Original Research

# Mechanisms of abemaciclib, a CDK4/6 inhibitor, induced apoptotic cell death in prostate cancer cells *in vitro*

Gamze Guney Eskiler [a],[*], Asuman Deveci Ozkan [a], Ayten Haciefendi [b], Cemil Bilir [c]

[a] *Department of Medical Biology, Faculty of Medicine, Sakarya University, Korucuk Campus, Sakarya 54290, Turkey*
[b] *Department of Medical Biology, Institute of Health Sciences, Sakarya University, Sakarya, Turkey*
[c] *Department of Medical Oncology, Faculty of Medicine, Istinye University VMMedical Park Pendik Hospital, Istanbul, Turkey*

A B S T R A C T

The therapeutic effects of abemaciclib (ABE), an inhibitor of cyclin- dependent kinases (CDK) 4/6, on the proliferation of two types of prostate cancer (PC) cells were revealed. In this study, *in vitro* cytotoxic and apoptotic effects of ABE on metastatic castration-resistant prostate cancer (mCRPC) androgen receptor (AR) negative PC-3 and AR mutant LNCaP PC cells were analyzed with WST-1, Annexin V, cell cycle, reactive oxygen species (ROS), mitochondrial membrane potential, RT-PCR, western blot, and apoptosis protein array. ABE considerably inhibited the growth of PC cells in a dose-dependent manner ($p<0.01$) and caused significant apoptotic cell death through the suppression of CDK4/6-Cyclin D complex, ROS generation and depolarization of mitochondria membrane potential. However, PC-3 cells were more sensitive to ABE than LNCaP cells. Furthermore, the expression levels of several pro-apoptotic and cell cycle regulatory proteins were upregulated by ABE in especially PC-3 cells with the downregulation of apoptotic inhibitor proteins. Our results suggest that ABE inhibits PC cell growth and promotes apoptosis and thus ABE treatment may be a promising treatment strategy in especially mCRPC. Further preclinical and clinical studies should be performed to clarify the clinical use of ABE for the treatment of PC.

## Introduction

Prostate cancer (PC) is the second leading cause of cancer-associated death among men within an estimated approximately 248.530 new cases in the United States for 2021 and the second rate of incidence worldwide [1,2]. The 5-year survival rate is almost 100% in patients with local or regional PC. However, this survival rate is 30% for metastatic PC (mPC) patients when spread to other parts of the body. For PC patients, the androgen receptor (AR) deprivation therapy (ADT) is the primary therapy due to oncogenic features of AR causing cell growth, proliferation and metastasis. Not surprisingly, most PC patients will acquire resistance to ADT with a recurrence of 3–4 years and defined as castration-resistant prostate cancer (CRPC). Different FDA-approved therapies (docetaxel, abiraterone, enzalutamide, cabazitaxel, radioisotope treatment and Poly (ADP-ribose) polymerase inhibitors) have been used for the treatment of metastatic CRPC patients in the clinic. Unfortunately, the median survival rate of CRPC patients is nearly 35 months and therefore new treatment strategies are urgently needed for improving the overall survival rate and quality of life [3–6].

Cyclin-dependent kinases (CDK) 4/6 inhibitors (CDK4/6i) have been gained attention in recent years due to promising efficacy in the treatment of hormone receptor (HR) positive breast cancer patients and increased median progression-free survival (mPFS) and overall survival. Estrogen receptors (ER) and androgen receptors (AR) regulate the cell cycle process from G1 to S phase transition through regulatory proteins. After induction of growth factor, Cyclin D binds to CDK4/CDK6 and phosphorylates retinoblastoma (Rb). Following hyperphosphorylation of Rb, E2 transcription factor (E2F) is activated and provides the cells into the S phase for DNA replication. Therefore, three CDK4/6i (palbociclib, abemaciclib (ABE) and ribociclib) are FDA approved and inhibit the activation of cyclin D-CDK4/6 complex in the treatment of breast cancer [7–9]. In PC, AR activates different signaling pathways including Src, Ras/Raf, protein kinase C, AKT/PI3K and mitogen-activated protein kinase (MAPK) pathways causing cell proliferation. The aberrant activation of cyclin D is mediated by particularly the upregulation of MAPK and Akt/mTOR signaling pathways. The amplification of cyclin D is detected in nearly 4.7% of mCRPC patients. The use of ADT affects the transcription of genes regulating the cell cycle

and eventually leads to androgen-independent castration resistance in patients [10–12]. Therefore, the use of CDK4/6i could be a new therapeutic modality for the inhibition of AR signaling pathways. On the other hand, the significant toxicity of the first generation of CDK4/6i can delay the clinical applicability of these inhibitors in PC treatment.

Some studies have been investigated the therapeutic potential of CDK4/6i in PC [13–15]. In one preclinical study, palbociclib inhibits the proliferation of HSPC and CRPC cells, *in vitro* and *in vivo*. Furthermore, Stice et al. (2017) state that newly described CDK4/6 inhibitors (G1T28 and G1T38) as an alternative to taxane can potentially decrease hormone-sensitive and resistant prostate cancer cell proliferation, including wild-type AR (VCaP), AR-V7 (22Rv1 and LNCaP), and the activity of these inhibitors depends on Rb status, *in vitro* and *in vivo* [13]. Additionally, five phases Ib/II clinical trials (NCT02059213, NCT02905318, NCT02555189, NCT02494921, NCT03706365) are currently in the treatment of mCRPC or mHSPC, RB-positive patients. In these trials, the potential combination of palbociclib, abemaciclib, ribociclib with ADT, enzalutamide, docetaxel, and abiraterone has been investigated in patients [14,15]. In this context, further preclinical and clinical studies are warranted in the treatment of PC patients to identify the appropriate patient population according to genomic alterations and the best synergistic combination to overcome resistance.

Here, we, for the first time, investigated the inhibition of CDK4/6-Cyclin D complex by ABE in both hormone-sensitive PC and resistant mCRPC cell growth, *in vitro*. In addition, the underlying molecular mechanisms of ABE-induced cell death in PC cancer cell lines were identified at the molecular level.

## Materials and methods

### Cell culture

Two different human PC cell lines, PC-3 [hormone-independent (ATCC CRL-1435)] and LNCaP [hormone-dependent (ATCC, Rockville, MD, USA, CRL-1740)] were cultured in a humidified incubator containing 5% $CO_2$ at 37 °C. The cells were grown in Rosewell Park Memorial Institute (RPMI) 1640 media (Gibco, San Francisco, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, San Francisco, CA, USA) and 1% penicillin–streptomycin (Gibco San Francisco, CA, USA).

### Cell viability assay

The PC cells were cultured in 96-well plates ($2 \times 10^4$ cells/well) and treated with different concentrations (0.25, 0.5, 1, 1.5 and 2 μM) of ABE (Biovision, San Francisco, CA, USA for 24 h. ABE was dissolved in DMSO and stored at −20 °C. PC-3 and LNCaP cells were incubated in a growth medium without ABE, as a negative control. Cell viability percentages were measured by WST-1 assay according to the manufacturer instructions (Biovision, San Francisco, CA, USA). The absorbance value was measured using an ELISA reader at 450 nm (Allsheng, China). The WST-1 assay was used to determine the most effective treatment dose or doses for further analysis.

