Effects of combined use of ribociclib with PARP1 inhibitor on cell kinetics in breast cancer

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Abstract. In the present study, antiproliferative and anticancer effects of Valamor (VLM), which contains the active component ribociclib, and DPQ, a poly(ADP-ribose) polymerase 1 inhibitor, alone and in combination were evaluated in the MCF-7 and MDA-MB-231 breast cancer cell lines *in vitro*. VLM was applied at concentrations of 40, 80 and 160 μg/ml, and DPQ was used at concentrations of 3, 6 and 9 μg/ml. The proliferation rate, cell index obtained from the real-time cell analysis system, mitosis activity, bromodeoxyuridine cell proliferation and caspase activity parameters were determined. In conclusion, the results obtained from cell kinetics parameters demonstrated the anticancer and antiproliferative effects of the combination of VLM and DPQ on breast cancer cells.

Introduction

In the past, breast cancer (BC) was the most prevalent illness affecting women globally (1-4). Since the time of the ancient Egyptians, attempts have been made to eradicate BC. More advanced surgical procedures are now being used to decrease the psychological toll of treatment, while medicinal therapies such as mastectomy and chemotherapy have significantly enhanced patient survival (5-7). However, preventative effectiveness and treatment options can never be fully effective without a thorough grasp of the pathophysiology and underlying mechanisms.

BC is a form of cancer that manifests itself differently in various individuals (8). Women all across the world are affected by BC, a prevalent type of cancer (9). BC can be classified into three groups based on molecular and histological evidence: BC expressing human epidermal growth factor receptor 2 (HER2+), triple-negative BC [TNBC; estrogen receptor (ER)-, progesterone receptor (PR)- and HER2-] and BC expressing hormone receptors, ER+ or PR+ (10,11).

The methods of treatment should be determined by the molecular features of BC. TNBC has also been classified into six groups: Luminal androgen receptor, immunomodulatory, mesenchymal, mesenchymal stem cell-like, basal-like 1 (BL-1) and BL-2 (10). These categories are not always useful because a single BC may have a variety of distinct cell types (4,12-15).

The majority of BC-related fatalities result from metastases. At 3 years after the main tumor was first discovered, 10-15% of patients with BC had distant metastases (16). At 10 years after the first diagnosis, it is common to observe the appearance of micrometastases in distant places. As a result, individuals with BC have a lifetime risk of developing metastases (16-18).

Third-generation cyclin-dependent kinase (CDK)4/6 inhibitor ribociclib (LEE011) is highly selective and inhibits CDK4/6 by competitively interacting with its ATP binding sites. By inhibiting the CDK4/6-cyclin D-retinoblastoma (Rb)-E2F axis, this strategy may stop unchecked cell division and tumor progression. CDK4 and 6 are crucial for the development and division of cancer cells, and ribociclib may limit the development of cancerous cells and stop the spread of the disease by inhibiting these enzymes (19).

Poly(ADP-ribose) polymerase (PARP) has crucial roles in DNA repair, apoptosis, cell regulation, cell division, differentiation, transcriptional regulation and chromosome maintenance (20,21). PARP1 repairs single-strand breaks in DNA through a base truncation repair mechanism. PARP-1 inhibition is a potent cancer death pathway (21). DNA damage occurs with cancer treatments such as temozolamide, platinum compounds, topoisomerase inhibitors and radiation therapy. Treatment resistance develops due to PARP, which is involved in DNA repair. Therefore, inhibiting the PARP enzyme may increase the treatment efficacy. Tumor suppressor genes, such as mutated BRCA1 and BRCA2, are highly sensitive to PARP1 inhibition, leading to cell cycle arrest and apoptosis (22). It has been proposed that PARP limits the spread of improperly repaired DNA, serving as a connection between severe DNA damage and cell death. Berger was the first to postulate this idea, also referred to as the 'PARP suicide hypothesis' (23).

In this study, the antiproliferative and anticancer effects of Ribociclib and PARP1 inhibitor alone and together on the Luminal A type breast cancer MCF-7 cell line and triple negative cancer type MDA-MB-231 cell line were investigated.

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Materials and methods

Cell culture. The TNBC cell line MDA-MB-231 and the luminal A BC cell line MCF-7 (European Cell Culture Collection) were both employed. The two cell lines were kept in DMEM (Gibco: Thermo Fisher Scientific, Inc.) tissue culture medium containing 100 μg/ml Streptomycin sulfate (I.E. Ulugay), 100 IU/ml penicillin (Pronapen; Pfizer), Amphotericin B (Sigma-Aldrich; Merck KGaA), 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) with the pH adjusted to 7.2 with 4.4% $NaHCO_3$.

