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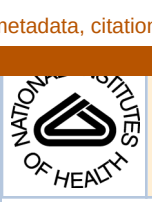

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Rapamycin potentiates the effects of paclitaxel in endometrial cancer cells through inhibition of cell proliferation and induction of apoptosis[1]

Aaron Shafer[2], **Chunxiao Zhou**[2,3], **Paola A. Gehrig, MD**[2], **John F. Boggess**[2], and **Victoria L. Bae-Jump**[2,4]

[2] Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, University of North Carolina, Chapel Hill, NC

Abstract

mTOR inhibitors modulate signaling pathways involved in cell cycle progression, and recent phase II trials demonstrate activity in endometrial cancer patients. Our objective was to examine the effects of combination therapy with rapamycin and paclitaxel in endometrial cancer cell lines. Paclitaxel inhibited proliferation in a dose-dependent manner in both cell lines with IC_{50} values of 0.1–0.5 nM and 1–5 nM for Ishikawa and ECC-1 cells, respectively. To assess synergy of paclitaxel and rapamycin, the combination index (CI) was calculated by the method of Chou and Talalay. Simultaneous exposure of cells to various doses of paclitaxel in combination with rapamycin (1 nM) resulted in a significant synergistic anti-proliferative effect (CI <1, range 0.131–0.920). Rapamycin alone did not induce apoptosis, but combined treatment with paclitaxel increased apoptosis over that of paclitaxel alone. Treatment with rapamycin and paclitaxel resulted in decreased phosphorylation of S6 and 4E-BP1, two critical downstream targets of the mTOR pathway. Rapamycin decreased hTERT mRNA expression by real-time RT-PCR while paclitaxel alone had no effect on telomerase activity. Paclitaxel increased polymerization and acetylation of tubulin, and rapamycin appeared to enhance this effect. Thus, in conclusion, we demonstrate that rapamycin potentiates the effects of paclitaxel in endometrial cancer cells through inhibition of cell proliferation, induction of apoptosis and potentially increased polymerization and acetylation of tubulin. This suggests that the combination of rapamycin and paclitaxel may be a promising effective targeted therapy for endometrial cancer.

Keywords

endometrial cancer; rapamycin; paclitaxel; mTOR pathway; telomerase

INTRODUCTION

Endometrial cancer is the most common gynecologic malignancy in the United States, accounting for an estimated 40,100 new diagnoses and 7,470 deaths in 2008 [1]. While most

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[4]Manuscript Correspondence: Victoria L. Bae-Jump, MD, PhD, Assistant Professor, University of North Carolina, Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, CB #7572, Chapel Hill, NC 27599-7572, Phone: 919-843-4899, Fax: 919-966-2646, vbae@unch.unc.edu.

[3]This author contributed an equal amount of work as first author.

CONFLICT OF INTEREST STATEMENT

We, the authors of this manuscript, have no financial or personal relationships to disclose that could inappropriately influence or bias this work.

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women with endometrial cancer will be diagnosed with early stage disease and have a high cure rate with surgery alone, up to 25% of women will have advanced stage or recurrent disease and require additional treatment. Traditional therapies with cytotoxic or hormonal regimens have had limited success; and thus, attention has focused on the potential therapeutic benefit of novel agents that target specific cellular signaling pathways thought to be essential in endometrial cancer progression and metastasis. Some of the most promising of these for endometrial cancer are the mammalian target of rapamycin (mTOR) inhibitors.

Rapamycin is a macrolide antibiotic that is a product of the bacterium *Streptomyces hygroscopicus* and was initially used as an immunosuppressant in transplant patients. Rapamycin binds to mTOR and is the prototypical drug in the class of mTOR inhibitors. mTOR is a serine/threonine kinase which phosphorylates S6K1 and 4E-BP1, resulting in the transcription of critical mRNAs involved in cell cycle progression from G1 to S phase [2–4]. By binding mTOR, rapamycin decreases the phosphorylation of S6K1 and 4E-BP1; and thus, interferes with signals required for cell cycle progression, ultimately causing G1 arrest [2]. mTOR inhibitors are currently under evaluation in phase I, II and III clinical trials for a broad range of cancers including endometrial cancer [5, 6]. Loss of PTEN expression is one of the most prevalent molecular abnormalities associated with endometrial cancers and given that wild-type PTEN downregulates the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling pathway [7, 8], it is reasonable to postulate that mTOR inhibitors would help restore some of the inhibitory effects of normal PTEN. Thus, this class of drugs may represent a compelling treatment strategy for this disease.

We have previously demonstrated that rapamycin potently inhibits cell growth in endometrial cancer cell lines [4]. In addition to being used as single agent therapy, rapamycin and its analogues have been shown to enhance the efficacy of several cytotoxic chemotherapeutic agents in a variety of different cancers that rely on the mTOR pathway [9–14]. Paclitaxel is a widely used chemotherapeutic agent that exerts its cytotoxic effects by promoting the polymerization of microtubules, resulting in disruption of microtubule dynamics and subsequent mitotic arrest and apoptotic cell death [15]. It has proven to be one of the most clinically active agents for the treatment of a variety of solid tumors, including endometrial carcinoma [16, 17]. Thus, our goal was to determine if rapamycin would potentiate the effects of paclitaxel on endometrial cancer cells in regards to inhibition of cell growth and induction of apoptosis, in the hope that these two agents used in combination may be a more effective treatment option for women with recurrent or advanced stage endometrial cancer.

