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ORIGINAL ARTICLE

Targeting cell cycle and hormone receptor pathways in cancer

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The cyclin/cyclin-dependent kinase (CDK)/retinoblastoma (RB)-axis is a critical modulator of cell cycle entry and is aberrant in many human cancers. New nodes of therapeutic intervention are needed that can delay or combat the onset of malignancies. The antitumor properties and mechanistic functions of PD-0332991 (PD; a potent and selective CDK4/6 inhibitor) were investigated using human prostate cancer (PCa) models and primary tumors. PD significantly impaired the capacity of PCa cells to proliferate by promoting a robust G_1-arrest. Accordingly, key regulators of the G_1-S cell cycle transition were modulated including G1 cyclins D, E and A. Subsequent investigation demonstrated the ability of PD to function in the presence of existing hormone-based regimens and to cooperate with ionizing radiation to further suppress cellular growth. Importantly, it was determined that PD is a critical mediator of PD action. The anti-proliferative impact of CDK4/6 inhibition was revealed through reduced proliferation and delayed growth using PCa cell xenografts. Finally, first-in-field effects of PD on proliferation were observed in primary human prostatectomy tumor tissue explants. This study shows that selective CDK4/6 inhibition, using PD either as a single-agent or in combination, hinders key proliferative pathways necessary for disease progression and that RB status is a critical prognostic determinant for therapeutic efficacy. Combined, these pre-clinical findings identify selective targeting of CDK4/6 as a *bona fide* therapeutic target in both early stage and advanced PCa and underscore the benefit of personalized medicine to enhance treatment response.

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INTRODUCTION

Prostate cancer (PCa) is a leading cause of cancer mortality in men and presents an ongoing therapeutic challenge.[1] PCa is one of the most prevalent cancers diagnosed in men with a lifetime risk of 1-in-6 according to the American Cancer Society. Localized disease can be effectively managed through surgery and/or radiation;[1,2] however, advanced disease represents a major clinical challenge as standard chemotherapeutics are not typically effective.[1] Treatment for advanced PCa is based primarily on its dependence of the androgen receptor (AR) for development, growth and survival. Therefore, the mainstay of treatment for advanced disease involves androgen deprivation therapies (ADTs) that result in cell cycle arrest and/or death.[1] These ablative therapies are effective on average for 2–3 years, whereupon lethal castration-resistant PCa (CRPC) develops, because of re-activation of AR signaling.[1] Despite recent progress, the ability to effectively treat CRPC remains limited; therefore, additional therapeutic options are needed.

Owing to the importance of androgen signaling, PCa at all stages remains reliant on AR to promote growth and/or survival.[1] AR is a nuclear hormone receptor and upon activation by androgens (for example, dihydrotestosterone, (DHT)) induces a complex transcriptional program that includes proliferation and expression of prostate-specific genes such as *kallikrein-related peptidase 3* (*KLK3*) (prostate-specific antigen, PSA). As an AR-target, *KLK3*/PSA is not only used as a surrogate for AR activity, but is also used in the clinical setting to monitor disease.[1] Treatment of advanced PCa entails limiting AR activity either through inhibiting androgen levels or through the use of direct AR antagonists like bicalutamide.[1] Recurrent disease invariably ensues as a result of re-activated AR, indicated by resurgent *KLK3*/PSA. Multiple mechanisms have been described to explain restored AR activity including: amplification, activating mutations or splice variants, altered post-translational modifications, aberrant expression of cofactors, and intracrine androgen synthesis.[1] Regardless of the mechanism of restoration, AR continues to promote proliferation in advanced disease. Thus, a concerted effort has been undertaken to determine how AR governs cell cycle progression in order to target the cell cycle machinery and improve therapy.

The mechanism by which androgen/AR induces the cell cycle to instigate proliferation has been recently reviewed.[3] Briefly, androgen promotes enhanced translation, through mammalian target of rapamycin, of the D-type cyclins and the induction of $p21^{Cip1}$ mRNA. These inductive events combine to assist the formation of an active complex between D-cyclins, $p21^{Cip1}$ and the cyclin-dependent kinases 4/6 (CDK4/6) that are important for cell cycle progression. The combined kinase functions of early G_1 cyclin-D/CDK4 or 6 and late G_1 cyclin-E/CDK2 serve to phosphorylate the retinoblastoma protein (RB), allowing E2F transcription factors to control downstream cyclin expression (for example, cyclin A) required for S-phase transition. Given the importance of the cyclin/CDK/RB-axis in controlling the G_1-S transition in the majority of cancers, including PCa, a prime

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therapeutic candidate has been CDK activity.[4–7] In PCa, the majority of early pre-clinical studies designed to modulate CDK activity with flavopiridol, a pan-CDK inhibitor, showed antitumor activity in xenografts.[8–10] However, enthusiasm waned as phase II clinical trials of flavopiridol were disappointing, due largely to off-target effects and toxicity.[11] Recently, a clinical grade, orally active CDK inhibitor (PD-0332991, PD) has been developed that selectively and reversibly inhibits CDK4/6 at low nanomolar concentrations.[12,13] Pre-clinical studies in other cancer models have shown that PD induces a robust cytostatic G_1-arrest, delays or prevents xenograft formation with minimal cell death, and may hinder metastatic potential. PD has been shown preliminarily to limit disease progression in patients with inoperable RB-positive teratomas, which are commonly resistant to chemo- and radiation therapy, with minimal adverse events.[14] Importantly, clinical trials have been initiated based on pre-clinical data from both hematological and solid tumors (for example, lymphoma,[15] leukemia,[16,17] myeloma[18,19], breast,[20–25] colon,[26–28] lung,[29] esophageal[30] and glioblastoma[31,32]).

Currently, no study has assessed the efficacy of PD in PCa, despite the preliminary pre-clinical analyses and few recently completed phase I trials.[33–35] Here, a pre-clinical evaluation of PD was undertaken to determine its therapeutic potential in PCa. Using well-established hormone-dependent and CRPC cell models, PD showed remarkable single-agent activity with regard to limiting cellular proliferation and growth. Subsequent analyses demonstrated the feasibility of combinatorial therapy between PD and existing treatments such as AR antagonists and radiation. The potential therapeutic effect of PD was revealed using both *in vivo* mouse xenografts and a recently developed novel *ex vivo* assay using primary human tumors obtained by radical prostatectomy. These pre-clinical findings, using PD, suggest selective CDK4/6 inhibition as a potential node of intervention in PCa, and warrant future studies to evaluate its clinical efficacy.