Ribociclib and PARP1 concentration. In the experiments, for both cell lines, 40, 80 and 160 μg/ml concentrations of VLM (FARMANOVA), which contains the active ingredient ribociclib, and 3, 6 and 9 μg/ml concentrations of DPQ (Sigma-Aldrich; Merck KGaA), a PARP1 inhibitor, were used. These concentrations are based on preliminary experiments performed by our group.

Cell viability assay. The MTT assay was used to investigate the cytotoxicity of VLM and DPQ on the cells as a consequence of the application of the planned doses. For this application, MCF-7 cells were seeded into 96-well plates at a density of $2x10^4$ cells per well and incubated overnight. The cells were then treated with VLM and DPQ concentrations for 24 h. At the end of the experimental period, the medium in each well was removed and 40 μl fresh MTT solution (Sigma-Aldrich; Merck KGaA) was added into each well and cells were incubated at 37˚C for 4 h. Subsequently, 160 μl DMSO was added. By using the 690 nm wavelength as a reference, a spectrophotometer was used to measure the absorbance values of the experimental groups at 570 nm.

Cell index. Cell index values determine the cytotoxic effect of agents applied to the cell by monitoring cell reproduction, cell size or morphology in real time. The cytotoxic impact of VLM and DPQ on the designated cell lines was assessed by xCELLigence DP (Acea Biosciences, Inc.). Before taking any measurements, each well of a 16-well E-plate was filled with 100 μl of the appropriate medium. Subsequently, 100 μl cell suspension was added to each well of the E-plate. In each well, 10.000 cells for the MCF-7 cell line and 5.000 cells for the MDA-MB-231 cell line were seeded. The E-plates were kept at room temperature in a sterile environment for 20 min and then placed in the stations in the device and the experiment was continued at 37˚C and 5% CO_2 under saturated humidity ambient conditions. The device was set to measure every 15 min. After an overnight incubation, drugs were added and measurements were continued (24).

Bromodeoxyuridine (BrdU) incorporation assay. VLM and DPQ were used in the experiments in line with the kit's protocol (BrdU Cell Proliferation Assay Kit; cat. no. 2750; EMD Millipore) at the optimum combination concentrations.

Mitotic index. Experimental results were obtained following the kit protocol with VLM and DPQ at the specified doses (Mitotic assay kit; cat. no. 18021; Active Motif).

Caspase activity. In line with the kit instructions (CaspaTag Caspase 3, 7 *in situ* assay kit; cat. no. APT403; EMD Millipore), experimental findings were obtained.

Statistical analysis. All parameters of cell kinetics were evaluated comparing multiple independent groups. Comparisons between groups were performed using one-way ANOVA and Dunnett's tests. Statistical evaluations of the cell index were made by the xCelligence device. Dunnett's post-hoc test was used for all other parameters. Statistical analyses were reviewed twice in line with referee opinions. Experiments were performed in triplicate. The statistical analyses were performed using SPSS statistics software (v22.0; IBM). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell viability. When the MCF-7 cell absorbance levels following treatment for 40, 80 and 160 μg/ml VLM for 24 h were analyzed, it was observed that the 40 μg/ml VLM decreased the viability of MCF-7 cells to 85%, the 80 μg/ml VLM concentration decreased cell viability to 49.19% and the 160 μg/ml VLM concentration decreased cell viability to 13.79% compared to the control group, which was set as 100% (Fig. 1A).

Analysis of the absorbance values following DPQ treatment of MCF-7 cells at concentrations of 3, 6 and 9 μg/ml for 24 h showed that the 3 μg/ml DPQ concentration decreased the viability of MCF-7 cells to 65%, the 6 μg/ml DPQ concentration decreased cell viability to 57% and the 9 μg/ml DPQ concentration decreased cell viability to 51% compared to the control group, which was set as 100% (Fig. 1B).

Analysis of the absorbance rates after 40, 80 and 160 μg/ml VLM treatment of MDA-MB-231 cells for 24 h indicated that the 40 μg/ml VLM concentration decreased the viability of MDA-MB-231 cells to 46.86% compared to the control group, which was considered 100%. The 80 μg/ml VLM concentration reduced the cell viability to 45.71%, while the 160 μg/ml VLM concentration reduced the cell viability to 25.42% (Fig. 1C).

When the absorbance values were analyzed after DPQ was applied to MDA-MB-231 cells at concentrations of 3, 6 and 9 μg/ml for 24 h, it was observed that 3 μg/ml MDA-MB-231 cells had a 63.52% vitality after exposure to DPQ, the 6 μg/ml DPQ concentration decreased cell viability to 25.93% and the 9 μg/ml DPQ concentration decreased cell viability to 12.48% compared to the control group, which was set as 100% (Fig. 1D).

