

# A novel sequential treatment of palbociclib enhances the effect of cisplatin in RB-proficient triple-negative breast cancer

**Yajing Huang**

Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology  
<https://orcid.org/0000-0001-7876-165X>

**Hao Wu**

Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology

**Xingrui Li** (✉ [lixingrui@tjh.tjmu.edu.cn](mailto:lixingrui@tjh.tjmu.edu.cn))

Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology  
<https://orcid.org/0000-0001-7640-9142>

---

## Primary research

**Keywords:** Palbociclib, Cisplatin, RB, Cell cycle, Triple-negative breast cancer

**Posted Date:** September 4th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-40994/v2>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

**Version of Record:** A version of this preprint was published on October 12th, 2020. See the published version at <https://doi.org/10.1186/s12935-020-01597-x>.

# Abstract

**Background:** Triple-negative breast cancer (TNBC) is a highly aggressive malignancy lack of sensitivity to chemo-, endocrine and targeted therapy. CDK4/6 inhibitors, combined with endocrine therapy, have been proven to be effective in postmenopausal women with HR-positive, HER2-negative advanced or metastatic breast cancer. So we investigated that whether CDK4/6 inhibitor palbociclib (PD) could enhance effects of cisplatin (CDDP) on TNBC.

**Methods:** The effects of different drug regimens of PD and CDDP on MDA-MB-231 and RB-knockdown MDA-MB-231 (sh-MDA-MB-231) cells were assessed in vitro and vivo. MDA-MB-468 and RB-overexpression MDA-MB-468 cells were used to assess the effect of PD-CDDP regimen in vitro. Immunoblotting illustrated cyclin D1/RB/E2F axis signaling pathway.

**Results:** PD induced G1 phase cell cycle arrest in MDA-MB-231 cell line. However, synchronous treatment with PD and CDDP for 24h, PD used for 24h and then followed by CDDP or CDDP used for 24h and then followed by PD all had no influence on cell apoptosis of MDA-MB-231 cells. We further investigated the effect of PD or CDDP withdrawal on sequential treatment and found that PD used for 48h and then withdrawn for 48h followed by CDDP (PD-CDDP) could significantly increase apoptosis, inhibit cell viability and colony formation of MDA-MB-231 cells, while in other regimens PD and CDDP represented additive or antagonistic response. Preferential use of PD could increase DNA damage by CDDP as measured through  $\gamma$ H2AX. These findings above were negative in sh-MDA-MB-231 cells and cell function experiments of MDA-MB-468 and RB-overexpression MDA-MB-468 cells could draw similar conclusions, which indicated that PD enhanced the sensitivity of TNBC cells to CDDP in a RB dependent manner. In vivo, this combination treatment inhibited tumor growth and Ki-67 expression compared with single drug treatments in MDA-MB-231 xenograft models. Western blotting analysis presented that PD enhanced sensitivity to CDDP through CDK4/6-cyclin D-RB-E2F pathway.

**Conclusions:** Pre-treatment with PD synchronized tumor cell cycle through CDK4/6-cyclin D1-RB-E2F pathway, which could increase anti-tumor effect of CDDP. PD-CDDP might be an effective treatment for RB-proficient TNBC patients.

## Background

Breast cancer (BC) is the most frequently diagnosed cancer in women worldwide, and it continues to be one of the leading causes of cancer-related deaths [1, 2]. The standard treatment for BC mainly depends on the molecular subtype of tumor. Triple-negative breast cancer (TNBC) accounts for about 15-20% of all BCs, which lacks estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). As a highly aggressive and heterogeneous tumor, it contributes significantly to tumorigenesis, resistance to chemotherapy, thus associates with increased risk of disease recurrence and death [3]. Moreover, improper interventions, both in timing and methods, will lead to earlier relapses and worse outcomes [4]. Hence, more effective strategies desire to be explored.

Over the last decade, advances in molecular translational research have heralded major breakthroughs in the understanding, diagnosis and management of breast cancer. Cell cycle progression undertakes a crucial role in cell proliferation, whose aberration has been acknowledged as a hallmark of cancer [5, 6]. CDK4 and CDK6 are cyclin-dependent kinases that function in a form of cyclin D1-bound compounds at cell cycle checkpoint, which could promote cell cycle in G1-S phase transition. A major target of CDK4 and CDK6 during cell-cycle progression is the tumor suppressor retinoblastoma protein (RB). When RB is phosphorylated, its growth-suppressive properties are inactivated, and then releasing the E2Fs. Amplification and overactivation of the CDK4/6-cyclin D-RB-E2F pathway have been observed in various malignancies including breast cancer [7-12]. Selective CDK4/6 inhibitors “turn off” these kinases and dephosphorylate RB, resulting in a block of cell-cycle progression in mid-G1 and preventing proliferation of cancer cells [13].

Although large amounts of pre-clinical and clinical trials based on different TNBC subtypes were conducted, we have identified no explicit target yet [14, 15]. Clinical studies have confirmed the efficacy of cisplatin (CDDP) for TNBC, either in single or combined administration. However, CDDP is highly toxic to the blood and nervous system and has limited survival benefits, so it is not routinely applied to chemotherapy regimens [16, 17]. Palbociclib (PD0332991, PD) is a selective CDK4/6 inhibitor, with the Food and Drug Administration (FDA) approving of it as first line of endocrine-based therapy for those postmenopausal women with hormone-receptor-positive (HR+), HER2-negative advanced or metastatic breast cancer [14, 18-20]. So we speculate whether PD alone or in combination with CDDP could be applied as a novel treatment for TNBC. For the purpose of solving this question we performed the current research to discover the effect of PD alone or combined with CDDP on TNBC cells and the corresponding mechanism.

## Methods

### Cell culture and treatments

TNBC cell line MDA-MB-231 was kindly donated by Professor Erwei Song, University of Sun Yat-Sen on 11/2018. The TNBC cell lines MDA-MB-468 and HCC1937 were purchased from Genechem Company (Shanghai, China) on 01/2019. All cells were free from mycoplasma contamination, and their identities were authenticated by short tandem repeat (STR) DNA profiling by Shanghai Biowing Applied Biotechnology Co., Ltd in 31/10/2019. All cells were used in experiments within 30 passages after thawing. MDA-MB-231 was cultured in DMEM with 10% FBS (Gibco). MDA-MB-468 was cultured in RPMI-1640 with 10% FBS (Gibco). Cells were incubated in a 37°C humidified atmosphere containing 5% CO<sub>2</sub>. In vitro, cells were treated with 500nM PD (PD-0332991, SelleckChem, Houston, TX, USA) or vehicle (PBS, BOSTER, Wuhan, China). MDA-MB-231 was treated with 50µM CDDP and MDA-MB-468 was treated with 1µM CDDP (SelleckChem, Houston, TX, USA).

### Apoptosis analysis

Cells ( $5 \times 10^4$ /well) were seeded in triplicate in 10% RPMI-1640/DMEM-FBS (full media) in 6-well plates and treated with PD and CDDP separately or combined at indicated concentrations. After being treated for defined times, cells were washed, resuspended in binding buffer, and stained with Annexin V-FITC/PI according to the manufacturer's instructions (BD Biosciences). The apoptotic cell populations were analyzed using flow cytometry (Beckman Coulter, CA). All assays were independently performed three times.

### **Cell cycle analysis**

For cell cycle analysis, cells were harvested, washed with PBS, fixed in pre-chilled 70% ethanol, and kept overnight at  $-20^\circ\text{C}$ . Fixed cells were then collected, washed, and resuspended in PBS. The cells were incubated with 1 mg/mL RNase and  $50\mu\text{g/mL}$  propidium iodide (PI) in the dark for 30 min at  $37^\circ\text{C}$ , and subjected to flow cytometry (Beckman Coulter, CA). The cell cycle results were analyzed using FlowJo version 7.6.1. All assays were independently performed in triplicate.

