# Reduced BCL2 and CCND1 mRNA expression in human cervical cancer HeLa cells treated with a combination of everolimus and paclitaxel

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**Aim of the study:** Cervical cancer is the second most common malignancy in women worldwide. Everolimus displays direct effects on growth and proliferation of cancer cells via inhibition of mammalian target of rapamycin (mTOR) protein, which is known to be associated with drug resistance. In this study, we aimed to investigate the effects of everolimus, gemcitabine, and paclitaxel in terms of cell viability and mRNA expression levels of GRP78, CCND1, CASP2, and BCL2 genes.

**Material and methods:** HeLa cells were treated with different doses of everolimus, gemcitabine, and paclitaxel. Cell viability was assessed using MTT assay, and obtained dose response curves were used for the calculations of inhibitory concentration (IC) values. At the end of the treatment times with selected doses, RNA isolation and cDNA synthesis were performed. Finally, GRP78, CCND1, CASP2, and BCL2 genes mRNA expression levels were analysed using quantitative PCR.

**Results:** The IC50 value of everolimus was 0.9 μM for 24-hour treatment. Moreover, the IC50 value of gemcitabine and paclitaxel was found to be around 18.1 μM and 7.08 μM, respectively. Everolimus, gemcitabine, and paclitaxel treatments alone did not change the GRP78, CCND1, BCL2 and CASP2 mRNA expression levels significantly. However, combined treatment of everolimus and paclitaxel significantly reduced BCL2 and CCND1 mRNA expression ($p < 0.05$). In contrast, this combination did not change GRP78 and CASP2 mRNA expression levels ($p > 0.05$).

**Conclusions:** Down-regulation of CCND1 and BCL2 expression may be an important mechanism by which everolimus increases the therapeutic window of paclitaxel in cervical cancers.

**Key words:** everolimus, gene expression, HeLa cells, paclitaxel, unfolded protein response, GRP78, BCL2, CCND1, CASP2.

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## Introduction

Cervical cancer is the second most common malignancy in women worldwide, and it remains a leading cause of cancer-related death for women in developing countries [1]. Although, the most pivotal aetiological agent is human papillomavirus (HPV) in the development of cervical cancer, extra molecular changes are also defined in the pathogenesis of cervical cancer [1, 2]. In recent years, autophagy has appeared as a substantial biological mechanism in targeting human cancers, including carcinoma of the cervix [3].

Autophagy, also known as type II programmed cell death, is one of the homeostatic and evolutionarily conserved processes found in cells. Activation of autophagy leads to the targeting of cellular proteins and organelles to lysosomes for degradation [4]. By impeding the increase of impaired proteins and organelles, autophagy prevents tumour formation. On the other hand, it can also encourage the growth of developed tumours via acting as a cell survival mechanism. Unfolded protein response (UPR) after endoplasmic reticulum (ER) stress and the mammalian target of rapamycin (mTOR) signalling pathway are two potent regulators of autophagy [5].

Endoplasmic reticulum stress, which results from accumulation of unfolded, misfolded, or damaged proteins in ER lumen, activates autophagy directly through upregulation of *GRP78* and through mechanisms downstream of *GRP78* release of the other UPR signal transducers [6]. *GRP78* was shown to be obligatory for autophagosome formation by gene knockdown experiment [7].

mTOR is activated downstream of phosphoinositide 3-kinase (PI3K)/ v-akt murine thymoma viral oncogene homolog (AKT) pathway. By associating several proteins, mTOR forms two different protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Predominantly, mTORC1 regulates cell growth, cell cycle progression, macromolecule biosynthesis, and autophagy. However, mTORC2 controls cell survival and metabolism, as well as organisation of cytoskeleton [7, 8].

Unusual activation of PI3K/AKT/mTOR signalling pathway has frequently been found in several cancers from different origins, including cervical tumours [10]. It has been associated with resistance to chemotherapeutics and worst prognosis [11]. Upregulation of *PI3K* gene in cervical cancer cell line (HeLa) was shown, and administration of PI3K inhibitor (LY294002) blocked cell proliferation [12].