RESULTS

PCa cell proliferation is attenuated by CDK4/6-specific inhibition

PD, a CDK 4/6-selective inhibitor, was evaluated in a comprehensive panel of hormone-sensitive PCa cells. Dose dependence studies for PD indicated an IC_{50} range of 44–91 nM (Supplementary Figure 1A) consistent with other hormone-dependent cancer cell systems.[20,36,37] PCa cells were treated with PD ($\sim$5–10X the IC_{50}) and assessed for active proliferation via pulse labeling with bromodeoxyuridine (BrdU) and quantified by flow cytometry (Figure 1a). As shown, BrdU incorporation in LNCaP, LAPC4 and VCaP cells was profoundly attenuated (treated vs control (%): 4.27 vs 23.1, 2.93 vs 28.5 and 2.32 vs 23.2, respectively). Cell cycle analyses revealed a strong G_0/G_1-phase arrest (data not shown) consistent with suppression of CDK4/6 activity.[5] VCaP cells treated with PD, which showed the strongest anti-proliferative response, displayed minimal cell death as indicated by sub-G1 accumulation (Supplementary Figure 1B) and cleaved poly ADP-ribose polymerase (PARP) (Supplementary Figure 1C) as compared with etoposide. Similarly, PD had minimal impact on extracellular signal-regulated kinase signaling (Supplementary Figure 1D). In addition, treatment of PD conferred a reduction in cell growth as indicated by crystal violet staining (Figure 1b). As the cyclin/CDK/RB pathway is implicated in oncogenic signaling in cancer,[38] protein expression of cell cycle components was monitored after PD treatment (Figure 1c). In all cells tested, protein levels of CDK4 and AR were unchanged by PD. In contrast, RB protein Ser780-phosphorylation, a known site of CDK4/6 activity,[38] was suppressed. Cyclin A, a well-characterized RB target gene and positive indicator of proliferation,[38,39] levels were attenuated by PD. Combined, the decreased RB phosphorylation and cyclin A protein levels strongly indicated that PD effectively inhibited CDK4/6 activity. Examination of the protein levels of key G_1-cyclins (cyclins D1 and E), required for the activation of CDKs (CDK4/6 and CDK2, respectively), revealed disparate and cell-specific changes on PD exposure. Cyclin E1 was unchanged or decreased only in LAPC4 cells, whereas cyclin D1 was modestly but significantly increased in LNCaP and LAPC4 but not VCaP cells. Elevated cyclin D1 was somewhat surprising, as many therapeutics that suppress proliferation and induce G_1-arrest are frequently associated with loss of cyclin D1.[40] As cyclin D1 binds and initiates CDK4/6 activity,[38,41,42] co-immunoprecipitation analyses were performed (Supplementary Figure 1E) to determine if PD altered the cyclin D1–CDK4 complex. Immunoprecipitation of CDK4 from PD-treated LNCaP cells resulted in a modest increase in co-immunoprecipitated cyclin D1 (compare lanes 2 and 5), suggesting that PD may stabilize an inactive cyclin D1–CDK4 complex and hinder the turnover of cyclin D1. Combined, these data indicate that PD inhibits CDK4/6-dependent phosphorylation of RB resulting in suppression of proliferation/growth in multiple hormone-sensitive PCa cells.

Efficacy of AR-directed therapeutics is retained in combination with CDK4/6 inhibition

Virtually all stages of PCa are dependent on androgen/AR signaling.[1] Consequently, advanced PCa is treated with hormone-based therapies that block AR signaling.[1] It has been shown that aberrant cyclin D1 levels can selectively modulate androgen-dependent AR activity.[43] Therefore, the impact of PD on androgen-dependent AR activity and/or potential response to AR-directed therapies (i.e., casodex, Csdx) was assessed via gene expression analyses of AR-target genes (*KLK3*/PSA, *TMPRSS2* and *KLK2*) with known clinical relevance (Figure 2). To measure androgen/AR-dependent target gene expression, LNCaP (Figure 2a), LAPC4 (Figure 2b) and VCaP (Figure 2c) cells were cultured in steroid hormone-depleted (charcoal dextran-treated serum) media, then stimulated with DHT in the presence of the AR antagonist Csdx, PD or a combination thereof. As expected, DHT resulted in a robust increase in the mRNA expression of all AR-target genes. Conversely, Csdx significantly reduced DHT-induced AR-target gene expression. Addition of PD (or in combination with Csdx) had no impact on DHT-induced gene expression in VCaP cells and had minimal cooperative impact in LNCaP and LAPC4 cells, consistent with the established observation that cyclin D1 modulates AR activity independent of CDK function. These data indicate that PD acts in a manner distinct from AR-directed therapeutics, and that suppression of CDK4/6 activity does not antagonize standard of care AR-directed therapies.

CDK4/6-specific inhibition sensitizes PCa cells to ionizing radiation (IR)

Radiation therapy in conjunction with novel therapeutics is frequently used to treat locally advanced PCa.[1,2,44] Therefore, the capacity of PD to cooperate with IR was assessed (Figure 3). As expected, single-agent treatment with PD or IR (compared with vehicle) suppressed long-term cell growth (at day 7) indicated by *in vitro* cell growth kinetics of LNCaP, LAPC4 and VCaP cells (Figure 3a) and parallel colony formation assays (Supplementary Figure 2). Concurrent treatment with PD and IR resulted in a significant attenuation in cell growth (compared with either single-agent alone) for LNCaP, LAPC4 and VCaP cells. All cell lines (Table 1, data calculated from the raw data in Figure 3a) demonstrated prolonged doubling times (compared with vehicle) either on individual (PD: 1·2- to 1·6-fold or IR: 1·4- to 2.0-fold) or combined treatments (PD + IR: 2.4- to 3.7-fold). PD treatment alone substantiated the above findings that CDK4/6-specific inhibition suppresses proliferation. In addition, the ability of PD to cooperate with IR was determined using a clonogenic survival assay (Figure 3b). Importantly, these studies further demonstrated

