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Dual mTORC1/2 inhibition in a preclinical xenograft tumor model of endometrial cancer☆

Sharmilee Bansal Korets [a], Fernanda Musa [a], John Curtin [a,b], Stephanie V. Blank [a,b], Robert J. Schneider [b,*]

[a] Department Obstetrics and Gynecology, NYU Langone Medical Center, New York, NY 10016, USA
[b] NYU Cancer Institute, NYU School of Medicine, New York, NY 10016, USA

HIGHLIGHTS

- Catalytic mTORC1/2 inhibition demonstrates clear efficacy over rapamycin approaches in endometrial tumor models.
- Targeting mTORC1/2 inhibits Akt activation in endometrial tumors, aiding tumor control.
- mTORC1/2 kinase inhibitors warrant further investigation as a treatment for endometrial cancer.

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ABSTRACT

Objectives. Up to 70% of endometrioid endometrial cancers carry PTEN gene deletions that can upregulate mTOR activity. Investigational mTOR kinase inhibitors may provide a novel therapeutic approach for these tumors. Using a xenograft tumor model of endometrial cancer, we assessed the activity of mTOR and downstream effector proteins in the mTOR translational control pathway after treatment with a dual mTOR complex 1 and 2 (mTORC1/2) catalytic inhibitor (PP242) compared to that of an allosteric mTOR complex 1 (mTORC1) inhibitor (everolimus, RAD001).

Methods. Grade 3 endometrioid endometrial cancer cells (AN3CA) were xenografted into nude mice. Animals were treated with PP242, PP242 and carboplatin, carboplatin, RAD001, and RAD001 and carboplatin. Mean tumor volume was compared across groups by ANOVA. Immunoblot analysis was performed to assess mTORC1/2 activity using P-Akt, P-S6 and P-4E-BP1.

Results. The mean tumor volume of PP242 + carboplatin was significantly lower than in all other treatment groups, $P < 0.001$ (89% smaller). The RAD001 + carboplatin group was also smaller, but this did not reach statistical significance ($P = 0.097$). Immunoblot analysis of tumor lysates treated with PP242 demonstrated inhibition of activated P-Akt.

Conclusions. Catalytic mTORC1/2 inhibition demonstrates clear efficacy in tumor growth control that is enhanced by the addition of a DNA damage agent, carboplatin. Targeting mTORC1/2 leads to inhibition of Akt activation and strong downregulation of effectors of mTORC1, resulting in downregulation of protein synthesis. Based on this study, mTORC1/2 kinase inhibitors warrant further investigation as a potential treatment for endometrial cancer.

Background

Endometrial cancer is the most common gynecologic malignancy, with almost 50,000 new diagnoses and more than 8,000 deaths estimated to occur in the United States in 2013 [1]. While most women with endometrial cancer are diagnosed at an early stage owing to symptoms of irregular bleeding, approximately 15% of diagnoses are made at stages III or IV, with five-year survival rates ranging from 20 to 50%. Patients with advanced or recurrent disease have limited treatment options. Despite many recent advances in cancer therapy, there has been little improvement in survival for this patient population over the past 30 years [2]. Current treatment standards include surgical cytoreduction followed by adjuvant chemotherapy and/or radiation, with a possible addition of hormonal therapy. In recurrent disease, a variety of cytotoxic chemotherapy agents with or without radiation can be used for systemic or local disease control. Few, if any, of the treatments presently regarded as "standard of care" exploit known molecular alterations common to endometrial cancer as a target for therapy [2,3], with the exception of hormonal therapy. However, hormonal agents tend to have limited efficacy in poorly differentiated cancers, which comprise the majority of advanced and recurrent cases [2,3].

☆ All authors declare no conflicts of interest.

* Corresponding author at: NYU School of Medicine, Alexandria Center for Life Sciences, 450 East 29th street, 3rd floor, New York, NY 10016, USA. Fax: +1 646 501 4541.
E-mail address: Robert.schneider@nyumc.org (R.J. Schneider).

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Most endometrial cancers are of endometrioid histology [4]. Endometrioid and non-endometrioid endometrial cancers have distinct molecular alterations that provide potential new therapeutic targets [2,3]. Up to 83% of endometrioid endometrial cancers have mutations in the tumor suppressor phosphatase and tensin homologue (PTEN) pathway [4], making proteins in this pathway natural targets in the treatment of these cancers. The protein phosphatase encoded by the PTEN gene has multiple anti-cancer activities. It maintains cell cycle arrest at the G1/S checkpoint, upregulates pro-apoptotic pathways controlled by the protein kinase Akt, and downregulates pro-survival anti-apoptotic pathways. When functioning normally, PTEN also prevents focal adhesion formation and cell spread, and serves as an inhibitor of mTOR pathway activation [5]. Therefore, loss of normal PTEN function results in aberrant cell proliferation, apoptotic escape, and abnormal cell spread [6], as well as increased mTOR activation [7–9]. This increase in mTOR activation subsequently increases protein synthesis necessary to sustain these aberrant, pro-proliferative activities in endometrial cancer [5]. In molecular terms, loss of PTEN activity results in increased phosphorylated and activated Akt, which can hyper-activate mTOR and stimulate mRNA translation. This in turn results in an overall moderate increase in protein synthesis, and a selective larger increase in the translation of angiogenic, DNA damage and repair, survival and pro-proliferative mRNAs [10]. Thus, restoring normalcy to the mTOR pathway, which is upregulated or hyperactivated in many endometrioid endometrial cancers, represents an attractive molecular target for treatment.

mTOR forms two protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [11]. mTORC1 directly regulates mRNA translation. Rapalogs such as sirolimus and temsirolimus are allosteric mTOR inhibitors that block only mTORC1 activity. Rapalogs have been shown to have activity in endometrial cancer in both cell culture [9,12,13] and clinical studies [14–16]. However, rapalogs as anti-cancer agents represent a compromised approach. They only weakly inhibit mTORC1, and they have been linked to increased Akt activity by positive feedback regulation from loss of mTORC1 suppression of PI3K/Akt by IRS1, and from mTORC2 itself [14,15]. Thus, suppression of only mTORC1 may ultimately upregulate cancer cell proliferative pathways. An agent that dually inhibits both mTORC1 and 2, such as a catalytic mTOR inhibitor, would theoretically result in improved anti-tumor activity compared to a rapalog [5].

