

Predominance of mTORC1 over mTORC2 in the Regulation of Proliferation of Ovarian Cancer Cells: Therapeutic Implications

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Abstract

mTOR is a serine/threonine kinase that acts by binding different sets of proteins forming two complexes, termed mTORC1 and mTORC2. mTOR is deregulated in a substantial proportion of ovarian tumors. Despite the use of drugs directed to mTOR in ongoing clinical trials, the functional relevance of the individual mTORC branches in ovarian cancer is not known. Here, we show that mTORC1 and mTORC2 were constitutively active in ovarian cancer cell lines. Knockdown of raptor or rictor, proteins required for the function of mTORC1 or mTORC2, respectively, resulted in profound inhibition of ovarian cancer cell proliferation. The knockdown of raptor had a more important inhibitory effect than the knockdown of rictor, indicating mTORC1 had a predominant role over mTORC2 in the control of ovarian cancer cell proliferation. Rapamycin decreased the proliferation of ovarian cancer cells, and this was accompanied by inhibition of the phosphorylation of S6, a protein used as readout of mTORC1 function. However, rapamycin had only a marginal effect on the phosphorylation status of 4E-BP1, another mTORC1 substrate. Therefore, mTORC1 probably controls p4E-BP1 along two distinct pathways, one of them sensitive to rapamycin and another insensitive. The dual PI3K/mTOR inhibitor BEZ235 was more efficient than rapamycin in its inhibitory action on ovarian cancer cell proliferation. Biochemically, BEZ235 completely inhibited pS6, p4E-BP1, and pAkt. Our results suggest that broad-spectrum mTOR inhibitors that block mTORC1 and mTORC2 are more desirable for their clinical development in ovarian cancer than agents exclusively targeting one of the mTOR branches. *Mol Cancer Ther*; 11(6); 1342–52. ©2012 AACR.

Introduction

Ovarian cancer represents the leading cause of death from gynecologic neoplasias and the fifth cause of cancer death among women (1). Although surgical and chemotherapeutic strategies have improved the outcome of patients with this disease, in the metastatic setting, it remains incurable. Therefore, new therapies are required.

The mTOR serine/threonine kinase is an important regulator of cell growth and body size in different organisms (2). mTOR acts in concert with other proteins forming 2 complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 includes 5 proteins: mTOR, raptor, PRAS40, mLST8/GβL, and deptor; where-

as mTORC2 includes 6 proteins: mTOR, rictor, mLST8/GβL, Sin1, protor-1, and deptor (3). mTORC1 is sensitive to growth factor stimulation, oxygen levels, or nutrient availability and acts by regulating the phosphorylation of the ribosomal S6 kinase and the elongation factor-binding protein 4E-BP1, proteins involved in the control of protein synthesis, translation initiation, and cell mass. mTORC2 has been reported to participate in the control of cell survival and proliferation at least partially due to its regulation of Akt activity, through control of the phosphorylation of Akt at serine 473 (4). In mice, disruption of mTOR causes embryonic death in early developmental stages (E5.5–6.5 refs. 5, 6). Raptor disruption has a similar effect (7). However, in rictor knockout mice, death is delayed (E11.5) suggesting a less important role of mTORC2 in early stages of development (7, 8).

The contribution of the individual mTORC1 and mTORC2 routes to ovarian cancer has not been addressed. In ovarian cancer cells, mTOR is frequently phosphorylated (9). Moreover, treatment with rapamycin, a drug used as an mTOR inhibitor, resulted in G₁ arrest. However, several studies have questioned the ability of rapamycin to act as a bona fide inhibitor of mTOR. Thus, rapamycin may provoke Akt activation in some cells, probably due to a negative feedback of mTORC1 exerted over mTORC2, which is mechanistically still obscure but

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may involve regulation of the adapter protein IRS-1 by the S6 kinase or Grb10 stabilization (10, 11). Furthermore, in some cell types, rapamycin blocks phosphorylation of S6 but fails to affect phosphorylation of 4E-BP1 (12). These results still leave open the important question of which component of the mTOR pathway should be targeted to get the highest antitumor activity.

Here, by using knockdown of specific components of mTORC1 or mTORC2, we have evaluated the role of these complexes in ovarian cancer. We show that mTORC1 has a predominant role over mTORC2 in controlling the proliferation of ovarian cells. We also evaluated the action of several agents that target distinct molecules along the mTOR route to explore which one exerted the highest antitumoral action on ovarian cancer cells. Our findings, in addition to revealing the role of mTORC1 and mTORC2 in control of proliferation of ovarian cancer cells, support the value of targeting both mTOR routes as a novel therapeutic strategy in ovarian cancer.

Materials and Methods

Reagents and antibodies

Generic chemicals were purchased from Sigma-Aldrich, Roche Biochemicals, or Merck. The anti-protor-1, anti-GAPDH, anti-cyclin E, anti-CDK2, anti-CDK4, anti-PARP, and anti-p27 antibodies were purchased from Santa Cruz Biotechnology. The anti-mTOR, anti-raptor, anti-pS6 (S^{240/244}), anti-S6, anti-p4E-BP1 (T^{37/46}), anti-4E-BP1, pP70S6K (T³⁸⁹) and (T⁴²¹/S⁴²⁴), anti-pGSK-3 α/β (S^{21/9}), anti-pFoxO1 (T²⁴)/FoxO3a (T³²), anti-Akt, anti-pRb (S⁷⁸⁰), and anti-pNDRG1 (T³⁴⁶) antibodies were from Cell Signalling Technologies. The anti-cyclin A, anti-cyclin B, anti-cyclin D1, anti-cyclin D3, and anti-Rb were purchased from BD Biosciences. The anti-deptor, anti-mLST8, anti-Sin1, and anti-PRAS40 antibodies were from Millipore Corporation. The anti-riCTOR was from Bethyl laboratories. Horseradish peroxidase (HRP) conjugates of anti-rabbit and anti-mouse IgG were from Bio-Rad Laboratories. The rabbit polyclonal anti-calnexin antibody was from Stressgen Biotechnologies Corporation. The anti-phosphorylated Akt (serine 473) antibody was described previously (13).