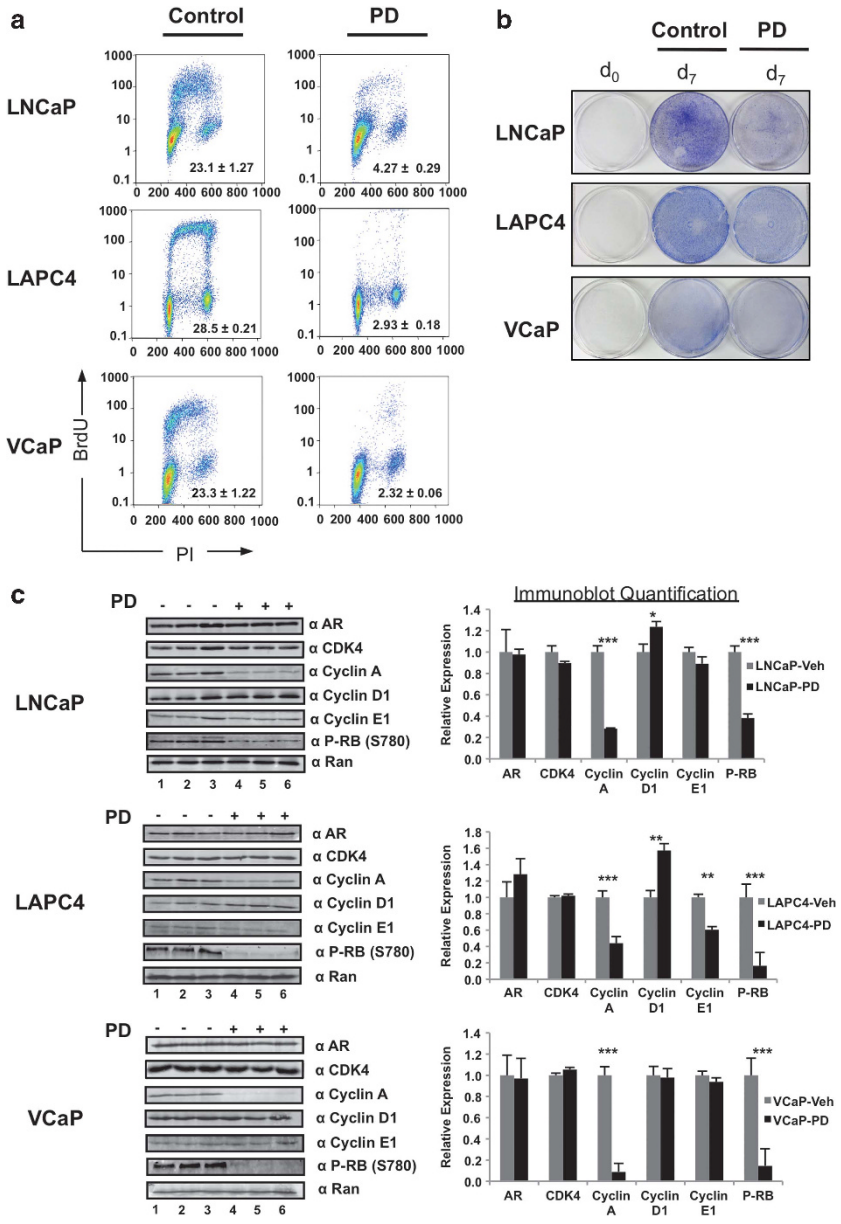

Figure 1. CDK4/6-specific inhibition suppresses proliferation of androgen-dependent PCa cells. The impact of the CDK4/6-specific inhibitor (PD) on proliferation and cell cycle components was characterized in multiple androgen-dependent PCa cell model systems. (**a**) Bivariate flow cytometry analyses of: LNCaP (upper), LAPC4 (middle) and VCaP (lower) cells treated 24 h with 0.1% dimethylsulphoxide control (left column) or 0.5 μM PD (right column). Profiles are representative of three independent experiments. The x axis denotes relative DNA content as indicated by propidium iodide (PI) staining. The y axis denotes cells undergoing active S-phase as indicated by 2-h pulse-label of BrdU. Inset values: % BrdU incorporation (mean ± s.d., from an experiment performed in biological triplicate). (**b**) Crystal violet staining at day 7 (d_7) relative to plating at day 0 (d_0) from LNCaP, LAPC4, and VCaP cells initially treated with control or 0.5 μM PD for 24 h. Data are representative of three independent experiments. (**c**) Immunoblot analyses, from parallel treated cells in panel (**a**) for the indicated prostate and cell cycle components (left panels) and quantified by LI-COR image analyses (right panels). Loading and quantification are relative to Ran. Grey and black bars = control and PD treatments, respectively. *; **; *** indicates *P*-values: <0.05; 0.01; 0.001, respectively.

that the combination of PD and IR (at 2 or 4 Gy, doses higher than 6 Gy yielded no colonies (data not shown)) significantly reduced the number of colonies formed as compared with IR treatment alone. Combined, these data indicate that PD cooperates with and/or sensitizes PCa cells to the effects of IR.

CRPC cells are amenable to CDK4/6-specific inhibition, dependent on RB status

Based on the findings above that PD showed a remarkable capacity to not only inhibit proliferation but also act in concert with IR to limit growth of hormone-sensitive PCa cells, it was surmised that CDK4/6-specific inhibition could be advantageous in the treatment of CRPC, which is typically associated with increased proliferative/survival capacity and poor outcome.[1] To challenge this hypothesis, a collection of diverse CRPC cells were treated with PD, and flow cytometry and gene expression analyses were performed to evaluate the overall response of CRPC cells to therapy (Figure 4). Cell cycle analyses of PC3M cells (Figure 4a), a variant of the PCa-derived PC3 cell line with bone homing potential, indicated that PD treatment resulted in an increased percentage of cells in G_0/G_1 with a concomitant decrease in S and

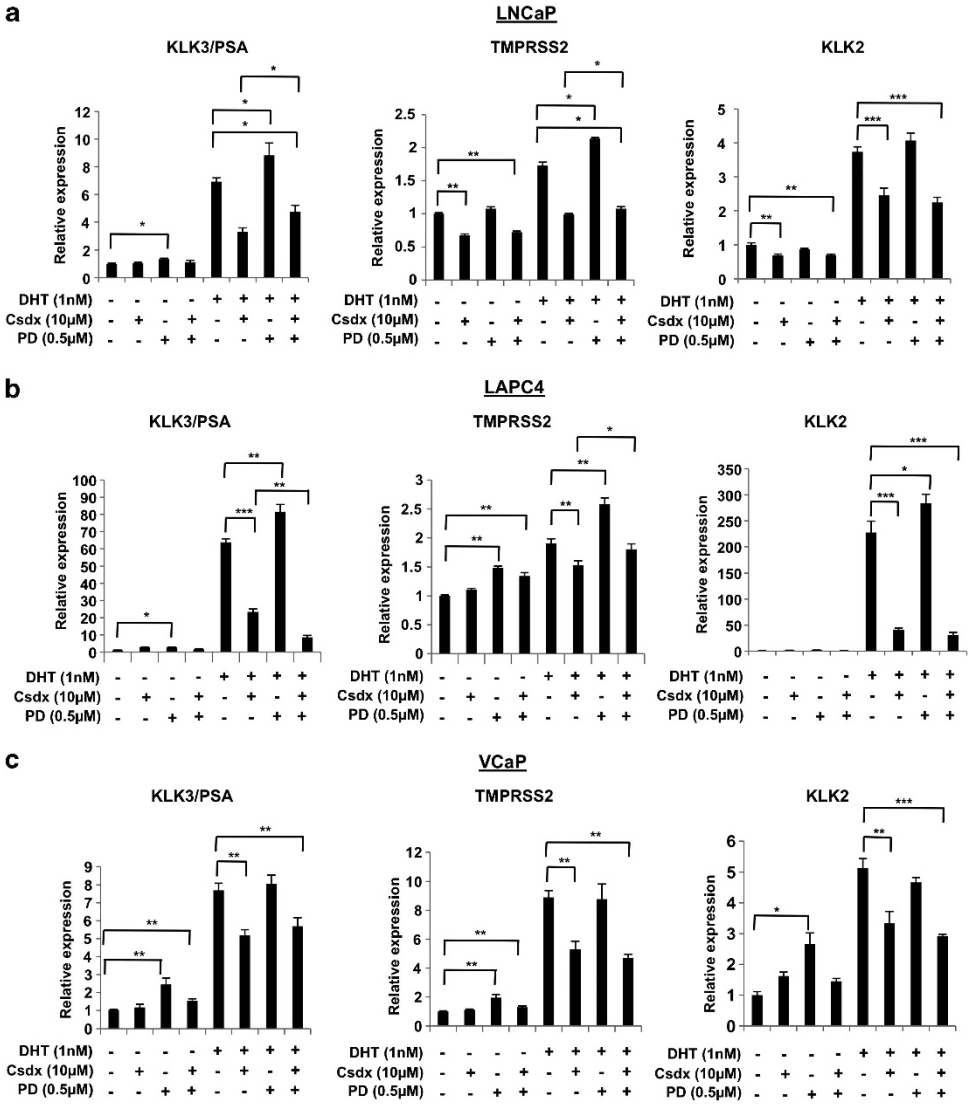

Figure 2. AR-directed therapies are effective in the presence of CDK4/6-specific inhibition. To assess AR activity, androgen-dependent PCa cells: (**a**) LNCaP (**b**) LAPC4 and (**c**) VCaP were cultured 72 h in media containing steroid-deprived serum (5% charcoal-dextran treated (CDT)) then stimulated 24 h with (or without) DHT (1 nM) in the presence of PD (0.5 μM), Csdx (10 μM) or combination of PD and Csdx. Relative mRNA expression normalized to glyceraldehyde 3-phosphate dehydrogenase was determined by quantitative PCR (qPCR) for the known AR-target genes: *KLK3*/PSA (left), *TMPRSS2* (middle) and *KLK2* (right). Indicated treatments for each gene are relative to non-DHT and non-drug treated cells. *; **; *** indicates *P*-values: <0.05; 0.01; 0.001, respectively.

