Mitogenic Action of the Androgen Receptor Sensitizes Prostate Cancer Cells to Taxane-Based Cytotoxic Insult

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Abstract

Prostate cancer cells are dependent on androgen for growth and survival; as such, inhibition of androgen receptor (AR) activity is the first line of intervention for disseminated disease. Recently, specific cytotoxic agents have been shown to extend survival times in patients with advanced disease. Given the established ability of androgen to modify cell survival in prostate cancer cells, it is imperative to determine the effect of the hormonal environment on cytotoxic response. Here, we show that the response of prostate cancer cells to taxane-induced cell death is significantly enhanced by androgen stimulation in AR-positive, androgen-dependent prostate cancer cells. Similar results were observed on androgen-independent AR activation. By contrast, AR-positive yet androgen-independent or AR-negative cells were refractory to androgen influence on taxane function. The ability of androgen to potentiate taxane activity was dependent on its mitogenic capacity and was separable from overall AR activity, as coadministration of AR antagonists, G_1 cyclin-dependent kinase inhibitors, or high-dose (growth inhibitory) androgen nullified the proapoptotic function of androgen. Observed induction of cell death was attributed to caspase-dependent apoptosis and correlated with p53 activation. Combined, these data indicate that the cytotoxic effects of taxanes are substantially influenced by the hormonal environment and/or status of AR activity in prostate cancer cells and provide the foundation for refinement and optimization of cytotoxic intervention in prostate cancer. (Cancer Res 2006; 66(24): 11998-2008)

Introduction

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths among U.S. men (1). Locally confined tumors are treated by radical prostatectomy or radiation therapies; however, treatment for disseminated disease remains a major clinical challenge. Conventional therapy for metastatic disease is reliant on the androgen dependence of prostatic adenocarcinomas, as this tumor type requires androgen for growth and survival (2, 3).

Androgens mediate their action through activating the androgen receptor (AR), a ligand-dependent transcription factor. The most prevalent AR ligand in serum is testosterone, which is converted through the action of 5-α-reductase to dihydrotestosterone (DHT) in prostatic epithelia or adenocarcinoma cells (4). DHT binding stimulates displacement of heat shock proteins from AR, receptor dimerization, and rapid translocation of AR into the nucleus. Activated AR associates with specific DNA sequences, termed androgen-responsive elements, and subsequently recruits coactivators to initiate target gene transcription (2, 5). Through these events, androgen elicits numerous biological outcomes dependent on cellular context, including proliferation, survival, and differentiation (6).

To exploit the dependence of prostate cancer on AR function, androgen deprivation therapy is implemented by either surgical (bilateral orchiectomy) or pharmacologic (gonadotropin-releasing hormone agonists) methodologies (3, 5, 7). These therapies are initially effective and induce both cell cycle arrest and apoptosis in tumor cells (3). However, recurrent tumors ultimately arise wherein AR activity has been restored (5, 7–9). Until recently, no therapeutic strategy had been identified that yielded a significant survival advantage for patients with recurrent prostate cancer (7). Recently completed clinical trials showed that microtubule-stabilizing agents (e.g., taxanes) improve clinical outcome in recurrent disease (10). Although encouraging, the benefits were relatively modest. As such, recent attention has been directed toward the optimization of treatment strategies using these cytotoxic agents (10).

Given that androgens and the AR play significant roles in regulation of proliferation and apoptosis in prostatic epithelium (11–14), we investigated the role of AR in the response to cytotoxic insult induced by taxanes. Our data show that in androgen-dependent prostate cancer cells, AR activation synergizes with paclitaxel to enhance cell death. This function of AR is exquisitely dependent on its mitogenic capacity as shown through multiple analyses. By contrast, efficacy of paclitaxel was severely diminished when AR activity was nullified or under conditions of forced cell cycle arrest. Similarly, in AR-deficient cells, or AR-proficient but hormone-independent cells, the response of paclitaxel was refractory to the hormonal milieu. Combined, these data indicate that the cytotoxic effects of paclitaxel are dependent on the mitogenic function of AR and provide the foundation for further refinement of combinatorial therapy for prostate cancer.