Cell index. Cell index values obtained from the xCelligence real-time cell analysis system (Acea Biosciences Inc.) monitor cell reproduction, cell size or morphology in real time. Acquired cell index values from the real-time cell analysis (RTCA) after treatment with VLM in MCF-7 and MDA-MB-231 cells at 40, 80 and 160 μg/ml showed that the drug had antiproliferative effects in both cell lines. When the curves obtained from the cell index graphs of MCF-7 cells are examined, different effects are observed at different concentrations. While no effect was observed at a concentration of 40 μg/ml, an antimitotic effect was observed at a concentration of 80 μg/ml and DNA damage was observed at a concentration

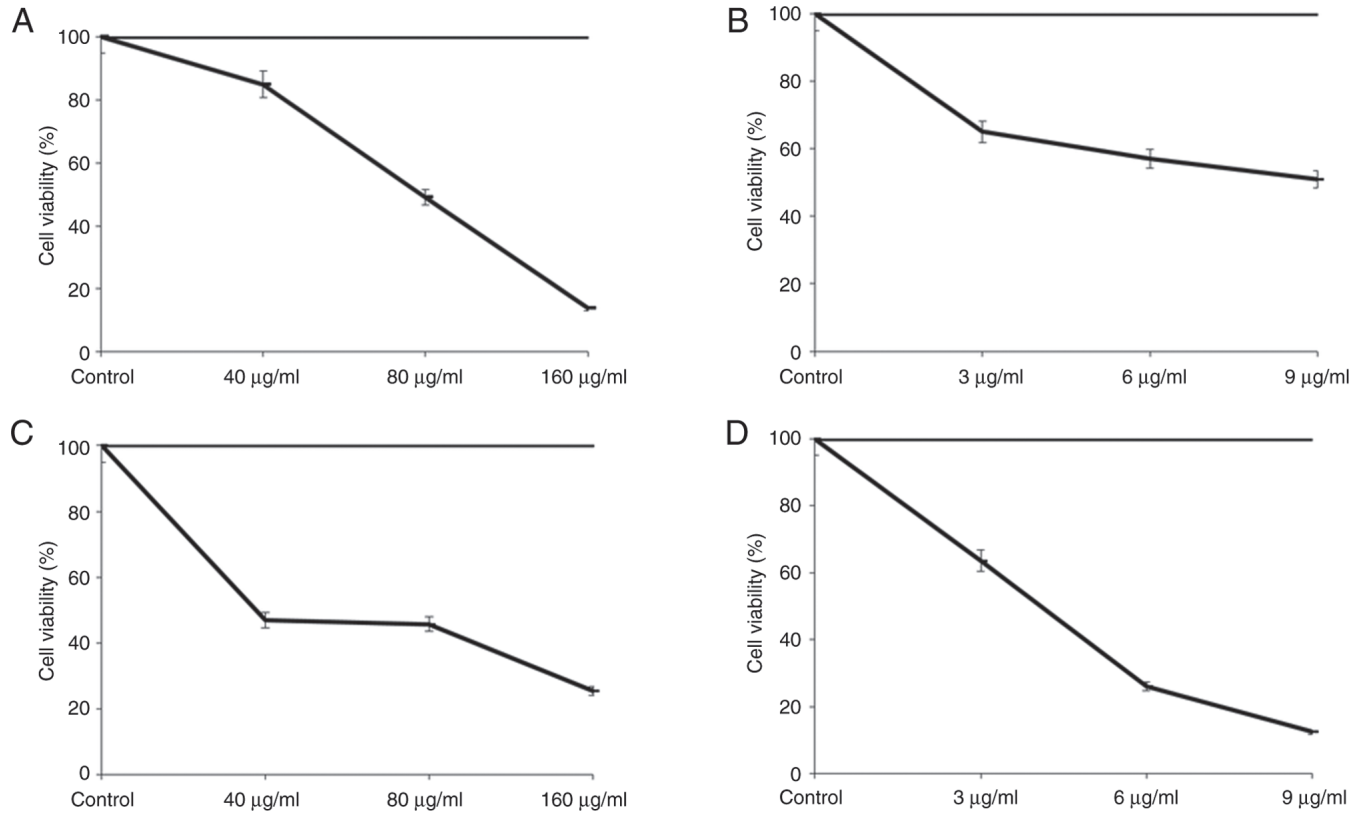

Figure 1. Viability values of MCF-7 cells treated with (A) VLM and (B) DPQ; % viability values of MDA-MB-231 treated with (C) VLM and (D) DPQ. VLM, Valamor.

