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ORIGINAL PAPER

Inhibition of CDK4 sensitizes multidrug resistant ovarian cancer cells to paclitaxel by increasing apoptosiss

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Abstract
Purpose Overexpression of cyclin-dependent kinase (CDK) 4 has been observed in a variety of cancers and has been found to contribute to tumor cell growth and proliferation. However, the effect of inhibition of CDK4 in ovarian cancer is unknown. We investigated the therapeutic effect of the CDK4 inhibitor palbociclib in combination with paclitaxel in ovarian cancer cells.
Methods Cell viabilities were determined by MTT assay after exposure to different dosages of palbociclib and/or paclitaxel. Western blot, immunofluorescence, and Calcein AM assays were conducted to determine the mechanisms underlying the cytotoxic effects of palbociclib in combination with paclitaxel. CDK4 siRNA was used to validate the outcome of targeting CDK4 by palbociclib in ovarian cancer cells.
Results We found that combinations of palbociclib and paclitaxel significantly enhanced drug sensitivity in both Rb-positive (SKOV3TR) and Rb-negative (OVCAR8TR) ovarian cancer-derived cells. When combined with paclitaxel, palbociclib induced apoptosis in both SKOV3TR and OVCAR8TR cells. We also found that palbociclib inhibited the activity of P-glycoprotein (Pgp), and that siRNA-mediated CDK4 knockdown sensitized multidrug resistant (MDR) SKOV3TR and OVCAR8TR cells to paclitaxel.
Conclusions Inhibition of CDK4 by palbociclib can enhance paclitaxel sensitivity in both Rb-positive and Rb-negative MDR ovarian cancer cells by increasing apoptosis. CDK4 may serve as a promising target in the treatment of ovarian cancer.

Keywords Ovarian cancer · CDK4 · Palbociclib · Rb · Paclitaxel · Apoptosis · MDR

1 Introduction

Ovarian cancer is the most common gynecological cancer, is usually diagnosed at an advanced stage, and accounts for more deaths than any other gynecological malignancy. Approximately 21,980 women were newly diagnosed with ovarian cancer in 2014, and more than 14,000 patients succumb each year to this disease in the United States alone [1]. Although standard surgical debulking and chemotherapy regimens have been developed for several decades, the overall five-year survival rates of ovarian cancer patients have not significantly improved [2–4]. Currently, the microtubule stabilizing agent paclitaxel is widely used in the clinical management of ovarian cancer [5]. However, most ovarian cancer patients who are initially responsive to paclitaxel will inevitably develop multidrug resistance (MDR). Therefore, therapeutic exploitation of more potent agents to sensitize MDR cells to paclitaxel is essential to improve the treatment outcome of ovarian cancer.

Cyclin-dependent kinases (CDKs) are members of the serine/threonine family of protein kinases and play important roles in tumor cell cycle progression and apoptosis. Among them, CDK4 regulates the G1-S transition phase of the cell

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cycle by deactivating the tumor suppressor retinoblastoma (Rb) protein [6, 7]. Specifically, in response to pro-proliferative stimuli, CDK4 can complex with D-type cyclin1 (Cyclin D1) to induce the phosphorylation of Rb (pRb) and, by doing so, to switch off the tumor suppressive function of Rb. Consequently, once phosphorylated, pRb will not be able to bind the transcription factor E2F, which allows cancer cells to progress through the cell cycle via the transcription of various cell cycle and anti-apoptotic genes [8–10]. Overexpression and activation of the CDK4/Cyclin D1/Rb pathway has amply been shown to be correlated with unrestricted tumor cell growth and proliferation. CDK4 expression has become a hallmark of various types of cancer, including ovarian cancer [11–14], and CDK4 targeting in cancer cells has become a promising therapeutic strategy [15–18].

Recently, the CDK4 inhibitor palbociclib (Ibrance, Pfizer) has been approved by the U.S. FDA as a first-line therapeutic agent for the treatment of ER positive, HER2 negative advanced breast cancer [19, 20]. Palbociclib is a potent and specific CDK4 inhibitor causing G1 cell cycle arrest and inhibiting cancer cell growth. Although previous studies have uncovered an important role of CDK4 in different types of cancer, the significance of CDK4 expression in ovarian cancer and the effects of palbociclib on ovarian cancer cell growth are largely unknown. Therefore, we set out to investigate the effects of palbociclib alone or in combination with paclitaxel on ovarian cancer cells in vitro as well as its potential underlying mechanisms.

2 Materials and methods

2.1 Cell lines, cell culture and reagents

Previously, we characterized and described the drug sensitive ovarian cancer cell lines SKOV3 and OVCAR8 and its MDR derivatives SKOV3TR and OVCAR8TR that were used in this study [21–26]. Both SKOV3TR and OVCAR8TR were selected from its respective parental cell lines by exposure to stepwise increases in paclitaxel concentrations. The resistant derivatives were found to be 100-fold more resistant to paclitaxel compared to the sensitive parental cell lines [25]. All cell lines were cultured in RPMI-1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA) at 37 °C in a 5% CO_2 - 95% air humidified atmosphere. The highly selective CDK4/6 inhibitor palbociclib (PD-0332991) HCl (Chemical Name: 6-acetyl-8-cyclopentyl-5-methyl-2-(5-(piperazin-1-yl)pyridin-2-ylamino)pyrido[2,3-d]pyrimidin-7(8H)-one hydrochloride), was purchased from Selleck Chemicals (Houston, TX, USA). The anticancer drug paclitaxel was obtained from Teva Phamaceuticals (Sellersville, PA, USA). The human nonspecific siRNA and CDK4 targeting siRNA (Genebank Accession Number: NM_000075.3, 5′-CUCUUAUCUACAUAAGGAU-3′) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The CDK4 siRNA (Cat# SASI_Hs01_00122488) was validated to effectively knock down the expression of CDK4. Lipofectamine® RNAiMAX was purchased from Life Technologies. The monoclonal rabbit anti-human CDK4 antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). The Rb pathway associated antibodies, including anti-Rb and anti-phospho-Rb Ser780, 795, and 807/811, were also purchased from Cell Signaling Technology.

