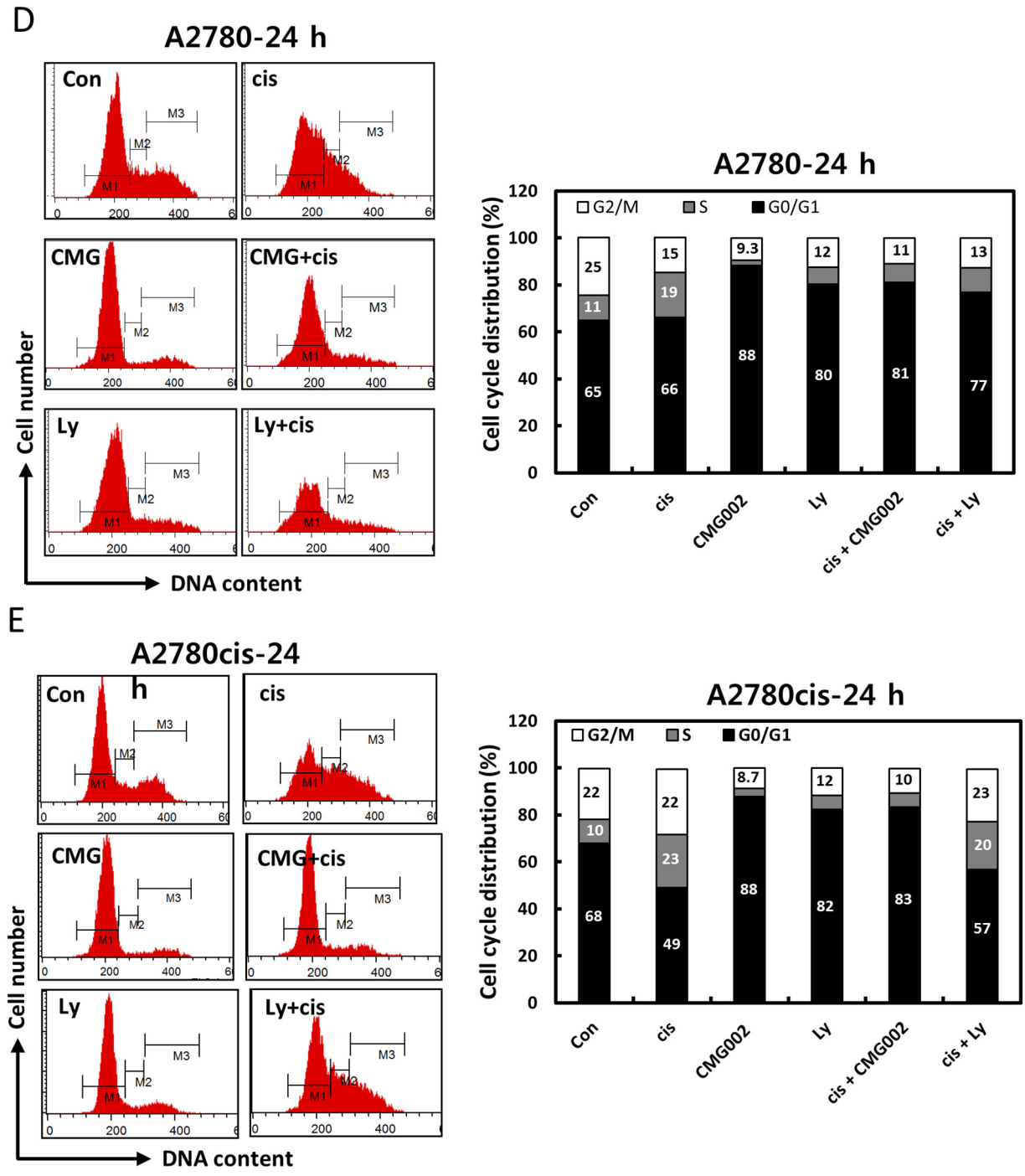
Taken together, CMG002 induces G1 arrest even in chemoresistant SKpac17 and A2780cis cells by decreasing expression of cyclin D3 and cyclin E, which was not observed after treatment with PTX or cis alone.

3.4. CMG002 inhibits the PI3k/Akt/mTOR signaling pathway

We, then, monitored expression of downstream proteins, and found that phosphorylated Akt (Ser473 and Thr308), phosphorylated mTOR (Ser2448), and phosphorylated S6 (at Ser235/236) in all cell lines was inhibited by CMG002 (when used alone and in combination with PTX;

Fig. 4. Effect of CMG002 on cell cycle arrest of paclitaxel or cisplatin-sensitive or -resistant ovarian cancer cells. A, B. Cell cycle analysis for SKOV3 and SKpac17 cells. SKOV3 (A) and SKpac17 (B) cells were treated with PTX (40 nM), CMG002 alone (1 μM), CMG002 + PTX (40 nM), Ly (10 μM), or Ly (10 μM) + PTX (40 nM) for 24 h (con: control). Cells were harvested and analyzed by flow cytometry. Histograms show the percentage of cells in G1, S, and G2/M phase (A and B, right panels). C. Western blot analysis using antibodies specific for cyclin D3, cyclin B1, cyclin E, and β-actin (loading control). D and E. Cell cycle analysis for A2780 and A2780cis. A2780 (D) and A2790cis (E) cells were treated cis (15 μM), CMG002 (1 μM), CMG002 + cis, Ly (10 μM), or Ly + cis, for 24 h. Histogram shows the percentage of cells in G0/G1, S, and G2/M (D and E, right panels). F. Western blot analysis using antibodies specific for cyclin D3, cyclin B1, and β-actin (loading control).