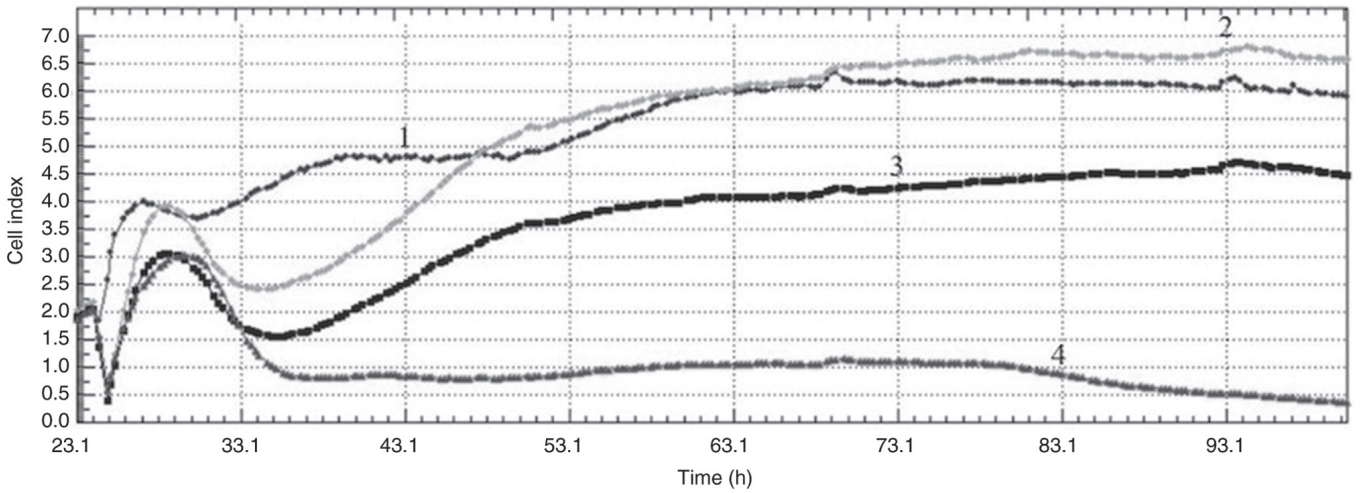

Figure 2. Cell index graph of MCF-7 cells treated with 40, 80 and 160 μg/ml VLM (1: Control; 2: 40 μg/ml VLM; 3: 80 μg/ml VLM; and 4: 160 μg/ml). VLM, Valamor.

of 160 μg/ml (Fig. 2). When the curves obtained from the cell index graphs of MDA-MB-231 cells are examined, all concentrations applied appeared to have an antiproliferative effect and cause DNA damage to the cells (Fig. 3).

The cell index values obtained from the RTAC system after DPQ treatment of cells at concentrations of 3, 6 and 9 μg/ml showed that the PARP inhibitor had antiproliferative effects on both cell lines. When the curves obtained from the cell index graphs of MCF-7 cells were examined, DNA damage was observed at all concentrations (Fig. 4). When the curves obtained from the cell index graphs of MDA-MB-231 cells were examined, it was observed that all concentrations applied had antiproliferative effects and caused DNA damage to the cells (Fig. 5).

Cell index values obtained from the RTAC system showed antiproliferative effects on both MCF-7 and MDA-MB-231 cells as a result of the combined application of VLM and DPQ at concentrations of 2.5 μg/ml VLM + 1.5 μg/ml DPQ, 20 μg/ml