2.2 MTT assay

The cytotoxicities of palbociclib and paclitaxel in ovarian cancer cells were assessed by MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. In brief, 3×10^3 cells per well were seeded into a 96-well microplate and exposed to different concentrations of palbociclib with or without paclitaxel. After a 5-day incubation, 20 μl MTT (Sigma-Aldrich) was added after which the cells were incubated for 4 h at 37 °C in a 5% CO_2 humidified atmosphere. Subsequently, the resulting intracellular formazan crystals were solubilized in acid-isopropanol. The absorbances were assessed using a SpectraMax Microplate® Spectrophotometer (Molecular Devices LLC, Sunnyvale, CA, USA) at 490 nm, and normalized to those of untreated cells. In all cases, the MTT assay was conducted in triplicate.

2.3 Western blotting

Protein lysates were extracted using 1 × RIPA lysis buffer (Upstate Biotechnology, Charlottesville, VA, USA) supplemented with a complete protease cocktail and phosphatase inhibitor set I/II (Roche Applied Science, IN, USA). The protein concentrations were evaluated using Protein Assay Reagents (Bio-Rad, Hercules, USA) and quantified using a SPECTRAmax Microplate® Spectrophotometer. Equal amounts of protein were separated using NuPAGE® 4–12% Bis-Tris Gel (Life Technologies) and transferred onto nitrocellulose membranes (Bio-Rad). Next, the membranes were incubated with specific primary antibodies at 4 °C overnight, and then probed with its respective secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA). Finally, the bands were scanned by Odyssey® CLx equipment and quantified using Odyssey software 3.0.

2.4 Apoptosis assay

To investigate how CDK4 sustains ovarian cancer cell growth and survival, potential apoptotic events elicited by CDK4 expression knockdown in drug sensitive and resistant ovarian

cancer-derived cells were assessed. To this end, whole-cell lysates were probed with antibodies directed against PARP (Cell Signaling Technologies) and its cleavage products, as well as other apoptosis-associated proteins, including Survivin (Cell Signaling Technologies) and Bcl-xL (Cell Signaling Technologies). Western blotting was performed as described above.

2.5 Drug uptake assay

Drug uptake was determined using a Vybrant™ Multidrug Resistance Assay Kit (Molecular Probes Inc., Eugene, OR, USA). For visualization of the intracellular accumulation of the P-glycoprotein (Pgp) substrate Calcein AM, 2×10^4 cells were seeded in a 12-well plate in triplicate the day before the assay. Next, the cells were incubated with either 10 μM palbociclib or 10 μM verapamil in RPMI-1640 medium for 2 h at 37 °C. Verapamil, which was included as a positive control, is a calcium channel blocker that inhibits Pgp activity noncompetitively. As a negative control untreated ovarian cancer-derived drug sensitive cells were used. Next, 0.25 mM Calcein AM was added to each well after which the cells were incubated at 37 °C for another 15 min. After washing the cells with phosphate buffered saline (PBS), 1 μg/ml Hoechst 33342 (Life Technologies) was used to stain nuclei. Finally, the cells were photographed using a Nikon Eclipse Ti-U fluorescence microscope (Nikon Instruments Inc., NY, CA, USA) equipped with a SPOT RT™ digital camera.

2.6 Immunofluorescence assay

After siRNA-mediated knockdown in ovarian cancer-derived cells, CDK4 was visualized using an immunofluorescence assay. To this end, cells were incubated 48 hr post transfection in 4% paraformaldehyde, fixed in ice-cold methanol, and blocked with 1% bovine serum albumin (BSA). Next, immunostaining was performed using an anti-CDK4 antibody and an Alexa Fluor 594 conjugated goat anti-rabbit antibody (Life Technologies). Nuclei were counterstained with 1 μg/ml Hoechst 33342. Images were acquired using a Nikon Eclipse Ti-U fluorescence microscope equipped with a SPOT RT™ digital camera. Red color represents CDK4 protein, and blue color represents nuclei.

2.7 Statistic analyses

The data were analyzed using Prism 5.0 software (Graph Pad Software Inc., San Diego, CA, USA). Statistical significance was assessed using independent two-tailed Student t-tests for independent data. Differences with $p < 0.05$ were considered significant for all statistical tests.

3 Results

3.1 Palbociclib and paclitaxel combinations significantly affect the proliferation of ovarian cancer-derived cells

To explore the effect of CDK4 inhibition on ovarian cancer cells, we first examined the expression of several CDK4 pathway-associated proteins. Based on Western blot data, CDK4 and Cyclin D1 were found to be uniformly expressed in all four cell lines tested (Fig. 1). Both OVCAR8 and OVCAR8TR cells carry Rb deletions and lack Rb phosphorylation (Ser807–811, 795, and 780), while SKOV3 and SKOV3TR cells express wild-type and phosphorylated Rb. Therefore, SKOV3 and SKOV3TR are defined as Rb-positive cell lines, and OVCAR8 and OVCAR8TR as Rb-negative cell lines. In addition, we found that the MDR cell lines SKOV3TR and OVCAR8TR exhibit higher Pgp expression levels than their parental drug sensitive counterparts (Fig. 1).