It is known that a natural mTOR inhibitor named rapamycin and its analogues (rapalogues), including everolimus, selectively target mTORC1 to stimulate autophagy [5]. Studies have revealed striking clinical therapeutic

results for rapalogues in several tumours as single-agent therapies or in combination with other therapeutics. Thus, a combination of mTOR inhibitors and classical chemotherapeutics was suggested as an effective approach to treat cancer, based on remarkable findings obtained from both preclinical studies and clinical observations [13, 14].

mTOR inhibition displays direct effects on the growth and proliferation of cancer cells via downregulation of genes having roles in cell cycle progression, such as cyclin D1 (CCND1) and cyclin dependent kinase 4 (CDK4) [15]. There is also consistent evidence that the AKT/mTOR signalling pathways may be associated with resistance to taxanes and other drugs acting on microtubules [16]. For this reason, *in vivo* combinations of everolimus with paclitaxel showed additive and synergistic effects in tumour models [17].

Some cytoskeletal disrupting agents, including paclitaxel, have potent anticancer activities against a variety of tumours from different origins [18]. These agents kill cancer cells eventually by inducing apoptotic pathway, but the mechanism is not clearly understood. However, some studies have underlined the proapoptotic function of caspase-2 (CASP2) [19, 20] and the antiapoptotic function of B-cell CLL/lymphoma 2 (*BCL2*) molecules in cell death induced by cytoskeletal disruption [21–23].

In this study, we aimed to investigate the effects of everolimus, gemcitabine, and paclitaxel on cervical cancer cell line (HeLa) viability. Moreover, changes of relative mRNA expression levels of *GRP78*, *CCND1*, *CASP2*, and *BCL2* genes were also investigated after administration of these agents, alone or in combination, to the cells by real-time PCR analysis.

## Material and methods

### Cell culture

HeLa cells (HÜKÜK #90061901) were obtained from the Animal Cell Culture Collection in the Foot and Mouth Disease Institute, Turkey. Cells were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS), 200 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin (all obtained from HyClone, USA) in a humidified atmosphere containing 5% $CO_2$ at 37°C.

### Cell viability assay

Cell viability was assessed using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. Cells were seeded into 96-well plates at a density of $1 \times 10^4$ cells/well in medium containing 1% FBS and incubated overnight to attach. Then the cells were treated with 10% FBS medium containing various concentrations of everolimus (25–500 nM), gemcitabine (0.125–8 µM), and paclitaxel (25–500 nM) and incubated for 24 hours (all obtained from Sigma-Aldrich, USA). At the end of the treatment times MTT mixture was added to each well and incubated for an additional four hours. Then the medium was discarded and formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance values were measured at 570 nm using a Spectramax M3 microplate reader (Molecular Devices, USA). Control wells for absorbance readings contained DMSO and MTT mixture. All experiments were repeated five times for each dose and the mean absorbance values were calculated. The inhibitory concentration values ($IC_{10}$, $IC_{25}$, $IC_{50}$, $IC_{75}$, and $IC_{90}$) for each agent were calculated from the dose-response curves by means of GraphPad Software PRISM 5 (GraphPad Software, USA).

After analysis of the response of the cells to the agents, selected doses of agents (400 nM for everolimus, 8 µM for gemcitabine, and 100 nM for paclitaxel) were given to the cells as single agent or in dual combinations (i.e. everolimus + gemcitabine and everolimus + paclitaxel) and incubated for 24 hours to perform mRNA expression analysis.

### RNA isolation and cDNA synthesis

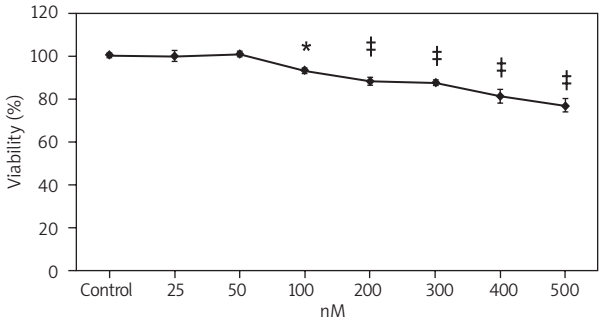
At the end of the incubation time, total RNA was isolated using High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. The quantity and quality of the isolated RNA was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). One microgram of total RNA from each sample was reverse transcribed with random hexamers using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The cDNA reaction was performed in an Eppendorf Mastercycler EP gradient S thermal cycler (Eppendorf, Hamburg, Germany).