Cell culture and infection with lentivirus

All cell lines were cultured at 37°C in a humidified atmosphere in the presence of 5% CO₂ and 95% air. The cell lines were provided by Dr. Faustino Mollinedo (CSIC-Salamanca, Salamanca, Spain), who obtained them from the American Type Culture Collection. No authentication was conducted in the author's laboratory. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; OVCAR-8 and SKOV-3) or in RPMI medium (A2780 and IGROV-1) containing a high glucose concentration (4,500 mg/L) and antibiotics (penicillin at 100 mU/mL, streptomycin at 100 μ g/mL) and supplemented with 10% FBS.

The lentiviral vectors containing short hairpin RNA (shRNA) for raptor, rictor, and mTOR (4) were obtained from Addgene. A minimum of 2 different lentiviral vec-

tors were tested for each target mRNA, and the one that produced higher knockdown levels of the respective proteins was used for the proliferation experiments. Preparation of lentiviral vectors was carried out as described previously (14).

Immunoprecipitation and Western blotting

Cells were washed with PBS and lysed in ice-cold lysis buffer [20 mmol/L Tris-HCl (pH 7.0), 140 mmol/L NaCl, 50 mmol/L EDTA, 10% glycerol, 1% Nonidet P-40, 1 μ mol/L pepstatin, 1 μ g/mL aprotinin, 1 μ g/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1 mmol/L sodium orthovanadate]. After scraping the cells from the culture dishes, samples were centrifuged at 10,000 \times g at 4°C for 10 minutes. Cleared cell lysates were used for Western blotting or immunoprecipitated with the corresponding antibody and protein A-Sepharose at 4°C for at least 2 hours. Samples were then boiled in electrophoresis sample buffer and subjected to SDS-PAGE. After electrophoresis, proteins in gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation), which were blocked for 1 hour in TBS with Tween-20 [TBST; 100 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 0.05% Tween-20] containing 1% of bovine serum albumin and then incubated for 2 to 16 hours with the corresponding antibody. Filters were incubated with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies for 30 minutes and bands were visualized by enhanced chemiluminescence (ECL).

Cell proliferation, apoptosis, and cell-cycle assays

Cells were plated in 24-well plates at 20,000 cells per well and cultured overnight in DMEM or RPMI + 10% FBS. The next day, medium was replaced with DMEM or RPMI containing the drugs. Cell proliferation was analyzed 2 days later by an MTT-based assay (15). Unless otherwise indicated, the results are presented as the mean \pm SD of quadruplicates. To determine whether the combination of BEZ235 and Taxotere, or BEZ235 and cisplatin was synergistic, additive, or antagonist, we used the CalcuSyn v2.0 software program (Biosoft; ref. 16), as described previously (17).

For apoptotic analysis, cells were plated and treated with the drugs as indicated. Then, cells were collected by trypsinization, washed with PBS, and resuspended in 100 μ L of binding buffer [10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl₂] containing 5 μ L of Annexin V-fluorescein isothiocyanate (FITC; BD Biosciences) and 5 μ L of 50 μ g/mL propidium iodide (PI; Sigma-Aldrich Inc.) for 15 minutes in the dark. After adding another 300 μ L of binding buffer, labeled cells were read in a FACSCalibur flow cytometer (BD Biosciences). For cell-cycle profile, after permeabilization by ice-cold 70% ethanol on ice, cells were washed, resuspended in 1 mL of PBS containing 50 μ g PI and 200 μ g DNase-free RNAase A (Sigma-Aldrich Inc.), and incubated at room temperature in the dark for 1 hour, then labelled cells were read in a FACSCalibur flow cytometer.

Xenograft studies

Female BALB/c nude mice, 7-week-old, were obtained from Charles River Laboratories. A total of 7×10^6 IGROV-1 cells in 100 μ L of DMEM and 100 μ L of Matrigel (BD Biosciences) were subcutaneously injected into the right and left flank of each mice. When the tumors were measurable, the mice were randomly assigned into 2 groups [with equal average tumor volumes (50 mm³) before initiation of treatments]—vehicle ($n = 8$), and BEZ235 ($n = 8$). The mice were treated daily and orally with 30 mg/kg BEZ235 dissolved in 10% *N*-methyl-2-pyrrolidone (Sigma-Aldrich) and 90% PEG300. Analyses of tumor diameters and volumes were calculated as reported (17). For biochemical analyses, tumor samples were obtained after sacrifice, by CO₂ inhalation, of the animals at day 32, 8 hours after being treated with BEZ235, and immediately frozen in liquid nitrogen. Mice were handled at the University's animal facility (Servicio de Experimentación Animal, Salamanca, Spain), and all treatments were in accordance with the legal and institutional guidelines.

Statistical analysis

Comparison of continuous variables between 2 groups for xenograft tumor model experiments was done with a 2-sided Student *t* test. At least 2 independent experiments were carried out for the *in vivo* studies. Differences were considered to be statistically significant when *P* values were less than 0.05. All data were analyzed with the statistical software SPSS 15.0 (SPSS Inc.).

Results

Constitutive activation of mTORC1 and mTORC2 pathways in ovarian cancer cells

To investigate the role of mTOR and its 2 branches in the proliferation of ovarian cancer cells, we first analyzed the expression of the different components of the mTOR complexes in 4 ovarian cancer cell lines, as well as the activation status of the mTORC1 and mTORC2 routes. mTOR, mLST8, raptor, rictor, PRAS40, Sin1, and p130Cas were expressed in all the cell lines, but p130Cas was undetectable (Fig. 1A). p130Cas was readily detected in the multiple myeloma cell line MM1S, in which it is overexpressed (3). The functionality of mTORC1 and mTORC2 was explored by analyzing the phosphorylation status of several downstream components. Phosphorylation of S6, 4E-BP1, and P70S6K was observed in the 4 ovarian cancer cell lines, indicating constitutive activation of mTORC1. IGROV-1 and SKOV-3 cells contained phosphorylated forms of the mTORC2 downstream components Akt, GSK3 α/β , NDRG1, and FoxO1 and FoxO3a. In the OVCAR-8 cell line, phosphorylation of Akt, FoxO1, FoxO3, and GSK3 α/β was very low or undetectable. A2780 cells presented Akt and GSK3 α/β phosphorylation. No statistically significant relationship between the level of activation of the mTOR branches and the prolif-

eration of the 4 cell lines was observed (Supplementary Fig. S1).

mTORC1 and mTORC2 control the proliferation of ovarian cancer cells

The strategy that we contemplated to separately analyze the contribution of mTORC1 and mTORC2 in ovarian cancer was based on inhibition of each of these 2 branches by RNA interference. We selected raptor and rictor as targets to decrease mTORC1 and mTORC2 signaling, as well as direct knockdown of mTOR. Western blotting of cell extracts from A2780 or IGROV-1 cells showed the knockdown of the different components of the mTOR pathway (Fig. 1C). Interestingly, knockdown of raptor caused a small but reproducible decrease in mTOR, indicative of a positive feedback exerted by the mTORC1 complex on the levels of mTOR. Raptor and mTOR knockdown decreased pS6 and p4E-BP1. Raptor knockdown provoked an increase in pS⁴⁷³-Akt, indicative of a negative feedback exerted by mTORC1 over mTORC2 in these cell lines. Rictor knockdown decreased pS⁴⁷³-Akt in IGROV-1 and also decreased pS6 levels in A2780 cells.