A
SKOV3-24 h
Con
PTX
CMG
CMG+PTX
Ly
Ly+PTX
M1
M2
M3
0
200
400
Cell number
DNA content
SKOV3-24 h
G2/M
S
G0/G1
Cell cycle distribution (%)
0
20
40
60
80
100
120
24
11
64
94
9.5
88
12
10
77
56
24
19
91
Con
PTX
CMG002
Ly
PTX + CMG002
PTX + Ly
B
SKpac17-24 h
Con
PTX
CMG
CMG+PTX
Ly
Ly+PTX
M1
M2
M3
0
200
400
Cell number
DNA content
SKpac17-24 h
G2/M
S
G0/G1
Cell cycle distribution (%)
0
20
40
60
80
100
120
29
16
55
28
23
59
14
80
20
14
66
17
78
19
18
63
Con
PTX
CMG002
Ly
PTX + CMG002
PTX + Ly
C
SKOV3, 24 h
Skpac17, 24 h
Con
PTX
CMG002
Ly
CMG+PTX
Ly+PTX
cyclinD3
cyclinB1
cyclinE
β-actin


D
A2780-24 h
Con
cis
CMG
CMG+cis
Ly
Ly+cis
M1
M2
M3
Cell number
DNA content
A2780-24 h
G2/M
S
G0/G1
Cell cycle distribution (%)
Con
cis
CMG002
Ly
cis + CMG002
cis + Ly
E
A2780cis-24 h
A2780cis-24 h
F
A2780, 24 h
A2780cis, 24 h
control
Cis 15 uM
CMG 1 uM
Ly 10 uM
CMG + cis
Ly + cis
cyclinD3
cyclinB1
β-actin