MATERIALS AND METHODS

Cell Culture and Reagents

The endometrial cancer cell lines, Ishikawa and ECC-1, were used. The Ishikawa and ECC-1 cell lines are both derived from well-differentiated, estrogen receptor positive (ER+) adenocarcinomas of the endometrium [18]. We have previously documented functional ER and progesterone receptor (PR) status among the endometrial cancer cell lines present in our laboratory [19]. Ishikawa and ECC-1 cells were shown to be ER- and PR-positive as determined by estrogen- and progesterone-induced progesterone response element (PRE) chloramphenicol acetyltransferase (CAT) activity [19]. The Ishikawa cells were grown and maintained in MEM supplemented with 5% bovine serum. The ECC-1 cells were grown in RPMI media containing 5% fetal bovine serum, 200 pg/ml estrogen and 6 mM sodium bicarbonate. All media was supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin under 5% CO_2. Paclitaxel and rapamycin were purchased from Sigma (St. Louis, MO) and dissolved in DMSO. The methyl thiazolyl-diphenyl-tetrazolium (MTT) dye was purchased from Sigma (St. Louis, MO). The ssDNA apoptosis ELISA kit was obtained

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from Chemicon/Millipore International (Billerica, MA). The anti-phosphorylated-S6 antibody, anti-pan-S6 antibody, anti-phosphorylated-4E-BP1 antibody and anti-pan-4E-BP1 antibody were purchased from Cell Signaling (Danvers, MA). The anti-acetylated tubulin antibody, anti-α-tubulin antibody and the anti-β-actin antibody used for Western immunoblotting were purchased from Sigma (St. Louis, MO). The anti-α-tubulin antibody used for immunofluorescent staining was purchased from Millipore (Billerica, MA).

Cell Proliferation Assay

The ECC-1 and Ishikawa cells were plated and grown in 96-well plates at a concentration of 6000 cells/μL and 8000 cells/μL respectively for 24 hours. Cells were then treated with varying doses of paclitaxel or rapamycin alone or paclitaxel plus rapamycin at a concentration of 1 nM. Cells were incubated with the treatment drug or drugs for 24, 48, 72 and 96 hours. Viable cell densities were determined by metabolic conversion of the dye (MTT). MTT was added to the 96 well plates at 10 μL/well, and the plates were then incubated for an additional 2 hours. The MTT assay results were read by measuring absorption at 595 nm. The effect of paclitaxel and rapamycin were calculated as a percentage of control cell growth obtained from DMSO (1%) treated cells grown in the same 96 well plates. Each experiment was performed in triplicate and repeated three times to assess for consistency of results.

Western Blot Analysis

The Ishikawa and ECC-1 cells were plated at 1×10^5 cells/well in 6 well plates in their corresponding media. After 24 hours, cells were treated with paclitaxel, rapamycin or both combined for 48 hours. Cell lysates were prepared in RIPA buffer (1% NP40, 0.5 sodium deoxycholate and 0.1% SDS). Equal amounts of protein were separated by gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk and then incubated with a 1:1000 dilution of primary antibody overnight at 4°C. The membrane was then washed and incubated with a secondary peroxidase-conjugated antibody for 1 hour after washing. Antibody binding was detected using an enhanced chemiluminescence detection system (GE Healthcare Life Sciences, Piscataway, NJ). After developing, the membrane was stripped and reprobed using antibody against β-actin to confirm equal loading. Western blot films were digitized, and band net intensities were quantified by a densitometer using the Genegynome Image System (Sygene, MD). Each experiment was repeated three times to assess for consistency of results.

Apoptosis Assay

The Ishikawa and ECC-1 cells were grown and plated in a 96 well plate at a concentration of approximately 6000 cells/μL. After 24 hours, the cells were treated with paclitaxel, rapamycin or both in combination. After 48 hours of treatment, the plates were dried, and the wells were treated with formamide. S1 nuclease was added to the control wells. Subsequently, 3% nonfat dry milk was added to block non-specific binding. After one hour, the milk was removed, and the wells were incubated with primary anti-dsDNA antibody for 30 minutes. The cells were then treated with ABTS solution for 60 minutes. The stop solution was then added, and the 96 well plates were read at 405 nm. The amount of apoptosis was calculated relative to that of the control wells. Each experiment was repeated three times to assess for consistency of results.

hTERT Expression

Total RNA was isolated using the RNAeasy kit (Qiagen, Valencia, CA). The reverse transcription and PCR reactions were performed using the TaqMan Gold one-step RT-PCR kit in the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City,

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CA). Reverse transcription was carried out at 48°C for 30 min. The PCR conditions consisted of a 10 min step at 95°C, 40 cycles at 95°C for 15 sec each and 1 min at 65°C. A housekeeping control gene acidic ribosomal phosphoprotein P0 (RPLP0, also known as 36B4) was used as an internal control to correct for differences in the amount of RNA in each sample. Primers and fluorogenic probes for hTERT and RPLP0 have been described previously [20]. The standard curve for hTRERT was generated by using dilutions of a known amount of cRNA synthesized by *in vitro* transcription of a cloned fragment. The normalized level of hTERT in each sample was estimated by a ratio of the hTERT level to the RPLP0 level, as described previously [20]. Each experiment was performed in triplicate and repeated three times to assess for consistency of results.

α-tubulin Expression/Labeling

After plating the Ishikawa and ECC-1 cells at a concentration of 8000 and 6000 cells/μL, respectively, on an 8-well glass slide, the cells were incubated at 37°C for 24 hours. Cells were then treated with paclitaxel, rapamycin or both for 48 hours. The cells were then fixed with 3.7% formaldehyde and permeabilized with Triton X-100. The cells were then incubated with anti-α-tubulin (Millipore, Billerica, MA), clone DM1A, Alexa® 488 conjugate for 1 hour. A nuclear counterstain with Topro-3 (Invitrogen, Carlsbad, CA) was then added. The cells were then examined under a fluorescence microscope and pictures taken with a computerized digital camera.

Statistical Analysis

Results for experiments were normalized to the mean of the control and analyzed using the student t-test. Differences were considered significant if the p value was less than 0.05 ($p<0.05$) with a confidence interval of 95%. STATA software (StataCorp, College Station, TX) was used to perform the statistical analyses.

Statistical analysis on synergy was used to evaluate the effects of combined drug treatments. The results from the MTT assays were calculated by the CalcuSyn for Windows computer program (Biosoft, Cambridge, United Kingdom) to determine the presence of synergy between rapamycin and paclitaxel. The software uses a median-effect method, which is a well-established procedure to quantify the effects of drug combinations and to determine whether they produce greater effects together than expected from simple summation of their individual effects. The combination index (CI) values are obtained from the data and reflect the nature of the interaction between rapamycin and paclitaxel, i.e. < 1, synergistic activity; = 1, additive; > 1, antagonism.

