

P53, Bcl2 and Bax Expression and Apoptosis in Perifosine and Vitamin D-Treated Endometrial Cancer Cell Line (HEC1A) [†]

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[†] Presented at the 2nd International Cell Death Research Congress, Izmir, Turkey, 1–4 November 2018.

Published: 10 December 2018

Abstract: Endometrial cancer is the most common cancer of the female reproductive system. Perifosine has shown anti-tumor activity in a variety of cancers by inhibition of AKT phosphorylation. The active metabolite of vitamin D (1,25(OH)₂D₃) has anti-proliferative and pro-apoptotic properties and is recognized as an agent with the great potential for cancer intervention. Combination treatment with specific agents has been widely used as targeted therapy for cancer. In this study, we aimed to investigate the anti-apoptotic effect of varying concentrations of perifosine and vitamin D on endometrial cancer cell line (HEC1A). The expression levels of P53, BCL2 and BAX were assessed in cultured HEC1A cells at 10 and 30 µM concentrations of perifosine, 50 and 200 nM of vitamin D and combination groups using qRT PCR. Apoptosis evaluation in HEC-1A cells was performed using flow cytometry after 48 and 72 h of treatment with perifosine, vitamin D and combinations of both. According to the obtained data, treatment with different doses of perifosine and vitamin D significantly induced apoptosis in a time and dose-dependent manner in HEC-1A cells. The mRNA expression levels of P53, BAX, BCL2 in HEC-1A cells were found to be significant in the experimental groups as compared with the control. Perifosine displayed substantial anti-tumor activity in HEC-1A. These effects were increased with vitamin D. Therefore, perifosine and vitamin D combinations should be further evaluated in clinical studies in endometrial cancer.

Keywords: endometrial cancer; perifosine; vitamin D; apoptosis; P53; BCL2; BAX

1. Introduction

Endometrial carcinoma is the most common cancer of the female reproductive system, and it present as two different clinicopathologic conditions (an estrogen-dependent type and an estrogen-independent type) [1]. Perifosine is a synthetic alkylphospholipid which has demonstrated anti-tumor activity in a variety of cancers by inhibition of AKT phosphorylation [2]. It downregulates the anti-apoptotic mitogene activated protein kinase extracellular signal regulated kinase (MEK-ERK 1/2) pathway and activates the pro-apoptotic c-jun-N-kinase (JNK) network, therefore modulating the balance between the survival and death signaling cascades, thus inducing programmed cell death [3]. Vitamin D is a hormone that can be acquired from the diet or produced endogenously by a series of reactions that culminate in the most active metabolite of vitamin D, 1,25(OH)₂-vitamin D₃ (1,25(OH)₂D₃) [4]. The active metabolite of 1,25(OH)₂D₃ has anti-proliferative and pro-apoptotic features [2]. 1,25(OH)₂D₃ shows anti-proliferative effects in a variety of cancer cell types including cell lines derived from prostate, ovarian, endometrial and breast [5]. The anticancer mechanisms of

1,25(OH)₂D₃'s action contain induction of cell cycle arrest, promotion of differentiation, in addition to inhibition of invasive and migratory potential of cancer cells [6]. Apoptosis plays a part in the development and maintenance of homeostasis in the variety of tissues including the female reproductive tract. It is an important regulator in the reconstruction of endometrium during the menstrual cycle. Apoptosis is regulated by extracellular signals as well as an intracellular autonomous genetic program [7]. Inactivation of tumor-suppressor genes (TSG) is a molecular target for the development of neoplasia. P53 is the most commonly involved TSG in a wide variety of human neoplasms including those of the endometrium. Strong expression of the mutant P53 protein in endometrial cancer has been associated with advanced stage and high-grade tumors. BCL2 is an anti-apoptotic protein and preserve cells from apoptosis by regulating mitochondrial membrane function. BCL2-associated X protein (BAX) encode pro-apoptotic proteins that are responsible for inducing cell death. An excess of BAX results in cell death, whereas excess BCL2 inhibits programmed cell death (apoptosis). Therefore, the BCL2/BAX ratio within a cell is a crucial determinant of apoptosis and a decreased BCL2/BAX ratio is a factor in predisposing endometrial cells to apoptosis. It has been suggested that dysfunction of the P53/BCL2/BAX apoptosis signaling pathway act a role in tumorigenesis and tumor progression [7,8]. The current study investigates a potential anti-tumor activity of perifosine and vitamin D on endometrial cancer cell (HEC-1A) using perifosine either on its own or in combination with an established vitamin D.