Next, we set out to assess the effects of palbociclib on the different ovarian cancer-derived cell lines using a MTT assay, after normalization to the respective untreated cell lines (Fig. 2). We found that all four (Rb-positive/negative) cell lines tolerated low concentrations of palbociclib, i.e., after a 5-day incubation with 1 μM palbociclib the cell viabilities were ~80–90% of the untreated control group ($p > 0.05$). However, a higher concentration of palbociclib (10 μM) reduced the cell survival to 50%, which may be due to the long-term treatment. Nevertheless, when palbociclib was added alone, no significant difference was observed in the sensitivity to palbociclib between the paired sensitive and resistant cell lines ($p > 0.05$), i.e., the inhibitor concentration - cell viability curves of the drug sensitive and drug resistant cell lines were found to be nearly identical (Fig. 2a and b). Next, we set out to assess the synergistic effect of paclitaxel and palbociclib. We found that the MDR cell lines, both Rb-positive SKOV3TR and Rb-negative OVCAR8TR, were able to survive a 5-day incubation with 0.1 μM paclitaxel. However, when these MDR cells

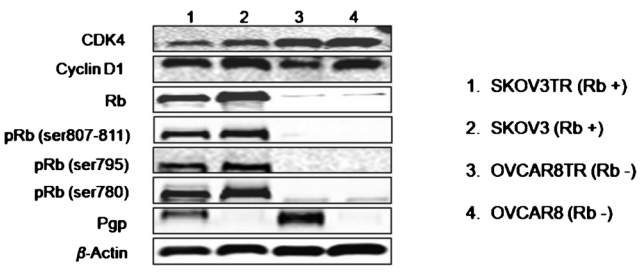

Fig. 1 CDK4/Cyclin D1/Rb/Pgp expression profiles in ovarian cancer-derived cell lines. Western blots showing protein levels of CDK4, Cyclin D1, Rb, pRb (Ser807–811, 795, and 780), and Pgp in SKOV3TR, SKOV3, OVCAR8TR, and OVCAR8 cells