### Real-time quantitative PCR

Real-time PCR was carried out using a Light Cycler® 480 (Roche Diagnostics GmbH, Mannheim, Germany). Light Cycler® 480 Probe Master reaction mix in combination with human Universal Probe Library (UPL) probes was used to determine gene expression levels (Roche Diagnostics, Mannheim, Germany). Expression levels of the target genes were normalised to mRNA level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene. Intron-spanning primers and specific probes for *GRP78*,

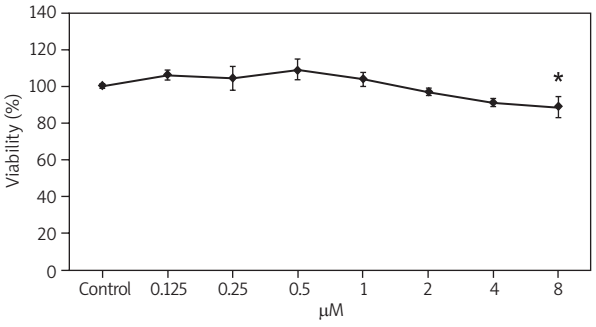
**Table 1.** Primer sequences and probe numbers used in real-time quantitative PCR for the detection of target mRNAs.

| Gene | mRNA accession number | Forward primer | Reverse primer | UPL probe number |
|---|---|---|---|---|
| *GRP78* | NM_005347 | 5'-ACTGTTACAATCAAGGTCTATGAAGG-3' | 5'- CAAAGGTGACTTCAATCTGTGG-3' | 15 |
| *BCL2* | NM_000633 | 5'-AGTACCTGAACCGGCACCT-3' | 5'-GGCCGTACAGTTCCACAAA-3' | 75 |
| *CASP2* | NM_032982 | 5'-CGCCATCTATGGTGTGGAT-3' | 5'-CAGTTGGCGTTGTCAAAGAG-3' | 78 |
| *GAPDH* | NM_002046 | 5'-AGCCACATCGCTCAGACAC-3' | 5'-GCCCAATACGACCAAATCC-3' | 60 |



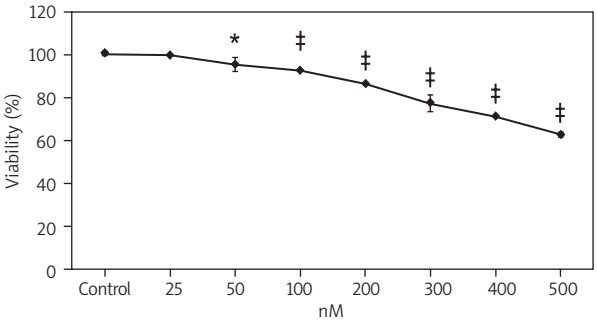

* indicates $p < 0.05$, ‡ denotes $p < 0.001$

**Fig. 1.** Effects on cell viability of everolimus treatment on HL-60 cells measured by MTT assay. The results are expressed as a percentage of control. Data shown are the means of five independent experiments




* indicates $p < 0.05$

**Fig. 2.** Effects of gemcitabine treatment on HeLa cell viability measured by MTT assay. The results are expressed as a percentage of control. Data shown are the means of five independent experiments




* indicates $p < 0.05$; ‡ denotes $p < 0.001$

**Fig. 3.** Effects of paclitaxel treatment on HeLa cell viability measured by MTT assay. The results are expressed as a percent of control. Data shown are the means of five independent experiments

*CCND1*, *CASP2*, *BCL2*, and *GAPDH* transcripts were selected using the online Universal Probe Library Assay Design Centre (http://www.universalprobelibrary.com). Primers and probes used in the real-time quantitative PCR for the detection of target mRNAs were given in Table 1. The quantitative polymerase chain reaction (qPCR) conditions were as follows: after initial denaturation at 94°C for 5 minutes, 45 cycles consisting of 95°C for 15 seconds, and 60°C for 20 seconds, and finally samples were cooled down to 40°C. The qPCR was repeated three times for each gene.

### Statistical analysis

Changes of *GRP78*, *CCND1, CASP2*, and *BCL2* mRNA expression levels were compared by Relative Expression Software Tool (REST 2009 v2.013) [24]. Other parameters were analysed by one-way analysis of variance (ANOVA) with Tukey's post hoc tests using SigmaStat 3.5 software. Data were expressed as the mean ± SD (standard deviation) from a representative experiment. *P* values lower than 0.05 were considered as significant.