2. Materials and Methods

RNA isolation and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR):

The protein expression of BAX and BCL2 and P53 were evaluated by qRT-PCR. Total RNA was isolated from HEC-1A cells using the High Pure RNA Isolation Kit (1828665, Roche, Istanbul, Turkey), according to the manufacturer's instructions. The Transcriptor First Strand c-DNA Synthesis Kit (4896866, Roche, Istanbul, Turkey) was used to generate c-DNA from RNA, according to the manufacturer's instructions; 10 µL total RNA were used for amplification. PCR reactions were performed using specific primers for BAX, BCL2, and P53. Amplification reactions were set up in a reaction volume of 20 µL using the LightCycler 480 PCR Master Mix (04707494001, Roche, Istanbul, Turkey). PCR primers and TaqMan probes were synthesized and preoptimized. qRT-PCR was performed using PCR primers (BAX forward primer: 5'-GCCCTTTTGCTTCAGGGTTT-3'; BAX Reverse primer: 5'-TCCAATGTCCAGCCCATGAT-3'; BCL2 Forward primer: 5'-CGGAGGCTGGGATGCCTTTG-3'; BCL2 Reverse primer: 5'-TTTGGGGCAGGCATGTTGAC-3'; p53 forward primer: CCCCTCCTGGCCCCTGTCATCTTC; P53 reverse primer: GCAGCGCCTCACAACTCCGTCAT; ACTB Forward primer: 5'-GAGACCTTCAACACCCCAGCC-3'; ACTB Reverse primer: 5'-AGACGCAGGATGGCATGGG-3').

Apoptosis analysis by flow cytometry:

HEC-1A were incubated with perifosine (10 µM, 30 µM), vitamin D (50 nM, 200 nM), and combinations of both (10 µM + 50 nM, 10 µM + 200 nM, 30 µM + 50 nM and 30 µM + 200 nM) for 48 h and 72 h. HEC-1A cells without treatment were used as a control. Apoptosis of HEC-1A cells was analyzed by flow cytometry using an Annexin V-fluorescein isothiocyanate (AV-FITC) apoptosis detection kit (640932, BioLegend, San Diego, CA, USA). After the incubation period, different groups of HEC-1A cells were harvested and further processed according to the protocol of manufacture for flow cytometry analysis. The samples were analyzed using fluorescence-activated cell sorter can flow cytometry with at least 10,000 events recorded for each condition.

Statistical analysis:

The statistical calculations were performed using STATISTICA Version 13.3 software. All in vitro studies were carried out in triplicate and results are expressed as means ± SD. The repeated measures ANOVA test was used as multiple comparison tests to compare the statistical differences between group and time interactions. Statistical significance between groups was evaluated with Tukey-HSD for post-hoc multiple comparisons. $p < 0.05$ was considered statistically significant.

