MolecularMedicine

Dual Targeting of mTOR Activity with Torin2 Potentiates Anticancer Effects of Cisplatin in Epithelial Ovarian Cancer

Azhar R Hussain,[1] Maha Al-Romaizan,[2] Maqbool Ahmed,[1] Saravanan Thangavel,[1] Fouad Al-Dayel,[3] Shaham Beg,[1] Shahab Uddin,[1] Abdul K Siraj,[1] and Khawla S Al-Kuraya[1,2]

[1]Human Cancer Genomic Research, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia; [2]AlFaisal University, Riyadh, Saudi Arabia; and [3]Department of Pathology, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia

Mammalian target of rapamycin (mTOR) and phosphatidylinositol 3-kinase (PI3K) are two key components of PI3K/Akt/mTOR signaling pathway. Dysregulation of these pathways have been found in many cancers, including epithelial ovarian cancer (EOC), however, the role of mTOR has not been fully elucidated in Middle Eastern EOC. Therefore, we investigated the activation of mTOR complexes (mTORC1 and mTORC2) in a cohort of 156 EOC from Saudi Arabia by immunohistochemistry in a tissue microarray format. mTORC1 and mTORC2 were found to be activated in 55 of 146 (37.7%) and 63 of 140 (45%) of EOC samples, respectively. mTORC1 was significantly associated with mTORC2 ($p < 0.0001$) activation and both mTOR complexes were significantly associated with p-AKT ($p = 0.0205$ and 0.0298) and p-P70S6 ($p < 0.0001$ and 0.0035), respectively. Interestingly, mTOR activation incurred a poor progression-free survival (PFS) ($p = 0.0188$) in EOC. Next, the *in vitro* effect of inactivation of mTOR complexes was evaluated using a second-generation mTOR inhibitor, Torin2, on a panel of EOC cell lines. Torin2 treatment decreased cell viability and induced apoptosis in a dose-dependent manner via inactivation of mTORC1 and mTORC2 and their downstream targets in EOC cell lines. Furthermore, treatment of EOC cells with a subtoxic dose of Torin2 potentiated a cisplatin-induced apoptotic response in EOC cell lines. Finally, we studied the *in vivo* effect of a combination of Torin2 and cisplatin and found that this combination synergistically inhibited tumor growth in nude mice. These studies highlight the importance of targeting the mTOR survival pathway and suggest that cotreatment with cisplatin and Torin2 may be beneficial for the management of EOC.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2014.00238

INTRODUCTION

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy in the world (1). EOC is a heterogeneous disease that spreads rapidly if untreated (2). EOC usually presents as a late-stage disease due to a lack of symptoms to diagnose the cancer at an early stage (3). As EOC usually presents as a late-stage disease, the treatment protocol commonly used is cytoreduction and debulking of the tumor by surgery followed by platinum-based chemotherapy along with paclitaxel (4). Even though the surgical protocols as well as the treatment for EOC have improved tremendously over the last decade (4), 50% of the patients that present with late-stage disease will eventually relapse or die (5). Therefore, there is an urgent need to improve the overall survival of patients diagnosed with EOC.

Mammalian target of rapamycin (mTOR) is a serine-threonine kinase that controls cell survival and growth and is often found to be dysregulated in many diseases (6,7,8). mTOR functions by forming two different protein complexes; mTORC1 and mTORC2 (9). mTORC1 is rapamycin sensitive and is dependent upon changes in oxygen levels, stimulation by growth factors and changes in nutrients status (10). A critical function of mTORC1 is to regulate protein synthesis via a number of substrates, including p70S6 kinase, the inhibitory eIF4E-binding proteins (4E-BPs) and the eIF4G initiation factors (11,12). mTORC2 is rapamycin resistant and is not dependent on nutrients and is responsible for cancer cells growth and proliferation even in extreme conditions such as lack of nutrients and energy (9). The functionality of the mTORC2 complex is facilitated mainly by activation of AKT at site Ser473. Once AKT is activated, it leads to cell survival, proliferation and growth (12,13,14). It is an accepted fact that AKT

Address correspondence to *Khawla S Al-Kuraya, Human Cancer Genomic Research, Research Center, King Faisal Specialist Hospital and Research Cancer, MBC#98-16, P.O. Box 3354, Riyadh 11211, Saudi Arabia. Phone: +966-11-205-5167; Fax: +966-11-205-5170; E-mail: Kkuraya@kfshrc.edu.sa.*
Submitted December 1, 2014; Accepted for publication May 25, 2015; Published Online (www.molmed.org) May 26, 2015.

is found to be constitutively activated in a variety of cancers (15,16,17), therefore, for efficient treatment of cancer, it is very important that both mTOR complexes are targeted simultaneously to achieve an anticancer effect (13).

Clinical trials using newer generation mTOR inhibitors have shown the efficacy and utility of targeting mTOR pathways for the management of various cancers (18,19,20). These trials have paved the way for using mTOR inhibitors for the treatment of advanced stage renal cell carcinoma and breast cancer (21,22). Even though there has been success in treating advanced stage cancers with mTOR inhibitors, most of the first generation mTOR inhibitors have the propensity to target the mTORC1 complex and it has been shown that by not targeting the mTORC2 complex, resistance against these inhibitors quickly develops via activation of AKT at phosphorylation site Ser473 (23,24,25). Torin2, a second generation mTOR inhibitor, has the ability to target and inhibit both the mTOR complexes efficiently and therefore has an edge over other first generation inhibitors in effectively inhibiting mTOR activity and inducing apoptosis in cancer cells (26,27,28).

Platinum resistance is a major obstacle in the treatment of ovarian cancer. Even though most ovarian cancers respond to initial platinum-based chemotherapy, more than 50% of these cancers eventually relapse. Relapse in ovarian cancer cases can be classified in three groups: "platinum refractory" if the cancer relapses within a month of treatment or if the disease progresses despite platinum-based therapy; "platinum resistant" if the cancer relapses within 6 months of treatment; and "platinum sensitive" if the cancer relapses after 6 months of initial treatment (29). There are several reasons for cancer cells to develop resistance to platinum-based chemotherapy (30,31). Constitutive activation of the pro-survival protein AKT also can confer resistance to platinum-based chemotherapy (32).

In this study, we have investigated the activation of the mTOR proteins mTORC1 and mTORC2 and their clinical and molecular associations in a cohort of epithelial ovarian cancer samples. Next, we investigated the effect of Torin2 treatment on a panel of EOC cell lines to identify whether this second-generation mTOR inhibitor is able to inactivate the mTOR pathway as well as to induce apoptosis. Finally, we investigated the combined effects of Torin2 and cisplatin *in vitro* and *in vivo* to evaluate whether Torin2 can potentiate the anticancer effect of cisplatin in EOC cells as they undergo apoptosis.

MATERIALS AND METHODS

Patient Selection and Tissue Microarray (TMA) Construction

One hundred fifty-six patients with ovarian carcinoma diagnosed between 1991 and 2007 were selected from the files of the King Faisal Specialist Hospital and Research Center. All samples were analyzed in a tissue microarray (TMA) format. TMA construction was performed as described earlier (33,34). The Institutional Review Board of the King Faisal Specialist Hospital and Research Center approved the study. The histological subtype of each tumor sample was determined by accepted criteria (35). The histological subtypes of tumors were as follows: serous, 125 (80.1%); endometroid, 22 (14.1%); clear cell, 4 (2.6%); and undifferentiated/mixed epithelial, 5 (3.2%). The median age of patients was 56 years (range 19 to 86 years). The majority of patients underwent primary surgical staging or cytoreduction and those unfit for primary surgery underwent primary neoadjuvant chemotherapy followed by interval debulking surgery. FIGO stage at diagnosis was stage I–II in 8 patients (5.1%), stage III–IV in 137 (87.8%), and unknown in 11(6.1%). The median follow-up time was 14.9 months (range, 2 to 130 months). PFS was computed from date of surgery for patients who underwent primary cytoreduction and from date of diagnosis by biopsy or cytology in those who underwent primary neoadjuvant chemotherapy.

Immunohistochemistry (IHC)

TMA slides were processed and stained manually. The streptavidin-biotin peroxidase technique with diaminobenzidine as chromogen was applied. For antigen retrieval, Dako Target Retrieval Solution (pH 9.0; catalog number S2368) was used, and the slides were boiled in a pressure cooker (Pascal pressure cooker, Dako Cytomation, model S2800). Details of primary antibodies used are given in Supplementary Table 1. Endogenous peroxidase activity was quenched using 3% hydrogen peroxidase. Endogenous biotin was blocked and all slides were counterstained with hematoxylin, dehydrated, cleared, and cover slipped with premount. For negative control, primary antibody was omitted and replaced by nonreacting antibody of the same species. Only fresh cut TMA slides were stained to minimize the influence of slide aging and to maximize repeatability and reproducibility of the experiment.

Each TMA spot was assigned an intensity score from 0–3 (I0, I1, I2, I3) and the proportion of the tumor staining for that intensity was recorded as 5% increments from a range of 0–100 (P0, P1–3). A final H score (range 0–300) was obtained by adding the sum of scores obtained for each intensity and proportion of area stained (H score = I1XP1 + I2XP2 + I3XP3). X-tile plots were constructed for assessment of biomarker and optimization of cutoff points based on outcome. The X-tile plots allowed determination of an optimal cutpoint while correcting for the use of minimum *P* statistics. By using the X-tile program, an optimal cutpoint for p-mTOR (Ser2481) was 150. Tumors with an H-score ≥150 were categorized as high expression tumors and those with an H-score of <150 were categorized as low expression. Similarly, cutoff values for each marker p-mTOR (Ser2448), p-AKT, p70s6, p-4EBP1 and Bcl-Xl was calculated and tumors were grouped into two groups as high and low expression (Table 1).