### **Assessment of cell viability**

Viability of cells were assessed by Cell Counting Kit-8 reagent (CCK8, Dojindo, Tokyo, Japan). 5000 to 10000 cells per well depending on growth characteristics of each cell line were seeded in 96-well plates in triplicate. After adhering overnight, different concentrations and/or sequences of the designated drug were added to the wells. After defined times, supernatants were removed and  $100\mu\text{l}$  CCK8 solution (1:10 dilution) was added to the cells. After 2h incubation at  $37^\circ\text{C}$  in dark, optical density (OD) at 450nm was measured by a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Each experiment was performed three times. The half maximal inhibitory concentration ( $\text{IC}_{50}$ ) was determined from dose-response curves generated by GraphPad Prism version 6.0. The combination index (CI) value was calculated with CompuSyn version 1.0 for the combined treatments of PD and CDDP [21].

### **Colony formation assay**

Cells (500-1000/well) were seeded in 6-well plates and treated with indicated drugs. After combination treatments, media was replenished every 3 days until control wells reached 80-100% confluence. Monolayers were then fixed and stained with a solution of 4% paraformaldehyde and 0.5% crystal violet for 30 min severally at room temperature, washed with water, and dried. Colonies greater than or equal to 50 cells were visually identified and counted. Assays were performed with three independently treated cell populations.

## Tumor xenograft studies

The study was approved by the Ethics Committees of Tongji Hospital, and performed in accordance with the Guide for the Care and Treatment of Laboratory Animals of Tongji Hospital. Four-week-old female BALB/c nude mice were bought from Beijing Vital River Laboratory Animal Technology Co., Ltd., and spent a week of quarantine period in a separate condition before experiment. For MDA-MB-231 cell lines xenograft models,  $7 \times 10^6$  cells were suspended in 100 $\mu$ l PBS plus 50 $\mu$ l matrigel (BD Biosciences) and subcutaneously injected into the left axilla. One week later, mice bearing engrafted tumors of 50mm<sup>3</sup> were randomized to oral treatment with 150mg/kg PD (n=4), intraperitoneal injection with 5mg/kg CDDP (n=4), PD-CDDP treatment (n=4) or vehicle (PBS) treatment (n=4), according to the dosing schedule provided in Fig. 4a. The perpendicular tumor diameters were measured with calipers. Tumor volumes were calculated as (length  $\times$  width<sup>2</sup>)/ 2 every three days. The tumor weight was weighed when mice were euthanized by cervical dislocation. All mice were sacrificed when the tumor burden of vehicle group was equal to 1000mm<sup>3</sup>. Tumors were fixed in 4 % paraformaldehyde for paraffin embedding and used for immunohistochemical staining.

## Immunohistochemistry

Tissue sections were incubated with antibody Ki-67 (#9027, 1:400) (Cell-signaling Technology) overnight at 4°C and stained by 3, 3'-diaminobenzidine (DAB). Densitometry analysis was performed using Image J version 1.48v.

## Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, California, USA). RB expression was measured in triplicate using SYBR Green qPCR Mix (Toyobo, Shanghai, China) according to the manufacturer's instructions. Primer sequences were as follows: RB Forward: 5'-CTCTCGTCAGGCTTGAGTTTG-3', RB Reverse: 5'-GACATCTCATCTAGGTCAACTGC-3'; GAPDH Forward: 5'-GGAGCGAGATCCCTCCAAAAT-3', GAPDH Reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'. The comparative Ct method was used to calculate the relative mRNA expression and GAPDH was used as an internal control.

## Cell transfection

The plasmids contained small hairpin RNA (shRNA) targeting RB and negative-control shRNA (shNC), RB-overexpression plasmid and empty plasmid were purchased from RiboBio (Guangzhou, China). ShRNA were transfected into MDA-MB-231 cells, and RB-overexpression plasmid and empty plasmid were

transfected into MDA-MB-468 cells by X-tremeGENE HP DNA Transfection Reagent (Roche, CHE) according to the manufacturer's instructions. ShRNA sequences were as follows: shNC, 5'-TTCTCCGAACGTGTCACGT-3', shRB, 5'-CGGCTAAATACACTTTGTGAA-3'.

### **Immunofluorescence assay**

Breast cancer cells were subjected to indirect immunofluorescence staining with  $\gamma$ H2AX (Ser139, #9718, 1:400) and then labeling with FITC Goat Anti-Rabbit IgG (#AS007, 1:200). Nuclei were stained with DAPI (Life Technology). Fluorescence images were acquired using an inverted fluorescence microscope (Olympus). Image J version 1.48v was utilized for foci measurement and image analysis.

### **Western blot analysis**

Cell lysates were separated by SDS-PAGE and then transferred to PVDF membranes. Proteins were detected using the following antibodies: RB (ab181616, 1:2000), Cyclin D1 (ab40754, 1:1000), E2F1 (ab179445, 1:1000) (Abcam), p-RB (S780) (#8180, 1:1000), PARP/Cleaved PARP (#9542, 1:1000) (Cell Signaling Technology),  $\beta$ -Actin (AC026, 1:100000) (ABclonal). Specific bands were visualized by ECL (Advansta, USA) and detected with an imaging system (Bio-Rad, USA).

### **Statistical analysis**

Statistical significance, means and standard deviation were calculated by GraphPad Prism version 6.0. All analyses were performed in triplicate and  $P < 0.05$  were considered statistically significant. Data were expressed as the mean  $\pm$  SD or mean. The statistical differences between two groups were analyzed by two-tailed Student's *t* test. Chou-Talalay method was performed to calculate combination index (CI).

## **Results**

### **Mutations and dysregulation of genes in the CDK4/6 pathway are common in TNBC**

TNBC genomic mutations in the CDK4/6 pathway from The Cancer Genome Atlas (TCGA) were obtained and visualized by the cBioPortal browser ([www.cbioportal.org](http://www.cbioportal.org)) [22, 23]. CDKN2A, also known as multiple tumor suppressor I (MTS1), serves as a brake on cell cycle progression by inhibiting CDK4 and CDK6. As was shown in Fig. 1a, 15% of TNBC patients suffered CDKN2A amplifications or deep deletions. 23% patients showed significant amplifications, deep deletions or other mutations in RB gene. Other genes in the CDK4/6 pathway were mainly amplified in TNBC patients. All these classes of mutations would contribute to disorder of CDK4/6 pathways and uncontrolled cell cycle. In general, these data indicated that therapy with CDK4/6 inhibitor would likely benefit a part of TNBC patients. Therefore, we

investigated the effects of CDK4/6 inhibitor PD on TNBC cells. Since previous studies have shown that RB gene plays an important role in the mechanism of CDK4/6 inhibitors [18-20], we detected the mRNA levels of RB in different breast cancer cell lines. As a result, only the mRNA level of RB in MDA-MB-231 cells was significantly higher in TNBC cell lines and was basically as high as HR+ cell lines (MCF-7 and T47-D) (Fig. 1b). So we chose MDA-MB-231 cells for the following experiments.

### **PD could lead to G1 phase arrest in MDA-MB-231 cells and three drug regimens were initially established**

PD, as expected, could significantly block MDA-MB-231 cells in G1 phase (Fig. 1c). Then, we found that PD had no significant effect on apoptosis of MDA-MB-231 cells after being applied continuously for 24h, 48h or 72h (Additional file 1: Figure S1A). So we turned to investigate the possibility of PD enhancing the sensitivity of TNBC cells to CDDP. We established three common drug regimens based on literatures: PD and CDDP, synchronous treatment with PD and CDDP for 24h; PD to CDDP, PD was used for 24h and then followed by CDDP for 24h; CDDP to PD, CDDP was used for 24h and then followed by PD for 24h (Additional file 2: Figure S2A-C). However, none of them could significantly increase cell apoptosis of MDA-MB-231 cells compared with CDDP alone group (Additional file 1: Figure S1B).