## Results

The dose-response curve for everolimus-treated HeLa cells is given in Fig. 1. Everolimus did not show a significant growth inhibitory effect on HeLa cells at 25- and 50-nM concentrations for 24 hours ($p > 0.05$). However, cell viability was significantly affected at 100 nM everolimus concentration ($p < 0.05$). Furthermore, at doses higher than 100 nM, everolimus remarkably decreased cell viability ($p < 0.001$) and 76% of the cells were determined as viable at the highest dose used in our study (i.e. 500 nM). As shown in Table 2, the $IC_{50}$ value of everolimus was calculated as 907.5 nM (or 0.9 μM) for 24-hour treatment.

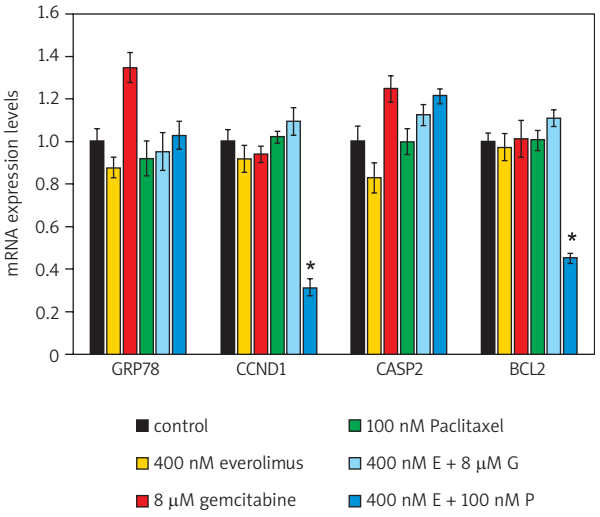
No significant viability changes were found for gemcitabine-treated cells at doses between 0.125 and 4 μM. On the other hand, the highest gemcitabine dose tested in our study significantly affected HeLa cell viability ($p < 0.05$), and 88% of the cells were determined as viable after treatment with 8 μM gemcitabine (Fig. 2). The $IC_{50}$ value of gemcitabine was found to be around 18.1 μM for this treatment duration (Table 2).

The most striking anti-proliferative effect was seen in paclitaxel-treated cells when compared to the everolimus- and gemcitabine-treated cells. Significant dose-dependent decreases in viability were obtained at 50 nM ($p < 0.05$) and ≥ 100 nM ($p < 0.001$) paclitaxel concentrations. After incubation with 500 nM paclitaxel for 24 hours, HeLa cell viability decreased to 62% compared to untreated cells (Fig. 3). The $IC_{50}$ value of paclitaxel was found to be about 708 nM (or 7.08 μM) as calculated from the concentration-response curve (Table 2).

**Table 2.** Calculated IC values of everolimus, gemcitabine, and paclitaxel

| | $IC_{10}$ | $IC_{25}$ | $IC_{50}$ | $IC_{75}$ | $IC_{90}$ |
|---|---|---|---|---|---|
| Everolimus (nM) | 224.9 | 451.7 | 907.5 | 1823.1 | 3662.5 |
| Gemcitabine (μM) | 4.9 | 9.4 | 18.1 | 35 | 67.7 |
| Paclitaxel (nM) | 141.3 | 316.4 | 708.8 | 1587.7 | 3556.3 |

In order to analyse whether treatment with these agents alone or in combination for 24 hours affected key molecules having roles in unfolded protein response, cell cycle regulation, and antiapoptotic and proapoptotic genes including *GRP78*, *CCND1*, *BCL2*, and *CASP2*, we evaluated changes in the expression of these genes using quantitative real-time PCR. The results of mRNA expression analysis of the *GRP78*, *CCND1*, *BCL2*, and *CASP2* genes are given in Fig. 4. We found that everolimus, gemcitabine, and paclitaxel alone did not significantly change the *GRP78, CCND1, BCL2, and CASP2* mRNA expression levels. Moreover, combined treatment with everolimus and gemcitabine did not significantly change mRNA expressions ($p > 0.05$). However, combined treatment of everolimus and paclitaxel significantly reduced *BCL2* and *CCND1* mRNA expression ($p < 0.05$). Conversely, this combination did not change *GRP78* and *CASP2* mRNA expression levels ($p > 0.05$). *CCND1* and *BCL2* mRNA expression were decreased to 3.2-fold and 2.2-fold, respectively, at 400 nM everolimus plus 100 nM paclitaxel combination as compared to the control cells ($p < 0.05$).