Cell Culture

EOC cell lines OVSAHO and OVISE were Japanese Collection of Research Bioresources products and MDAH2774 and OVCAR were American Type Culture Collection (ATCC) products. Cells were grown in RPMI 1640 medium supplemented with 20% (vol/vol) fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin at 37°C in humidified atmosphere containing 5% CO_2. All *in vitro* experiments were performed in media supplemented with 5% FBS.

Reagents and Antibodies

Torin2 was a Tocris Bioscience product. zVAD-fmk was a Calbiochem product. Antibodies against p-mTOR (Ser2448), p-mTOR (Ser2481), p-p70 S6 Kinase (Ser389), p70S6 Kinase, p-4E-BP1 (Thr37/46), Bcl-Xl, GAPDH, Cytochrome c and PARP, were Cell Signaling Technologies products as were mTOR and scrambled siRNA. Antibodies against caspase-9, caspase-3, p-AKT and Bax were Santa Cruz Biotechnology products. Bax clone 6A7 and cisplatin were Sigma-Aldrich products. Survivin, cIAP1 and cIAP2 antibodies were R&D Systems products. β-actin and XIAP antibodies were Abcam products. Annexin V was a Molecular Probes product.

Cell Lysis and Immunoblotting

EOC cells were treated with and without Torin2 for the indicated time period following which cells were lysed and immunoblotted using different antibodies as described in the figure legends. The immunoblots were developed and visualized using an enhanced chemiluminescence (ECL) system (Amersham) as described previously (15).

Cell Growth Studies by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

To determine cell viability of EOC cells following treatment with Torin2 and cisplatin, 10^4 cells were treated and incubated in 96-well plates in a final volume of 0.2 mL for 48 h. Inhibition of cell viability was determined by MTT cell viability assays as described earlier (16). Replicates of six wells for each dosage including vehicle control were analyzed for each experiment. Statistical significance was $p < 0.05$.

Table 1. Clinicopathological correlation of p-mTOR Ser2448 (mTORC1) expression in EOC.

	Total		>180		≤180		
Epithelial group	N	%	N	%	N	%	*P* value
Total number of cases	146[a]		55	37.7	91	62.3	
Age							
≤50 years	60	41.1	21	35.0	39	65.0	0.5773
>50 years	86	58.9	34	39.5	52	60.5	
Tumor grade							
Stage I–II	8	5.8	4	50.0	4	50.0	0.4484
Stage III–IV	129	94.2	47	36.4	82	63.6	
Histopathology							
Clear cell	4	2.7	3	75.0	1	25.0	0.2191
Endometriod	21	14.4	5	23.8	16	76.2	
Serous	117	80.1	45	38.5	72	61.5	
Undifferentiated	4	2.7	2	50.0	2	50.0	
Histologic grade							
Well-differentiated	25	17.1	14	56.0	11	44.0	0.0717
Moderately differentiated	78	53.4	29	37.2	49	62.8	
Poorly differentiated	43	29.4	12	27.9	31	72.1	
p-MTOR ser2481							
>150	62	44.6	34	54.8	28	45.2	<0.0001
≤150	77	55.4	17	22.1	60	77.9	
p-P70S6							
>30	67	53.6	36	53.7	31	46.3	<0.0001
≤30	58	46.4	11	19.0	47	81.0	
p-AKT (473)							
>110	68	51.1	32	47.1	36	52.9	0.0205
≤110	65	48.9	18	27.7	47	72.3	
Bcl-Xl							
>180	115	82.1	39	33.9	76	66.1	0.0945
≤180	25	17.9	13	52.0	12	48.0	
p-4E-BP1							
>25	89	69.5	35	39.3	54	60.7	0.5172
≤25	39	30.5	13	33.3	26	66.7	
PFS-Median (months)			11.1		17.7	0.1505	

[a]Out of 156 TMA spots, IHC results were available for only 146 cases. Data was not available (NA) for some cases as follows: Tumor Grade (NA = 09), p-MTOR ser 2481 (NA = 07), p-7056 (NA = 21), p-AKT-473 (NA = 13), BcL-XL (NA = 06) & p-4E-BP-1 (NA = 18).

Live Dead Assay

Live/Dead viability/cytotoxicity kit (Invitrogen) was used to determine cell death in EOC cells following treatment with Torin2. Cells (1×10^6) were treated with the indicated doses of Torin2 for 48 h following which they were washed with PBS and stained with 50 μmol/L calcein AM and 8 μmol/L ethidium homodimer for 30 min in the dark at 37°C. Cell death was determined by the number of green-stained cells (Live cells) as compared with red-stained cells (Dead cells) visualized by an Olympus fluorescent microscope using a long pass filter.

Cell Cycle and Apoptosis Analysis by Flow Cytometry

Following treatment, cells were washed in phosphate buffer saline (PBS) and stained with hypotonic propidium iodide (PI) buffer and analyzed by flow cytome-

try for cell cycle fractions. For detection of apoptosis, cells were washed with PBS and incubated with fluorescein-conjugated annexin V/PI at room temperature for 30 min in the dark using an apoptosis detection kit (Molecular Probes). Following incubation, cells were analyzed for apoptosis by flow cytometry.

Assay for Cytochrome c Release

Cytochrome c release from mitochondria was assayed as described earlier (15). Twenty μg of proteins from cytosolic and mitochondrial fractions of each samples were analyzed by immunoblotting using an anti-cytochrome c antibody.

Measurement of Mitochondrial Membrane Potential

EOC cells (1×10^6) were treated with indicated doses of Torin2 for 48 h. Cells were washed twice with PBS and resuspended in mitochondrial incubation buffer (Alexis Corp.). Cells were then stained with 10 μmol/L JC1 (5,5′,6,6′-teterachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) and incubated at 37°C in the dark for 30 min. Cells were washed with PBS and resuspended in 500 μl of mitochondrial incubation buffer and mitochondrial membrane potential (% of green and red aggregates) was measured by flow cytometry as described earlier (15).

Estimation of Combination Doses using Calcusyn Software

To determine the subtoxic doses of Torin2 and cisplatin that would induce a synergistic apoptotic response, we treated MDAH2774 and OVSAHO cells with various combinations of the two drugs for 48 h. Apoptosis and cell viability assays were performed and the results were plotted using the Chou and Talalay method on Calcusyn software (36). The data calculated the combination doses of Torin2 and cisplatin that would induce a synergistic apoptotic response in both the cell lines.

Gene Silencing using siRNA

For siRNA knockdown studies, mTOR siRNA and scrambled control siRNA were transfected using lipofectamine 2000 (Invitrogen) for 6 h. After incubation, the siRNA complex was removed and fresh media containing 20% fetal bovine serum was added for 48 h. Cells were harvested and lysed and immunoblotted using specific antibodies.

Animals and Xenograft Study

Six-week-old female nude mice were obtained from Jackson Laboratories and maintained in a pathogen-free animal facility for at least 1 wk before use. All animal studies were done in accordance with institutional guidelines. For the xenograft study, female mice were inoculated subcutaneously in the right abdominal quadrant with 10 million MDAH2774 cells in 200 μL PBS. After 1 wk, the animals were randomly divided into five groups (n = 4): The first group received 10% dimethyl sulfoxide (DMSO) and two groups received 2 mg/kg and 10 mg/kg of Torin2, respectively. The remaining two group received either 3 mg/kg cisplatin alone or a combination of 3 mg/kg cisplatin + 2 mg/kg of Torin2 per body weight intraperitoneally. Mice were injected with these drugs twice weekly. The body weight and tumor volume of each mouse also was monitored weekly. The tumor volume was measured as described previously (37). After 5 wks of treatment, mice were euthanized and individual tumors were weighed and then snap frozen in liquid nitrogen for storage.

Statistical Analysis

All Statistical analysis was performed using the Statview JMP software (version 10.0). Fisher exact chi-square (χ^2) test was used to assess associations between categorical variables. Kaplan-Meier survival analyses were carried out for PFS using the log-rank test for differences between groups. Results were considered statistically significant when p from a two-tailed test was <0.05.

All supplementary materials are available online at www.molmed.org.

RESULTS

mTORC1 and mTORC2 Activation Status in EOC Samples and Association with Clinical and Molecular Markers

Initially, we investigated the activation of mTORC1 and mTORC2 in a cohort of EOC samples from Saudi Arabia by immunohistochemistry in a tissue microarray format. The activation of mTORC1 and mTORC2 was determined by evaluation of phosphorylation status of mTOR1 (Ser2448) and mTOR2 (Ser2481), respectively (Figure 1A). We found that 37.7% (55/146) EOC samples had activation of mTORC1 while 45.0% (63/140) had activation of mTORC2 (Tables 1 and 2). Interestingly, mTORC1 and mTORC2 were significantly associated with each other ($p < 0.0001$). mTORC1 was significantly associated with p-P70S6 and p-AKT; on the other hand, mTORC2 was also found to be significantly associated with p-P70S6, p-AKT and p-4E-BP1. EOC patients with a high expression of p-mTORC2 (Ser2481) had a poor PFS of 13.2 months as compared with 18.1 months with a low expression of mTORC2, which was statistically significant ($p = 0.0188$) (Table 2, Figure 1B).