### Annexin V analysis

To explore the rate of apoptotic cell death induced by ABE, Annexin V analysis was conducted. PC cells were cultured in 6-well plates ($5 \times 10^5$ cells/well) and treated with 1 and 2 μM ABE, selected as the most effective doses according to the WST-1 results. The cells were washed twice with cold phosphate buffer solution (PBS, Gibco, San Francisco, CA, USA) and stained with Annexin V- Dead Cell Assay kit (Millipore, Germany). After staining, the cells were examined with Muse Cell Analyzer (Millipore, Germany).

### Acridine orange/propidium iodide (AO/PI) dual staining

To determine the effect of ABE on the morphological changes of PC cells, AO/PI dual staining was performed. The PC cells were cultured in 6-well plates ($5 \times 10^5$ cells/well) and treated with 1 and 2 μM ABE. After treatment, the cells were fixed with 4% paraformaldehyde (PFA) then stained by AO and PI. The stained cells were captured with EVOS FL Cell Imaging System (Thermo Fisher Scientific, Waltham, MA, USA).

### Mitochondrial membrane potential assay

To evaluate the changes in mitochondrial transmembrane potential, PC cells were cultured in 6-well plates ($5 \times 10^5$ cells/well) and treated with 1 and 2 μM ABE. After treatment, the cells were collected with centrifugation and washed twice with PBS. The collected pellet was incubated with Muse MitoPotential Kit (Millipore, Germany) for 25 min at 37 °C. After staining, the cells were analyzed with Muse Cell Analyzer (Millipore, Germany).

### Intracellular reactive oxygen species (ROS) determination assay

To determine the ROS level induced by ABE, oxidative stress assay was performed. The PC cells were cultured in 6-well plates ($5 \times 10^5$ cells/well) and treated with 1 and 2 μM ABE. After treatment, the cells were collected with centrifugation and incubated with Muse Oxidative Stress Assay Buffer (Millipore, Germany) for 30 min at 37 °C. After incubation, the cells were analyzed with Muse Cell Analyzer (Millipore, Germany).

### RNA extraction, cDNA synthesis, and real-time RT-PCR

To determine the mRNA level of *Bax, Bcl-2, CCDN1, Rb, Caspase-3* and *Caspase-8*, RT-PCR analysis was used. Total RNA was isolated with E.Z.N.A. Total RNA Kit (Omega Bio-Tek, Norcross, GA) according to the manufacturer instructions. Then isolated RNA purity and concentrations were measured by Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA synthesis was performed with a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). The changes in mRNA levels of selected genes were measured by The StepOnePlus Real- Time PCR (Applied Biosystems) using Universal Master mix II (Applied Biosystems, Foster City, CA). The commercial primer sets of *Bax* (Hs00180269_m1), *Bcl-2* (Hs00236808_s1), *CCDN1* (Hs00765553_m1), *Rb* (Hs01078066_m1), *Caspase-3* (Hs00234387_m1), *Caspase-8* (Hs01018151_m1) and *β-Actin* (Hs99999903_m1) (Applied Biosystems, Foster City, CA) were used for RT-PCR. *β-Actin* was accepted as an endogenous reference gene.

### Western blot analysis

To further analyze the protein expression level of pRB and CCDN1 genes, protein isolation and western blot analysis were conducted. Total protein was isolated with RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) and concentrations of isolated proteins were measured with Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). For blotting, isolated protein lysates were separated at 12% SDS-PAGE and transferred to the nitrocellulose membranes (Bio-Rad, San Diego, CA). After separation, the membranes were blocked in nonfat milk in tris-buffered saline with Tween 20 (TBS-T). After blocking, Anti-p-Rb (sc-377,539), Anti-CCDN1 (sc-8396) and Anti-β-Actin (sc-47,778) (Santa Cruz Biotechnology, CA, USA) primary antibodies were used for detection and β-Actin was used as an internal control. After primary antibody incubation, the membranes were washed and incubated with horseradish peroxidase–conjugated anti-mouse IgG secondary antibody (Bio-Rad, San Diego, CA). Protein detection was conducted using chemiluminescence detection (Bio-Rad, San Diego, CA) and chemiluminescent bands were captured and analyzed with the detection

system (Syngene, USA).

*Apoptotic protein antibody array analysis*

To determine the effects of ABE on the expression levels of apoptosis related proteins in PC cells, a Human Apoptosis Array (Raybiotech, GA, USA) was used. The PC cells were cultured in 6-well plates ($5 \times 10^5$ cells/well) and treated with 2 µM ABE for 24 h. After treatment, the proteins were extracted from the collected pellet and apoptosis array protocol was performed according to the manufacturer instructions. Finally, protein detection was conducted using a chemiluminescence detection system (Syngene, USA).

*Statistical analysis*

Obtained results were statistically analyzed by using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and presented as means ± standard deviation (SD). Significant differences were determined using One-way analysis of variance (ANOVA) followed by Tukey's test. The web-based statistical analysis software (https://www.qiagen.com/tr/shop/genes-and-pathways/ data-analysis-center-overview-page/other-real-time-pcrprobes-or-primers-data-analysis-center/) was used to determine the relative expression of the selected genes. p<0.05 was considered statistically

significant.

## Results

*Effects of ABE on the cell viability and apoptosis in PC cells*

To evaluate the viability of PC cells following treatment with 0.1, 0.25, 0.5, 1 and 2 µM ABE for 24 and 48 h, WST-1 analysis was conducted (Fig. 1A and B). A dose-dependent significant decrease was detected in both PC cells for 24 and 48 h (*p*<0.05; Fig. 1). We determined that ABE could considerably suppress PC-3 cell viability at doses of 1 and 2 µM (56.2 ± 0.2% and 35.1 ± 0.1%, respectively) for 24 h (*p*<0.01, Fig. 1A). Additionally, LNCaP cell viability followed by 1 and 2 µM ABE treatment showed a dose-dependent significant decrease (59.5 ± 2.3% and 43.2 ± 2.9%, respectively) after 24 h as indicated in Fig. 1B (*p*<0.01). The inhibition of PC cell viability was more profound in 24 h treatment than 48 h treatment (85.4 ± 1.8% and 57.7 ± 1.5%in PC-3 and 60.8 ± 0.9% and 49.3 ± 2.3% in LNCaP cells at 1 and 2 µM ABE, respectively). Thus, ABE exhibited greater anti-proliferative activity in both PC cells at 1 and 2 µM doses for 24 h and we used this treatment procedure for further analysis. The IC$_{50}$ value of ABE for PC-3 and LNCaP cells was 1.30 and 1.59 µM, respectively.

