RESEARCH COMMUNICATION

Combination Effects of Paclitaxel with Signaling Inhibitors in Endometrial Cancer Cells

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Abstract

This study was conducted to evaluate and compare molecular and cellular effects of paclitaxel in combination with epidermoid growth factor receptor (EGFR) or/and mammalian target of rapamycin (mTOR) inhibitors with two endometrial cancer lines HEC-1A and Ishikawa. Treatment was with the EGFR inhibitor RG14620, the mTOR inhibitor rapamycin, and the conventional cytotoxic drug paclitaxel, alone or in combination. The 50% inhibitory concentration (IC50) and cell viability were determined by the MTT assay. Multiple drug effect/combination indexes (CI) analysis was applied to assess interactions between paclitaxel and the two inhibitors. Apoptosis and cell cycling were evaluated by flow cytometry analysis. Western blotting was performed to evaluate the related protein alteration in PI3K/AKT signaling pathway. RG14620, rapamycin and paclitaxel showed obvious dose-dependent growth inhibition with time. The IC50 of paclitaxel at 24 hours decreased significantly when pretreated with low doses of RG14620 and Rapamycin alone or in combination. Moreover, combination index (CI) of paclitaxel with each inhibitor was larger than 1, indicating a synergistic effect between pairs of drugs in these two cell lines. FACS analysis showed the cell apoptosis rate increased with a synergistic effect. On Western blotting, activation of PI3K/AKT pathway was detected in both two cell lines in the control case. When paclitaxel was used as a single-agent or in combinations, the protein expression of PI3K/AKT pathway totally abated, especially in HEC-1A cells, suggesting a role in chemoresistance. The combination of three drugs induced the greatest over-expression of caspase-3. Combining targeted inhibitors with cytotoxic chemotherapy appears to be a promising strategy for the effective treatment of endometrial cancer which merits further clinical investigation.

Keywords: Endometrial cancer - PI3K/AKT pathway - inhibitor - paclitaxel - combination therapy

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Introduction

Endometrial cancer is the most common gynecologic cancer and ranks as the fourth most common neoplasm and the eighth leading death cause of cancer in females. (Amant et al., 2005). Although lots of improvement had been made in the treatment of endometrial cancer, the overall survival of patients has not significantly improved because considerable refractory to these therapies. Compared to other gynecologic malignancies, endometrial cancer has relatively poor chemo-sensitivity.

Paclitaxel is an antimicrotubule agent which is the most single chemotherapeutic agents. Paclitaxel binds to the h-tubulin subunit and stabilizes the microtubules, resulting in disruption of normal microtubule dynamics during cell division (Diaz and Andreu, 1993). Failure of microtubule separation during the G2-M phase blocks cell mitosis and results in apoptosis. The overall response rate of paclitaxel is 35.7% when used in advanced or recurrent endometrial cancer (Ball et al., 1996; Humber et al., 2007). Unfortunately, therapeutic dose of paclitaxel often result in serious adverse effects including myelosuppression, gastrointestinal toxicity, and peripheral neuropathy (Lincoln et al., 2003).

Another potential strategy for the treatment of advanced and/or recurrent human cancers is using molecular targeting agents to against particular signaling molecules. The PI3K/Akt pathway plays a central role in maintaining the aggressive malignant phenotype of endometrial cancer, and its activation mediates treatment resistance by promoting cellular survival and inhibiting the induction of apoptosis. The apparent dependence of cancer cells on this pathway makes it an attractive target for new therapies. The most common strategies used to block this pathway are EGFR- inhibitors and mTOR-inhibitors. Activation of EGFR signaling in tumor cells will promote its proliferation, angiogenesis, and metastasis and decrease apoptosis, collectively making EGFR as an attractive target for anticancer therapy (Pfeiffer et al., 1997). It is reported that EGFR over-expressed in 43–67% endometrial cancer patients and the disease-free and overall survival rate of these patients was...
significant the stimulus for PI3K activation. Preclinical data from various studies support that combining targeted therapeutics against growth factor receptors or downstream signal mediators of the PI3K pathway; especially mTOR (Jimeno et al., 2007). mTOR is a central integrator of multiple signal transduction effectors, including the Ras/ MAPK and PI3K pathways. And it is also the monitor’s environmental factors, such as nutrient status (ATP, amino acids and glucose levels); serving to integrate mitogenic signals with protein synthesis and cell growth (Asnaghi et al., 2004; Vignot et al., 2005). mTOR has been extensively evaluated in malignant tumor. However, trials using targeted therapeutics against growth factor receptors or downstream signal mediators of the PI3K pathway alone have demonstrated only modest clinical benefit (Oza et al., 2008; Slivovitz et al., 2010; Dedes et al., 2011). It is considered that single-agent inhibition may induce tumor cells to activate alternative pathways (Chaturvedi et al., 2009; Zuo et al., 2010). Multiple strategies to inhibit PI3K/AKT pathway have been proposed and are in ongoing clinical trials (Buck et al., 2006; Fainvre et al., 2006; Masiello et al., 2007; Wang et al., 2007).