### **Three novel drug regimens were established according to the effect of PD on cell cycle**

To discover the effective drug regimen on MDA-MB-231 cells, we further investigate the effect pattern of PD on cell cycle. With the prolonged treatment of PD, the blocking effect on cell cycle of MDA-MB-231 cells was gradually strengthened, which was manifested as that the proportion of G1 phase cells gradually increased, and the G2 and S phases gradually decreased. When PD was continued for 48h, the proportion of G1 phase cells reached the maximum, which did not increase significantly when the duration was prolonged to 72h (Fig. 1d). We continued to investigate the withdrawal effects on cell cycle after 48h continuous treatment of PD, and found that when PD was continuously treated on MDA-MB-231 cells for 48h and then was withdrawn for 48h, the ratio of cells in G1 phase began to decrease (Fig. 1e), indicating that the blocked cells reenter cell cycle. According to the above data, we set three novel drug regimens: PD+CDDP, CDDP-PD, PD-CDDP. PD+CDDP treatment was performed by treating cells with PD for 48h and with CDDP concomitantly for the first 24h. CDDP-PD treatment was performed by treating cells with CDDP for 24h, then withdrawing CDDP for 48h before PD exposure for 48h. PD-CDDP treatment was performed by treating cells with PD for 48h, then withdrawing PD for 48h before CDDP exposure for 24h (Additional file 2: Figure S2D-F).

### **PD-CDDP treatment in vitro has synergistic effect on inhibiting cell functions in MDA-MB-231 cells**

There was no significant difference in apoptosis of MDA-MB-231 cells between PD+CDDP group and CDDP alone group, so was CDDP-PD group and CDDP alone group. However, the apoptosis of MDA-MB-231 cells treated with PD-CDDP was significantly increased compared with CDDP alone (Fig. 2a). Previous studies have demonstrated  $\gamma$ H2AX foci to be an accurate readout for double strand DNA breaks. PD+CDDP group and CDDP-PD group had no obvious effect on DNA damage compared with CDDP alone group (Fig. 2b, c), while MDA-MB-231 cells treated with PD-CDDP had increased  $\gamma$ H2AX positivity (Fig. 2d). Moreover, PD-CDDP group had lower cell viability and less number of colony formation compared with CDDP alone (Fig. 3a, b). The IC<sub>50</sub> of CDDP showed that PD sensitized MDA-MB-231 cells to CDDP, when sequentially administered before CDDP (Fig. 3c), which indicated that PD-CDDP treatment could reduce the dosage of CDDP. CompuSyn software was used to calculate combinational index (CI). CI values = 1 or > 1 are indicative of an additive or antagonistic response, respectively, whereas CI values < 1 indicates a synergistic response. The CI values further indicated that PD-CDDP was the optimal order for the combination of the two drugs, which could maximize the inhibitory effect on MDA-MB-231 cells (Fig. 3d).

### **PD-CDDP treatment in vivo can effectively delay the proliferation of MDA-MB-231 xenograft**

Sixteen female BALB/c nude mice were given  $7 \times 10^6$  suspended MDA-MB-231 cells at the fifth week of age. After 1 week, they were randomly divided into four groups, and the treatment schema was shown in Fig. 4a. As a result, the tumor volumes and weights in PD-CDDP group were significantly less than those in control group (Fig. 4b). Immunohistochemistry analysis showed that the expression of Ki-67 in PD-CDDP group was significantly lower than that in control group (Fig. 4c). Collectively, these results indicated that the application of PD before CDDP could significantly delay tumor growth, which was consistent with in vitro experiments.

### **PD synergizes CDDP-mediated cytotoxicity in a RB-dependent manner**

The mRNA level of RB in MDA-MB-231 cells was dramatically decreased by shRB (Fig. 5a). PD treatment on MDA-MB-231 cells could result in a prominent G1 cell cycle arrest as described above, while there were no such changes in RB-knockdown MDA-MB-231 (sh-MDA-MB-231) cells (Fig. 5b). Cell apoptosis of sh-MDA-MB-231 cells with PD-CDDP treatment showed no significant difference compared with CDDP alone (Fig. 5c). Likewise, there were no obvious difference between PD-CDDP and corresponding CDDP alone group in cell viability, colony formation and DNA damage of sh-MDA-MB-231 cells (Fig. 5d). The IC<sub>50</sub> of CDDP showed that PD could not change the sensitivity of sh-MDA-MB-231 cells to CDDP (Fig. 5e). Since the relative mRNA expression of RB in MDA-MB-468 cells was the lowest, we constructed RB-overexpression MDA-MB-468 (OE) cells for further verification. Vector referred to MDA-MB-468 cells transfected with empty plasmid. As was shown in Fig. 6a, RB expression was significantly higher in OE cells than in vector. PD treatment on OE resulted in obvious G1 cell cycle arrest as in MDA-MB-231 cells

(Fig.6b). In PD-CDDP group, cell apoptosis of OE cells was significantly higher compared with CDDP alone, while there was no such difference in vector (Fig.6c). Similarly, PD-CDDP treatment could significantly inhibit cell viability, colony formation, and increase DNA damage in OE cells compared with corresponding CDDP alone group, while these differences above did not exist in vector (Fig.6d). So we speculated that PD-CDDP treatment had inhibiting effects on RB-proficient TNBC cells, but no such effects on RB-deficient TNBC cells.

### **PD-CDDP treatment enhances the inhibiting effect through cyclin D1/RB/E2F axis**

Consistent with the above result, the protein level of total RB was obviously higher in MDA-MB-231 cells. Furthermore, western blot analysis revealed that compared with CDDP alone group, the phospho-RB (p-RB) of MDA-MB-231 cells in PD-CDDP group was significantly reduced, and the downstream transcription factor E2F1 was also significantly reduced, while there was no such change in sh-MDA-MB-231 cells (Fig. 7a). This indicated that PD inhibited the cyclin D1/RB/E2F pathway of RB-proficient cells and increased CDDP-induced apoptosis and DNA damage of MDA-MB-231 cells, which resulted in increased cleaved PARP and  $\gamma$ H2AX. Proposed mechanism model was shown in Fig. 7b.

## **Discussion**

In this study, we explored the effect of various combination strategies of PD and CDDP in the treatment of TNBC. We initially established three common drug regimens, and discovered that simply combined or sequential use of PD and CDDP showed no more effectiveness on MDA-MB-231 cells compared with CDDP alone group. Therefore, we performed a deeper investigation about the drug regimens and found that the synergism occurred in PD-CDDP group. According to the effect of PD on cell cycle, when PD is used for 48h and then withdrawn for 72h, its blocking effect on cells is weakened, and some cells have entered S and G2 phase. Therefore, it is reasonable to speculate that when PD is used for 48h and then withdrawn for 48h, the cycle of most cells is in the synchronous state, and is ready to enter S phase, in which cells are more sensitive to CDDP, thereby achieving synergistic effect in PD-CDDP group. While in CDDP-PD group, tumor cells are in various cell cycle with quite different sensitivity to CDDP. Sequential use of PD following CDDP may induce partial cells with DNA damage to recover due to cell cycle extension, so CDDP-PD treatment finally produced antagonistic or the same effect as CDDP alone.

Combination therapy that rely on complementary mechanisms of antitumor activity has increasingly becoming the current trend in cancer treatment [24, 25]. Nowadays, more and more targeted therapies like CDK inhibitors, combined with conventional chemotherapy regimens have been applied to improve their anti-tumor effects and inhibit tumor resistance. For example, pre-treatment of PD could sensitize myeloma cells to bortezomib-induced apoptosis [26]. While another study found that simultaneous combination of abemaciclib with paclitaxel or CDDP could achieve better efficacy compared to chemotherapy group [27]. In contrast with above findings, Patrick et al. demonstrated that simultaneous combination of carboplatin with PD decreased antitumor activity compared with carboplatin alone in Rb-

proficient mice and coadministration of PD with carboplatin had no effect on tumor growth in vivo [28]. The above results indicate that it requires careful design when planning CDK inhibitors plus chemotherapy drugs. Different tumor types, different chemotherapeutic agents, and even different time points, as in our study, may emerge totally different effects. Improper strategy may lead to ineffective or even opposite results.

In addition, we demonstrate that PD synergizes CDDP-mediated cytotoxicity in RB-dependent manner. In RB-knockdown or RB-deficient cell lines, PD could not induce cell cycle arrest and PD-CDDP could not promote cytotoxic effect of CDDP, while overexpression of RB could restore the sensitivity of RB-deficient cells to PD. Some literatures and our study all demonstrated that RB could act as a hallmark to select patients suffered cancer who would be likely to benefit from PD treatment and the loss of RB function may be the main cause of primary and secondary drug resistance [29-31].