The effect of mTOR, rictor, and raptor knockdowns on the proliferation of A2780 and IGROV-1 cells was evaluated by MTT metabolization assays. mTOR knockdown exerted the largest effect on the MTT metabolization values (Fig. 1D). Knockdown of raptor had a higher inhibitory effect than the knockdown of rictor. In the IGROV-1 cell line, the effect of the raptor knockdown on the MTT metabolization values was very similar to the effect obtained by mTOR knockdown, indicating that mTORC1 is likely responsible for the cell growth signals channeled through the mTOR pathway in this cell line. Moreover, as pS⁴⁷³-Akt levels are increased upon raptor knockdown, these data suggest that augmented signaling through the mTORC2 route cannot complement the loss of mTORC1.

mTORC1 and mTORC2 knockdowns inhibit cell-cycle progression

To study a potential induction of cell death by each of the 3 knockdowns, cells were stained with Annexin V-FITC/PI and analyzed by flow cytometry. No evidence of increased Annexin V-FITC/PI staining was observed in the raptor, rictor, or mTOR knocked down IGROV-1 or A2780 cells, as compared with control cells (Fig. 2A).

Cell-cycle profiling of ovarian cancer cells that were interfered with by shRNAs against raptor or mTOR defined blockade at the G₀–G₁ cell-cycle phases, and an ensuing decrease in S and G₂–M phases (Fig. 2B). As expected from the higher effect of the knockdown of raptor and mTOR in IGROV-1 versus A2780, the effect of those knockdowns on the cell cycle was more pronounced in IGROV-1 cells. Biochemical analyses of cell-cycle proteins indicated a substantial decrease in the presence of phosphorylated Rb in IGROV-1 cells, when raptor or mTOR had been knocked down (Fig. 2C). In addition, we also detected a decrease in

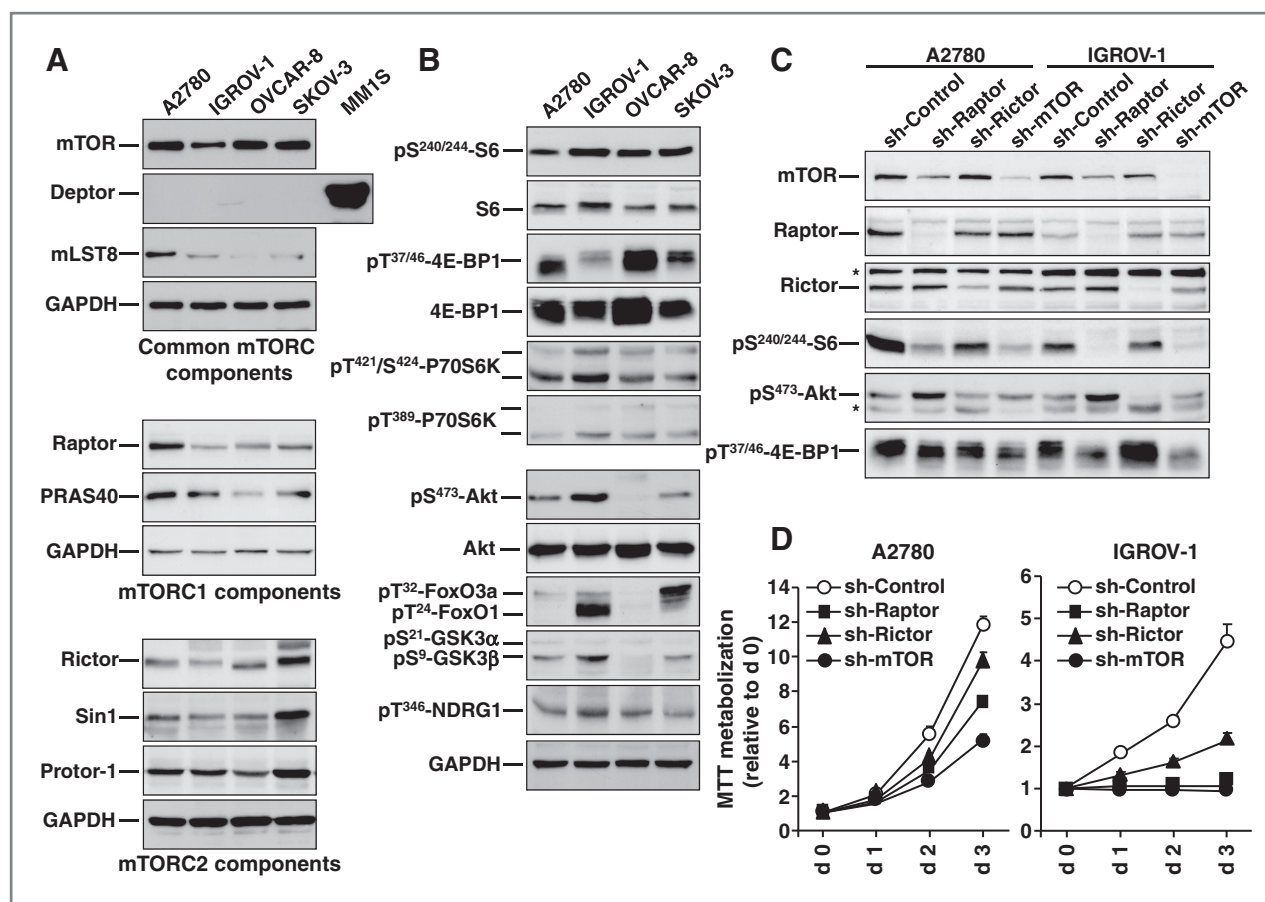


Figure 1. Expression of different mTOR complex components in ovarian cancer cells and the effects of knocking down raptor, rictor, and mTOR. **A**, expression of different mTOR complex components in ovarian cancer cells. Extracts from the different cell lines were run on SDS-PAGE gels, and the blots probed with the indicated antibodies. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. **B**, activation status of different mTORC1 and mTORC2 pathway intermediates in the 4 ovarian cancer cell lines used. **C**, knockdown of raptor, rictor, or mTOR in A2780 and IGROV-1 cell lines. Cells were infected with lentiviral vectors containing shRNA sequences directed to raptor, rictor, or mTOR. As a control, we used a vector containing a scrambled unrelated sequence. Forty-eight hours after infection, cells were placed in puromycin-supplemented media and then selected for 2 days. Cell extracts were obtained and analyzed for the indicated proteins by Western blotting. *, nonspecific bands. **D**, effect of the knockdown of raptor, rictor, or mTOR on the proliferation of ovarian cancer cells. Cell proliferation results are plotted as the mean \pm SD of quadruplicates of an experiment that was repeated 3 times with similar results.