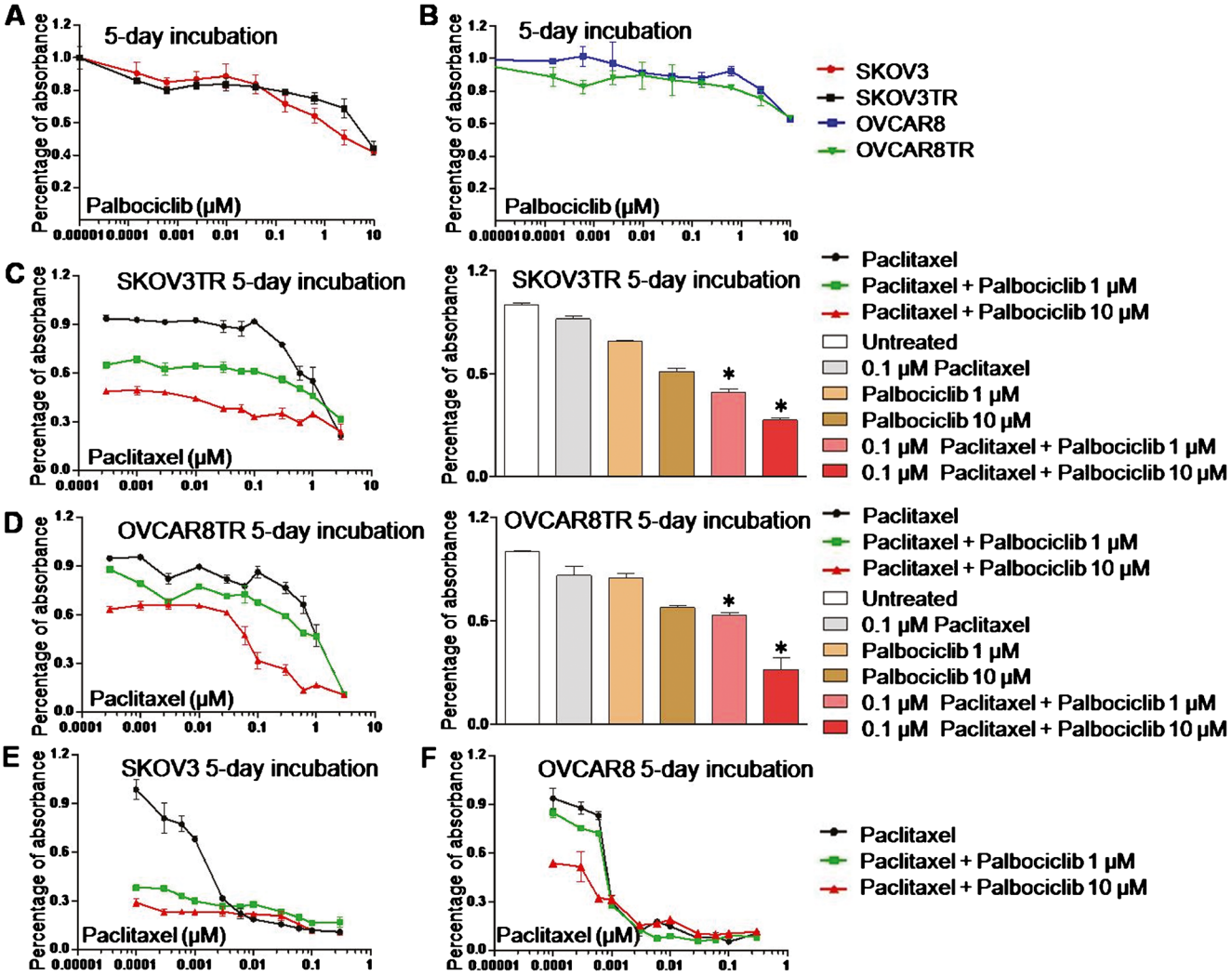

Fig. 2 Palbociclib and paclitaxel combinations significantly suppress the viability of Rb+/− ovarian cancer-derived cells. **a** relative cell viability of SKOV3 and SKOV3TR cells after incubation with different concentrations of palbociclib for five days. The black line represents palbociclib alone (0–10 μM) treated SKOV3TR cells. **b** relative cell viability of OVCAR8 and OVCAR8TR cells after incubation with different concentrations of palbociclib for five days. The blue line represents palbociclib alone (0–10 μM) treated OVCAR8TR cells. **c** relative cell viability of SKOV3TR cells after incubation with paclitaxel alone or in combination with palbociclib for five days. The black line represents paclitaxel alone treated SKOV3TR cells. **d** relative cell viability of OVCAR8TR cells after incubation with paclitaxel alone or in combination with palbociclib for five days. The black line represents paclitaxel alone treated OVCAR8TR cells. **e** relative cell viability of SKOV3 cells after incubation with paclitaxel alone or in combination with palbociclib for five days. The black line represents paclitaxel alone treated SKOV3 cells. **f** relative cell viability of OVCAR8 cells after incubation with paclitaxel alone or in combination with palbociclib for five days. The black line represents paclitaxel alone treated OVCAR8 cells. *$p < 0.05$

were treated with combinations of paclitaxel and 1 μM or 10 μM palbociclib, the SKOV3TR survival rates decreased to ~50% and ~30% (Fig. 2c, $p < 0.05$) and those of OVCAR8TR to ~60% and ~30% of the control groups, respectively (Fig. 2d, $p < 0.05$). Also in the drug sensitive SKOV3 and OVCAR8 cells we found that co-administration of palbociclib and paclitaxel restored their chemosensitivity (Fig. 2e and f, $p < 0.05$). From these results we conclude that, when combined with palbociclib, the responses to paclitaxel are partially increased in both drug resistant and drug sensitive ovarian cancer-derived cells.

3.2 The CDK4/cyclin D1/Rb/apoptosis axis is altered in ovarian cancer-derived cells after palbociclib and/or paclitaxel treatment

To investigate the molecular mechanisms underlying the cytotoxic effects of palbociclib and/or paclitaxel, we set out to quantitatively determine the expression of the CDK4/Cyclin D1/Rb/apoptotic axis-related proteins by Western blotting. To this end, SKOV3TR and OVCAR8TR were incubated with different doses of paclitaxel alone or in combination with palbociclib for 48 h. Consistent with our previous results, we found that both drug resistant SKOV3TR cells and

OVCAR8TR cells survived paclitaxel when administered at concentrations as high as 1 μM. In contrast, we found that SKOV3TR cells did not tolerate 0.06 μM paclitaxel when administered together with 10 μM palbociclib. In addition, we found that the cell viability did not significantly change during a 48 h incubation with 10 μM palbociclib. Very similar results were obtained with OVCAR8TR cells.

We did not observe any overt alterations in CDK4, Cyclin D1, Rb, or pRb protein expression levels when SKOV3TR cells were treated with paclitaxel alone (0.06, 0.3, and 1 μM, $p > 0.05$, Fig. 3 and S1). Although the Bcl-xL levels were slightly decreased in SKOV3TR cells treated with 1 μM paclitaxel, the levels of PARP cleavage and Survivin expression were not found to be affected. When SKOV3TR cells were incubated with 1, 2.5, or 10 μM palbociclib alone, the CDK4, Cyclin D1, and Rb protein levels were not found to be changed compared to the control group ($p > 0.05$). The phosphorylation of Rb (Ser807–811, 795, and 780) was down-regulated by palbociclib in a dose-dependent manner ($p < 0.05$). The apoptosis-related PARP cleavage and Survivin expression levels were also found to be substantially altered along with increasing palbociclib concentrations ($p < 0.05$, Fig. 3 and S1). We also assessed the respective protein levels in cells incubated with both palbociclib (10 μM) and paclitaxel (0.06, 0.3, and 1 μM). Consistent with the palbociclib-only treated cells, no significant differences were observed in CDK4, Cyclin D1, and Rb protein levels when the paclitaxel concentrations were increased ($p > 0.05$). Rb phosphorylation was, however, almost undetectable and significantly lower than that in untreated cells and cells treated with paclitaxel alone ($p < 0.05$). Furthermore, increased PARP cleavage was observed in the combined palbociclib (10 μM) and paclitaxel treatment group compared to the palbociclib alone (10 μM) treatment group ($p < 0.05$), whereas Survivin and Bcl-xL continued to exhibit low expression levels (Fig. 3 and S1, $p < 0.05$). The same treatment was applied to OVCAR8TR (Rb-negative) cells. We found that both the CDK4 and Cyclin D1 protein levels were not significantly altered by either type of treatment ($p > 0.05$), and that neither Rb nor pRb was detectable by Western blotting. In contrast, increased PARP cleavage and decreased Bcl-xL expression levels were observed after combined paclitaxel and palbociclib treatment ($p < 0.05$), whereas no overt changes in Survivin expression were noted (Fig. 4 and S2).

