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Cyclin D1 repressor domain mediates proliferation and survival in prostate cancer

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Abstract

Regulation of the androgen receptor (AR) is critical to prostate cancer (PCa) development; therefore, AR is the first line therapeutic target for disseminated tumors. Cell cycle dependent accumulation of cyclin D1 negatively modulates the transcriptional regulation of the AR through discrete, CDK4-independent mechanisms. The transcriptional co-repressor function of cyclin D1 resides within a defined motif termed ther repressor domain (RD), and it was hypothesized that this motif could be utilized as a platform to develop new strategies for blocking AR function. Here, we demonstrate that expression of the RD peptide is sufficient to disrupt AR transcriptional activation of multiple, prostate-specific AR target genes. Importantly, these actions are sufficient to specifically inhibit S-phase progression in AR-positive PCa cells, but not in AR-negative cells or tested AR-positive cells of other lineages. As expected, impaired cell cycle progression resulted in a suppression of cell doubling. Additionally, cell death was observed in AR-positive cells that maintain androgen dependence and in a subset of castrate-resistant PCa cells, dependent on Akt activation status. Lastly, the ability of RD to cooperate with existing hormone therapies was

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examined, which revealed that RD enhanced the cellular response to an AR antagonist. Together, these data demonstrate that RD is sufficient to disrupt AR-dependent transcriptional and proliferative responses in PCa, and can enhance efficacy of AR antagonists, thus establishing the impetus for development of RD-based mimetics.

Keywords

Androgen Receptor; Testosterone; Prostatic Adenocarcinoma; Cell Cycle

Introduction

Prostate cancer (PCa) is the second leading cause of male cancer mortality in the US (Jemal *et al.*, 2007). Development, progression, and maintenance of disease are dependent upon the action of the androgen receptor (AR), a ligand activated transcription factor Upon ligand (testosterone or dihidydrotestosterone, DHT) binding, AR is released from inhibitory heat-shock proteins, translocates to the nucleus, and binds to DNA at androgen response elements within the regulatory regions of target genes (Feldman and Feldman, 2001). Therein, AR induces a program of gene transcription that results in diverse activities dependent on cellular context (Balk and Knudsen, 2008; Burnstein, 2005; Pienta and Bradley, 2006; Shand and Gelmann, 2006). Prostate specific antigen (PSA) is the best characterized AR target gene whose expression is utilized as a biomarker for disease progression (Lilja *et al.*, 2008). Proliferation of PCa cells require a functional AR; therefore, first line treatment for disseminated disease targets the AR pathway (Salesi *et al.*, 2005). Advanced PCa is treated with hormone therapy (Sharifi *et al.*, 2005) as accomplished by: (1) pharmacological or surgical blockade of androgen production (androgen ablation), (2) use of direct AR antagonists that compete for DHT binding (Petrylak, 2005) and induce co-repressor recruitment (Hadaschik and Gleave, 2007), or (3) a combination of both regimens (combined androgen blockade). These therapies all impinge on the AR carboxy-terminal ligand-binding domain (LBD) as a mechanism to ablate AR action. The validity of AR as an appropriate biochemical target is apparent, as these regimens initiate cell cycle arrest and/or cell death in vivo, resulting in tumor regression (Agus *et al.*, 1999). These outcomes are almost invariably accompanied by concomitant PSA reduction (Lilja *et al.*, 2008), indicating that AR can be effectively inhibited through carboxy-terminal directed therapies. Unfortunately, relapse occurs within a median time of 24–36 months (Feldman and Feldman, 2001). This “castration-resistant” phenotype represents an incurable stage of disease (Hadaschik and Gleave, 2007; Petrylak, 2005), and there is a significant need to develop new modes of therapeutic intervention to treat castration-resistant PCa (CRPC) (Petrylak, 2005). Remarkably, it is evident that these androgen independent tumors typically arise as a result of restored AR activity (Burnstein, 2005; Feldman and Feldman, 2001; Pienta and Bradley, 2006), as noted by the observations that visible tumor recurrence is almost always preceded by rising PSA, and that AR is active in CRPC (Lilja *et al.*, 2008). Mechanisms that restore AR activity include gain-of-function mutations, AR amplification, ligand-independent AR activation, deregulation of AR co-factors, and intracrine androgen production (Chmelar *et al.*, 2007; Feldman and Feldman, 2001; Locke *et al.*, 2008;

Montgomery *et al.*, 2008; Stanbrough *et al.*, 2006). Thus, targeting the AR in CRPC remains a major goal of current investigations.

Mechanisms that control AR-dependent cell cycle progression are increasingly well understood (Balk and Knudsen, 2008). In part, androgen-dependent activation of mTOR induces translation of cyclin D1 (Xu *et al.*, 2006), which can bind to cyclin dependent kinase 4 (CDK4) to initiate cell cycle progression (Knudsen *et al.*, 1998; Matsushime *et al.*, 1992; Quelle *et al.*, 1993). However, only a subset of cyclin D1 is typically associated with CDK4 (James *et al.*, 2008), and cyclin D1 has multiple CDK4-independent functions that control transcription factor action (Bienvenu *et al.*, 2001; Cheng *et al.*, 1998; Coqueret, 2002; Ewen and Lamb, 2004; Ewen *et al.*, 1993; Fu *et al.*, 2004; Ganter *et al.*, 1998; Inoue and Sherr, 1998; Kato *et al.*, 1998; Parry *et al.*, 1999; Skapek *et al.*, 1995). In prostatic cells, accumulated cyclin D1 binds directly to the AR N- terminal domain and inhibits ligand-dependent AR activity through well-defined mechanisms (Burd *et al.*, 2005; Knudsen *et al.*, 1999; Petre *et al.*, 2002; Reutens *et al.*, 2001). First, cyclin D1 binds to the AR FxxLF motif and therein prevents the formation of conformational changes (intra-molecular N-C interactions) (Burd *et al.*, 2005) required for AR stabilization on chromatin (Li *et al.*, 2006; Wong *et al.*, 1993). Second, the ability of cyclin D1 to bind histone deacetylase 3 (HDAC3) is required for AR inhibition (Li *et al.*, 2002; Lin *et al.*, 2002; Petre *et al.*, 2002). These activities of cyclin D1 map to a repressor domain (RD) within the protein which is distinct from the CDK4-regulatory motif (Petre-Draviam *et al.*, 2005). Thus, it was hypothesized that segregation of the cyclin D1 functional domains may provide a means to develop new strategies in targeting AR activity in PCa.

