Dual mTORC1/2 Inhibition as a Novel Strategy for the Resensitization and Treatment of Platinum-Resistant Ovarian Cancer

Fernanda Musa1, Amandine Alard2, Gizelka David-West1, John P. Curtin1,3, Stephanie V. Blank1,3, and Robert J. Schneider2,3

Abstract

There is considerable interest in the clinical development of inhibitors of mTOR complexes mTORC1 and 2. Because mTORC1 and its downstream mRNA translation effectors may protect against genotoxic DNA damage, we investigated the inhibition of mTORC1 and mTORC1/2 in the ability to reverse platinum resistance in tissue culture and in animal tumor models of serous ovarian cancer. Cell survival, tumor growth, platinum resistance in tissue culture and in animal tumor models, responsible for over 14,000 deaths annually in the United States (1), Seventy-five percent of patients have advanced disease at presentation and face an approximate 30% 5-year survival despite surgery and chemotherapy (1). Response to platinum-based chemotherapy, the standard of care for ovarian cancer, is excellent upfront, even in high-grade serous cancer (HGSC) (reviewed in ref. 2), although most patients with HGSC will ultimately relapse, with approximately 25% of patients acquiring de novo resistance during primary treatment or relapsing within 6 months (reviewed in ref. 3). In the recurrent setting, effective options are lacking for women with platinum-resistant disease. Recent research efforts such as the Cancer Genome Atlas (TCGA) project have highlighted the need for better molecular targets to improve treatment (4–6).

Resistance to platinum-based chemotherapy is thought to involve three mechanisms: reduced drug uptake or increased export, increased DNA repair mechanisms, or alterations in pro-apoptotic mechanisms or genes (reviewed in ref. 7). A primary mechanism for platinum/carboplatin resistance is the inactivation of the p53 gene (TP53; refs. 8, 9). Analysis of the TCGA data indicates that over 95% of HGSC tumors contain mutations in TP53 (3–6). HGSC ovarian cancers also harbor a variety of extensive copy number changes, a variety of genetic alterations that disrupt homologous DNA recombination repair, and a high level of intratumoral heterogeneity in the absence of prevalent oncogenic driver mutations, apart from TP53 mutation (4, 5, 11). One notable exception, however, is the high prevalence (15%–20%) of germline or somatic mutation of the DNA repair BRCA-1 or -2 genes (reviewed in refs. 3, 5, 6) or hypermethylation (down-regulation) of the BRCA-1 transcriptional promoter (5). However, whereas BRCA-1/2-mutated ovarian cancers are generally more metastatic, they are also typically more responsive to platinum-based chemotherapy (12, 13). A recent whole-genome analysis of HGSC ovarian cancers found that platinum resistance in a subset of patients was associated with either reversion of BRCA-1 or BRCA-2 mutations or loss of BRCA-1 promoter methylation (5). Treatment failure was also associated with a higher level of

Introduction

Ovarian cancer is the leading cause of mortality in gynecologic malignancies, responsible for over 14,000 deaths annually in the United States (1). Seventy-five percent of patients have advanced disease at presentation and face an approximate 30% 5-year survival despite surgery and chemotherapy (1). Response to platinum-based chemotherapy, the standard of care for ovarian cancer, is excellent upfront, even in high-grade serous cancer (HGSC) (reviewed in ref. 2), although most patients with HGSC will ultimately relapse, with approximately 25% of patients acquiring de novo resistance during primary treatment or relapsing within 6 months (reviewed in ref. 3). In the recurrent setting, effective options are lacking for women with platinum-resistant disease. Recent research efforts such as the Cancer Genome Atlas (TCGA) project have highlighted the need for better molecular targets to improve treatment (4–6).

Resistance to platinum-based chemotherapy is thought to involve three mechanisms: reduced drug uptake or increased export, increased DNA repair mechanisms, or alterations in pro-apoptotic mechanisms or genes (reviewed in ref. 7). A primary mechanism for platinum/carboplatin resistance is the inactivation of the p53 gene (TP53; refs. 8, 9). Analysis of the TCGA data indicates that over 95% of HGSC tumors contain mutations in TP53 (3–6). HGSC ovarian cancers also harbor a variety of extensive copy number changes, a variety of genetic alterations that disrupt homologous DNA recombination repair, and a high level of intratumoral heterogeneity in the absence of prevalent oncogenic driver mutations, apart from TP53 mutation (4, 5, 11). One notable exception, however, is the high prevalence (15%–20%) of germline or somatic mutation of the DNA repair BRCA-1 or -2 genes (reviewed in refs. 3, 5, 6) or hypermethylation (down-regulation) of the BRCA-1 transcriptional promoter (5). However, whereas BRCA-1/2-mutated ovarian cancers are generally more metastatic, they are also typically more responsive to platinum-based chemotherapy (12, 13). A recent whole-genome analysis of HGSC ovarian cancers found that platinum resistance in a subset of patients was associated with either reversion of BRCA-1 or BRCA-2 mutations or loss of BRCA-1 promoter methylation (5). Treatment failure was also associated with a higher level of
long and/or more highly structured 5'UTR, and other key pathways (15). Many of these mRNAs contain "oncogenic" mRNAs that promote cell proliferation, survival, and/or cell-cycle progression in the ovarian cancer cell, resulting in selective translation of more than 70% of the protein kinase mTOR, a key regulator of cellular protein synthesis at the level of the protein kinase mTOR, a key regulator of cellular metabolism and mRNA translation. As has been seen in other solid tumors, in ovarian cancer, the PI3K/AKT/mTOR pathway is often hyperactivated and promotes translational reprogramming of the ovarian cancer cell, resulting in selective translation of more "oncogenic" mRNAs that promote cell proliferation, survival, DDR, and other key pathways (15). Many of these mRNAs contain long and/or highly structured 5' untranslatable regions (5'UTR) and/or common sequence motifs, and benefit the greatest from increased mTOR activity (16–20). In fact, the mTOR pathway is hyperactivated in approximately 70% of epithelial ovarian cancers, where it contributes to tumorigenesis and chemoresistance (21).