the levels of cyclin E, and an increase in the amount of p27 in the mTOR, raptor, and rictor knockdowns. The p27 increase was higher in cells in which mTOR had been knocked down than in the raptor or rictor knockdowns. A decrease in cyclin B and cyclin A was also observed in the mTOR knocked down cells.

Efficacy of drugs that target different components of the mTOR route

We next evaluated the effect on cell proliferation of drugs that affect several branches of the PI3K/mTOR pathway to assess their effectiveness as potential anti-ovarian cancer treatments. For these experiments, we used the phosphoinositide 3-kinase (PI3K) inhibitor PX866 (18), the mTORC1 inhibitor rapamycin (19), the mTOR inhibitor Ku0063794 (20), and the dual PI3K and mTOR inhibitor BEZ235 (21). Their structures are shown in Fig. 2D. The most potent of these drugs was rapamycin,

which achieved 50% to 60% inhibition of MTT metabolization between 1 and 10 nmol/L (Fig. 3A). However, its efficiency was below that of BEZ235, which reached inhibition of MTT metabolization between 70% and 90%. Of the inhibitors tested, the less efficient and less potent was the PI3K inhibitor PX866.

The action of these inhibitors on the activation status of markers of mTORC1 and mTORC2 pathways was evaluated in IGROV-1, A2780, and OVCAR-8 cells (Fig. 3B). Rapamycin provoked a strong decrease in the phosphorylation of S6 at 1 nmol/L in all the cell lines. In contrast, rapamycin only slightly affected the phosphorylation status of 4E-BP1, another signaling intermediate of the mTORC1 route. In fact, 4E-BP1 appeared as several phosphorylated bands in the Western blotting, representative of phosphorylation at 4 sites by mTORC1 (22). Rapamycin only affected the phosphorylation status of the lower migrating band. We interpret these data to

