Cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with ralaniten analogues for the treatment of androgen receptor-positive prostate and breast cancers

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ABSTRACT

Androgen receptor (AR) has essential roles in the growth of prostate cancer and some breast cancers. Inhibition of AR transcriptional activity by targeting its N-terminal domain with ralaniten or an analogue such as EPI-7170 causes accumulation of cells in the G1 phase of the cell cycle. Inhibition of cyclin-dependent kinases 4/6 with palbociclib also leads to accumulation of cells in the G1 phase. Here a combination of EPI-7170 with palbociclib attenuated the in vivo growth of human castration-resistant prostate cancer xenografts that are resistant to antiandrogens. Cell-cycle tracing experiments in cultured cells revealed that EPI-7170 targeted cells in S phase, possibly through inducing DNA damage or impairing the DNA damage response, whereas palbociclib targeted the G1-S transition to delay the cell cycle. Combination treatment prevented cells in G1 and G2/M from progressing in the cell cycle and caused a portion of cells in S phase to arrest which contributed to a two-fold increase in doubling time to >63 hours compared to 25 hours in control cells. Importantly, sequential combination treatments with palbociclib administered first then followed by EPI-7170, resulted in more cells accumulating in G1 and less cells in S phase than concomitant combination which was presumably because each inhibitor has a unique mechanism in modulating the cell cycle in cancer cells. Together these data support that the combination therapy was more effective than individual monotherapies to reduce tumor growth by targeting different phases of the cell cycle.
INTRODUCTION

The cell cycle is precisely controlled in benign cells by cyclin-dependent kinases (CDK), regulatory cyclins, and checkpoint regulators. However, in cancer the cell cycle is frequently altered due to somatic mutations in genes encoding cell cycle regulators and defects in checkpoint proteins (1, 2). CDK4/6 inhibitors used clinically include palbociclib, abemaciclib and ribociclib and these are administered in combination with hormone therapy for the treatment of breast cancer. When the retinoblastoma (RB) pathway is intact, these CDK 4/6 inhibitors block the transition of cells from G1 to S phase which leads to accumulation of cells in G1 (3).

Androgen receptor (AR) is a transcription factor that regulates the expression of thousands of genes including those involved in cell growth and survival. Altered expression and transcriptional activity of AR contribute to the development and progression of castration-resistant prostate cancer (CRPC) and some triple-negative breast cancers (TNBC). AR associates with regulators of the cell cycle (4). Over-expression of CDK6 occurs in approximately 50% of prostate cancer and enhances AR transcriptional activity to promote the progression of the disease (5). Increased AR transcriptional activity is associated with disruption of cyclin D1 repression in prostate cancer (6-8). In LNCaP human prostate cancer cells, AR interacts with the pre-replication complex during mid-G1 phase and associates with DNA synthesis, indicating its essential role during G1-S transition (9). Luminal AR is a subtype of TNBC that is particularly sensitive to CDK4/6 inhibitors and this sensitivity is associated with AR expression and low cyclin E1 levels (10).

Full-length AR consists of N-terminal domain (NTD), DNA-binding domain, a hinge region, and a ligand-binding domain (LBD) to which androgens and antiandrogens bind. Deletion of LBD results in constitutively active AR variants (AR-Vs), of which AR-V7 is most clinically relevant. AR-V7 is associated with the failure of CRPC to therapies targeting the full-length AR such as antiandrogens and androgen deprivation therapies (11, 12). AR-V7 is also detected in human breast cancer cell lines as well
as in clinical samples of breast cancer (13-16). AR-V7 mediates a growth response and regulates expression of a subset of genes that is unique from those regulated by the full-length AR (15, 17).

Ralaniiten (aka EPI-002) and its analogues specifically bind to transactivation unit-5 (Tau-5) within activation function 1 (AF-1) of the intrinsically disordered AR NTD (18-22). Uniquely, ralaniten compounds inhibit the transcriptional activities of full-length AR, AR-Vs as well as gain-of-function mutations in AR LBD that are found in CRPC patients that are resistant to antiandrogens and abiraterone (18, 20, 22). Antiandrogens and AR-NTD inhibitors cause prostate cancer cells that express full-length AR to arrest in G1 and not progress to S phase of the cell cycle (9, 20, 23). Thus, a functional AR is required for G1 to S progression. Cyclin D1-CDK4/6 and late G1 cyclin E-CDK2 kinases phosphorylate the RB protein which leads to E2F-regulation of expression of cyclins that are required for the transition to S-phase (24). Based upon the importance of CDK4/6 in controlling G1-S progression, here we propose a therapy of inhibiting CDK4/6 combined with an AR-NTD antagonist that blocks transcription activities of all AR species (full-length AR and AR-Vs).

Monotherapies for metastatic cancers rarely lead to durable responses or cures primarily due to tumor plasticity and heterogeneity with the survival and outgrowth of resistant clones. The goals of combination therapies include deeper and more durable responses and can include approaches such as targeting pathway reactivation and/or targeting multiple components of a signaling pathway. In this study, we examined the effects of targeting both AR-NTD and CDK4/6 in CRPC and AR-expressing breast cancer with a combination of a second-generation ralaniten analogue and palbociclib. EPI-7170 is a more potent analogue of ralaniten that specifically blocks AR transcriptional activity (23, 25, 26). Here we show that EPI-7170 disrupted the cell cycle in CRPC and AR-expressing breast cancer cell lines, and when combined with palbociclib had improved antitumour activity. As expected, palbociclib caused G1 accumulation, whereas EPI-7170 caused both G1 accumulation and also delayed the cell cycle. Sequential versus concomitant combinations resulted in differential effects upon the cell cycle.
MATERIALS AND METHODS

Cell Lines, Cell Culture and Inhibitor Treatment

LNCaP cells were obtained from Dr. Leland Chung (Cedars-Sinai Medical Center, Los Angeles, California, USA) and maintained in RPMI-1640 supplemented with 5% (v/v) FBS. LNCaP95 cells were obtained from Dr. Stephen Plymate (University of Washington, Seattle, Washington, USA) and maintained in RPMI-1640 supplemented with 10% (v/v) charcoal-stripped serum (CSS). SUM159PT cells were obtained from Dr. Sharon Gorski (Canada’s Michael Smith Genome Sciences Centre at BC Cancer, Vancouver, British Columbia, Canada) and maintained in Ham’s F-12 Nutrient Mix supplemented with 5% (v/v) FBS, L-Glutamine, HEPES, 1μg/mL hydrocortisone and 0.2U/mL bovine insulin. MFM-223 cells were purchased from DSMZ (DSMZ #ACC422) and maintained in MEM supplemented with 10% (v/v) FBS, 1% (v/v) L-Gln and insulin-transferrin-selenium. MDA-MB-453 cells were purchased from ATCC (ATCC HTB-131TM) and maintained in RPMI-1640 supplemented with 10% (v/v) FBS. MDA-MB-468 cells were purchased from ATCC (ATCC HTB-132TM) and maintained in MEM supplemented with 10% (v/v) FBS and 1% (v/v) L-Gln. All cell lines were incubated at 37 °C with 5.0% CO₂. All cell lines have been authenticated using Short Tandem Repeat profiling and were routinely tested every 6 months to ensure mycoplasma-free using the Venor GeM Mycoplasma Detection kit (Sigma-Aldrich). The last test for mycoplasma was done July 23, 2021. All treatments with inhibitors or vehicle controls were carried out in culture maintaining media supplemented with FBS for each of the cell lines except LNCaP95 cells which were in 5% charcoal-stripped serum (CSS). All repeated experiments were conducted using cells within 5-10 passages. EPI-7170 was synthesized by Dr. Raymond Andersen (University of British Columbia) (23, 25). Enzalutamide (ENZA) was purchased from Omega Chem. Palbociclib (PD-0332991) HCl was purchased from Selleckchem (Catalog #S1116). These 3 inhibitors were dissolved in DMSO. Vehicle controls were DMSO (final = 0.02 - 0.04% v/v). For combination treatments, concomitant regimens
(two inhibitors were mixed in media and added to the cultures at the same time) were performed unless a sequential regimen was otherwise stated.

**Cell Viability Assay**

LNCaP (3,000 cells per well), LNCaP95 (3,000 cells per well) and SUM159PT (2,000 cells per well) were plated in 96-well culture plate (Corning® Primaria™). Next day (as Day 0) cells were treated with inhibitors at the indicated concentrations or with vehicle control. On days 2 and 3 of treatment, alamarBlue was added to the treated cells and incubated at 37 °C for 2-4 hours prior to fluorescence measurement (560nm excitation and 590nm emission) by Infinite® M1000 (Tecan). EPI or enzalutamide in medium without cells did not change alamarBlue fluorescence measurement as compared to medium only. GraphPad Prism 8 was used for plotting data and statistical analyses.