Cap-dependent mRNA translation is largely regulated by the complex mTORC1, consisting of mTOR, Raptor, and GβL proteins (22). mTORC1 is responsible for the phosphorylation (inactivation) of 4E-BP1, the negative regulator of cap-binding protein eIF4E. 4E-BP1 binds and blocks the activity of the translation initiation factor eIF4E by competing for interaction with translation initiation factor eIF4G, a molecular scaffold for the translation initiation machinery (23). eIF4E binds the methyl-7 GTP cap on mRNAs, recruiting the translation initiation complex consisting of eIF4G and the mRNA helicase eIF4A, which are required for mRNA translation (15, 24–27). We and others have shown that hyperactivated mTORC1 and its downstream effectors (eIF4E, eIF4G) drive the selective translation of specific oncogenic mRNAs encoding proteins responsible for survival factors (survivin, Mci1, XIAP), tumor angiogenesis (VEGF-A, FGF2), and the DDR (BRCA1, 53BP1, γH2AX, others), among other pathways (28).

Despite compelling molecular reasons for their clinical use, currently available mTORC1 rapamycin-based allosteric inhibitors (rapalogs) including everolimus, temsirolimus, and others have shown limited anticancer activity in clinical trials, marked by poor response or rapid development of tumor resistance. Moreover, rapalogs such as everolimus only weakly inhibit mTORC1, which can increase AKT activity by positive feedback regulation from loss of mTORC1 suppression of PI3K/AKT by S6K1/IRS1, and from mTORC2 itself, that is not a target of rapalog inhibition (29, 30). mTORC2 consists of mTOR, Rictor, and GβL proteins. mTORC2 acts upstream from mTORC1 to phosphorylate and activate pro-oncogenic AKT at serine 473, leading to increased cancer cell proliferation and survival (31). In addition, rapalogs only poorly block mTORC1 phosphorylation of certain downstream targets, particularly 4E-BP1 (32, 33), and thus, have limited activity as translation downregulators. Moreover, many cancers have intrinsic or acquired resistance to rapalogs, in many cases paralleling the acquisition of resistance to genotoxic therapies (34, 35). Ultimately, rapalog resistance reflects molecular and biochemical pathway alterations that maintain cancer cell proliferative pathways that are also targets of genotoxic therapies. The recent development of catalytic (ATP active site) mTOR inhibitors capable of blocking both mTORC1 and mTORC2 activity has stimulated interest in this pathway as a means of bypassing mechanisms of rapalog resistance and possibly increasing chemotherapeutic sensitivity. Emerging data suggest that these compounds efficiently inhibit the feedback upregulation of AKT by mTORC2, and are also superior inhibitors of mTORC1 activity and its downstream inactivation of 4E-BP1 (32). In this regard, while the anticancer cell and anti-cell proliferation effects of mTORC1 inhibition involve the downregulation of protein synthesis, possibly selectively, mTORC1 also impacts on other key pathways and proteins that regulate cell proliferation and survival. These include mTOR regulation by phosphorylation of the autophagy pathway (36) through the ULK1–mAtg13–FIP200 complex (37), the growth factor receptor bound protein 10 (Grb10) protein, which blocks growth factor mitogenic signaling (38, 39), and the phosphorylation of mRNA-binding proteins such as LARP1 that regulates cell division (40), among others. Consequently, if chemotherapy resistance can be reversed through mTOR inhibition, it might or might not directly involve mTORC1 phosphorylation of protein synthesis effectors S6K and 4E-BP1.

We therefore investigated the use of mTORC1-only and mTORC1/2 dual inhibitors in reversing platinum-resistant ovarian cancer and the role of selective cap-dependent mRNA translation. Using tissue culture and animal tumor models of high-grade platinum-resistant serous ovarian cancer, we demonstrate that only mTORC1/2 inhibition significantly restores platinum sensitivity, which is associated with both inhibition of activating AKT phosphorylation and downregulation of cap-dependent mRNA translation of key proteins involved in survival and DDR pathways. This results in reduced cell proliferation and cell survival responses, platinum resensitization, increased tumor cell killing, improved tumor control, reduced metastasis, and increased survival in an animal model. Several key mRNAs are involved in these responses and are sensitive to mTORC1/2 inhibition, including CHK1 and BRCA-1. As hyperactivation of mTOR is an integrative step between several oncogenic pathways (AKT, MAPK, RAS, and others) that are commonly altered in ovarian cancer, we believe there is rationale to pursue mTORC1/2 inhibition as a novel therapeutic target for this disease.