3.3 Palbociclib inhibits the activity of Pgp in both Rb-positive and Rb-negative ovarian cancer-derived cells

Both SKOV3TR and OVCAR8TR are cell lines that exhibit high levels of Pgp expression. Pgp is also characterized as one

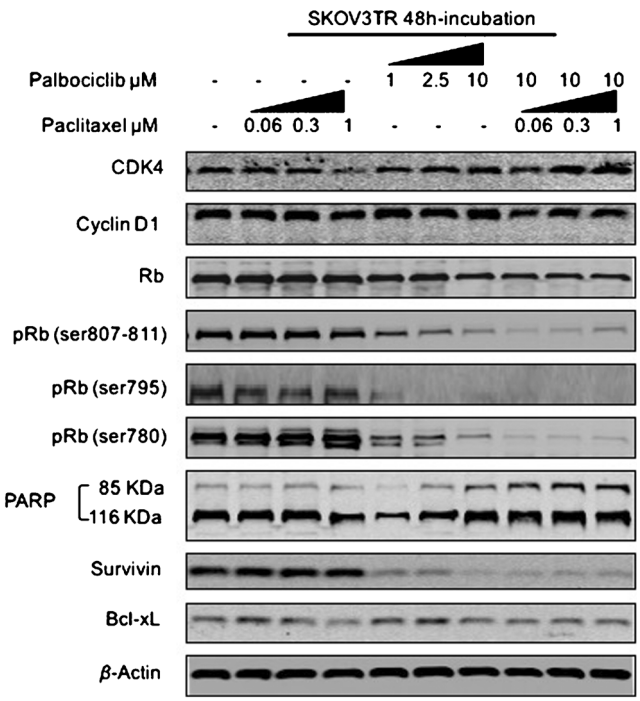

Fig. 3 Palbociclib induces apoptosis in SKOV3TR (Rb-positive) cells via increased Bcl-xL expression and PARP cleavage, and decreased Survivin expression. Western blots showing protein levels of CDK4, Cyclin D1, Rb, pRb (Ser807–811, 795, and 780), PARP, Survivin, and Bcl-xL in SKOV3TR cells after treatment. Quantitative data of the Western blots are shown in Fig. S1

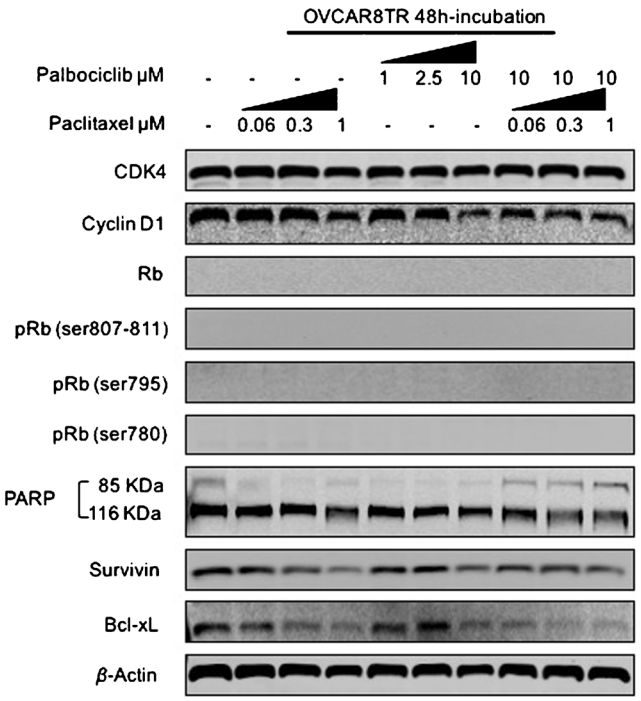

Fig. 4 Palbociclib induces apoptosis in OVCAR8TR (Rb-negative) cells via increased Bcl-xL expression and PARP cleavage, and decreased Survivin expression. Western blots showing protein levels of CDK4, Cyclin D1, Rb, pRb (Ser807–811, 795, and 780), PARP, Survivin, and Bcl-xL in OVCAR8TR cells after treatment. Quantitative data of the Western blots are shown in Fig. S2

of the classic MDR mediators in ovarian cancer. We found that during different paclitaxel-palbociclib combination treatments, the Pgp protein levels in the two cell lines remained constant (Fig. 5a, $p > 0.05$). In order to subsequently assess whether palbociclib can inhibit the function of Pgp, a Calcein AM assay was carried out during the aforementioned treatment regimens. By doing so, we found that in the parental drug sensitive SKOV3 and OVCAR8 cell lines, non-fluorescent Calcein AM readily diffused across the cell membrane after which fluorescent Calcein accumulated in the cytoplasm after cleavage of Calcein AM by endogenous esterase (Fig. 5b). In contrast, we found that in SKOV3TR and OVCAR8TR cells upregulation of Pgp extruded Calcein AM from the cell membrane before it could be hydrolyzed by esterase, therefore reducing the accumulation of fluorescent Calcein. Verapamil is a calcium channel blocker that inhibits Pgp activity noncompetitively and was included as the positive control. Compared to Verapamil treated cells, a strong fluorescence was also observed in palbociclib treated SKOV3TR and OVCAR8TR cells. These results indicate that palbociclib may act as a Pgp suppressor.