To investigate the ABE-induced cell death, we conducted the
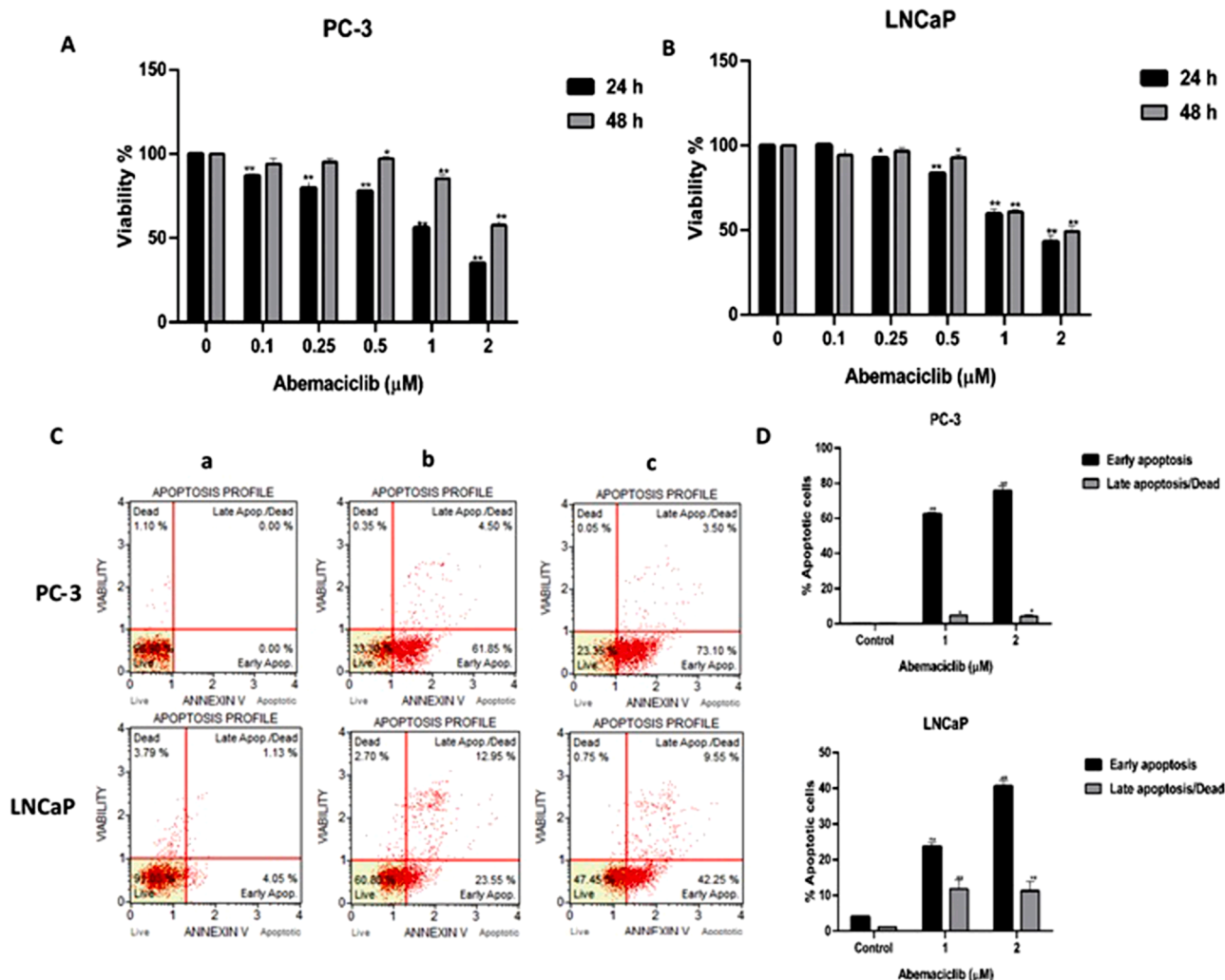


**Fig. 1.** Effects of ABE on the proliferation and the apoptotic cell death of PC cells. The cell viability of PC-3 (A) and LNCaP (B) cells in response to ABE treatment was evaluated through WST-1 analysis for 24 and 48 h. (C) The histograms a) Control, (b) 1 µM and (c) 2 µM ABE and (D) statistical analysis of the percentage of early and late apoptotic cells in response to ABE treatment was evaluated through Annexin-V and death cell assay for 24 h. These results were performed at least in triplicate and expressed as mean ± SD (**p*<0.05 and ***p*<0.01).

Annexin-V and dead cell assay (Fig. 1C and D). Our results showed that 1 and 2 μM of ABE treatment markedly increased the percentage of early apoptotic cells ($62.3 \pm 0.6\%$ and $75.5 \pm 2.2\%$, respectively) in PC-3 cells compared to the control ($p<0.01$). On the other hand, ABE treatment significantly increased both the percentage of early and late apoptotic cells in a dose-dependent manner in LNCaP cells ($p<0.01$, Fig. 1D). In 2 μM of ABE treated group, the percentage of the early and late apoptotic cells increased from $4.03 \pm 0.1\%$ and $1.21 \pm 0.1\%$ to $40.6 \pm 1.5\%$ and $11.3 \pm 2.6\%$, respectively, in LNCaP cells (Fig. 1C and D). Thus, particularly 2 μM ABE treatment could result in significant apoptotic cell death in both PC cells.

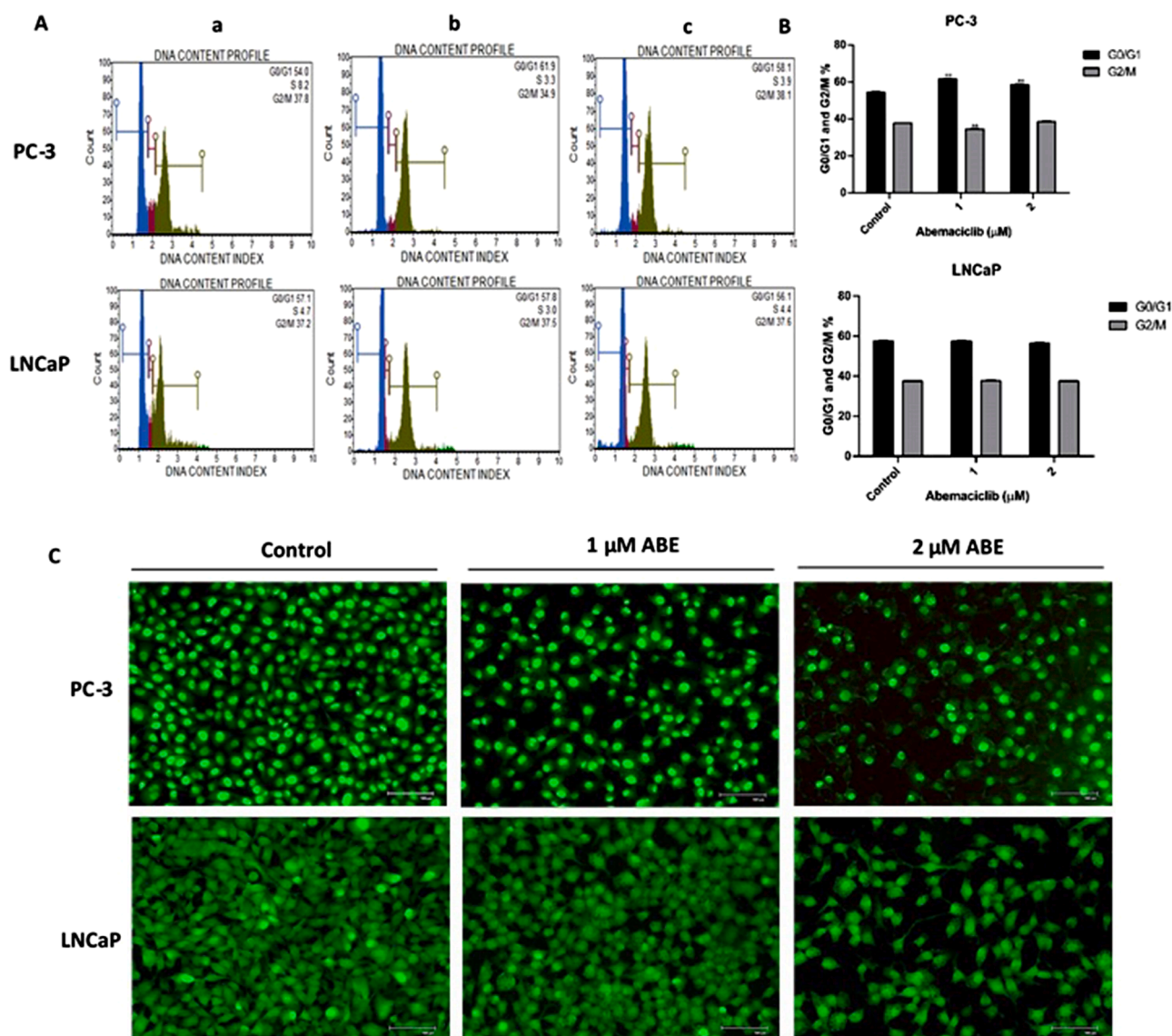### Effects of ABE on cell cycle arrest and cell morphology in PC cells