Herein, the discrete influence of cyclin D1 RD function was assessed in androgen-dependent PCa and CRPC cells. This study demonstrates that RD is sufficient to suppress ligand-dependent AR transcriptional activity. In AR-positive, androgen-dependent PCa cells. RD inhibited cell cycle progression and induced cell death. By contrast, no cytostatic or cytotoxic effects were observed in AR-negative PCa cells or in tested AR-positive cells of other lineages. RD also elicited cell death in a subset of CRPC cells, dependent on Akt status. Lastly, RD sensitized cells to the therapeutic AR antagonist bicalutamide (Casodex). Combined, these studies demonstrate that the AR-inhibitory motif of cyclin D1 is sufficient to prevent AR activity, specifically inhibit PCa cell growth, and enhance the efficacy of existing therapeutics. These studies provide the impetus for the development of RD mimetics for targeted therapeutic intervention.

Materials and Methods

Cell culture and treatment

LNCaP, C4-2, CV1, MCF7, 22Rv1, PC3, and PC12(Q10) cells were cultured as previously described (Petre *et al.*, 2002; Petre-Draviam *et al.*, 2003; Walcott and Merry, 2002). For steroid-free conditions charcoal-dextran-treated FBS (CDT) (Hyclone Laboratories) was utilized. LY290042 was obtained from Calbiochem, and used at 3.125μM.

Transfection and transcription assays

CV1 cells were transfected as previously described (Chen and Okayama, 1987; Knudsen *et al.*, 1999). Following transfection, cells were treated with either 1nM DHT or 0.1% ethanol vehicle. Relative luciferase activity was assessed using the Promega luciferase assay kit and Galacto-Star reagent (Applied Biosystems) was used to detect β-galactosidase activity (transfection normalization).

Expression vectors

Most plasmids were previously described (Petre *et al.*, 2002; Petre-Draviam *et al.*, 2003; Petre-Draviam *et al.*, 2005). RD-Flag construct was generated by removing RD from the GST-RD construct (Petre-Draviam *et al.*, 2005) via BamH1 digestion. The insert was then ligated into the BamHI site of the pcDNA-Flag plasmid and screened for orientation. Adenoviral GFP-RD was generated utilizing the AD-Easy system (Qbiogene, Irvine, CA, USA). Briefly, GFP-RD (Petre-Draviam *et al.*, 2005) was excised with NheI and XbaI and inserted into the XbaI site of pShuttle-CMV. After recombination with Ad-easy1, adenovirus was generated and purified as per manufacturer's protocol.

Immunoblot Analysis

SDS-PAGE was performed as previously described (Knudsen *et al.*, 1998), and immunoblotting achieved using antisera against: GFP (sc-9996), FLAG (sc-7787), AR (sc-816), Lamin B (sc-6217), PARP (Cell Signaling, 9542), phosphorylated Akt (Cell Signaling, 193H12), total Akt (Cell Signaling, 9272), cdk4 (sc-601) or β-tubulin (sc-5274). Signal was visualized using either the Odyssey IR Imaging System (LI-COR, Biosciences) or chemiluminescence (Perkin Elmer, Boston, MA).

Cell Proliferation Assays

To monitor BrdU incorporation, cells were cultured on coverslips and transfected with H_2B-GFP (transfection marker) and either vector (pCDNA3.1), cyclin D1, DRD or RD-Flag. BrdU staining and counting was performed as described (Knudsen *et al.*, 1998; Petre-Draviam *et al.*, 2005). For growth assays, indicated cells were seeded into poly-L-lysine coated 6-well dishes (except for PC3 cells, which are strongly adherent), infected after 24h with virus encoding either GFP or RD-GFP, and cell number counted using trypan blue exclusion and a hemocytometer. Total cell number was determined from at least three independent experiments each with three biological replicates per time point. For flow cytometry, parallel studies using these conditions were used, but at 96h post-infection, cells were fixed in ethanol, stained with propidium iodide (0.2μg/mL) and subjected to flow cytometry. Samples were quantified on a BD FACS-Calibur using BD CellQuestPro software (BD Biosciences), and analyzed using FlowJo software (Tree Star Inc.).

Reverse transcriptase PCR

LNCaP cells were seeded on poly-L-lysine coated 6cm dishes, infected 24h later, and the media was replaced 24h post-infection. RNA was harvested 48h post-infection using Trizol reagent (Life Technologies, Gaithersburg, MD, USA) as per manufacturer's protocols.

Amplification of TMPRSS2, GAPDH and PSA was performed as previously described (Hess-Wilson *et al.*, 2006; Wilson *et al.*, 2005).

Results

Cyclin D1 repressor domain inhibits AR transcriptional activity *in vitro*

The region of cyclin D1 required for AR association and repression (RD, repressor domain) has been previously described (Figure 1A, left panel) (Petre-Draviam *et al.*, 2005). To segregate this activity away from the cell cycle regulatory functions of cyclin D1, RD was fused to an epitope (either GFP; green fluorescent protein, or 3× Flag), and expression validated after transient transfection into LNCaP (AR-positive, androgen-dependent) PCa cells. As shown, the peptide of expected molecular weight (Flag-RD: 18 kDa) was detected. H2B-GFP was used as a control for transfection efficiency (Figure 1A, right panel).

Although RD is required for cyclin D1-mediated suppression of AR activity (Petre-Draviam *et al.*, 2005), sufficiency of this domain to inhibit AR had not been determined. To test this, transient transfection assays were performed wherein CV1 cells (spontaneously immortalized epithelial cells lacking endogenous AR) (Rundlett *et al.*, 1990) were cotransfected in the absence of hormone with plasmids encoding either empty vector, wild type cyclin D1, an RD-deficient mutant (cyclin D1-ΔRD), or RD alone (Flag-RD) and the ARR2-Luc reporter, pSG5-AR, and CMV-β-galactosidase (as an internal transfection control). Following transfection, cells were stimulated with 1nM 5-α-dihydrotestosterone (DHT) or 0.1% ethanol (vehicle control, and relative luciferase activity determined. As shown in Figure 1B, cyclin D1 inhibited the ligand-dependent transactivation function of AR by 56%, while cyclin D1-ΔRD had no significant effect. These findings are consistent with previous reports wherein RD was required for cyclin D1-mediated AR inhibition (Petre-Draviam *et al.*, 2005). Additionally, RD expression significantly reduced AR activity (40% suppression), demonstrating for the first time that RD is sufficient to inhibit AR function in transient transfection assays.