RESULTS

Synergistic anti-proliferative effects of rapamycin and paclitaxel

We examined the effects of rapamycin used in combination with paclitaxel in two endometrial cancer cell lines (Ishikawa and ECC-1), with respect to cell proliferation and cytotoxicity. As expected, paclitaxel potently inhibited growth in a dose-dependent manner in both of these cell lines with IC_{50} values of 0.5 nM (Ishikawa) and 0.1 (ECC-1) nM (Figure 1). The combination of paclitaxel and rapamycin showed greater inhibition of cell proliferation than that of paclitaxel alone (Figure 1). The IC_{50} values of paclitaxel with rapamycin (1nM) were 0.1–0.5 nM ($p = 0.0004 – 0.0097$) and 0.01–0.001 nM ($p = 0.0001–0.0357$) for Ishikawa and ECC-1 cells, respectively.

Median-effect plot analyses and calculation of the multiple drug effect/combination index (CI) was performed using the well-established method of Chou and Talalay [21]. Simultaneous exposure of various doses of paclitaxel in combination with 1 nM of

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rapamycin on these endometrial cancer cell lines resulted in a significant synergistic anti-proliferative effect with a CI of < 1, with a range of 0.131 to 0.920 (Figure 1 & Table 1). Synergistic effects on cell proliferation were also observed when different concentrations of paclitaxel were combined with 0.1 nM and 10 nM rapamycin in both of these cell lines (data not shown).

Rapamycin enhanced paclitaxel-induced apoptosis

An apoptosis assay using an antibody to dsDNA was performed after treatment with paclitaxel alone or in combination with rapamycin, in both the Ishikawa and ECC-1 cell lines. Paclitaxel induced apoptosis in a dose-dependent manner in both endometrial cancer cell lines ($p = 0.0097–0.05$ for Ishikawa cells; $p = 0.0036–0.0045$ for ECC-1 cells). In addition, rapamycin increased paclitaxel-mediated apoptosis of both cell lines for each concentration of paclitaxel, over that of paclitaxel alone ($p = 0.04–0.05$ for Ishikawa cells; $p = 0.03–0.04$ for ECC-1 cells) (Figure 2). Rapamycin alone did not induce apoptosis over that of the control. These results indicate that rapamycin enhances the efficacy of paclitaxel by increasing paclitaxel-induced apoptosis.

Effect of rapamycin and paclitaxel on the mTOR pathway

To investigate the mechanisms underlying the synergistic anti-proliferative effect between rapamycin and paclitaxel, we characterized the effect of this combination treatment on relevant cell signaling targets. Previous studies suggest that p70S6K is a downstream target of the mTOR pathway [22]. p70S6K kinase directly phosphorylates the 40S ribosomal protein S6, which results in enhanced synthesis of proteins that contain a polypyrimidine tract in the 5′-untranslated region [22]. Therefore, we studied the effect of rapamycin and paclitaxel on the phosphorylation of the S6 ribosomal protein in both cell lines. After 24–48 hours of treatment, rapamycin alone and in combination with paclitaxel dramatically decreased the phosporylation of S6 (Figure 3A and 3B). Paclitaxel alone also decreased phosphorylation of S6, with the greatest effect seen after 72 hours of exposure (Figure 3C and 3D). Expression of pan-S6 was not affected by paclitaxel or rapamycin.

mTOR kinase regulates protein synthesis by phosphorylating the translation repressor 4E-BP1 on multiple serine/threonine sites [23]. The phosphorylation status of 4E-BP1 regulates binding to eukaryotic initiation factor 4E (eIF4E). Hyperphosphorylation of 4E-BP1 disrupts its binding to eIF4E, activating cap-dependent protein synthesis [24]. After 24–48 hours of treatment, rapamycin alone and in combination with paclitaxel decreased phosphorylation of 4E-BP1 and pan-4E-BP1 in the Ishikawa and ECC-1 cell lines (Figures 4A and 4B). Treatment with paclitaxel alone had no effect on the phosphorylation of 4E-BP1 in either cell line (Figures 4C and 4D). These experimental data suggest that rapamycin in combination with paclitaxel may exert their synergistic effect through the mTOR pathway by regulating phosphorylation of both the S6 and 4E-BP1 proteins.

Effect of rapamycin and paclitaxel on hTERT mRNA expression

The hTERT gene encodes the catalytic subunit of telomerase. hTERT expression is the rate-limiting determinant of the enzymatic activity of human telomerase and is thought to be a sensitive marker of telomerase function. Real-time RT-PCR was used to quantify hTERT mRNA expression in the endometrial cancer cell lines. Treatment with paclitaxel alone did not affect hTERT mRNA expression (Figure 5). However, rapamycin alone ($p = 0.04$ for Ishikawa cells; $p = 0.0067$ for ECC-1 cells) and the combination of rapamycin with paclitaxel ($p = 0.05–0.06$ for Ishikawa cells; $p = 0.01–0.04$ for ECC-1 cells) did decrease hTERT mRNA expression (Figure 5). Varying the dose of paclitaxel with 1 nM rapamycin did not effect the suppression of hTERT expression (Figure 5), implying that the effect on telomerase activity was primarily due to rapamycin.

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Effect of rapamycin and paclitaxel on tubulin expression and acetylation

Paclitaxel-treated Ishikawa and ECC1 cells had increased levels of polymerized tubulin arranged along the cell axis, as evidenced by immunofluorescent staining with the anti-α-tubulin antibody (Figure 6). This effect appeared to be dose-dependent. In contrast, untreated or control cells had extensive fine microtubules throughout the cytoplasm. Rapamycin alone had little effect on α-tubulin expression. However, the combination of paclitaxel with rapamycin (1 nM) appeared to potentiate the effect of paclitaxel alone on α-tubulin expression and organization in both cell lines, primarily through increased polymerization of the cellular microskeleton in the dual-treated cells (Figure 6). This promotion of microtubule polymerization may be another possible underlying mechanism for the synergy seen between rapamycin and paclitaxel in endometrial cancer cells.