Overall, this is the first study to investigate the inhibition of TNBC cells by a combination strategy of PD and CDDP under specific drug regimen. However, there are several limitations in this study. We mainly used only one RB-proficient cell line in this study, so we can't rule out that the synergistic effect observed may only specific to MDA-MB-231 cells. Additionally, adding validation of CDDP-resistant cell lines in this study could be more clinically meaningful.

## Conclusions

Our data has proven that PD-CDDP treatment could significantly suppress viability, induce apoptosis and DNA damage of RB-proficient TNBC cells than CDDP alone, and this anti-tumor effect could be ascribed to the synchronization of cell cycle by PD through CDK4/6-cyclin D1-RB-E2F pathway. PD-CDDP may be a promising antineoplastic scheme in the future, however, challenges warrant further validation in prospective studies.

## Abbreviations

PD: Palbociclib; CDDP: Cisplatin; PD-CDDP: treating cells with PD for 48h, then withdrawing PD for 48h before CDDP exposure for 24h; CDDP-PD: treating cells with CDDP for 24h, then withdrawing CDDP for 48h before PD exposure for 48h; PD+CDDP: concomitant treatment with PD and CDDP for 24h; BC: Breast cancer; TNBC: Triple-negative breast cancer; ER: Estrogen receptor; PR: Progesterone receptor; HER2: Human epidermal growth factor receptor 2; RB: Retinoblastoma; STR: Short tandem repeat; CI: Combination index

## Declarations

### Ethics approval and consent to participate

The study was approved by the Ethics Committees of Tongji Hospital, and performed in accordance with the Guide for the Care and Treatment of Laboratory Animals of Tongji Hospital.

## Consent for publication

Not applicable.

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Competing interests

The authors declare that they have no competing interests.

## Funding

There are no funders to report for this submission.

## Authors' contributions

YH and XL designed experiments. YH performed experiments. YH and HW performed data analysis. YH and HW wrote the manuscript. All authors read and approved the final manuscript.

## Acknowledgements

Not applicable.

## References

1. DeSantis CE, Ma J, Gaudet MM, Newman LA, Miller KD, Goding Sauer A, Jemal A, Siegel RL: **Breast cancer statistics, 2019**. *CA: a cancer journal for clinicians* 2019, **69**(6):438-451.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A: **Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries**. *CA: a*

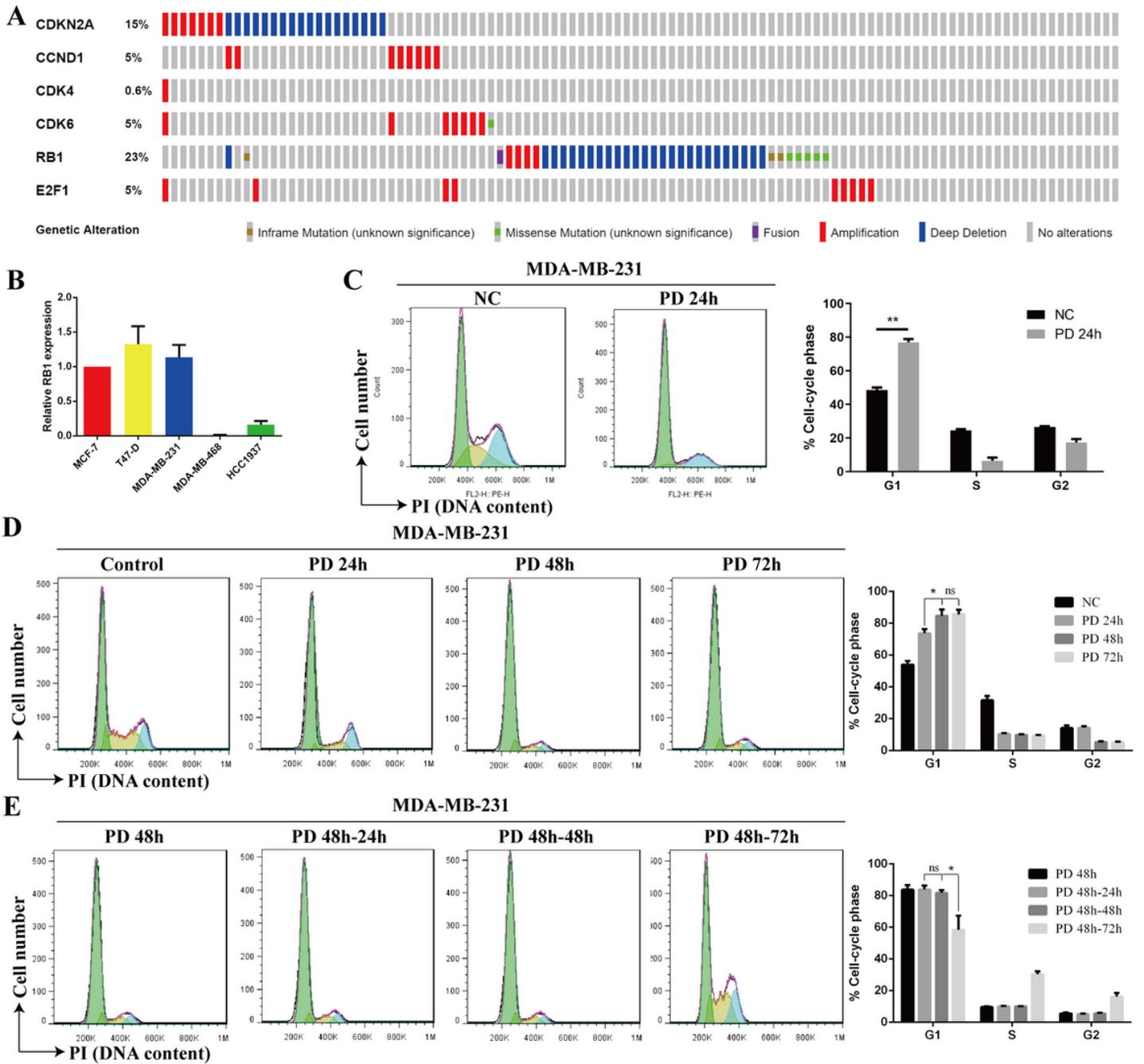
- cancer journal for clinicians* 2018, **68**(6):394-424.
3. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA *et al*: **Molecular portraits of human breast tumours**. *Nature* 2000, **406**(6797):747-752.
  4. Agarwal G, Nanda G, Lal P, Mishra A, Agarwal A, Agrawal V, Krishnani N: **Outcomes of Triple-Negative Breast Cancers (TNBC) Compared with Non-TNBC: Does the Survival Vary for All Stages?** *World journal of surgery* 2016, **40**(6):1362-1372.
  5. Hanahan D, Weinberg RA: **Hallmarks of cancer: the next generation**. *Cell* 2011, **144**(5):646-674.
  6. Sutherland RL, Musgrove EA: **Cyclins and breast cancer**. *Journal of mammary gland biology and neoplasia* 2004, **9**(1):95-104.
  7. Lee HJ, Lee WK, Kang CW, Ku CR, Cho YH, Lee EJ: **A selective cyclin-dependent kinase 4, 6 dual inhibitor, Ribociclib (LEE011) inhibits cell proliferation and induces apoptosis in aggressive thyroid cancer**. *Cancer letters* 2018, **417**:131-140.
  8. Li X, Seebacher NA, Garbutt C, Ma H, Gao P, Xiao T, Hornicek FJ, Duan Z: **Inhibition of cyclin-dependent kinase 4 as a potential therapeutic strategy for treatment of synovial sarcoma**. *Cell death & disease* 2018, **9**(5):446.
  9. Iyengar M, O'Hayer P, Cole A, Sebastian T, Yang K, Coffman L, Buckanovich RJ: **CDK4/6 inhibition as maintenance and combination therapy for high grade serous ovarian cancer**. *Oncotarget* 2018, **9**(21):15658-15672.
  10. Xiong Y, Li T, Assani G, Ling H, Zhou Q, Zeng Y, Zhou F, Zhou Y: **Ribociclib, a selective cyclin D kinase 4/6 inhibitor, inhibits proliferation and induces apoptosis of human cervical cancer in vitro and in vivo**. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* 2019, **112**:108602.
  11. Naz S, Sowers A, Choudhuri R, Wissler M, Gamson J, Mathias A, Cook JA, Mitchell JB: **Abemaciclib, a Selective CDK4/6 Inhibitor, Enhances the Radiosensitivity of Non-Small Cell Lung Cancer In Vitro and In Vivo**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2018, **24**(16):3994-4005.
  12. Olmez I, Brenneman B, Xiao A, Serbulea V, Benamar M, Zhang Y, Manigat L, Abbas T, Lee J, Nakano I *et al*: **Combined CDK4/6 and mTOR Inhibition Is Synergistic against Glioblastoma via Multiple Mechanisms**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2017, **23**(22):6958-6968.
  13. Baker SJ, Reddy EP: **CDK4: A Key Player in the Cell Cycle, Development, and Cancer**. *Genes & cancer* 2012, **3**(11-12):658-669.
  14. Kwapisz D: **Cyclin-dependent kinase 4/6 inhibitors in breast cancer: palbociclib, ribociclib, and abemaciclib**. *Breast Cancer Res Treat* 2017, **166**(1):41-54.
  15. Asghar US, Barr AR, Cutts R, Beaney M, Babina I, Sampath D, Giltnane J, Lacap JA, Crocker L, Young A *et al*: **Single-Cell Dynamics Determines Response to CDK4/6 Inhibition in Triple-Negative Breast Cancer**. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2017, **23**(18):5561-5572.