We conducted the cell cycle assay for determining the inhibitory effects of ABE on CCDN1/CDK4/6 complex formation (Fig. 2). Our results indicated that ABE treatment could result in G0/G1 arrest for 24 h in both PC cells. The accumulation of PC-3 cells in the G0/G1 phase significantly increased from $54.4 \pm 0.4\%$ to $61.5 \pm 0.3\%$ and $58.5 \pm$

$0.4\%$ at 1 and 2 μM of ABE, respectively (Fig. 2A-B). However, there was no significant difference (from $57.5 \pm 0.4\%$ to $57.4 \pm 0.4\%$ and $56.5 \pm 0.5\%$) in LNCaP cells at G0/G1 phase following treatments with 1 and 2 μM of ABE, respectively (Fig. 2B).

To further reveal the ABE-induced apoptotic or necrotic cell death, AO/PI dual staining was performed (Fig. 2C). The results demonstrated that ABE treatment exhibited apoptotic morphological changes in PC cells. Following administration of 1 and 2 μM of ABE for 24 h, rounded cells, the formation of nuclear blebbing, and some DNA fragmentation were observed closely related to induction of apoptosis in especially PC-3 cells dose-dependently (Fig. 2C). Furthermore, particularly large cytoplasmic vacuoles were detected in both PC cells following both 1 and 2 μM ABE. However, these vacuoles were more obvious in LNCaP than PC-3 cells at particularly 1 μM ABE.

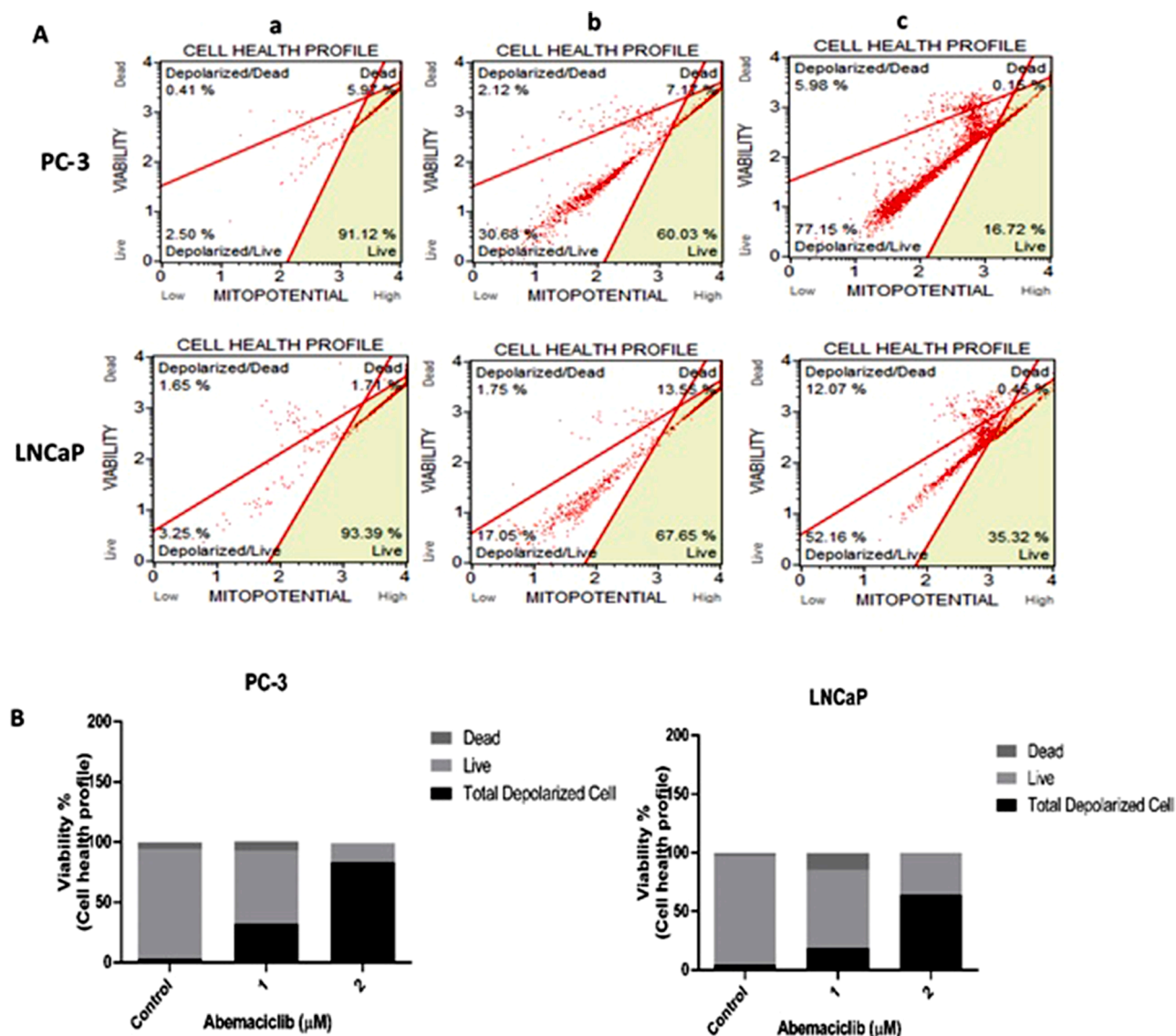### Effects of ABE on the mitochondrial membrane potential of PC cells

To further evaluate the ABE-induced apoptosis, we conducted the Muse MitoPotential assay (Fig. 3). After treatment with 1 and 2 μM ABE



**Fig. 2.** Effects of ABE on the cell cycle arrest and cell morphology of PC cells. (A) The DNA content profile a) Control, (b) 1 μM and (c) 2 μM ABE and (B) statistical analysis of the accumulation in G0/G1 and G2/M phase in response to ABE treatment were evaluated through cell cycle assay for 24 h. (C) Morphological evaluation in response to ABE treatment was evaluated with AO/PI dual staining for 24 h. These results were performed at least in triplicate and expressed as mean $\pm$ SD ($*p<0.05$ and $**p<0.001$).