Materials and Methods

Reagents. DHT, 17β-estradiol (E2), cholesterol, bisphenol A (BPA; 4,4′-isopropylidenediphenol), paclitaxel, and docetaxel were purchased from Sigma Chemical Co. (St. Louis, MO). Casodex (bicalutamide) was a generous gift from AstraZeneca Pharmaceuticals (London, United Kingdom). Cholesterol was dissolved in chloroform to 1 mol/L and then to 10^{-2} mol/L in 100% ethanol (EtOH) for storage at −20°C. Paclitaxel and docetaxel were dissolved in DMSO to 10^{-2} mol/L. All other compounds were reconstituted to 10^{-2} mol/L in EtOH. Recombinant human heregulin β1 protein (HRG) was purchased from NeoMarkers (Fremont, CA) and stored at −20°C. Ac-VAD-CHO caspase

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inhibitor, roscovitine, and aphidicolin were purchased from Calbiochem (La Jolla, CA), reconstituted in DMSO, and used at the indicated concentrations. The following antibodies were used: rabbit polyclonal poly(ADP-ribose) polymerase (PARP) and Ser^{15} p53 phosphorylated specific (Cell Signaling, Danvers, MA), Bax (N-20) and cyclin-dependent kinase (CDK) 4 (H-22; Santa Cruz Biotechnology, Santa Cruz, CA), E2F-1 (Ab-3; NeoMarkers), p53 (Ab-6; Calbiochem), and bcl-2 and Hsp27 (Stressgen, Ann Arbor, MI).

Cell culture and treatment. PC-3 and LNCaP cells were obtained from the American Type Culture Collection (Rockville, MD) and used between passages 30 and 45. 22Rv1 cells were the generous gift of Dr. J. Jacobberger (Case Western Reserve University, Cleveland, OH). LNCaP cells were maintained in Iscove's modified Eagle's medium (IMEM; Cellgro, Mediatech, Herndon, VA) containing 5% heat-inactivated fetal bovine serum (FBS; Biofluids, Rockville, MD). PC-3 and 22Rv1 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS. For growth in steroid-free conditions, cells were seeded in phenol red–free IMEM (LNCaP) or DMEM (PC-3 or 22Rv1) containing charcoal/dextran-treated FBS (CDT serum, 5% for LNCaP cells and 10% for PC-3 and 22Rv1 cells; BioSource, Rockville, MD). All media for cell types were supplemented with 100 units/mL penicillin-streptomycin and 2 mmol/L L-glutamine (Mediatech). Cells were cultured at 37°C in a 5% CO_2 humidified incubator.

Cell growth and survival assessment. Cells were seeded to approximately 3.5×10^5 per well in six-well dishes into appropriate medium with indicated concentration of hormone. For cell growth assays, cells were cultured in designated conditions for 48 hours. For survival assays, cells were seeded as above and indicated doses of paclitaxel, docetaxel, or DMSO control were added to each well after 24 hours in culture and challenged for indicated hours (24 or 72 hours). After treatment, viable cells were counted using a hemacytometer and trypan blue exclusion. To define the effect of taxanes on cell survival, the number of cells remaining after taxane treatment was set relative to each condition without cytotoxic challenge (100% survival). For experiments analyzing the effect of bicalutamide (Casodex) or aphidicolin/roscovitine on paclitaxel-mediated cell death, 10^{-6} mol/L Casodex or 2 μg/mL aphidicolin or 5 μg/mL roscovitine was added to indicated medium after cells had adhered to surface (before administration of paclitaxel). The Ac-VAD-CHO pan-caspase inhibitor was included at a final concentration of 50 μmol/L and administered 1 hour before paclitaxel exposure. Following 24 hours of 10^{-6} mol/L paclitaxel treatment, cell survival was determined as above. Total cell number was determined for each condition in triplicate samples, and each experiment was replicated at least thrice.