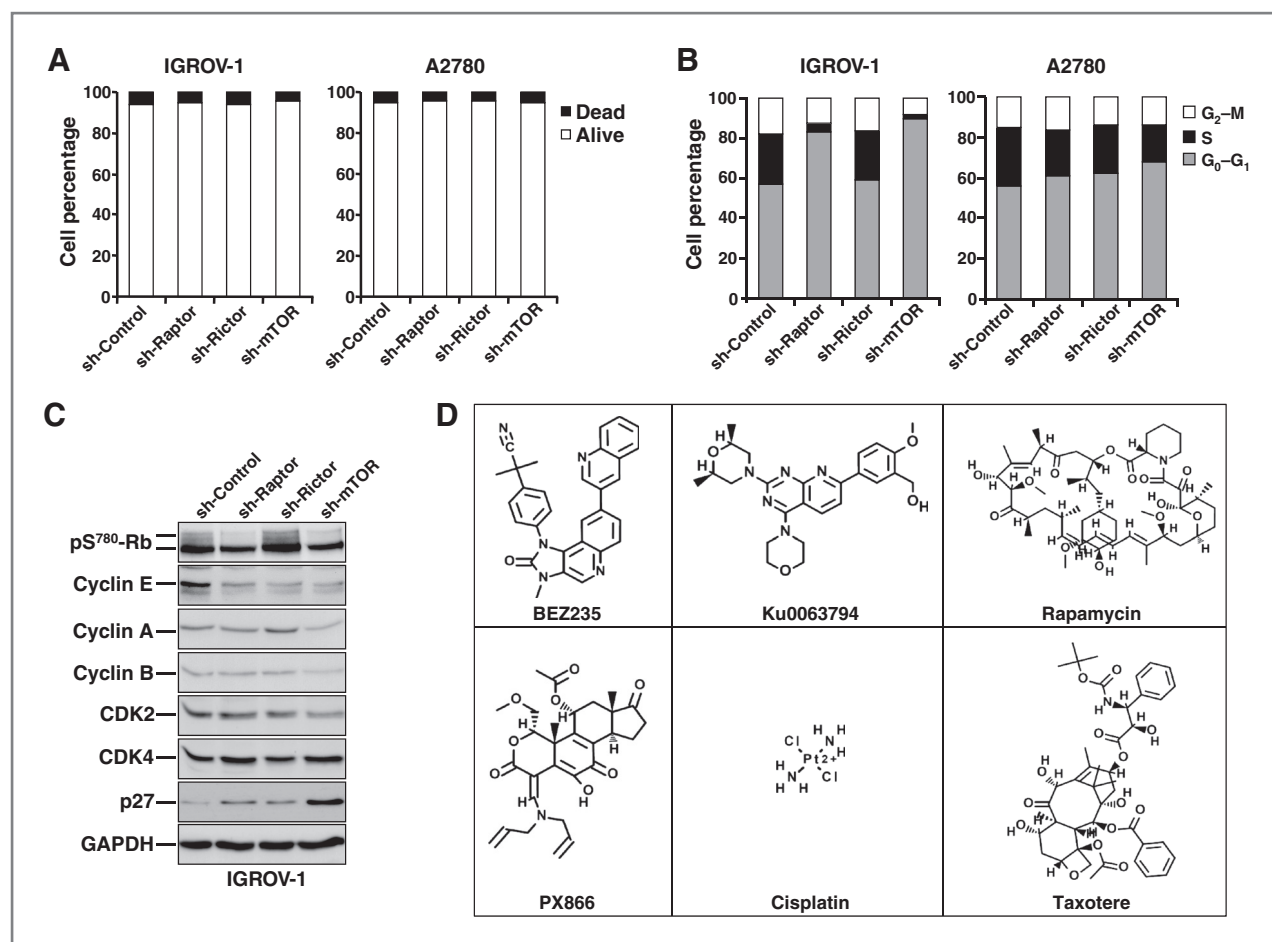


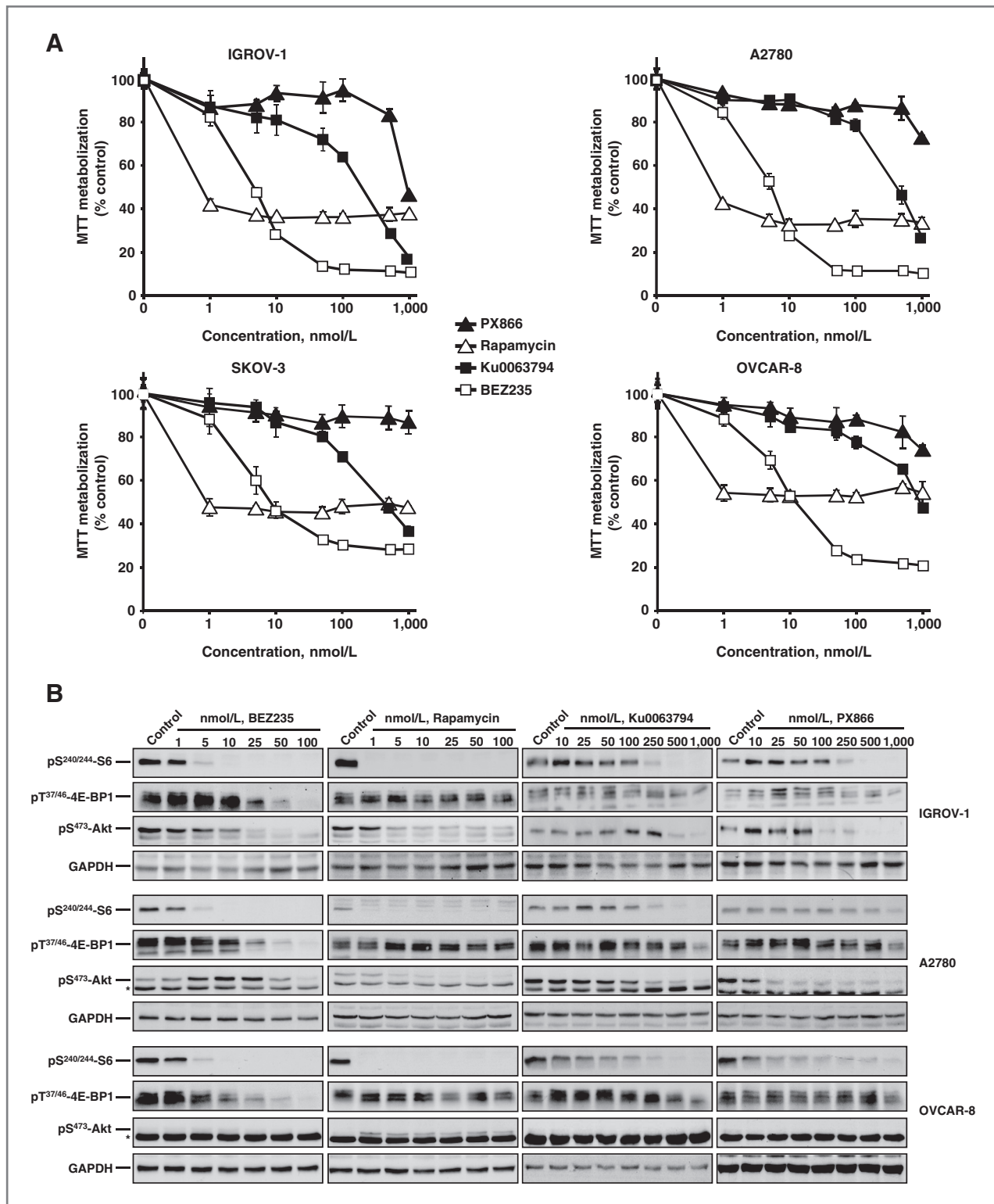
Figure 2. Effect of raptor, rictor, or mTOR knockdowns on apoptosis and cell cycle. A, IGROV-1 or A2780 cells infected with the lentiviral vectors were analyzed by Annexin V-FITC/PI staining by fluorescence-activated cell sorting. B, cell cycles were analyzed by PI staining and quantitation of the different phases using the ModFit program. C, Western blotting analyses of proteins involved in cell-cycle progression of IGROV-1 cells interfered for raptor, rictor, or mTOR. D, chemical structures of BEZ235, Ku0063794, rapamycin, PX866, cisplatin, and Taxotere. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

indicate that mTORC1 probably controls pS6 and p4E-BP1 along 2 distinct pathways, one of them sensitive to rapamycin and the other insensitive. In IGROV-1 and A2780 cells, rapamycin decreased the level of pSer⁴⁷³-Akt. In contrast, in OVCAR-8, in which resting pSer⁴⁷³-Akt levels were very low, rapamycin provoked upregulation of pSer⁴⁷³-Akt levels. The phosphorylation status of p4E-BP1, pS6, and pSer⁴⁷³-Akt was sensitive to inhibition by BEZ235, PX866, and Ku0063794. The latter 2 were less potent than BEZ235. In A2780 cells, BEZ235 increased the levels of pSer⁴⁷³-Akt at intermediate doses (5–25 nmol/L), but at higher doses (>50 nmol/L), its action on pSer⁴⁷³-Akt was inhibitory. This fact, together with the upregulation of pS⁴⁷³-Akt by rapamycin, suggests that in fact mTORC1 negatively controls the activity of mTORC2. Of note, at these low doses of BEZ235 (5–25 nmol/L), which efficiently reduced pS6, indicative of inhibition of mTORC1, BEZ235 increased pAkt. These findings also indicate the existence of 2 distinct thresholds for mTORC1 and mTORC2 inhibition by BEZ235. In IGROV-1 cells, which

present high resting levels of pSer⁴⁷³-Akt, BEZ235 had an inhibitory action on Akt phosphorylation, even at low (1 nmol/L) doses. The effect of BEZ235 on pS6 was also inhibitory, even though its potency was below that of rapamycin, requiring 10 times more BEZ235 to achieve the same inhibitory effect than rapamycin.