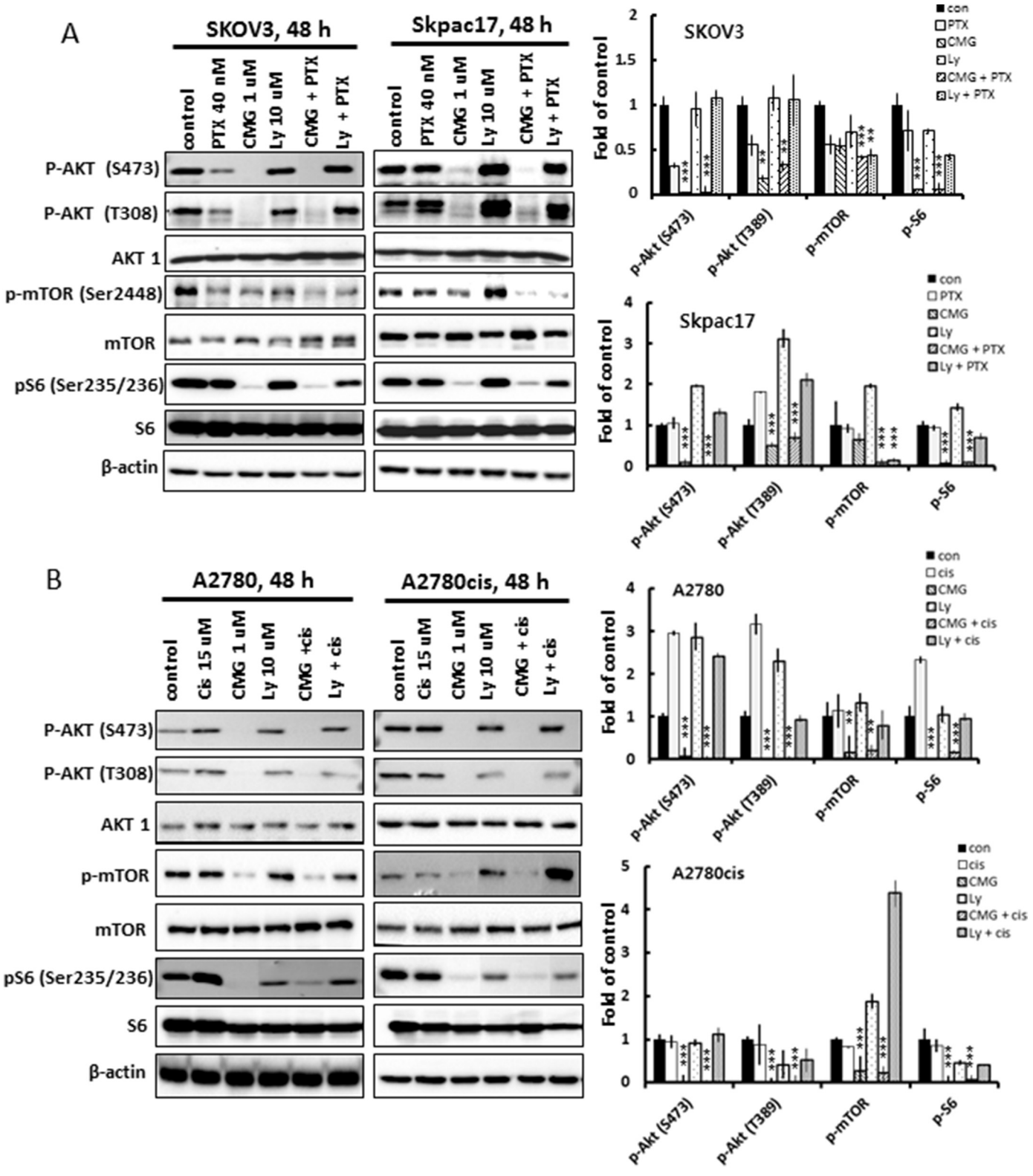
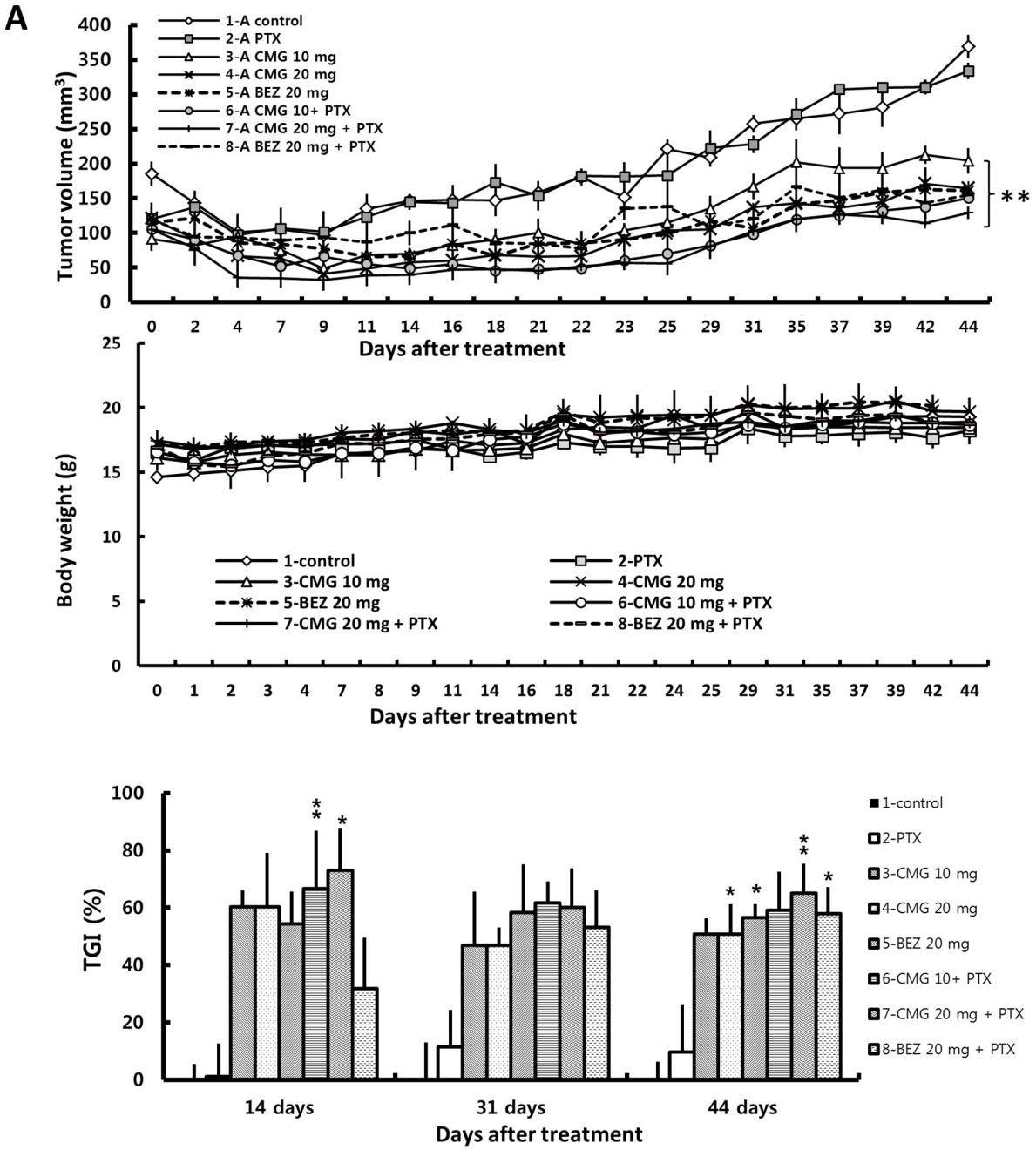
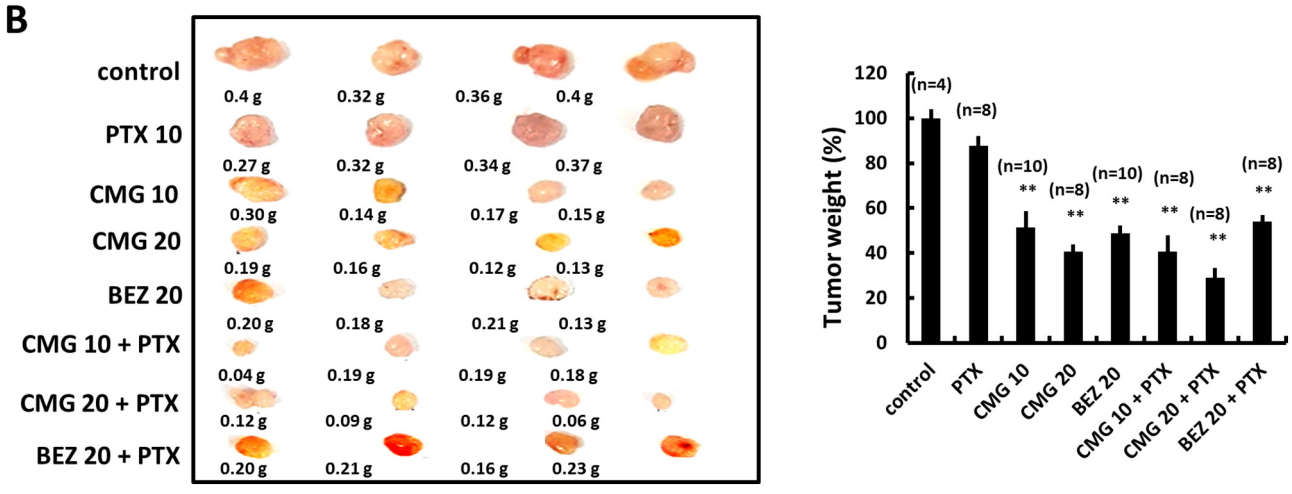