3. Results

The effect of perifosine and vitamin D on the expression levels of P53, BCL2 and BAX Statistically significant differences in fold change of the mRNA expression levels of Bax, Bcl-2, p53 in HEC-1A cells as compared with control cells is shown ($p < 0.05$) (Figure 1). The mRNA expression level of P53 remained unchanged in vitamin D groups. After 48 h and 72 h, a significant decrease in the mRNA expression level of P53 was observed in the perifosine groups and combined treatment of perifosine + vitamin D (10 μ M + 50 nM, 10 μ M + 200 nM, 30 μ M + 50 nM) (Figure 1a). Real-time PCR results showed that combined-treatment (10 μ M + 50 nM, 30 μ M + 50 nM, 30 μ M + 200 nM) caused a significant down-regulation of Bcl-2 than in cells treated with each reagent alone (Figure 1b). After 24 h of treatment, the mRNA expression level of BAX increased significantly under treatment with perifosine at 10 μ M and 30 μ M, vitamin D at 50 nM, and combined-treatment (10 μ M + 50 nM, 10 μ M + 200 nM), as well as decreased significantly in combined-treatment (30 μ M + 50 nM, 30 μ M + 200 nM) (Figure 1c). Perifosine and Vitamin D induces cell apoptosis in HEC-1A cells Asymmetry and permeability of the cell membrane were analyzed by Annexin V-FITC in HEC-1A cells. Cells were incubated with perifosine (10 μ M, 30 μ M), vitamin D (50 nM, 200 nM), and combinations of both (10 μ M + 50 nM, 10 μ M + 200 nM, 30 μ M + 50 nM and 30 μ M + 200 nM) for 48 h and 72 h. After the incubation period, cells were harvested and further processed according to the protocol of manufacture for flow cytometry analysis. As shown in Figure 2, induction of apoptosis occurred in cancer cells. Time course experiments revealed that long-term exposure to perifosine, vitamin D, and combinations of both resulted in significant apoptosis of HEC-1A cells.

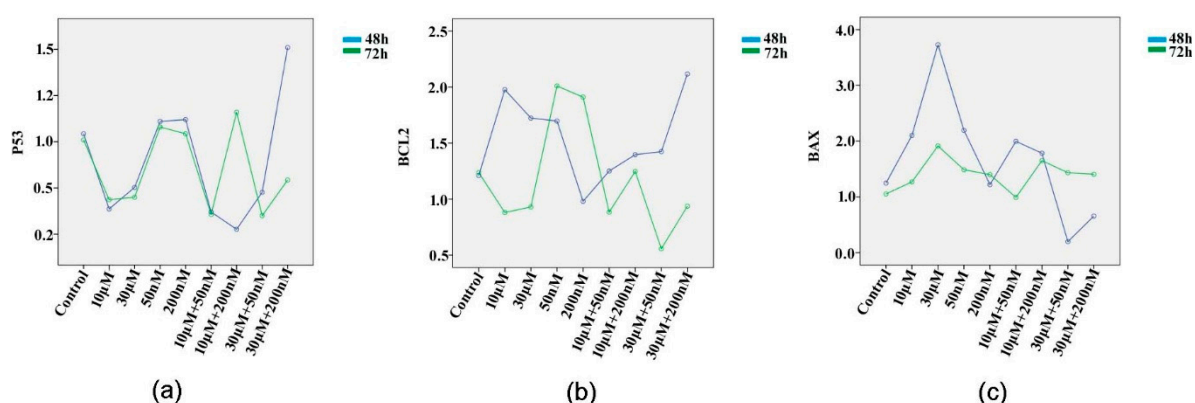


Figure 1. Evaluation of the effects of perifosine (10 μ M, 30 μ M), vitamin D (50 nM, 200 nM), and combinations of both (10 μ M + 50 nM, 10 μ M + 200 nM, 30 μ M + 50 nM and 30 μ M + 200 nM) for 48 and 72 h on the P53 (a), BCL2 (b) and BAX (c) expression level using qRT-PCR.