G_2M (treated vs control (%): 86.1 vs 61.7, 8.27 vs 28.8, 3.24 vs 8, respectively). PC3M cells, because of a lack of detectable AR, represent a rare form of CRPC.[45] These data not only demonstrate the effectiveness of PD in multiple forms of PCa, but also are consistent with the above findings (Figure 2) that the effects of PD on proliferation are independent of AR.

Although PD was effective at limiting AR-negative CRPC cell proliferation, the vast majority of CRPC cases remain AR positive.[1] Therefore, it is essential to evaluate PD in the context of CRPC cells that maintain AR. Based on the mechanism of action of PD, it was hypothesized that the capacity of PD to limit CDK4/6-dependent proliferation is dictated by the status of RB. Recently, isogenic PCa tumor models of RB loss were developed, wherein it was shown that RB loss is a critical mediator of the transition to castration resistance, and promotes lethal phenotypes through enhanced AR levels and activity.[46] Thus, using isogenic pairs derived from LNCaP cells with and without RB knockdown, the notion that RB is required for PD action was assessed. Importantly, cell cycle analysis of this CRPC model system treated with PD (Figure 4b) indicated that RB loss is sufficient to promote PD resistance. Moreover, the ability of RB loss to promote therapeutic bypass of PD was confirmed by stable transduction of the shRB1 construct into PC3 (which lack AR), LAPC4 and VCaP cell model systems (Supplementary Figure 3). Thus, in these diverse cellular contexts, the data overwhelmingly support the concept that the effectiveness of PD is dependent on the integrity of RB.

Additional studies were performed using two common CRPC cells (22Rv1 and LNCaP-derived C4-2, both positive for RB and AR) that represent different pathways of acquired resistance to hormone-based therapy. For these studies, 22Rv1 and C4-2 cells (Figure 4; panels c and d, respectively) were cultured and treated in conditions that mimic ADT. As expected, both cell models proliferated with control treatments as indicated by the percentage of cells in S-phase. Treatment of both 22Rv1 and C4-2 cells with PD resulted in an increased percentage of cells in G_0/G_1 (treated vs control (%)—22Rv1: 85.6 vs 55, C4-2: 85.1 vs 69.2)

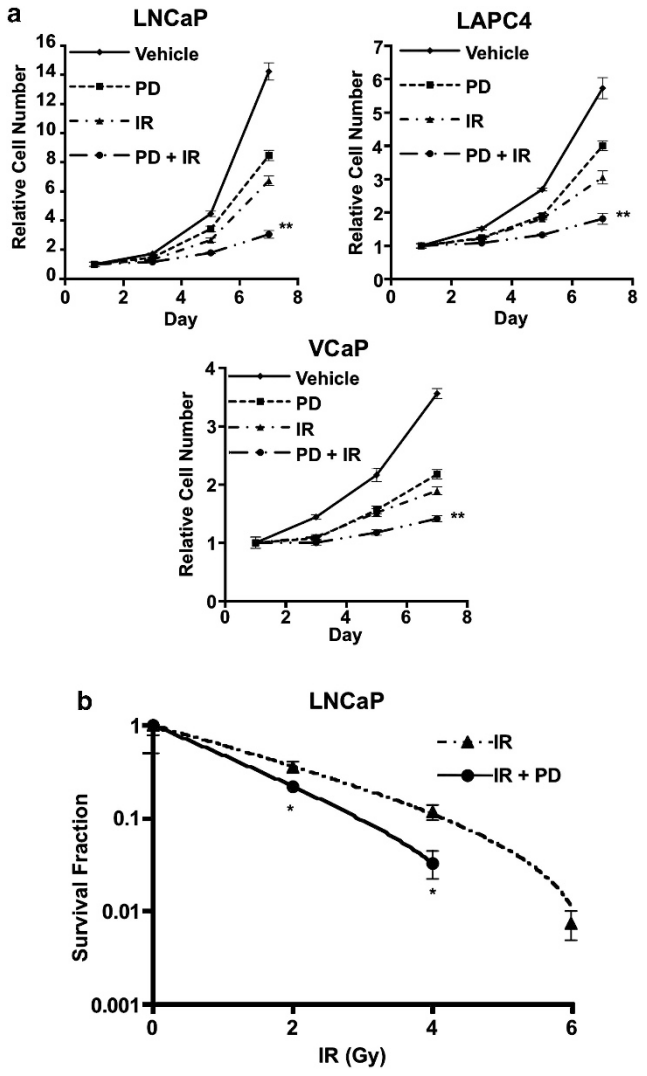

Figure 3. CDK4/6-specific inhibition cooperates with IR to attenuate PCa cell growth. IR was administered in conjunction with PD to evaluate the effect of combinatorial therapy, as described in the Material and methods section. (**a**) Cell growth analyses of LNCaP, LAPC4 and VCaP cells treated with single-dose IR (2 Gy), PD (0.5 μM) or a combination of both. Cell number, for the indicated times and treatments, was determined by Trypan blue exclusion and was normalized to the initial day of treatment (day 1). **Indicates a *P*-value <0.01 relative to individual treatments alone. Data shown are representative of three independent experiments. (**b**) Clonogenic assay using LNCaP cells treated with the indicated dose (Gy) of IR or in combination with PD (0.5 μM). Colonies were stained with crystal violet and counted 14 days post-treatment. Studies were performed in biological triplicate and are representative of three independent experiments. *Indicates a *P*-value <0.05 relative to IR treatment alone.

Table 1. Doubling times

	LNCaP	*LAPC4*	*VCaP*
Veh	1.57[a]	2.38	3.28
PD	1.95	3.00	5.36
IR	2.18	3.73	6.62
PD + IR	3.72	7.00	12.0

Abbreviations: IR, ionizing radiation; PD, PD-0332991. [a]Values in days.

paralleled by decreased S (treated vs control (%)—22Rv1: 10.2 vs 34.1, C4-2: 6.77 vs 18) and G_2M (treated vs control (%)—22Rv1: 4.27 vs 10.9, C4-2: 6.21 vs 8.47). To evaluate the impact of PD on AR signaling and potential utility in combination with ADT in CRPC disease, gene expression analyses were performed using C4-2 cells (Figure 4e). AR-target (that is, *KLK3*/PSA and *TMPRSS2*) gene expression was similar to hormone-sensitive LNCaP cells (Figure 2), suggesting that PD does not interfere with ADT in the CRPC setting. These data demonstrate that PD effectively attenuated proliferation of multiple CRPC models, independent of AR status and/or standard ADT therapy. Moreover, PD action is dependent on RB, suggesting the need to stratify patients based on RB status.