To validate these observations using endogenous AR target genes, LNCaP cells were infected in the presence of androgen with an adenovirus encoding GFP, wild type cyclin D1 (positive control), or GFP-RD. As shown, full-length cyclin D1 reduced endogenous PSA expression by 61% relative to the GFP control (set to 1), while RD inhibited PSA mRNA production by 32% (Figure 2A). A second AR target gene of clinical importance, TMPRSS2 (Mehra *et al.*, 2007a; Mehra *et al.*, 2007b; Perner *et al.*, 2007; Tomlins *et al.*, 2007), was analyzed to determine the specificity of RD-mediated inhibition. Similar to PSA, both cyclin D1 and RD reduced TMPRSS2 mRNA production (by 68% and 55%, respectively, Figure 2B). There was no detectable change in AR protein levels between mock, GFP, or RD infected cells (Figure 2C), indicating that the observed changes in transcriptional regulation are not due to a reduction in AR levels. Together, these data demonstrate that RD is sufficient to reduce AR transcriptional regulation of clinically relevant AR target genes.

RD inhibits cell cycle progression in AR-positive PCa cells

Since PCa cell proliferation is dependent upon AR, the capacity of RD to block cell cycle progression was determined after transient transfection. As shown, neither full-length cyclin D1 nor RD impacted S-phase progression in CV1 (AR-negative) cells, compared to control (Figure 3A). Validation of RD expression is provided in the lower panel, with GFP provided as a control for transfection efficiency. By contrast, RD inhibited cell cycle progression in a dose-dependent manner in LNCaP (Figure 3B), with full-length cyclin D1 as positive control and cyclin D1-ΔRD as negative control. These data demonstrate that in PCa cells dependent upon AR for cell cycle progression, RD is sufficient to inhibit S-phase progression. To determine if the effect of RD is reliant on functional AR in the context of PCa, AR-negative, androgen-independent PC3 cells (Kaighn *et al.*, 1979) were analyzed in parallel. Similar to the results observed with CV1 cells, neither cyclin D1 nor derivatives thereof induced a detectable effect on cell cycle progression (Figure 3C), further suggesting that the cell cycle inhibitory function of RD is dependent on AR. Taken together, these data demonstrate that RD of cyclin D1 is sufficient to selectively attenuate cell cycle progression in AR-positive PCa cells.

RD is both cytostatic and cytotoxic in AR-positive, androgen-dependent PCa cells

To determine if the observed effects resulted in durable changes in proliferation, LNCaP (Fig. 4A, left panel) or PC3 (right panel) cells were infected with adenovirus encoding either GFP-RD or GFP alone (>95% infection efficiency), and cell number was determined at the indicated time points. In parallel, immunoblots were performed to assess relative expression of GFP-RD and GFP (bottom panels). As shown, in Figure 4A (left panel), LNCaP cells infected with the GFP-RD adenovirus demonstrated substantially slower growth kinetics when compared to GFP infected controls, indicating that RD not only slowed cell cycle progression but also blocked cell proliferation. At the later timepoint, LNCaP cells incurred a decrease in cell number, suggesting the possibility of cell death. By contrast, no measurable change in PC3 cell doubling was noted under any condition.

To address the impact on cell death, flow cytometry was performed in parallel studies. Representative cell cycle traces are shown (Figure 4B left panel), and the sub-G1 population was quantified (middle panel). In GFP-RD infected LNCaP cells, a significant increase in the sub-G1 population indicated that RD induced cell death. This premise was further supported by concurrent analyses of PARP (Poly(ADP-ribose) polymerase) cleavage, wherein RD induced this event (Figure 4C compare lanes 3 and 5). Cisplatin treated cells served as positive control (lane 6). To determine if the observed affects were due to the tumor-derived AR mutant harbored by LNCaP cells (T877A), LAPC4 (wild-type AR) PCa cells were examined. As shown, RD inhibited LAPC4 proliferation (Figure 4C, left panel), and induced death as determined by PARP cleavage (right bottom panel). Specificity was addressed using AR-negative PC3 cells, wherein there was no change in cell cycle distribution, sub-G1 (<2N) accumulation, or PARP cleavage were detected (Figure 4D). Similarly, whereas GFP-RD accumulation was readily observed in CV1 (4E), MCF7 (AR-positive breast cancer) (Birrell *et al.*, 1995) (4F), or PC12(Q10) (rat pheochromocytoma with tet-inducible human AR) cells (Walcott and Merry, 2002) (4G), no PARP cleavage was

detected (left panels). In sum, these data demonstrate that RD-induced cell death appears specific to AR-positive, androgen-dependent PCa.

The cytotoxic effects of RD in CRPC are sensitive to PI3K/Akt status

Given the selectivity of the cytotoxic response to AR-positive cells of prostatic origin, parallel studies were performed in CRPC cells (22Rv1 and C4-2) cells. To determine the impact of RD in this subclass of cells, parallel studies were performed. Accumulation of RD (Figure 5A, left panel) in 22Rv1 elicited a cytotoxic effect, as demonstrated by PARP cleavage (right panel, lanes 2 and 3). However, RD expression in C4-2 cells (Figure 5B, left panel) failed to induce PARP cleavage (right panel), thus indicating that not all CRPC cells maintain the capacity to undergo RD-mediated cell death. Subsequent proliferation assays revealed that RD attenuated cell growth in C4-2 cells when compared to control (Figure 5C) with no detectable change in sub-G1 content (data not shown). These data indicate that CRPC cells are differentially responsive to the cytotoxic (but not cytostatic) effects of RD.

Given the disparity in CRPC response, the underlying mechanisms were investigated. It is known that AR in C4-2 is more stable when compared to the parental line (LNCaP) (Gregory *et al.*, 2001), but alteration in AR levels are unlikely to underlie the observed resistance to RD-induced cell death, as further elevation in RD expression failed to alter cellular outcome (data not shown). By contrast, it is known that although LNCaP cells lack functional PTEN and therefore harbor activated Akt (Davies *et al.*, 1999), the C4-2 derivative exhibits a marked enhancement of Akt activity as compared to the parental cell line (Ghosh *et al.*, 2005). This observation was confirmed in the present study wherein the ratio of phosphorylated (active) Akt to total Akt was significantly higher in C4-2 versus parental LNCaP (Figure 5D, compare lanes 1 and 2). Given the importance of Akt in modulating cell survival, its role in resistance to RD-mediated cell death was challenged by pre-treatment of C4-2 cells with the PI3K inhibitor LY294002. LY294002 pre-treatment alone resulted in modest PARP cleavage (Figure 5E lane 1), consistent with previous reports that this compound can elicit moderate cell death (Gottschalk *et al.*, 2005). Importantly, LY294002 treated cells were sensitized to RD-induced PARP cleavage (Figure 5E lane 3), whereas no induction of PARP cleavage was observed in control infected cells (Figure 5E lanes 4,5). Parallel studies with the MEK inhibitor UO126 failed to sensitize cells to RD (data not shown). Combined, these data indicate that while RD can induce a cytostatic response in all AR-positive PCa cells tested, the cytotoxic effects of RD in CRPC are sensitive to PI3K/Akt activation status.