Fig. 5. Effect of CMG002 on the PI3K/mTOR signaling pathway. A and B. Western blot analysis for PI3K/mTOR signaling proteins. The SKOV3, SKpac17 (A), A2780, and A2780cis cells (B) were seeded at 5×10^4 cells/mL and treated with PTX (40 nM), cis (15 μM), CMG002 (1 μM), CMG002 + PTX, CMG002 + cis, Ly, Ly + PTX, or Ly + cis, for 48 h. Western blots were performed with antibodies specific for p-Akt (Ser473), p-Akt (Thr308), Akt1, p-mTOR (Ser2448), p-S6 (Ser235/236), S6, and β-actin (loading control). Experiments were repeated three times, with similar results. Protein bands (A and B, left panels) were quantitated by densitometric analysis. The ratio of the intensity of protein bands relative to β-actin was calculated (A and B, right panels). Experiments were repeated tree times with similar results. $^{**}p < 0.01$; $^{***}p < 0.001$, *versus* the control group.

$p < 0.001$); however, treatment with PTX or cis alone had no effect on expression of these proteins in PTX-resistant SKpac17 cells or A2780cis cells (Fig. 5A & B). Treatment with Ly did not cause a significant reduction in expression of these proteins. These data demonstrate that CMG002 suppresses expression of downstream proteins of the PI3k/Akt/mTOR signaling pathway, including mTORC1 and mTORC2.


A
1-A control
2-A PTX
3-A CMG 10 mg
4-A CMG 20 mg
5-A BEZ 20 mg
6-A CMG 10+ PTX
7-A CMG 20 mg + PTX
8-A BEZ 20 mg + PTX
Tumor volume (mm3)
Days after treatment
**
Body weight (g)
1-control
2-PTX
3-CMG 10 mg
4-CMG 20 mg
5-BEZ 20 mg
6-CMG 10 mg + PTX
7-CMG 20 mg + PTX
8-BEZ 20 mg + PTX
Days after treatment

TGI (%)
1-control
2-PTX
3-CMG 10 mg
4-CMG 20 mg
5-BEZ 20 mg
6-CMG 10+ PTX
7-CMG 20 mg + PTX
8-BEZ 20 mg + PTX
14 days
31 days
44 days
Days after treatment


B
control
PTX 10
CMG 10
CMG 20
BEZ 20
CMG 10 + PTX
CMG 20 + PTX
BEZ 20 + PTX
0.4 g
0.32 g
0.36 g
0.4 g
0.27 g
0.32 g
0.34 g
0.37 g
0.30 g
0.14 g
0.17 g
0.15 g
0.19 g
0.16 g
0.12 g
0.13 g
0.20 g
0.18 g
0.21 g
0.13 g
0.04 g
0.19 g
0.19 g
0.18 g
0.12 g
0.09 g
0.12 g
0.06 g
0.20 g
0.21 g
0.16 g
0.23 g
Tumor weight (%)
(n=4)
(n=8)
(n=10)
(n=8)
(n=10)
(n=8)
(n=8)
(n=8)
control
PTX
CMG 10
CMG 20
BEZ 20
CMG 10 + PTX
CMG 20 + PTX
BEZ 20 + PTX