Quantification of micronucleated cells. Cells were seeded on poly-L-lysine–coated coverslips under conditions indicated. After a 24-hour attachment period, 10^{-6} mol/L paclitaxel or DMSO control was added to the cells. Cells were treated for 16 hours and subsequently fixed in 3.7% formaldehyde. Cells were permeabilized in 0.3% Triton X-100 at room temperature for 20 minutes before addition of Hoechst 33258 (Sigma-Aldrich, St. Louis, MO). Hoechst was added to a final concentration of 0.1 μg/mL in PBS and incubated for 30 minutes at 37°C. Coverslips were mounted on glass slides, and nuclei were visualized by indirect immunofluorescence. Experiments were done twice in triplicate, and at least 200 cells were scored per condition.

Immunoblotting. Cells were treated as described for survival assays. For protein analyses, total cells were harvested and lysis was done in radioimmunoprecipitation assay buffer supplemented with protease inhibitor mixture and phenylmethylsulfonyl fluoride. Lysates were subjected to brief sonication and clarified by centrifugation. Protein concentration was determined using Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA), and equal protein was loaded and subjected to SDS-PAGE. Proteins were transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA) and immunoblotted for the indicated proteins. Antigen-antibody complexes were visualized using enhanced Western Lightning chemiluminescence (Perkin-Elmer Life Sciences, Wellesley, PA). Ser^{15}-p53 fluorescence was determined using Alexa Fluor 680 rabbit secondary (Molecular Probes/Invitrogen, Carlsbad, CA) and imaged and quantified on Odyssey IR Imaging System (LI-COR Biosciences, Lincoln, NB).

Reverse transcription-PCR. 22Rv1 cells were seeded in 6-cm dishes in either 10% CDT, 10% FBS, or 10% CDT plus 10^{-10} mol/L DHT. After 48 hours, cells were harvested and total RNA was isolated via Trizol reagent (Life Technologies, Gaithersburg, MD) as recommended by the manufacturer. Reverse transcription-PCR was done followed by PCR of the cDNA using primers for prostate-specific antigen (PSA; primer pair: 5′-CTTGTAGCCTCTCGTGGCAG-3′ and 5′-GACCTTCATAGCATCCGTGAG-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; loading control; primer pair: 5′-CCACCCATGGCAAATTCCATGGCA-3′ and 5′-TCTAGACGG-CAGGTCAGGTCCACC-3′). PCR conditions were as follows: PSA, 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds; and 72°C for 10 minutes; GAPDH, 94°C for 2 minutes; 25 cycles of 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds; and 72°C for 10 minutes.

Flow cytometry. LNCaP cells were seeded in 6-cm dishes in either 5% CDT or FBS medium. After ~24 hours, indicated concentrations of aphidicolin or roscovitine or DMSO control were added to each culture condition for 24 hours. Cells were harvested and fixed in 80% ice-cold EtOH. Following fixation, cells were stained with propidium iodide (0.2 μg/μL) and subjected to flow cytometry to detect propidium iodide intensity. Samples were analyzed and quantified on a Beckman Coulter (Fullerton, CA) Cell Lab Quanta SC flow cytometer. Histograms represent ~10,000 cells.

Statistical assessment. Quantitative results are expressed as mean ± SD. Statistical analyses were done using one-way ANOVA followed by Newman-Keuls' multiple comparison post test. The criterion for statistical significance was $P < 0.05$.