RD enhances the effect of currently used AR targeted therapies

Since RD induced both cytostatic and cytotoxic response, the potential of RD as a novel adjuvant therapeutic was assessed in combination with androgen ablation or AR antagonists. After androgen withdrawal in LNCaP cells, BrdU incorporation was minimal (Figure 6A left striped bar), verifying the reliance of these cells on androgen for cellular proliferation. Prior introduction of RD further reduced S-phase progression (Figure 6A), thus indicating that RD can cooperate with androgen ablative strategies. To determine the effect of RD in combination with bicalutamide, experiments were performed in parallel using cells cultured in the presence of androgen. As expected, bicalutamide alone reduced BrdU incorporation

by 52% (Figure 6B, compare white bars) (Maucher and von Angerer, 1993), and RD alone significantly reduced BrdU incorporation by 37% (Figure 6B left black bar), in the presence of androgen. Cooperative effects were observed when these treatments were combined (26% of control). Further studies were performed to assess dose response, wherein it was observed that RD sensitized cells to low dose bicalutimide (Figure 6C). Taken together, these data demonstrate that RD acts to enhance the cellular response to existing hormone therapy regimens

Discussion

It has previously been established that cyclin D1 modulates nuclear receptor function independent of its cell cycle regulatory role. Here, we show that the transcriptional co-repressor activity can be segregated from the cyclin regulatory region, and that this motif (RD) is sufficient to suppress AR activity. Decreased AR activity was observed in both transient assays and through analysis of endogenous AR target gene expression. Consistent with the observation that AR activity is required for PCa cell growth, RD inhibited PCa cell proliferation *in vitro* in AR-positive cells, and surprisingly induced death in cells that retain androgen dependence. In CRPC, it was revealed that the cytotoxic response is sensitive to PI3K/Akt status. No effect was seen in AR-negative PCa cells, or non-prostatic cells which harbor AR, showing specificity. Lastly, RD sensitized cells to the inhibitory action of clinically relevant AR antagonists (bicalutamide) to induce a significant decrease in cell cycling. These data suggest that mimetics of RD function could serve as a novel PCa therapeutic and/or in combination with current therapeutic regimens.

Functional motifs of cyclin D1 that are needed for cell cycle and CDK4 regulation have been well defined (Coqueret, 2002; Knudsen, 2006) and remarkably, these functions appear to be distinct from the region of cyclin D1 that exerts transcriptional repression (Fu *et al.*, 2005; Knudsen *et al.*, 1999; Lin *et al.*, 2002; Wang *et al.*, 2003). The present data demonstrate for the first time that the RD motif is sufficient to impede AR activity and induce cell cycle arrest and/or cell death, thus indicating that cdk regulation is not required for this function. Not all transcriptional regulatory actions of cyclin D1 occur through this motif. For example, the ability of cyclin D1 to modulate the estrogen receptor occurs through the nuclear receptor interaction motif (LxxLL, a.a. 254-259) (Neuman *et al.*, 1997; Zwijsen *et al.*, 1998) which is dispensable for AR regulation (Petre *et al.*, 2002). By contrast, transcriptional repression of AR, PPARγ, and DMP1 requires the RD (Fu *et al.*, 2005; Inoue and Sherr, 1998; Petre-Draviam *et al.*, 2005). This domain is known to associate with HDACs, and indeed, cyclin D1 mediated suppression of AR (Petre *et al.*, 2002), PPARγ (Li *et al.*, 2002), and TR (Lin *et al.*, 2002) can be partially reversed by HDAC inhibitors. A secondary function of RD is manifest through interaction of RD with the AR FxxLF domain, causing blockade of ligand-induced confromational changes required for transactivation. While the present data show that RD is sufficient to inhibit AR activity and induce cell cycle arrest and/or cell death, it cannot be formally ruled out that selected RD effects are mediated by interaction with other transcription factors. Cellular effects do appear to require AR, as AR independent cells are refractory to RD action. It is unlikely that PPARγ plays a part, as RD refractory PC3 cells express high levels of this receptor (Subbarayan *et al.*, 2005). It is equally unlikely that the potential interaction

between cyclin D1 and DMP1 results in the observed cellular consequence, as cyclin D1 suppresses the cell cycle inhibitory capacity of DMP1 (Inoue and Sherr, 1998). However, TR can induce LNCaP proliferation (Esquenet *et al.*, 1995), and thus the contribution of TR to the observed effects could be of interest. Although future studies will likely reveal additional transcription factors that interact with RD, the present data are strongly suggestive that AR is the critical determinant of the observed cellular response.

The demonstrated capacity of RD to inhibit AR transcriptional activity is of importance, as both early PCa and CRPC cells remain dependent on AR signaling for survival and proliferation (Burnstein, 2005) and AR knockdown inhibits growth of both PCa and CRPC cells *in vivo* (Cheng *et al.*, 2006). Herein, RD not only impinged on AR transcriptional activity and inhibited proliferation of AR-positive PCa cells but also induced cell death, dependent on PI3K/Akt status. It is well established that Akt is a pro-survival factor (Franke *et al.*, 1997), and PTEN inactivation (upstream negative regulator of PI3K/Akt) has been implicated in the transition to CRPC (Shen and Abate-Shen, 2007). Loss of PTEN is a frequent event in PCa, and has been correlated with poor prognosis (McMenamin *et al.*, 1999). The present observation that CRPC with Akt hyperactivity acquire resistance to the cytotoxic effects of RD is of translational importance, and provides the foundation for future studies aimed at coordinated targeting of AR N/C interaction and Akt inhibition.

Previous studies using “decoy” peptides derived from the AR N-terminus resulted in phenotypes of some similarity to RD. These decoy peptides (encoding a.a. 1–558 of AR) acting in part by blocking N to C interaction, suppressed cell cycle progression of AR-positive, androgen-dependent cells *in vitro* (Minamiguchi *et al.*, 2004), and *in vivo*, and an increase in apoptotic cell death was observed (Quayle *et al.*, 2007a; Quayle *et al.*, 2007b). Thus, the ability of peptides that bind the AR N-terminus to induce cell death is not without precedent. As observed in unbiased analysis (Comstock et al, in preparation), only a subset of AR target genes are affected by cyclin D1, and interrogation of these should provide clues as to the basis of cell death. Additionally, recently published microarray data demonstrate that D-type cyclins exert distinct transcriptional regulation in liver (Mullany *et al.*, 2008), suggesting that the effects of RD of cyclin D1 are likely different than those of RD of cyclin D3. Lastly, it was previously demonstrated that cells lacking cyclin D1 have increased mitochondrial size and function (Wang *et al.*, 2006), thus leading to the as-of-yet unchallenged possibility that these cyclin D1 actions may in part underlie the effects seen in androgen-dependent PCa cells. As specific induction of tumor cell death is the ultimate goal of cancer therapy, it will be critical to discern the mechanism by which RD elicits the cytotoxic response.