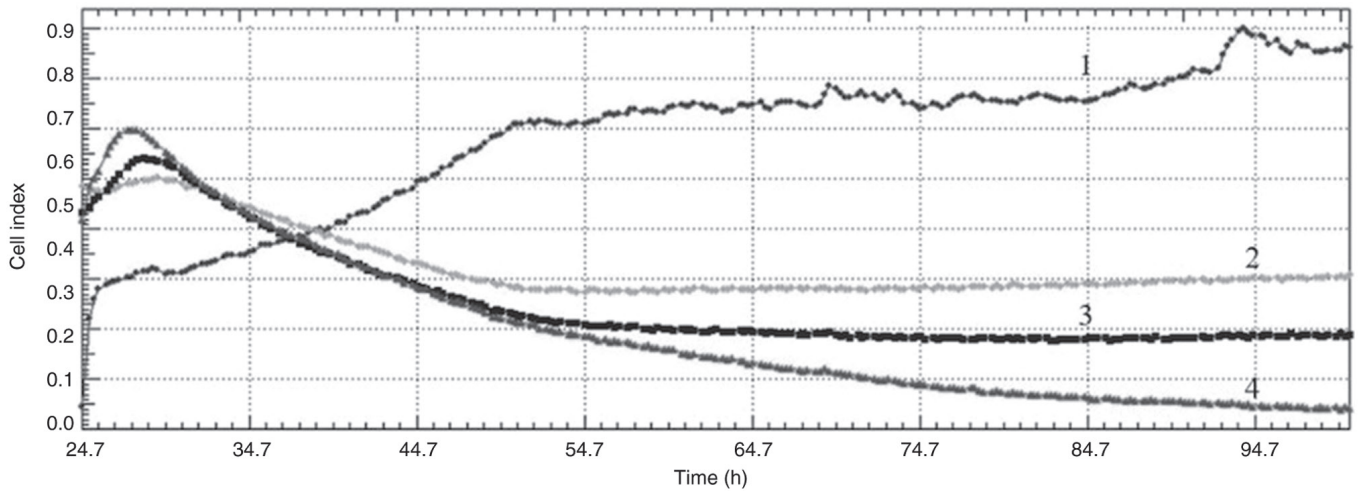

Figure 3. Cell index graph of MDA-MB-231 cells treated with 40, 80 and 160 μg/ml VLM (1: Control; 2: 40 μg/ml VLM; 3: 80 μg/ml VLM; and 4: 160 μg/ml). VLM, Valamor.

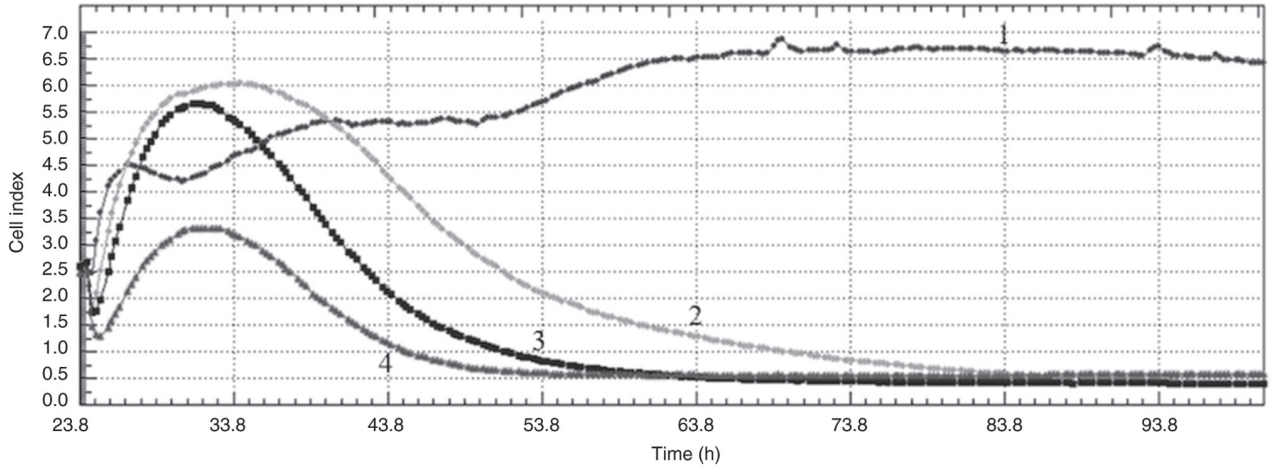

Figure 4. Cell index graph of MCF-7 cells treated with 3, 6 and 9 μg/ml DPQ (1: Control; 2: 3 μg/ml DPQ; 3: 6 μg/ml DPQ; and 4: 9 μg/ml DPQ).