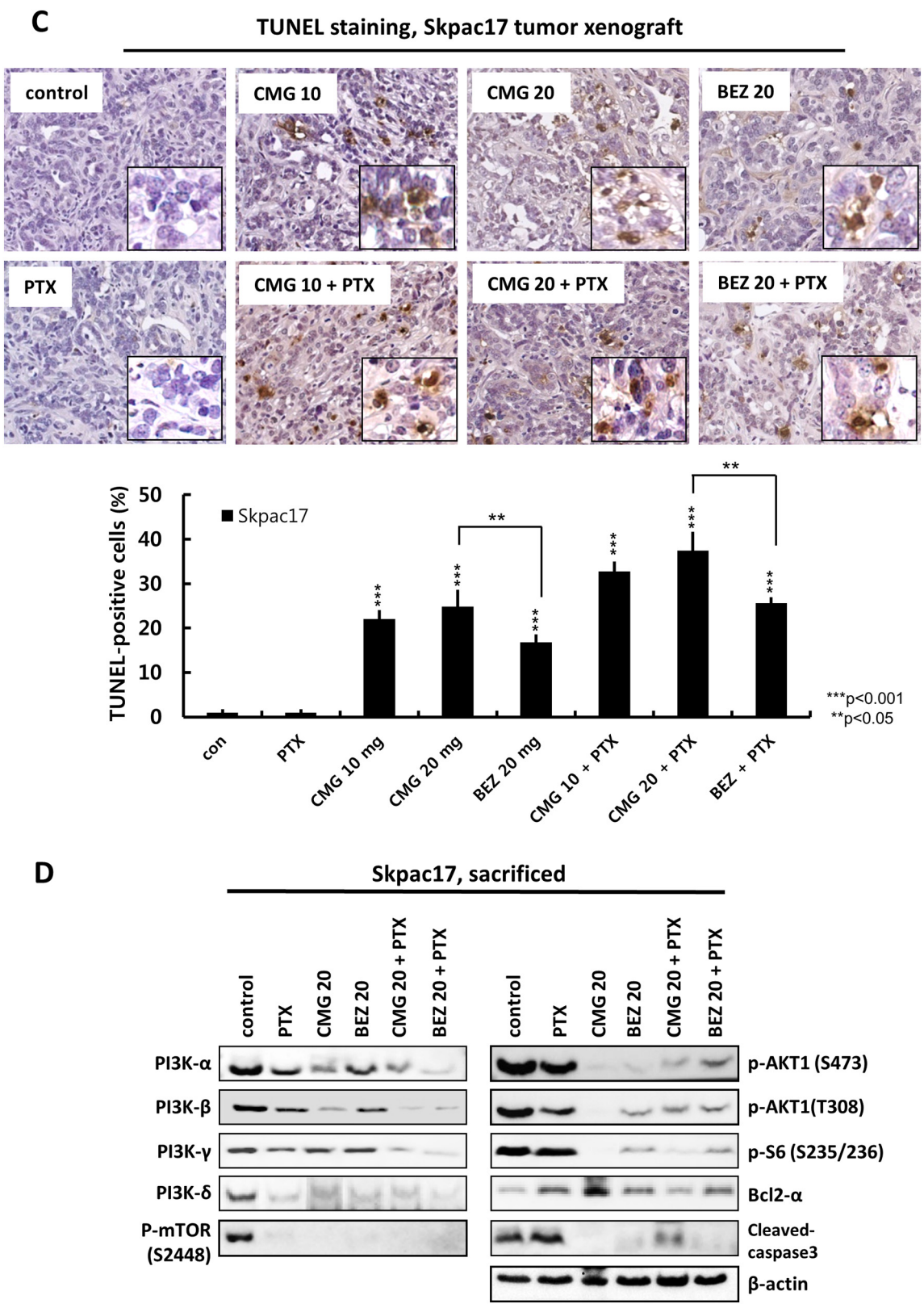


Fig. 6. Effect of CMG002 and NVP-BEZ235 on growth of PTX-resistant SKpac xenografts. A. Tumor growth inhibition effect of CMG002 in comparison with NVP-BEX235 on SKpac xenografts. Changes in tumor volume (A, upper panel). There was no difference in body weight between the groups (A, middle panel). Tumor growth inhibition (TGI) was quantified and expressed as a percentage relative to the control. $^{*}p < 0.05$; $^{**}p < 0.01$, *versus* control (A, lower panel). B. Tumor size and weight at the end of the experiment. The graph represents the tumor weight of each group relative to control. Results are expressed as the mean ± SEM (SKpac17: control (n = 4), PTX (n = 8), CMG002 [10 mg/kg] (n = 10), CMG002 [20 mg/kg] (n = 8), NVP-BEZ235 [20 mg/kg] (n = 10), CMG002 [10 mg/kg] + PTX (n = 8), CMG002 [20 mg/kg] + PTX (n = 8), and NVP-BEZ235 [20 mg/kg] + PTX (n = 8)). C. TUNEL stain for extracted tumor tissue. Original magnification, ×100 (C, upper panel). The graph represents the percentage of apoptotic cells of each group. The number of TUNEL-positive cells in five high power fields was counted and data expressed as the percentage of apoptotic cells within the total tumor cell population (mean ± S.D.; 10 fields in two slides) (C, lower panel). $^{***}p < 0.001$, *versus* control group. D. Western blot analysis of p-Akt (Sre473), p-Akt (Thr308), p-S6 (Ser235/236), and β-actin expression in SKpac17 xenograft tumors.

3.5. Antitumor effects of CMG002 in a chemoresistant xenograft model

To assess the *in vivo* efficacy of CMG002, we treated female nude mice bearing SKpac17 or A2780cis xenografts with vehicle, CMG002 (20 mg/kg) alone, or a combination of CMG002 + PTX (10 mg/kg) (SKpac17 xenografts) or CMG002 + cis (2 mg/kg) (A2780cis xenografts). Compared with vehicle, CMG002 alone inhibited growth of SKpac17 xenografts by 78% ($p < 0.01$) and that of A2780cis xenografts by 60% ($p < 0.05$); these values increased further when CMG002 was used in combination with PTX (SKpac17: 89.6% inhibition; $p < 0.001$) or cis (A2780cis: 79% inhibition; $p < 0.001$) for 9–11 days (Supplementary Fig. S4A & B). Consistent with the above data, the weights of extracted tumors from the CMG002 alone or combined treatment groups were significantly lower than those from the control group [Skpac17 ($p < 0.05$): CMG002, 38.1%; CMG + PTX, 11.8% of the control value; A2780cis ($p < 0.05$): CMG002, 40.2%; CMG002 + cis, 22% of the control value].

TUNEL staining of A2780cis xenograft tumor tissue revealed that CMG002, either alone (42.1%) or in combination with cis (52.3%), led to a significant increase in the percentage of TUNEL-positive cells when compared with the control (16.3%) or cis only (17.7%) groups ($p < 0.001$; Supplementary Fig. S4C). As expected, Western blotting revealed that expression of phosphorylated Akt (Ser473 and Thr308) and phosphorylated mTOR (Ser2448) in tumors treated with CMG002 alone or in combination with cis was much lower than that in the control and cis-only groups (Supplementary Fig. S4D). It was not possible to perform TUNEL staining and Western blotting of SKpac xenografts because not enough tumor tissue could be extracted.