CDK4/6-specific inhibition impacts PCa tumor proliferation both *in vivo* and in *ex vivo* primary human tumors

Although CDK4/6-specific inhibition is effective against androgen-dependent and CRPC cell proliferation or growth *in vitro*, additional analyses were performed to discern the *in vivo* benefit of PD treatment in PCa systems (Figure 5). For these studies, VCaP cells were used as they harbor two clinically relevant aberrations: (1) elevated AR that is frequently associated with disease progression, and (2) chromosomal rearrangements that fuse ETS oncogenes (*ERG* or *ETV*) under androgen/AR control via the *TMPRSS2* regulatory locus as seen in 50–75% of PCa.[47] Mice harboring VCaP xenografts were treated with PD (150 mg/kg) or lactate vehicle, consistent with known dosage regimens.[18,22,25,28,48] VCaP tumor proliferation was determined using immunohistochemistry against endogenous Ki-67 (Figure 5a, left panel). As expected, VCaP tumors in control treated animals were proliferative. In contrast, treatment with PD resulted in a reduction in Ki-67. Quantification (Figure 5a, right panel) revealed a significant reduction (65.8%) in VCaP proliferation upon PD treatment. As the data in Figure 4 indicated that RB is a critical factor for PD action, *in vivo* xenograft growth was evaluated using the PC3 cells (described in Supplementary Figure 3) treated with a short course of PD. As shown in Supplementary Figure 4A, PC3-shNS cells had a delayed growth response following treatment with PD. These data were expected based on previous studies in other model systems.[13,22,24,25,32] In contrast, PC3-shRB1 cells showed a growth profile similar to that of vehicle-treated PC3-shNS cells. Together, these *in vivo* data indicate that PD is sufficient to reduce xenograft proliferation and delay growth in an RB-dependent manner.

Encouraged by the xenograft data, additional studies were performed using a novel *ex vivo* culture system.[49] Human prostatectomy tissues (processed into $\sim 1\,mm^3$ explants) were cultured, using standard cell culture growth media and components, on sterile dental sponges to allow for efficient media/oxygen exchange. Using these conditions the tumor tissue maintains many of its characteristics including: histology, AR status, proliferative capacity and stromal environment.[49] This *ex vivo* assay affords the potential to assess novel therapies in tissue that is one-step removed from the patient without the difficulty and expense of implanting tissue into animals. In proof of concept, we obtained tissue specimens from five patients that were subsequently determined to be RB proficient (Figure 5b, left panel; representative RB-positive tumor and corresponding hematoxylin and eosin). Explant tissues were treated, as described in the Materials and methods section, for a short- and long-term duration (days 2 and 6, respectively) in the presence of two different concentrations of PD (0.5 and 1.0 μM). As shown (Figure 5b, right panel; same specimen as left panel), *ex vivo* culture for 6 days in control or two concentrations of PD did not overtly disrupt the glandular structure as indicated by hematoxylin and eosin staining. Similarly, minimal cell death was observed as determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, in both long-term control and treated

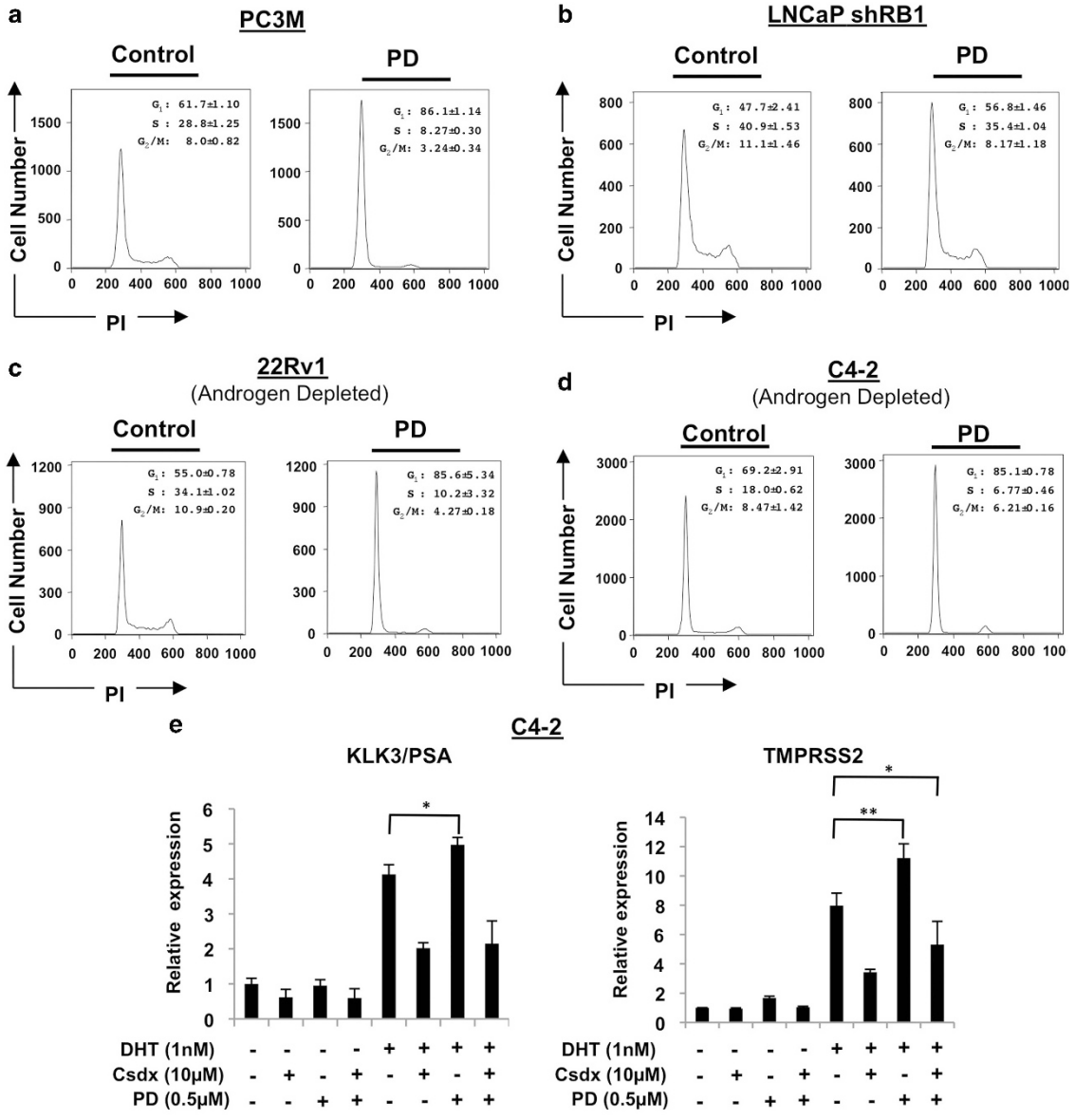

Figure 4. CRPC cells are sensitive to CDK4/6-specific inhibition, dependent on RB status. The relevance of PD treatment to advanced PCa was determined using multiple CRPC cell model systems. Flow cytometry was performed on CRPC cells: (**a**) PC3M; (**b**) LNCaP shRB1 (stable LNCaP cells depleted of the RB protein); (**c**) 22Rv1; and (**d**) C4-2 treated with control (left panels) or PD (right panels), as described in Figure 1a. Representative flow cytometry profiles for each cell model system are shown. The y axis denotes the number of fixed cells stained with propidium iodide (PI). Inset values: % PI-stained cells (mean ± s.d., from an experiment performed in biological triplicate) in the G_1-, S-, G_2M-phases as determined using the cell cycle algorithm in FlowJo. Note: 22Rv1 and C4-2 cells were treated in media containing 5% charcoal-dextran treated (CDT) serum, as described in the Materials and methods section. (**e**) Gene expression analyses from C4-2 cells was performed, as described in Figure 2, to assess the impact of indicated therapies on AR activity. Representative AR target genes: *KLK3*/PSA (left) and *TMPRSS2* (right) are shown. *; ** indicates *P*-values: <0.05; 0.01, respectively.