**Fig. 3.** Effects of ABE on the mitochondrial membrane potential of PC cells. (A) The histograms of cell profile (a) Control, (b) 1 μM and (c) 2 μM ABE and (B) the graphs in response to ABE treatment was evaluated through Muse MitoPotential assay for 24 h. These results were performed at least in triplicate and expressed as mean ± SD (*$p<0.05$ and **$p<0.001$).

for 24 h, a total depolarized live cell percentage increased ($32.1 \pm 0.8\%$ and $83.0 \pm 0.1\%$, respectively) in a dose-dependent manner due to the reduction of the mitochondrial membrane potential in PC-3 cells (Fig. 3A-B). In addition, the percentage of total depolarized live cells increased followed by 1 and 2 μM ABE treatment ($18.4 \pm 0.4\%$ and $64.1 \pm 0.2\%$, respectively) in LNCaP cells. Thus, ABE treatment could result in a change in the mitochondrial membrane potential in PC cells and PC-3 cells were more sensitive to ABE treatment than LNCaP cells.

*Effects of ABE on the intracellular level of ROS production in PC cells*

Excess ROS levels in cells resulting from oxidative stress, can lead to the activation of cell death processes such as apoptosis. To confirm and support the ABE-induced apoptotic cell death, we performed the ROS determination assay (Fig. 4). After treatment with 1 and 2 μM ABE for 24 h, the intracellular ROS positive cell percentage significantly increased (from $4.7 \pm 0.2\%$ to $12.7 \pm 0.3\%$ and $39.7 \pm 0.6\%$, respectively) in PC-3 cells ($p<0.01$, Fig. 4A and B). Furthermore, the intracellular ROS positive cell percentage was $17.9 \pm 0.7\%$, followed by 2 μM ABE treatment compared with control ($5.1 \pm 0.4\%$) in LNCaP cells

(Fig. 4A and B). Thus, ABE treatment caused an increase in the intracellular level of ROS in PC cells and the induction of ROS by ABE was higher in PC-3 cells than LNCaP cells inconsistent with the mitochondrial membrane potential assay results.

*Effects of ABE on the alteration of gene and protein expression of PC cells*

To evaluate the potential mechanism of ABE-induced apoptotic cell death, the mRNA and protein expression levels of pro-apoptotic and anti-apoptotic genes were analyzed by RT-PCR and apoptosis antibody array (Fig. 5A–C). In Fig. 5A, the *Bax, Bcl-2, caspase-3* and *caspase-8* expression levels considerably increased to 2.1-, 5.6-, 1.8-, and 2.9-fold, respectively at 2 μM ABE in PC-3 cells, whereas only the expression of caspase-8 level was significantly up-regulated following 2 μM ABE treatment in LNCaP cells compared with control (Fig. 5A). To further confirm our findings, 43 apoptosis-related proteins were analyzed. We found that the effects of ABE on apoptosis-related proteins were more profound in PC-3 cells than LNCaP cells. ABE treatment resulted in the upregulation of pro-apoptotic proteins including Bid, Bim, Caspase-3 and Caspase-8 expressions and cell cycle regulatory proteins (p53/
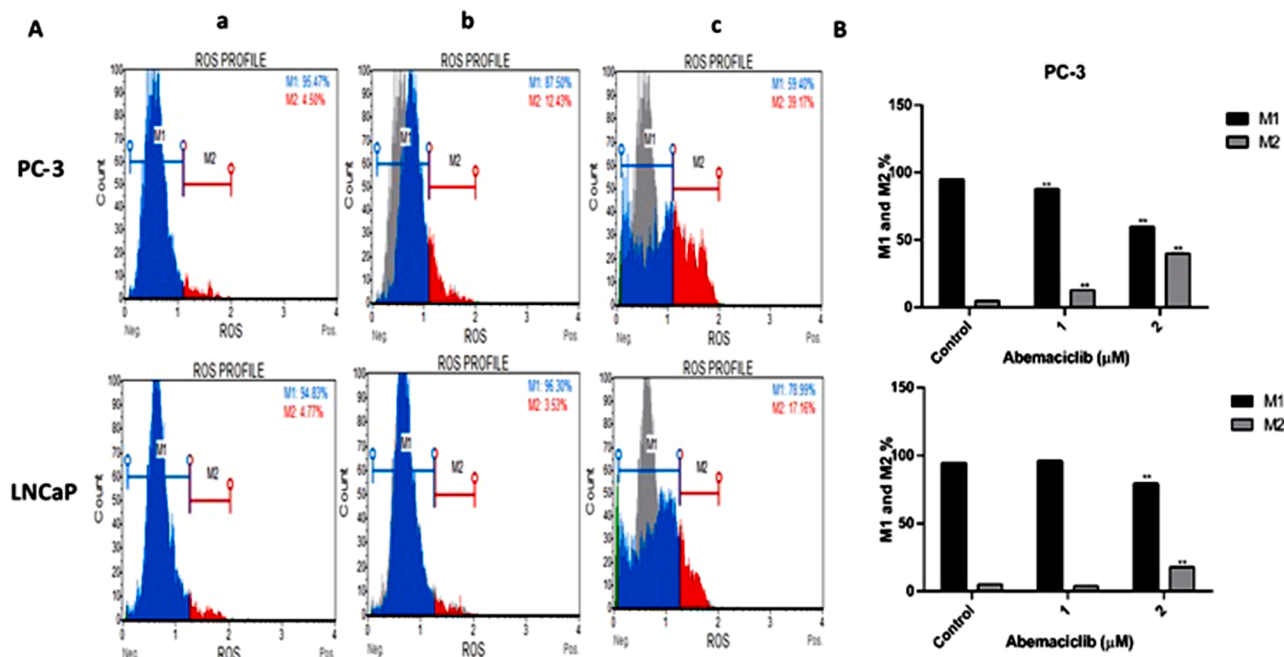
**Fig. 4.** Effects of ABE on the ROS production in PC cells. (A) The ROS profile of cells (a) Control, (b) 1 μM and (c) 2 μM ABE and (B) the statistical analysis of M1 and M2 cells in response to ABE treatment was evaluated through ROS assay for 24 h. These results were performed at least in triplicate and expressed as mean ± SD (M1: ROS negative, M2: ROS positive, *$p < 0.05$ and **$p < 0.001$).

p21/p27) and high temperature requirement protein A2 (HTRA2) in particularly PC-3 cells compared to the control group. (Fig. 5B and C). Additionally, the expression level of several inhibitors of apoptosis proteins (cIAP-2, XIAP, HSP60 and survivin) was down-regulated in ABE treated PC cells.

Additionally, we also explored the potential mechanism of ABE-induced G0/G1 arrest, the mRNA and protein expression levels of *CCDN1* (Cyclin D) and *Rb1* were analyzed by RT-PCR and western blot analysis (Fig. 5D and E). According to our results, ABE treatment suppressed the mRNA and protein levels of CCDN1 in PC cells. Furthermore, the mRNA level of *Rb1* was significantly up-regulated by 1.6- and 2.0-fold at 1 and 2 μM ABE, respectively in PC-3 cells ($p < 0.001$). In Fig. 5E, the expression level phospho Rb (pRb) protein was down-regulated by ABE in a dose-dependent manner in PC cells due to G0/G1 arrest induction.