Materials and Methods

Cell lines

Platinum-resistant OVCAR-3 cells were obtained from the ATCC. OVCAR-3 cells were authenticated by the ATCC using short tandem repeat (STR) profiling and used within 6 months after resuscitation. OVCAR-3 cells were derived from a patient with a platinum-resistant recurrence of high-grade serous ovarian cancer and shown to have acquired increased resistance to platinum drugs (41) and at dose levels similar to those used here (42).
An OVCAR-3 cell line engineered to express GFP and Firefly luciferase (FLuc) genes by retroviral transduction was kindly provided by Dr. Renier Brentjens (Memorial Sloan Kettering Cancer Center, New York, NY, ref. 43). Cells were isolated by FACS and sorted for high GFP expression and firefly luciferase activity to enable animal studies using bioluminescent imaging (IVIS Lumina II). These cells were not independently validated by us. OVCAR-3 cells were cultured at 37°C in 5% CO₂ in RPMI1640 supplemented with 20% FBS, penicillin/streptomycin, amphotericin B, and plasmidum prophylaxis.

Platinum-resistant SKOV-3 cells were obtained from the ATCC. SKOV-3 cells were authenticated by the ATCC using STR profiling and used within 6 months after resuscitation. SKOV-3 cells were originally derived from the ascites of a patient with platinum-resistant epithelial ovarian cancer and shown to have acquired increased resistance to platinum drugs at dose levels similar to those used here (44, 45). SKOV-3 cells were cultured at 37°C in 5% CO₂ in McCoy 5a medium modified (ATCC cat. #30-3007) supplemented with 10% FBS, penicillin/streptomycin, amphotericin B, and plasmidum prophylaxis.

Cells were routinely tested and found to be mycoplasma free. Prior to cell culture and tumor growth into animals, cells were cultured in media enriched with FBS but free of antibiotics, antifungals, or other additives. Cells were tested for mycoplasma every 2 months and found to be free of infection.

**Drugs**

PP242 (Chemdeca) is a dual mTORC1/2 inhibitor. PP242 is highly selective for both mTOR complexes, and from 10-to 100-fold more specific than for other members of the P38K family. At dose levels used here, PP242 does not significantly target kinases other than mTORC1/2 (46). Everolimus (RAD001, BioTang) is an allosteric mTORC1 inhibitor. For tissue culture studies, PP242 and everolimus were diluted into 10% DMSO to the indicated concentrations. For animal experiments, PP242 was suspended in 15% polyvinyl-pyrollidone (PVP) + 5% N-methylpyrrolidone. Everolimus was suspended in PBS with 10% DMSO. Control groups in tissue culture experiments were treated with PBS + 10% DMSO. For animal studies, control groups were treated with sterile pH 7.2 buffered water in 15% PVP-DMSO. Carboplatin was chosen as the DNA-damaging agent given its conventional use in the treatment of ovarian cancer and favorable toxicity profile. Carboplatin was used at a stock dose of 10 mg/mL diluted in PBS to the desired concentration.

**Clonogenic assays**

Cells (100,000) were plated into 6-well plates and allowed to settle over 24 hours, studies were repeated five times. Cells were treated as follows: (i) PP242 alone, (ii) everolimus alone, (iii) carboplatin alone, (iv) PP242 and carboplatin, (v) everolimus and carboplatin, (vi) control. Treatment included PP242 or everolimus over 24 hours versus control (PBS + 10% DMSO). At the end of 24 hours, carboplatin-treated groups were treated for 5 hours with carboplatin, and control groups were mock treated for 5 hours with PBS. After treatment, cells were washed thoroughly with PBS and the media replaced, then incubated for 10 days during which the media were changed every 3 days. Cells were fixed and stained with crystal violet dye, and colonies counted by light microscopy from 6 randomly chosen fields twice and averaged. Colony diameters were assessed using ImageJ64 software as described previously (47).

**Dose finding and growth inhibitory 50% (GI₅₀) experiments**

Cells were plated into 96-well plates and treated with increasing doses of PP242, everolimus, and carboplatin as single agents (Supplementary Fig. S1). Cells were treated for 24 hours with everolimus or PP242, or 5 hours with carboplatin. After 72 hours, cells were harvested and growth quantified using MTT assays as described by the manufacturer (Promega #G4000). GI₅₀ doses were established from these data as follows: tissue culture: PP242, 2.5 μmol/L; everolimus, 20 nmol/L; and carboplatin, 1.0 μmol/L. The same doses were maintained for treatment combinations.