Targeting of mTOR Signaling Pathway Using Torin2 Inhibits Cell Viability and Induces Apoptosis in EOC Cell Lines

Clinically, our data identified a group of EOC samples that had constitutive activation of the mTOR signaling complex. Keeping this in mind, we first sought to determine the presence of mTORC1 and mTORC2 activity in our panel of EOC cell lines. Our data showed that both mTOR complexes, mTORC1 and mTORC2, were constitutively activated in our panel of EOC cell lines. Next, we serum starved our EOC cells for 36 h and compared activation of mTORC1 and mTORC2 with EOC cells grown in 20% FBS. As shown in Supplementary Figure 1A, there was no difference between activation of mTORC1 and mTORC2 in serum-starved EOC cells as compared with cells grown in 20%

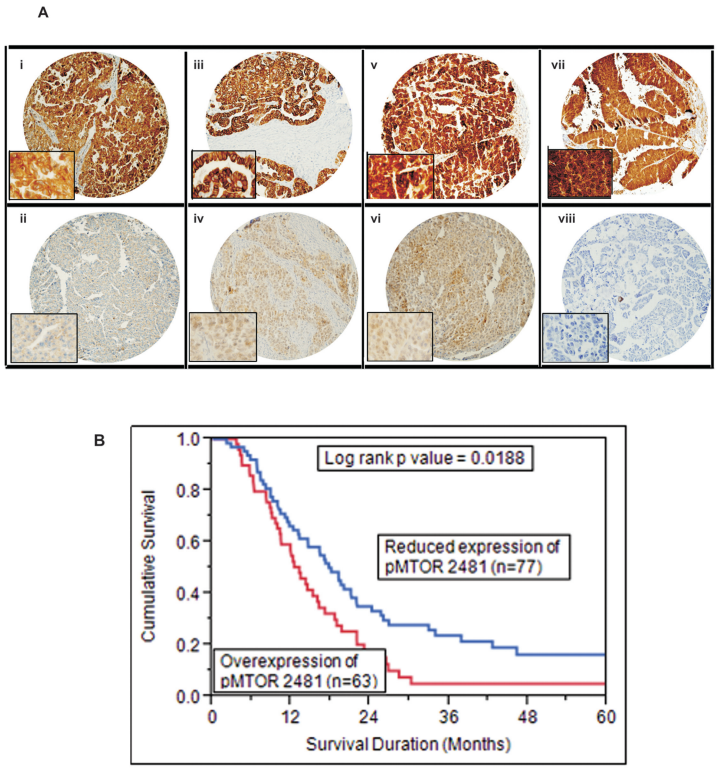

Figure 1. (A) Immunohistochemical analysis of p-mTORSer2481, p-mTOR Ser2448, p-AKT and p-4EBP1 expression in epithelial ovarian carcinoma. TMA spots showing overexpression of (i) p-mTOR (ser2481), (iii) p-mTOR (Ser2448), (v) p-AKT and (vii) p-4EBP1. In contrast, different TMA spots showing reduced/absent expression of (ii) p-mTOR (Ser2481), (iv) p-mTOR (Ser2448), (vi) p-AKT and (viii) p-4EBP1. 20 × magnification on Olympus BX-51 microscope (Olympus America) with inset showing 40 × magnifications of the same TMA spot. (B) Kaplan-Meier survival curve in EOC patients showing poor survival in p-mTOR Ser2481 overexpressed cases compared with those with reduced expression (p = 0.0188).

serum. This data clearly indicates that there is constitutive activation of mTORC1 and mTORC2 in our panel of EOC cell lines. We also examined activation of mTORC1 and mTORC2 in five normal ovarian epitheliums by immunohistochemistry and peripheral blood mononuclear cells (PBMNC) from five healthy female donors by immunoblotting. There was minimal-to-no activation found in normal ovarian epithelium (data not shown) by IHC and activation of these complexes in normal PBMNCs was not detected by immunoblotting (Supplementary Figure 1B). For *in vitro* experiments, we investigated whether Torin2, a second-generation inhibitor of mTOR, could inhibit cell viability in these cell lines. All four EOC cell lines were treated with increasing doses of Torin2 for 48 h and the cells were analyzed for cell viability by MTT assay. As shown in Figure 2A, all the EOC cells had inhibition of cell viability following Torin2 treatment in a dose-dependent manner that reached significance from doses of 100 nmol/L and onwards for OVISE and OVSAHO and from 200 nmol/L for MDAH2774 and OVCAR. From these data, we estimated the working dose of Torin2 to be 200 and 400 nmol/L and continued using these doses for the rest of the experiments. Next, we determined whether Torin2 treatment could lead to cell cycle arrest in these cell lines. EOC cell were treated with 200 and 400 nmol/L Torin2 for 48 h and cells were analyzed by flow cytometry for cell cycle fractions. As shown in Figure 2B, there was increase in the subG1 (Apo) fraction in cells treated with 200 nmol/L and 400 nmol/L Torin2. We confirmed cell death in these cells by staining the cells with calcein AM and ethidium homodimer and visualizing the cells under a microscope. Cells that were not treated were stained green (depicting live cells) while cells that were treated with 200 and 400 nmol/L Torin2 stained red (depicting dead cells) (Figure 2C). Finally, apoptosis was confirmed by dual staining with annexin V/PI and analyzed by flow cytometry following treatment with 200 and 400 nmol/L Torin2 for 48 h. As shown in Figure 2D, there was an increase in apoptosis in cells treated with Torin2 as compared with control. To confirm specificity of Torin2 against mTORC1 and mTORC2, we also treated three PBMNCs from healthy female donors with 200 and 400 nmol/L Torin2 for 48 h. PBMNCs from healthy donors did not undergo apoptosis as detected by annexin V/PI dual staining and immunoblotting of caspase-3 (Supplementary Figures 2A and B).

Torin2 Inactivates mTORC1, mTORC2 and Their Downstream Targets in EOC Cells

Once we established that Torin2 treatment of EOC cells induced apoptosis, we wanted to investigate whether Torin2 treatment was inducing apoptosis via inactivation of mTORC1 and mTORC2 and their downstream targets. Therefore, we treated MDAH2774, OVCAR and OVSAHO cell lines with 200 and 400 nmol/L Torin2 for 48 h and, following treatment, cells were immunoblotted with antibodies that were involved in this pathway. We found that Torin2 treatment inactivated mTORC1 and mTORC2 in EOC cell lines as shown in Figure 3A. In addition, Torin2 treatment also dephosphorylated the downstream targets, P70S6 and 4E-BP1, at doses of 200 and 400 nmol/L in these cell lines (Figure 3A). These data were confirmed by targeting MDAH2774 and OVSAHO cell lines with siRNA targeted against mTOR, clearly indicating specificity of Torin2 in de-

Table 2. Clinicopathological correlation of p-mTORC Ser2481 (mTORC2) expression in EOC

	Total		>150		≤150		
Epithelial group	N	%	N	%	N	%	*P* value
Total number of cases	140[a]		63	45.0	77	55.0	
Age							
≤50 years	59	42.1	27	45.8	32	54.2	0.8770
>50 years	81	57.9	36	44.4	45	55.6	
Tumor Grade							
Stage I-II	8	6.1	3	37.5	5	62.5	0.6886
Stage III-IV	123	93.9	55	44.7	68	55.3	
Histopathology							
Clear cell	4	2.9	2	50.0	2	50.0	0.2357
Endometriod	20	14.3	5	25.0	15	75.0	
Serous	113	80.7	55	48.7	58	51.3	
Undifferentiated	3	2.1	1	33.3	2	66.7	
Histologic Grade							
Well-differentiated	25	17.9	9	36.0	16	64.0	0.3397
Moderately Differentiated	74	52.9	32	43.2	42	56.8	
Poorly Differentiated	41	29.3	22	53.7	19	46.3	
p-mTOR ser2448							
>180	51	36.7	34	66.7	17	33.3	<0.0001
≤180	88	63.3	28	31.8	60	68.2	
p70S6							
>30	66	53.2	40	60.6	26	39.4	0.0035
≤30	58	46.8	20	34.5	38	65.5	
pAKT (473)							
>110	68	52.3	37	54.4	31	45.6	0.0298
≤110	62	47.7	22	35.5	40	64.5	
Bcl-Xl							
>180	114	83.2	54	47.4	60	52.6	0.1304
≤180	23	16.8	7	30.4	16	69.6	
p-4E-BP1							
>25	86	69.96	44	51.2	42	48.8	0.0119
≤25	37	30.04	10	27.1	27	72.9	
PFS-Median (months)				13.2		18.1	0.0188

[a]Out of 156 TMA spots, IHC results were available for only 140 cases. Data was not available (NA) for some cases as follows: Tumor Grade (NA = 09), p-MTOR ser 2448 (NA = 01), p-7056 (NA = 16), p-AKT-473 (NA = 10), BcL-XL (NA = 03) & p-4E-BP-1 (NA = 17)

phosphorylating proteins involved in the mTOR pathway (Figure 3B). AKT is closely associated with the mTOR pathway and cross-talk between the mTOR and PI3-kinase/AKT pathways has been shown in various cancers (38,39). In our cohort of clinical samples, a significant association between activation of mTORC1 and mTORC2 with AKT was detected. Therefore, we were interested in determining whether Torin2 treatment could also inactivate AKT and its downstream targets in EOC cells. As shown in Figure 3C, Torin2 treatment led to dephosphorylation of AKT at Ser473 in all the cell lines examined. In addition, Torin2 inactivated the downstream targets of AKT (FOXO1, GSK3 and Bad) in EOC cell lines (Figure 3C). Specificity of Torin2 in inactivating AKT and its downstream targets also was confirmed by siRNA knockdown studies that showed similar results (Figure 3D).