**Clonogenic Assay**

LNCaP (400-600 cells per well), LNCaP95 (500 cells per well) and SUM159PT (400 cells per well) were plated in 24-well culture plates. The next day (as Day 0) cells were treated with inhibitors at indicated concentrations or with vehicle control without medium change. For consistency, the same cell numbers were plated before treatments, instead of plating the same cell number after treatments which would require adjusting cell numbers after collecting cells from each of the treatments. For experiments with a one-time medium change: on day 3 (LNCaP95) or day 4 (SUM159PT) of treatment, the media from one set of treated cells was changed to fresh media without inhibitors. On day 15 (LNCaP and LNCaP95) or day 7 (SUM159PT) of treatments, cells were fixed with 4% paraformaldehyde, washed with PBS and stained with 0.01% (w/v) crystal violet. Images of colonies were captured by ChemiDoc XRS+ Imaging System with a trans-white conversion screen and Image Lab 5.1 software (BioRad).
Cell Cycle Analysis

For cell cycle with BrdU incorporation, non-synchronized cells were plated (200,000 or 300,000 cells per dish for LNCaP; 300,000 cells per dish for LNCaP95 and MDA-MB-453; 150,000 cells per dish for SUM159PT; 500,000 for MFM-223; 250,000 for MDA-MB-468) in 60-mm culture dishes. The next day (as Day 0), cells were treated with inhibitors at the indicated concentrations or with vehicle control. At the indicated time points, cells were labeled with 10μM BrdU for 2 hours. After BrdU incorporation, culture media containing both non-attached cells and attached cells that were trypsinized by TrypLE were collected. Cells were then passed through a 50-μm filter to obtain single cells and fixed by cold 70% (v/v) ethanol. Fixed cells were stained with anti-BrdU-FITC antibody (clone B44, BD Biosciences Catalog #347583) and 7-aminoactinomycin D (7-AAD from Sigma Catalog #A9400), analyzed by flow cytometry using FACSCalibur™ and data was acquired by CellQuest™ Pro software (BD Biosciences). For cell-cycle-tracing with BrdU incorporation, experimental procedures were the same as described above except cells were labeled with BrdU for 2 hours prior to inhibitor treatments. Cells were harvested and collected at the indicated time points. All cell cycle data were analyzed with FlowJo V.10. Single cell gating was performed on FSC-H vs. FL3-W profiles. Data calculations for bar or line graphs used GraphPad Prism 8.

For analysis of LNCaP95 cell cycle with EdU incorporation and marker expression, Click-iT™ EdU Alexa Fluor™ 647 Flow Cytometry assay kit (Invitrogen) was used and manufacturers’ protocol was followed with modifications on fixation and intracellular marker staining. Cells (1,200,000 per dish) were plated on 100-mm culture dish. The next day (as Day 0), cells were treated with inhibitors or with vehicle control. On day 3 of treatments, cells were labeled with 10μM EdU for 2 hours prior to collecting non-attached and attached cells. Collected cells were fixed with 70% cold ethanol. Incorporated EdU was detected using Click chemistry. Antibody against phospho-histone H2A.X (Ser 139) (clone: 20E3; Cell Signaling Technology CST#9718) was used for intracellular marker staining.
with staining carried out using TBS/FBS (4% v/v). Secondary antibody conjugated with Alexa Fluor 488® was used (Invitrogen A11008 for anti-rabbit IgG). After intracellular marker staining, cells were stained with 7-AAD, analyzed by LSRFortessa™ and data was acquired by FACSDiva™ software (BD Biosciences). All cell cycle data was analyzed using FlowJo V.10. Single-cell gating was performed on SSC-A vs. SSC-W profiles. Data calculations for bar graphs were done using GraphPad Prism 8.

Cell number counting was carried out using an automatic cell counter (EVE™ Automated Cell Counter, NanoEnTek USA Inc.). The same number of cells from different treatment conditions at different time points were used for analyses of the cell cycle (BrdU) and EdU/γH2AX except for cell-cycle-tracing experiments where all collected cells were used for BrdU staining to show cell number changes using 7AAD staining.

**Tumor Xenograft**

Animal studies were approved by the University of British Columbia Animal Care Committee. Mice were maintained in Animal Resource Centre at BC Cancer Research. Six-week-old male NSG mice were castrated. Two weeks after castration, 2 x 10⁶ cells of LNCaP95-D3 cells prepared in 1:1 ratio of RPMI and Matrigel (Corning, Product 354234) were inoculated subcutaneously on the back of mice. When tumor volume reached 50-90 mm³, mice were randomly separated into 4 treatment groups: vehicle control, EPI-7170 (30mg/kg body weight), palbociclib (75mg/kg body weight) and combination (EPI-7170 at 30mg/kg body weight and palbociclib at 75mg/kg body weight). Treatments were given daily by oral gavage for 21 days. Treatment solutions were freshly prepared each day. A working solution of palbociclib (isethionate salt purchased from LC Laboratories P-7766) was prepared daily from powder by dissolving in sterile Milli-Q water. To be completely soluble, each treatment solution was prepared in the following order of DMSO or EPI-7170 (dissolved in DMSO), Tween-80, methyl cellulose solution (Sigma M0512; 0.5% w/v prepared in sterile Milli-Q water), and sterile Milli-Q water.
or palbociclib solution to have final treatment concentrations in 5% DMSO/1.5% Tween-80/0.5% methyl cellulose. Body weight was measured every day. Tumor volume was measured every 3 days and calculated using formula: length x width x height x 0.5236.

**Protein Expression Analysis**

Whole cell lysates were extracted from LNCaP95 cells treated with inhibitors or vehicle control after 3 days by lysing cells in lysis buffer RIPA buffer containing protease inhibitors (cOmplete EDAT-free protease inhibitor cocktail and PhosStop from Roche). Xenograft tumors were homogenized in lysis buffer in Lysing Matrix M with FastPrep-24 homogenizer (MP Biomedicals). Protein concentrations in cell lysates were quantified by the bicinchoninic acid assay (Pierce BCA Protein assay kit). An equal amount of protein (40 µg) of each sample was loaded and resolved on 10% or 12.5% SDS-PAGE, and transferred to nitrocellulose membrane for immunoblotting analyses. Antibodies were diluted in TBS (pH 8.0)/Tween20 (0.05%) containing 5% milk or 5% BSA (w/v). Washes were done using TBS (pH8.0)/ Tween20 (0.05%). Antibodies included: AR (clone: ER179[2]; abcam ab108341), RB (clone: 4H1; Cell Signaling Technology CST#9309), phospho-RB at Ser780 (clone: D59B7; CST#8180), CDK4 (clone: D9G3E; CST#12790), CDK6 (clone: DCS83; CST#3136), CDK2 (clone: D-12; Santa Cruz Biotechnology sc-6248), Cyclin D1 (clone: 92G2; CST#2978), Cyclin A2 (clone: BF683; CST#4656), p27 Kip1 (clone: D69C12; CST#3686), Skp2 (clone: D3G5; CST#2652), RAD51 (clone: D4B10; CST#8875) and β-actin (clone: AC-15; Sigma-Aldrich A5441). Secondary antibodies conjugated with HRP were used (CST#7074 for anti-rabbit IgG and CST#7076 for anti-mouse IgG) and enhanced ECL (GE Healthcare Lifescience™ Amersham™ ECL™ Prime Western Blotting Detection Reagent) was applied for chemiluminescence detection by ChemiDoc XRS+ Imaging System and Image Lab 5.1 software (BioRad). Relative protein levels were quantified by Image Lab 5.1 software. Data and bar graphs were calculated and generated by GraphPad Prism 8.
Immunofluorescence Staining

LNCaP95 cells were plated on 8-well chamberslide (15,000 cells per well). Next day cells were treated with vehicle control, EPI-7170, palbociclib or EPI-7170 combined with palbociclib as indicated. After 3 days of treatments, cells were fixed with 4% paraformaldehyde (v/v) and washed with TBS, pH8.0. Fixed cells were penetrated with TBS/0.1% TritonX-100 (v/v), blocked with TBS/0.05% Tween20 (v/v)/5% BSA (w/v) and stained in the same blocking buffer with antibodies against phospho-histone H2AX (Ser 139) (clone: D7T2V; CST#80312) and 53BP1 (CST#4937S). Secondary antibody conjugated with Alexa Fluor 488® (Invitrogen A11008 for anti-rabbit IgG) and Alexa Fluor 568® (Invitrogen A11031 for anti-mouse IgG) were used. Secondary antibodies only were used as negative controls. After staining, cells were washed and mounted with mounting media containing DAPI. Stained cells were imaged by Axio Imager.M2 (ZEISS) and images of two-dimensional planes were acquired and processed by ZEN blue edition 2.6.