16. Silver DP, Richardson AL, Eklund AC, Wang ZC, Szallasi Z, Li Q, Juul N, Leong CO, Calogrias D, Buraimoh A *et al*: **Efficacy of neoadjuvant Cisplatin in triple-negative breast cancer.** *J Clin Oncol* 2010, **28**(7):1145-1153.
17. Sikov WM, Berry DA, Perou CM, Singh B, Cirrincione CT, Tolaney SM, Kuzma CS, Pluard TJ, Somlo G, Port ER *et al*: **Impact of the addition of carboplatin and/or bevacizumab to neoadjuvant once-per-week paclitaxel followed by dose-dense doxorubicin and cyclophosphamide on pathologic complete response rates in stage II to III triple-negative breast cancer: CALGB 40603 (Alliance).** *J Clin Oncol* 2015, **33**(1):13-21.
18. Beaver JA, Amiri-Kordestani L, Charlab R, Chen W, Palmby T, Tilley A, Zirkelbach JF, Yu J, Liu Q, Zhao L *et al*: **FDA Approval: Palbociclib for the Treatment of Postmenopausal Patients with Estrogen Receptor-Positive, HER2-Negative Metastatic Breast Cancer.** *Clinical cancer research : an official journal of the American Association for Cancer Research* 2015, **21**(21):4760-4766.
19. Finn RS, Crown JP, Lang I, Boer K, Bondarenko IM, Kulyk SO, Ettl J, Patel R, Pinter T, Schmidt M *et al*: **The cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with letrozole versus letrozole alone as first-line treatment of oestrogen receptor-positive, HER2-negative, advanced breast cancer (PALOMA-1/TRIO-18): a randomised phase 2 study.** *The Lancet Oncology* 2015, **16**(1):25-35.
20. Walker AJ, Wedam S, Amiri-Kordestani L, Bloomquist E, Tang S, Sridhara R, Chen W, Palmby TR, Fourie Zirkelbach J, Fu W *et al*: **FDA Approval of Palbociclib in Combination with Fulvestrant for the Treatment of Hormone Receptor-Positive, HER2-Negative Metastatic Breast Cancer.** *Clinical cancer research : an official journal of the American Association for Cancer Research* 2016, **22**(20):4968-4972.
21. Chou TC: **Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies.** *Pharmacological reviews* 2006, **58**(3):621-681.
22. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E *et al*: **The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data.** *Cancer discovery* 2012, **2**(5):401-404.
23. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E *et al*: **Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal.** *Science signaling* 2013, **6**(269):pl1.
24. Ghosh D, Nandi S, Bhattacharjee S: **Combination therapy to checkmate Glioblastoma: clinical challenges and advances.** *Clinical and translational medicine* 2018, **7**(1):33.
25. Bhattacharjee S, Nandi S: **Synthetic lethality in DNA repair network: A novel avenue in targeted cancer therapy and combination therapeutics.** *IUBMB life* 2017, **69**(12):929-937.
26. Huang X, Di Liberto M, Jayabalan D, Liang J, Ely S, Bretz J, Shaffer AL, 3rd, Louie T, Chen I, Randolph S *et al*: **Prolonged early G(1) arrest by selective CDK4/CDK6 inhibition sensitizes myeloma cells to cytotoxic killing through cell cycle-coupled loss of IRF4.** *Blood* 2012, **120**(5):1095-1106.
27. Liu Y, Zhao R, Fang S, Li Q, Jin Y, Liu B: **Abemaciclib sensitizes HPV-negative cervical cancer to chemotherapy via specifically suppressing CDK4/6-Rb-E2F and mTOR pathways.** *Fundamental &*

*clinical pharmacology* 2020.

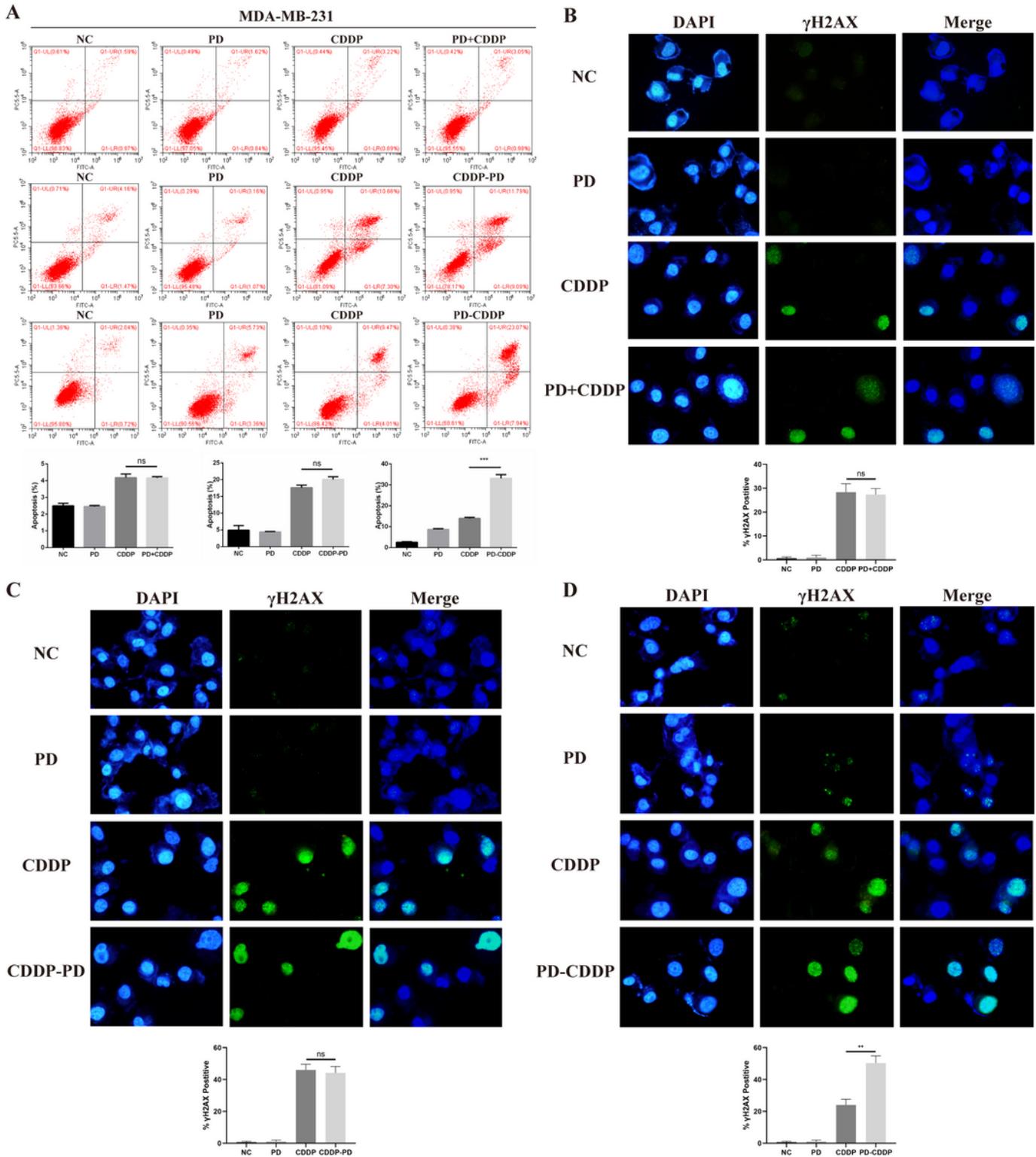
28. Roberts PJ, Bisi JE, Strum JC, Combest AJ, Darr DB, Usary JE, Zamboni WC, Wong KK, Perou CM, Sharpless NE: **Multiple roles of cyclin-dependent kinase 4/6 inhibitors in cancer therapy.** *Journal of the National Cancer Institute* 2012, **104**(6):476-487.
29. Fry DW, Harvey PJ, Keller PR, Elliott WL, Meade M, Trachet E, Albassam M, Zheng X, Leopold WR, Pryer NK *et al.*: **Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts.** *Molecular cancer therapeutics* 2004, **3**(11):1427-1438.
30. Condorelli R, Spring L, O'Shaughnessy J, Lacroix L, Bailleux C, Scott V, Dubois J, Nagy RJ, Lanman RB, Iafrate AJ *et al.*: **Polyclonal RB1 mutations and acquired resistance to CDK 4/6 inhibitors in patients with metastatic breast cancer.** *Annals of oncology : official journal of the European Society for Medical Oncology* 2018, **29**(3):640-645.
31. Wander SA, Cohen O, Gong X, Johnson GN, Buendia-Buendia JE, Lloyd MR, Kim D, Luo F, Mao P, Helvie K *et al.*: **The Genomic Landscape of Intrinsic and Acquired Resistance to Cyclin-Dependent Kinase 4/6 Inhibitors in Patients with Hormone Receptor-Positive Metastatic Breast Cancer.** *Cancer discovery* 2020, **10**(8):1174-1193.