Inhibition of mTOR Signaling Pathway Leads to Activation of Intrinsic Apoptotic Pathway in EOC Cells

For the intrinsic apoptotic pathway to be activated, the downstream target of AKT (Bad) has to be dephosphorylated to allow the apoptotic signal to reach the mitochondria via causing conformational changes in the Bax protein by exposing the epitope that is required for homodimerization (40). We found that Torin2 inactivated Bad in EOC cell lines, thereby allowing the apoptotic signal to reach the mitochondria via causing conformational changes and upregulation of the Bax protein (41). Our data showed that there was upregulation and conformational change in the Bax protein starting at 8 h of Torin2 treatment that peaked in 24 h in the OVSAHO cell line using the Bax 6A7 antibody that only detects the N-terminal of Bax required for conformational change (Figure 4A). Once the Bax protein is dimerized, it leads to changes in the mitochondrial membrane potential, allowing release of cytochrome c into cytosole. To determine whether these events take place in EOC cells treated with Torin2, we treated all four cell lines with 200 and 400 nmol/L Torin2 for 48 h and then examined the cells for changes in mitochondrial membrane potential by flow cytometry using JC1 dye. Most of the untreated cells stained red suggesting an intact mitochondria while there was an increase in green-stained cells treated with Torin2 clearly indicating a damaged mitochondria (Figure 4B). This led to a release of cytochrome c from mitochondria into cytosole as detected by immunoblotting on mitochondrial-free cytosolic extracts after treatment with Torin2 (Figure 4C).

Inhibitor of apoptosis proteins (IAP) plays an important role in inhibiting apoptosis via interfering with the proapoptotic proteins and rendering them ineffective. Therefore, for efficient apoptosis to occur, IAP needs to be downregulated. To determine whether Torin2-induced apoptosis occurred via inhibition of IAP, we treated EOC cells with 200 and 400 nmol/L Torin2 for 48 h. Cells were lysed and immunoblotted with antibodies against XIAP, cIAP1 and survivin. As shown in Figure 4D, Torin2 treatment downregulated expression of these proteins indicating that downregulation of IAPs is coincident with activa-

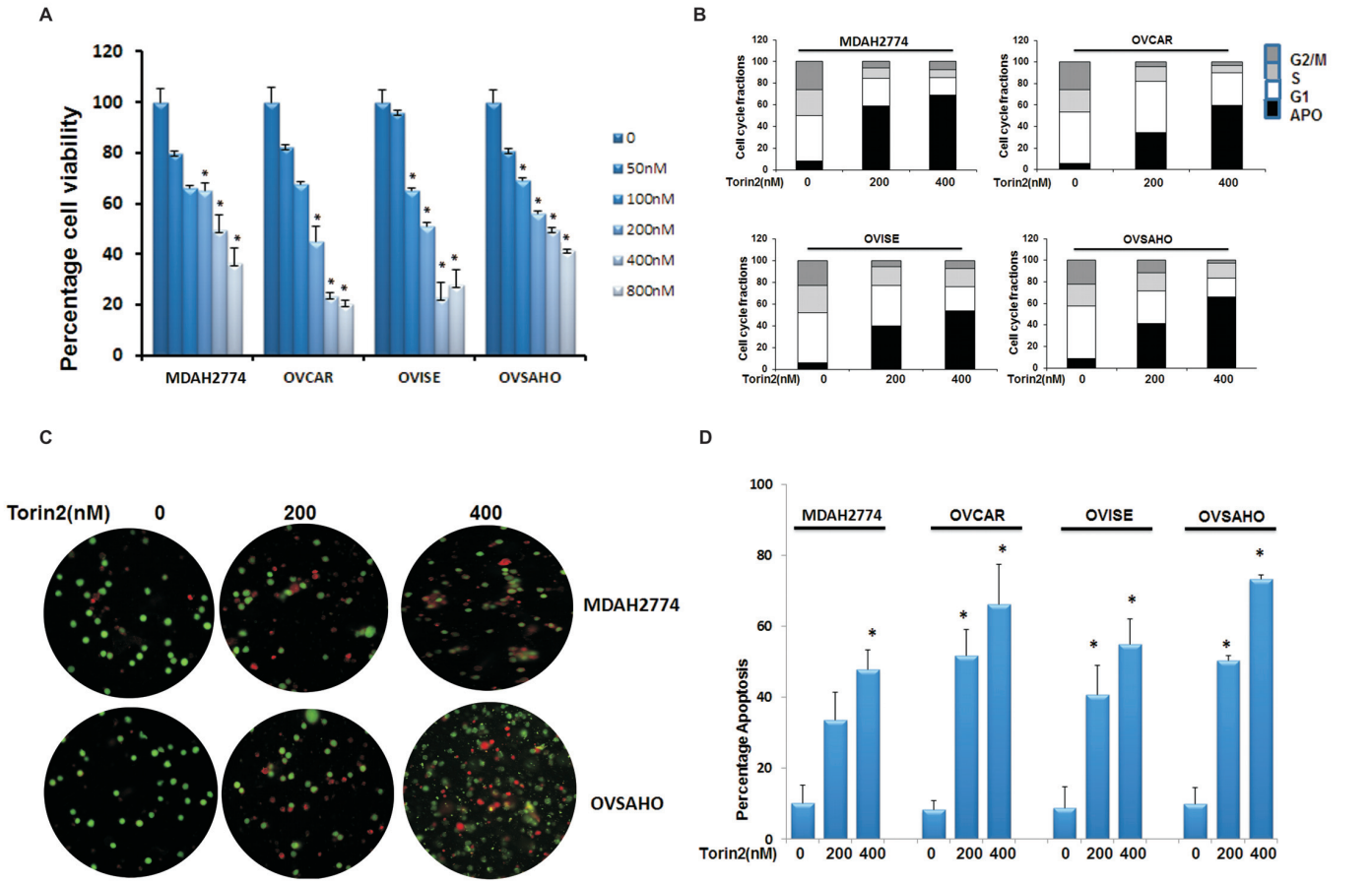

Figure 2. Torin2 treatment inhibits cell viability and induces apoptosis in EOC cells. (A) Torin2 treatment ThiostreptonTh inhibits cell viability in a dose-dependent manner in EOC cells. MDAH2774, OVCAR, OVSAHO and OVISE cells were incubated with 0, 50, 100, 200, 400 and 800 nmol/L Torin2 for 48 h. Cell viability assays were performed using MTT as described in Materials and Methods. The graph displays the mean ± SD (standard deviation) of three independent experiments with replicates of six wells for all the doses and vehicle control for each experiment, $*p < 0.01$, statistically significant (Student *t* test). (B) Torin2 increases Apo fraction of cells in EOC cell lines. EOC cells were treated with 200 nmol/L and 400 nmol/L Torin2 for 48 h. Thereafter, the cells were washed, fixed and stained with propidium iodide (PI), and analyzed for cell cycle fractions by flow cytometry as described in Materials and Methods. There was an increase in Apo fractions (also known as sub-G1 population) in cells treated with 200 nmol/L and 400 nmol/L Torin2 for 48 h. The bar graph is an average of three independent experiments. (C) Torin2 treatment causes cell death in EOC cells. EOC cells (1×10^6) were treated with 200 nmol/L and 400 nmol/L Torin2 for 48 h. Following incubation, cells were resuspended in 1 ml PBS containing 50 μmol/L calcein AM and 8 μmol/L ethidium homodimer and cells were incubated in the dark for 20 min. Fifty μl of suspension was transferred on slides and visualized under an Olympus fluorescent microscope using a longpass filter and cell death was measured as described in Materials and Methods. (D) Torin2-induced apoptosis detected by annexin V/PI dual staining. EOC cells were treated with 200 nmol/L and 400 nmol/L Torin2 for 48 h and cells were subsequently stained with flourescein-conjugated annexin-V and PI and apoptotic cells were analyzed by flow cytometry. The bar graph is an average of four independent experiments performed on different days. $*p < 0.01$, statistically significant (Student *t* test).

tion of mitochondrial apoptotic pathway secondary to Torin2 treatment for induction of apoptosis in EOC cells.