Foci numbers were quantified by ImageJ 1.45s and cell nuclei identified by ImageJ were manually confirmed. Cell clusters without clear separation or boundary were excluded. Thresholds (or noise tolerance in ImageJ) to identify foci of both 53BP1 and γH2AX were set to have 1:1 ratio of both proteins in Control within each independent experiment to allow comparison among experiments. At least 3 different fields from each condition and total number of cells (Control: 1753, EPI-7170: 854, palbociclib: 1207, and E+P: 617) were analyzed from n=4 independent experiments.

Statistical Analysis

Statistics analyses were facilitated using GraphPad Prism 8. Two-way ANOVA with Tukey’s, Dunnett’s or Sidak’s multiple comparisons test and One-way ANOVA with Tukey’s multiple comparisons test were used for calculate statistical significance amongst treatments. T-test was used to
determine statistical significance for tumor weight, Ki67 and TUNEL analyses in xenograft study. For estimation of cell doubling-times, the exponential growth model (Malthusian model) was used to estimate the best-fit values.

Data Availability

All data are available within the article and its supplementary data.
RESULTS

Combination of palbociclib and EPI-7170 affects cell viability, cell number, and colony formation

A combination of palbociclib with EPI-7170 was tested in CRPC and AR-expressing breast cancer cells to determine their effective concentrations to reduce tumor growth, colony formation, and cell viability. Enzalutamide was included as a control because it targets the full-length AR by binding to the AR-LBD and has no effect on AR-Vs. Antiandrogens including more potent second generation compounds such as enzalutamide are routinely used in cell culture at a concentration of 10 μM (27) presumably to saturate the AR (28).

LNCaP human prostate cancer cells express full-length AR, are negative for AR-Vs, and are sensitive to androgen and enzalutamide. These cells exhibited a dose-dependent decrease in cell viability in response to palbociclib when used as a monotherapy and when combined with EPI-7170 or enzalutamide (Fig. 1A). As expected, enzalutamide caused an approximately 25-40% decrease in the viability of LNCaP cells that plateaued at 2.5μM (Fig. 1B, orange line). The addition of 0.25μM palbociclib resulted in a synergistic effect with enzalutamide (Fig. 1B, light orange line, and Supplementary Fig. S1) in LNCaP cells.

LNCaP95 cells express full-length AR and AR-Vs, but their proliferation is driven by AR-Vs and hence they are resistant to enzalutamide (22). The addition of 5μM enzalutamide had no effect on its own nor did it improve the response achieved with palbociclib in LNCaP95 cells, whereas 5μM EPI-7170 had a profound inhibitory effect on its own in the absence of palbociclib which could not be improved with addition of higher doses of palbociclib (plateaued) (Fig 1A). Maintaining the concentration of palbociclib at 0.25 μM and adding increasing amounts of EPI-7170 yielded dose-dependent decreases in the viability of LNCaP and LNCaP95 cells (Fig. 1B, light blue line) that were synergistic at lower concentrations of EPI-7170 in LNCaP cells but at higher concentrations of EPI in
LNCaP95 cells (Supplementary Fig. S1). No increase in cell density or in cell number was detected with increased concentrations of EPI-7170 with or without palbociclib (Supplementary Fig. S2-S3).

The TNBC cell line, SUM159PT, exhibited a dose-dependent decrease in cell viability in response to EPI-7170 monotherapy (Fig. 1C, dark blue line) and its combination with palbociclib resulted in synergistic decreases at higher concentrations of EPI-7170 (Fig. 1C, green and light blue lines, and Supplementary Fig. S1).

Interestingly, LNCaP cells were unable to form colonies in the presence of 0.25 μM palbociclib (Fig. 1D) even though cell viability and cell number were not reduced to zero on day 3 (Fig. 1A and Supplementary Fig. S2). On the contrary, LNCaP95 cells were able to form colonies that were reduced in a dose-dependent manner by EPI-7170 and by palbociclib, whereas enzalutamide had no effect (Fig. 1E, left panel: No Medium Change, and Supplementary Fig. S4A). Holding the concentrations of EPI-7170 and enzalutamide constant at 5μM, palbociclib inhibited colony formation in a dose-dependent manner regardless of inhibitor in LNCaP95 cells. A combination of EPI-7170 with palbociclib resulted in a synergistic effect at higher concentrations of EPI-7170 (Supplementary Fig. S4B-C). In addition, the inhibitory effect was reversible as indicated by increased colonies after removal of both inhibitors after 3 days of treatments compared to conditions without removal of the inhibitors (Fig. 1E, right panel: Medium Change on Day 3). The reversible effect was not 100% complete as colony numbers were less at the higher concentrations tested (10μM or 15μM EPI-7170; 500nM palbociclib) than vehicle control, which may suggest that some of the cells were either dead or permanently affected in their ability to form colonies or proliferate. Similar results were observed in SUM159PT cells (Fig. 1F).

**Combination of EPI-7170 and palbociclib alters the cell cycles of CRPC and breast cancer cells**

Attenuation of AR transcriptional activity and inhibition of CDK4/6 activity both independently block cell cycle progression from G1 to S phase (4, 9, 29-31). Hence, we next examined whether EPI-
7170 that targets AR NTD would affect the cell cycle in both prostate cancer and breast cancer cells and whether it would deepen any response mediated by palbociclib. Non-synchronized cells were used to reflect what would be encountered in tumor tissues with all phases of the cell cycle present. In addition, one particular method of synchronizing cells may result in different phases in differing types of cells. Single cells were selected for cell cycle analyses as shown in Supplementary Fig. S5. Based on the above results from colony formation assays and cell viability, concentrations of 5μM and 10μM of EPI-7170 were chosen with 10μM being approximately 10-fold its IC50 for blocking AR transcriptional activity in LNCaP cells (23). Consistent with previous reports (23, 25), EPI-7170 significantly inhibited the transcriptional activities of androgen-induced full-length AR as well as androgen-independent AR-V7 transcriptional activity as demonstrated by luciferase reporter gene assays (Supplementary Fig. S6A-B) and measurement of expression of endogenous genes regulated by AR and AR-V7 with quantitative RT-PCR (Supplementary Fig. S6C-D). Palbociclib had no effect on the transcriptional activities of androgen-induced full-length AR or AR-V7 as measured using two reporter gene constructs (Supplementary Fig. S6A-B). As expected palbociclib had no effect on androgen-regulated genes (PSA and KLK2) while impacting the cell cycle genes UBE2C, CDC20, and CCNA2 that are also known AR-V7 target genes (17) (Supplementary Fig. S6C-D). These data support that the synergistic effects observed on cell viability and colony formation measured with the combination therapy of palbociclib with EPI-7170 are not due to palbociclib impacting AR transcriptional activity.

As expected in a cell line that is dependent upon a transcriptionally active full-length AR for proliferation, the percentages of cells in S phase were reduced when LNCaP cells were treated with enzalutamide and EPI-7170 (Fig. 2A and Supplementary Fig. S7A). At 10μM EPI-7170, LNCaP cells in S phase decreased to 1.71±0.52% (mean±SEM) on day 2 and 3.75±0.59% on day 3, and importantly decreased to almost 0% when EPI-7170 was combined with palbociclib. Unique to treatment with 10μM EPI-7170, either alone or with palbociclib, was the increase in the percentage of cells in G2/M
(approximately 2-fold compared to controls) that was not observed with enzalutamide thereby differentiating these two inhibitors of AR in a cell line that expresses solely full-length AR. A combination of 5\(\mu\)M EPI-7170 and palbociclib resulted in a similar effect on S phase, but not on G1 or G2/M, as measured with 10\(\mu\)M EPI-7170 monotherapy (i.e. reduced S percentages compared to controls). Palbociclib monotherapy increased the percentage of LNCaP cells in G1 on day 2 as expected, with little to no effect on day 3 compared to controls. However, the combination on day 3 with either enzalutamide or EPI-7170 had significant effect compared to the individual treatments. Similar cell distribution patterns (i.e., ratios among different phases of cell cycle) were detected between day 2 and day 3 of treatments when comparing amongst the different treatment conditions. However, S percentage increased on day 3 (compared to day 2) under EPI-7170 treatment with or without palbociclib, whereas S percentage decreased on day 3 (compared to day 2) for enzalutamide treatment with or without palbociclib.

