Combination of palbociclib with enzalutamide shows in vitro activity in RB proficient and androgen receptor positive triple negative breast cancer cells

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Abstract

Objectives
Triple negative breast cancer (TNBC) lacks specific drug targets and remains challenging. Palbociclib, a cyclin-dependent kinases 4 and 6 (CDK4/6) inhibitor is approved for metastatic estrogen receptor (ER)-positive and human epidermal growth factor 2 (HER2)-negative breast cancer. The nature of cell cycle inhibition by palbociclib suggests its potential in TNBC cells. Retinoblastoma (RB, a known substrate of CDK4/6) pathway deregulation is a frequent occurrence in TNBC and studies have revealed that pharmacological CDK4/6 inhibition induces a cooperative cytostatic effect with doxorubicin in RB-proficient TNBC models. In addition, recent studies reported that anti-androgen therapy shows preclinical efficacy in androgen-receptor (AR)-positive TNBC cells. Here we examined the effect of palbociclib in combination with an anti-androgen enzalutamide in TNBC cells.

Method
MDA-MB-453, BT-549, MDA-MB-231 and MDA-MB-468 TNBC cell lines were used for in vitro studies. Protein expressions were assessed by Western blot analysis. Cytostatic effect was examined by MTT assay. Cell cycle and apoptosis were examined by flow cytometry.

Results
Palbociclib showed inhibitory effect in RB-proficient TNBC cells, and enzalutamide inhibited cell viability in AR-positive TNBC cells. Enzalutamide treatment could enhance the palbociclib-induced cytostatic effect in AR-positive/RB-proficient TNBC cells. In addition, palbociclib-mediated G1 arrest in AR-positive/RB-proficient TNBC cells was attenuated by RB knockdown.
Conclusion
Our study provided a preclinical rationale in selecting patients who might have therapeutic benefit from combining CDK4/6 inhibitors with AR antagonists.

Introduction
Triple-negative breast cancer (TNBC) remains a challenging breast cancer subtype for its higher risk of distant recurrence, and poorer outcome after recurrence or metastasis than other types of breast cancer [1–3]. Targeted therapy for TNBC is emerging in clinical trials and recent molecular profiling studies have revealed molecular heterogeneity of TNBC [4], highlighting the importance of finding biomarkers for targeted therapy guidance for TNBC.

Palbociclib is a highly selective cyclin-dependent kinases 4 and 6 (CDK4/6) inhibitor, which blocks the phosphorylation of retinoblastoma protein (pRB) and subsequently arrests cell cycle at G1-phase [5, 6]. Previous study in vitro showed that palbociclib in combination with hormone therapy (tamoxifen) or target therapy (trastuzumab) had an effectively inhibitory effect on ER-positive and HER2-amplified breast cancer, respectively [7]. In clinical, palbociclib in combination with letrozole (aromatase inhibitor) has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of patients with ER-positive and HER2-negative advanced breast cancer [8, 9]. However, the effects of palbociclib in TNBC are not well-documented.

Enzalutamide, an androgen receptor antagonist, has been approved by the FDA for the treatment of patients with metastatic prostate cancer [10, 11]. Cumulative evidences showed that enzalutamide has potent anti-tumor effects on TNBC cells, and suggested that androgen receptor (AR) might be a promising target for treatment of TNBC [12–14]. However, the effect of combination palbociclib with enzalutamide in TNBC cells is still unclear.

In present study, we tested the combination effect of palbociclib with enzalutamide in TNBC cells. Cytostatic effects of enzalutamide, palbociclib or combined treatment and effects of treatments on AR and pRB proteins expressions were examined. Moreover, the influences on cell cycle distribution and apoptosis were also evaluated.