As current therapies for disseminated PCa are only transiently effective, determining means by which to improve upon current treatment strategies is of paramount importance. As shown, RD enhanced the cytostatic response to androgen withdrawal, but was more effective as a cooperating factor for bicalutamide. This disparity in the magnitude of cooperation is not unexpected, as AR is deprived of ligand under conditions of androgen ablation, and cyclin D1 impinges predominantly on ligand-bound AR. However, bicalutamide acts through competing for binding to the AR LBD, and induces co-repressor recruitment (Zhu *et al.*, 2006). Thus the capacity of RD to interact with the AR N-terminal

FxxLF motif and thereby block the N to C terminal interactions likely underlies its ability to enhance the action of bicalutamide. Thus, the present studies provide proof of principle evidence that simultaneous targeting of multiple AR functional domains may have therapeutic benefit.

As the present study indicates that mimetics of RD action could be of therapeutic benefit, the concept of rational drug design must be addressed. Important precedent exists in the design of imatinib (Sharifi and Steinman, 2002), and nutlins (Hu *et al.*, 2007). A significant hurdle with regard to RD as a therapeutic includes the lack of a crystal structure for the cyclin D1 protein (or domains thereof). RD is proposed to form a five alpha helix structure (Petre-Draviam *et al.*, 2005); importantly, this domain is conserved in cyclin D3 (Olshavsky *et al.*, 2007), which also retains the ability to bind AR and block N to C interaction, but harbors distinct transcriptional modulatory activities. Future development of structural investigations will assist in the long-term goal of generating RD mimetics.

In summary, the studies herein demonstrate that the RD motif induced AR-specific effects in PCa cells, is sufficient to inhibit AR activity, which results in both cytostatic and cytotoxic responses. The capacity of RD to cooperate with and enhance the efficacy of a currently utilized AR antagonist further advocates the importance of the findings, and provide evidence that concurrent targeting of both N and C terminal AR functions may be of clinical benefit. Future studies will determine if this small peptide can be translated into a clinically relevant treatment for PCa.

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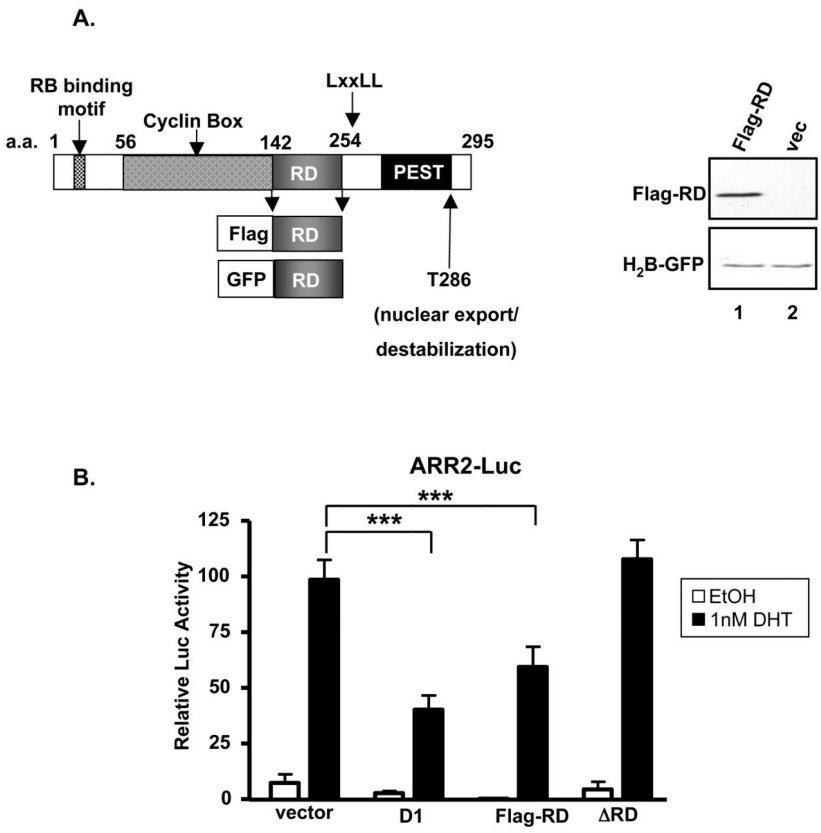


Figure 1. Cyclin D1 repressor domain is sufficient to inhibit AR transcription in vitro

A) Left: Schematic representing cyclin D1 domains and RD constructs. Amino acid positions are shown. Right: LNCaP cells were transfected with expression plasmids encoding H2B-GFP (as a control for transfection efficiency), RD-flag or vector control. Post-transfection, immunoblots were performed for FLAG (bottom panel) or GFP (top panel). **B)** CV1 cells in six well dishes in the absence of steroid hormones and transfected with a total of 4 μg of DNA (0.25 μg CMV-b-galactosidase, 0.25 μg of ARR2-Luc, 0.25 μg of pSG5-AR, and either pcDNA vector, 0.25 μg, 0.5 μg, 1.0 μg RD, 1.0 μg D1, or 1.0 μg of ΔRD. Post-transfection, cells were treated with 1nM DHT or 0.1% ethanol vehicle, and relative luciferase activity determined. Normalized AR activity in the presence of ligand was set to 100 with the average fold induction by DHT presented. Error bars represent standard deviation. Statistical analyses were performed using ANOVA followed by a Newman-Kuels multiple comparison post test. ***$p<0.001$ Data shown reflects at least 9 independent data points.