## Discussion

The inhibition of hyper-activated Cyclin D/CDK4/6 complex by CDK4/6i has been shown promising results in the treatment of hormone-sensitive breast cancer patients. Due to the similar action of AR and ER in cell growth, we focus on the effects of ABE as a CDK4/6i on PC. Here, for the first time, we compared the efficacy of ABE in AR- PC-3 mCRPC and AR+ mutant LNCaP PC cells. Our preliminary findings showed that ABE potentially suppressed the proliferation of PC cells through apoptosis. However, the response of PC cells to ABE was different. The anti-cancer effects of ABE could be influenced by AR status and molecular features.

The limited number of preclinical studies have addressed the therapeutic potential of CDK4/6i in PC [13–15]. In these studies, palbociclib and two new CDK4/6i, G1T28 and G1T38, effectively suppress the proliferation of PC cell lines [wild-type AR (VCaP), AR-V7 (22Rv1 and LNCaP)] and this effect is only mediated by Rb status rather than AR expression. However, we found that ABE was more effective in PC-3 cells than LNCaP cells in this study. The effects of AR on Cyclin D are regulated by the activation of MAPK, Akt and mammalian target of rapamycin (mTOR). Furthermore, the over-expression of androgens can

result in the down-regulation of CDK inhibitors (p21, p27 and p16). These changes lead to the inactivation of Rb and transition from G1 to S phase in PC. Therefore, ADT treatment promotes the transition from AR-sensitive PC cells to AR-mutated PC cells through the aberrant activation of these pathways. At this stage, CDK4/6i could be an alternative therapeutic strategy to overcome resistance regulating by aberrant expression of AR [10,11,16–19]. On the other hand, palbociclib treatment exerts limited efficacy in hormone-sensitive and CRPC cells *in vitro, in vivo* and *ex vivo*, in the previous studies [11,20]. These results could be associated with some genomic alterations including the loss of Rb, higher expression of p16INKa and the amplification of cycle E1/E2 or E2F leading to CDK4/6i resistance [11,21]. Among three CDK4/6i, ABE exerts a higher selectivity for inhibiting the CDK4/cyclin D complex than palbociclib and ribociclib [22]. In our study, ABE exhibited significant anti-cancer activity in AR- mCRPC cells. Therefore, further studies should focus on the effects of new generation CDK4/6i on AR+ wild-type, AR mutant and AR- PC cells.

Furthermore, ABE treatment caused particularly early apoptosis in PC-3 cells through a significant decrease in the mitochondrial membrane potential, increased ROS production, and the over-expression of pro-apoptotic genes. Interestingly, the inhibition of the cell cycle by ABE would be expected to more promote G0/G1 arrest in PC cells. Cdk inhibitors (flavopiridol, UCN-01) lead to G1 arrest by inhibiting Cdk2 and p53-independent induction of p21Waf1/Cip1 and p27Kip1 in cells expressing wild-type Rb [23–25]. Cdk2 /Cyclin E plays a crucial role in the initiation of DNA replication [26]. Additionally, p21 and p27 activation inhibits G1/S phase transition when the cells undergo senescence [27]. Furthermore, our findings showed that the expression of *cyclin D* level was up-regulated upon treatment with ABE in PC cells despite of the down-regulation of protein level. The mechanism of ABE-induced *cyclin D* gene expression could be associated with an inactive Cdk4/Cyclin D complex, thereby protecting cyclin D from turnover as in the study of Comstock et al. (2013). Therefore, further investigation is needed to explore the molecular mechanism of the cell cycle following treatment with ABE in PC cells.

CDK4/6i induce senescence or apoptosis in cancer cells [28–34]. In preclinical studies, the efficacy of ABE in different types of cancer
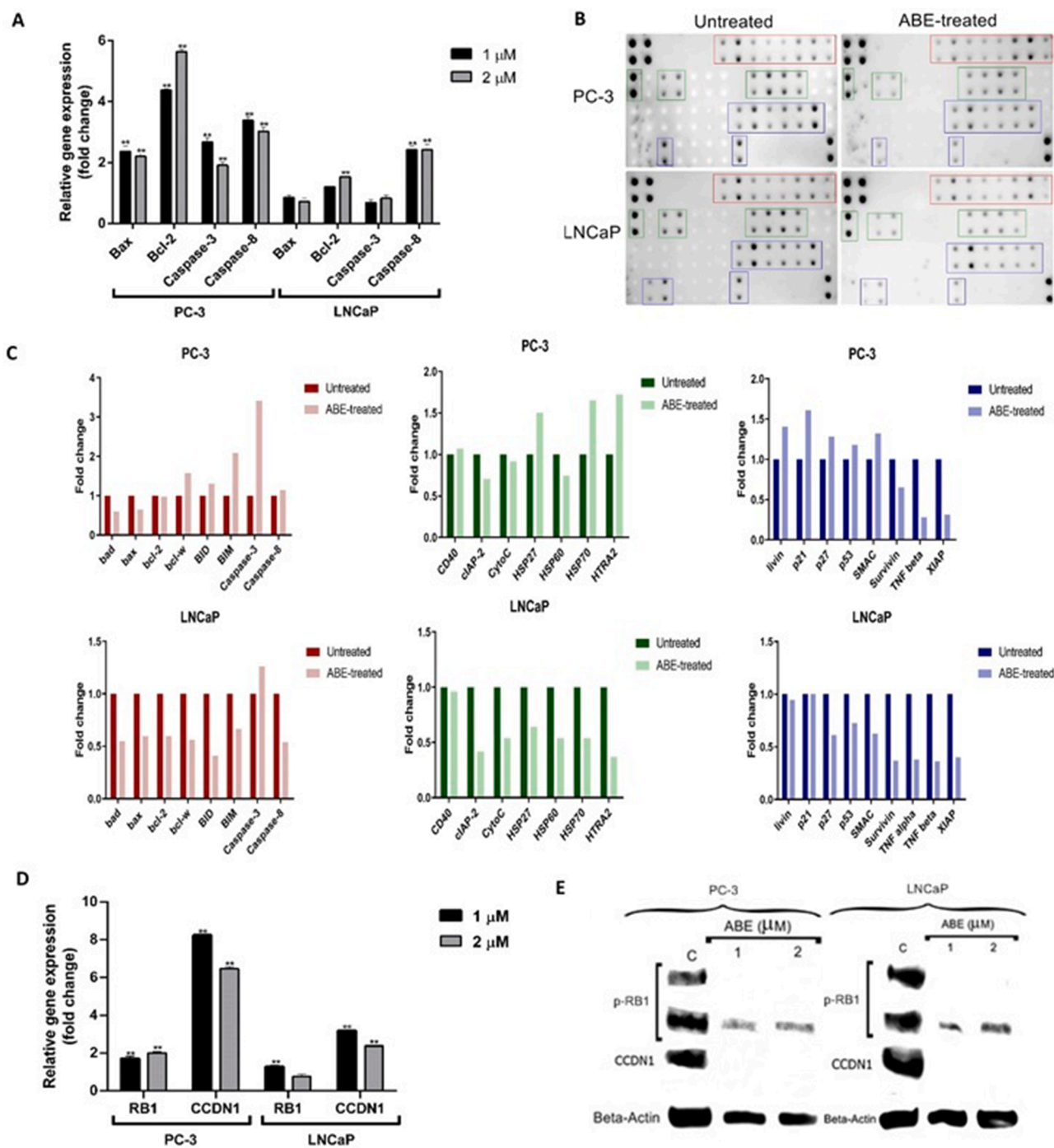
**Fig. 5.** Effects of ABE on the alteration of gene and protein expression levels in PC cells. (A) The mRNA expression of *Bax, Bcl-2, Caspase-3,* and *Caspase-8* in PC cells in response to ABE treatment (B) and (C) the results of apoptosis antibody array, (D) the mRNA and (E) protein expression levels of CCDN1 and RB1 in PC cells after treatment with 1 and 2 μM ABE for 24 h. These results were performed at least in triplicate and expressed as mean ± SD (*$p < 0.05$ and **$p < 0.001$).