Another important finding was the unique increase of percentages of cells in S phase without BrdU incorporation (S BrdU-) seen in cells treated with 10\(\mu\)M EPI-7170 and with a combination of EPI-7170 (5 and 10\(\mu\)M) with palbociclib that was not observed with enzalutamide monotherapy or combination. This S BrdU- population was more profoundly increased in cells treated with a combination of 10\(\mu\)M EPI-7170 plus palbociclib (day 2: 3.72±0.69% in combination and 0.24±0.03% in control; day 3: 3.38±0.61% in combination and 0.34±0.06% in control).

Proliferation of LNCaP95 cells is driven by AR-Vs and in these cells EPI-7170 had similar impact on the cell cycle profile as seen with LNCaP cells, but this was not the case for enzalutamide that had no effect when compared to controls (Fig. 2B and Supplementary Fig. S7B). Interestingly, even though higher S percentages were detected in LNCaP95 controls (day 2: 38.95±0.68%; day 3: 29.70±1.10%) than in LNCaP controls (day 2: 18.66±0.62%; day 3: 14.52±0.59%), the combination treatment of EPI-7170 and palbociclib caused reduction in the S population of LNCaP95 cells to almost
0% as observed in LNCaP cells. The G1 accumulation induced by palbociclib in LNCaP95 was consistent with a previous report using other AR-V-driven CRPC cells treated with this compound (29). In addition, the percentages of S BrdU- cells increased to approximately 13% with 10µM EPI-7170 plus palbociclib compared to controls.

The impact of these inhibitors on the cell cycle of several breast cancer cell lines was next examined. SUM159PT TNBC cells exhibited two distinct S-phase populations (early S and late S) on days 3 and 4 (Supplementary Fig. S8A). EPI-7170 treatment with or without palbociclib in these cells did not result in substantial changes in total percentages of cells in S phase (Fig. 2C) as compared to that measured in LNCaP or LNCaP95. However, the percentages of SUM159PT cells in early S phase were reduced by EPI-7170 on day 4 (Supplementary Fig. S8A and red bars in Fig. S8B). Enzalutamide (10µM) reduced the percentages of cells in early S phase but to a lesser extent than EPI-7170. As expected, palbociclib increased the percentage of cells in G1 phase (Fig. 2C and Supplementary Fig. S8A). However, palbociclib reduced the S BrdU- population (gray bars on Fig. 2C and Supplementary Fig. S8A) and reduced the number of tetraploid cells which are the cells with doubled DNA content (i.e. non-gated cells, higher 7AAD intensity, and excluded from calculation in bar graphs).

In MFM-223 TNBC cells that express both full-length AR and AR-V7 (15), the percentages of cells in S phase were reduced by EPI-7170 but there were no distinct S-phase populations (Fig. 2D and Supplementary Fig. S8C). The combination of 10µM EPI-7170 with palbociclib reduced the percentage of MFM-223 cells in S phase to almost zero which was consistent to that observed in LNCaP and LNCaP95 cells as shown above.

MDA-MB-453 breast cancer cells are negative for both estrogen and progesterone receptors but importantly express AR-V7 (15). These cells responded to EPI-7170 more robustly compared to enzalutamide especially with respect to accumulation in G1 and reduction of cells in S phase (Fig. 2E and Supplementary Fig. S8D).
MDA-MB-468 TNBC cells were included as a negative control because they do not express either AR or RB (Supplementary Fig. S9A). As predicted, there was no impact on the cell cycle phases of MDA-MB-468 cells in response to EPI-7170, enzalutamide, or palbociclib (Fig. 2F and Supplementary Fig. S9B), thereby supporting the specificity of these inhibitors.

Taken together, these results indicate EPI-7170 disrupted the cell cycle in prostate cancer and breast cancer cells that express AR and that its effects were greater in combination with palbociclib. Enzalutamide did not disrupt cell cycle as profoundly as EPI-7170 for cell lines that express AR-Vs such as in LNCaP95, MFM-223, and MDA-MB-453.

**Combination of EPI-7170 and palbociclib improves in vivo antitumor activity**

To investigate the effectiveness of combination treatments in AR-V7-driven tumors, the LNCaP95-D3 human CRPC xenograft model was employed. This enzalutamide-resistant cell line expresses AR-Vs and is less heterogeneous than parental LNCaP95 cells (32). Reduced tumor growth was observed in animals receiving EPI-7170 monotherapy and palbociclib monotherapy (Fig. 3A-C). Whereas the combination treatment significantly reduced the tumor growth compared to both control group and EPI-7170 monotherapy (Fig. 3A-B). Body weight was not significantly altered amongst the different treatment groups (Fig. 3D). The xenograft tumors from 3 individual best responders under combination treatment exhibited decreased percentage of proliferating cells (i.e. Ki67-positive cells) and increased percentage of apoptotic cell death (TUNEL) (Supplementary Fig. S10).

The essential role of AR on cell-cycle progression has been shown by experiments examining androgen depletion, antiandrogens, and knockdown of AR (9, 33-36). Knockdown of AR or inhibition of AR transcriptional activity by antiandrogens or androgen depletion blocks progression from G1 to S by mechanisms involving decreased levels of CDK2, and cyclin A and increased levels of p27Kip1 (34-36). Here we examined *in vivo* and *in vitro* levels of AR and cell cycle-associated proteins in response to
individual and combination treatments. Levels of these proteins detected from the harvested xenografts were not statistically significantly different between treatment groups and control group due to a wide range of variability (Fig. 3E and Supplementary Table S1). However, individual tumors from the best responders in each of the treatment groups appeared to be distinct from the average values as shown for AR, AR-V, pS780-RB, CDK6, cyclin A2, p27^Kip1 and Skp2 (labeled orange in Fig. 3E). The original immunoblot images are provided in Supplementary Fig. S11 and S12.

Levels of these cell cycle-associated proteins were also measured from in vitro studies using LNCaP95 cells that were treated with EPI-7170 and palbociclib. Consistent with previous studies in enzalutamide-resistant prostate cancer cell lines (25), EPI-7170 treatment was associated with decreased protein levels of AR-V7 whereas not altering levels of full-length AR (Fig. 3F). EPI-7170 altered the protein levels of pS780-RB, CDK2, cyclin A2, p27^Kip1, Skp2 and RAD51 which was consistent with its on-target effects on AR and mechanism of action in blocking cell cycle progression from G1 to S (34-36). Palbociclib significantly altered the protein levels of CDK4, cyclin D1 and CDK2 (Fig. 3F; original immunoblot images are provided in Supplementary Fig. S13). The increased levels of CDK4 and cyclin D1 proteins induced by palbociclib are consistent with stabilization of CDK4-cyclin D1 complex as demonstrated by others with this compound (37). Interestingly, when compared to the control, the combination treatment significantly decreased the protein levels of total RB, pS780-RB, CDK2, cyclin A2, Skp2 and RAD51, but significantly increased the levels of p27^Kip1 protein (Fig. 3F). This increased level of p27^Kip1 protein was also detected in LNCaP95-D3 xenografts from the best responders treated with EPI-7170 monotherapy and the combination (Fig. 3E); whereas palbociclib did not alter the level of p27^Kip1. The differential effects of EPI-7170 and palbociclib on the levels of these cell cycle-associated molecules supports their distinct inhibitory mechanisms on the cell cycle. Importantly, EPI-7170, palbociclib and the combination reduced the levels of CDK2 and cyclin A2 proteins which have critical roles in DNA replication (38). These reduced protein levels were consistent with decreased percentages
of cells in S-phase with EPI-7170, palbociclib and combination treatments. EPI-7170, palbociclib, and the combination significantly reduced the protein levels of RAD51 which is important for DNA damage repair.