Antagonism of cell surface receptor and nonreceptor kinases could potentially disrupt the PI3K pathway by eliminating the stimulus for PI3K activation. Preclinical data from various studies support that combining targeted inhibitors with cytotoxic chemotherapy or other novel agents could effectively inhibit tumor growth and overcome intrinsic resistance to single-agent therapy (Dancey and Chen, 2006). Combining cytotoxic agents with an inhibitor of the PI3K pathway to overcome resistance has been shown to be effective in preclinical testing (de Groot et al., 2008; Nakajima et al., 2010; Ren et al., 2010). The future of personalized cancer therapy based on the characteristics of individual tumors will probably reside in efficacious and rational combinations of targeted and traditionally used agents and therapeutic modalities. Unfortunately, the molecular determinants of response and outcome from these combinations are not known and are only now being evaluated retrospectively.

So we intended to investigate the effects of cytotoxic agent-paclitaxel in combination with an EGFR inhibitor -RG14620 or/and an mTOR inhibitor-rapamycin functioned in different PTEN status endometrial cancer cells HEC-1A (PTEN- positive) and Ishikawa (PTEN-negative). Furthermore, we evaluated whether an activation of the PI3K/AKT/mTOR pathway might be detectable in endometrial cancer cells so as to potentially define biomarkers that might serve to select patients who may profit from a therapy with targeted inhibitors.

Materials and Methods

Cells and reagents

The human endometrial malignant tumor cell lines Ishikawa and HEC-1A were gifted from Dr Zhimin Li (Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College, HUST, China). DMEM and 10% fetal bovine serum were purchased from Gibco Co. (USA). Paclitaxel was purchased from Sigma (St. Louis, MO) and reconstituted in DMSO. RG-14620 (Enzo Life Sciences International, USA), Rapamycin (Cell Signaling Technology, USA) were soluted and stored in DMSO. Rabbit anti-phospho-EGFR, anti-EGFR, anti-phospho- mTOR, anti-mTOR, anti- phospho-AKT, anti-AKT, anti-caspase-3 were from Cell Signaling Technology (USA); Mouse anti-PTEN was from Santa Cruz Biotechnology (USA).

Cell viability assay

Cell viability was measured by the 3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) method. HEC-1A and Ishikawa cells were incubated in 96-well plates at a density of 5×103 cells per well in DMEM with 10% fetal bovine serum (FBS) for 24h. To examine the growth inhibitory effects of RG14620, rapamycin and paclitaxel, cells were exposed to fresh medium containing various concentrations(RG14620: 0.1, 1, 10, 20, 50, 100, 200µmol/l; Rapamycin: 0.1, 1, 10, 20, 50, 100, 200nmol/l) for up to 72h. To explore the combination effects, after being pretreated with RG14620 or rapamycin, singly or in combination at the recommended doses for 24 h, cells were treated with different concentrations of paclitaxel (0.1, 1, 5, 10, 20, 50, 100µg /ml) for another 72 h. At the time point, 20µl of sterile MTT dye (5 mg/ml; Sigma-Aldrich Corp) was added to one plate and incubated for another 4h at 37°C. Then the supernatant was discarded and a 150µl of dimethyl sulfoxide was added to each well and thoroughly mixed for 10 min. Spectrometric absorbance at a wave length of 492 nm was measured on an enzyme immunoassay analyzer (model 680; Bio-Rad, American). IC50 values were calculated from the linear regression line of the plot of percentage inhibition versus log inhibitor concentration. The IC50 values were calculated using SigmaPlot (Systat Software, Point Richmond, CA) sigmoidal dose response equation with variable slopes. Means and SEs were calculated from at least three experiments. The results of MTT assay represented the average of three individual experiments.

The combination index (CI) of different drugs

The combination index(CI) was calculated using the following equation: combination index = (Am)50/(As)50 + (Bm)50/(Bs)50, where (Am)50 is the concentration of drug A necessary to achieve a 50% inhibitory effect in the combination; (As)50 is the concentration of the same drug that will produce the identical level of effect by itself; (Bm)50 is the concentration of drug B that will produce a 50% inhibitory effect in the combination; and (Bs)50 is the concentration of drug B that will produce the same level of effect by itself. Combination index > 1 indicates antagonism; combination index < 1 indicates synergy; and combination index=1 indicates an additive effect (Ollikainen et al., 2007).