explants (data not shown). Ki-67 staining, described in the Materials and methods section, indicated that both PD concentrations reduced proliferation compared with the control. As shown (Figure 5c), short-term treatment with 1 μM PD significantly inhibited proliferation (82.1%), and long-term treatment demonstrated that the inhibition is dose dependent (46.8 and 85.7%). Interestingly, long-term proliferation of explant tissues at the higher PD concentrations was equally attenuated as the short-term treatment, suggesting a response with PD can be achieved early in treatment. Having defined the proliferative response of explant tissues to PD, additional studies were performed to evaluate the capacity of PD to enhance the therapeutic potential of IR (Supplementary Figure 4B). These *ex vivo* data, similar to the data obtained in Figure 3, indicated that PD has the potential to cooperate with IR. As the data above indicated that the response of PCa cells is dependent on RB, explant tissues were transduced with the shNS and shRB1 constructs and treated with or without PD (Supplementary Figure 4C). As expected, based on the cell and xenograft data, PD reduced the proliferation of explant tissue that had been transduced with shNS. Importantly, PD was ineffective in explant tissues that had been transduced with shRB1; thus, these data are consistent with the notion that RB is a critical determinant for the response to PD in PCa. These studies, using xenografts *in vivo* and human PCa tissues *ex vivo*, demonstrate that CDK4/6

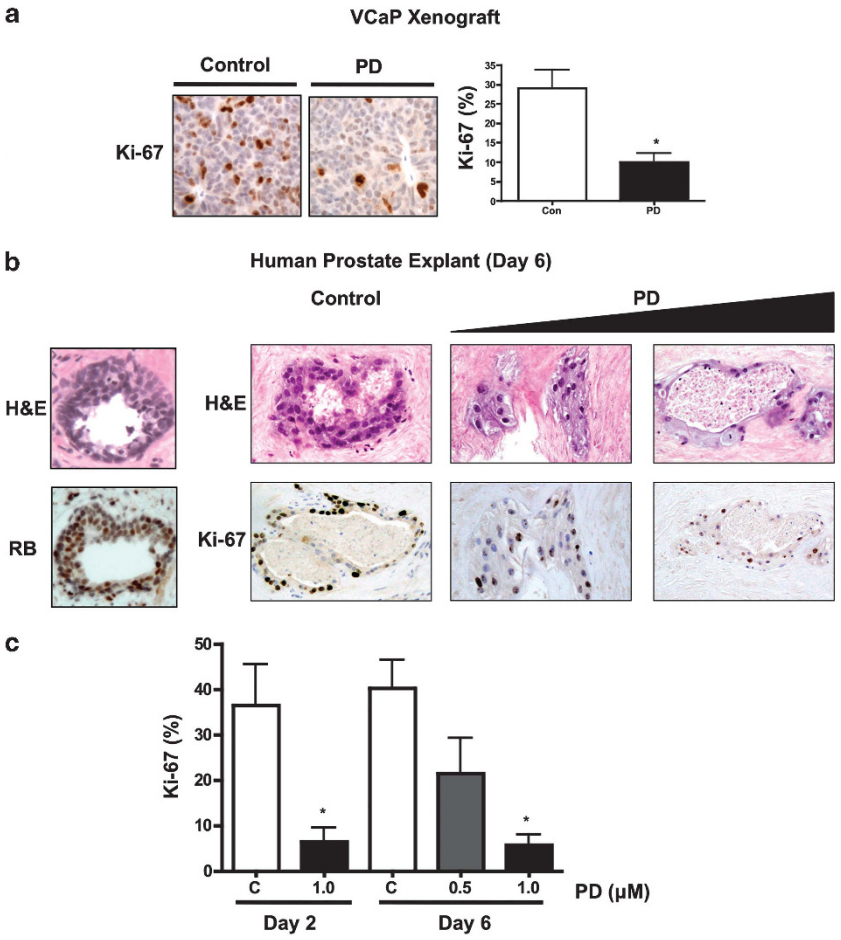

Figure 5. CDK4/6-specific inhibition suppresses proliferation of xenografts *in vivo* and primary human prostate tissue *ex vivo*. The efficacy of PD on PCa was determined by: (**a**) Proliferative Ki-67 marker analysis from VCaP xenografts, grown in SCID mice, treated with control ($n=4$ mice) or PD ($n=4$ mice). Mice were treated with 150 mg/kg of PD, as described detail in the Materials and methods section. Representative images (20 ×) are shown (left panel) and Aperio-based quantification of % Ki-67 (right panel). *Indicates a *P*-value <0.05. (**b**) Representative images from histo- and immunohistological analyses of human prostate tissues used for *ex vivo* tissue explant culture: left panel, images (20 ×) from hematoxylin and eosin (H&E) and RB-stained tissue before processing for culture; right panel, H&E images (20 ×, upper row) and Ki-67 images (20 ×, lower row) from *ex vivo* explant tissues treated 6 days with control (left column) and 0.5 (middle column) or 1 μM PD (right column). (**c**) Aperio-based quantification of % Ki-67 from human explant tissues cultured *ex vivo* for a short (day 2) and long (day 6) treatment with control (C) or 0.5 or 1 μM PD. Bars represent (mean ± s.e.m.) from five patient specimens. *Indicates a *P*-value <0.05.

can be selectively targeted in an RB-dependent manner to limit proliferation and growth. Overall, these data suggest that PD can be developed as an adjuvant means to suppress tumor growth in RB-positive tumors.

DISCUSSION

Proliferation and growth are hallmark phenotypes in cancer and are frequently regulated by the activity of CDK4/6.[7] In the past, therapeutic targeting of CDK4/6 activity has been limited to pan-CDK inhibitors, often resulting in adverse effects.[6] Most notably, for PCa, the broad spectrum CDK inhibitor flavopiridol resulted in adverse effects and toxicity in phase I trials.[10,50] During phase II trials, the dosage was reduced, patients were removed because of unacceptable toxicity, and increased adverse effects were observed, indicating that drug toxicity was a contributing factor for the poor efficacy.[11] Recently, a selective CDK4/6 inhibitor (PD) has been developed with a more favorable phase I toxicity profile;[12,13] however, the impact of PD in PCa has not been explored. Herein, it was demonstrated that PD: (1) effectively limited proliferation of hormone-sensitive and CRPC cells *in vitro*, xenografts *in vivo* and primary human tumor tissues *ex vivo*; (2) cooperated with existing treatments to enhance therapeutic impact without negating standard ADT; and (3) was mechanistically dependent on the RB tumor suppressor, suggesting the necessity for precision medicine. These pre-clinical findings represent the most comprehensive assessment of PD as a viable and potential combination therapy for PCa.