BEZ235 provokes cell-cycle arrest

Because BEZ235 was the most efficient agent of the 4 tested, we decided to explore its mechanism of antitumoral action on ovarian cancer cells. BEZ235 caused a significant increase in the amount of cells in the G₀-G₁ phase of the cell cycle, and an ensuing decrease in S- and G₂-M phases (Fig. 4A). The effect was more pronounced in IGROV-1 than in the A2780 cell line, in agreement with the results obtained with the knockdowns of mTOR, raptor, and rictor. Of note, no sub-G₀ fraction was observed, which was indicative of lack of cell death caused by BEZ235 (data not shown). Moreover, the failure to observe an increase in Annexin V-FITC staining



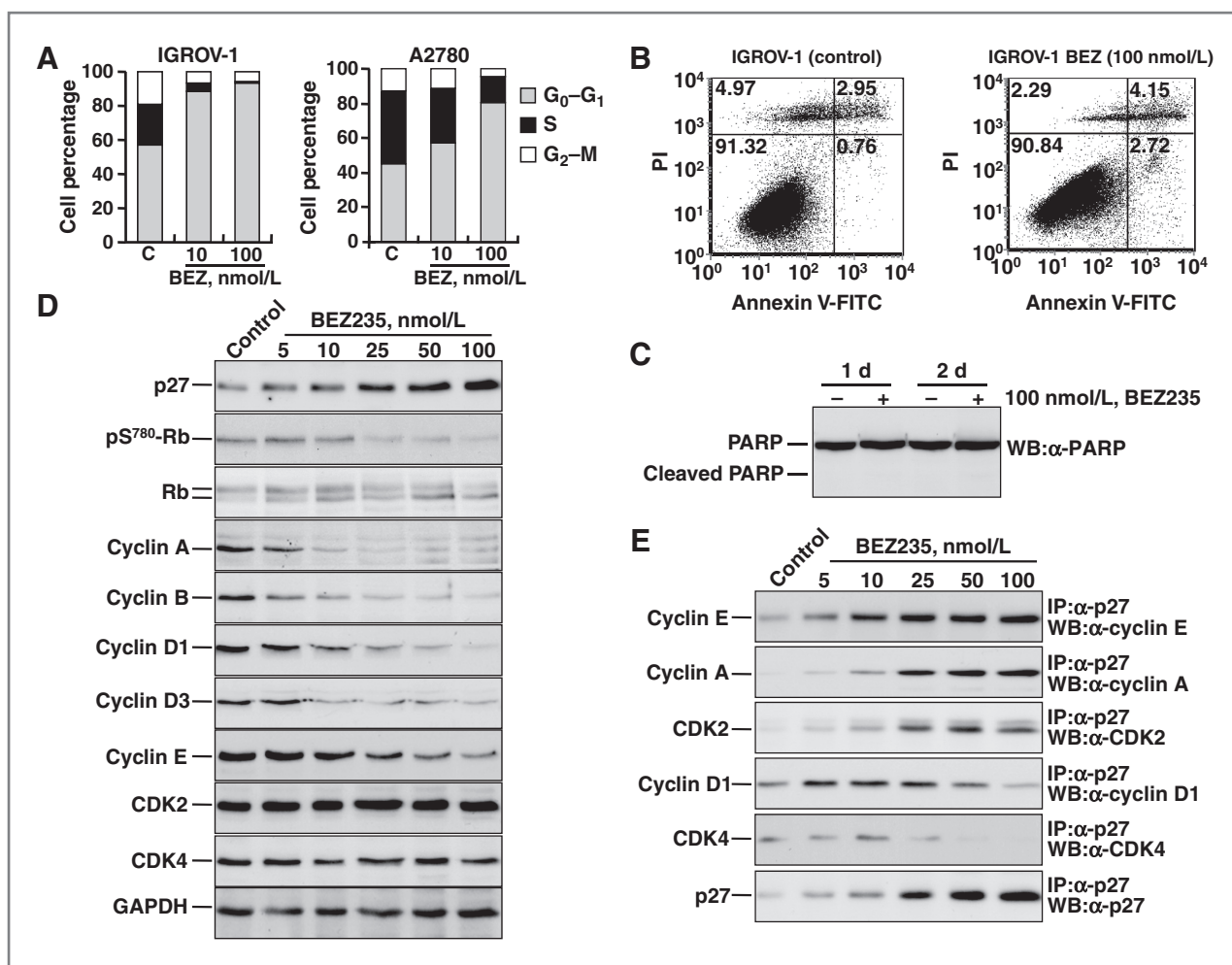


Figure 4. Action of BEZ235 on apoptosis and cell cycle of ovarian cancer cells. **A**, cells were treated with the indicated concentrations of BEZ235 for 24 hours, and then cell-cycle phases quantitatively analyzed by PI staining and FACS. **B**, double Annexin V-FITC/PI staining to analyze apoptosis. IGROV-1 cells were treated with BEZ235 (100 nmol/L) for 24 hours and double stained. **C**, IGROV-1 cells treated for the indicated times with BEZ235 were lysed and PARP cleavage analyzed by Western blotting (WB). **D**, analyses of proteins involved in the control of cell-cycle progression in IGROV-1 cells treated with BEZ235. **E**, co-immunoprecipitation (IP) of cell-cycle proteins with p27. IGROV-1 cells were grown in 100-mm dishes and treated for 24 hours with BEZ235. One milligram of cell lysates was immunoprecipitated with anti-p27 antibody. The blots underwent several rounds of probing with different antibodies to the indicated proteins. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(Fig. 4B) and the lack of PARP processing (Fig. 4C) confirmed that the action of BEZ235 on IGROV-1 cells did not include apoptotic cell death.