Results

AR activity reduces cell survival in response to taxanes. Given the influence of AR activity on cell survival and proliferation in prostate cancer, the effect of androgen on the response to taxanes in prostate cancer cells was determined. For these initial studies, PC-3 (AR negative, androgen independent) and LNCaP (AR positive, androgen dependent) cells were cultured in the absence or presence of steroid hormones (CDT or FBS sera, respectively). After 24-hour pretreatment, parallel cultures were treated with either 10^{-6} mol/L paclitaxel or vehicle (DMSO) control. Following a 24-hour incubation, cell viability was assessed using trypan blue exclusion. As shown in Fig. 1*A* (*left*), cell viability was unaffected by hormone background in PC-3 cells, wherein ~50% of cells survived in either CDT or FBS ($P > 0.05$). This magnitude of cell death and survival is consistent with previous reports (15) and indicates that sensitivity of PC-3 cells to paclitaxel is unaffected by steroid hormones. Conversely, LNCaP cells cultured in steroid hormones (FBS) showed substantially reduced cell survival in the presence of paclitaxel (25% survival) compared with those depleted of steroid hormone (55–60% survival; $P < 0.001$; Fig. 1*A, right*). These data indicate that the sensitivity of AR-dependent prostate cancer cells to paclitaxel may be strongly influenced by the hormonal milieu and implicate AR activity as a potential effector of paclitaxel-mediated cell death. To challenge this concept directly, a specific AR antagonist, bicalutamide (Casodex), was used. For these studies, LNCaP cells pretreated with either CDT or FBS were cosupplemented with 10^{-6} mol/L Casodex and subsequently challenged with paclitaxel using the strategy outlined in Fig. 1*A*. As shown, Casodex had no significant effect on cell survival in the presence of CDT, wherein AR activity is already inhibited as a function of ligand depletion (Fig. 1*B*). By contrast, Casodex increased cell survival in the presence of steroid hormone, improving cell survival from 25% to 50% ($P < 0.01$).

To further confirm that AR activity augments taxane cytotoxicity, we sought to define whether the specific taxane, dose, and/or timing affect the change in cytotoxic efficacy imparted by AR