The molecular target of PD is exceedingly clear, in that it is a highly selective CDK inhibitor with a well-documented potency for CDK4/6 (IC_{50} ~10–15 nM) as compared with CDK2 (IC_{50} >5 μM).[12] CDK4/6 activity serves a specialized role, to initiate proliferation of a number of diverse tumor types, through the phosphorylation and inactivation of the RB tumor-suppressor family.[38] In PCa, it has been shown that RB-directed CDK4 activity is important for androgen-dependent proliferation.[3] Importantly, PD-inhibited PCa proliferation/growth and RB phosphorylation at Ser780 (a known CDK4/6 phosphorylation site), providing additional evidence that the mechanism underpinning androgen-dependent proliferation is mediated by CDK4. Interestingly, CDK4 levels are maintained in PCa,[51] suggesting that CDK4 activity has a significant role in proliferation. Androgen withdrawal or stimulation does not alter

CDK4 protein levels, suggesting the possibility of targeting CDK4 in combination with standard ADT. Previous investigation revealed that androgen-stimulated CDK4 activity, in part, is regulated by mammalian target of rapamycin-dependent induction of D-type cyclins.[3] Thus, it would be of future interest to simultaneously target CDK4/6 and mammalian target of rapamycin. It has been recently shown that mammalian target of rapamycin inhibition confers sensitivity of PCa cell proliferation/growth to IR.[44] For patients with locally advanced PCa, addition of radiotherapy has become standard-of-care;[52] therefore, the ability of PD to cooperate with IR in the PCa models tested suggests that CDK4/6 inhibition alone or in combination with other treatments may be beneficial. Consistent with this, others have shown that PD may cooperate with radiation to suppress glioblastoma multiforme tumor cell growth.[32] However, additional studies are needed to reveal the full potential of PD in the context of locally advanced PCa. Overall, these data support a model wherein cyclin D1–CDK4/ are integral to PD-mediated cell cycle inhibition in PCa cells and suggest the feasibility for combinatorial therapy.

In accordance with a cyclin D1–CDK4/6-dependent pathway, PD modestly upregulated cyclin D1 protein levels in the LNCaP and LAPC4 cells. The mechanism of PD-mediated cyclin D1 induction is somewhat enigmatic as therapeutics that result in G1-arrest often exhibit increased cyclin D1 turnover.[40] Initially, it was thought that PD could be inducing a compensatory induction of cyclin D1 expression; however, the levels of cyclin D1 induction were not sufficient to override the PD-mediated cell cycle inhibition. Co-immunoprecipitation analysis of CDK4 indicated a modest increase in cyclin D1 association, suggesting a potential mechanism whereby PD may retain cyclin D1 and CDK4 into an inactive complex, thereby protecting cyclin D1 from turnover. A recent crystallographic study of cyclin D1/CDK4 indicated that CDK4 was in an inactive configuration that resembled other inactive CDKs or p19-inhibited CDK6.[53–56] Although speculative, PD could be inducing a similar inactive conformation of CDK4. Unfortunately, no structural analysis is available for cyclin D1/CDK4 in the presence of PD and low-resolution (3 Å) analysis of cyclin V/CDK6 with PD does not indicate an inactive conformation.[57] Nonetheless, based on the well-characterized ability of cyclin D1, independent of CDK4, to control AR activity, it was hypothesized that PD-induced cyclin D1 accumulation/ sequestration with CDK4 might limit the ability of cyclin D1 to hinder genomic AR activity resulting in a modest increase in AR target genes. Consistent with this, AR target gene expression (that is, *KLK3*/PSA) with PD was, in general, modestly increased in the absence or presence of DHT in cells that exhibited increased cyclin D1 (that is, LNCaP and LAPC4). Interestingly, PD exposure in VCaP cells did not show evidence of cyclin D1 accumulation; yet, AR target gene expression was modestly elevated but only in the absence of DHT, suggesting additional factors are involved. One factor may be CDK6 as it has been shown to interact with and enhance AR activity, independent of its kinase activity or association with cyclin D1.[58] In addition, CDK6 overexpression in LNCaP cells displayed increased *KLK3*/PSA expression as well as increased secreted PSA protein in the absence or presence of androgen. Alternatively, although phospho- or total extracellular signal-regulated kinase levels were unchanged by PD (Supplementary Figure 1D), it is possible that PD influences other non-genomic AR targets. Thus, it will be important to discern the broader implications of PD-induced cyclin D1 accumulation with regard to regulating AR activity. It will also be of interest to determine if cyclin D1 and/or CDK6 has any predictive value concerning the response to PD or the potential bypass to therapy in PCa.

As with many drug interventions, a major hurdle is to accurately assess the probability of treatment response or development of resistance. To this point, a number of potential mechanisms have been implicated that may circumvent PD action. For example, elevated cyclin E1 levels could confer downstream CDK2 activation, as has been suggested by gene expression profiling from a panel of PD-resistant ovarian cancer cells.[37] However, cyclin E1 protein levels in PCa cells after PD treatment did not reveal any induction, suggesting that other mechanisms may exist or longer-term treatments are needed to observe changes in potential mechanisms of PD resistance. It is generally held that the majority of CDK4/6 activity targets RB;[38] therefore, based on initial and continuing reports describing PD action, the most probable candidate to nullify the response to PD is RB loss. This study demonstrates that PD-mediated inhibition of PCa proliferation requires RB. Moreover, it was recently identified that disruption of RB is frequently observed in late-stage, human CRPC.[46] These observations suggest that RB disruption might predict the development of resistance to therapeutic agents that inhibit CDK4/6 activity. In addition to allelic loss, RB inactivation occurs through a host of mechanisms that retain RB protein but cripple its tumor-suppressor function.[38] Thus, it is hypothesized that tumors stratified based on gene expression 'signatures' indicative of functional RB could significantly impact therapeutic potential.[59] To this end, we tailored a robust cohort of genes using multiple model systems that reflect functional RB and have validated its application using gene expression data from human PCa and CRPC specimens.[39] Based on the knowledge that RB is generally inactivated during the transition to late-stage CRPC, it is postulated that early-stage PCa patients stratified according to functional RB would benefit from PD-mediated CDK4/6 inhibition.

In this study, a number of well-characterized CRPC cells maintained responsiveness to PD implying that a subset of patients with CRPC may have functional RB and could potentially benefit from targeted CDK4/6 inhibition. Therefore, understanding the timing and response to cell cycle therapy in CRPC is of the utmost importance as the vast majority of patients who succumb to disease have CRPC.[1] In addition, recent observations have shown that CRPC cells have developed unique alterations in cell cycle.[60] It will be of interest to determine if PD alters the 'rewired' cell cycle program in the CRPC setting. Finally, it was shown that CRPC cells were amenable to PD treatment when grown in conditions that mimic standard ADT. Therefore, it will be of interest to examine the combination of PD with more recently developed second-generation, hormone-based therapies[61] (for example, CYP17A1 inhibitors, abiraterone; or AR antagonists, MDV3100) or approved anti-mitotic chemotherapies[62] (for example, microtubule stabilizers, docetaxel) in the context of CRPC as well as hormone-sensitive PCa.