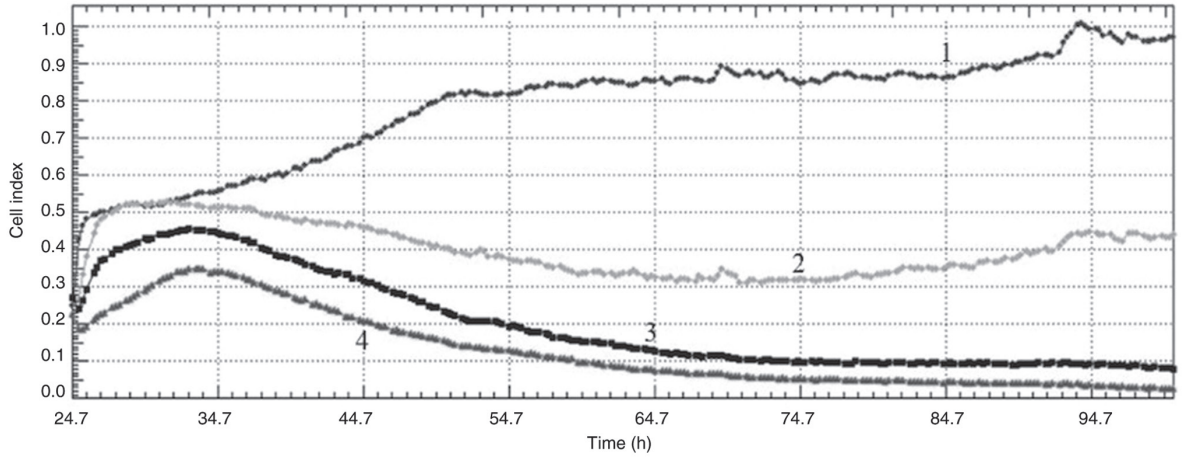

Figure 5. Cell index graph of MDA-MB-231 cells treated with 3, 6 and 9 μg/ml DPQ (1: Control; 2: 3 μg/ml DPQ; 3: 6 μg/ml DPQ; and 4: 9 μg/ml DPQ).

VLM + 4.5 μg/ml DPQ and 10 μg/ml VLM + 4.5 μg/ml DPQ. The combined concentrations showed DNA damage in MCF-7 cells (Fig. 6) and DNA damage in MDA-MB-231 cells (Fig. 7).

BrdU incorporation. Combining VLM with DPQ to treat MCF-7 and MDA-MB-231 cells (2.5 μg/ml VLM + 1.5 μg/ml DPQ) for 0-72 h resulted in labeling of cells with BrdU at

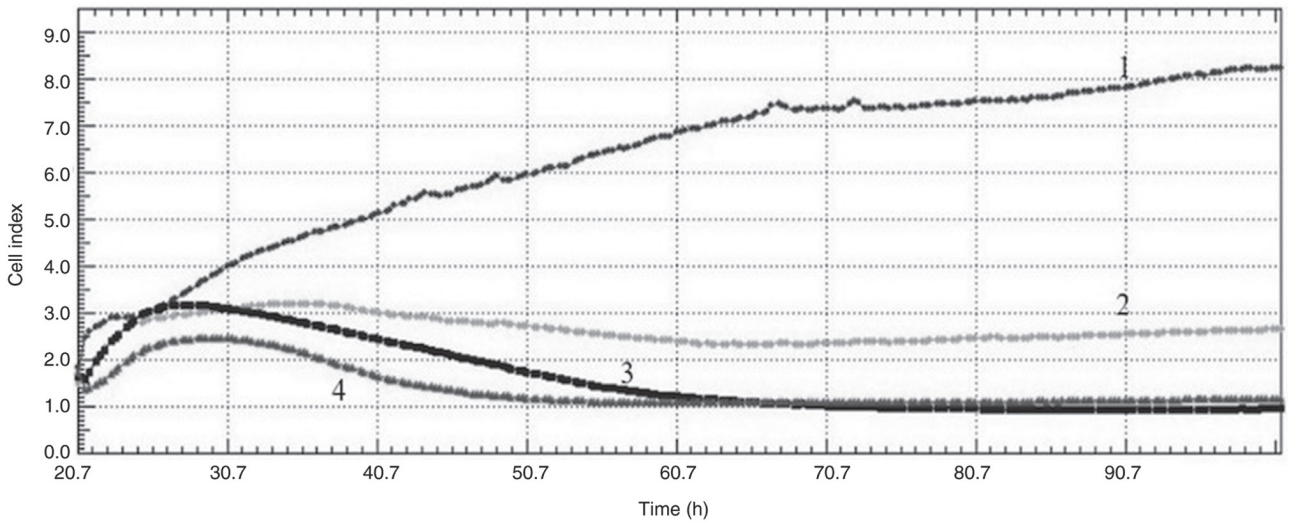

Figure 6. Cell index graph of MCF-7 cells treated with 2.5 μg/ml VLM + 1.5 μg/ml DPQ, 20 μg/ml VLM + 4.5 μg/ml DPQ and 10 μg/ml VLM + 4.5 μg/ml DPQ (1: Control; 2: 2.5 μg/ml VLM + 1.5 μg/ml DPQ; 3: 20 μg/ml VLM + 4.5 μg/ml DPQ; and 4: 10 μg/ml VLM + 4.5 μg/ml DPQ). VLM, Valamor.