Materials and methods
Cell culture and transfection
Human TNBC cell lines MDA-MB-453, MDA-MB-231, MDA-MB-468, BT-20 and HCC1937 cells and human breast epithelial cell line MCF 10A cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM). Human TNBC cell lines BT-549 cells were cultured in RPMI 1640 medium with 0.023 UI/ml insulin. The complete growth medium was supplemented with 10% FBS, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 100 units/ml penicillin G, 100 μg/mL streptomycin sulphate and 25 μg/mL amphotericin B in 37˚C humidified incubator and an atmosphere of 5% CO₂ in air. Cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). For transfection, cells were seeded into 6-well for 24 h and transiently transfected by Lipofectamine 3000 Reagent (Thermo Fisher Scientific). For knockdown validation, ON-TARGETplus Human RB1 siRNA (GE Healthcare Dharmacon, E-003296-00-0005) was used to knockdown the endogenous RB1, and ON-TARGETplus Non-targeting Pool (GE Healthcare Dharmacon, D-001810-10-20) as a negative control.
Western blot analysis

Whole cell extracts were prepared using RIPA buffer (Thermo Scientific) with a Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). The protein concentrations were determined using the Bradford assay (Sigma-Aldrich). Samples were diluted in 5X Laemmli buffer (300 mM Tris-HCl pH 6.8, 10% SDS (w/v), 5% 2-mercaptoethanol, 25% glycerol (v/v), 0.1% bromphenol blue w/v) and boiled for 5 min. 35 μg of proteins were separated by 8–15% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (PALL Life Science). Unspecific binding sites on the PVDF membranes were blocked with 5% non-fat milk in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 1% Tween-20). Membranes were hybridized with antibodies against anti-AR (Santa Cruz), anti-CDKN2A/p16INK4α (Abcam), anti-RB, anti-phospho-Rb (Ser780), anti-p21 Waf1/Cip1, anti-cyclin D1 and anti-β-actin (Cell Signaling Technology) for overnight at 4˚C, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. The membranes were then developed using Immobilon Western chemiluminescence HRP substrates (Millipore). Images were captured by Luminescence/Fluorescence Imaging System (GE Healthcare).

MTT (3-[4,5-Dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide) assay

Cells were seeded into 96-well plates allowed to attach for 24 h and treated with indicated concentrations of enzalutamide or palbociclib for 72 h. The treated cells were added 0.5 mg/mL MTT (Sigma-Aldrich) to each well and incubated for 4 h at 37˚C. The violet MTT formazan precipitates were subsequently dissolved in 100 μL DMSO. The absorbance at 570 nm was measured on an UQuant reader.

Cell cycle analysis

The cell cycle assay was carried out by flow cytometry. Cells were collected and fixed with 70% chilled ethanol for overnight. Then, the cells were stained with 0.2 mg/mL Propidium Iodide (PI) for 30 min in the presence of 100 μg/mL RNase and protected from light. The stained cells were suspended in 500 μL phosphate buffered saline (PBS) and analyzed by flow cytometry. Data was gated and analyzed by the CellQuest software.

Flow cytometry analysis for apoptosis detection

The apoptotic cells were measured by APC Annexin V (BD Biosciences) and PI double staining assay. Cells were seeded onto 6-well plates for overnight and then treated with enzalutamide or palbociclib for 48 h. The treated cells were harvested and suspended 1 x 10^6 cells in 1 mL 1X Binding Buffer (BD Biosciences). 5 μL APC Annexin V and 4 μL PI were added into each sample and mixed. Cells were incubated for 15 min at room temperature prevented from light and then analyzed by flow cytometry.

Results

Effect of palbociclib and enzalutamide on AR and RB protein expressions in TNBC cell lines

To develop precision medicine for TNBC subtypes, we first examined endogenous AR and RB expressions in TNBC cell lines and human breast epithelial cell line MCF 10A cells. The result showed that MDA-MB-453 and BT-549 is AR-positive cell lines, and RB and phosphorylated RB could be detected in MDA-MB-453 and MDA-MB-231 (Fig 1A). Moreover, we also
confirmed that enzalutamide and palbociclib were effective against AR and RB protein expression, respectively (Fig 1B–1E). These results indicated that these TNBC cell lines could be classified into four categories, including AR-positive/RB-proficient (MDA-MB-453), AR-positive/RB-deficient (MDA-MB-231), AR-negative/RB-proficient (MDA-MB-468), and AR-negative/RB-deficient (HCC1937, BT-20).