In order to better quantify the effect of rapamycin and paclitaxel on microtubule dynamics, tubulin acetylation was assessed by Western immunoblotting. Tubulin acetylation is an established marker of microtubule stability [25]. Rapamycin (1 μM) enhanced the ability of paclitaxel (0.1 μM) to induce tubulin acetylation (Figure 7). Treatment with paclitaxel and rapamycin increased acetylated tubulin expression by 3.1 and 4.0 fold for the Ishikawa and ECC-1 cell lines, respectively. Paclitaxel alone increased tubulin acetylation by 1.5 and 1.3 fold for the Ishikawa and ECC-1 cell lines, respectively. Rapamycin alone had no effect on acetylated tubulin. At higher concentrations of paclitaxel, the extent of tubulin acetylation was so high that it was difficult to discern whether rapamycin had any additional effect. It may be that tubulin acetylation reaches a plateau at concentrations greater than 0.1 μM of paclitaxel; and thus, its acetylation level cannot be further enhanced by rapamycin. Expression of α-tubulin was not affected by low dose paclitaxel or rapamycin.

DISCUSSION

We have demonstrated a synergistic relationship between paclitaxel and rapamycin in regards to inhibition of cell proliferation and induction of apoptosis in human endometrial cancer cell lines. Treatment with rapamycin and paclitaxel resulted in decreased phosphorylation of S6 and 4E-BP1, two critical downstream targets of the mTOR pathway. Rapamycin decreased hTERT mRNA expression while paclitaxel alone had no effect on telomerase activity. In addition, rapamycin enhanced paclitaxel's effect on ordered microtubule structure as was evidenced by increased levels of polymerized α-tubulin staining. This suggests that the combination of rapamycin and paclitaxel may be a promising effective targeted therapy for endometrial cancer.

Synergy in this study was quantified using the combination index equation of Chou and Talalay which allows for the evaluation of two or more chemotherapeutic agents at different concentrations and effect levels [21]. Through this methodology, combinations of drugs can be analyzed for synergy versus antagonism as well as their maximal anti-tumor efficacy. Rapamycin and its derivatives have been proposed as potentially potent chemotherapeutic chemosensitizers. Paclitaxel and rapamycin have exhibited synergy through inhibition of cell proliferation and induction of apoptosis in many types of cancer cells, including breast cancer, cervical cancer and head and neck cancer [11, 13, 26]. We have demonstrated that even low doses of rapamycin (1 nM) in combination with low doses of paclitaxel (0.01–0.1 μM) resulted in a strong synergistic effect (Figure 1, Table 1). Although rapamycin did not significantly induce apoptosis in these endometrial cancer cell lines, we found that treatment with rapamycin and paclitaxel together increased induction of apoptosis, well above the effects of paclitaxel alone (Figure 2).

p70S6K is a mitogen-activated serine/threonine kinase that is a potent regulator of protein synthesis, and thus, plays a crucial role in cell growth and survival. Cell cycle progression

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necessitates a steady increase in the rate of protein synthesis, resulting in the coordinated activation of cyclins, cyclin-dependent kinases (CDK) and CDK inhibitors. Rapamycin is known to block cell cycle progression through G1 by inhibition of p70S6K [22, 27]. The effect of paclitaxel on p70S6K has only been recently explored. In breast and ovarian cancer cell lines, paclitaxel has been shown to simultaneously decrease the activity of p70S6K and induce the phosphorylation of p70S6K at threonine 421 (T421) and serine 424 (S424) [28]. This seemingly contradictory effect implies that paclitaxel-induced $p70S6K^{T421/S424}$ phosphorylation and kinase inactivation may be regulated by different mechanisms and that phosphorylation of p70S6K may not always be associated with kinase activation [28]. Interestingly, T421 and S424 are sites on p70S6K which do not appear to be phosphorylated by activated mTOR [22]. Rapamycin is thought to exert its effects on p70S6K via phosphorylation at threonine 229 (T229) and threonine 389 (T389), as mutating these amino acids confers resistance to rapamycin [22, 29].

We found that paclitaxel alone inhibited phosphorylation of the S6 ribosomal protein, with the greatest effect seen after 72 hours of exposure (Figure 4). p70S6K directly phosphorylates the 40S ribosomal protein S6 which results in enhanced synthesis of proteins that contain a polypyrimidine tract in the 5′-untranslated region [22]. Thus, for this study, we did not focus on either the T421/S424 or T229/T389 sites of p70S6K but rather its immediate downstream target. As compared to treatment with paclitaxel alone, exposure to rapamycin alone or in combination with paclitaxel completely abolished phosphorylation of S6 (Figure 3). This suggests that paclitaxel may have its own independent effect on phosphorylation of S6, but it is unclear the extent of its contribution in the presence of rapamycin.

Although rapamycin and its derivatives clearly act as cytostatic agents by arresting cells in the G1 phase, these agents are generally not thought to induce apoptosis. However, we [30] and others [11, 13] have demonstrated that rapamycin in combination with cytotoxic agents results in increased induction of apoptosis that surpasses that of the cytotoxic agents alone. This has been shown for paclitaxel as well as cisplatin and carboplatin. The mechanism by which rapamycin enhances apoptosis induced by cytotoxic agents is poorly understood but some theories exist. S6K1 is known to phosphorylate and inactivate the pro-apoptotic molecule BAD, a process which can be reversed by treatment with rapamycin [31]. Interestingly, paclitaxel may exert its anti-tumor effects partially through inhibition of S6K1, as found previously by Le et. al. [28] and validated in this study (Figure 3C and 3D).

The other downstream target of mTOR, 4E-BP-1, may also be important in rapamycin's chemosensitization to paclitaxel. Once phosphorylated, 4E-BP-1 releases the cap-binding protein eIF-4E from a functionally inactive complex, which subsequently leads to initiation of translation. In cells undergoing apoptosis, 4E-BP-1 undergoes caspase-dependent cleavage, resulting in a NH2-terminally truncated polypeptide that is unable to become phosphorylated and dissociate from eIF-4E [31]. Thus, induction of apoptosis by paclitaxel and inhibition of mTOR by rapamycin both simultaneously decrease the availability of 4E-BP-1, and this may be a plausible explanation for the potentiation of apoptosis by mTOR inhibition in the setting of cytotoxic injury.