**EPI-7170 affects the DNA damage response**

Antiandrogens and abiraterone have been reported to increase DNA-damage in LNCaP, LNCaP-AR, and VCaP cells (39, 40). Decreased levels of RAD51 protein as measured here with EPI-7170 are also consistent with studies using antiandrogens and androgen ablation (41, 42). Hence, we examined whether EPI-7170 behaved similarly to other AR antagonists and would increase DNA-damage. Indeed, phosphorylated H2AX (γH2AX) was detected in LNCaP95 cells treated with EPI-7170 and these levels could be further increased by combination treatment (Fig. 4A-B). Interestingly, with EPI-7170 or the combination treatment, the majority of S phase EdU- (same as S BrdU-) cells expressed γH2AX (approximately 60-70% in Fig. 4A lower panel and 4C). On the contrary, only a small portion (approximately 20%) of S phase EdU- cells expressed γH2AX in the control or palbociclib-treated cells. Cells in G2/M also expressed γH2AX at a significantly higher level with the combination than cells treated with control or solely palbociclib (Fig. 4C). The expression trend of γH2AX in cells in G1 was similar to the trend in G2/M cells but to a lesser extent (Fig. 4C). Immunofluorescence staining demonstrated that the foci of γH2AX and 53BP1 were increased in cells treated with EPI-7170 and the combination as compared with control and indicated by the number of foci per cell and the percentage of cells with more than 5 foci in the nucleus (Fig. 4D-G). Even though the number of foci for both proteins was correlated in a linear manner in each cell regardless of treatment (Fig. 4G) they did not always co-localize (Fig. 4D). The increase of DNA double-strand breaks was also observed in LNCaP cells treated with EPI-7170 or combination (Supplementary Fig. S14). Taken together, these data suggest that EPI-
7170 may induce DNA double-strand breaks or impair the DNA-damage response similar to other inhibitors of the androgen axis.

**EPI-7170 and palbociclib target different phases of the cell cycle**

Cell-cycle-tracing experiments were conducted in LNCaP95 cells to reveal a more detailed mechanism of the inhibition mediated by EPI-7170 and palbociclib on the cell cycle. Each phase of the cell cycle from either BrdU-positive or BrdU-negative populations was measured after selecting single cells (Supplementary Fig. S15A). Compared to control cells, the cells treated with the combination had increased granularity in SSC and slightly decreased cell size in FSC over a 4-day treatment (left panel on Fig. 5A). Cells treated with the combination exhibited distinct BrdU-positive and BrdU-negative populations with decreased BrdU intensities to a much lesser extent over time compared to controls (right panel on Fig. 5A). This suggests a slower rate of division/proliferation for the combination-treated cells compared to the control cells. Surprisingly, a portion of cells in S phase from the BrdU-positive population were sustained in S phase with a 4-day combination treatment, as shown in the diagonally distributed population in Fig. 5B (red gates). However, another portion of cells in S phase continued to progress through the cell cycle and had already entered a new G1 phase on day 1 of treatment albeit at a slower rate; i.e. it took slightly longer to reach regular cell cycle profiles as control as demonstrated in BrdU-positive cells (Fig. 5B-C and upper panel of Supplementary Fig. S15B). For BrdU-negative cells, the combination treatment stopped the cells from continuing the cell cycle over 4 days which was unique from control cells (Fig. 5B-C and lower panel of Supplementary Fig. S15B). Measurement of the number of live cells indicated that the control cells were proliferating more rapidly than the cells treated with the combination which appeared to stop proliferating over the 4 days (Supplementary Fig. S15C).

To further understand how each inhibitor affected different phases of the cell cycle, we examined shorter intervals of treatment durations. BrdU-labeled cells were collected and analyzed at several time
points during a course of 36-hour treatments. Consistent with the observation under a 4-day treatment, the combination treatment caused a portion of cells in S phase to be sustained in S phase over 36 hours (Supplementary Fig. S16). This sustained population was only observed with the combination and was not observed in the control or either monotherapy. Next we examined BrdU-positive and -negative populations separately. Interestingly, for BrdU-positive cells, at 6 hours of treatment with inhibitors there was approximately 8% of new G1 population with the control or palbociclib while there was no new G1 population with EPI-7170 or the combination (Fig. 5D and Supplementary Fig. S17; note: cells at t=0 were all in S phase and gates were drawn as reference only). Approximately 2% of new G1 cells started to form at 9 hours with EPI-7170 or the combination. After 18 hours, control cells reached a plateau with approximately 60% of new G1 population as cells were trying to reach a ‘disrupted’ tumor cell cycle distribution. However, palbociclib-treated cells resulted in a higher percentage of new G1 population compared to the control. This suggests that these cells were accumulating in G1 phase. Cells treated with EPI-7170 or the combination also displayed a higher percentage of new G1 cells than the control although this accumulation was delayed until 30 hours of treatment. These data indicate that for S-phase cells, palbociclib resulted in accumulation of cells in a new G1 population, whereas EPI-7170 delayed entry into a new G1 phase as well as caused cells to accumulate in a new G1 population.

Moreover, G2/M cells were likely to be affected by each of the inhibitors because the percentage changes were different from controls. Possibly the small populations with lower BrdU intensity within G2/M gates were new G2/M cells which were detected in early time points (within 12 hours) and detected in combination treatments during 36-hours as well as 4-day time courses (Supplementary Fig. S17 and S15B upper panel). This new population of G2/M cells were neither detected in later time points over a 36-hours course nor any other time point over 4-days in control cells. The BrdU intensities in the new G2/M populations were similar to those in the new G1 populations in these control cells which was more obvious after 18 hours. Note that the gates for the new G2/M population of cells could
not be drawn precisely to calculate the percentages due to the fact that late S and G2/M cells were not shown as distinct populations as clearly as the new G1 cells (Supplementary Fig. S17). Nevertheless, it is possible that with the combination treatment there were sustained populations of cells in late-S and new-G2/M phases of the cell cycle.

For the BrdU-negative cells at t=0, the majority of the cells were in G1 and G2/M with approximately 1% of the cells in S phase (Fig. 5E and Supplementary Fig. S18). After 6 hours of treatment with inhibitors or in the control, G2/M cells had already started moving to the G1 phase as indicated by increased percentages of cells in G1 and decreased percentages of cells in G2/M compared to t=0 (left panel and right panel in Fig. 5E and Supplementary Fig. S18). In control cells, G1 cells started to enter S phase after 9 hours (left panel and middle panel in Fig. 5E). This S phase entry slowed down after 18 hours as the percentage of cells in G1 started increasing whereas those in S phase started decreasing. At the same time, new cells in the G2/M phase, from the new cells in S phase, started to increase until 24 hours after which new G2/M cells started entering into G1 phase. These data indicate that over 36 hours there were two events of G2/M cells entering G1 phase and one time of G1 cells entering S phase. Both monotherapy with EPI-7170 or palbociclib altered these cell cycle distributions but with slightly different patterns. EPI-7170 altered G2/M cells at the beginning of treatment, i.e. different from control prior to 6 hours, whereas palbociclib altered G2/M cells after 12 hours of treatment, i.e. different from control after 12 hours. Both EPI-7170 and palbociclib caused different G1 and S percentages after 9 hours when compared to controls. However, EPI-7170 caused different G1 and S patterns after 9 hours when compared to palbociclib. Combination treatment disrupted the cell cycle to a greater degree than the monotherapies as determined by the percentages of cells in G1 and G2/M compared to the single treatments over time (Fig. 5E). These results suggest that the cell cycle did not progress, but rather arrested with combination treatment and this cell cycle arrest was observed in nearly constant cell number counts as shown in the cell cycle histograms (BrdU-negative cells in Fig. 5F).
BrdU-positive cells slowed down in the cell cycle with EPI-7170 alone or in combination, whereas with palbociclib monotherapy the cells accumulated in the G1 phase. Of note, 7-AAD intensities decreased over time in the control cells as shown in the shift to the left in the histograms from 6 to 36 hours (Fig. 5F) and from 1 to 4 days (Fig. 5C). This suggests that the control cells were proliferating. As noted in the Methods, all collected cells were used for DNA staining without cell number adjustment for cell-cycle-tracing experiments thereby the DNA dye intensity may decrease as the cell number increases. The decreases in 7-AAD intensities were also detected with both monotherapies and the combination albeit to lesser extent. Consistent with these observations, the numbers of total live cells from the cells treated with either monotherapy or the combination were significantly lower compared to control cells (Fig. 5G). This was consistent with the greater than 2-fold increase in cell doubling-time determined for the combination therapy at approximately 63 hours compared to the control at approximately 25 hours.

Collectively, these cell-cycle tracing studies revealed that EPI-7170 delayed the cell cycle for S-phase cells even though EPI-7170 treated cells eventually accumulated in the G1 phase. With the combination treatment, BrdU-negative cells were stopped in the cell cycle; however, BrdU-positive cells were able to continue in the cycle but with delayed entry into G1 phase, and with only a portion of cells sustained in S and G2/M phases, thereby suggesting a partial cell cycle arrest.