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Fig 1. Effect of palbociclib and enzalutamide on AR and RB protein expressions in TNBC cell lines. (A) The whole cell extracts of MCF 10A, BT-549, BT-20 MDA-MB-231, MDA-MB-453, MDA-MB-468 and HCC1937 cells were analyzed by Western blot using antibodies against anti-AR, anti-phospho-RB, anti-RB and anti-β-actin. (B-D) The whole cell extracts of MDA-MB-453, BT-549, MDA-MB-231 and MDA-MB-468 were treated with DMSO (-), 1.25 and 2.5 μM palbociclib, 10 and 20 μM enzalutamide and combination of 2.5 μM palbociclib and 20 μM enzalutamide for 48 h which prepared for Western blot analysis using antibodies against anti-AR, anti-phospho-RB, anti-RB and anti-β-actin.
RB-negative (BT-549), AR-negative/RB-proficient (MDA-MB-231) and AR-negative/RB-negative (MDA-MB-468, HCC1937 and BT-20), respectively.

Enzalutamide in combination with palbociclib enhances the cytostatic effect in AR-positive/RB-proficient TNBC cells

Since palbociclib is a selective CDK4/6 inhibitor, which blocks the phosphorylation of RB and subsequently leads to inhibition of cell growth, we evaluated the effect of palbociclib in three TNBC cell lines. The result showed that palbociclib significantly inhibited the cell growth in RB-proficient cells (MDA-MB-453 and MDA-MB-231) but not in RB-negative cells (MDA-MB-468) (Fig 2A). Enzalutamide-suppressed cell viability of AR-positive cells is nearly 50% (Fig 2B). To examine whether enzalutamide enhanced palbociclib-induced cytostatic effect, we used 0.156 or 2.5 μM palbociclib combined with various concentrations of enzalutamide in TNBC cell lines. The results revealed that enzalutamide enhanced the palbociclib-induced cytostatic effect in AR-positive/RB-proficient (MDA-MB-453) cells rather than AR-positive/RB-negative (BT-549), AR-negative/RB-proficient (MDA-MB-231), AR-negative/RB-negative (MDA-MB-468) cells

![Graphs showing cell viability](https://doi.org/10.1371/journal.pone.0189007.g002)
Presence of AR and RB enhance palbociclib-induced G1 arrest in TNBC cells

It is known that palbociclib decreases the phosphorylation of RB by inhibiting CDK4/6 and subsequently promotes cell cycle arrest at G1 phase, we next examined whether combined treatment of enzalutamide would alter the pattern of cell cycle in the presence of palbociclib. Enzalutamide treatment did not alter cell cycle distribution in TNBC cell lines. Palbociclib treatment slightly increased the cells in G1 phase of AR-negative/RB-proficient (MDA-MB-231) cells. However, palbociclib treatment significantly induced G1 arrest in AR-positive/RB-proficient (MDA-MB-453) cells but not in AR-positive/RB-negative (BT-549) and AR-negative/RB-negative (MDA-MB-468) cells (Fig 3A–3D). Palbociclib-induced G1 phase is nearly plateau (~90%) in MDA-MB-453 cells which might partly interpret that enzalutamide treatment did not further enhance palbociclib-induced G1 arrest in MDA-MB-453 cells.

To explore whether concurrent expression of AR and RB was involved in palbociclib-induced G1 arrest, we analyzed the effects of palbociclib treatment in RB-knockdown MDA-MB-453 cells. Palbociclib treatment reduced cell viability in MDA-MB-453 cells. Knockdown of RB1 attenuated palbociclib-suppressed cell viability (Fig 4A). Palbociclib-induced G1 arrest was also attenuated by RB knockdown (S2 Fig). We further analyzed the effects of combination of palbociclib and enzalutamide in cell cycle regulation. Results showed G1 arrest was attenuated by RB knockdown (Fig 4B) Treatment both of palbociclib and enzalutamide upregulated p21 and downregulated p16 whereas the effects were reduced by RB knockdown (Fig 4C). These results might suggest that concurrent expression of AR and RB was contributed for palbociclib-induced G1 arrest in TNBC cells.