In both endometrial cancer cell lines, rapamycin alone and in combination with paclitaxel decreased phosphorylation of 4E-BP1 with a concomitant reduction in total 4E-BP1 protein (Figure 4). The association between rapamycin and suppression of total 4E-BP1 is unclear. One could postulate that a decrease in phosphorylation of 4E-BP1 results in destabilization of this protein, leading to the loss of total 4E-BP1. Another possibility is that a decrease in phosphorylation of 4E-BP1 may have a feedback effect to repress transcription of total 4E-BP1 mRNA. Further work is needed to elicit the mechanism underlying this relationship.

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In most normal somatic cell types, telomerase activity is usually undetectable; however, the endometrium is one exception [32]. It is thought that telomerase plays a critical role in the ability of normal endometrium to repeatedly proliferate from the onset of menarche to menopause. Furthermore, activation of telomerase has also been implicated as a fundamental step in cellular immortality and oncogenesis in many cancers[32], including gynecologic malignancies [33, 34]. Telomerase is comprised of an RNA template (hTR) and the catalytic protein hTERT which has reverse transcriptase activity. hTERT is considered to be the rate-limiting factor in the formation of functional telomerase. We have previously demonstrated that rapamycin profoundly suppresses telomerase activity via inhibition of hTERT mRNA expression in endometrial, ovarian and cervical cancer cell lines [4, 30]. Rapamycin's effect on regulation of hTERT expression was independent of its ability to induce cell cycle arrest [30].

In this study, rapamycin was once again found to decrease hTERT mRNA expression in the endometrial cancer cell lines (Figure 5). Paclitaxel had no effect on hTERT mRNA expression (Figure 5), confirming the work of others [35, 36]. Although paclitaxel is not thought to regulate telomerase activity, it has been shown to induce telomere erosion, occurring before the onset of apoptosis [35, 36]. The consequence of telomere erosion by paclitaxel is poorly understood; however, telomerase inhibitors used in combination with paclitaxel have been found to enhance the anti-tumor activity of paclitaxel both *in vitro* and *in vivo* [35]. These authors suggest that the combination of therapeutic agents that target both telomeres and telomerase activity may be particularly efficacious for tumors that rely on telomerase for telomere maintenance. Thus, rapamycin and its unique ability to suppress telomerase activity may be logical therapeutic partner to paclitaxel.

Rapamycin appeared to enhance paclitaxel's effect on polymerization of α-tubulin (Figure 6), suggestive of another underlying mechanism of the synergistic relationship between these two chemotherapeutic agents. Rapamycin alone had little effect on α-tubulin expression. To our knowledge, there has been only one other study that examined the effect of rapamycin on α-tubulin polymerization [37]. In contrast to our findings, rapamycin was shown to have no effect on microtubule stabilization in either the presence or absence of paclitaxel [37]. However, in this previous study, human follicular B-cell lymphoma cell lines were used as opposed to solid tumor cell lines, and the cells were pre-treated for 24 hours with rapamycin prior to paclitaxel instead of simultaneous treatment [37]. Furthermore, this prior study found that pre-treatment with rapamycin protected cells from apoptosis induced by paclitaxel which is in stark contrast to the conclusions that we and others have derived in various cancer cell types [11, 13, 38]. These conflicting results may be secondary to intrinsic differences in response to rapamycin among cancer cell types or alternatively imply that the sequencing of administration of the two drugs may be paramount to the overall effect of survival versus cell death.

Rapamycin was also shown to enhance the acetylating activity of paclitaxel (Figure 7). The state of tubulin acetylation is thought to play a crucial role in regulating microtubule stability [25]. The capability to induce acetylation of tubulin has been documented for histone deacetylase inhibitors (HDI), a class of potent anti-neoplastic agents that induce growth arrest and apoptosis [39, 40], but has not been previously linked to rapamycin. It has been hypothesized that drugs which enhance paclitaxel effects on microtubule stability would improve its anti-tumorigenic potential, allowing for lower dosing of paclitaxel and subsequent reduction of toxicities for patients. Suppression of microtubule dynamics is the mechanism through which paclitaxel blocks mitosis and induces apoptotic cell death. Given that rapamycin alone had no effect on acetylation of tubulin, we postulate that rapamycin may facilitate more efficient binding of paclitaxel to microtubules as opposed to a direct effect on microtubule stability. This may also contribute to the potentiation of paclitaxel-induced apopotosis by rapamycin.

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The mTOR pathway has been implicated in the apoptotic signaling cascade activated by microtubule-damaging agents, such as paclitaxel. Paclitaxel inhibits microtubule depolymerization, leading to phosphorylation and inhibition of the anti-apoptotic Bcl-2 protein and ultimately lowering the threshold of programmed cell death. Signaling through mTOR is thought to be critical for phosphorylation of Bcl-2 by paclitaxel. Inhibition of mTOR signaling, by inducing the expression of a dominant negative mutant of the Akt kinase (DN-Akt) in human embryonic kidney cells (HEK 293), results in increased levels of Bcl-2 phosphorylation and a lower threshold of apoptosis in those cells treated with paclitaxel [41]. Opposite effects are seen in HEK 293 cells expressing consitutively active Akt (CA-Akt) and exposed to paclitaxel [41]. These authors postulate that the down-regulation of the Akt-mTOR signaling pathway may strengthen the death signals summoned by the microtubule damage induced by paclitaxel[41, 42].

The mTOR signaling pathway has been implicated in chemotherapeutic drug resistance in a variety of cancers, including breast, ovarian, lung, prostate and endometrial cancer [43]. It has been postulated that mTOR activation in cancer cells culminates in survival and anti-apoptotic signals which ultimately can lead to drug resistance. Thus, logically, mTOR inhibitors such as rapamycin have been shown to overcome drug resistance to cytotoxic agents such as cisplatin, paclitaxel and doxorubicin as well as hormonal agents such as letrozole and tamoxifen [43, 44]. This ability of mTOR inhibitors to restore sensitivity to chemotherapeutic agents is predominantly found in those cancer cells with aberrant mTOR activity, through such mechanisms as overexpression of Akt and PI3K or loss of PTEN.