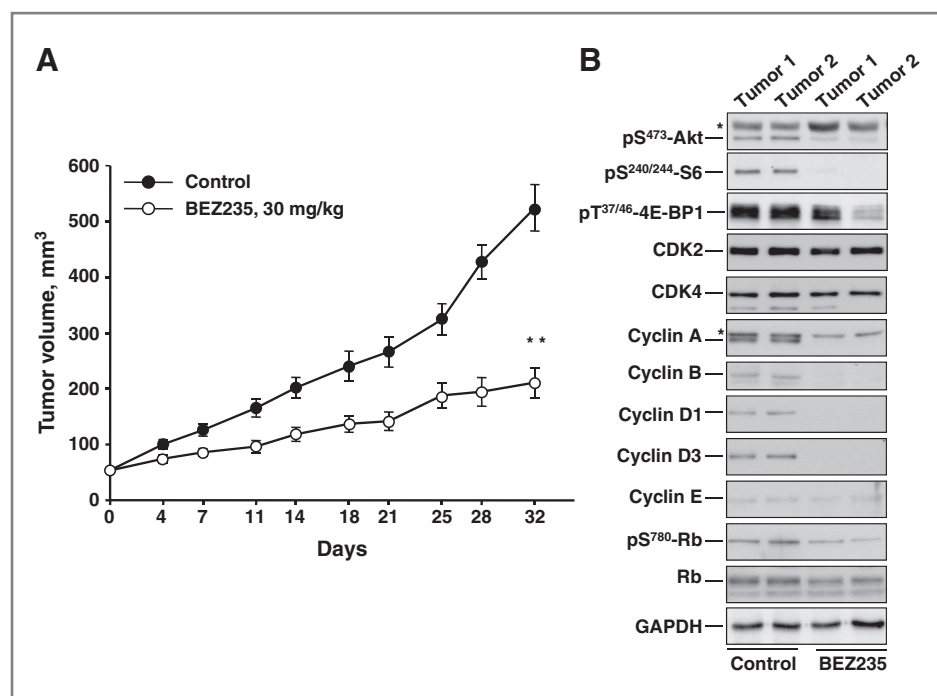
As the action of BEZ235 appeared to depend on a blockade of the cell cycle, we analyzed the expression of several proteins that participate in cell-cycle progression. p27 was upregulated by treatment with BEZ235 in IGROV-1 (Fig. 4D). Treatment with BEZ235 also decreased the amount of phosphorylated Rb and cyclins A, B, D1, D3, and E. No changes in the amount of cyclin-dependent kinase (CDK)2 or CDK4 was observed. Coprecipitation experiments indicated that even though the total amounts of cyclins A and E were decreased by BEZ235, the amount that associated to p27 was increased (Fig. 4E). Analogously, CDK2 bound to p27 also increased in cells treated with BEZ235. At low BEZ235 concentra-

tions (5–25 nmol/L), cyclin D1 slightly increased its association to p27. A decrease in CDK4 associated to p27 was observed at concentrations of BEZ235 of 25 nmol/L or higher.

In vivo efficacy of BEZ235

The *in vivo* action of BEZ235 was next analyzed. To this end, IGROV-1 cells were injected subcutaneously in the back of nude mice, at the level of the hip joint. Tumor growth was evaluated along 32 days after the beginning of the treatment, which started when the tumors of the mice reached 50 mm³. Daily treatment with BEZ235 (30 mg/kg) resulted in a substantial decrease in tumor growth, as compared with vehicle-treated controls (Fig. 5A). Biochemical analyses of the tumor samples obtained from mice indicated that the level of pS6 was very low in the

Figure 5. *In vivo* effect of BEZ235. A, tumors generated by injecting IGROV-1 cells were measured and the mean \pm SEM plotted. **, significance ($P < 0.001$). B, Western blotting of the indicated proteins after tissue extraction of tumors treated with vehicle or BEZ235. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. The asterisks shown in the Western blots indicate nonspecific bands.



tumors obtained from BEZ235-treated mice (Fig. 5B). The levels of pAkt, p4E-BP1, cyclins A, B, D1, and D3, and pRb were also decreased in the BEZ235-treated mice with respect to the vehicle-treated mice.

BEZ235 synergizes with standard-of-care treatments used in ovarian cancer

As most anticancer therapies are based on drug combinations, we next tested whether BEZ235 could augment the action of conventional standard-of-care treatments used in ovarian cancer. We therefore analyzed whether BEZ235 could increase the action of platinum and taxanes, whose combination represents the first-line treatment for advanced ovarian cancer (23). Combination of BEZ235 with cisplatin was synergistic in SKOV-3 and OVCAR-8 (Fig. 6). The combination of BEZ235 with taxanes was synergistic in SKOV-3 and IGROV-1.

Discussion

In this article, we have analyzed the role of mTOR and its 2 branches, mTORC1 and mTORC2, in ovarian cancer. This is important as ongoing clinical studies are evaluating different drugs against this pathway in ovarian cancer without a clear understanding of the importance of the components of this route in the pathophysiology of that disease. Moreover, genomic and biochemical studies indicate that activation of the PI3K/mTOR pathway may be present in up to 50% of patients with ovarian cancer (9,24).

We used a genetic approach to block mTOR as well as its 2 branches, mTORC1 and mTORC2. Knockdown of mTOR resulted in a profound inhibition of MTT metabo-

lization, provoked by the inhibition of cell-cycle progression. This was supported by the augmented levels of cells in the G₀-G₁ phase of the cell cycle, together with a decrease in S- and G₂-M. A decrease in the amount of cyclin E and hyperphosphorylated Rb and an increase in p27 was observed. Cyclin E together with CDK2 sustain Rb phosphorylation in late G₁, allowing cells to progress to the S-phase of the cell cycle (25). The latter biochemical data are consistent with an effect of mTOR on the cell cycle, likely through blockade at late G₁.

Knockdown of raptor or rictor allowed the evaluation of the importance of each of the 2 branches of mTOR in ovarian cancer cell proliferation. These experiments indicated that knockdown of raptor was more effective in preventing cell proliferation than knockdown of rictor, indicating a predominant role of mTORC1 over mTORC2 in the control of ovarian cancer cell proliferation. In fact, knockdown of raptor in IGROV-1 cells caused inhibition of cell proliferation with a magnitude analogous to the mTOR knockdown. These results remind the similar effect of knocking out mTOR, raptor, or rictor in mice (26). In IGROV-1 and A2780 cells, raptor knockdown caused an increase in pS⁴⁷³-Akt indicative of upregulation of mTORC2. Interestingly, this increase in mTORC2 activity cannot rescue cell proliferation, suggesting that mTORC1 activity is required and that most of the action of mTOR on cell proliferation is channeled through mTORC1. Yet, the fact that knockdown of rictor also affected cell proliferation indicates that the activity of the mTORC2 branch of the mTOR pathway has also a role in the regulation of ovarian cancer cell number, even if the mTORC1 route is functional. Interestingly, rictor