Apoptosis analysis

After Ishikawa and HEC-1A cells were incubated in 6-well plates at a density of 3x10^3 cells per well in DMEM with 10% FBS for 24h, cells were pretreated with a 10 µmol/L RG14620 and 10nmol/l rapamycin for 24 hours respectively, before incubation with 6µg/ml paclitaxel for another 24 hours. Cells were subjected to apoptosis
Results were analyzed using SPSS software 11.0 and compared using one-way analysis of variance (ANOVA) with Fisher’s post hoc test. Data were presented as mean ± standard deviation (SD) of separate experiments (n ≥ 3). P values less than 0.05 were considered to be significant.

**Results**

**Antineoplastic effect of RG14620, rapamycin and paclitaxel on endometrial cancer cells**

RG14620, rapamycin and paclitaxel showed grow inhibition effect in both two endometrial cancer cell lines single. RG14620, rapamycin and paclitaxel significantly induced cell death in a time and dose-dependent manner as measured by MTT assay after 72 h of treatment (Figure 1). At 48-hour time point, the IC50 of RG14620 for Ishikawa cells was 139.5±19.2 μmol/L compared to 28.0±3.3 μmol/L for HEC-1A cells after these two cell lines were exposed to a single dose. At 24-hour time point, the IC50 of paclitaxel for Ishikawa cells was 29.3±5.8 μg/ml compared to 20.3±3.8 μg/ml for HEC-1A cells after these two cell lines were exposed to a single dose. At 48-hour time point, the IC50 of rapamycin for Ishikawa cells was 32.4±4.2nmol/L compared to 179.7±22.8 nmol/L for HEC-1A cells after these two cell lines were exposed to a single dose.

**Cell viability effect of combination treatment of paclitaxel and inhibitors**

Cells were pretreated with a single dose of 10 μmol/L RG14620 or/and 10nmol/l rapamycin for 24 hours before incubation with paclitaxel for another 24 hours. In HEC-1A cells, The IC50 of paclitaxel decreased from 20.3±3.8 μg/ml to13.6±2.5ug/ml in HEC-1A cells and from 29.3±5.8 μg/ml to 16.7±3.3ug/ml in Ishikawa cells when these cells were pretreated with RG14620, P<0.05. IC50 decreased from 20.3±3.8 μg/ml to 15.2±1.9ug/ml in HEC-1A cells and 29.3±5.8 μg/ml to 13.9±2.1 μg/ml in Ishikawa cells when pretreated with rapamycin.

IC50 decreased sharply from 20.3±3.8 μg/ml to 7.5±1.0 μg/ml in HEC-1A cells and 29.3±5.8 μg/ml to 9.1±1.2 μg/ml in Ishikawa cells (P<0.05) when were

![Figure 1. Influence of RG14620 and Rapamycin on the Cytotoxic Effect of Paclitaxel in Both Cancer Cells](image-url)
pretreated with RG14620+rapamycin.

The combination index (CI) of paclitaxel and RG14620, paclitaxel and rapamycin were all less than 1 (Table 2) in both two cell lines, suggesting a synergistic effect between every two drugs in this two cell lines.

Cells were pretreated with a single dose of 10 umol/L RG14620 or/and 10nmol/l rapamycin for 24 hours before incubation with 6ug/ml paclitaxel for another 24 hours. Cell viability curves were performed for each single chemotherapeutic drug and in combinations (Figure 1 and 2). The result indicated that the inhibitors can decrease the proliferation of both Ishikawa and HEC-1A cells and increase the cells’ sensitivity to paclitaxel treatment.

**Cell apoptosis induced by paclitaxel and targeted inhibitors combination treatments**

FACS analysis was performed to detect DNA fragmentation in apoptotic cells following combined use of inhibitors and paclitaxel in HEC-1A and Ishikawa human endometrial cancer cells. Paclitaxel, RG14620 and rapamycin were independently successful in inducing apoptosis to varying degrees in both two cancer cells compared with control cells (Percentages of apoptotic cells: Ishikawa: from 5.06±1.1% to 33.26±5.2%, 11.49±2.7%, 16.54±4.3%, respectively, P<0.05; HEC-1A: from 9.68±3.7% to 38.63±5.9%, 22.53±4.6%, 19.87±5.1%, respectively, P<0.05). Compared with single treatment, the combination of paclitaxel with RG14620, paclitaxel with rapamycin, paclitaxel with both RG14620 and rapamycin induced a significant (p < 0.05)increase amount of apoptotic death(Figure 3) in both two cell lines, suggesting that an synergistic effect of apoptosis developed in the cells being treated with paclitaxel and targeted inhibitors. Moreover, as a single chemotherapy, paclitaxel and RG14620 might be more efficacious in HEC-1A cells (PTEN-positive) than in Ishikawa cells (PTEN-negative), but for rapamycin, the growth inhibition effect that observed in this study in HEC-1A cells was much weaker than in Ishikawa cells (comparison HEC-1A vs. Ishikawa for each treatment, P < 0.05).