Neither of the endometrial cancer cell lines used in this work were resistant to paclitaxel or cisplatin [45]. However, overexpression of the Akt isoforms, Akt2 and Akt3, has been associated with cisplatin chemoresistance in the endometrial cancer cell lines, KLE and HEC-1-A [46]. Interestingly, knockout of the Akt isoforms through silencer RNA (siRNA) increased the sensitivity of the cisplatin-resistant KLE cell line to the apoptotic effects of cisplatin [46]. Clinically, this could be very important for women with endometrial cancer where inevitable chemoresistance to cisplatin/carboplatin and paclitaxel remains a significant barrier to successful treatment of this disease.

Combination therapy with mTOR inhibitors is not novel, and has been shown to increase the effectiveness of traditional cytotoxic and hormonal chemotherapeutic regimens both *in vitro* and *in vivo* [9–14, 38]. The data from these studies as well as our own work gleam the potential promise of combining mTOR inhibitors with standard cytotoxic chemotherapies for many cancers, including endometrial cancer. Advanced or recurrent endometrial cancer is notoriously difficult to treat with poor response rates and dismal overall survival. Traditional cytotoxic agents also have significant toxicities, especially hematologic and neurologic, which can be distressing for both the patient and the physician. mTOR inhibition by rapamycin may have the potential to inhibit tumor growth as well as enhance the effects of paclitaxel at lower doses, culminating in overall decreased toxicities for endometrial cancer patients. The combination of rapamycin and paclitaxel seems to be a logical therapeutic strategy for endometrial cancer and deserves further investigation in clinical trials.

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ABBREVIATIONS

mTOR	mammalian target of rapamycin
PTEN	phosphatase and tensin homolog
PI3K	phosphatidylinositol 3-kinase
eIF4E	eukaryotic initiation factor 4E
ER	estrogen receptor
PR	progesterone receptor
MTT	methyl thiazolyl-diphenyl-tetrazolium
CI	combination index
DN-Akt	dominant negative mutant of Akt kinase
HEK 293	human embryonic kidney cells 293
CA-Akt	constitutively active Akt
HDI	histone deacetylase inhibitors

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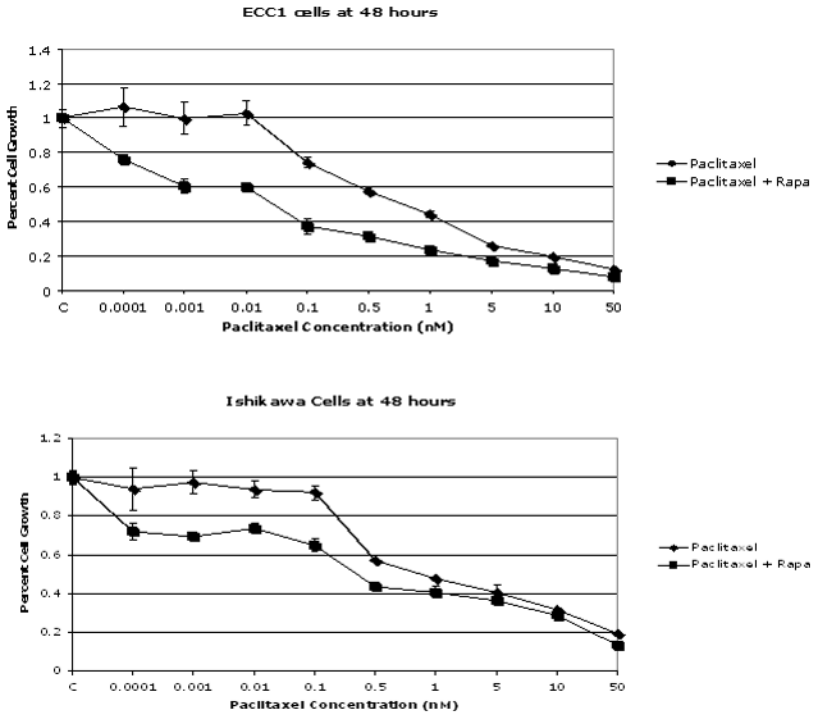

Figure 1.

Effect of paclitaxel with and without rapamycin on proliferation of endometrial carcinoma cells. Ishikawa and ECC-1 cells were cultured in the presence of varying concentrations of paclitaxel and rapamycin. Rapamycin enhances sensitivity to paclitaxel after 48 hours of exposure in both cell lines. The relative growth of cells was determined by MTT. The IC_{50} values of paclitaxel with rapamycin (1nM) were 0.1–0.5 nM ($p = 0.0004 – 0.0097$) and 0.01–0.001 nM ($p = 0.0001–0.0357$) for Ishikawa and ECC-1 cells, respectively. The results are shown as the mean ± SE of triplicate samples and are representative of three independent experiments.

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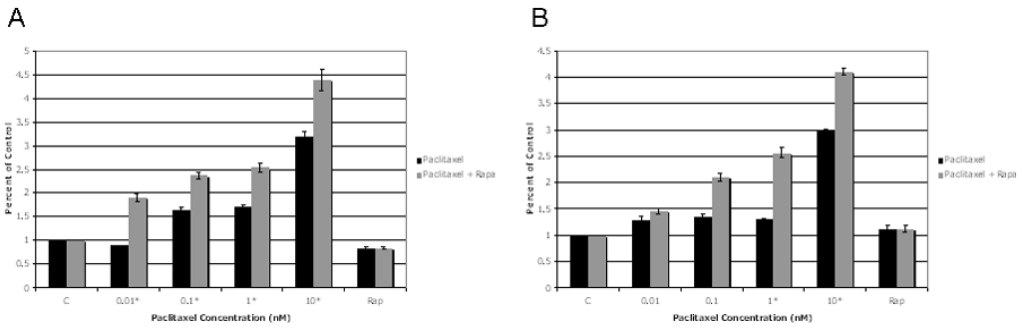

Figure 2.

Paclitaxel and rapamycin in combination increased apoptosis compared to paclitaxel alone. Rapamycin alone did not induce apoptosis. The (A) Ishikawa and (B) ECC-1 cell lines were grown for 24 hours and then treated with the indicated concentrations of rapamycin, paclitaxel or paclitaxel in combination with 1 nM rapamycin. Apoptosis was assessed using an antibody to dsDNA. Data shown are representative of at least two independent experiments (* indicates statistically significant difference).