**Immunoblotting**

For immunoblotting, the following antibodies from Cell Signaling Technology were used unless otherwise indicated: rabbit anti-S6K (#2217), rabbit anti-P-S6K (Thr389, #2211), rabbit anti-4E-BP1 (#9452), rabbit anti-P-4E-BP1 (Ser65, #9451), rabbit anti-AKT (#2272), rabbit anti-P-AKT (Ser473, #9271), all used at 1:1,000. For DNA repair targets: mouse anti-CHK1 (#2360), rabbit anti-P-CHK1 (Ser345, #2348), rabbit anti-CHK2 (#2662), rabbit anti-P-CHK2 (Thr68, #2661), rabbit anti-BRCA1 (#9010), rabbit anti-P-BRCA1 (Ser1524, #9009), mouse anti-ATM (#2873), mouse anti-ATM (Ser1981, #4526), rabbit anti-ATR (#2790), rabbit anti-mTOR (#2983), rabbit anti-p-mTOR (Ser2448, #2448) rabbit anti-P-TOR (Ser248, #2853), and anti-PARP (#9542). Loading controls were mouse anti-GAPDH (#2118) and mouse anti-β tubulin (#2146). Other targets were survivin (#2802) and caspase 3 (#9662).

After treatments of tissue culture cells, cells were washed twice in ice-cold PBS and lysed in 0.5% NP-40 lysis buffer at 4°C or 0.5% SDS lysis buffer. NP-40 lysates were clarified by centrifugation at 13,000 x g for 10 minutes and protein concentrations determined by Bradford method (Bio-Rad). To determine the total levels and phosphorylation status of specific proteins, equal amounts of protein were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). The phosphorylation status of proteins was determined either by immunoblotting the membrane first with P-specific antibody then stripping the membranes using Restore Western blot stripping buffer (Pierce), followed by reprobing the membranes with non-P-specific antibodies, or by running two separate gels. Tumor immunoblotting was conducted as above by combining two tumors for each condition surgically removed at day 14 after treatment and soluble proteins extracted using 0.5% SDS lysis buffer. Equal protein amounts were used for SDS-PAGE.

**Immunofluorescence**

Cells were grown and treated on chamber slides, fixed, and immunostained for anti-53BP1 (Abcam, ab36823) and γH2AX (Cell Signaling Technology, #9718). Secondary antibodies were conjugated with fluorescent marker (FITC, Jackson) for 1 hour. After washing, slides were allowed to air dry. Fluorescent foci were counted from 6 fields chosen at random as a surrogate measure of DNA double-strand breaks.

**Assessment of mRNA levels by qRT-PCR**

Forward and reverse primers were designed to detect the following mRNAs: 4E-BP1, AKT, BRCA1, BRCA2, ATM, ATR, CHK1, CHK2, rps6, GAPDH (control). RNA was extracted from cells treated as follows: (i) PP242 alone, (ii) everolimus alone, (iii) carboplatin alone, (iv) PP242 and carboplatin, (v) everolimus and carboplatin. cDNA was synthesized from the extracted
and quantified sample of RNA (GoScript, Promega). Real-time PCR was performed in triplicate using SYBR Green (Life Technologies) on a 7500 Fast Dx-RT-PCR Instrument (Applied Biosystems). The ΔΔCt was calculated using the 7500 software and the data were further normalized to control (GAPDH). Fold change values relative to the GAPDH control were calculated and reported as graphs designed on GraphPad Prism.

Metabolic protein labeling and protein synthesis rates

The effect of PP242 on mRNA translation was assessed using [35S]-methionine metabolic labeling into nascent proteins. OVCAR-3 cells were cultured and treated for 24 hours with: (i) DMSO control; (ii) carboplatin at 10 μmol/L; (iii) PP242 at 2.5 μmol/L; and (iv) carboplatin and PP242 in combination. After treatment, cells were labeled with 25 μCi of [35S]-methionine/cysteine/mL (EasyTag Express Protein Labeling Mix, Perkin Elmer) in Me/Cys-free DMEM supplemented with gentamicin at 0.04 mg/mL, 5% FBS, and bovine insulin at 0.01 mg/mL and incubated at 37°C for 30 minutes. Lysates were prepared using NP-40 buffer and specific activity of [35S]-methionine/cysteine incorporation into nascent protein was determined by trichloroacetic acid (TCA) precipitation onto GF/C filters and liquid scintillation counting. Studies were repeated three times and data were presented as means, normalized to the control, with SEM.

Intraperitoneal animal model

All studies were approved by the NYU School of Medicine Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with IACUC guidelines. Female SCID/scid mice aged 5 to 6 weeks (Taconic Farms, Inc.) were implanted intraperitoneally with 6 × 106 cells (OVCAR-3 expressing firefly luciferase and GFP). Tumor growth was assayed by bioluminescent imaging (IVIS Lumina II) every 2 weeks until tumor size reached the defined region of interest (ROI) of the entire mouse abdomen was approximately 105 photons/second. Treatment allocation was made randomly in groups of 10: (i) PP242, (ii) everolimus, (iii) carboplatin, (iv) PP242 and carboplatin, (v) everolimus and carboplatin, and (vi) control vehicle. As the GI50 responses of OVCAR-3 and SKOV cells were within the dosing range of many other cell lines, for animal studies, established dosing regimens were used consistent with the human equivalent drug and doses were used across treatment groups using nonparametric ANOVA methods. Paired tests used Student t tests. Statistical analyses were performed on SPSS and GraphPad Prism. Median survival was calculated for each treatment according to the Kaplan–Meier method (SPSS version 21).