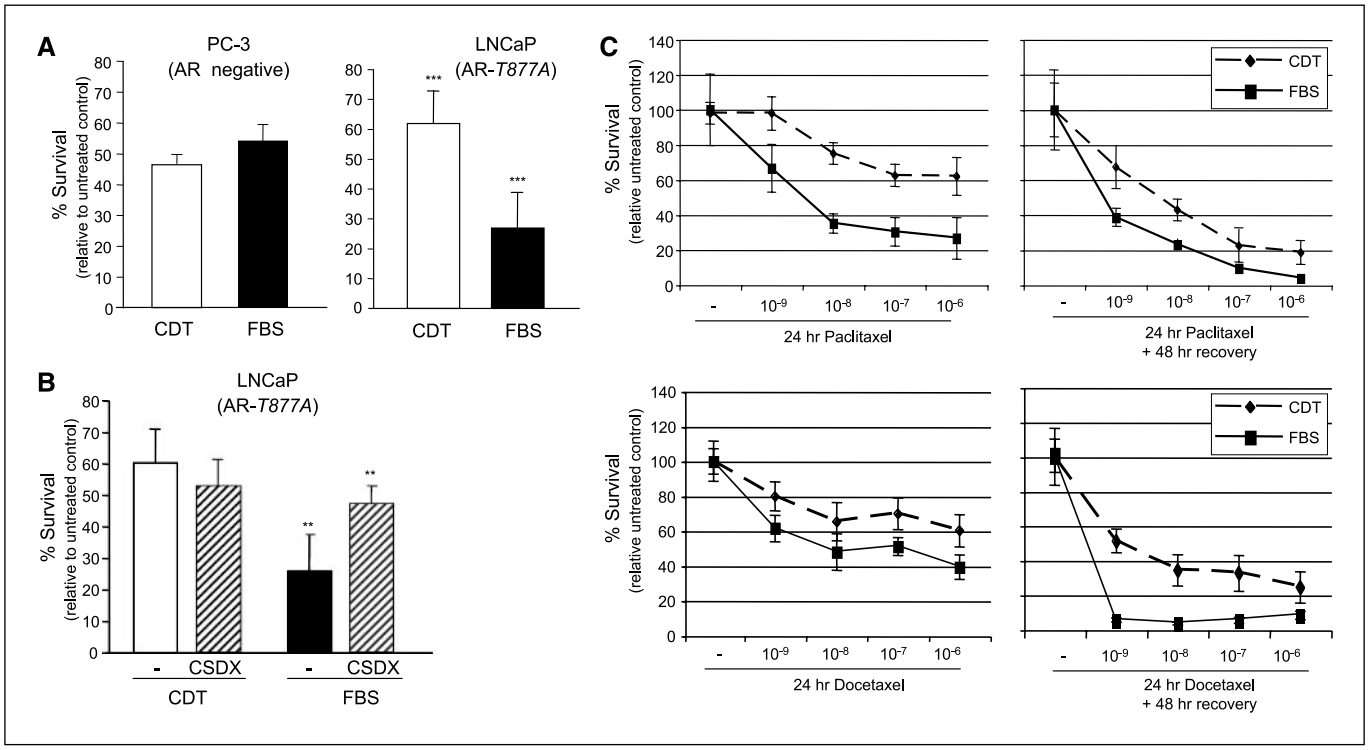


Figure 1. AR activity reduces cell survival in response to taxanes. *A,* PC-3 or LNCaP cells were seeded either in CDT or FBS medium. After 24 hours, 10^{-6} mol/L paclitaxel or DMSO vehicle control was added to each set of cells. Percentage cell survival for each cell type after 24 hours of paclitaxel treatment was determined by trypan blue exclusion comparing cell number after paclitaxel exposure with cell number in DMSO control for each condition. *B,* percentage survival following paclitaxel treatment in LNCaP cells was determined as in (*A*); however, cells were initially seeded in CDT or FBS $\pm$ 10^{-6} mol/L Casodex (*CSDX*). *C,* percentage survival following paclitaxel or docetaxel treatment at 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} mol/L in LNCaP was determined as in (*A*) for 24 hours of exposure and also at 72 hours after addition of cytotoxic challenge, wherein after 24 hours of exposure fresh medium was replaced and cells were allowed to propagate for an additional 48 hours. The number of viable cells was set relative to each condition without taxane. All experiments were done at least thrice in triplicate. ***, $P < 0.001$; **, $P < 0.01$.

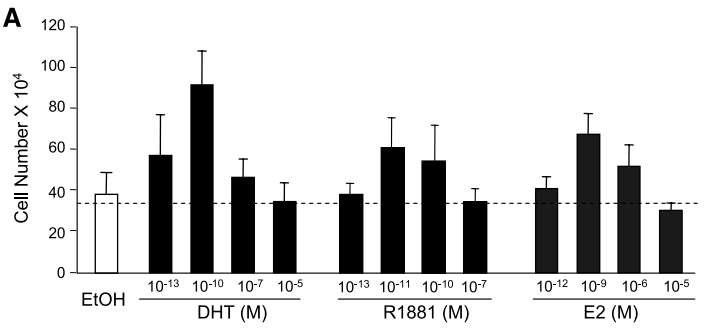
action. For these studies, LNCaP cells were seeded as described in Fig. 1*A* in either CDT or FBS conditions. The following day, various doses of paclitaxel (10^{-9}, 10^{-8}, 10^{-7}, or 10^{-6} mol/L) or DMSO vehicle control were added. After 24 hours of exposure, cells were either counted and scored for viability, as in Fig. 1*A*, or placed into fresh CDT or FBS medium and cultured for an additional 48 hours and then counted as described above. As can be seen in Fig. 1*C* (*top*), paclitaxel-mediated cell death was enhanced in the presence of hormone (FBS) for all doses tested, and this disparity in survival persisted for up to 72 hours after the recovery period (Fig. 1*C, top*). In parallel experiments, LNCaP cells were challenged with docetaxel to determine the effect of hormone on this cytotoxic response. As can be seen in Fig. 1*C* (*bottom*), hormone exacerbated docetaxel-mediated cell death at each dose tested. LNCaP cells proved to be generally more sensitive to docetaxel cytotoxicity compared with paclitaxel especially when combined with hormone. Together, these data indicate that AR activation synergizes with taxanes to enhance cell death.