Torin2 Treatment Led to Caspase-Dependent Apoptosis in EOC Cells

Caspases are critical and vital proteins for apoptosis to occur and can activate and cleave once the mitochondrial protein cytochrome c is released in cytosole. To determine whether Torin2 treatment can cleave caspases-9 and -3, we treated EOC cells with 200 and 400 nmol/L Torin2 for 48 h and immunoblotted the proteins with antibodies against caspases-9 and -3. As shown in Figure 4E, there was cleavage of caspases-9 and -3 in all cell lines treated with Torin2. PARP cleavage is necessary for the cells to undergo apoptosis and is cleaved once caspase-3 is activated. Our data showed that there was efficient cleavage of PARP following Torin2 treatment in all EOC cell lines. To confirm the role of caspases

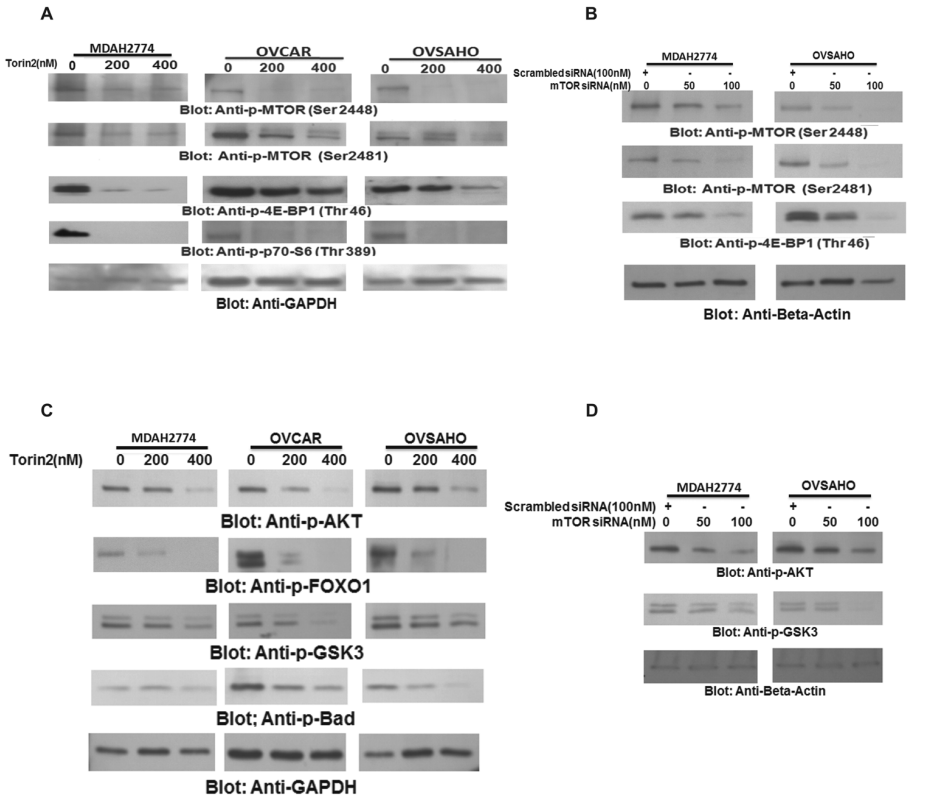

Figure 3. Torin2 treatment inactivates mTOR and AKT survival pathway in EOC cells. (A) After treating with 200 nmol/L and 400 nmol/L Torin2 for 48 h, MDAH2774, OVCAR and OVSAHO cells were lysed and immunoblotted with antibodies against p-mTOR Ser2448, p-mTOR Ser2481, p-4E-BP1 Thr46, p-P70S6 Thr389 and GAPDH as indicated. (B) MDAH2774 and OVSAHO were transfected with either scrambled siRNA (100 nmol/L) or mTOR siRNA (50 and 100 nmol/L) with lipofectamine as described in Materials and Methods. After 48 h of transfection, cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to immobilon membrane, and immunoblotted with antibodies against p-mTOR Ser2448, p-mTOR Ser2481, p-4E-BP1 and β-actin as indicated. (C) EOC cells were treated with 200 nmol/L and 400 nmol/L Torin2 for 48 h. After treatment, cells were lysed and immunoblotted with antibodies against p-AKT, p-FOXO1, p-GSK3 and p-Bad. GAPDH was used for equal loading. (D) MDAH2774 and OVSAHO were transfected with either scrambled siRNA (100 nmol/L) or mTOR siRNA (50 and 100 nmol/L) with lipofectamine as described in Materials and Methods. After 48 h of transfection, cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to immobilon membrane, and immunoblotted with antibodies against p-AKT, p-GSK3 and β-actin as indicated.

in Torin2-induced apoptosis, we pretreated EOC cells with a universal inhibitor of caspases, zVAD-fmk, for 2 h followed by Torin2 treatment. As shown in Figure 4F, zVAD-fmk pretreatment inhibited Torin2-induced cleavage of caspase-3 and PARP as well as inhibited apoptosis (data not shown), confirming the role of caspases in Torin2-induced apoptosis in EOC cells.

Torin2 Potentiates Anticancer Effect of Cisplatin in EOC Cells to Induce Efficient Apoptosis

Platinum resistance is a major obstacle in the treatment of EOC as platinum-based regimens are used as a first line of treatment for ovarian cancer (29). To identify newer regimens where small molecular inhibitors used in conjunction with platinum drugs can synergize EOC cells to undergo apoptosis, we used multiple combinations of Torin2 and cisplatin to determine the appropriate dose required to induce a synergistic apoptotic response in EOC cells. Using Calcusyn software and the Chou and Talalay methodology (36), we found that 50 nmol/L Torin2 and 10 μmol/L cisplatin in combination had a combination index of 0.218 and 0.140 in MDAH2774 and OVSAHO cell lines, indicating a strong synergistic response (Supplementary Figure 3 and Supplementary Table 2). Using these doses, we treated MDAH2774 and OVSAHO cell lines for 48 h and found that there was significant inhibition of cell viability in both cell lines (Figure 5A). In addition, this combination dose was able to induce apoptosis in both cell lines treated (Figures 5B,C). Finally, combination treatment of EOC cell lines with suboptimal doses of Torin2 and cisplatin downregulated expression of antiapoptotic proteins Bcl-Xl and XIAP and cleaved caspases-9, -3 and PARP (Figure 5D). These data clearly indicate that Torin2 has the ability to potentiate the effect of cisplatin to induce apoptosis in EOC cells.

Inactivation of mTOR Activity by Torin2 Suppresses EOC Xenograft Alone or in Combination with Cisplatin at Suboptimal Doses

Our *in vitro* data showed that Torin2 treatment alone could inhibit cell viability via induction of apoptosis at doses of 200 and 400 nmol/L. In addition, we found that Torin2 at a dose of 50 nmol/L and cisplatin at 10 μmol/L could synergistically induce apoptosis in EOC cells. To confirm this data *in vivo*, xenografts were generated in female nude mice using the MDAH2774 cell line. Once tumors were developed, they were divided into different groups. The first set of experiments was conducted using two doses of Torin2, 2 mg/kg and 10 mg/kg body weight. As shown in Supplementary Figures 4A and B, there was a decrease in tumor volume and weight in animals treated with 10 mg/kg body weight. Naked eye