3.6. CMG002 inhibits growth of chemoresistant tumors more effectively than BEZ

To confirm CMG002-mediated inhibition of tumor growth, we repeated the *in vivo* study by treating the SKpac17 xenografts with CMG002 alone (10 mg/kg and 20 mg/kg) or in combination with PTX (Fig. 6). BEZ (20 mg/kg), another dual PI3K/mTOR inhibitor, was used as a reference compound. After 14 days, we noted that the tumor growth-inhibiting effect of CMG002, either alone or in combination with PTX, was similar to the result in the previous experiments (CMG002 alone [10 mg/kg], 52%; CMG002 [10 mg/kg] + PTX, 66.7% ($p < 0.01$); CMG002 alone [20 mg/kg], 60.3%; CMG002 [20 mg/kg] + PTX, 73% ($p < 0.01$); BEZ [20 mg/kg], 54.3%; and BEZ [20 mg/kg] + PTX, 31% ($p < 0.01$, compared with the control and PTX only groups). Next, treatment was stopped for 15 days (from Day 15 to Day 30) to observe rebound phenomena. At Day 31, tumor growth was still inhibited relative to that in control mice. Drugs were then re-administered from Day 32, and mice were sacrificed at Day 44. At this point, tumor growth inhibition reached 35.4% and 47.9% (CMG002 and CMG002 + PTX, respectively, $p < 0.01$ compared with the control and PTX-only groups) in CMG002 [10 mg/kg] treated group; 52.7% and 56% (CMG002 and CMG002 + PTX, respectively, $p < 0.01$) in CMG002 [20 mg/kg] treated group; 44.2% and 41.8% (BEZ and BEZ + PTX respectively, $p < 0.01$) in BEZ [20 mg/kg] treated group. The weights of tumors in the CMG002 alone and CMG002 + PTX groups were significantly smaller than those in the control group: CMG002 [10 mg/kg], 51.3%; CMG002 [10 mg/kg] + PTX, 40.5%; CMG002 [20 mg/kg], 40.5%; CMG002 [20 mg/kg] + PTX, 29%; and PTX only; 87% of the control) (Fig. 6B; $p < 0.001$). The weights of tumors treated with BEZ [20 mg/kg] were 48.6% and 54% of the control (BEZ alone and BEZ + PTX, respectively).

These data indicate that CMG002 significantly inhibits the growth of chemoresistant xenografts even by a lower dose (10 mg/kg), which effects were comparable with those afforded by a higher dose of BEZ (20 mg/kg). In addition, combined treatment with CMG002 and PTX had a synergistic effect, however, BEZ did not have this effect.

Finally, we examined the role of apoptosis on CMG002-mediated tumor inhibition (Fig. 6C). CMG002-treated (10 or 20 mg/kg) tumor tissues showed a significant increase in the percentage of TUNEL-positive cells (22% and 24.8% at 10 and 20 mg/kg, respectively; $p < 0.001$), compared with that of control of PTX alone treated group (1%). BEZ at 20 mg/kg also induced apoptosis (16.8%, $p < 0.001$), which was less than that of CMG002 [20 mg/kg] group ($p < 0.05$). Notably, combination treatment led to an even more significant increase in the percentage of apoptotic cells (CMG002 [10 mg/kg] + PTX, 32.8%; CMG [20 mg/kg] + PTX, 37.4%; and BEZ [20 mg/kg] + PTX, 25.6%; $p < 0.001$). Next, we performed Western blotting (Fig. 6D) and immunohistochemistry (Supplementary Fig. S5), found that phosphorylated Akt, mTOR, and S6 were significantly downregulated in CMG002-treated tumor tissues compared with tissues treated with vehicle or PTX alone. We evaluated the liver histology as well as body weight monitoring. There was no necrotic area on H&E sections suggesting liver toxicity.

4. Discussion

The high mortality rate of ovarian cancer is mainly related to its recurrence from the adoption of chemoresistance. The development of chemoresistance is reportedly associated with activation of the PI3K/AKT/mTOR pathway in cancer cells [15,16]. Therefore, strategies aimed at inhibiting the PI3K/AKT/mTOR pathway might overcome chemoresistance [17] and increase disease free survival.

The PI3K/Akt/mTOR pathway is activated constitutively in many human cancers, providing a unique opportunity for intervention using chemotherapeutics. Compared with the other types of PI3K pathway inhibitor, PI3K/mTOR dual inhibitors target all catalytic forms of PI3K, as well as mTORC1 and mTORC2. To do this, they must overcome feedback inhibition normally associated with mTORC1 inhibitors, which effectively blocks this pathway and limits efficacy [18].

This study is the first to provide evidence for the efficacy of a newly developed PI3K/mTOR dual inhibitor, CMG002, in preclinical models of PTX-resistant or cis-resistant ovarian cancer cells. We investigated whether this PI3K/mTOR dual inhibitor exerts a significant antitumor effect when used either alone or in combination with conventional chemotherapeutic agents. We found that treatment with CMG002 caused a significant reduction in the viability of chemoresistant ovarian cancer cells as well as chemosensitive cells. We also compared the effect of CMG002 with that of other PI3K/mTOR inhibitors, Ly and BEZ [19,20]. Low concentrations of CMG002 inhibited proliferation of chemosensitive SKOV3 and A2780 cells more effectively than the other PI3K inhibitors. In addition, CMG002 reduced the viability of PTX-resistant SKpac17 cells and cis-resistant A2780cis cells; the other PI3K inhibitors had less significant effect on these resistant cells when used at similar concentrations.

CMG002 induced apoptosis in PTX-resistant SKpac cells; this was not the case for PTX alone. Combined treatment with CMG002 and PTX led to even greater apoptosis induction, suggesting that CMG002 re-sensitizes PTX-resistant cells to PTX. A similar effect was seen in cis-resistant A2780cis cells co-treated with CMG002 and cis. This response was accompanied by a significant increase in expression of cleaved caspase 3 in cells co-treated with CMG002 and PTX or cis. Bim and Mcl-1 were the major pro- and anti-apoptotic proteins regulated by CMG002.