Paclitaxel induced a significant increase amount of apoptotic death(p < 0.05) after pretreated with RG14620 and rapamycin in both two cell lines, suggesting that an synergistic effect of apoptosis developed in the cells being treated with paclitaxel and targeted inhibitors. Untreated cells served as a negative control.

**Synergistic effects of targeted inhibitors and paclitaxel on cell cycle analysis**

In HEC-1A cells, when RG14620 used as a single agent, the percentage of cells in G1 phase increased from 71.97±4.45% to 77.03±3.89% (P<0.05). However, the G1 phase cells induced by rapamycin only increase from 71.97±4.45% to 75.36±4.34 % (P>0.05). Paclitaxel blocked the HEC-1A cell cycle at the G2-M phase but RG14620 or rapamycin did not show effect on the G2-M phase. Paclitaxel increased the percentage of cells at G2-M phase from 11.73±2.1% to 16.65±3.2% (P<0.05) compared with control cells (Figure 4). The percentage of cells in G2-M phase increased significantly from 16.65±3.2% to 23.80±3.4% (P<0.05). When paclitaxel pretreated with RG14620 compared to single treatment. However, paclitaxel did not show obviously effect with pretreatment of rapamycin (16.65±3.2% to 17.59±2.7%, P> 0.05). Paclitaxel showed a greater increase in the population of cells that were in G2-M phase from 16.65±3.2% to 29.8±4.2 % after pretreating with two inhibitors together (P<0.05). Combination of paclitaxel with both two inhibitors exerted synergistic effects on cell cycle progression.

Paclitaxel alone or in combination with two inhibitors blocked theIshikawa endometrial cancer cell cycle at the G2-M phase but RG14620 or rapamycin show effect on the G1 phase. In Ishikawa cells, although there were no significant differences, RG14620 (53.16±6.2%, P>0.05) and rapamycin (54.46±7.1%, P>0.05) could increase...
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The G1 population separately in Ishikawa cancer cells compared to control cells (50.85±6.9%) (Figure 5). It is worth noting that compared with paclitaxel (41.87±4.9%) used alone, the combination treatment of paclitaxel with RG14620 (61.62±4.5%, P<0.05), paclitaxel with rapamycin (72.53±5.4%, P<0.05), paclitaxel with RG14620 and rapamycin (84.97±6.7%, P<0.05) produced both a greater percentage of S and G2-M phase cells, suggesting a synergistic effect of the combination therapy on cell cycle progression.

Protein alteration in PI3K/AKT signaling pathway after treatment

Ishikawa cells lacking PTEN protein showed stronger level of EGFR, AKT, p-AKT, downstream mTOR, p-mTOR expression on contrast to HEC-1A cells (Figure 6). It suggests that PI3K/AKT pathway do exist in HEC-1A and Ishikawa endometrial cancer cells.

After a single dose of 10 umol/L RG14620 treatment for 48h, Western blot analysis showed that there was obvious decrease in EGFR, p-EGFR and total AKT expression in both two cell lines, its downstream mTOR expression in HEC-1A cells decreased slightly while there was no significant change in Ishikawa cells; p-AKT, p-mTOR remained stable in both two cell lines throughout the experiment.

After a single dose of 10 nmol /L rapamycin treatment for 48h, the expression of AKT, mTOR, p-mTOR all decreased in both two cell lines, in contrast, EGFR, p-EGFR, p-AKT increased while might compensate for the anti-proliferative function of rapamycin. Results showed that rapamycin inhibits the growth of Ishikawa cells and to a lesser extent in the growth of HEC-1A cells. In combination treatment of RG14620 and rapamycin, EGFR, p-EGFR, total AKT, mTOR, p-mTOR decreased significantly in both two cell lines, a more significant expression was observed in the combination therapy.