## Figures



**Figure 1**

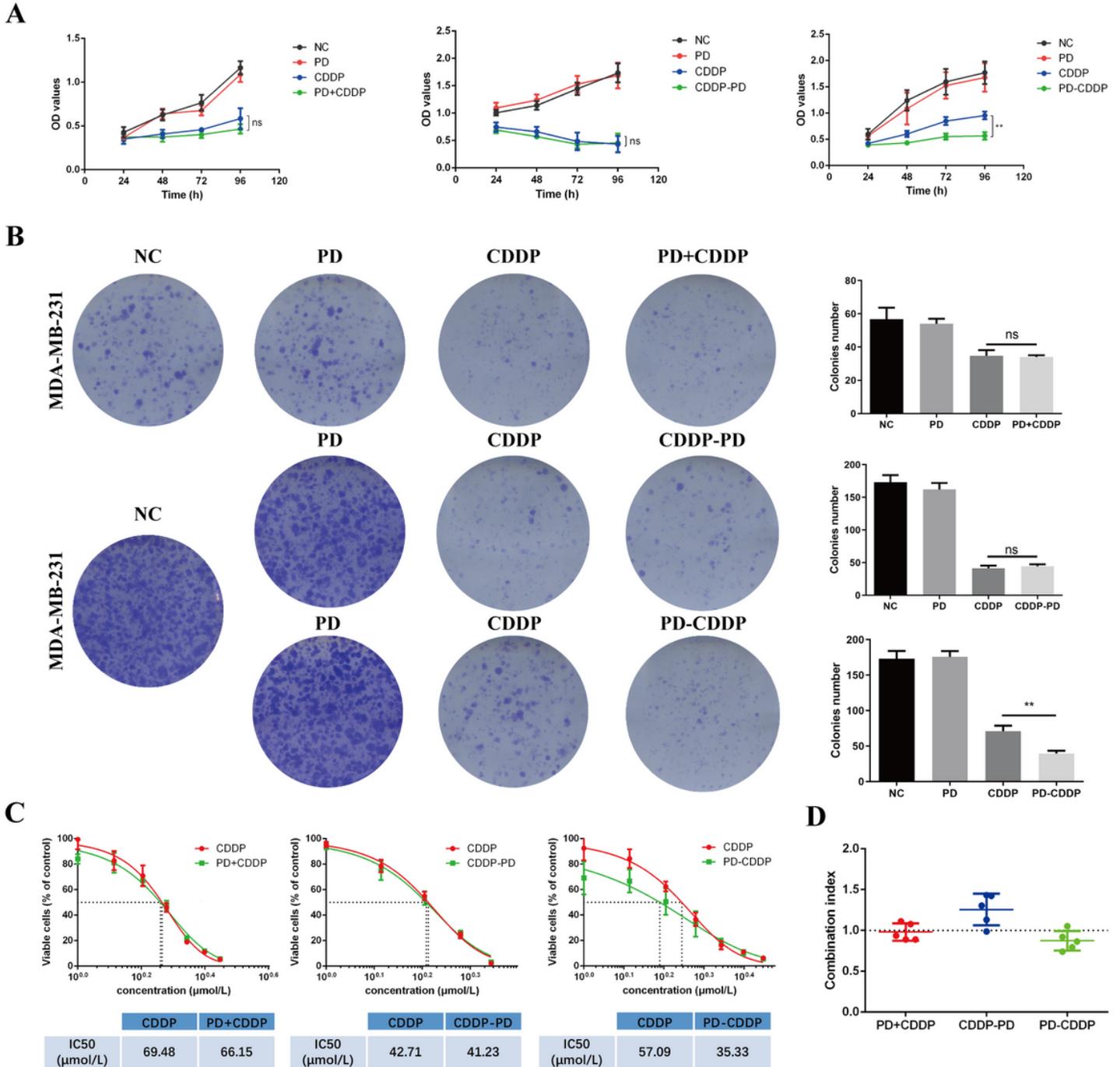
PD could be a potential therapeutic treatment for TNBC. a Analysis of 171 tumors from the TCGA database showing mutations of genes in the CDK4/6 pathway. b The mRNA expression of RB in different breast cancer cell lines. c Cell cycle of MDA-MB-231 cells treated with PD for 24h. d MDA-MB-231 cells were treated with PD for 24h/48h/72h, e Treating MDA-MB-231 cells with PD for 48h and then withdrawing for 24h/48h/72h. The changes of cell cycle were observed by Flow cytometry. The Student t test (two-tailed, equal variance) was used to derive the P values: \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.



**Figure 2**

PD enhances CDDP-induced apoptosis and DNA damage in MDA-MB-231 cells in PD-CDDP group. a Flow cytometry showed that PD enhanced CDDP-induced apoptosis of MDA-MB-231 cells in PD-CDDP group, and average percent apoptosis was quantified. b, c and d Immunofluorescence analysis was used to evaluate the expression of  $\gamma$ H2AX (green) in MDA-MB-231 cells treated with PD, CDDP, PD+CDDP, PD-

