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 which acts by binding different sets of proteins forming two complexes, termed mTORC1 and mTORC2. mTOR is deregulated in a substantial proportion of ovarian tumours. 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 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.
Introduction

Ovarian cancer represents the leading cause of death from gynecological 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 mammalian target of rapamycin (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 two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 includes five proteins: mTOR, Raptor, PRAS40, mLST8/GβL, and Deptor; while mTORC2 includes six 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) (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 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 the 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 (St. Louis, MO), Roche Biochemicals, or Merck (Darmstadt, Germany). 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 (Santa Cruz, CA). The anti-mTOR, anti-Raptor, anti-pS6 (S^{240/244}), anti-S6, anti-p4E-BP1 (T^{37/46}), anti-4E-BP1, pP70S6K (T^{389}) and (T^{421}/S^{424}), anti-pGSK-3α/β (S^{21/9}), anti-pFoxO1 (T^{24}), FoxO3a (T^{32}), anti-Akt, anti-pRb (S^{780}), and anti-pNDRG1 (T^{346}) antibodies were from Cell Signalling Technologies (Beverly, MA). The anti-cyclin A, anti-cyclin B, anti-cyclin D1, anti-cyclin D3 and anti-Rb were purchased from BD Biosciences (San Jose, CA). The anti-Deptor, anti-mLST8, anti-SIN1, and anti-PRAS40 antibodies were from Millipore Corporation (Bedford, MA). The anti-Rictor was from Bethyl laboratories (Montgomery, TX). Horseradish peroxidase conjugates of anti-rabbit and anti-mouse immunoglobulin G (IgG) were from Bio-Rad Laboratories (Hercules, CA). The rabbit polyclonal anti-calnexin antibody was from Stressgen Biotechnologies Corporation (British Columbia, Canada). The anti-phosphorylated Akt (Serine 473) antibody was described (13).

*Cell Culture and infection with lentivirus*

All cell lines were cultured at 37°C in a humidified atmosphere in the presence of 5% CO₂–95% air. The cell lines were provided by Dr. Faustino Mollinedo (CIC-Salamanca), who obtained them from the ATCC. No authentication was performed in the author’s laboratory. Cells were grown in DMEM (OVCAR-8 and SKOV-3) or in RPMI medium (A2780 and IGROV-1) containing a high glucose concentration (4,500 mg/liter) and antibiotics (penicillin at 100 mU/ml, streptomycin at 100 μg/ml) and supplemented with 10% FBS.
The lentiviral vectors containing shRNA for raptor, rictor and mTOR (4) were obtained from Addgene. A minimum of two different lentiviral vectors were tested for each target mRNA, and the one which produced higher knockdown levels of the respective proteins was used for the proliferation experiments. Preparation of lentiviral vectors was performed as described (14).

Immunoprecipitation and Western blotting

Cells were washed with PBS and lysed in ice-cold lysis buffer (20 mM Tris-HCl [pH 7.0], 140 mM NaCl, 50 mM EDTA, 10% glycerol, 1% Nonidet P-40, 1 μM pepstatin, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM PMSF, and 1 mM 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 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 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). After electrophoresis, proteins in gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Bedford, MA), which were blocked for 1 hour in TBST (100 mM Tris [pH 7.5], 150 mM NaCl, 0.05% Tween 20) containing 1 % of bovine serum albumin (BSA) and then incubated for 2 to 16 h with the corresponding antibody. Filters were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies for 30 minutes and bands were visualized by ECL.

Cell Proliferation, apoptosis and cell cycle assays

Cells were plated in 24-well plates at 20,000 cells/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 ± standard deviation (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, Ferguson, MO) (16), as described (17).

For apoptotic analysis, cells were plated and treated with the drugs as indicated. Then cells were collected by trypsination, washed with PBS, and resuspended in 100 μL of Binding Buffer (10 mmol/L HEPES/NaOH (pH7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl2) containing 5 μL of Annexin V-FITC (BD Biosciences, San Jose, CA) and 5 μL of 50 μg/ml propidium iodide (PI, Sigma-Aldrich Inc, St. Louis, MO) for 15 min in the dark. After adding another 300 μL of Binding Buffer, labeled cells were read in a FACScalibur flow cytometer (BD Biosciences, San Jose, CA). For cell cycle profile, after permeabilization by ice-cold 70% ethanol on ice, cells were washed, resuspended in 1 ml of PBS containing 50 μg PI and 200 μg DNase-free RNAase A (Sigma-Aldrich Inc, St. Louis, MO) and incubated at room temperature in the dark for 1 h; then label cells were read in a FACScalibur flow cytometer.