Results

Proliferation of carboplatin-resistant ovarian cancer cells is only modestly inhibited by long-term or short-term carboplatin treatment with mTORC1 or mTORC1/2 inhibition

We first determined the GI50 for OVCAR-3 cells using an MTT assay for single-agent everolimus, PP242, and carboplatin (Supplementary Fig. S1). The values established are in keeping with the literature for OVCAR-3 cells (PP242, 2.5 μmol/L; everolimus, 20 nmol/L; carboplatin, 1.0 μmol/L; e.g., 49, 50–52). Using the GI50 dose level for each agent, as well as a low dose of carboplatin (1.0 μmol/L) that does not affect cell proliferation or survival, we conducted a single-agent dose response 10-day clonogenic cell survival analysis. Carboplatin at subphysiologic (1.0 μmol/L) levels also established a baseline for combinatorial testing to determine whether synergistic cell killing can be established. Clonogenic analysis utilizes cell survival over an extended period time, providing a more relevant and biologically functional determination of cell proliferation and survival. Cells were treated with each agent for 24 hours prior to plating. Viable cell colonies ≥40 cells in size were counted by light microscopy and total colony counts compared across all treatment groups. Inhibition of mTORC1 (everolimus) and mTORC1/2 (PP242) showed similar single-agent inhibition of cell proliferation and colony size at the physiologic GI50 doses as first established by MTT assay (Fig. 1A).

We next determined whether the failure to observe a significant difference between inhibition of mTORC1 and mTORC1/2 in resensitization to carboplatin in resistant ovarian cancer cells is a result of toxicity from long-term (24-hour) exposure to carboplatin. Clonogenic 10-day cell survival studies were performed as above but after 24-hour exposure to mTOR inhibitors, with only 5-hour carboplatin treatment. Results are shown for the platinum-resistant ovarian cancer cell lines OVCAR-3 (Fig. 1B) and SKOV-3 (Supplementary Fig. S2). Again there was a significant decrease in both colony counts for both cell lines with carboplatin at sub-GI50 levels (1 μmol/L) when combined with PP242 or everolimus, but...
similar to carboplatin alone at its GI50 level (10 \mu mol/L). To account for cytostatic cell growth–inhibitory effects of mTOR inhibitors that may not necessarily lead to a decrease in colony number, the average colony diameter in two dimensions was estimated using ImageJ software to develop a colony burden score, calculated by multiplying colony counts by colony diameter (47). Inhibition of mTORC2 provided no additional decrease in colony diameter per 40-cell number compared with inhibition of mTORC1 only (Fig. 1B). The similar decrease in colony counts and average colony diameter score for both mTORC1 (everolimus) and mTORC1/2 (PP242) inhibition is understandable given the fact that mTORC1 inhibition by either agent impairs cell proliferation, which would be expected to have a much greater impact on colony size (dominated by cell number) than inhibition of cell growth (size) by mTORC2 inhibition (19). In summary, the antiproliferative effects of the mTOR inhibitors on platinum-resistant OVCAR-3 cells by clonogenic assay were modest and just slightly less than single-agent carboplatin at its GI50 dose.

Catalytic active site inhibition of mTORC1 is a more effective inhibitor of 4E-BP1 activation than allosteric everolimus

Only the catalytic (PP242) mTOR inhibitor was associated with decreased target ribosomal S6K (S6K) and 4E-BP1 protein phosphorylation at GI50 dose levels (2.5 \mu mol/L PP242) with no change in total protein levels (Fig. 2A). At the GI50 dose level (20 nmol/L), everolimus did not reduce phosphorylation of S6K or 4E-BP1 but did so at 2.5 times the GI50 (50 nmol/L), suggesting that OVCAR-3 cells are either intrinsically resistant or have acquired resistance to rapalogs coincident with resistance to carboplatin. It is not surprising that inhibition of cell proliferation by everolimus can be uncoupled from mTORC1 phosphorylation of S6K and 4E-BP1 targets. Studies have shown that rapalog resistance can arise in a number of ways,
downregulate activating phosphorylation of mTOR, whereas high doses (50 nmol/L) of everolimus were also necessary to highly resistant to everolimus, but not to PP242 (Fig. 2A). Very characteristic phosphorylation of AKT in cancer cells was also transformation cells lose responsiveness to rapalog inhibition. This is consistent with the observation that with increased FKBP51, which regulates proproliferative AKT activity (55). Which confer differential mTOR and other target specificity, which confer differential mTOR and other target specificity (54). Among these, for example, is the control of the PHLPP by FKBP51, which regulates proproliferative AKT activity (55). This is consistent with the observation that with increased transformation cells lose responsiveness to rapalog inhibition of S6K phosphorylation but not cell-cycle inhibition (56). The characteristic phosphorylation of AKT in cancer cells was also highly resistant to everolimus, but not to PP242 (Fig. 2A). Very high doses (50 nmol/L) of everolimus were also necessary to downregulate activating phosphorylation of mTOR, whereas GI50 levels of PP242 (2.5 μm) were sufficient. At 2.5 times the GI50 levels of everolimus, there was only modest evidence for cell killing, indicated by PARP cleavage, compared with catalytic mTOR inhibition. Consistent with the resistance to everolimus, OVCAR-3 cells were only very slightly reduced in protein synthesis (~10%) by GI50 levels of everolimus, but more strongly impaired (~30%) by PP242, regardless of carboplatin treatment (Fig. 2B). Carboplatin treatment alone had no effect on mTOR signaling or protein synthesis. These findings indicate that catalytic inhibition of mTOR is more effective than allosteric inhibition in reducing mTOR signaling and protein synthesis, yet in tissue culture there is no gain in ovarian cancer cell killing or resensitization to carboplatin by mTORC1/2 inhibition.