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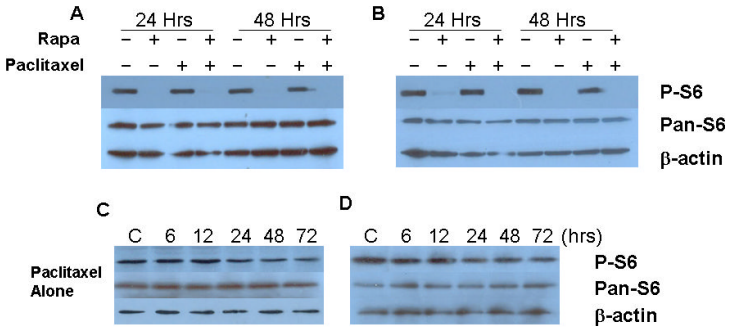

Figure 3.

The effect of paclitaxel and rapamycin on phosphorylation of S6 in (A) Ishikawa and (B) ECC-1 cells. The cells were treated with rapamycin (1 nM), paclitaxel (1 nM) or both in combination for 24–48 hours. Rapamycin alone and in combination with paclitaxel completely abolished phosphorylation of S6. Treatment with paclitaxel alone also reduced phosphorylation of the S6 protein in Ishikawa (C) and ECC-1 cells (D). This effect was best seen after 72 hours of exposure to paclitaxel. Phosphorylated S6, pan-S6 and β-actin were determined by Western immunoblotting.

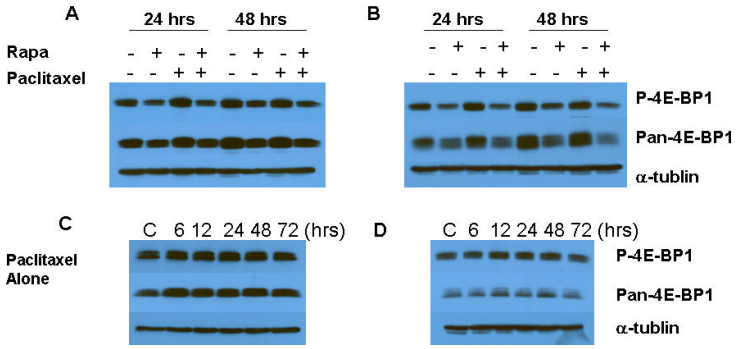

Figure 4.

The effect of paclitaxel and rapamycin on phosphorylation of 4E-BP1 in (A) Ishikawa and (B) ECC-1 cells. The cells were treated with rapamycin (1 nM), paclitaxel (1 nM) or both in combination for 24–48 hours. Rapamycin alone and in combination with paclitaxel decreased phosphorylation of 4E-BP1 and pan-4E-BP1. Treatment with paclitaxel alone had no effect on the phosphorylation of 4E-BP1 in either cell line (C & D). Phosphorylated 4E-BP1, pan-4E-BP1 and α-tubulin were determined by Western immunoblotting.

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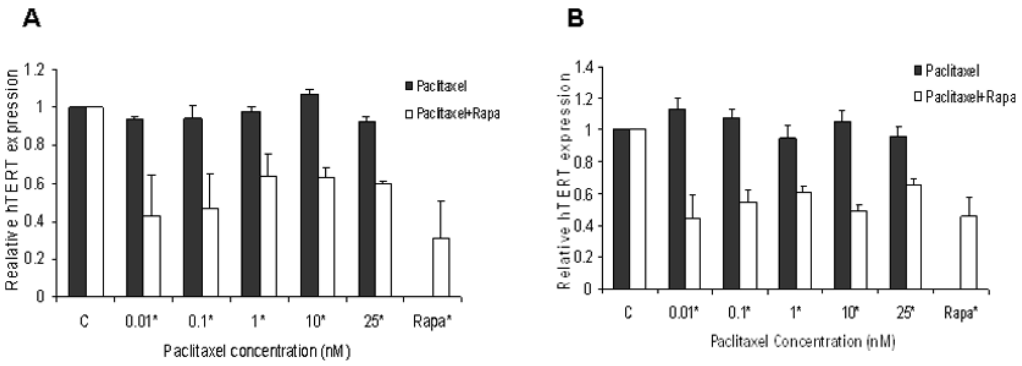

Figure 5.

Paclitaxel did not affect hTERT mRNA expression in the (A) Ishikawa and (B) ECC-1 cell lines. Treatment with rapamycin alone ($p = 0.04$ for Ishikawa cells; $p = 0.0067$ for ECC-1 cells) and in combination with paclitaxel ($p = 0.05–0.06$ for Ishikawa cells; $p = 0.01–0.04$ for ECC-1 cells) resulted in decreased hTERT mRNA expression. Both cell lines were cultured for 24 hours and then treated with the indicated concentrations of paclitaxel alone or in combination with rapamycin (1 nM) for 48 hours. hTERT expression was determined by real-time PCR. The results are shown as the mean ± SE of two independent experiments (* indicates statistically significant difference).