We also demonstrated that the anti-proliferative activity of CMG002 is also due to induction of G1 cell cycle arrest. Ly reportedly inhibits PI3K activity and reduces proliferation of ovarian cancer cells by inducing G1 cell cycle arrest; this is accompanied by decreased expression of G1-associated proteins, including cyclin D1 [11]. Inhibiting mTOR or PI3K in different cell types shows similar effects [21,22]. The mTORC1 increases S6 kinase activity, which then phosphorylates S6 and plays an important role in cell cycle progression *via* G1 phase [23]. Here, we found that CMG002 induced G1 arrest even in PTX-resistant SKpac cells, whereas PTX did not induce G2 arrest in PTX-resistant SKpac cells (it usually did in PTX-sensitive SKOV3 cells). This change was accompanied by reduced expression of cyclin D3 and cyclin E. Intriguingly, PTX is a microtubule

inhibitor that binds to tubulins or microtubules to suppress microtubule dynamics and function; this induces G_2/M cell cycle arrest [24]. Here, we found that PTX increased expression of cyclin B1 in SKOV3 cells; this was also the case after combination treatment with PTX and CMG002. CMG002 also induced G1 arrest in A2780 and cis-resistant A2780cis cells, which was accompanied by decreased expression of cyclin D3; this was not observed after treatment with cis alone.

A number of PI3K pathway inhibitors have been developed and are being evaluated in preclinical studies. Rapamycin analogs that specifically inhibit mTORC1 are the most clinically advanced, however, the activity of traditional mTORC1 inhibitors is limited [25], because it may cause feedback-mediated activation of the PI3K/Akt pathway [18,26]. Several studies demonstrated that a previously developed PI3K/mTOR dual inhibitor, BEZ, is an effective treatment option for various cancers [19,27–29]; indeed, the drug has entered phase I testing [30,31]. Montero et al. [32] showed that BEZ effectively suppresses proliferation of four ovarian carcinoma cell lines, and previous preclinical studies demonstrate that BEZ sensitizes nasopharyngeal carcinoma cells to the antitumor effects of cis [33].

Here, our *in vivo* study demonstrated that CMG002 was a more potent inhibitor of PTX-resistant SKpac tumor growth than BEZ, even when used at a low dose (10 mg/kg); indeed, the effects of low-dose CMG002 were equivalent to those of BEZ at 20 mg/kg. In addition, CMG002 re-sensitized resistant cells to the effects of PTX or cis, whereas BEZ did not re-sensitize PTX-resistant cells to PTX. We showed that CMG002 plus PTX or cis induced a synergistic increase in apoptotic cell death, suggesting that CMG002 re-sensitizes PTX or cis-resistant ovarian cancer cells to these drugs; therefore, CMG002 might overcome resistance to taxol or platinum agents. *In vivo* testing revealed that CMG002 induced marked suppression of tumor growth, either alone or in combination with PTX or cis; indeed, combination treatment had a more pronounced effect (with no change in body weight). This supports our notion that CMG002 re-sensitizes resistant cells to the effects of PTX or cis.

In summary, we demonstrate here for the first time that a newly developed novel PI3K/Akt/mTOR dual inhibitor, CMG002, inhibits growth of chemosensitive and chemoresistant ovarian cancer cells (both *in vivo* and *in vitro*) by inducing apoptosis and G1 arrest. In addition, CMG002 re-sensitizes resistant cells to the effects of PTX or platinum agents. Thus, CMG002 (either alone or in combination with PTX or cis) is a promising therapeutic option for chemoresistant ovarian cancer.

Conflict of interest statement

The authors have no potential conflicts of interest to declare.

Acknowledgements

This research was supported by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute, funded by the Ministry of Health and Welfare, Republic of Korea (grant number: HI16C1559).

Author contribution

Hye Joung Choi: Write the first draft of manuscript

Jin Hyung Heo, Se Hwa Kim, Yong Wha Moon: Review and discuss the study

Ju Yeon Park, Ju Yeon Jeong, Hyeon Ju Cho, Kyung Soon Park: Perform the experiments

Jin Sung Kim: Production of CMG002

Hee Jung An: Study design, review the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2019.01.012.