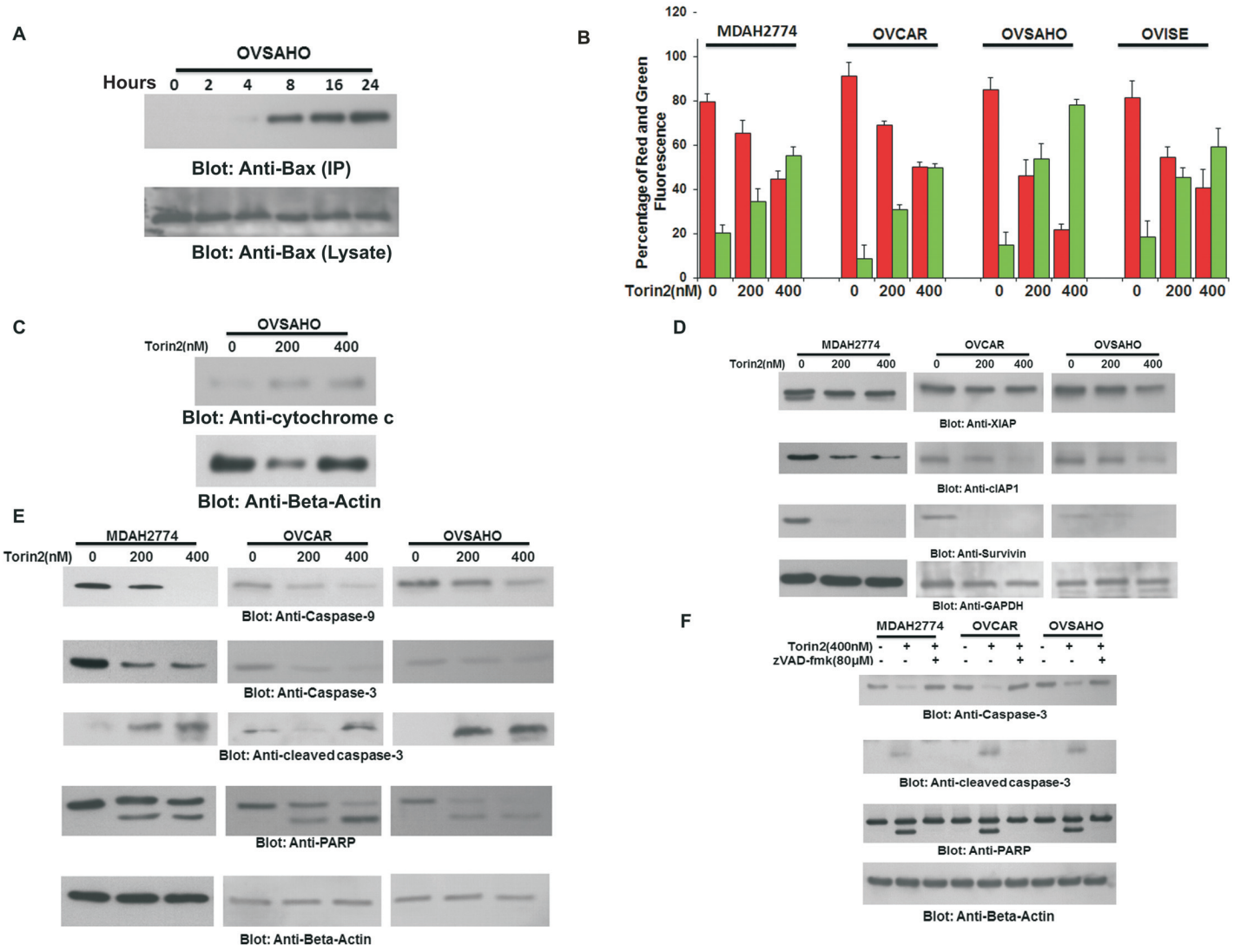

Figure 4. Torin2 activates the mitochondrial apoptotic pathway and induces caspase-dependent apoptosis in EOC cells. (A) OVSAHO cells were treated with 400 nmol/L Torin2 for indicated time points. Following treatment, cells were lysed and immunoprecipitated with Bax monoclonal antibody (clone 6A7) and probed with polyclonal Bax antibody (Sc493). Cell lysate of the same experiment was probed with polyclonal Bax antibody to ensure equal loading. (B) EOC cell lines were treated with 200 nmol/L and 400 nmol/L Torin2 for 48 h. Following incubation, cells were washed twice with PBS and stained with 10 μmol/L JC1 dye in 500 μl mitochondrial incubation buffer and incubated at 37°C in the dark for 30 min. After the incubation, cells were washed in PBS and resuspended in mitochondrial incubation buffer and change in mitochondrial membrane potential was detected by flow cytometry. (C) Torin2 treatment causes release of cytochrome c from mitochondria into cytosole. OVSAHO cells were treated with 200 nmol/L and 400 nmol/L Torin2 for 48 h. Mitochondrial free cytosolic fractions were isolated and immunoblotted with antibodies against cytochrome c and β-actin. (D) Torin2 treatment downregulates expression of IAPs in EOC cells. MDAH2774, OVCAR and OVSAHO cells were treated with 200 and 400 nmol/L Torin2 for 48 h. Following treatment, cells were lysed and immunoblotted with antibodies against XIAP, cIAP1, survivin and GAPDH. (E) Activation of caspases induced by Torin2 treatment in EOC cells. MDAH2774, OVCAR and OVSAHO cells were treated with and without 200 and 400 nmol/L for 48 h. Cells were lysed and proteins were immunoblotted with antibodies against caspase-9, caspase-3, cleaved caspase-3, PARP, and β-actin. (F) zVAD/fmk pretreatment abrogates Torin2-induced caspase-3 and PARP cleavage in EOC cells. MDAH2774, OVCAR and OVSAHO cells were pretreated with 80 μM z-VAD/fmk for 2 h and subsequently treated with 400 nmol/L Torin2 for 48 h. Following treatment, cells were lysed and 10 μg proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with antibodies against caspase-3, cleaved caspase-3, PARP, and β-actin.

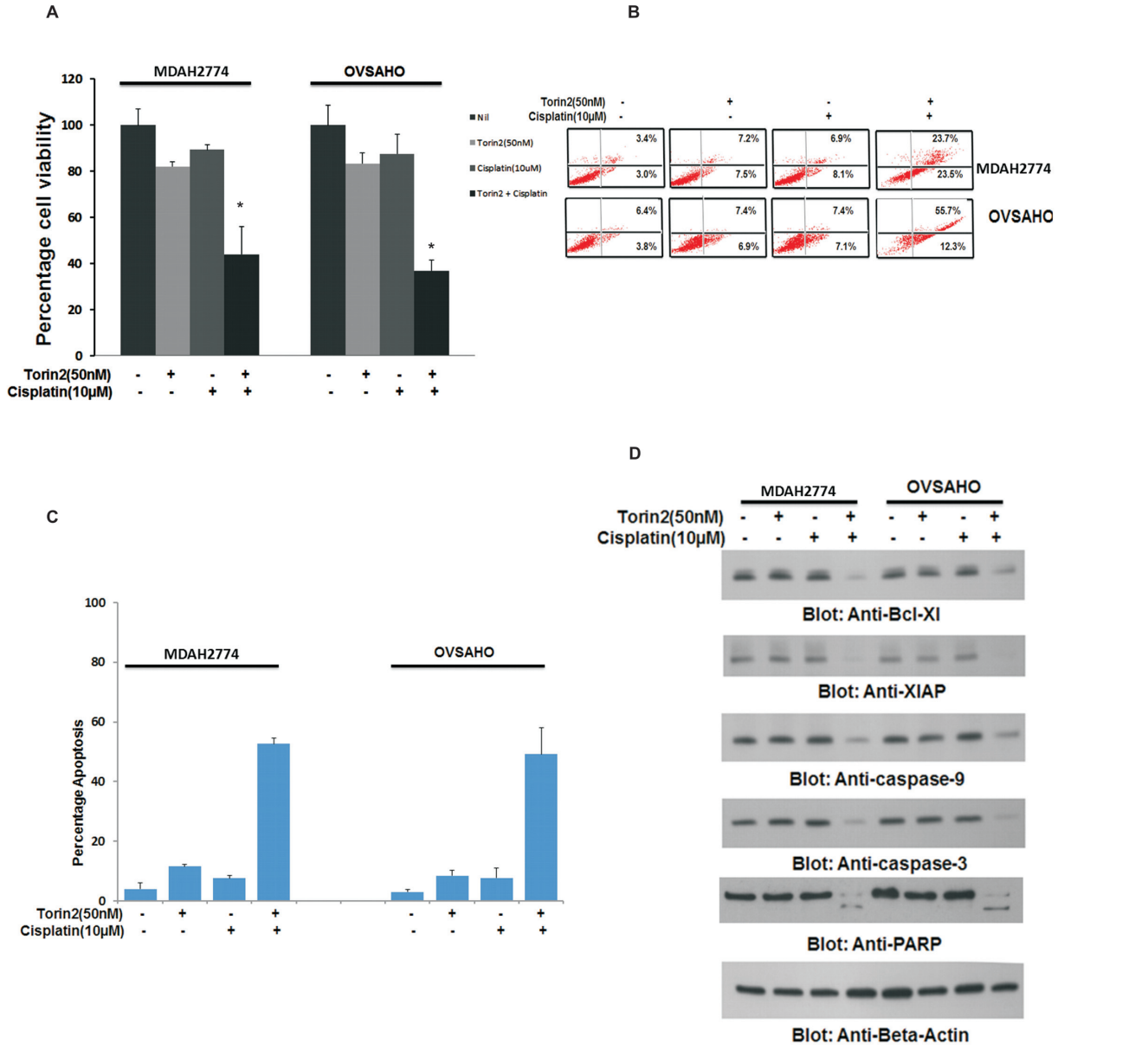

Figure 5. Cotreatment with Torin2 and cisplatin inhibit cell viability and induce caspase-dependent apoptosis in EOC cells. (A) MDAH2774 and OVSAHO cells were treated with either 50 nmol/L Torin2 in the presence and absence of 10 μmol/L cisplatin for 48 h. Following treatment, cells were analyzed for cell viability by MTT assay as described in Materials and Methods. Bar graph displays the mean ± SD (standard deviation) of three independent experiments, *$p < 0.05$, statistically significant (Student *t* test). (B and C) MDAH2774 and OVSAHO cells were treated with either 50 nmol/L Torin2 in the presence and absence of 10 μmol/L cisplatin for 48 h. Following treatment, cells were stained with fluorescent-conjugated annexin V/PI and apoptosis was analyzed by flow cytometry. Figure 5C is a bar graph with an average of three independent experiments. (D) EOC cells were treated with either 50 nmol/L Torin2 in the presence and absence of 10 μmol/L cisplatin for 48 h. Following treatment, cells were lysed and equal amounts of proteins were immunoblotted with antibodies against Bcl-Xl, XIAP, caspase-9, caspase-3, PARP and β-actin.

observation of animals also clearly showed response to 10 mg/kg Torin2 treatment (Supplementary Figure 4C). This response of Torin2 was elicited by inactivation of mTORC1, mTORC2, P70S6, 4E-BP1 and AKT as well as downregulation of Bcl-Xl and cleavage of caspase-3 (Supplementary Figure 4D). In the next set of experiments, we used suboptimal doses of Torin2 (2 mg/kg) and cisplatin (3 mg/kg) either alone or in combination. As shown in Figures 6A