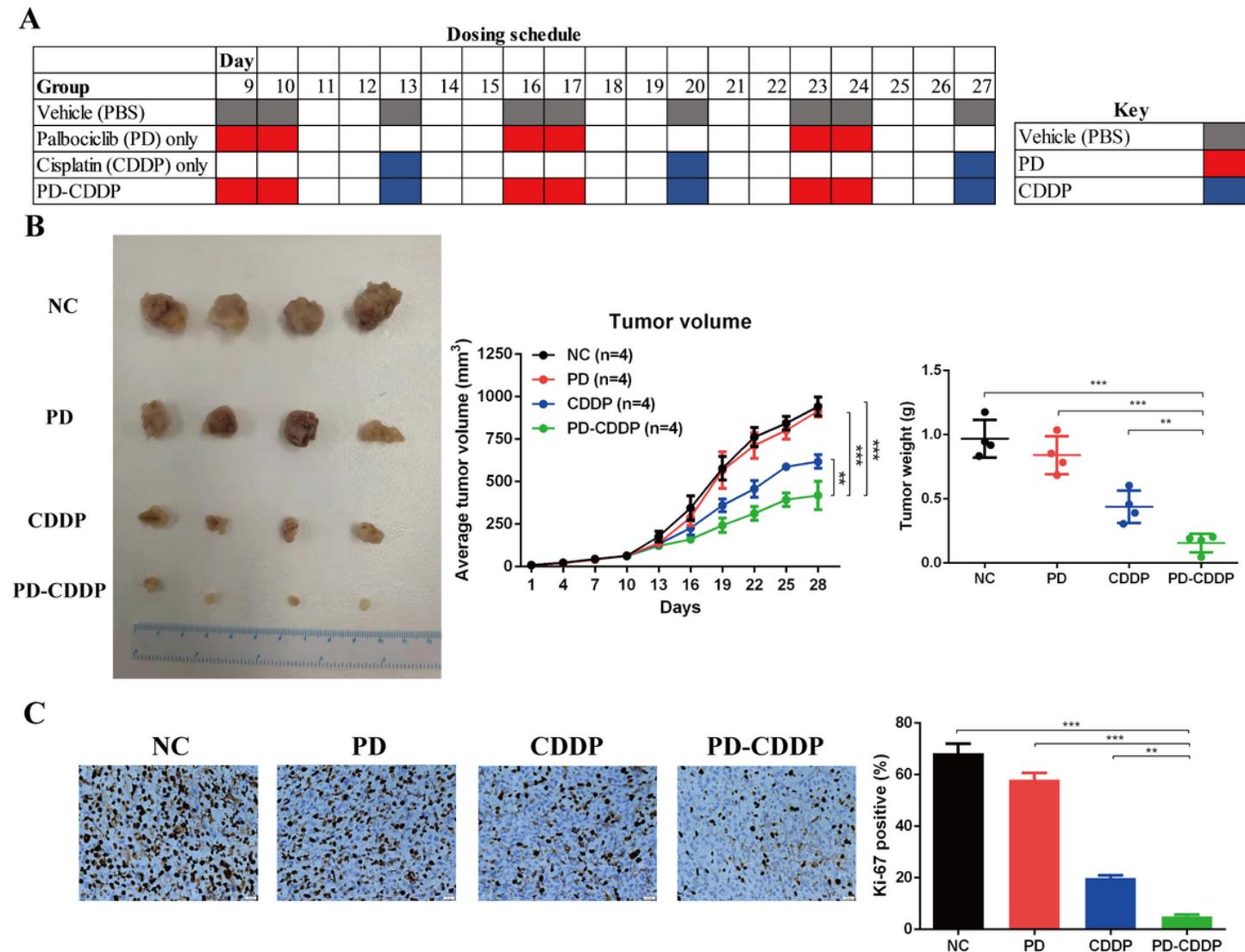
CDDP, CDDP-PD. DAPI was used to detect nuclei. Images were captured at x400 with an Olympus microscope.



**Figure 3**

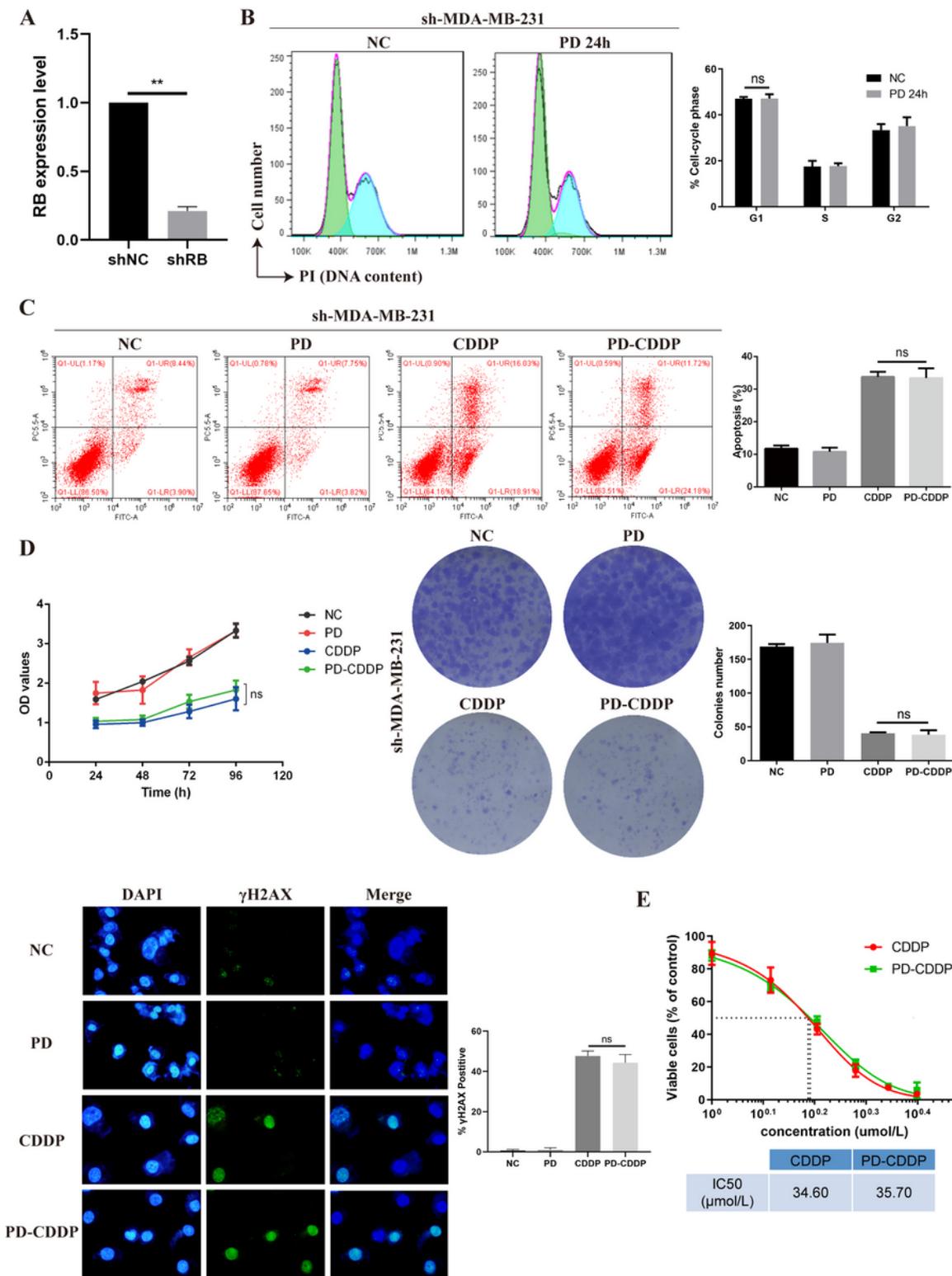
PD enhances the sensitivity of MDA-MB-231 cells to CDDP. a Cell viability was determined by CCK8 assay of MDA-MB-231 cells after exposure to each treatment. b Colony formation assay and its quantification of MDA-MB-231 cells with each treatment were performed. c The IC<sub>50</sub> of CDDP (drug concentration required to inhibit growth by 50%) was calculated in MDA-MB-231 cells treated with PD+CDDP, PD-CDDP or CDDP-PD. d Combination index (CI) resulted from the dose-response analysis of CDDP and PD

treatments. Data, analyzed with CompuSyn software, were used to measure if the two drugs had a synergistic ( $CI < 1$ ), additive ( $CI = 1$ ) or antagonistic ( $CI > 1$ ) effect.