**Sequential treatments caused differential effects on the cell cycle compared to concomitant treatments**

The unique inhibitory effects on each phase of the cell cycle mediated by EPI-7170 and palbociclib suggest that sequential treatments may yield different responses from concomitant treatments. To investigate this, LNCaP95 cells were used with two treatments that were 24 hours apart so that the first inhibitor would have shown its effect on the cell cycle or have altered the cell cycle pattern before the 2nd inhibitor was applied. Of note, day 1 and day 2 after the second treatments are day
2 and day 3, respectively, after the first treatments. Single treatments and concomitant combination were the same treatment durations as in Fig. 2B. Although the percentages of cells in each phase of the cell cycle were not identical in both sets of data (Fig. 2B and Fig. 6A), the trends were the same. As shown in Fig. 6A-B, the distribution patterns of cell cycle phases were similar between day 1 and day 2 after the second treatment. When 10μM EPI-7170 was applied first (i.e. E10+D, E10/P+D and E10+P; symbol “+” is used to denote sequential and symbol “/” is used to denote concomitant), the S BrdU-population increased substantially (range of 12-17%) as compared to the control and other treatment conditions (range of 0.7-5.1%). Interestingly, the G2/M population was also increased by approximately 2-fold with these three treatment conditions that used 10μM EPI-7170. As expected, the percentages of cells in S phase decreased to almost 0 regardless of the sequence of addition of 10μM EPI-7170.

Compared to E5+P treatment (day1: 4.25±0.72%; day2: 9.64±1.47%), P+E5 caused a greater reduction of cells in S phase (day1: 3.12±0.95%; day2: 6.35±2.17%). The treatment of P+E5 also caused a greater increase of cells in G1 phase compared to E5+P even though all three conditions (E5/P, E5+P and P+E5) caused a substantial increase of cells in G1 phase compared to the control and to the single treatments (D+D, E5+D, E10+D and P+D). The percentages of cells in G1 phase were higher when cells were first treated with palbociclib before the addition of EPI-7170. Overall, the cell cycle distribution patterns for P+E10 and E10+P were very different, whereas the patterns for P+E5 and E5+P were quite similar. The total number of live cells significantly increased from day 1 to day 2 in the control, E5+D, and P+D, but not with any other treatments (Fig. 6C). Monotherapy with 10μM EPI-7170 stopped the cells from proliferating. Similarly, for the combination treatments both concentrations of EPI-7170 blocked the cell numbers from increasing regardless of the order of addition. Taken together, these data revealed that a lower concentration of 5μM EPI-7170 can be used when it was administered in combination with palbociclib to prevent cells from proliferating (i.e. reducing cells in S phase) and to induce G1 arrest.
DISCUSSION

Most benign/non-malignant cells are able to proliferate in the absence of CDK4/6 activities. Thus, inhibitors of these kinases were predicted to have selectivity against cancer cells to cause cell cycle arrest in G1, senescence, and induce apoptosis while sparing benign cells. There are three CDK4/6 inhibitors that are FDA-approved for use in combination with hormonal therapies for the treatment of advanced and metastatic breast cancer patients that have estrogen receptor-positive and human epidermal growth factor 2 (HER2)-negative disease. Palbociclib is highly specific for inhibiting CDK4 and CDK6 (43, 44). Dose-limiting toxicity to palbociclib is neutropenia. Ribociclib has a similar structure and toxicity profile to palbociclib, whereas abemaciclib has preference to inhibit CDK4 but also inhibits CDK6 and 9 (45) with toxicities being diarrhea and fatigue. The success of CDK4/6 inhibitors for some breast cancers has spurred interest for their potential application for other types of cancers including its combination with androgen-deprivation therapy for prostate cancer that expresses full-length AR (NCT02059213). However, to date there are no studies that have examined the feasibility of a combination of a CDK4/6 inhibitor with an AR-NTD inhibitor that targets both full-length AR and AR-Vs for the treatment of CRPC and TNBC. Expression of AR-V7 is considered a major mechanism of resistance to clinically approved therapies that target the full-length AR (11, 12).

Here we investigated the combination of palbociclib with EPI-7170 in breast cancer and prostate cancer and reveal that the combination: 1) blocked the in vivo growth of CRPC xenografts; 2) maximized the reduction of cells in S phase with accumulation in G1 phase compared to monotherapies; 3) caused a portion of cells in S phase to not continue in the cell cycle; and 4) caused cells in G1 and G2/M to arrest. Palbociclib and EPI-7170 have differential effects on the progression of the cell cycle thereby supporting their unique mechanisms of action. EPI-7170 monotherapy delayed cells in S phase to continue the cell cycle thereby resulting in accumulation of cells in G1. EPI-7170 also induced DNA
damage and/or impaired the DNA repair response. The order in which the combination was applied as either sequential or concomitant had differing impact on the cell cycle.

Combining a CDK4/6 inhibitor with another targeted therapy may overcome the resistance that can develop when using CDK4/6 inhibitors as monotherapies (1, 3). These resistance mechanisms include RB status, amplified cyclin E1, PIK3CA mutations, and CDK2 activation (3, 37, 46). AR regulates the expression and activities of CDK1 and CDK2 in prostate cancer cells in response to androgen (47, 48). Consistent with these reports, here the AR-NTD inhibitor, EPI-7170 attenuated the level of CDK2 protein which may be beneficial in preventing resistance to palbociclib by a mechanism of upregulation of this kinase. CDK4/6 inhibitors have shown some efficacy against full-length AR-positive prostate cancer cells that are resistant to antiandrogens due to a gain-of-function point mutation in the LBD of AR such as AR F876L (49). A combination of palbociclib with inhibition of the full-length AR by application of either enzalutamide or shRNA knock-down of AR have shown in vitro efficacy in breast cancer (50, 51). However, clinical samples of breast cancer can express both full-length AR and AR-V7 (13-16). Recently it was shown that expression of AR-V7 measured in circulating tumor cells of patients with metastatic breast cancer was associated with bone metastases and also failure of TNBC to therapies (13, 52). Thus, application of AR-NTD inhibitors such as EPI-7170 that inhibit the transcriptional activities of both AR-V7 and full-length AR (23, 25, 26) may be beneficial for the treatment of TNBC as well as CRPC.

This is the first report to examine a CDK4/6 inhibitor in combination with an AR-NTD inhibitor for the treatment of AR-expressing breast cancer and prostate cancer. Palbociclib inhibited the phosphorylation of RB at Ser780 in LNCaP95 cells and this is consistent with previous studies in LNCaP, LAPC4 and VCaP cell lines (29). Ser780 on RB is specifically phosphorylated by CDK4 and not CDK2 and it is a robust marker of mitosis. Palbociclib may stabilize CDK4/6-cyclin D1 complexes as interpreted from the increased levels of these proteins in palbociclib-treated LNCaP95 cells. These
results are consistent with reports of palbociclib stabilizing the activated CDK4/6-cyclin D complex (37) to suggest an incomplete cell cycle arrest that may lead to resistance when using a discontinuous treatment regime (3-weeks on therapy and 1-week off) (37, 53). The moderately decreased levels of CDK4, CDK6 and cyclin D1 proteins shown here in cultured LNCaP95 cells in response to EPI-7170 may be beneficial when using a combination with palbociclib to prevent drug resistance.

AR has an important role in the cell cycle with its transcriptional activity dependent upon the phase of the cell cycle. Full-length AR is transcriptionally active at G0 and S phases but its activity is markedly reduced during G1-S transition when levels of cyclin D1 and CDK4 are at their peak in a mouse fibroblast model that measured endogenous AR transcriptional activity on integrated promoters (54). In human prostate cancer cells, AR transcriptional activity also varies during cell cycle and it is required for cells to progress from G1 to S phase and also for DNA synthesis during S phase (9).

The p27Kip1 protein blocks activation of the cyclin D-CDK4 complex to regulate progression of the cell cycle at G1. Levels of this protein are regulated by proteasomal degradation in prostate cancer cells by AKT and mTOR complex 2 in response to a transcriptionally active AR (55). The loss of expression of p27Kip1 is associated with increased tumor grade and increased proliferative index in prostate cancer (56) which is consistent with the resumed transactivation of the AR axis in CRPC.

Hence inhibition of AR transcriptional activity as shown here with EPI-7170, either as a monotherapy or in combination with palbociclib, was expected to lead to increased levels of p27Kip1 protein as shown in both cultured cells and in xenografts harvested from the best responders. The consequence of EPI-7170 enhanced protein levels of p27Kip1 would be cell cycle arrest or slowing down the cell division cycle.