(pancreatic ductal adenocarcinomas (PDAC), breast cancer, lung cancer, cervical cancer) has been evaluated [29–34]. The study of Dhir et al. (2019) states that 0.5 μM ABE treatment during 3 days can results in the over-expression *caspase-3*, the induction of early apoptosis and G1 arrest through the down-regulation of p-Rb in PDAC cells. Furthermore, the levels of senescence-associated secretion (SASP) markers are significantly increased supported by β-galactosidase staining after 7 days ABE treatment (29). The inhibition of CDK4/6 causes elevated levels of ROS for senescence induction (28). However, ABE-induced senescence is not mediated by ROS generation in PDAC cells in that study [29]. In the study of Liu et al. (2021), ABE inhibits the proliferation of HPV-negative

cervical cancer cells and induces apoptosis via the repression of CDK4/6-Rb-E2F and mTOR pathways (30). In breast cancer, ABE treatment significantly inhibits the proliferation of both hormone-sensitive and triple negative breast cancer (TNBC) through the induction of apoptosis and senescence *in vitro* and *in vivo* [33,34]. Furthermore, the inhibition of CDK4 induces translocation of RelA and suppresses NFkB-driven transcription prior to apoptosis in SW480 colon cancer cells [31]. On the other hand, CDK4 inhibitor causes TRAIL-mediated apoptosis through the down-regulation of Survivin in pancreatic cancer cells and suppresses NFkB activation via degradation of IkB [32]. In our study, ABE treatment caused a significant increase in

the rate of particularly early apoptosis through the suppression of CDK4/6/Cyclin D complex, the over-expression of *caspase-3* level, pro-apoptotic (Bid, Bim) and cell cycle regulatory proteins (p53/p21/p27) and the down-regulation of cIAP-2, XIAP, HSP60 and survivin in PC-3 cells. Therefore, further investigations are required to elucidate the role of senescence and the contribution of apoptosis-associated signaling pathways into ABE induced cell death in PC cells.

On the other hand, the mRNA levels of *caspase-3* and *caspase-8* were not correlated with the protein expression according to apoptosis antibody array in PC cells as well as *Bcl-2* level in PC-3 cells. In the literature, the regulatory process including post-transcriptional, protein synthesis, translational modifications and protein degradation as well as the spatial and temporal variations of mRNAs can affect the relationship between coding transcript and protein levels. Therefore, the dynamic relationship between transcript levels and protein abundance should be better clarified by understanding genotype-phenotype relationships through transcriptomic and proteomic analysis [35–37].

Finally, our preliminary results indicated that ABE caused many cytoplasmic vacuoles in PC cells and these effects were more profound in LNCaP cells than PC-3 cells. The study of Hino et al. (2020) states that ABE induces atypical cell death in A549 human non-small cell lung cancer cells and multiple cytoplasmic vacuoles are derived from lysosomes but are not associated with autophagy [38]. Additionally, ABE treatment does not induce apoptosis and/or necrosis in A549 cells compared to previous studies. In our previous study, we observed the same vacuoles in MDA-MB-231 triple negative breast cancer cells upon treatment with ABE and we found that these vacuoles did not derive from autophagy. Therefore, lysosomal vacuolation could be related to the cytocidal effect of ABE as in the study of Hino et al. (2020). However, further studies should be performed to identify the interaction of apoptosis and atypical cell death with ABE treatment and the underlying molecular mechanism of these death types in different cancer types.

Our results demonstrated for the first time that ABE treatment significantly inhibited PC cell growth through apoptosis, depolarization of mitochondria membrane potential, the generation of ROS and the over-expression of pro-apoptotic and caspase protein levels. Therefore, further preclinical and clinical investigations are warranted to reveal the potential clinical use of ABE for the treatment of PC. In addition, the combination therapy of ABE with chemotherapy drugs may offer a promising advantage, particularly mCRPC therapy. Thus, further investigations will provide additional insight into the clinical use of CDK4/6i for the treatment of both hormone-sensitive and metastatic castration resistant PC.

## CRediT authorship contribution statement

**Gamze Guney Eskiler:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Asuman Deveci Ozkan:** Investigation, Writing – original draft, Visualization. **Ayten Haciefendi:** Conceptualization, Methodology, Investigation. **Cemil Bilir:** Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgments

## References

[1] J. Ferlay, M. Colombet, I. Soerjomataram, D.M. Parkin, M. Piñeros, A. Znaor, F. Bray, Cancer statistics for the year 2020: an overview, Int. J. Cancer (2021), https://doi.org/10.1002/ijc.33588.

[2] S. Pilleron, E. Soto-Perez-de-Celis, J. Vignat, J. Ferlay, I. Soerjomataram, F. Bray, D. E. Sarfati, Estimated global cancer incidence in the oldest adults in 2018 and projections to 2050, Int. J. Cancer 148 (2020) 601–608.

[3] A.M. Kase, J.A. Copland III, W. Tan, Novel therapeutic strategies for CDK4/6 inhibitors in metastatic castrate-resistant prostate cancer, Onco Targets Ther. 13 (2020) 10499–10513.

[4] N. Brighi, V. Conteduca, C. Lolli, G. Gurioli, G. Schepisi, M. Palleschi, U. De Giorgi, The cyclin-dependent kinases pathway as a target for prostate cancer treatment: rationale and future perspectives, Crit. Rev. Oncol. Hematol. 157 (2020), 103199.

[5] S. Sumanasuriya, J. De Bono, Treatment of advanced prostate cancer—a review of current therapies and future promise, Cold Spring Harb. Perspect. Med. 8 (2018), a030635.

[6] D.K. Leung, P.K. Chiu, C.F. Ng, J.Y. Teoh, Novel strategies for treating castration-resistant prostate cancer, Biomedicines 9 (2021) 339.

[7] Q. Du, X. Guo, M. Wang, Y. Li, X. Sun, Q. Li, The application and prospect of CDK4/6 inhibitors in malignant solid tumors, J. Hematol. Oncol. 13 (2020) 1–12.

[8] S. Pernas, S.M. Tolaney, E.P. Winer, S. Goel, CDK4/6 inhibition in breast cancer: current practice and future directions, Ther. Adv. Med. Oncol. 10 (2018), 1758835918786451.

[9] F. Lynce, A.N. Shajahan-Haq, S.M. Swain, CDK4/6 inhibitors in breast cancer therapy: current practice and future opportunities, Pharmacol. Ther. 191 (2018) 65–73.

[10] N. Brighi, V. Conteduca, C. Lolli, G. Gurioli, G. Schepisi, M. Palleschi, U. De Giorgi, The cyclin-dependent kinases pathway as a target for prostate cancer treatment: rationale and future perspectives, Crit. Rev. Oncol. Hematol. 157 (2021), 103199.