Palbociclib and enzalutamide do not result in apoptosis in TNBC cells

Due to the fact that palbociclib and enzalutamide have been reported to induce apoptosis in cancer, we further investigated the effect of palbociclib and enzalutamide on apoptosis in TNBC cells. We found that neither treatment alone nor combined treatment induced apoptosis, but cisplatin treatment increased the apoptotic cells in TNBC cell lines (Fig 5A–5D). These results indicated that the anti-cancer effect of palbociclib and enzalutamide was dependent on repression of cell growth rather than induction of apoptosis in TNBC cells.

Clinical significances of AR and RB1 gene expression in TNBC

To determine the clinical significances of AR and RB1 gene expression in TNBC, we first examined the expression of AR in TNBC samples. We downloaded the tumor genome atlas (TCGA) data from cBioPortal website (http://www.cbioportal.org/) [15, 16], and analyzed the association between the levels of AR and RB1 and clinical variables. The results showed that expression levels of AR or RB1 did not link to overall survival of patients with TNBC (Fig 6A and 6B). However, we divided breast cancer patients into AR/RB1 double-strong expression and AR/RB1 double-weak expression cohorts. The results showed that TNBC patients with double-strong expression of AR/RB1 significantly associated with poor overall survival (Fig 6C). The expression of AR was not correlated with age and tumor stage (S1 Table). Moreover, the expression of AR is correlated with RB1 in patients with TNBC (Fig 6D) and breast cancer (S4 Fig). In addition, the tissue array was analyzed by IHC, AR expression in TNBC samples is about 11.6% (S3 Fig) whereas the expression of AR was not associated with recurrence-free
survival (S3 Fig). Results from tissue array were consistent with TCGA database. There results indicated that concurrent expression of AR and RB1 might be the biomarker in selecting

**Fig 3. Palbociclib-induced G1 arrest is increased in AR-positive/RB-proficient cells.** (A) MDA-MB-453, (B) BT-549, (C) MDA-MB-231 and (D) MDA-MB-468 cells were treated with DMSO (-), 1.25 and 2.5 μM palbociclib, 10 and 20 μM enzalutamide and combination of 2.5 μM palbociclib and 20 μM enzalutamide for 48 h, the cell cycle analysis by DNA content using flow cytometry. The means ± SEM of three independent experiments performed in triplicate are shown.

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**Fig 4. The effects of combination of palbociclib and enzalutamide are attenuated by RB knockdown.** (A) MDA-MB-453 cells were transfected with siRNA against control (siCtrl) or RB1 (siRB) for 24 h, and the transfected cells were further treated with 2.5 μM palbociclib for 48 h. The treated cells were analyzed by MTT assay. *P < 0.05. (B, C) MDA-MB-453 cells were transfected with siRNA against control (siCtrl) or RB1 (siRB) for 24 h, and the transfected cells were further treated with 2.5 μM palbociclib and 20 μM enzalutamide for 48 h. The treated cells were analyzed by flow cytometry analysis (B) and Western blot (C) using antibodies against anti-phospho-RB, anti-RB, anti-cyclin D1, anti-p21, anti-p16 and anti-β-actin. G0/G1 subpopulation; **P < 0.01.

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patients who have therapeutic benefit from combining CDK4/6 inhibitors with AR antagonists.

**Discussion**

In this study, we demonstrated that both AR and RB status were important for the response to palbociclib in TNBC cells. In addition, palbociclib in combination with enzalutamide enhanced the cytostatic effect in AR-positive/RB-proficient (MDA-MB-453) TNBC cells but not in AR-positive/RB-negative (BT-549), AR-negative/RB-proficient (MDA-MB-231) and AR-negative/RB-negative (MDA-MB-468) TNBC cells. Moreover, concurrent expression of AR and RB was contributed for palbociclib-induced G1 arrest in TNBC cells.