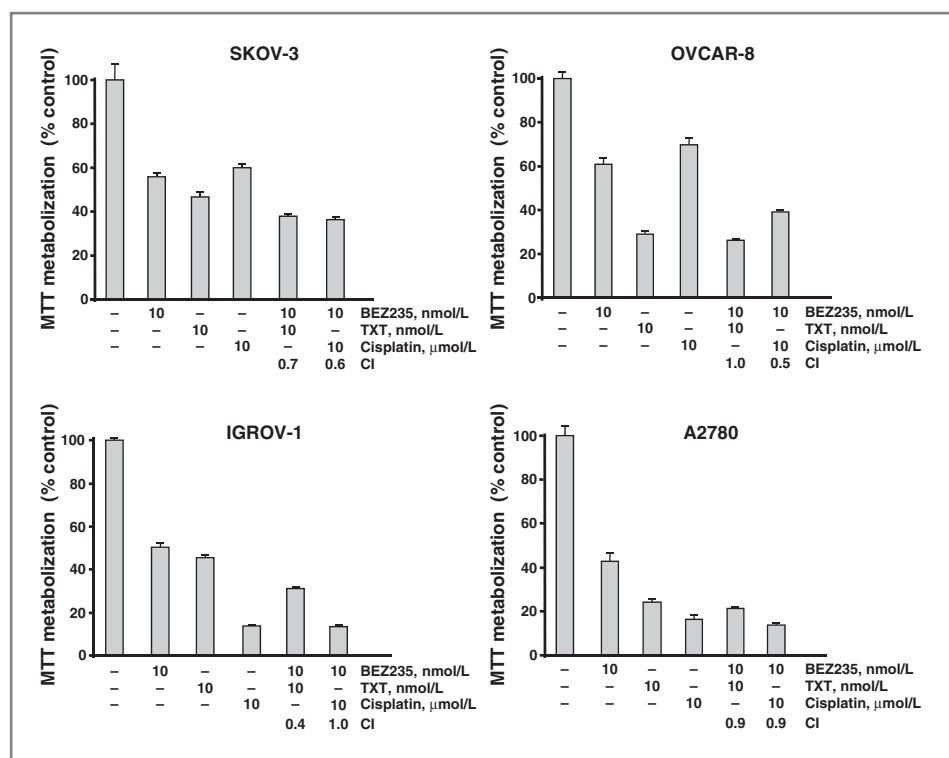


Figure 6. Analyses of the combination of BEZ235 with cisplatin and Taxotere (TXT). Drugs were added at the indicated concentrations to the different cell lines and then incubated for 2 days. MTT metabolization analyses were then carried out and the data were analyzed with the CalcuSyn program. CI, combination indexes.

knockdown also decreased pS6 levels in A2780 cells, suggesting the existence of some degree of cross-regulation between both mTOR branches in that cell line. Therefore, it is likely that the effect of mTOR on ovarian cancer cell proliferation requires coordinate actions that depend on both mTORC1 and mTORC2.

This concept of cooperativity between mTORC1 and mTORC2 was also substantiated by the experiments with drugs that target distinct components of the PI3K/mTOR pathway. Rapamycin potently inhibited the proliferation of all the ovarian cancer cell lines tested. However, its efficacy reached a plateau at concentrations of 10 nmol/L or above, and such plateau consisted in no more than 60% inhibition of cell proliferation. Rapamycin blocked S6 phosphorylation in all the cell lines with a potency that correlated with its effect in cell proliferation. However, the phosphorylation of 4E-BP1 was only partially affected by rapamycin, with phosphorylation of some residues fully resistant to the action of this drug. These data suggest the existence of 2 independent mTORC1 subroutes, one sensitive to rapamycin, which controls S6 phosphorylation, and second rapamycin-insensitive route that controls phosphorylation of 4E-BP1. It is therefore possible that phosphorylation of 4E-BP1 or other mTORC1 downstream targets may sustain the residual proliferation observed in ovarian cancer cells treated with rapamycin. In line with our data, others have reported that rapamycin-insensitive mTORC1 complexes are involved in the growth and survival of leukemic BCR-ABL-expressing cells (12). Moreover, it has recently been reported that phosphorylation of S6 is dispensable for lymphomagen-

esis, but 4E-BP1 exerts substantial control on this process through its action in controlling cap-dependent translation and cell growth (27).

BEZ235 achieved higher degrees of inhibition of proliferation than rapamycin. This may be due to the better inhibitory action of BEZ235 on both mTORC branches, but also on PI3K. At low doses, BEZ235 inhibited S6 phosphorylation without substantially affecting pAkt or p4E-BP1. This represents an additional indication that the pathway controlling S6 phosphorylation by BEZ235 is more sensitive than the one regulating the function of the 4E-BP1 and mTORC2 branches. Mechanistically, the action of BEZ235 on ovarian cancer cells resembled the effect of mTOR knockdown. Treatment with BEZ235 caused a decrease in the expression levels of several cyclins. BEZ235 also provoked an increase in the amount of p27, which was accompanied by augmented association of that protein with CDK/cyclin complexes. Given the inhibitory role of p27 on the kinase activity of these complexes (28), it is possible that this mechanism contributes to the cell-cycle arrest caused by BEZ235.

In addition to these functional and mechanistic concepts, our results also offer important conclusions that may have clinical relevance. The efficacy of rapamycin *in vitro* supports the clinical development of agents that target mTORC1 for the treatment of ovarian cancer. However, the fact that knocking down raptor increased pSer⁴⁷³-Akt levels and that rapamycin increased such levels in OVCAR-8 cells must be taken into account, as activation of the mTORC2 branch may contribute to escape from the antitumoral action of mTORC1 inhibitors.

This fact, together with the partial antitumoral action of rapamycin may help in explaining the relatively limited success of rapalogs in the treatment of solid neoplasias, especially if they are to be used as single agents (29). These facts should be considered when designing clinical studies using rapalogs in ovarian cancer.

Treatment of ovarian cancer is based on drug combinations with platinum and taxanes. Importantly, BEZ235 had a synergistic effect when combined with these compounds. This indicates that BEZ235 could be added to the therapeutic armamentarium to fight ovarian cancer. Moreover, the *in vivo* studies conducted in mice indicate that BEZ235 is active *in vivo* against ovarian cancer xenografts.

In summary, our article described the predominant role of mTORC1 over mTORC2 in the control of ovarian cancer cell proliferation. Our data also suggest that the mTOR pathway can represent an interesting target in ovarian cancer, especially considering the novel generation of mTOR inhibitors that inhibit both mTOR complexes. It should be interesting to evaluate the clinical relevance of

our findings by developing trials that combine mTOR inhibitors to standard-of-care treatments used in the ovarian cancer clinic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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