References

[1] D.M. Parkin, Global cancer statistics in the year 2000, Lancet Oncol. 2 (2001) 533–543.
[2] S.A. Cannistra, Intraperitoneal chemotherapy comes of age, N. Engl. J. Med. 354 (2006) 77–79.
[3] S.L. Cooke, J.D. Brenton, Evolution of platinum resistance in high-grade serous ovarian cancer, Lancet Oncol. 12 (2011) 1169–1174.
[4] T.A. Yap, C.P. Carden, S.B. Kaye, Beyond chemotherapy: targeted therapies in ovarian cancer, Nat. Rev. Cancer 9 (2009) 167–181.
[5] J.Q. Cheng, C.W. Lindsley, G.Z. Cheng, H. Yang, S.V. Nicosia, The Akt/PKB pathway: molecular target for cancer drug discovery, Oncogene 24 (2005) 7482–7492.
[6] M. Cully, H. You, A.J. Levine, T.W. Mak, Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis, Nat. Rev. Cancer 6 (2006) 184–192.
[7] I. Vivanco, C.L. Sawyers, The phosphatidylinositol 3-kinase AKT pathway in human cancer, Nat. Rev. Cancer 2 (2002) 489–501.
[8] S. Wullschleger, R. Loewith, M.N. Hall, TOR signaling in growth and metabolism, Cell 124 (2006) 471–484.
[9] B. Vanhaesebroeck, M.D. Waterfield, Signaling by distinct classes of phosphoinositide 3-kinases, Exp. Cell Res. 253 (1999) 239–254.
[10] S. Kang, A.G. Bader, P.K. Vogt, Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 802–807.
[11] N. Gao, D.C. Flynn, Z. Zhang, X.S. Zhong, V. Walker, K.J. Liu, et al., G1 cell cycle progression and the expression of G1 cyclins are regulated by PI3K/AKT/mTOR/p70S6K1 signaling in human ovarian cancer cells, Am. J. Phys. Cell Physiol. 287 (2004) C281–C291.
[12] N. Pullen, G. Thomas, The modular phosphorylation and activation of p70s6k, FEBS Lett. 410 (1997) 78–82.
[13] S.D. Westfall, M.K. Skinner, Inhibition of phosphatidylinositol 3-kinase sensitizes ovarian cancer cells to carboplatin and allows adjunct chemotherapy treatment, Mol. Cancer Ther. 4 (2005) 1764–1771.
[14] H. Kang, J.Y. Jeong, J.Y. Song, T.H. Kim, G. Kim, J.H. Huh, et al., Notch3-specific inhibition using siRNA knockdown or GSI sensitizes paclitaxel-resistant ovarian cancer cells, Mol. Carcinog. 55 (2016) 1196–1209.
[15] C.P. Carden, A. Stewart, P. Thavasu, E. Kipps, L. Pope, M. Crespo, et al., The association of PI3 kinase signaling and chemoresistance in advanced ovarian cancer, Mol. Cancer Ther. 11 (2012) 1609–1617.
[16] Z.C. Dobbin, C.N. Landen, The importance of the PI3K/AKT/MTOR pathway in the progression of ovarian cancer, Int. J. Mol. Sci. 14 (2013) 8213–8227.
[17] J.Y. Jeong, K.S. Kim, J.S. Moon, J.A. Song, S.H. Choi, K.I. Kim, et al., Targeted inhibition of phosphatidyl inositol-3-kinase p110beta, but not p110alpha, enhances apoptosis and sensitivity to paclitaxel in chemoresistant ovarian cancers, Apoptosis 18 (2013) 509–520.
[18] K.E. O'Reilly, F. Rojo, Q.B. She, D. Solit, G.B. Mills, D. Smith, et al., mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt, Cancer Res. 66 (2006) 1500–1508.
[19] M.C. Manara, G. Nicoletti, D. Zambelli, S. Ventura, C. Guerzoni, L. Landuzzi, et al., NVP-BEZ235 as a new therapeutic option for sarcomas, Clin. Cancer Res. 16 (2010) 530–540.
[20] V. Serra, B. Markman, M. Scaltriti, P.J. Eichhorn, V. Valero, M. Guzman, et al., NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations, Cancer Res. 68 (2008) 8022–8030.
[21] M. Fekete, C. Santiskulvong, C. Eng, O. Dorigo, Effect of PI3K/Akt pathway inhibition-mediated G1 arrest on chemosensitization in ovarian cancer cells, Anticancer Res. 32 (2012) 445–452.
[22] C. Gong, H. Liao, J. Wang, Y. Lin, J. Qi, L. Qin, et al., LY294002 induces G0/G1 cell cycle arrest and apoptosis of cancer stem-like cells from human osteosarcoma via down-regulation of PI3K activity, Asian Pac. J. Cancer Prev. 13 (2012) 3103–3107.
[23] S. Volarevic, G. Thomas, Role of S6 phosphorylation and S6 kinase in cell growth, Prog. Nucleic Acid Res. Mol. Biol. 65 (2001) 101–127.
[24] M.A. Jordan, L. Wilson, Microtubules and actin filaments: dynamic targets for cancer chemotherapy, Curr. Opin. Cell Biol. 10 (1998) 123–130.
[25] E. Vilar, J. Perez-Garcia, J. Tabernero, Pushing the envelope in the mTOR pathway: the second generation of inhibitors, Mol. Cancer Ther. 10 (2011) 395–403.
[26] X. Wan, B. Harkavy, N. Shen, P. Grohar, L.J. Helman, Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism, Oncogene 26 (2007) 1932–1940.
[27] P. Baumann, S. Mandl-Weber, F. Oduncu, R. Schmidmaier, The novel orally bioavailable inhibitor of phosphoinositol-3-kinase and mammalian target of rapamycin, NVP-BEZ235, inhibits growth and proliferation in multiple myeloma, Exp. Cell Res. 315 (2009) 485–497.
[28] D.W. McMillin, M. Ooi, J. Delmore, J. Negri, P. Hayden, N. Mitsiades, et al., Antimyeloma activity of the orally bioavailable dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235, Cancer Res. 69 (2009) 5835–5842.
[29] C. Santiskulvong, G.E. Konecny, M. Fekete, K.Y. Chen, A. Karam, D. Mulholland, et al., Dual targeting of phosphoinositide 3-kinase and mammalian target of rapamycin using NVP-BEZ235 as a novel therapeutic approach in human ovarian carcinoma, Clin. Cancer Res. 17 (2011) 2373–2384.
[30] C. Garcia-Echeverria, W.R. Sellers, Drug discovery approaches targeting the PI3K/Akt pathway in cancer, Oncogene 27 (2008) 5511–5526.
[31] S.M. Maira, F. Stauffer, J. Brueggen, P. Furet, C. Schnell, C. Fritsch, et al., Identification and characterization of NVP-BEZ235, a new orally available dual

phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity, Mol. Cancer Ther. 7 (2008) 1851–1863.
[32] J.C. Montero, X. Chen, A. Ocana, A. Pandiella, Predominance of mTORC1 over mTORC2 in the regulation of proliferation of ovarian cancer cells: therapeutic implications, Mol. Cancer Ther. 11 (2012) 1342–1352.
[33] F. Yang, X.J. Qian, W. Qin, R. Deng, X.Q. Wu, J. Qin, et al., Dual phosphoinositide 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235 has a therapeutic potential and sensitizes cisplatin in nasopharyngeal carcinoma, PLoS One 8 (2013), e59879.