Enzalutamide, which is a second-generation AR antagonist, has a higher anticancer potency than first-generation AR inhibitors (bicalutamide and flutamide), including higher affinity for AR, repression of AR nuclear translocation, decrease in DNA binding and coactivator recruitment [17–19]. Enzalutamide has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of patients with metastatic castration-resistant or chemo-resistant prostate cancer [10, 11, 20–22]. In addition to prostate cancer, growing evidences showed that enzalutamide also exerted anticancer effect on TNBC [12–14]. At least 3 clinical trials of neoadjuvant enzalutamide in combination with or without chemotherapy in TNBC patients are currently ongoing or just-completed (ClinicalTrials.gov Identifier: NCT02689427, NCT01889238 and NCT02457910).

In our present study, we found that enzalutamide enhanced the palbociclib-induced cytostatic
effect in AR-positive/RB-proficient (MDA-MB-453) cells rather than AR-positive/RB-negative (BT-549), AR-negative/RB-proficient (MDA-MB-231) and AR-negative/RB-negative (MDA-MB-468) cells (Fig 2). Our results provided a preclinical rationale in selecting patients who might have therapeutic benefit by combining CDK4/6 inhibitors with AR antagonists.

Palbociclib is a specific CDK4/6 inhibitor, which decreases the phosphorylation of RB and subsequently inhibits cancer cell growth [5, 6]. Although palbociclib was believed to exhibit anti-cancer potencies in an RB-dependent manner, increasing evidences showed that another potential biomarker was necessary for the response of RB-proficient cancer cells to palbociclib [23]. For example, either deletion of CDKN2A (p16INK4A) or low-expression of E2F transcription factor 1 (E2F1) was the significant predictor of the response to palbociclib in renal cell carcinoma (RCC) cell lines [24], and ER-positive breast cancer cell lines with high-expression of cyclin D1 were more sensitive to palbociclib [7]. Moreover, RB-proficient ovarian cancer cell lines with low-expression of p16INK4A were more responsive to palbociclib [25]. Our data indicated that enzalutamide promoted palbociclib-induced G1 arrest in AR-positive/RB-proficient

Fig 6. Clinical significances of AR and RB1 gene expression in patients with TNBC. (A-D) The level 3 data of mRNA RSEM (RNA-Seq by Expectation Maximization) from patients with TNBC were selected and downloaded from the TCGA and Broad GDAC Firehose data portal. The mean of AR or RB1 gene expressions were the chosen as cut-off value for separating tumors with strong and weak expression. Overall survival rates of TNBC patients were plotted against time in month for different parameters: the level of AR gene (A), the level of RB1 gene (B), and the level of AR/RB1 genes (C). The correlation between AR and RB1 mRNA expressions was analyzed by Pearson correlation analysis (D).

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cells (Fig 3). RB is a transcriptional repressor which required for G1 to S phase transition. Previous studied demonstrated that RB interacts with AR in an androgen-independent manner and acts as a coactivator for AR [26]. In addition, AR could stimulate DNA replication via hyperphosphorylated RB indirectly in prostate cancer cells [27]. Palbociclib decreases the phosphorylation of RB, and enzalutamide might decrease the RB coactivator recruitment leading to RB-mediated cell cycle arrest.