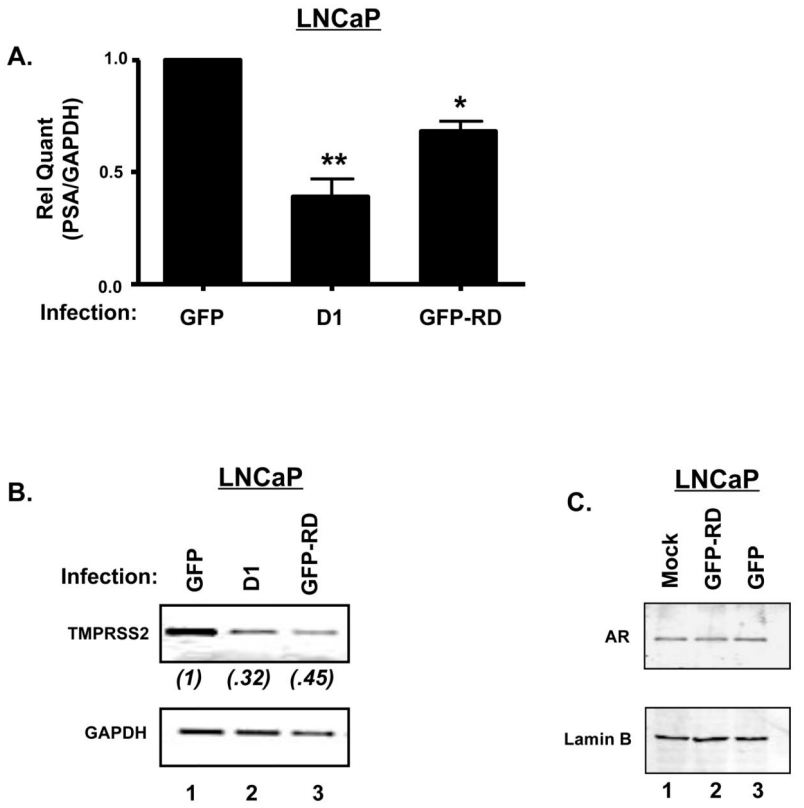


Figure 2. RD inhibits endogenous AR activity

A) LNCaP cells were seeded in steroid containing media, and after 24 h were infected with adenovirus encoding GFP, cyclin D1 wild-type (D1), or GFP-RD. 48h post-infection, RNA was isolated and quantitative real time PCR performed to quantify PSA and GAPDH. The GFP condition was used as a control and set to 1. Relative expression and standard deviations are shown. *p<0.05 and **p<0.01 **B)** LNCaP cells were treated as in (A) and TMPRSS2 mRNA detected by RT-PCR. Bands were quantified using Image J Software and relative levels are indicated (set to TMPRSS2/GAPDH in the GFP control). **C)** LNCaP cells were treated as in (A) immunoblotting for AR and Lamin B (loading control) was performed.

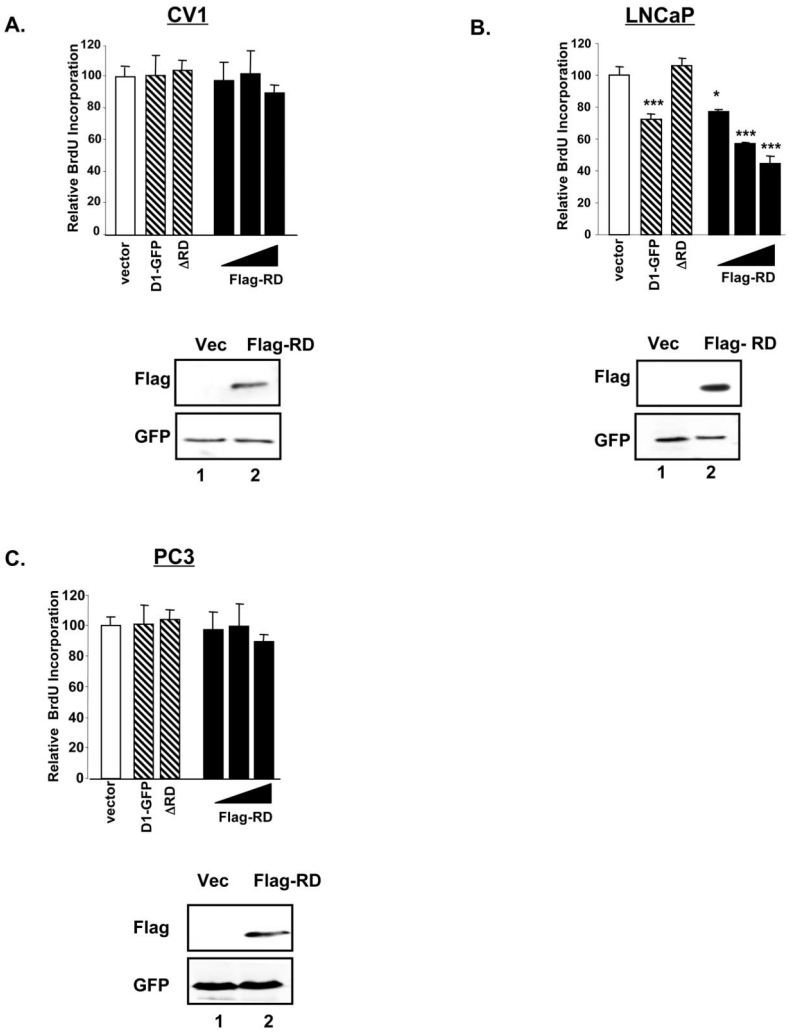
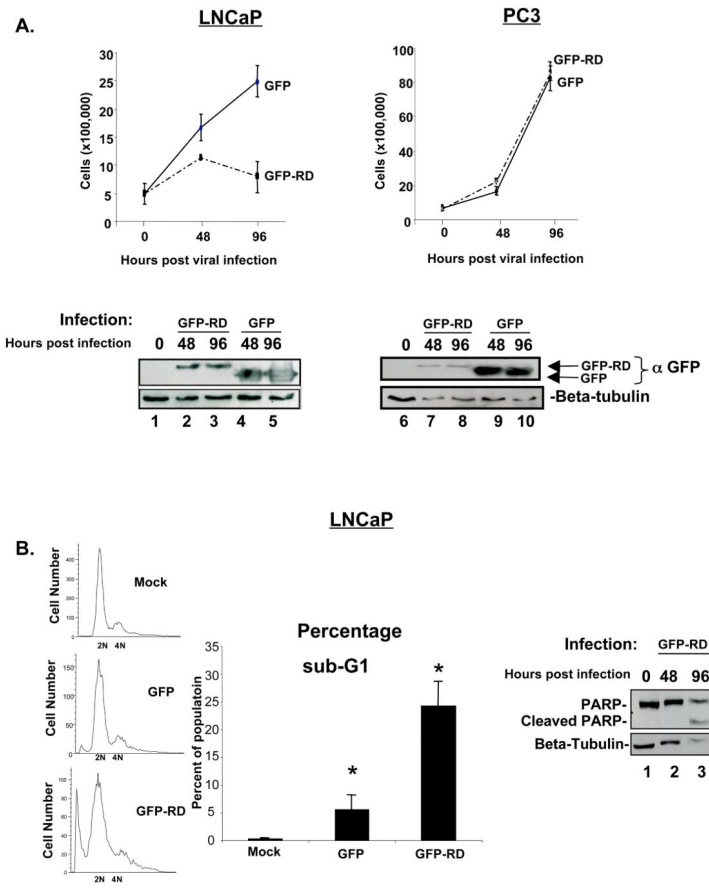


Figure 3. RD specifically inhibits cell cycle progression in AR-positive PCa cells

CV1 (panel A), LNCaP (panel B), or PC3 (panel C) cells were seeded in steroid containing media and transfected with expression plasmids encoding wild-type cyclin D1-GFP, cyclin D1-ΔRD, increasing amounts of the repressor domain (RD), or vector control. 48h post-transfection, cells were labeled for 16h with BrdU (PC3 cells were labeled for 8h), fixed, and immunostained. A minimum of 200 H2B-GFP positive cells per experimental condition were assessed for BrdU incorportation in at least three independent experiments, each with three biological replicates. The results are plotted relative to vector alone (set to 100%). Data are from at least three independent experiments each with three biological replicates. Averages and standard deviation are shown. Lower panels: Cells were treated as above and lysates were subjected to SDS-PAGE and immunoblotted with the indicated antisera. *$p<0.05$ and ***$p<0.001$.