Xenograft Studies

Female BALB/c nude mice, 7 weeks old, were obtained from Charles River Laboratories (Barcelona, Spain). 7 × 10^6 IGROV-1 cells in 100 μL of DMEM and 100 μL 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 two groups (with equal average tumor volumes (50 mm^3) 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, St Louis, MO)- 90% PEG300. Analyses of tumor diameters and volumes were calculated as reported (17). For biochemical analyses, tumour samples were obtained after sacrifice by CO2 inhalation of the animals at day 32, 8 hour 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 two groups for xenograft tumor model experiments was done using a two-sided Student’s t-test. At least of two independent experiments were performed for the in vivo studies. Differences were considered to be statistically significant when $P$ values were less than .05. All data were analyzed using the statistical software SPSS 15.0 (SPSS Inc., Chicago, IL).
Results

Constitutive activation of mTORC1 and mTORC2 pathways in ovarian cancer cells.

To investigate the role of mTOR and its two branches in the proliferation of ovarian cancer cells, we first analyzed the expression of the different components of the mTOR complexes in four ovarian cancer cell lines, as well as the activation status of the mTORC1 and mTORC2 routes. mTOR, mLST8, Raptor, Rictor, PRAS40, Sin-1, and Protor-1 were expressed in all the cell lines, but Deptor was undetectable (Figure 1A). Deptor 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 four ovarian cancer cell lines, indicating constitutive activation of mTORC1. IGROV-1 and SKOV-3 cells contained phosphorylated forms of the mTORC2 downstream components Akt, GSK3α/β, NDRG1, and FoxO1 and FoxO3a. In the OVCAR-8 cell line, phosphorylation of Akt, FoxO1, FoxO3, and GSK3α/β was very low or undetectable. A2780 cells presented Akt and GSK3α/β phosphorylation. No statistically significant relationship between the level of activation of the mTOR branches and the proliferation of the four cell lines was observed (Supplementary Figure 1).

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 two 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 demonstrated the knockdown of the different components of the mTOR pathway (Figure 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 using MTT metabolization assays. mTOR knockdown exerted the largest effect on the MTT metabolization values (Figure 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 three knockdowns, cells were stained with Annexin V-FITC/propidium iodide (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 to control cells (Figure 2A).

Cell cycle profiling of ovarian cancer cells interfered with shRNAs against Raptor or mTOR defined blockade at the G0/G1 cell cycle phases, and an ensuing decrease in S and G2/M phases (Figure 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 (Figure 2C). In addition we also detected a decrease in 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 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 Figure 2D. The most potent of these drugs was rapamycin, which achieved 50-60% inhibition of MTT metabolization between 1 and 10 nM (Figure 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 (Figure 3B). Rapamycin provoked a strong decrease in the phosphorylation of S6 at 1 nM 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 westerns, representative of phosphorylation at four sites by mTORC1 (22). Rapamycin only affected the phosphorylation status of the lower migrating band. We interpret these data to indicate that mTORC1 probably controls pS6 and p4E-BP1 along two distinct pathways, one of them sensitive to rapamycin and the other insensitive. In IGROV-1 and A2780 cells rapamycin decreased the level of pSer^{473}-Akt. In contrast, in
OVCAR8, in which resting pSer\textsuperscript{473}-Akt levels were very low, rapamycin provoked up-regulation of pSer\textsuperscript{473}-Akt levels. The phosphorylation status of p4E-BP1, pS6, and pSer\textsuperscript{473}-Akt were sensitive to inhibition by BEZ235, PX866 and Ku0063794. The latter two were less potent than BEZ235. In A2780 cells, BEZ235 increased the levels of pSer\textsuperscript{473}-Akt at intermediate doses (5-25 nM), but at higher doses (>50 nM) its action on pSer\textsuperscript{473}-Akt was inhibitory. This fact, together with the up-regulation of pS\textsuperscript{473}-Akt by rapamycin suggests that in fact mTORC1 negatively controls the activity of mTORC2. Of note, at these low doses of BEZ235 (5-25 nM) which efficiently reduced pS6, indicative of inhibition of mTORC1, BEZ235 increased pAkt. These findings also indicate the existence of two distinct thresholds for mTORC1 and mTORC2 inhibition by BEZ235. In IGROV-1 cells, which present high resting levels of pSer\textsuperscript{473}-Akt, BEZ235 had an inhibitory action on Akt phosphorylation, even at low (1 nM) doses. The effect of BEZ235 on pS6 was also inhibitory, even though its potency was below that of rapamycin, requiring ten times more BEZ235 to achieve the same inhibitory effect than rapamycin.