Inhibition of mTORC1/2 is superior to mTORC1 in treatment and control of platinum-resistant ovarian cancer in a xenotransplant model

We therefore examined the ability of allosteric mTORC1 and catalytic mTORC1/2 inhibitors to control ovarian tumor development and metastasis in a xenotransplant model of OVCAR-3 cells. We sought to determine whether the ability to block both mTORC1 and mTORC2 as well as or in addition to a greater ability to block 4E-BP1 might provide for resensitization to carboplatin and decreased tumor growth not observable in cell culture studies. Mice were injected intraperitoneally (i.p.) with OVCAR-3 cells that were previously engineered to constitutively express firefly luciferase and a GFP reporter to enable bioluminescence tracking (43) and imaged once a week. A strong metastatic signal could be seen by bioluminescence imaging by 80 days (Fig. 3A). Using Living Image software, the flux (photons/second) produced by tumor cell luciferase was calculated across the entire mouse. When the flux approximated 10^8 photons/second, mice were randomly allocated into treatment groups, at which point treatment was initiated. Treatment arms consisted of: (i) PP242, 100 mg/kg daily by oral gavage; (ii) everolimus, 2.5 mg/kg daily by oral gavage; (iii) carboplatin, 30 mg/kg every 4 days, i.p.; (iv) PP242 and carboplatin at same doses; (v) everolimus and carboplatin at same doses; or (vi) control vehicle. Bioluminescence imaging data taken at day 8 after treatment and day 28 (Fig. 3A and B) showed high tumor burden and significant metastasis for animals in the everolimus and carboplatin treatment groups, and a slight improvement by PP242 alone. Combined treatment of carboplatin and everolimus (mTORC1 inhibition) was marginally better than carboplatin alone, whereas carboplatin and PP242 (mTORC1/2 inhibition) demonstrated superior tumor control and significantly reduced metastasis compared with other treatments. To analyze the target specificity of treatments, two animals from each group were chosen at random at day 14 after treatment, tumors removed, combined, and extracted proteins analyzed by immunoblotting (Fig. 3C). Only the group treated with mTORC1/2 inhibitor PP242 demonstrated strong inhibition of P-S6K and 4E-BP1 phosphorylation (the latter by electrophoretic mobility). Moreover, inhibition of mTORC1/2 by PP242 but not mTORC1 inhibition by everolimus blocked activating AKT phosphorylation, which can promote tumor progression. Survival of animals in each treatment arm was determined over a period of 50 days of treatment. Superior survival was observed for the carboplatin and PP242 group, followed by PP242 alone and carboplatin alone.
Mice in the carboplatin plus PP242 (mTORC1/2 inhibition) group showed a near doubling in median survival compared with vehicle alone, and three times better than everolimus plus carboplatin (Fig. 3E, \( P < 0.001 \)). The surprising worsening of survival mice in the everolimus plus carboplatin group (Fig. 3C and D) may be due to increased toxicity by this combination, and/or the inability of the rapalog to counter activating phosphorylation of pro-oncogenic AKT. The poor survival of animals in both the everolimus, and everolimus plus carboplatin arms is consistent with this possibility, demonstrating an increase in metastatic activity despite treatment, which is also evident in the bioluminescence results (Fig. 3A). We can conclude...
that mTORC1/2 inhibition is superior to mTORC1 inhibition when combined with carboplatin in a model of platinum-resistant ovarian cancer. We therefore examined whether in addition to inhibition of AKT activity, superior platinum resensitization, increased tumor control and superior inhibition of metastasis by mTORC1/2 blockade also involves selective suppression of mRNA translation that cannot be achieved by everolimus.