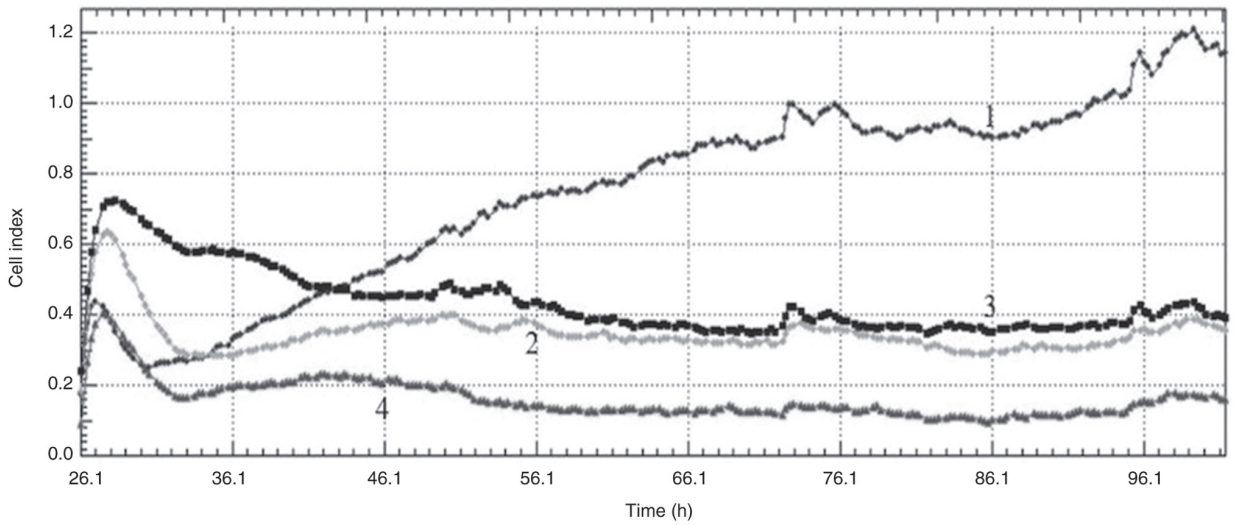

Figure 7. Cell index graph of MDA-MB-231 cells treated with 2.5 μg/ml VLM + 1.5 μg/ml DPQ, 20 μg/ml VLM + 4.5 μg/ml DPQ and 10 μg/ml VLM + 4.5 μg/ml DPQ (1: Control; 2: 2.5 μg/ml VLM + 1.5 μg/ml DPQ; 3: 20 μg/ml VLM + 4.5 μg/ml DPQ; and 4: 10 μg/ml VLM + 4.5 μg/ml DPQ). VLM, Valamor.

the synthesis stage and the absorbance values obtained are presented in Tables I and II, respectively. There was a significant difference between the proliferation of control and experimental groups ($P<0.05$).

Mitotic activity. The absorbance values reflecting mitotic activity of MCF-7 and MDA-MB-231 cells treated with a combination of VLM and DPQ (2.5 μg/ml VLM + 1.5 μg/ml DPQ) for 0-72 h are presented in Tables III and IV, respectively. There was a significant decrease in the experimental group according to the control ($P<0.05$).

Caspase activity. In Tables V and VI, the respective fluorescence amounts of caspase activity of MCF-7 and MDA-MB-231 cells after treatment with a combination of VLM and DPQ (2.5 μg/ml VLM + 1.5 μg/ml DPQ) for 0-72 h are presented. There was a significant increase in experimental group according to control ($P<0.05$).

Discussion

The primary goal of treatment for advanced or metastatic BC is to slow the disease's progression, ideally using patient-friendly anticancer medications that do not cause any undue damage (25). Cell cycle progression is deregulated in cancer, which is characterized by unchecked cell proliferation (26). The mitotic cell division cycle consists of four phases: Mitosis (M), the phase during which cellular DNA is synthesized (S), the first gap phase (G1) between the M and S phases, and the G2 phase between the S and M phases. To facilitate cell cycle progression, which is essential for mammalian cell cycle regulation, cell cycle-related proteins are phosphorylated by cyclins A, B, D and E, and their associated CDK1, -2, -4 and -6 (27,28). Although several distinct genes encode the 3 D-type cyclins, D1, D2 and D3 have a common set of amino acids (on average 57% across the coding area) (29). CDK4/6 inhibitors are a novel family of

Table I. Absorbance values at 450-655 nm (emission) of MCF-7 cells treated with 2.5 μg/ml VLM + 1.5 μg/ml DPQ in the bromodeoxyuridine assay (x10^{-3}).

Time, h	Control	Combination treatment
24	502±3	398±2[a]
48	507±4	257±3[a]
72	496±4	203±2[a]

[a]P<0.05 vs. control.

Table II. Absorbance values at 450-655 nm (emission) of MDA-MB-231 cells treated with 2.5 μg/ml Valamor + 1.5 μg/ml DPQ in the bromodeoxyuridine assay (x10^{-3}).