* indicates $p < 0.05$

E – everolimus; G – gemcitabine; P – paclitaxel

**Fig. 4.** Quantitative RT-PCR analysis results of *GRP78*, *BCL2*, *CASP2*, and *CCND1* mRNAs of HeLa cells treated with drugs for 24 hours. Gene expression levels were normalised to *GAPDH* mRNA expression level

## Discussion

Due to its antiapoptotic property, stress induction of *GRP78* mRNA represents an important pro-survival component of the UPR. Several agents have been shown to induce *GRP78* mRNA expression in cancer cells. Activation of mRNA synthesis of the *GRP78* gene in cell culture systems is considered as a sign for the presence of ER stress and the beginning of the UPR [25]. However, we did not show any significant changes for this gene in HeLa cells treated with everolimus alone or in combination with gemcitabine or paclitaxel. From this point of view, our results suggest that all the agents used in our study may not activate unfolded protein response at analysed concentrations.

The association of PI3K/mTOR signalling pathway in the development of cervical cancer was shown by analysing cervical tumour and non-neoplastic tissues. Researchers also indicated effective antigrowth effect of a PI3K inhibitor, LY294002, on HeLa cells [12]. In another study, PI3K inhibition was found to produce significant radiosensitisation in cervical cancer cells [26]. Similarly, rapamycin was shown to enhance the chemosensitivity of CaSki cells, derived from a cervical epidermoid carcinoma metastatic to the small bowel mesentery, to paclitaxel and cisplatin [27, 28]. In gastric cancer cell lines, everolimus had synergistic effects with 5-fuorouracil. Moreover, the growth inhibitory effect of everolimus was found to be related to G1 phase arrest and decreased expression of cyclins A, D, and E [29]. In HeLa cells, mTOR inhibition with rapamycin caused cell cycle arrest and increased accumulation of cells in G0/G1 [30]. In ovarian cancer cell lines (SK-OV-3 and IGROV1), treatment with rapamycin or everolimus was associated with a decrease in the expression of *CCND1* and *CDK4*, as well as G1 arrest. Moreover, *BCL2* expression was found to be a marker of resistance to programmed cell death induced by mTOR inhibitor in ovarian cancer cells [31]. In our study, we found that administration of everolimus, gemcitabine, and paclitaxel alone to the HeLa cells did not significantly change the mRNA expression of the *CCND1* and *BCL2* genes. However, combined treatment with everolimus and paclitaxel significantly diminished *CCND1* mRNA expression (about three-fold) (Fig. 4). With respect to *CCND1* in cell cycle regulation and its role in G1/S transition, this reduction can result in G1/S arrest.

Caspase-2 is considered as one of the initiator caspases, but its biological roles remain a matter of controversy [32]. *CASP2* gene deregulation in some cell lines suppress cell death pathway in response to antineoplastic drugs. The extent of caspase-2 involvement in apoptotic cell death depends on several of factors, including the nature of the cytotoxic agents, the origin of the cell type, and the intracellular abundance of caspase-2 relative to that of other pro-apoptotic molecules [33]. There have been a limited number of studies investigating the roles of *CASP2* in cancer development, including cervical cancer.

Significant elevation in caspase-2 activity was found in human cervical intraepithelial lesions, cervical cancer samples, and in the serum of donors, as compared to controls [32]. In our study, we showed the presence of *CASP2* mRNA expression in untreated HeLa cells. Moreover, mRNA expression pattern of *CASP2* did not significantly change after treatment with everolimus, gemcitabine, and paclitaxel alone or in combination. Although, *CASP2* was reported to be responsible for cell death induced by cytoskeletal poisons [20], we could not show any change in its mRNA levels after administration of paclitaxel alone or in combination with gemcitabine or everolimus.

In summary, mRNA expression of anti-apoptotic and proliferative genes did not change after paclitaxel alone, but *BCL2* and *CCND1* mRNAs expression decreased after a combination of paclitaxel with everolimus. Down-regulation of *CCND1* and *BCL2* expression may be an important mechanism by which everolimus increases the therapeutic window of paclitaxel in cervical cancer cells. A better un-

derstanding of the molecular response of the cells to both newly developed agents and chemotherapeutics will help to improve the therapy options of patients with cancer.

*The authors declare no conflict of interest.*

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