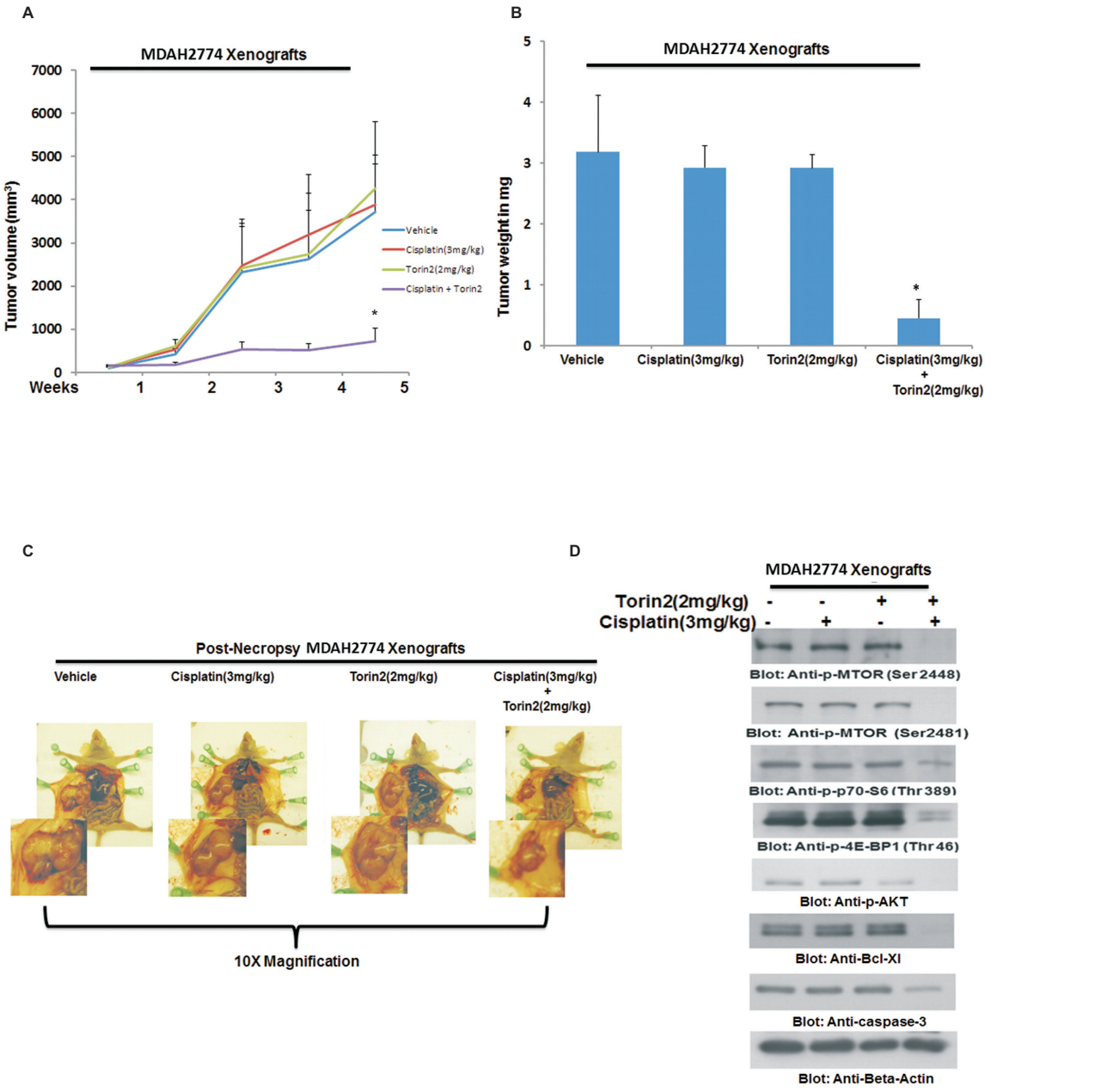

Figure 6. Inhibition of EOC-cell tumor xenografts growth by cotreatment with Torin2 and cisplatin. Nude female mice at 6 wks of age were injected subcutaneously with ten million MDAH2774 cells. After one week, the animals were divided into four groups, the first group received DMSO (vehicle) alone while the other three groups received Torin2 (2 mg/kg), cisplatin (3 mg/kg) and combination of both, injected twice weekly, intraperitoneally. After 5 wks of treatment, mice were euthanized and tumors were collected. (A) The volume of each tumor was measured every week. The average (n = 5) tumor volume in each group of mice was calculated, *$p < 0.05$ statistical significance. (B) After 5 wks of treatment, mice were euthanized and mean tumor weight (± SD) was calculated in each group. *$p < 0.05$ statistical significance. (C) Representative tumor images of each group of mice after necropsy. Inset picture showing 10× magnification. (D) Whole-xenograft homogenates from mice injected with MDAH2774 cells were immunoblotted with antibodies against p-mTOR Ser2448, p-mTOR Ser2481, p-P70S6, p-4E-BP1, p-AKT, Bcl-Xl, caspase 3 and β-actin.

and B, there was a significant decrease in tumor volume and weight in animals treated with the combination of Torin2 and cisplatin as compared with treatment alone. In addition, naked eye observation also showed regression of tumor in combination-treated animals (Figure 6C). Finally, we assessed expression of proteins that are involved in

mTOR signaling complex and found that there was inactivation of mTORC1, mTORC2, P70S6, 4E-BP1 and AKT in xenografts treated with a combination of Torin2 and cisplatin at suboptimal doses. Inactivation of these proteins also was associated with a downregulation of Bcl-Xl and caspase-3 in these xenografts (Figure 6D). These data reassert our findings that Torin2 has the ability to potentiate the anticancer effect of cisplatin *in vivo*.

DISCUSSION

The mTOR/PI3-kinase/AKT pathway is a well-characterized and researched survival pathway that plays an important role in cell survival and proliferation. This pathway has been found to be dysregulated in a variety of cancers (38,42,43). However, clinical characterization of the mTOR signaling pathway has not been fully elucidated on epithelial ovarian cancer from the Middle Eastern region. Therefore, in this study, we analyzed the activation of mTORC1 (2448) and mTORC2 (2481) in a cohort of 156 clinical samples of EOC of Middle Eastern origin by IHC. Our data showed that 37.7% and 45% of EOC cases had activation of mTORC1 and mTORC2, respectively. These data indicate that the mTOR pathway may be playing an important role in the pathogenesis in a subgroup of EOC that has activated mTOR complexes. The two complexes of mTOR have distinct functions, where mTORC1 is associated with translational activity of cells while mTORC2 is associated with cell survival. Interestingly, mTORC2-activated EOC clinical samples had a poor PFS of 13.2 months as compared with 18.1 months with low activity that was statistically significant ($p = 0.0188$) even when other aggressive clinical parameters such as old age, grade and stage were not associated with mTORC2. This data clearly suggests that targeting this pathway may be beneficial for the management of EOC.

Currently, there are several analogues of rapamycin that are being used either in clinical trials or in treatment regimens of various cancers to target the mTOR signaling pathway (13,44). Even though these experimental inhibitors have shown positive results, it has been found that cancer cells quickly develop resistance to these agents as they are only effective against the mTORC1 and have limited or no effect on mTORC2 (23,45,46). Torin2 has an advantage over other mTOR inhibitors as it can target both the mTOR complexes, mTORC1 and mTORC2, thereby effectively inhibiting the mTOR pathway completely (47). Our data indicated that Torin2 inhibits cell viability and induces apoptosis in EOC cells via inactivation of mTORC1 and mTORC2 and their downstream targets. By inactivating these proteins, Torin2 not only inhibits translation of proteins but also induces apoptosis in EOC via inactivating AKT and its downstream targets, FOXO1, GSK3 and Bad. Once Bad is dephosphorylated, the mitochondrial apoptotic pathway is activated, ultimately leading to apoptosis.

The end point of apoptosis occurs when the expression of proteins of two families, caspases and inhibitor of apoptosis (IAPs), are altered. IAPs act against induction of apoptosis via interfering with the activation and cleavage of the proapoptotic caspases family of proteins (48,49) . Therefore, for efficient apoptosis to occur, IAPs need to be downregulated, allowing the apoptotic signal to activate and cleave the involved caspases. Our data shows that the IAPs, XIAP, cIAP1 and survivin, are downregulated secondary to Torin2 treatment, leading to activation and cleavage of caspases, caspase-9 and caspase-3, ultimately leading to apoptosis in EOC cells.

In vivo experiments are usually conducted to confirm the *in vitro* results of a study. *In vivo* experiments also are important because, in addition to confirming *in vitro* results, they give an insight on toxicity that may be associated with inhibitors. Our *in vivo* data showed that Torin2 treatment alone caused regression of EOC xenografts via inactivation of mTORC1 and mTORC2 and their downstream targets. Additionally, our data showed that doses of Torin2 up to 10 mg/kg did not cause weight loss or lack of activity in mice (data not shown).

Our data also showed that Torin2 at a suboptimal dose also sensitized EOC cells to cisplatin-induced apoptosis. These experiments were conducted because one of the major drawbacks of treating EOC with chemotherapy is the development of resistance to platinum-based drugs (30,31). Unfortunately, platinum resistance of EOC cells usually dictate the response to treatment and more than 50% of EOC cases ultimately die of the disease or relapse due to this acquired resistance (50). This combinational effect of Torin2 and cisplatin is not only beneficial in inducing apoptosis in EOC cells, it also decreased the dose of cisplatin required to treat these cells, thereby decreasing the level of toxicity associated with cisplatin. Finally, we found that cotreatment with Torin2 and cisplatin at suboptimal doses was not only effective *in vitro* on EOC cells: even *in vivo* experiments confirmed that this mode of treatment efficiently regressed EOC xenograft tumors in nude mice.

CONCLUSION

Our study demonstrates that constitutive activation of the mTOR signaling complex can be targeted for therapeutic intervention using Torin2 in EOC cells. We provide ample evidence regarding the efficiency of using newer second-generation mTOR inhibitors for successful inhibition of cell growth and induction of apoptosis *in vitro* and *in vivo*. Additionally, inhibition of the mTOR signaling complex via Torin2 treatment potentiates EOC cells to platinum-based chemotherapy. Findings of this study highlight the importance of targeting the mTOR signaling pathway using second-generation inhibitors as a potential therapeutic intervention for the management of EOC.

ACKNOWLEDGMENTS

We would like to thank Roxanne Melosantos and Rafia Begum for their technical assistance and Zeeshan Qadri for statistical analysis.

DISCLOSURE

This study was supported by King AbdulAziz Centre for Science and Technology (KACST) under Project No. NSTIP 10-BIO963-20 (RAC 2140-033).