TNBC with genetic loss of PR/ER/HER2-amplification and molecular heterogeneity has no approved target therapy to date [28–30]. Recent studies have shown that the molecular profiling in TNBC has revealed a number of potential targets for TNBC patients, including epidermal growth factor receptor (EGFR), mitogen-activated protein kinase (MAPK), proteasome subunits, and poly (ADP-ribose) polymerases (PARPs). Several compounds of these candidates have entered clinical trials for TNBC patients [31]. TNBC patients with double-strong expression of AR/RB1 significantly associated with poor overall survival (Fig 6C). Gucalp et al. evaluated AR status using IHC in 424 patients with TNBC, 12% AR positive with > 10% nuclear staining [32]. The level of RB1 mRNA correlated with level of pRB [33] and RB is reported that lost in ~40% of TNBC [34]. Furthermore, pRB level was found that lost in most TNBC by IHC analysis [35]. Studies have identified that luminal androgen receptor (LAR) subtype of TNBC cell lines might be sensitive to CDK4/6 inhibition [36, 37]. Asghar et al. demonstrated that MFM223 and SUM185 cells were sensitive to palbociclib [37]. Indeed, these two LAR subtype cell lines harbored high level of pRB. The xenografts of LAR MDA-MB-453 cells were significantly reduced by palbociclib treatment [37]. These findings supported AR positive and RB proficient TNBC were suitable for palbociclib treatment. In fact, a clinical trial of palbociclib in combination with bicalutamide, a non-steroidal AR inhibitor, for the treatment of patients with AR-positive TNBC is ongoing (ClinicalTrials.gov Identifier: NCT02605486). In the present study, palbociclib plus enzalutamide was found to effectively repress AR-positive/RB-proficient TNBC cell growth suggesting that co-expression of AR and RB might be a biomarker for combined treatment of palbociclib with enzalutamide in TNBC.

Current study has some limitation, first of all only four TNBC cell lines were used and there might be other protein profile or mutants that can contribute to the differential effects of palbociclib and enzalutamide and combination in these cell lines. Furthermore, despite we showed that knockdown RB attenuated palbociclib-mediated effect on AR+/RB+ MDA-MB-453 cells, the notion that combination effect of palbociclib and enzalutamide is most significant in AR+/RB+ MDA-MB-453 cells needs further mechanistic studies to validate that AR is essential for palbociclib-mediated cell cycle arrest in AR+/RB+ TNBC cells. Last but not the least; our study is limited by lack of sufficient and consolidative clinical data to support the possible association between RB and AR. It is also not clear and inconclusive with regards to the role of AR and RB expressions in TNBC tumors. More clinical studies are needed to address or validate the biological association between AR and RB.

In conclusion, we found that palbociclib effectively inhibited RB-proficient TNBC cell growth and the expression of AR might contribute for palbociclib-mediated G1 arrest. Moreover, enzalutamide enhanced the palbociclib-induced cytostatic effect in AR-positive/RB-proficient TNBC cells indicating that palbociclib in combination with enzalutamide may be a therapeutic strategy for AR-positive/RB-proficient TNBCs.

Supporting information

S1 Table. The relationships between AR gene expression and clinical variables. The level 3 data of mRNA RSEM in patients with TNBC were downloaded from the TCGA and Broad GDAC Firehose data portal. The mean of AR or RB1 gene expressions were the chosen as cut-
off value for separating tumors with strong and weak expression which were analyzed for relationship between AR gene expression and clinical variables.

(DOCX)

S1 Fig. The effects of palbociclib and enzalutamide treatment in MCF 10A cells. (A) MCF 10A cells were treated with various concentrations of palbociclib, (B) enzalutamide and (C) combination of 2.5 μM palbociclib and 20 μM enzalutamide for 72 h, the cell viability was determined using MTT assay. The means ± SEM of three independent experiments performed in triplicate are shown.

(TIF)

S2 Fig. The effects of palbociclib are attenuated by RB knockdown. MDA-MB-453 cells were transfected with siRNA against control (siCtrl) and RB1 (siRB) for 24 h, and the transfected cells were further treated with 2.5 μM palbociclib for 48 h. The treated cells were analyzed by flow cytometry analysis.

(TIF)

S3 Fig. AR expression could be detected in patients with TNBC and did not link to recurrence-free survival. (A) Representative tissue microarray of immunohistochemical expression of AR in TNBC samples and the events of AR expression. (B) recurrence-free survival of TNBC patients were plotted against time in month for the level of AR gene.

(TIF)

S4 Fig. The correlation between AR and RB1 expression in patients with breast cancer. The level 3 data of mRNA RSEM in breast cancer were downloaded from the TCGA and Broad GDAC Firehose data portal. The correlation between AR and RB1 mRNA was analyzed by Pearson correlation analysis.

(TIF)

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