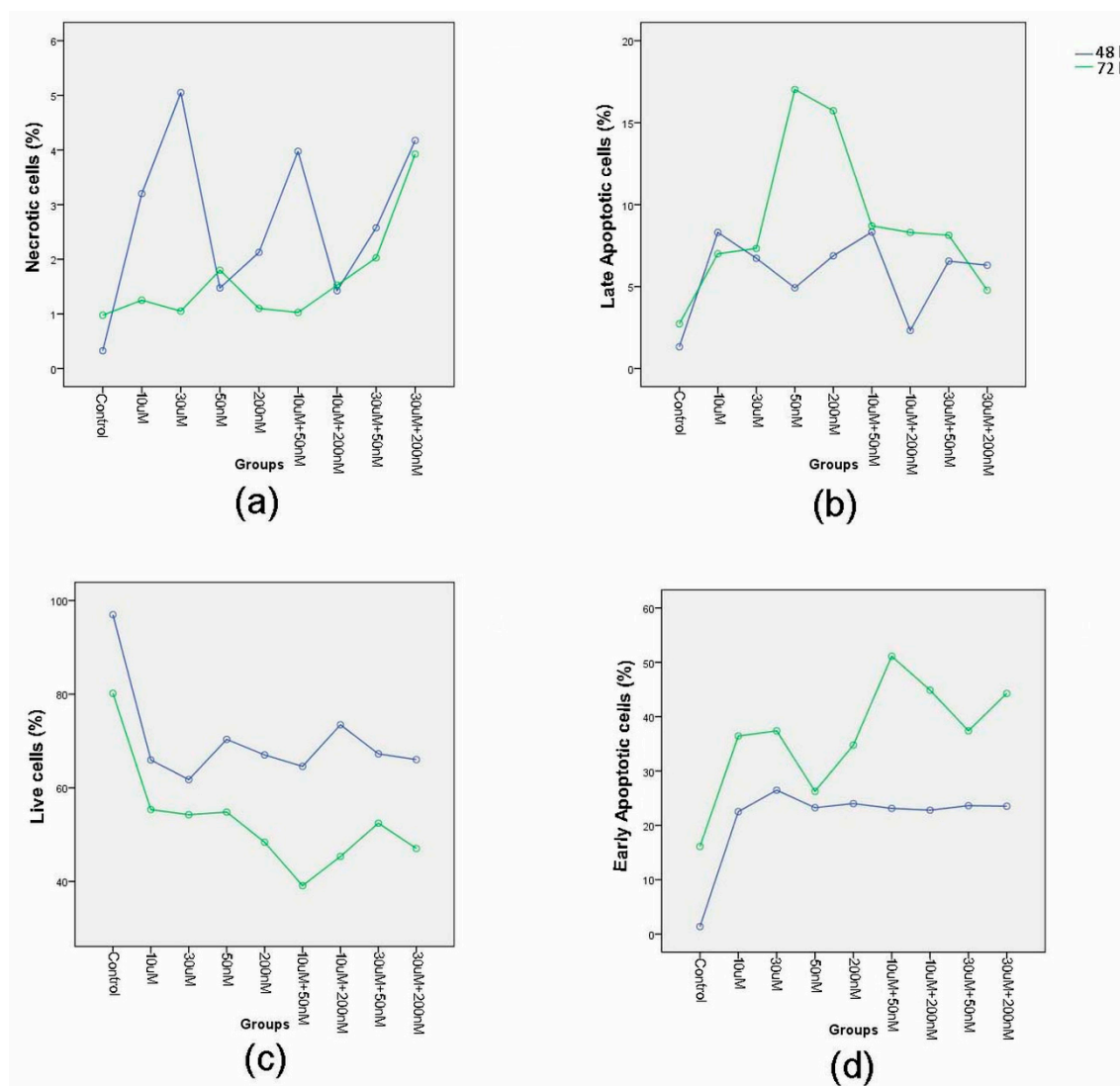


Figure 2. Perifosine and vitamin D induced apoptosis in a dose- and time-dependent manner. HEC-1A cells were treated with perifosine (10 μ M 30 μ M), vitamin D (50 nM, 200 nM), and combinations of both (10 μ M + 50 nM, 10 μ M + 200 nM, 30 μ M + 50 nM and 30 μ M + 200 nM) induced apoptosis in a dose-dependent manner after 48 h and 72 h. Necrotic cells (a), Late apoptotic cells (b), Live cells (c) and Early apoptotic cells (d).

4. Discussion

Treatment of advanced or repetitive endometrial cancer continues a major problem. Combination chemotherapy indicates objective responses in 40–60% of patients, but the survival is less than 1 year. We have investigated perifosine and vitamin D combination-treated on HEC-1A cells to explore whether it dominantly induces apoptosis. In addition, we evaluated the mRNA expression of P53, Bax and Bcl-2. The mRNA expression results suggested that the process triggered by perifosine and vitamin D combinations involved up-regulation of tumor suppressor P53, pro-apoptotic Bax, with a decrease of anti-apoptotic Bcl-2. These findings indicate new insights into the molecular mechanisms underlying the anti-cancer properties of perifosine and vitamin D combinations in endometrial cancer cells.

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