**Catalytic but not allosteric mTOR inhibition prevents translation of DDR, cell proliferation, and cell survival mRNAs**

We previously showed that high levels of mTORC1 activation and eIF4G1, a target and effector of mTORC1, promote the selectively increased translation of DDR and cell-cycle control mRNAs (28). We therefore examined the expression of proteins involved in the DDR and cell proliferation responses, including ATM, ATR, CHK1, CHK2, BRCA-1, BRCA-2, as well as AKT, rS6K, and their phosphorylated forms. We found that in addition to a previously observed strong decrease in phosphorylated (active) AKT, and phosphorylated (inactive) 4E-BP1 by treatment with the dual mTORC1/2 inhibitor, levels of key mediators of DNA repair responses were strongly reduced in platinum-resistant OVCAR-3 cells (Fig. 4A). These included total and phosphorylated forms of CHK1, ATR, and BRCA-1. There were no significant changes in the other proteins tested. Everolimus alone or in combination with carboplatin had no such effect (Fig. 4B and C). Of note, the substantial increase in BRCA-1 protein in response to carboplatin-induced DNA damage was blocked by dual mTORC1/2 inhibition with PP242, but not by mTORC1 only inhibition by everolimus (Fig. 4C). Corresponding mRNA levels were assessed by qRT-PCR for 4E-BP1, AKT, BRCA-1, BRCA-2, ATM, ATR, CHK1, CHK2, rS6K, and GAPDH (control; Supplementary Fig. S3). There were no reductions or only very minor reduction in mRNA levels relative to control for all except 4E-BP1, which was decreased by half with mTORC1/2 inhibition, and BRCA-1, which was reduced by approximately 20% (Supplementary Fig. S3). Notably, reduced levels of 4E-BP1 protein with mTORC1/2 inhibition is part of a described homeostatic mechanism for eIF4E/4E-BP1 protein interaction that increases eIF4E availability (57). We therefore sought evidence for reduced levels of CHK1, ATR, and BRCA-1 proteins in tumors of animals treated with mTORC1/2 or mTORC1 inhibitors. Tumor lysates corresponding to those shown in Fig. 3C were probed for CHK1, ATR, and BRCA-1 protein levels (Fig. 4D). Decreased levels were observed only in mTORC1/2 inhibited (PP242) and not in mTORC1-only inhibited (everolimus) tumors. While the downregulation of protein levels was not as strong as in tissue culture cells, although still substantial, this is possibly due to more limited drug exposure in animals. The reduction in CHK1, ATR, and BRCA-1 protein levels with mTORC1/2 but not mTORC1 inhibition, in the absence of equal reduction in mRNA levels, is particularly striking and indicative of translation inhibition. We note that the CHK1 mRNA contains a very long (~800 nt) and highly structured 5′ untranslated region (5′UTR, AG > -200 kcal/mol),
We observed much higher accumulation compared with carboplatin alone at GI 50 levels (Fig. 5A).

Figure 5.
Immunofluorescence analysis of double-strand DNA (dsDNA) damage response in platinum-resistant OVCAR-3 cells with mTORC1/2 inhibition and/or treatment with carboplatin. A, cells were grown and treated on chamber slides, in platinum-resistant OVCAR-3 cells with mTORC1/2 inhibition and/or treatment with carboplatin. A, cells were grown and treated on chamber slides, fixed, and immunostained for anti-53BP1 and H2AX, then stained with secondary antibodies conjugated to FITC. Fluorescent foci were counted from 6 fields chosen at random under 63× power. Representative images from three independent studies of untreated, carboplatin (Carbo)-treated, and PP242 plus carboplatin-treated cells are shown. B, quantification of 6 fields for DNA damage foci. Cells with >3 foci per cell (fpc) were scored. *, P < 0.001, by t test. C, sub-G0–G1 cell population from cell-cycle distribution analysis. Cells treated as in B were labeled with PI and subjected to flow cytometry to quantify G1 population, representative of dying cells with DNA fragmentation. The results of three studies were averaged = SEM. *, P < 0.001, by t test. Everol, everolimus.

Discussion
The mTOR pathway is a multifaceted integrator of extracellular signaling that regulates cell growth, proliferation, angiogenesis, and the metabolism of cancer cells (22). There is considerable evidence that the hyperactivation of mTOR signaling and the resulting aberrant upregulation of mRNA translation, both overall and selective, are critical for oncogenic processes in ovarian and other cancers (15, 27). Our work suggests that selectively increased translation of specific mRNAs due to mTOR hyperactivation also plays an important role in platinum resistance in ovarian cancer. Thus, targeting the mTOR-selective mRNA translation pathway may be an attractive novel strategy for therapy of platinum-resistant ovarian cancer.

Treatment of platinum-resistant OVCAR-3 cells with a dual mTORC1/2 inhibitor resulted in strong inhibition of cancer cell proliferation, improved tumor control, strikingly reduced metastasis, and significantly increased animal median survival. We were surprised by the exquisite sensitivity of ovarian cancer cells to mTORC1/2 inhibition in tumors, a reflection of the hyperactivated mTORC1 and mTORC2 state. In a growth factor–deprived state, hypophosphorylated 4E-BP1 is tightly bound to eIF4E, inhibiting from 15%–40% of cap-dependent mRNA translation. Published work from our group and others has demonstrated that hyperactivated mTORC1 drives the increased selective translation of specific mRNAs involved in the oncogenic process (15, 25–28, 58, 59). These include mRNAs encoding proteins responsible for cell survival (survivin, Mcl1, XIAP), tumor angiogenesis (VEGF-A, FGF2), and the DNA repair response (BRCA1, 53BP1, H2AX, H2AX) among others, in concert with an increase in the levels of mTOR and/or the activity of mTOR-targeted translation factors.