REFERENCES

1. Noske A. (2014) [Heterogeneity of epithelial ovarian carcinomas and their clinical significance]. *Praxis. (Bern 1994)*. 103:155–9.
2. Meinhold-Heerlein I, Hauptmann S. (2014) The heterogeneity of ovarian cancer. *Arch. Gynecol. Obstet*. 289:237–9.
3. Colombo PE, *et al*. (2014) Sensitivity and resistance to treatment in the primary management of epithelial ovarian cancer. *Crit. Rev. Oncol. Hematol*. 89:207–16.
4. Coleman RL, Monk BJ, Sood AK, Herzog TJ. (2013) Latest research and treatment of advanced-stage epithelial ovarian cancer. *Nat. Rev. Clin. Oncol*. 10:211–24.
5. Marcus CS, Maxwell GL, Darcy KM, Hamilton CA, McGuire WP. (2014) Current approaches and challenges in managing and monitoring treatment response in ovarian cancer. *J Cancer* 5:25–30.
6. Ma KL, *et al*. (2014) Activation of mTOR contributes to foam cell formation in the radial arteries of patients with end-stage renal disease. *Clin. Nephrol*. 81:396–404.
7. Perluigi M, *et al*. (2014) Neuropathological role of PI3K/Akt/mTOR axis in Down syndrome brain. *Biochim. Biophys. Acta*. 1842:1144–53.
8. Sun YX, *et al*. (2014) Differential activation of mTOR complex 1 signaling in human brain with mild to severe Alzheimer's disease. *J. Alzheimers Dis*. 38:437–44.
9. Zoncu R, Efeyan A, Sabatini DM (2011) mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat. Rev. Mol. Cell. Biol*. 12:21–35.
10. Dibble CC, Manning BD. (2013) Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. *Nat. Cell Biol*. 15:555–64.
11. Widlund AL, Baur JA, Vang O. (2013) mTOR: more targets of resveratrol? *Expert Rev. Mol. Med*. 15:e10.
12. Dobbin ZC, Landen CN. (2013) The importance of the PI3K/AKT/MTOR pathway in the progression of ovarian cancer. *Int. J. Mol. Sci*. 14:8213–27.
13. Zhang H, *et al*. (2013) A comparison of Ku0063794, a dual mTORC1 and mTORC2 inhibitor, and temsirolimus in preclinical renal cell carcinoma models. *PLoS One*. 8:e54918.
14. Wolin EM. (2013) PI3K/Akt/mTOR pathway inhibitors in the therapy of pancreatic neuroendocrine tumors. *Cancer Lett*. 335:1–8.
15. Uddin S, *et al*. (2006) Role of phosphatidylinositol 3′-kinase/AKT pathway in diffuse large B-cell lymphoma survival. *Blood*. 108:4178–86.
16. Hussain AR, *et al*. (2012) Cross-talk between NFκB and the PI3-kinase/AKT pathway can be targeted in primary effusion lymphoma (PEL) cell lines for efficient apoptosis. *PLoS One*. 7:e39945.
17. Uddin S, *et al*. (2010) Cyclooxygenase-2 inhibition inhibits PI3K/AKT kinase activity in epithelial ovarian cancer. *Int. J. Cancer* 126:382–94.
18. Wang Z, Jin W, Jin H, Wang X. (2014) mTOR in viral Hepatitis and hepatocellular carcinoma: function and treatment. *Biomed. Res. Int*. 2014:735672.
19. Inamoto T, *et al*. (2014) Favorable outcome in elderly Asian patients with metastatic renal cell carcinoma treated with everolimus: the Osaka Urologic Oncology Group. *Asian Pac. J. Cancer Prev*. 15:1811–5.
20. Jerusalem G, Rorive A, Collignon J. (2014) Use of mTOR inhibitors in the treatment of breast cancer: an evaluation of factors that influence patient outcomes. *Breast Cancer. (Dove Med Press)*. 6:43–57.
21. Anandappa G, Hollingdale A, Eisen T. (2010) Everolimus - a new approach in the treatment of renal cell carcinoma. *Cancer Manag. Res*. 2:61–70.
22. Lauring J, Park BH, Wolff AC. (2013) The phosphoinositide-3-kinase-Akt-mTOR pathway as a therapeutic target in breast cancer. *J. Natl. Compr. Canc. Netw*. 11:670–8.
23. Motzer RJ, *et al*. (2010) Phase 3 trial of everolimus for metastatic renal cell carcinoma : final results and analysis of prognostic factors. *Cancer*. 116:4256–65.
24. Motzer RJ, *et al*. (2008) Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial. *Lancet*. 372:449–56.
25. Harada K, Miyake H, Kumano M, Fujisawa M. (2013) Acquired resistance to temsirolimus in human renal cell carcinoma cells is mediated by the constitutive activation of signal transduction pathways through mTORC2. *Br. J. Cancer* 109:2389–95.
26. Liu Q, *et al*. (2011) Discovery of 9-(6-aminopyridin-3-yl)-1-(3-(trifluoromethyl)phenyl)benzo[h][1,6] naphthyridin-2(1H)-one (Torin2) as a potent, selective, and orally available mammalian target of rapamycin (mTOR) inhibitor for treatment of cancer. *J. Med. Chem*. 54:1473–80.
27. Liu Q, *et al*. (2013) Characterization of Torin2, an ATP-competitive inhibitor of mTOR, ATM, and ATR. *Cancer Res*. 73:2574–86.
28. Ahmed M, *et al*. (2014) High prevalence of mTOR complex activity can be targeted using Torin2 in papillary thyroid carcinoma. *Carcinogenesis*. 35:1564–72.
29. Kim A, Ueda Y, Naka T, Enomoto T. (2012) Therapeutic strategies in epithelial ovarian cancer. *J. Exp. Clin. Cancer Res*. 31:14.
30. Galluzzi L, *et al*. (2014) Systems biology of cisplatin resistance: past, present and future. *Cell Death Dis*. 5:e1257.
31. Ali AY, *et al*. (2012) Molecular determinants of ovarian cancer chemoresistance: new insights into an old conundrum. *Ann. N. Y. Acad. Sci*. 1271:58–67.
32. Fraser M, *et al*. (2003) Chemoresistance in human ovarian cancer: the role of apoptotic regulators. *Reprod. Biol. Endocrinol*. 1:66.
33. Bavi P, *et al*. (2006) Prevalence of fragile histidine triad expression in tumors from Saudi Arabia: a tissue microarray analysis. *Cancer Epidemiol. Biomarkers Prev*. 15:1708–18.
34. Kononen J, *et al*. (1998) Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat. Med*. 4:844–7.
35. Uddin S, *et al*. (2009) Bortezomib-mediated expression of p27Kip1 through S-phase kinase protein 2 degradation in epithelial ovarian cancer. *Lab. Invest*. 89:1115–27.
36. Chou TC, Talalay P. (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul*. 22:27–55.
37. Uddin S, *et al*. (2008) Fatty acid synthase and AKT pathway signaling in a subset of papillary thyroid cancers. *J. Clin. Endocrinol. Metab*. 93:4088–97.
38. Tasian SK, Teachey DT, Rheingold SR. (2014) Targeting the PI3K/mTOR pathway in pediatric hematologic malignancies. *Front Oncol*. 4:108.
39. Martini M, De Santis MC, Braccini L, Gulluni F, Hirsch E. (2014) PI3K/AKT signaling pathway and cancer: an updated review. *Ann. Med*. 1–12.
40. Samovski D, Kalderon B, Yehuda-Shnaidman E, Bar-Tana J. (2010) Gating of the mitochondrial permeability transition pore by long chain fatty acyl analogs in vivo. *J. Biol. Chem*. 285:6879–90.
41. Hsu YT, Youle RJ. (1997) Nonionic detergents induce dimerization among members of the Bcl-2 family. *J. Biol. Chem*. 272:13829–34.
42. Janku F, Kaseb AO, Tsimberidou AM, Wolff RA, Kurzrock R. (2014) Identification of novel therapeutic targets in the PI3K/AKT/mTOR pathway in hepatocellular carcinoma using targeted next generation sequencing. *Oncotarget*. 5:3012–22.
43. Houede N, Pourquier P. (2014) Targeting the genetic alterations of the PI3K-AKT-mTOR pathway: Its potential use in the treatment of bladder cancers. *Pharmacol. Ther*. 145:1–18.
44. Porta C, Paglino C, Mosca A. (2014) Targeting PI3K/Akt/mTOR signaling in cancer. *Front Oncol*. 4:64.
45. Rini BI, Atkins MB. (2009) Resistance to targeted therapy in renal-cell carcinoma. *Lancet Oncol*. 10:992–1000.
46. Hudes G, *et al*. (2007) Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma. *N. Engl. J. Med*. 356:2271–81.
47. Guilbert C, *et al*. (2013) Arsenic trioxide overcomes rapamycin-induced feedback activation of AKT and ERK signaling to enhance the anti-tumor effects in breast cancer. *PLoS One*. 8 e85995.
48. Saleem M, *et al*. (2013) Inhibitors of apoptotic proteins: new targets for anticancer therapy. *Chem. Biol. Drug Des*. 82:243–51.
49. Li J, Yuan J. (2008) Caspases in apoptosis and beyond. *Oncogene*. 27:6194–206.
50. Wong RR, Worley MJ Jr, Chung TK, Wong YF. (2014) An update on Mullerian-inhibiting substance: its potential application against ovarian cancer. *Endocr. Relat. Cancer*. 21:R227–33.

Cite this article as: Hussain AR, *et al*. (2015) Dual targeting of mTOR activity with Torin2 potentiates anticancer effects of cisplatin in epithelial ovarian cancer. *Mol. Med*. 21:466–78.