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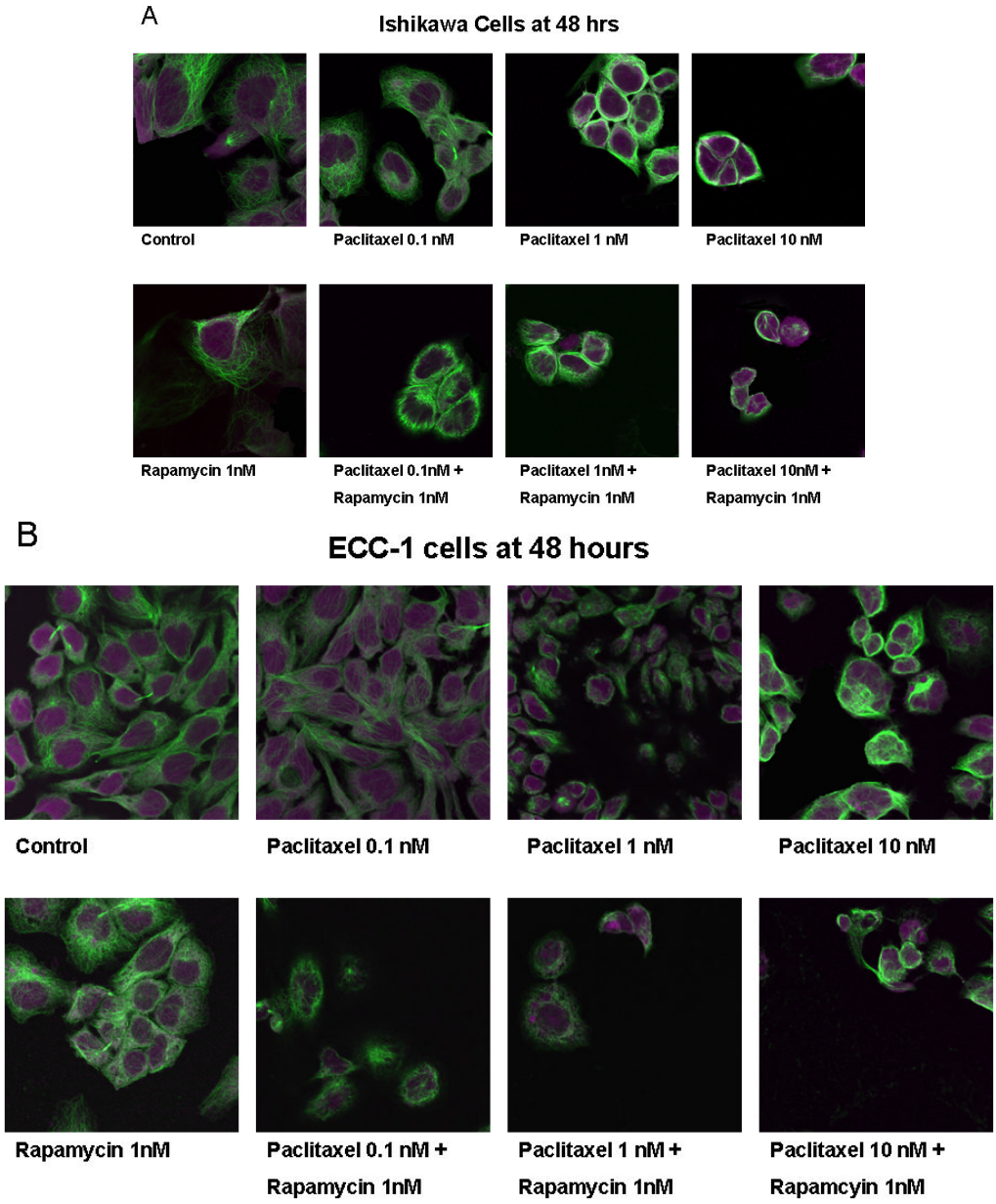

Figure 6.

Alterations in cellular microtubule structures resulting from exposure to paclitaxel and rapamycin in (A) Ishikawa and (B) ECC-1 cell lines. Microtubules were visualized by immunoflourescence using a primary antibody for α–tubulin. The combination of paclitaxel with rapamycin appeared to potentiate the effect of paclitaxel alone on α-tubulin polymerization and organization in both cell lines.

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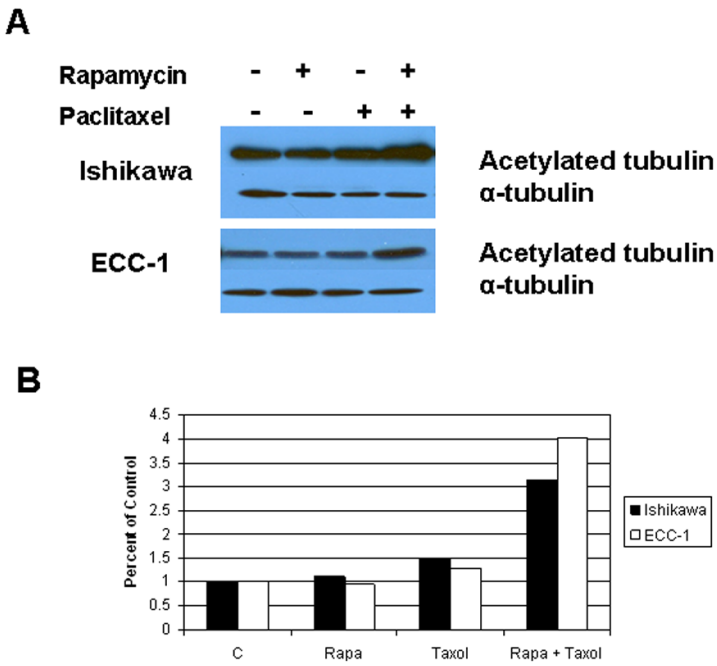

Figure 7.

The effect of paclitaxel and rapamycin on tubulin acetylation by Western immunoblotting. Ishikawa and ECC-1 cells were treated with paclitaxel (0.1uM), rapamycin (1 uM) or both in combination for 24 hours. (A) Increased tubulin acetylation was found in cells treated with both paclitaxel and rapamycin. (B) As quantified by densitometer analysis, treatment with paclitaxel and rapamycin increased acetylated tubulin expression by 3.1 and 4.0 fold for the Ishikawa and ECC-1 cell lines, respectively. Paclitaxel alone increased tubulin acetylation by 1.5 and 1.3 fold for the Ishikawa and ECC-1 cell lines, respectively. Rapamycin alone had no effect on acetylated tubulin. No effect was seen on α-tubulin expression.

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Table 1

Combination Index (CI) Values for ECC-1 and Ishikawa Cell Lines Treated with Paclitaxel and Rapamycin.

Paclitaxel (nM)	Rapamycin (nM)	CI for ECC-1 cells	CI for Ishikawa cells
0.0001	1	0.24	NA*
0.001	1	0.25	0.241
0.01	1	0.68	NA*
0.1	1	NA*	0.493
0.5	1	0.91	0.074
1	1	0.92	0.131
5	1	0.92	0.657
10	1	NA*	0.795

*Not applicable (NA) – the CI was not calculated for this dose.