**Figure 4**

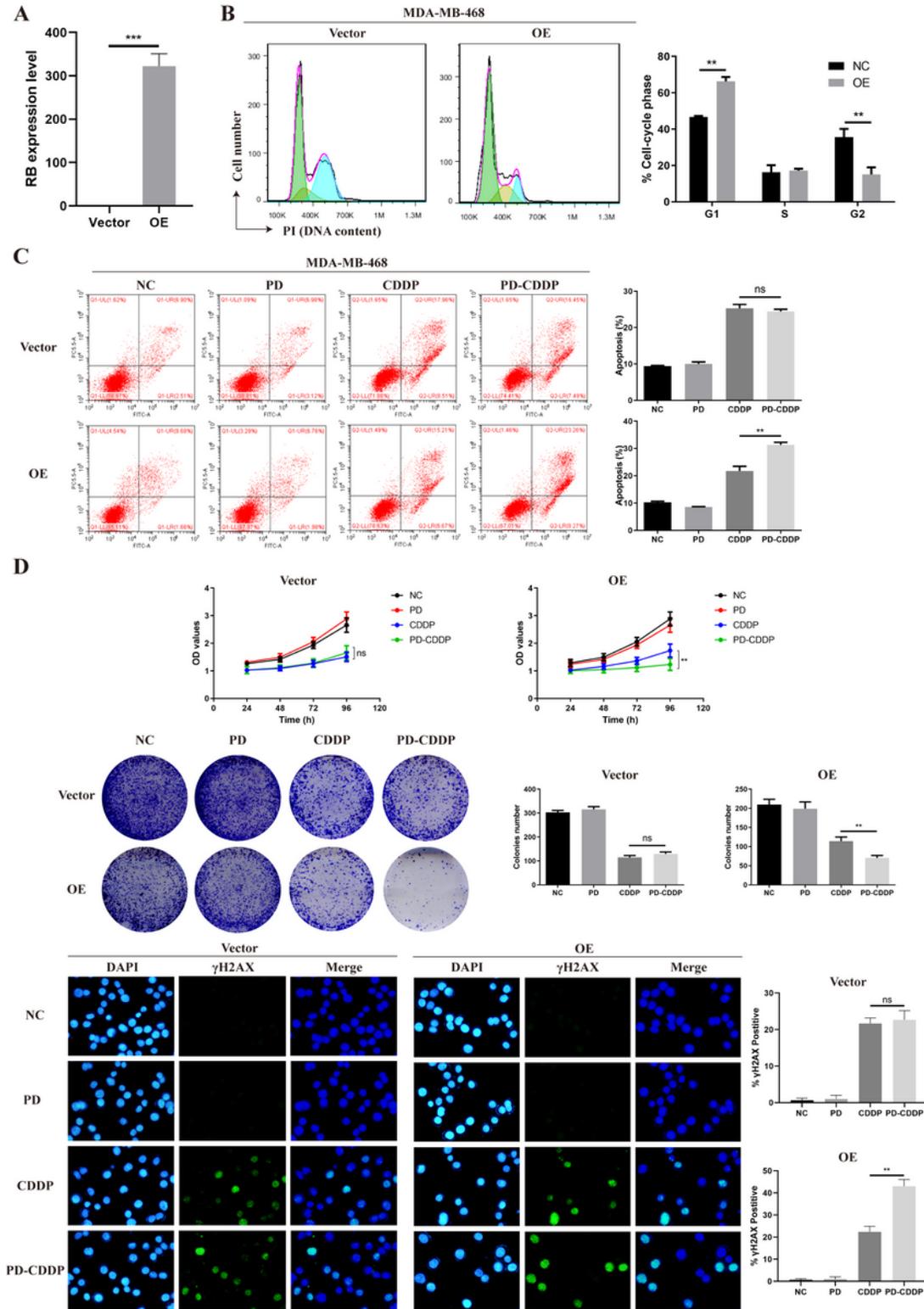
PD-CDDP treatment in vivo can effectively delay the proliferation of MDA-MB-231 xenograft. a Sample dosing schedule for PD and CDDP. b Randomly grouped nude mice bearing MDA-MB-231 tumors were treated with vehicle (PBS), PD (150mg/kg), CDDP (5mg/kg), or PD-CDDP treatment for three cycles. Tumor volume were determined every three days after the onset of treatment. c Harvested tumors were subsequently collected and representative immunohistochemistry results showed Ki-67 expression in MDA-MB-231 tumors. The percent of Ki-67 positive tumor cells and their staining intensity were assessed by Image J.



**Figure 5**

PD synergizes CDDP-mediated cytotoxicity potentially in a RB-dependent manner. a RB expression in MDA-MB-231 cells was dramatically decreased by shRB. b Cell cycle of sh-MDA-MB-231 cells treated with PD for 24h. c Flow cytometry showed that PD did not enhance CDDP-induced apoptosis of sh-MDA-MB-231 cells, and average percent apoptosis was quantified. d CCK8 assay, colony formation assay and immunofluorescence analysis were performed to determine that PD could not significantly enhance

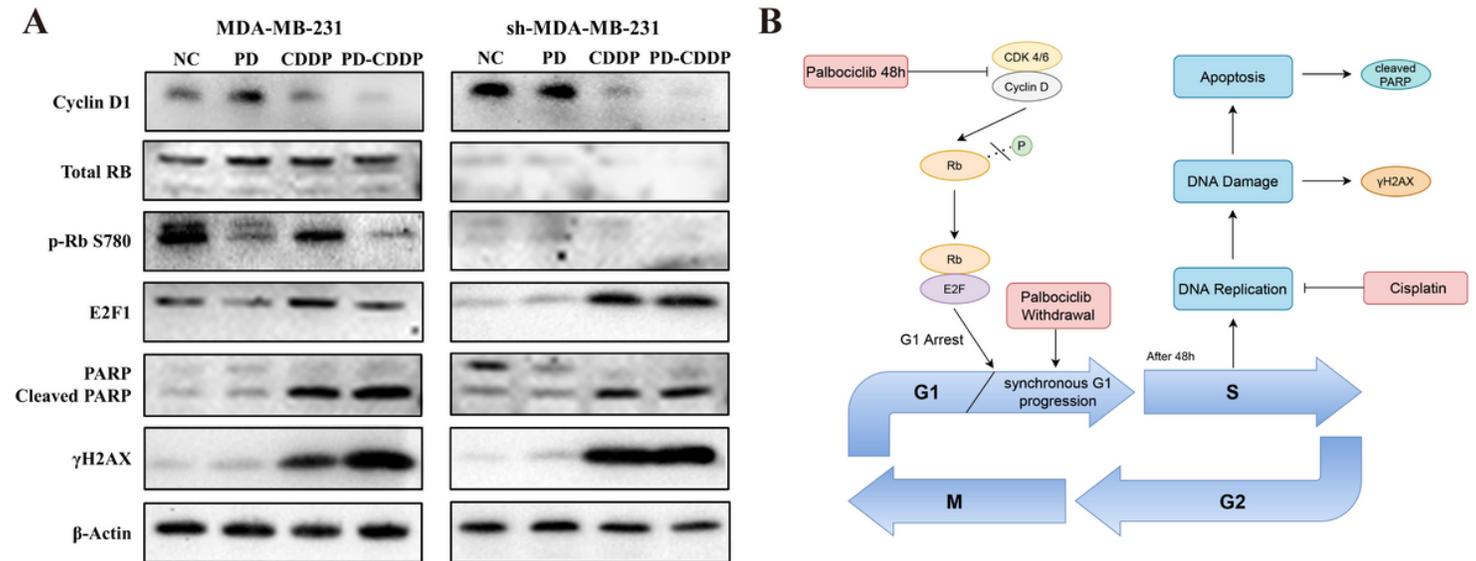
CDDP-induced inhibition of cell viability, colony formation and increase of DNA damage in sh-MDA-MB-231 cells. e The IC<sub>50</sub> of CDDP was calculated in sh-MDA-MB-231 cells treated with PD-CDDP.



**Figure 6**

Further validation of PD synergizing CDDP-mediated cytotoxicity in an RB-dependent manner through MDA-MB-468 cells. a RB expression was significantly higher in RB-overexpression MDA-MB-468 (OE) cells than in MDA-MB-468 cells transfected with empty plasmid (vector). b Cell cycle of OE and vector cells

treated with PD for 24h. c Flow cytometry showed that PD enhanced CDDP-induced apoptosis of OE cells but not of vector cells in PD-CDDP group, and average percent apoptosis was quantified. d CCK8 assay, colony formation assay and immunofluorescence analysis were performed to determine cell viability, colony formation and DNA damage of OE and vector cells.



**Figure 7**

a PD-CDDP treatment enhances the anti-tumor effect through cyclin D1/RB/E2F axis. Western blot was performed to assess cyclin D1, total RB, p-RB, E2F1, PARP/cleaved PARP, γH2AX and β-actin expression. b Proposed mechanism model for PD-CDDP treatment.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [FigureS1.tif](#)
- [FigureS2.tif](#)