Time, h	Control	Combination treatment
24	625±3	609±2[a]
48	639±4	355±3[a]
72	635±4	300±2[a]

[a]P<0.05 vs. control.

Table III. Absorbance values at 450-655 nm (emission) of MCF-7 cells treated with 2.5 μg/ml Valamor + 1.5 μg/ml DPQ reflecting mitotic activity (x10^{-3}).

Time, h	Control	Combination treatment
24	169±3	97±2[a]
48	165±04	83±3[a]
72	163±04	32±2[a]

[a]P<0.05 vs. control.

Table IV. Absorbance values at 450-655 nm (emission) of MDA-MB-231 cells treated with 2.5 μg/ml Valamor + 1.5 μg/ml DPQ reflecting mitotic activity (x10^{-3}).

Time, h	Control	Combination treatment
24	84±3	52±2[a]
48	85±4	28±3[a]
72	82±4	16±2[a]

[a]P<0.05 vs. control.

Table V. Fluorescence values at 450-655 nm (emission) of MCF-7 cells treated with 2.5 μg/ml Valamor + 1.5 μg/ml DPQ reflecting caspase activity.

Time, h	Control	Combination treatment
24	204±12	302±14[a]
48	212±11	328±15[a]
72	307±11	518±13[a]

[a]P<0.05 vs. control.

Table VI. Fluorescence values at 450-655 nm (emission) of MDA-MB-231 cells treated with 2.5 μg/ml Valamor + 1.5 μg/ml DPQ reflecting caspase activity.

Time, h	Control	Combination treatment
24	224±12	361±14[a]
48	226±11	427±15[a]
72	231±11	453±13[a]

[a]P<0.05 vs. control.

pharmaceuticals that decrease cell cycle progression. Tumor cell growth is halted in this manner. Palbociclib, ribociclib and abemaciclib are three such inhibitors that have been authorized lately for the treatment of BC in different contexts and combination regimens (30).

After having demonstrated noticeably improved progression-free survival outcomes in comparison to standard therapy, ribociclib is one of three selective small-molecule inhibitors of CDK4/6 that are currently approved for the treatment of advanced hormone receptor-positive, HER2-negative BC (31-33). Third-generation CDK4/6 inhibitor ribociclib (LEE011) is highly selective and inhibits CDK4/6 by competitively interacting with its ATP binding sites (19). Ribociclib can halt the spread of cancer by inhibiting these enzymes, which also reduce the proliferation of cancer cells (34).

Beyond the locally advanced/metastatic scenario, the introduction of PARP inhibitors may offer advantages for the treatment of BC (35). The oral version of PARP inhibitors has the potential to enhance patient experience and adherence (36).

The activity of CDKs is required for DNA end resection. Numerous investigations revealed that CDKs were crucial to PARP inhibitor resistance (37-42).

TNBC cells that had been resistant to niraparib were made sensitive again by the CDK inhibitor dinaciclib. The experiment, which included dinaciclib and niraparib, was effective not just in TNBC cells but also in cells from the pancreas, ovary, prostate, colon and lung cancers (43).

Furthermore, out of all the functioning cell-cycle complexes, the CDK4/6 complex had the strongest negative connection with mutations, indicating that combination suppression of CDK4/6 and PARP may work in concert. In addition, combined therapy demonstrated a reactive oxygen species-dependent synergy in both Rb-proficient and Rb-deficient BC cells. These results point to a possible therapeutic approach to increase the effectiveness of PARP and CDK4/6 inhibitors in the treatment of cancer (44).

In a study conducted with the HCC1937 cell line, a CDK4/6 inhibitor was used together with a PARP inhibitor. The results

of the study showed that the combination of CDK4/6 inhibitor and PARP inhibitor may expand the use of these inhibitors in patients with TNBC and potentially overcome PARP inhibitor resistance (45). Several recent studies have shown how CDK4/6 and PARP inhibitors work synergistically in various cancer cells (46-48).

In the current study, consistent with the studies mentioned above, VLM and DPQ showed antiproliferative effects on both MCF-7 and MDA-MB-231 cells at the lowest concentration combinations used. The combined concentrations were shown to induce DNA damage in both target cell lines. In conclusion, the findings of this research investigating the inhibitory effects of VLM and demonstrated significant efficacy to reduce the viability of MCF-7 and MDA-MB231 cell lines, the cell index, mitotic signs and BrdU labeling, as well as a significant increase in caspase activity in these cell lines. The lack of cell proliferation after 72 h of treatment may be due to the cells becoming stable, or it may be due to the cells entering the process of apoptosis when caspase activity is taken into account.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

EP and MT performed the experiments. EP and MT wrote and edited the manuscript. EP and MT confirm the authenticity of all the raw data. Both authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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