Here we provide a comprehensive analysis of the impact of inhibition of AR transcriptional activity by EPI-7170 and the CDK4/6 inhibitor palbociclib on the cell cycle of prostate cancer and breast cancer cells. These data reveal that EPI-7170 reduced the protein levels of cyclin D1 and CDK4/6, which caused accumulation of cells in G1, and prolonged the S phase, which resulted in a subset of cells
to delay the cell cycle. Accumulation of DNA damage likely occurred in cells in the prolonged S phase with EPI-7170 or with the combination since the majority of γH2AX-positive cells were in S phase (S EdU- in Fig. 4A and prolonged S in Fig. 5B). Full-length AR regulates DNA repair in prostate cancer (39-42). AR-Vs also regulate DNA repair (23, 57) and EPI-7170 has been proposed to enhance the sensitivity of prostate cancer cells to radiotherapy by targeting AR-V7 to block the expression of DNA damage repair genes (23). Here we show that EPI-7170 significantly decreased levels of both cyclin A2 and RAD51 proteins. Cyclin A2 is a regulator of homologous recombination in DNA repair that maintains protein levels of MRE11 and RAD51 (38). Increased expression of cyclin A2 has been found in many types of cancers and its reduction results in increased double-strand breaks in breast cancer cells and hypersensitivity to DNA-damaging agents (58). Thus, reduced levels of cyclin A2 (both xenograft and cultured LNCaP95) and RAD51 (in both xenograft and cultured LNCaP95 but more obvious in culture LNCaP95) proteins shown here in response to EPI-7170 were consistent with previous studies and support a role for functional AR in the DNA repair response. We noted that the combination therapy of EPI-7170 with palbociclib increased the % of γH2AX positive cells beyond levels achieved with the individual treatments. Thus, in addition to the AR playing an important role in DNA damage and repair, the cyclinD-CDK4/6-RB pathway is also involved in DNA repair (59) and inhibitors of CDK4/6 are being pursued as a strategy for tumor radiosensitization for numerous malignancies (for a review see Yang et al (60)). This is the first report of enhanced DNA damage in prostate cancer cells using a combination of an AR-NTD inhibitor with a CDK4/6 inhibitor which may have potential application with radiotherapy for high risk prostate cancer.

The beneficial inhibitory effect of EPI-7170 was optimally enhanced with the addition of palbociclib administered sequentially rather than concomitantly. Compared to control cells, EPI-7170 decreased the cells in S phase and increased the cells in G1 and G2/M, whereas palbociclib caused arrest in G1 to decrease the percentage of cells in S and G2/M. Moreover, sequential treatments, regardless of
whether the concentration of EPI-7170 was 5 or 10\(\mu\)M, caused more G1 accumulation and less cells in S phase than concomitant treatments. These effects were more profound, especially at saturating concentrations of EPI-7170 (10\(\mu\)M) and when palbociclib was administered before EPI-7170. Enzalutamide saturates AR in prostate cancer between 10.76 to 32 \(\mu\)M (5-15\(\mu\)g/mL) (28). The addition of EPI-7170 subsequent to palbociclib further enhanced the accumulation of cells in G1 with a reduction of cells in S phase to almost zero. These data are consistent with the inhibitory mechanism of each drug as seen previously with other cell cycle inhibitors and cells. The unique mechanisms of action of EPI-7170 and palbociclib to block the progression of the cell cycle support a strategy for sequential combination therapies for breast cancer and prostate cancer patients.
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FIGURE LEGENDS

**Figure 1. EPI-7170 and palbociclib inhibit cell growth and viability.** A-C, One day after plating, LNCaP or LNCaP95 cells were treated with (A) various concentrations of Palbo (dark blue), Palbo + 5μM EPI-7170 (light blue), or Palbo + 5μM ENZA (orange); (B) various concentrations of EPI-7170 (dark blue), EPI-7170 + 0.25μM Palbo (light blue), various concentrations of ENZA (orange), or ENZA + 0.25μM Palbo (light orange). One day after plating, (C) SUM159PT cells were treated with various concentrations of EPI-7170 (dark blue), EPI-7170 + 0.125μM Palbo (green), or EPI-7170 + 0.25μM Palbo (light blue). Cell viability was measured by alamarBlue after day 2 or day 3 of treatments. Measurements were normalized to vehicle controls in each of the independent experiments. Data are shown as mean ± SEM (n≥3 independent experiments). D-F, Single cells of LNCaP (D), LNCaP95 (E) or SUM159PT (F) were plated in low density to allow colony formation. The next day, cells were treated with vehicle control (DMSO), various concentrations of EPI-7170, ENZA or Palbo with a fixed concentration for one of the inhibitors. On day 3 (LNCaP95) or day 4 (SUM159PT) of treatments, media were changed to fresh medium without inhibitors in one set of treated cells. The media in the other set of treated cells was not changed. On day 15 (LNCaP and LNCaP95) or day 7 (SUM159PT) of treatments, cells were fixed for crystal violet staining. Representative data are shown (n=3 independent experiments). ENZA, enzalutamide; Palbo, palbociclib.

**Figure 2. EPI-7170 and palbociclib alter the cell cycle in AR-expressing cancer cells.** A-F, Cells were treated with different treatments as indicated and collected at two time points prior to 2-hour BrdU labeling for cell cycle analyses by flow cytometry. Distribution of each cell cycle phase for different cell lines are shown: LNCaP expressing AR (A), LNCaP95 expressing AR and AR-V (B), SUM-159PT expressing AR (C), MFM-223 expressing AR and AR-V (D), MDA-MB-453 expressing AR and AR-V (E), and AR-negative/RB-negative MDA-MB-468 (F). Data are presented as mean ± SEM (n≥3
independent experiments). * $p < 0.05$ for monotherapy treatment compared to control or combination treatment compared to palbociclib alone, # $p < 0.05$ for combination treatment compared to respective ENZA alone or EPI-7170 alone, + $p < 0.05$ for comparison between ENZA and EPI-7170 at the same concentrations in each cell cycle phase (two-way ANOVA); symbol color corresponds to bar color for each cell cycle phase. ENZA, enzalutamide; Palbo, palbociclib.

**Figure 3. Combination treatment blocks the growth of CRPC xenografts.**

A, Mice bearing LNCaP95-D3 xenografts were randomly separated into 4 treatment groups: vehicle control, EPI-7170 (30mg/kg body weight), palbociclib (75mg/kg body weight) and combination (EPI-7170 at 30mg/kg body weight and palbociclib at 75mg/kg body weight). Treatments were given daily by oral gavage for 21 days. Tumor size was measured every 3 days. Data are presented as mean ± SEM (n=9 tumors in each treatment group except n=8 for palbociclib group). **** $p < 0.0001$ compared to control, ### $p < 0.001$ compared to EPI-7170 monotherapy (two-way ANOVA); symbol color corresponds to treatment group. B, Weights of collected tumors were measured. Data are presented as mean ± SEM (n=9 tumors in each treatment group except n=8 for palbociclib group). ns, not significant, * $p < 0.05$, ** $p < 0.01$ compared between any two treatments (t-test). C, Images of collected xenograft tumors on day 22 were shown. D, Body weight was measured every day over the course of treatments. Data are presented as mean ± SEM (n=9 tumors in each treatment group except n=8 for palbociclib group). E, levels of AR and cell cycle related proteins were analyzed in whole cell lysates from xenograft tumors. Relative levels of proteins were quantified by normalization to β-actin levels in each sample and then normalization to the average of control samples on the same immunoblot membrane. Each data point represents one individual tumor. Three individual tumors from the best responders in each treatment groups were labeled with the color orange. Blue line indicates the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to control (two-way ANOVA). F, Protein levels in LNCaP95
cells treated with vehicle control DMSO, 10µM EPI-7170, 0.25µM palbociclib, or 10µM EPI-7170 + 0.25µM palbociclib for 3 days. Representative data are shown from n=3 independent treatments. Protein levels were quantified by normalization to β-actin levels in each of the treatments and to the average of control samples. Bar graphs are presented as mean ± SEM (n=3). * p < 0.05, ** p < 0.01, *** p < 0.001 compared to control (two-way ANOVA); asterisk colors correspond to types of treatments indicated on the graph.

Figure 4. DNA double-strand breaks are increased with EPI-7170 and combination treatments.