Platinum-resistant OVCAR-3 cells and tumors in animals treated with carboplatin and a dual mTORC1/2 inhibitor results in a notable increase in tumor cell killing, inhibition of cell proliferation, reduced metastasis, increased survival, and reduced mTORC1/2 and AKT activity (determined by surrogate phosphorylation), indicating a partial resequestration to platinum chemotherapy. In addition to the expected decrease in phosphorylation of 4E-BP1, S6K, and AKT with mTORC1/2 inhibition, we unexpectedly found decreased levels of CHK1, ATR, and BRCA1 proteins, consistent with the selective reduction observed in DDR pathway mRNA translation. It is likely that the reduction in activating AKT phosphorylation, in concert with the higher level of selective translational downregulation of DDR mRNAs, are in part responsible for the enhanced effects seen in tumors with mTORC1/2 inhibition compared with inhibition of mTORC1, and the reason that this was not detectable in 10-day clonogenic cell survival studies. The extended duration of tumor growth and treatment, extending for several months in animals compared with a single treatment in 10-day cell cultures, captures the increased need for higher level translation of these mRNAs and the impact of downregulation of AKT activity.

Multiple TCGA studies have now analyzed the exome of more than 500 epithelial ovarian tumors (4–6, 10). Other than a predominance of p53 mutations, there are no major other
mutations or mRNA expression profiles that have emerged to indicate a "typical" molecular signature of high-grade serous ovarian cancer, the conclusion being that ovarian cancer is very heterogeneous. Mutations (somatic or germline) found across many signaling and biochemical pathways leads to changes in ovarian cancer cell DNA repair capacity, tumor angiogenesis, and metastatic potential. However, mutational and gene expression analyses do not capture the vast posttranscriptional and translational alterations that are also involved in ovarian cancer growth and drug resistance. It is not a coincidence that most major oncogenic signaling pathways that are involved in human cancers, including ovarian cancer, converge to hyperactivate mTOR, a major regulator of mRNA translation, responsible for the increased synthesis of proteins directly involved in tumor cell growth, angiogenesis, and survival (15, 27). Analysis of alterations in DNA repair pathways of ovarian tumors in the TCGA reveals that these alterations in BRCA-1/2 genes (4–6, 10), as noted previously. Moreover, if they did, BRCA-1/2 mutations actually predispose to increased sensitivity to platinum-based therapy. Collectively, these data support the conclusion that there is an over-reliance of ovarian cancers on increased DDR pathway activity, which may partly explain the initial sensitivity to killing by platinum compounds. It also suggests the importance of incorporating an understanding of postmutational and posttranscriptional mechanisms such as mTOR hyperactivation, selective mRNA translation, and inhibition of AKT activity in overcoming platinum resistance. This is supported by our work. Specifically, ovarian cancers often harbor activating mutations in PIK3R1, PIK3C2G, and the MAPK pathway, which may be involved in platinum resistance (and are present in OVCAR-3 and SKOV-3 cells; ref. 60). These pathways hyperactivate mTOR and increase both overall and selective mRNA translation (15) and may inform a strategic direction in the development of new therapeutic approaches for this disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: F. Musa, A. Alard, R.J. Schneider
Development of methodology: F. Musa, A. Alard, R.J. Schneider
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Musa, A. Alard, G. David-West
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Musa, G. David-West, S.V. Blank, R.J. Schneider
Writing, review, and/or revision of the manuscript: F. Musa, J.P. Curtin, S.V. Blank, R.J. Schneider
Study supervision: J.P. Curtin, S.V. Blank, R.J. Schneider

Acknowledgments
The authors thank Dr. Renier Brentjens (Memorial Sloan Kettering Cancer Center, New York, NY) for the GFP/Firefly luciferase OVCAR-3 cells and B. Dinardo and R. Aziz for additional studies.

Grant Support
This work was supported by grants from the U.S. Department of Health and Human Services, NIH R01CA136593 (to S.V. Blank, R.J. Schneider); Avon Foundation for Women 02-2014-075 (to R.J. Schneider); U.S. Department of Health and Human Services, NIH, and National Center for Advancing Translational Sciences (NCATS) TR000036 (to B. Cronstein).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 10, 2015; revised April 12, 2016; accepted April 23, 2016; published online First May 16, 2016.

References
20. Shatsky IN, Dmitriev SE, Andreev DE, Terenin IM. Transcriptome-wide computational analysis:...
Molecular Cancer Therapeutics

Dual mTORC1/2 Inhibition as a Novel Strategy for the Resensitization and Treatment of Platinum-Resistant Ovarian Cancer

Fernanda Musa, Amandine Alard, Gizelka David-West, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-15-0926

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2016/05/14/1535-7163.MCT-15-0926.DC1

Cited articles
This article cites 60 articles, 24 of which you can access for free at:
http://mct.aacrjournals.org/content/15/7/1557.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.