Our study was designed to evaluate the anti-tumor activity of a catalytic mTORC1/2 inhibitor (PP242) compared to everolimus (RAD001), a rapalog, in an animal model of endometrioid endometrial cancer. Using a xenograft tumor model of endometrial cancer, we assessed the activity and expression of downstream proteins in the mTOR pathway after treatment with an mTORC1/2 inhibitor (PP242) or an allosteric mTORC1 (RAD001) inhibitor, with and without concurrent DNA damage chemotherapy with carboplatin. This paper presents our pre-clinical data in support of the clinical development of mTORC1/2 inhibitors for the treatment of endometrioid endometrial cancers.

Materials and methods

Cell lines

Pilot studies of multiple endometrial cancer cell lines were conducted to establish tumorigenicity of various lines. These studies demonstrated enhanced tumorigenicity of the AN3CA cell line compared to other endometrial cancer cell lines tested (Hec1A and Hec1B) based on increased mean tumor volume 26 days after injection (Hec1A 122 mm^3; Hec1B 123 mm^3; AN3CA 1232 mm^3). Further, AN3CA cells, isolated from a metastatic lesion in the lymph node of a patient with advanced endometrial cancer, produce poorly differentiated and platinum-resistant malignant tumors, and mirror more closely the clinical-pathologic characteristics of more aggressive endometrial cancers that would have a higher propensity toward recurrent or metastatic disease. Based on these findings, the human grade 3 endometrial adenocarcinoma cell line AN3CA was chosen for study. This cell line has previously been demonstrated to be PTEN negative, reflective of the majority of recurrent and metastatic endometrial cancers [16]. Cells were cultured at 37°, 5% CO_2 in minimum essential medium (Cellgro) supplemented with 10% fetal bovine serum, sodium pyruvate, and penicillin/streptomycin.

Tissue culture cell treatment protocols

AN3CA cells were plated in 6-well plates, then treated for 5 or 10 h with: (1) control vehicle; (2) carboplatin, 125 μg/ml; (3) PP242, 2.5 μM; (4) PP242 (2.5 μM) + carboplatin (125 μg/ml); and (5) RAD001, 100 nM; (6) RAD001 (100 nM) + carboplatin (125 μg/ml). After treatment, immunoblot analyses were performed on cell lysates to assess activation of the mTOR pathway.

Xenograft animal tumor 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 BALB/c nu/nu mice, aged 5 weeks, were obtained from Taconic Farms, Inc. 2×10^6 cells were injected subcutaneously in the right flank in a total volume of 100 μl (50 μl RPMI and 50 μl Matrigel), using a 26-gauge needle. When mean tumor volume was approximately 160 mm^3 calculated using external calipers and the standard formula for volume of an ellipsoid ($\pi/6 \times$ (larger diameter) $\times$ (smaller diameter)), mice were randomized into 6 groups, stratifying for average tumor volume. Each treatment group consisted of 7–8 mice and studies were repeated twice.

Xenograft tumor model treatment protocols

Treatment was conducted for 4 weeks. RAD001 and PP242 were administered on days 1–5 of each week. In groups in which carboplatin was also administered, this was injected on day 2 of the cycle. Mice were randomized into the following treatment groups: (1) control, treated by oral gavage with vehicle, 100 μl polyvinylpyrrolidone (PVP) + 5% N-methylpyrrolidone; (2) single agent PP242, 100 mg/kg PP242 in 100 μl by oral gavage (suspended in 15% PVP + 5% N-methylpyrrolidone as per manufacturer instructions); (3) PP242/carboplatin, treated as in group 2 with the addition of weekly carboplatin, 50 mg/kg injected on day 2 of weekly treatment cycle in total volume of 125 μl; (4) carboplatin, weekly at 50 mg/kg; (5) RAD001, at 2.5 mg/kg RAD001 in 100 μl by oral gavage (suspended in PBS + 10% DMSO); and (6) RAD001 + carboplatin, treated as in group 5 with the addition of weekly carboplatin as in group 4.

Assessment of xenograft tumor treatment response and toxicity

Tumor size was monitored twice per week as described above. Mice were weighed weekly and percentage weight change was used as a standard measure of toxicity.

Immunoblot analyses of tumor mTORC1/2 pathway response to treatment

Following treatments of tissue culture cells, cells were washed twice in ice-cold PBS and lysed at 4 °C or 0.5% SDS lysis buffer [17]. NP-40 lysates were clarified by centrifugation at 13,000 $\times g$ for 10 min and protein concentrations were determined for each sample by Bradford assay (Bio-Rad, Hercules, CA). To determine the total levels and phosphorylation status of specific proteins, equal amounts of protein were resolved by SDS–PAGE and analyzed by Western immunoblotting with specific antibodies as indicated. The phosphorylation status of most proteins was determined by immunoblotting membrane first with phospho-specific antibody then stripping the membranes using Restore Western blot stripping buffer (Pierce), followed by re-probing membranes with non-phospho-specific antibodies.