A, LNCaP95 cells were treated with vehicle control DMSO, 10µM EPI-7170, 0.25µM palbociclib, or 10µM EPI-7170 + 0.25µM palbociclib for 3 days. The levels of γH2AX were normalized to DNA content such that negative populations were at the same fluorescence intensity amongst the different treatments. The same gate (labeled as gH2AX, upper panel) was used to estimate the γH2AX positive population in all single cells. Levels of γH2AX in S EdU- population (red gates on middle panel) were analyzed by histograms (lower panel). Representative data are shown from n=4 independent experiments. B, Quantification of cells expressing γH2AX is presented as mean ± SEM (n=4 independent experiments). C, Quantifications of cells expressing γH2AX in S EdU-, G2/M or G1 populations are presented as mean ± SEM (n=4 independent experiments). Numbers shown on cell cycle profiles are in percentages. D, LNCaP95 cells were treated as described in (A). Localization and foci of 53BP1 and γH2AX were detected by immunofluorescence staining. Enlarged images of double stained cells are shown in inserts. E-F, Quantification of foci number per cell (E) and percentage of cells with more than 5 foci (F) are presented as mean ± SEM (n=4 independent experiments). G, Foci numbers of 53BP1 and γH2AX in each single cell under different treatments are shown in X-Y plot. Not significant, p > 0.05 (not indicated), * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 (two-way ANOVA). C, control; E, EPI-7170; P, palbociclib; E+P, EPI-7170 + palbociclib.
Figure 5. Different cell cycle phases are targeted by EPI-7170 and palbociclib. A-C, LNCaP95 cells were labeled with BrdU (2 hours) prior to combination treatment or vehicle control DMSO, and treated cells were collected after 1, 2, 3 and 4 days of treatment for cell cycle analyses. Profiles of FSC vs. SSC are shown on the left panel of (A). Profiles of FSC vs. BrdU expression are shown on the right panel of (A). Red reference lines are used to indicate the shift or non-shift of cell populations over 4 days. Changes of cell cycle phases in BrdU-positive and BrdU-negative populations over 4 days are shown in profiles of 7AAD vs. BrdU in (B). Sustained S phase cells are indicated in red gates (rectangles). BrdU-positive and BrdU-negative populations are separated by purple lines. Histograms in (C) were plotted to demonstrate the changes of cell numbers in different cell cycle phases over time (day 1 - day 4 are indicated on the graph). Representative data (with BrdU labeling) are shown from n=3 independent experiments. D-E, LNCaP95 cells were labeled with BrdU (2 hours) prior to being treated with EPI-7170, Palbo, combination or vehicle control DMSO. Treated cells were collected after 6, 9, 12, 18, 24, 30 and 36 hours of treatments for cell cycle analyses. BrdU-labeled and unlabeled cells at t=0 were collected as well. Percentages of cells in new G1 cells from BrdU-positive (D), G1 cells from BrdU-negative (E, left panel), S cells from BrdU-negative (E, middle panel), and G2/M cells from BrdU-negative (E, right panel) changed over 36 hours under different treatments. F, Histograms demonstrate the changes of cell numbers in different cell cycle phases over time (6 - 36 hours as indicated on the graph). G, Total live cells were counted at the time of cell harvest and fold-change of cell numbers compared to the cell numbers at t=0 were calculated and plotted over time. Estimated doubling times under different treatments were indicated on the graph. Curve data (D, E and G) are presented as mean ± SEM from n=3 independent experiments except n=4 for t=0 and t=24h. Representative data of histograms in (F) are shown. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 compared to control at each time point (two-way ANOVA); asterisk colors correspond to types of treatments.
indicated on the graph. Palbo, palbociclib; E+P, EPI-7170 + palbociclib; FSC, forward scatter; SSC, side scatter; H, height.

**Figure 6. Sequential treatments cause differences in cell cycle patterns and cell numbers compared to concomitant treatments.** A, LNCaP95 cells were treated with the first inhibitors or control DMSO for 24 hours before the second inhibitors or control DMSO were added. After 1 day (upper panel) and 2 days (lower panel) of the second treatments, cells were labeled with BrdU for 2 hours and then collected for cell cycle analyses. Representative data were shown (n=4 independent experiments). B, Percentages of each cell cycle phases under different treatments after 1 (left panel) or 2 (right panel) days of the 2nd treatments were presented as mean ± SEM from n=4 independent experiments. Not significant “ns” or no symbol indication, \( p > 0.05 \), \( * p < 0.05 \), \( *** p < 0.001 \), \( **** p < 0.0001 \) (two-way ANOVA); asterisk color corresponds to bar color for each cell cycle phase. C, Numbers of total live cells collected after 1 (left panel) or 2 (right panel) days of the 2nd treatments were plotted as mean ± SEM (n=4 independent experiments). \( * p < 0.05 \), \( *** p < 0.001 \), \( **** p < 0.0001 \) compared to day 1 in each treatment group (two-way ANOVA). D, DMSO; E5, 5\( \mu \)M of EPI-7170; E10, 10\( \mu \)M of EPI-7170; P, 0.25\( \mu \)M of palbociclib.
Figure 2

A

LNCaP

Day 2

Day 3

% 100
80
60
40
20
0
ENZA (µM) 0 5 10 0 0 5 10 0 0 5 10 0 0 5 10 0 0 5 10 0

EPI-7170 (µM) 0 0 5 10 0 0 5 10 0 0 5 10 0 0 5 10 0 0 5 10 0

0.25µM Palbo

S BrdU-

G2/M

S

G1

B

LNCaP95

Day 2

Day 3

% 100
80
60
40
20
0
ENZA (µM) 0 5 10 0 0 5 10 0 0 5 10 0 0 5 10 0 0 5 10 0

EPI-7170 (µM) 0 0 5 10 0 0 5 10 0 0 5 10 0 0 5 10 0 0 5 10 0

0.25µM Palbo

S BrdU-

G2/M

S

G1

C

SUM159PT

Day 3

Day 4

% 100
80
60
40
20
0
ENZA (µM) 0 5 10 0 0 5 10 0 0 5 10 0 0 5 10 0 0 5 10 0

EPI-7170 (µM) 0 0 5 10 0 0 5 10 0 0 5 10 0 0 5 10 0 0 5 10 0

0.25µM Palbo

S BrdU-

G2/M

S

G1

D

MFM-223

Day 5

Day 7

% 100
80
60
40
20
0
ENZA (µM) 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0

EPI-7170 (µM) 0 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0

0.1µM Palbo

S BrdU-

G2/M

S

G1

E

MDA-MB-453

Day 2

Day 3

% 100
80
60
40
20
0
ENZA (µM) 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0

EPI-7170 (µM) 0 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0

0.125µM Palbo

S BrdU-

G2/M

S

G1

F

MDA-MB-468

Day 2

Day 3

% 100
80
60
40
20
0
ENZA (µM) 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0

EPI-7170 (µM) 0 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0 0 10 0

0.25µM Palbo
Figure 5

A

Day 1 | Day 2 | Day 3 | Day 4
---|---|---|---
Control | Control | Control | Control
10 μM EPI-7170 | 10 μM EPI-7170 | 10 μM EPI-7170 | 10 μM EPI-7170
0.25 μM Palbo | 0.25 μM Palbo | 0.25 μM Palbo | 0.25 μM Palbo

B

Day 1 | Day 2 | Day 3 | Day 4
---|---|---|---
Control | Control | Control | Control
10 μM EPI-7170 | 10 μM EPI-7170 | 10 μM EPI-7170 | 10 μM EPI-7170
0.25 μM Palbo | 0.25 μM Palbo | 0.25 μM Palbo | 0.25 μM Palbo

C

Control | 10 μM EPI-7170 | 0.25 μM Palbo
Day 1 | Day 2 | Day 3 | Day 4
---|---|---|---
Cell number | Cell number | Cell number | Cell number
BrdU positive | BrdU positive | BrdU positive | BrdU positive

D

G1 | new G1 | G1 | G2/M
---|---|---|---
Control | Control | Control | Control
EPI-7170 | EPI-7170 | EPI-7170 | EPI-7170
Palbociclib | Palbociclib | Palbociclib | Palbociclib
E+P | E+P | E+P | E+P

E

G1 | S | G2/M
---|---|---
Control | Control | Control
EPI-7170 | EPI-7170 | EPI-7170
Palbociclib | Palbociclib | Palbociclib
E+P | E+P | E+P

F

Control | 10 μM EPI-7170 | 0.25 μM Palbo | 0.25 μM Palbo
---|---|---|---
BrdU positive | BrdU positive | BrdU positive | BrdU positive

G

Cell number fold change (normalized to E0)
---
Control: 25.06 h | EPI-7170: 43.48 h | Palbo: 39.57 h | E+P: 63.31 h
6 h | 9 h | 12 h | 18 h | 24 h | 30 h | 36 h

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