3.4 CDK4 silencing suppresses Rb phosphorylation and enhances drug sensitivity in vitro

To further evaluate whether suppression of CDK4 affects the growth and drug sensitivity of ovarian cancer-derived cells in vitro, we set out to knock down CDK4 expression by siRNA in SKOV3TR and OVCAR8TR cells. We found that CDK4 expression was markedly inhibited by 30 nM CDK4 siRNA ($p < 0.05$), whereas cells transfected with nonspecific siRNA expressed a constant level of CDK4 and other proteins compared to untreated cells (Fig. 6a and S3, $p > 0.05$). The effective knockdown was also confirmed by immunofluorescence (Fig. 6b). Although we found that the expression of Rb was not altered after CDK4 knockdown, the pRb (Ser807–811, 795, and 780) expression levels were significantly

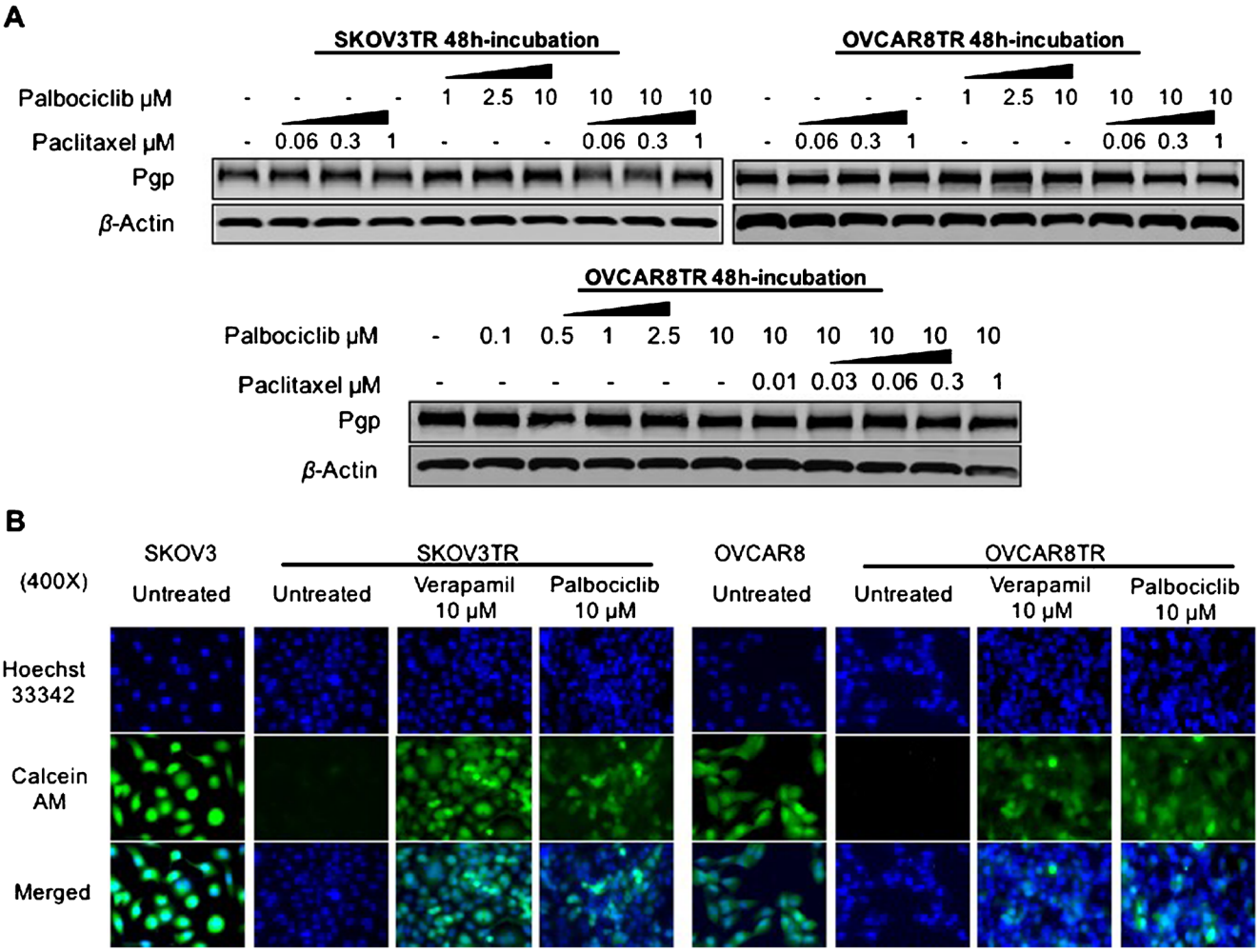

Fig. 5 Palbociclib inhibits the activity of Pgp in both Rb-positive and negative ovarian cancer-derived cells. **a** Western blots showing protein levels of Pgp in SKOV3TR and OVCAR8TR cells after treatment. **b** fluorescent Calcein AM accumulates in the cytoplasm of SKOV3TR and OVCAR8TR cells after treatment