[11] D.N. Rodrigues, G. Boysen, S. Sumanasuriya, G. Seed, A.M.D. Marzo, J. de Bono, The molecular underpinnings of prostate cancer: impacts on management and pathology practice, J. Pathol. 241 (2017) 173–182.

[12] S.P. Balk, K.E. Knudsen, AR, the cell cycle, and prostate cancer, Nucl. Recept. Signal. 6 (2008) e001.

[13] J.P. Stice, S.E. Wardell, J.D. Norris, A.P. Yllanes, H.M. Alley, V.O. Haney, D. P. McDonnell, CDK4/6 therapeutic intervention and viable alternative to taxanes in CRPC, Mol. Cancer Res. 15 (2017) 660–669.

[14] T. Scheinberg, J. Kench, M. Stockler, K.L. Mahon, L. Sebastian, P. Stricker, L. G. Horvath, Pharmacodynamics effects of CDK4/6 inhibitor LEE011 (ribociclib) in high-risk, localised prostate cancer: a study protocol for a randomised controlled phase II trial (LEEP study: LEE011 in high-risk, localised Prostate cancer), BMJ Open 10 (2020), e033667.

[15] P.L. Palmbos, S.A. Tomlins, N. Agarwal, P. Twardowski, A.K. Morgans, W. Kelly, M. Hussain, Cotargeting AR signaling and cell cycle: a randomized phase II study of androgen deprivation therapy with or without palbociclib in RB-positive metastatic hormone sensitive prostate cancer (mHSPC), J. Clin. Oncol. 36 (2018) 251.

[16] V. Conteduca, G. Gurioli, N. Brighi, C. Lolli, G. Schepisi, C. Casadei, U. De Giorgi, Plasma androgen receptor in prostate cancer, Cancers 11 (2019) 1719.

[17] M. Drobnjak, I. Osman, H.I. Scher, M. Fazzari, C. Cordon-Cardo, Overexpression of cyclin D1 is associated with metastatic prostate cancer to bone, Clin. Cancer Res. 6 (2000) 1891–1895.

[18] Z. Fang, T. Zhang, N. Dizeyi, S. Chen, H. Wang, K.D. Swanson, X. Yuan, Androgen receptor enhances p27 degradation in prostate cancer cells through rapid and selective TORC2 activation, J. Biol. Chem. 287 (2012) 2090–2098.

[19] D. Tilki, E.M. Schaeffer, C.P. Evans, Understanding mechanisms of resistance in metastatic castration-resistant prostate cancer: the role of the androgen receptor, Eur. Urol. Focus 2 (2016) 499–505.

[20] C.E.S. Comstock, M.A. Augello, J.F. Goodwin, R. De Leeuw, M.J. Schiewer, W. F. Ostrander, K.E. Knudsen, Targeting cell cycle and hormone receptor pathways in cancer, Oncogene 32 (2013) 5481–5491.

[21] A. McCartney, I. Migliaccio, M. Bonechi, C. Biagioni, D. Romagnoli, F. De Luca, A. Di Leo, Mechanisms of resistance to CDK4/6 inhibitors: potential implications and biomarkers for clinical practice, Front. Oncol. 9 (2019) 666.

[22] F. Schettini, I. De Santo, C.G. Rea, P. De Placido, L. Formisano, M. Giuliano, L. Del Mastro, CDK 4/6 inhibitors as single agent in advanced solid tumors, Front. Oncol. 8 (2018) 608.

[23] G.I. Shapiro, J.W. Harper, Anticancer drug targets: cell cycle and checkpoint control, J. Clin. Invest. 104 (1999) 1645–1653.

[24] A. Deep, R.K. Marwaha, M.G. Marwaha, R. Nandal, A.K. Sharma, Flavopiridol as cyclin dependent kinase (CDK) inhibitor: a review, New J. Chem. 42 (2018) 18500–18507.

[25] B.B. Mull, J.A. Livingston, N. Patel, T. Bui, K.K. Hunt, K. Keyomarsi, Specific, reversible G1 arrest by UCN-01 in vivo provides cytostatic protection of normal cells against cytotoxic chemotherapy in breast cancer, Br. J. Cancer 122 (2020) 812–822.

[26] S. Tadesse, A.T. Anshabo, N. Portman, E. Lim, W. Tilley, C.E. Caldon, S. Wang, Targeting CDK2 in cancer: challenges and opportunities for therapy, Drug Discov. Today 25 (2020) 406–413.

[27] A.M. Abukhdeir, B.H. Park, P21 and p27: roles in carcinogenesis and drug resistance, Expert Rev. Mol. Med. 10 (2008) e19.

[28] V. Wagner, J. Gil, Senescence as a therapeutically relevant response to CDK4/6 inhibitors, Oncogene 39 (2020) 5165–5176.

[29] T. Dhir, C.W. Schultz, A. Jain, S.Z. Brown, A. Haber, A. Goetz, J.R. Brody, Abemaciclib is effective against pancreatic cancer cells and synergizes with HuR and YAP1 inhibition, Mol. Cancer Res. 17 (2019) 2029–2041.

[30] Y. Liu, R. Zhao, S. Fang, Q. Li, Y. Jin, B. Liu, Abemaciclib sensitizes HPV-negative cervical cancer to chemotherapy via specifically suppressing CDK4/6-Rb-E2F and mTOR pathways, Fundam. Clin. Pharmacol. 35 (2021) 156–164.

[31] H.C. Thoms, M.G. Dunlop, L.A. Stark, CDK4 inhibitors and apoptosis: a novel mechanism requiring nucleolar targeting of RelA, Cell Cycle 6 (2007) 1293–1297.

[32] M. Retzer-Lidl, R.M. Schmid, G. Schneider, Inhibition of CDK4 impairs proliferation of pancreatic cancer cells and sensitizes towards TRAIL-induced apoptosis via downregulation of survivin, Int. J. Cancer 121 (2007) 66–75.

[33] R. Torres-Guzmán, B. Calsina, A. Hermoso, C. B.aquero, B. Alvarez, J. Amat, M. J. Lallena, Preclinical characterization of abemaciclib in hormone receptor positive breast cancer, Oncotarget 8 (2017) 69493.

[34] N. O'Brien, D. Conklin, R. Beckmann, T. Luo, K. Chau, J. Thomas, D.J. Slamon, Preclinical activity of abemaciclib alone or in combination with antimitotic and targeted therapies in breast cancer, Mol. Cancer Ther. 17 (2018) 897–907.

[35] T. Maier, M. Güell, L. Serrano, Correlation of mRNA and protein in complex biological samples, FEBS Lett. 583 (2009) 3966–3973.

[36] C. Vogel, E.M. Marcotte, Insights into the regulation of protein abundance from proteomic and transcriptomic analyses, Nat. Rev. Genet. 13 (2012) 227–232.

[37] Y. Liu, A. Beyer, R. Aebersold, On the dependency of cellular protein levels on mRNA abundance, Cell 165 (2016) 535–550.

[38] H. Hino, N. Iriyama, H. Kokuba, H. Kazama, S. Moriya, N. Takano, K. Miyazawa, Abemaciclib induces atypical cell death in cancer cells characterized by formation of cytoplasmic vacuoles derived from lysosomes, Cancer Sci. 111 (2020) 2132.