The in vitro sequence-specific functional inhibition of paclitaxel in endometrial cancer cells leads to increased caspase levels, followed by cell death. Both inhibitors and paclitaxel treatment alone depressed viability and caused caspase-3 up-regulation in both cell lines, a more significant expression was observed in the combination therapy.
**Discussion**

PI3K/Akt pathway plays a central role in maintaining the aggressive malignant phenotype of endometrial cancer, and its activation mediates treatment resistance by promoting cellular survival and inhibiting the induction of apoptosis (Xu et al., 2004). Growth factors (such as EGFR) stimulation of PI3K activity leads to AKT activation. Conversely, PI3K inhibition and PTEN results in inhibition of AKT.

In studies on mTOR inhibitor rapamycin, it was observed that the inhibitory efficacy of mTOR was maximal when cells exhibited no PTEN expression and therefore the AKT pathway was constitutively activated (Dedes et al., 2010). On the other hand, when cells showed high PTEN expression and therefore decreased AKT signaling, rapamycin had hardly effect on the proliferation of these cells. In this study, Ishikawa cells lacking PTEN protein showed stronger level of AKT, phosphorylation-AKT should be much sensitive to rapamycin treatment than HEC-1A cells and it is demonstrated by our findings. However, mTOR antagonists used as single agents are not likely to result in ideal responses, so that it is necessary to identify prospective agents that might be useful in combination. New insights into mechanisms of resistance to mTOR inhibitors have renewed interest in combination strategies to target the PI3K cascade and other pathways, as well as to use agents that block both PI3K and Mtor(Doherty et al., 2006). Based on the suppose that multikinase inhibitors that block multiple targets could potentially disrupt the PI3K pathway by eliminating the stimulus for PI3K activation, we treated HEC-1A and Ishikawa cancer cells with an mTOR inhibitor-rapamycin, or an EGFR inhibitor-RG14620, alone or in combination. As our study showed that although EGFR antagonists used in this study had minor anti-proliferative effects when used alone, once in combination, RG14620 demonstrated a significant increase in their anti-proliferative effects in the presence of rapamycin. However, it is still stressful that as a sequence of P-AKT increased obviously compared to single agent treatment, PI3K pathway will be activated again leading to treatment resistance by promoting cellular survival and inhibiting the induction of apoptosis even the combination of RG14620 and rapamycin.

Recent data suggest that combining targeted therapies with chemotherapy may counteract drug resistance. We have conducted a series of in vitro studies to investigate the therapeutic effects of inhibitors alone and in combinations with paclitaxel on these cell lines. Datas showed that when cells were pretreated with a low dose of RG14620 or rapamycin for 24 hours before incubation with paclitaxel, the IC50 of paclitaxel in both two cell lines reduced significantly, indicates that inhibitors enhances the cytotoxic effect on cancer cell growth. Inhibitors have also been shown to be synergistic with paclitaxel in the inhibition of cell growth, in the arrest of the cell cycle at the G2-M phase, and in the induction of apoptosis. As a combinational treatment, dosing and scheduling are important issues. We have shown that a single dose of 1/3 or 1/10 of inhibitors and 1/2-3 of paclitaxel IC50 values can achieve a 50% growth inhibition. Moreover, treatment of paclitaxel with both two inhibitors caused a notably down-regulation expression of the whole PI3K/AKT pathway. Thus, we may draw a conclusion that adding inhibitors to a paclitaxel regimen will significantly reduce the required dose of paclitaxel. Administrations of inhibitors may further reduce adverse effects of paclitaxel and possibly delaying the emergence of resistance.

The results from this study provide new rationales for novel combinational therapies using two signaling inhibitors to synergistically cooperate with paclitaxel in PTEN-positive and PTEN-negative cancer cell lines. As we see in this study, to be a single chemotherapy, paclitaxel and RG14620 might be more efficacious in HEC-1A cells(PTEN-positive) than in Ishikawa cells (PTEN-negative), but for rapamycin, the growth inhibition effect that observed in this study in HEC-1A cells was much weaker than in Ishikawa cells. These findings prompt the need for future evaluation of the therapeutic efficacy of PI3K/AKT-based combinational therapy in targeting high-grade/malignant tumors that with different PTEN status.

Taken together, these data indicate that the PI3K/AKT pathway is a critical target for cancer intervention in human endometrial carcinoma. Overall, these findings render the combined use of PI3K inhibitors with conventional cytotoxic drugs, a promising new strategy for cancer chemotherapy that should improve the response rate in resistant tumors which affect a large percentage of cancer patients.

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**References**


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MicroRNA-21 inhibitor sensitizes human glioblastoma cells U251 (pten-mutant) and Ln229 (pten-wild type) to taxol. BMC Cancer, 10, 27.


Wang LH, Chan JL, Li W (2007). Rapamycin together with herceptin significantly increased anti-tumor efficacy compared to either alone in erbB2 over expressing breast cancer cells. Int J Cancer, 121, 157-64.