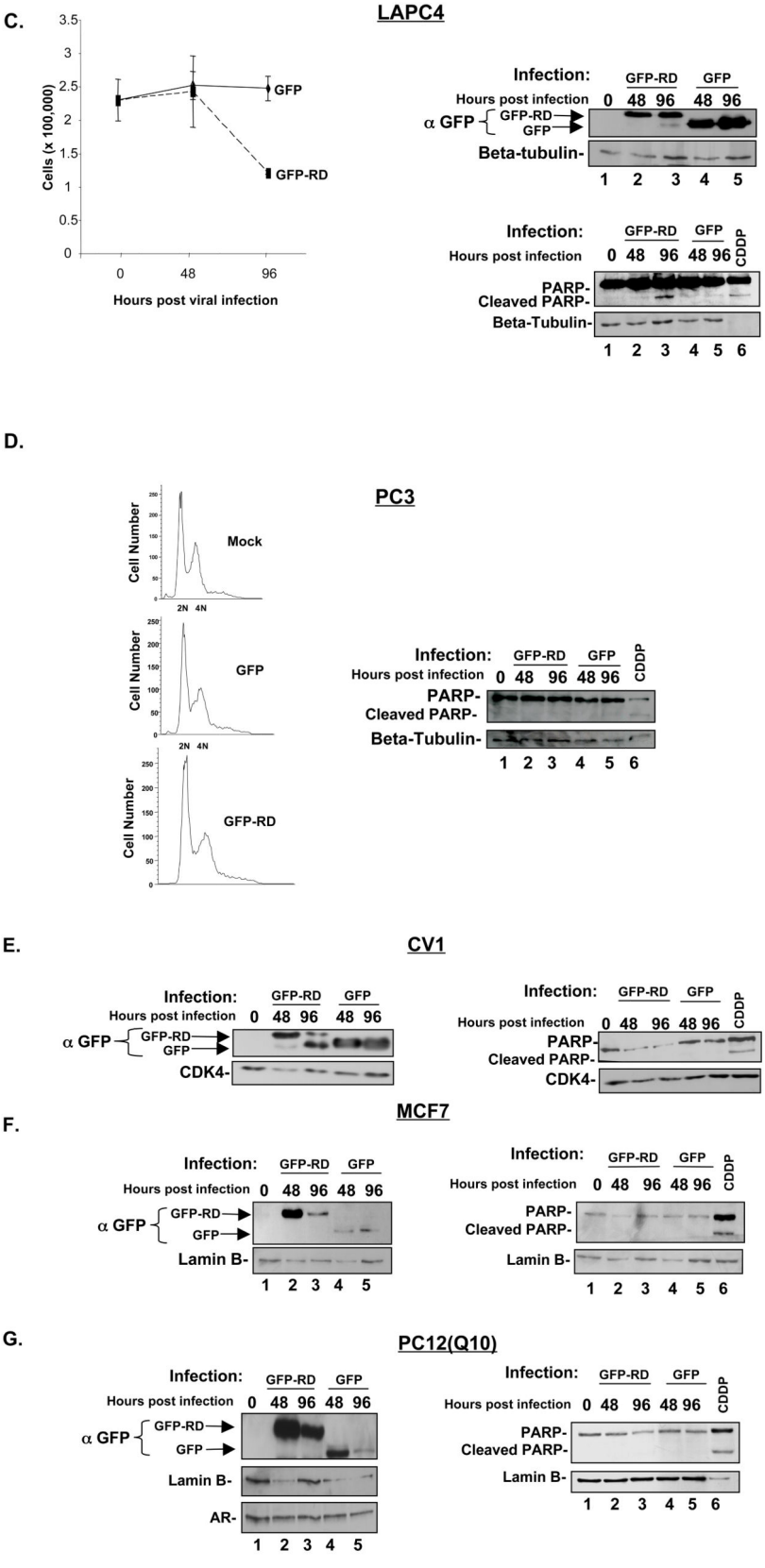


Figure 4. RD induces a specific cytostatic and cytotoxic response in AR-positive, androgen-dependent PCa

A) LNCaP and PC3 cells were infected with either RD-GFP or GFP adenovirus and cell number was monitored at the indicated time points (top panel). Data represent at least three experiments (each with at least 3 biological replicates). Cells were treated as above and lysates were subjected to SDS-PAGE for expression of the indicated proteins throughout the time course (bottom panel); β-tubulin was used as a loading control. **B)** LNCaP cells were treated as in (A) and 96h post- infection, cells and media were collected. Samples were fixed and stained with PI to detect DNA content. Traces shown represent the cell cycle profile of at least 10,000 infected (GFP positive) or uninfected (mock condition) events (left panels). Quantification of three independent experiments is shown (middle panel). Statistical analysis of indicated averages using Student's t-test was performed, wherein * $p<0.05$. Cells were treated as in (A) harvested at the indicated time points and the lysates were subjected to SDS-PAGE and immunoblotted to detect PARP and cleaved PARP. β-tubulin was used as a loading control (right panel). **C)** LAPC4 cells were treated as in (A). Left panel depicts cell number at indicated time points. Right top panel demonstrates expression of the constructs at given time points. Right bottom panel shows PARP cleavage in LAPC4 cells infected with either GFP or GFP-RD as shown at indicated time points, with CDDP treated cells serving as positive control. **D)** PC3 cells were treated as in (A) and 96h post-infection, cells and media were collected. Samples were fixed, stained with PI and quantified as in panel B. **E–G)** CV1 (panel E), MCF7 (panel F), or PC12(Q10) (panel G) cells were infected with either GFP or GFP-RD, harvested at the indicated time points, and lysates subjected to SDS-PAGE and immunoblotting for GFP (left panel), or PARP (right panel). Loading controls are shown (either CDK4 or lamin B).