**BEZ235 provokes cell cycle arrest.**

Since BEZ235 was the most efficient agent of the four 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 (Figure 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 4B) and the lack of PARP processing (Figure 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 up-regulated by treatment with BEZ235 in IGROV-1 (Figure 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 CDK2 or CDK4 were 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 (Figure 4E). Analogously, CDK2 bound to p27 also increased in cells treated with BEZ235. At low BEZ235 concentrations (5-25 nM) cyclin D1 slightly increased its association to p27. A decrease in CDK4 associated to p27 was observed at concentrations of BEZ235 of 25 nM 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. Tumour growth was evaluated along 32 days after the beginning of the treatment, which started when the tumours of the mice reached 50 mm³. Daily treatment with BEZ235 (30 mg/kg) resulted in a substantial decrease in tumour growth, as compared to vehicle-treated controls (Figure 5A). Biochemical analyses of the tumour samples obtained from mice indicated that the level of pS6 was very low in the tumours obtained from BEZ235-treated mice (Figure 5B). The level 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 (Figure 6). The combination of BEZ235 with taxanes was synergistic in SKOV-3 and IGROV-1.
Discussion

In this paper we have analyzed the role of mTOR and its two branches mTORC1 and mTORC2 in ovarian cancer. This is important since 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 two branches mTORC1 and mTORC2. Knockdown of mTOR resulted in a profound inhibition of MTT metabolism, provoked by inhibition of cell cycle progression. This was supported by 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 two 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 IGROV1 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 up-regulation 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 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 nM 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 two independent mTORC1 subroutes, one sensitive to rapamycin, which controls S6 phosphorylation, and a 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 lymphomagenesis, 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 performed in mice indicate that BEZ235 is active in vivo against ovarian cancer xenografts.

In summary, our paper 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 on 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.
References


Figure Legends.

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. GAPDH was used as a loading control. B, Activation status of different mTORC1 and mTORC2 pathway intermediates in the four 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 short hairpin RNA sequences directed to Raptor, Rictor, or mTOR. As a control, we used a vector containing a scrambled unrelated sequence. 48 hours after infection cells were placed in puromycin-supplemented media, and then selected for two days. Cell extracts were obtained and analyzed for the indicated proteins by Western blotting. The asterisks indicate non-specific 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 ± SD of quadruplicates of an experiment that was repeated three times with similar results.

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 FACS. 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.

Figure 3. Biological and biochemical effects of inhibitors of the PI3K-mTOR pathway. A, MTT metabolization of the four ovarian cancer cell lines treated with the indicated drugs at different concentrations, for two days. Results are plotted as mean ± SD of
quadruplicates from an experiment that was repeated twice. B, Action of the different drugs (24 hour treatment) on downstream signaling intermediates of the mTORC1 and mTORC2 branches. The asterisks indicate non-specific bands.

**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 nM) 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. D, Analyses of proteins involved in the control of cell cycle progression in IGROV-1 cells treated with BEZ235. E, Coimmunoprecipitation of cell cycle proteins with p27. IGROV-1 cells were growth in 100 mm dishes and treated for 24 hours with BEZ235. One mg of cell lysates was immunoprecipitated with anti-p27 antibody. The blots underwent several rounds of probing with different antibodies to the indicated proteins.

**Figure 5. In vivo effect of BEZ235.** A, tumours generated by injecting IGROV-1 cells were measured and the mean ± SEM is plotted. The double asterisk indicates significance (p<0.001). B, Western blotting of the indicated proteins after tissue extraction of tumours treated with vehicle or BEZ235.

**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 two days. MTT metabolism analyses were then performed and the data were analyzed using the CalcuSyn program. CI stands for combination indexes.
Figure 2

A. IGROV-1 and A2780 cell percentages showing dead and alive cells under different conditions.

B. IGROV-1 and A2780 cell cycle distributions showing G2/M, S, and G0/G1 phases.

C. Western blot analysis for pS780-Rb, Cyclin E, Cyclin A, Cyclin B, CDK2, CDK4, and p27 in IGROV-1.

D. Chemical structures of BEZ235, Ku0063794, Rapamycin, PX866, Cisplatin, and Taxotere.
Figure 3
Figure 4
Figure 5

A

Tumor volume, mm$^3$

Days

Control

BEZ235, 30 mg/kg

B

Tumor 1

Tumor 2

Tumor 1

Tumor 2

pS$^{473}$-Akt

pS$^{240/244}$-S6

pT$^{37/46}$-4E-BP1

CDK2

CDK4

Cyclin A

Cyclin B

Cyclin D1

Cyclin D3

Cyclin E

pS$^{780}$-Rb

Rb

GAPDH

Control

BEZ235
Figure 6
Predominance of mTORC1 over mTORC2 in the regulation of proliferation of ovarian cancer cells: therapeutic implications

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