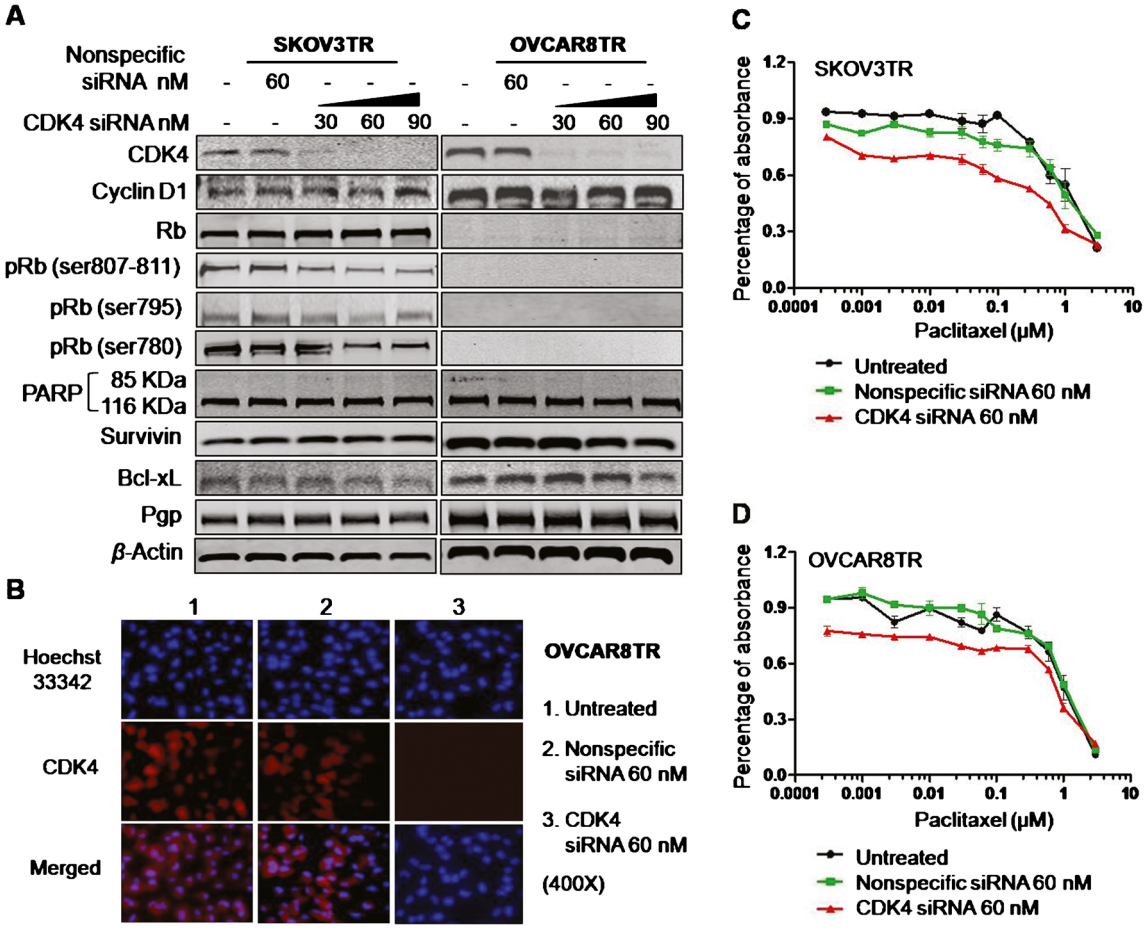

Fig. 6 CDK4 knockdown results in Rb phosphorylation suppression and drug sensitivity enhancement. **a** Western blots showing protein levels of CDK4, Cyclin D1, Rb, pRb (Ser807–811, 795, and 780), PARP, Survivin, and Bcl-xL in SKOV3TR and OVCAR8TR cells after CDK4 siRNA transfection. **b** immunofluorescence images of CDK4 in OVCAR8TR cells after CDK4 siRNA transfection. **c** and **d** sensitivity to paclitaxel of SKOV3TR and OVCAR8TR cells after CDK4 siRNA transfection

downregulated in SKOV3TR cells ($p < 0.05$). The apoptotic markers PARP cleavage, and Survivin and Bcl-xL expression, were not significantly changed in either cell line (Fig. 6a and S3, $P > 0.05$). Accordingly, we found by microscopic assessment that the cell viabilities were not altered. In addition, we found that, compared to untreated cells and nonspecific siRNA transfected cells, the sensitivity to paclitaxel was enhanced when CDK4 was knocked down (Fig. 6c and d, $p < 0.05$). Taken together, we conclude that CDK4 may play a critical role in maintaining drug sensitivity in ovarian cancer cells.

4 Discussion

In our current study, we found that a combined treatment with the cyclin dependent kinase 4 (CDK4) inhibitor palbociclib and paclitaxel serves as an effective co-treatment regimen in ovarian cancer-derived cells. We also found that siRNA-mediated CDK4 knockdown restored the sensitivity of these cells to paclitaxel.

Proteins governing cell cycle progression have long been a focus of cancer therapy development. During the G1-S phase, CDK4 associates with Cyclin D1, which acts as a subunit that promotes cell cycle progression. Rb is the main target of CDK4. In tumors, CDK4/Cyclin D1 pathway alterations may lead to Rb phosphorylation and overactivation of the cell cycle [27]. Therefore, we first assessed the expression of CDK4, Cyclin D1, Rb, and pRb in both drug sensitive and drug resistant ovarian cancer-derived cell lines, i.e., SKOV3/SKOV3TR and OVCAR8/OVCAR8TR, respectively. Each of these cell lines exhibits detectable CDK4 and Cyclin D1 expression levels. Rb and pRb were, however, only found to be expressed in the SKOV3/SKOV3TR cell lines. The observed lack of Rb and pRb expression in OVCAR8 cells is consistent with other reports [28, 29]. As expected, we also found that the MDR derivative cell line OVCAR8TR exhibited a lack of Rb and pRb expression. The different Rb statuses in these ovarian cancer-derived cell lines are also compatible with clinical studies that have reported that ~14% of the primary ovarian carcinomas are Rb-negative [30].

Palbociclib, a highly selective CDK4 inhibitor, has shown a reproducible activity and tolerated toxicity in different malignancies, including in breast cancer, liposarcoma, and mantle cell lymphoma [16, 19, 31]. Based on this information, we hypothesized that palbociclib might also be effective for the treatment of high CDK4 expressing MDR ovarian cancers. Although it has been found that palbociclib may exhibit a synergistic activity with anti-estrogens, limited data are available on the combination of palbociclib with conventional chemotherapeutic regimens. Here, the in vitro combined effects of palbociclib and paclitaxel in drug sensitive and drug resistant ovarian cancer cells were evaluated. Our results indicate that when combined with palbociclib, the sensitivity to paclitaxel was partially restored in MDR ovarian cancer-derived cells. In addition, we found that MDR cells exhibited similar responses as paclitaxel sensitive cells. Specifically, we found that the administration of 1 μM palbociclib alone did not significantly influence cell viability, but that the combination of 1 μM palbociclib with 0.1 μM paclitaxel significantly enhanced apoptosis compared to 0.1 μM paclitaxel treatment alone. Based on these results, we conclude that palbociclib can increase the sensitivity of ovarian cancer-derived cells to paclitaxel. Consistent with previous reports, we found that palbociclib was able to reverse paclitaxel resistance in SKOV3TR cells [26, 32]. Previously, prolonged responses were observed in a phase I trial of palbociclib and paclitaxel in metastatic breast cancer. In addition, it was found that the combination of palbociclib and paclitaxel was safe and well-tolerated [33].