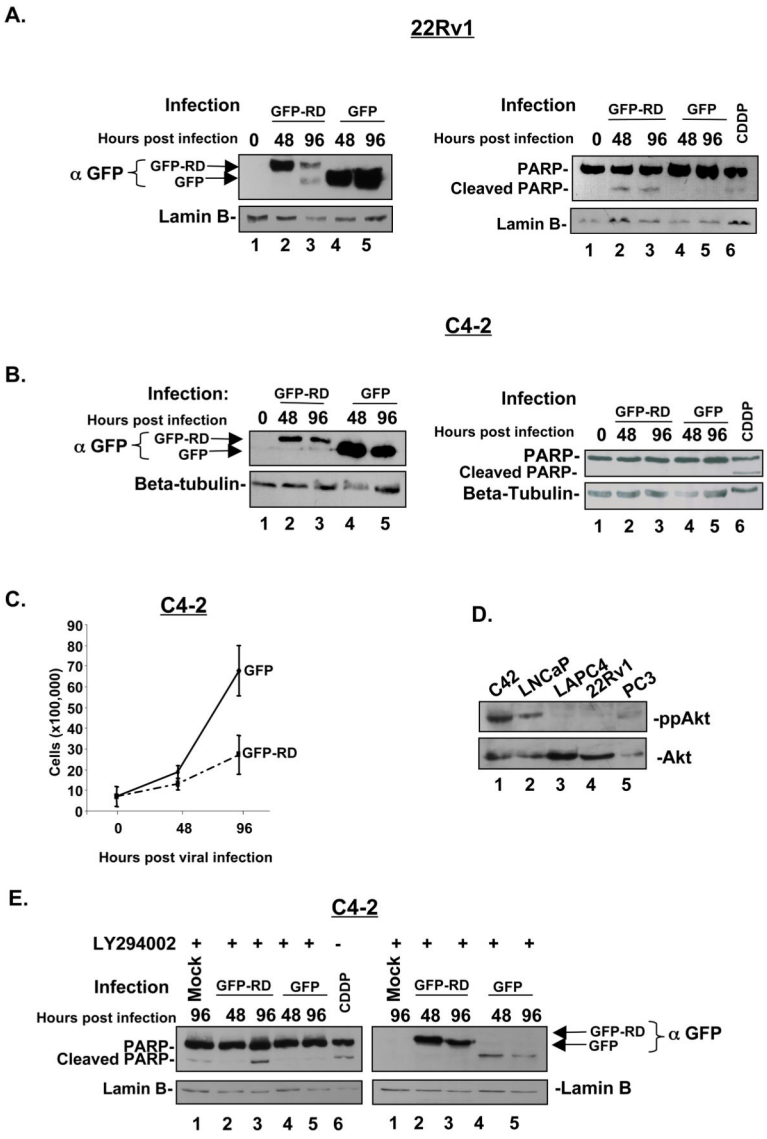


Figure 5. RD induces a cytostatic response in CRPC cells that is sensitive to Akt status

A,B) 22Rv1 (panel A) or C4-2 (panel B) cells were infected with either GFP-RD or GFP adenovirus, harvested, and whole cell lysates were subjected to SDS-PAGE and immunoblotting for the indicated proteins **C)** C4-2 cells were treated as in panel B and cell number was monitored at the indicated time points. Data represent at least three experiments, each with triplicate biological replicates. **D)** Asynchronous populations of the indicated cell types were harvested and lysates were subjected to SDS-PAGE and immunoblotted for phosphorylated Akt (top panel) and total Akt (bottom panel). **E)** Cells were pretreated with 3.12 μM LY290042 for 24h prior to infection with the indicated adenovirus. At indicated time points, cells and media were harvested and lysates were subjected to SDS-PAGE and immunoblotted to detect the GFP, PARP, and Lamin B as control.

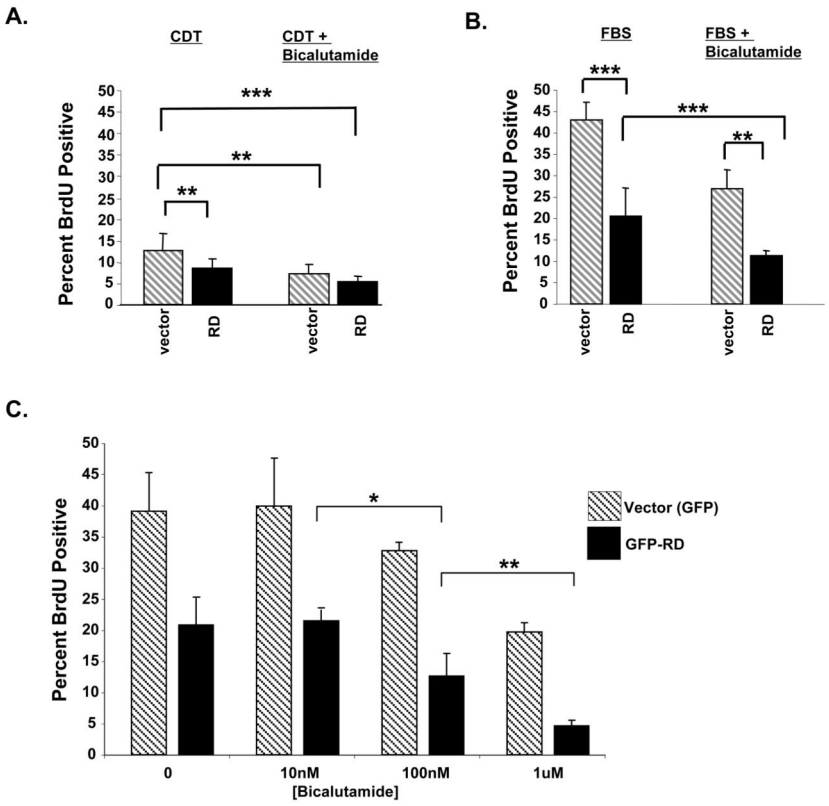


Figure 6. RD cooperates with AR antagonists

A) LNCaP cells were seeded in steroid free media (CDT) on poly lysine coated coverslips, transfected with vector or RD-Flag plasmids. Cells were then labeled with BrdU, fixed, stained and the percentage of GFP positive cells that incorporated BrdU determined by indirect immunofluorescence. Data shown is reflective of experiments performed in triplicate, each with multiple biological replicates. Statistical significance was determined by one way ANOVA followed by Newman-Kuels multiple comparison post test; ** $p<0.01$. **B)** LNCaP cells were seeded in steroid containing media (FBS), transfected as in panel A, and treated with 1μM bicalutamide for 24h. Cells were then processed and analyzed as in panel A; ** $p<0.01$ and *** $p<0.001$. **C)** Studies were performed as in panel B, but cells were subjected to indicated doses of bicalutamide prior to analysis; *$p<0.05$ and **$p<0.01$.