In summary, this study provides a compelling rationale for the use of CDK4/6-selective inhibitors, such as PD, in the treatment of PCa. These pre-clinical and mechanistic findings suggest that CDK4/6 inhibition may provide benefit for PCa patients through: (i) suppressed proliferation/growth in both hormone-sensitive and castration-resistant contexts, (ii) potential combinatorial therapy with IR or hormone-based therapeutics and (iii) approaches that use personalized medicine to evaluate functional RB status before treatment. Together, these studies not only highlight the clinical potential of PD to benefit PCa patient outcome, but also support a role for CDK4/6-specific inhibitors for the treatment of cancers that are dependent on the cyclin/CDK/RB-axis.

MATERIALS AND METHODS

Cell culture and treatments

Androgen-dependent PCa (LNCaP, LAPC4 and VCaP) and CRPC (LNCaP-shRB1, C4-2, 22Rv1 and PC3M) cells were maintained as previously described.[46,63] Unless otherwise indicated, cells ($1.8 \times 10^4/cm^2$) were plated 24 h in growth media using standard serum (5–10% fetal bovine serum) and then treated 24 h with 0.5 μM PD (Pfizer, New York, NY, USA) or 0.1% dimethylsulphoxide. C4-2 and 22Rv1 cells were treated as above, except cells were washed thrice in phosphate-buffered saline and plated in

phenol red-free growth media containing 5% charcoal-dextran-treated serum (HyClone, Thermo Fisher Scientific, Pittsburgh, PA, USA) to mimic castrate conditions.

Flow cytometry

PCa cells were challenged, as described above, and both adherent and non-adherent cells were harvested, gently re-suspended in 100% ethanol, and fixed overnight at $-20\,^{\circ}$C. Proliferation was measured by bivariate flow cytometry using a 2-h pulse-label of BrdU (Amersham (GE Healthcare Life Sciences), Pittsburgh, PA, USA, RPN201) before harvest and cell cycle position using propidium iodide staining, as described.[44] A Coulter Epics XL Flow Cytometer (Beckman Coulter, Indianapolis, IN, USA) was used to capture 20 000 BrdU/propidium iodide or 15 000 propidium iodide events for proliferation and cell cycle position, respectively. FlowJo software (TreeStar, Ashland, OR, USA) was used to gate for percent BrdU incorporation or cell cycle position using the cell cycle algorithm.

Immunoblotting

Control- and PD-treated cells, described above, were harvested to evaluate protein expression and interaction of cell cycle components. Briefly, total protein (30 μg) was separated by standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride and immunoblotted overnight at 4 °C. Antibodies and dilutions (1:1000) used are: AR (SC-816), CDK4 (SC-601); cyclins: A (SC-596), D1 (Neomarkers, Fremont, CA, USA, AB3) and E (SC-198); ppRB-pS780 (Cell Signaling, Beverly, MA, USA, 9307), Ran (BD Biosciences, San Jose, CA, USA, 610340). Immunoblots were quantified using a LI-COR Odyssey (Li-Cor, Lincoln, NE, USA).

Gene expression

To assess AR activity, cells were plated 72 h in phenol red-free growth media containing 5% charcoal dextran-treated serum then treated 24 h with various combinations of DHT (1 nM), Csdx (10 μM) and PD (0.5 μM). RNA was isolated and complementary DNA generated using the Trizol and Superscript methods, respectively. Quantitative PCR was performed for *KLK3*/PSA, *TMPRSS2* and *KLK2* and normalized to *glyceraldehyde 3-phosphate dehydrogenase* using published methodologies.[43]

Irradiation, clonogenic assay

Combinatorial therapy was determined by plating cells (day 0) in growth media containing standard serum conditions and treated with PD (day 1), described above, then 2 Gy of IR using a Pantak X-RAD orthovoltage X-ray irradiator (calibrated daily using a Victoreen dosimeter, Victoreen, Cleveland, OH, USA). Following treatment, growth media (lacking PD) was replaced (days 2 and 5) and cells were counted (days 3, 5 and 7) by a hemacytometer using the Trypan blue exclusion method. Cell doubling times were calculated as described.[64] Briefly, doubling time in days $= ((\log 2) \times (t_f - t_i))/(\log (q_f/q_i))$ where $t_i =$ initial time, $t_f =$ final time, $q_i =$ cell number at t_i, $q_f =$ cell number at t_f. Clonogenic survival was determined by plating LNCaP cells at varying densities in poly-L-lysine coated 50 ml flasks. After 24 h, cells were treated with 0.5 μM PD and IR or IR alone (at indicated IR doses). Cells were allowed to grow for 14 days, colonies were fixed in 100% cold ethanol and stained with 1% crystal violet in 2.5% acetic acid.

Xenografts

Xenograft studies were performed in accordance with NIH Guidelines and animal protocols were approved by Thomas Jefferson University. VCaP cells (1.5×10^6) were combined 1:1 with Matrigel (BD Biosciences, 354234) and inoculated subcutaneously into the flanks of 6 weeks, intact-male mice (NCI-Frederick, Frederick, MD, USA; NOD.SCID/NCr, 01N31). Tumors were measured with calipers and matched for an average size of 700 mm^3, oral gavage was initiated (day 0) with PD (150 mg/kg, $n = 4$ mice) or control (sodium lactate pH 8.0, $n = 4$ mice) and re-dosed twice (days 2 and 4). Tumors from control- and PD-treated mice were harvested (day 5), 24 h after the final treatment. Tumors were processed and sections stained for Ki-67 (1:250; Invitrogen, Carlsbad, CA, USA; 18-0191Z) using described methodology.[65]

Primary human prostate tumor explants

Primary tumor tissue was obtained from patients diagnosed with PCa who underwent radical prostatectomy at Thomas Jefferson University Hospital in accordance with Institutional Review Board standards and in compliance with federal regulations governing research on de-identified specimens and/or clinical data (45 CFR 46.102(f)). Tumors were dissected by a clinical pathologist under sterile conditions and collected in processing media: improved minimum essential medium (5% fetal bovine serum, 0.01 mg/ml insulin (Invitrogen, 12585-014), 30 μM hydrocortisone (Sigma-Aldrich, St Louis, MO, USA, H-0888) and penicillin/streptomycin). Tissue was minced into $\sim 1\,mm^3$ pieces and placed (2–3 pieces per well) in a 24-well plate on pre-soaked, 1 cm^3 dental sponges (Novartis Animal Health, Greensboro, NC, USA, Vetspon) containing 0.5 ml of processing media with either 0.5 or 1 μM PD. Treatments and controls were refreshed every 48 h and explants were harvested at early and late time points (days 2 and 6, respectively) for histological assessment. Control- and PD-treated explants ($n = 5$ patients) with evidence of glandular epithelial tissue, as determined by a clinical pathologist, were stained for Ki-67 using clinically approved protocols by the Thomas Jefferson University Hospital. Xenograft and explant stained slides were quantified for percent Ki-67 using an AperioScope AT and Spectrum software using the nuclear staining algorithm (Aperio Technologies, Vista, CA, USA).

ABBREVIATIONS

AR, androgen receptor; CDK, cyclin-dependent kinase; CRPC, castration-resistant prostate cancer; Csdx, casodex; DHT, dihydrotestosterone; KLK3/PSA, kallikrein-related peptidase 3/prostate-specific antigen; PCa, prostate cancer; PD, PD-0332991; RB, retinoblastoma.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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