To uncover the molecular mechanism(s) by which palbociclib inhibits the growth of ovarian cancer cells, we found that in the Rb-positive ovarian cancer-derived cell line SKOV3TR phosphorylation of Rb (Ser807–811, 795, and 780) was significantly suppressed due to distortion of the CDK4/Cyclin D1 complex by palbociclib in a dose-dependent manner. From a therapeutic point of view, apoptosis induction is of particular interest [34–36]. The nuclear enzyme PARP plays a well-established role in base excision and single strand DNA break repair [37]. Survivin is highly expressed in both hematologic and solid umors, and its expression has been correlated with resistance to therapy [38]. Bcl-xL plays a prominent role in cells exposed to paclitaxel, a prototype microtubule-stabilizing agent [39]. According to previous reports, the action of paclitaxel is mediated by downregulation of Bcl-xL and activation of PARP, resulting in apoptosis induction [40]. Paclitaxel can also cause an arrest at the G2/M phase of the cell cycle, again leading to apoptosis [41]. We found that upregulation of PARP cleavage and downregulation of Survivin and Bcl-xL expression were indicative of increased apoptosis after palbociclib and paclitaxel co-treatment. Previously, we found that CDK4 inhibition significantly enhanced the cytotoxic effect of paclitaxel in lung cancer cells by inducing apoptosis and increased PARP cleavage [42]. Therefore, we conclude that apoptosis induction serves as a key mechanism underlying the effect of the palbociclib/paclitaxel treatment combination in Rb-positive ovarian cancer cells. The combined treatment may overcome MDR in these cells.

It is well known that palbociclib inhibits E2F-mediated gene transcription in a Rb-positive setting by specifically preventing Rb phosphorylation [20, 43]. The data presented here are in line with these reports since we found that palbociclib, as a single agent and at concentrations below 10 μM, had no effect on the viability of Rb-negative cells [44, 45]. A synergistic anti-proliferative activity of paclitaxel and palbociclib was, however, noted in Rb-negative ovarian cancer-derived cells. Thus, it can be deduced that palbociclib may arrest Rb-negative ovarian cancer cells via a different pathway. In order to address this option, we studied the effects of palbociclib on Pgp activity. Increased expression of Pgp can induce MDR by preventing the intracellular accumulation of a large number of anticancer drugs that are chemically, structurally, and functionally unrelated [46]. Initially, Pgp was recognized as being responsible for the failure of conventional cytotoxic agents (e.g., vinblastine, doxorubicin, and paclitaxel). Currently, however, it is clear that this transporter can contribute to resistance to a broad range of more than 300 compounds [47], including targeted therapeutic drugs [48, 49]. Pgp is an ATP-dependent transport protein and, therefore, ATP inhibitors may have the potential to inhibit the efflux of drugs from cells [50]. The paclitaxel resistant ovarian cancer-derived cells investigated in the current study (SKOV3TR and OVCAR8TR) overexpress Pgp. Therefore, we also studied alterations in the expression and activity of Pgp. Based on our findings, we conclude that palbociclib may not serve as a substrate of Pgp but, instead, may inhibit the activity of Pgp. Specifically, we found that palbociclib increased the intracellular accumulation of the Pgp substrate Calcein AM by decreasing its efflux in resistant cells. Furthermore we found that, compared to the frequently used reversal agent verapamil, palbociclib exhibits similar Pgp efflux pump inhibition effects at the same concentration. Together, these findings may have implications for its therapeutic application and, therefore, a more detailed insight into the mechanism by which palbociclib inhibits Pgp activity will be the subject of future studies.

Deregulation of the CDK4 signaling pathway is frequently observed in human cancers, including ovarian cancer, and CDK4/Cyclin D1 pathway alterations have been reported to serve as significant predictors of a poor clinical outcome in ovarian cancer [12]. Previously, it was found that CDK4 is crucial for the proliferation of SKOV3 cells [13]. Although no significant alterations were observed in apoptosis-related proteins or cell death induction after CDK4 knockdown, an increased paclitaxel sensitivity underscores the important role of CDK4 in ovarian cancer. CDK4 may serve as a promising target for re-sensitizing paclitaxel resistant ovarian cancer

cells, as has been reported for lung cancer cells [42]. Our results support the notion that genetic knockdown or pharmacological inhibition of CDK4 may serve as a targeted therapeutic option in ovarian cancer.

Taken together, we found that inhibition of CDK4 by palbociclib or a CDK4-specific siRNA can increase the sensitivity to paclitaxel in both Rb-positive or Rb-negative MDR ovarian cancer-derived cells. Importantly, we noted proliferation arrest through two mechanisms in Rb-positive ovarian cancer cells, i.e., decreased Rb phosphorylation (Ser807–811, 795, and 780) or Pgp inactivation, resulting in enhanced apoptosis (i.e., upregulated PARP cleavage, downregulated Bcl-xL and Survivin expression) (Fig. S4). Our results are indicative for a therapeutic potential of targeting CDK4 to overcome MDR in patients with ovarian cancer.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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