Mitogenic doses of AR ligands synergize with paclitaxel to reduce cell survival. Because our data indicate that hormone enhances paclitaxel-mediated cytotoxicity, we hypothesized that this synergy is attributed to the ability of AR activation to stimulate proliferation in AR-dependent cells. The central cytotoxic action of paclitaxel is mediated through microtubule stabilization in mitosis (16). Thus, the ability of AR to bolster paclitaxel action could be dependent on its mitogenic capacity. Interestingly, although the transcriptional response of AR to ligand is linear, hormone-dependent cells (including LNCaP) exhibit a biphasic dose response to steroid stimulation (17). Specifically, physiologic doses of androgen (10^{-10} mol/L DHT) facilitate mitogenesis, and higher doses inhibit cellular proliferation, although still facilitate AR transactivation (17). Therefore, it was imperative to determine whether the ability of AR to enhance paclitaxel cytotoxicity was attributed to AR activation overall or was specific to the mitogenic function of AR. To assess this, mitogenic steroid hormone doses were validated to induce AR-dependent proliferation in prostate cancer cells. Cells were seeded in triplicate at a density of 3.5×10^5 per well under each condition (dotted line). As expected, under steroid-depleted conditions (CDT plus EtOH vehicle; Fig. 2*A, white columns*), LNCaP cells failed to undergo cell doubling during the growth period. Also consistent with previous results, DHT maximally enhanced proliferation at 10^{-10} mol/L, whereas higher doses failed to induce proliferation (10^{-7} and 10^{-5} mol/L, respectively; ref. 17). A similar response was observed with the commonly used DHT analogue R1881. For this compound, 10^{-11} mol/L was the optimal dose ($P < 0.05$), and higher concentration levels inhibited proliferation. Lastly, the effect of E2 was examined, as LNCaP cells express a somatic mutant of AR (AR-*T877A*) that commonly arises during prostate cancer disease progression. This mutant renders the receptor amenable to activation by alternate steroid hormones, especially estrogen (18). As shown, E2 induced maximal cellular proliferation at 10^{-9} mol/L ($P < 0.001$) and higher doses reduced proliferation.

Based on this information, the effect of individual AR ligands on the response to paclitaxel was monitored. LNCaP cells were pretreated for 24 hours with either CDT supplemented with vehicle control (EtOH), the steroid precursor cholesterol (negative control), mitogenic doses of AR ligands (10^{-10} mol/L DHT, 10^{-11} mol/L R1881, or 10^{-9} mol/L E2), or nonmitogenic doses (10^{-7} mol/L DHT, 10^{-7} mol/L R1881, or 10^{-6} mol/L E2; Fig. 2*B*). Cells were then challenged with 10^{-6} mol/L paclitaxel or DMSO control for 24 hours. As expected, cholesterol has no effect on paclitaxel-mediated cell death (Fig. 2*B, light gray columns*) and resulted in ~60% cell survival similar to paclitaxel effects in the presence of CDT plus vehicle treatment alone. However, when the mitogenic doses of AR ligands were used (10^{-10} mol/L DHT, 10^{-11} mol/L R1881, and 10^{-9} mol/L E2), cell death following 24-hour paclitaxel treatment was enhanced (35%, 45%, and 25% cell survival compared with no-paclitaxel treatment, respectively). Conversely, at the nonmitogenic doses (10^{-7} mol/L DHT, 10^{-7} mol/L R1881, and 10^{-6} mol/L E2), there was no change in cell survival following paclitaxel exposure (60%, 75%, and 60% cell survival, respectively).

These data indicate that AR ligands are capable of enhancing paclitaxel-mediated cell death only at doses in which cellular proliferation is induced. To validate this concept, an exogenous AR-*T877A* ligand was used, BPA. BPA is a prevalent environmental compound and activates AR-*T877A*, inducing dose-dependent AR-mediated gene transcription and cellular proliferation (19). We have previously shown that BPA optimally induces AR-mediated cellular proliferation at 10^{-9} mol/L, whereas BPA inhibits cell proliferation at micromolar doses (10^{-6} mol/L or higher; ref. 20). As shown in Fig. 2*C*, pretreatment with the mitogenic dose of BPA (10^{-9} mol/L) enhanced the effect of paclitaxel-mediated cytotoxicity compared with vehicle-treated cells (40% compared with 60% cell survival). High-level BPA (10^{-6} mol/L), which inhibits proliferation, did not statistically alter the paclitaxel-mediated reduction in cell survival (just under 70% compared with the 60% vehicle control). Collectively, these data strongly support a model wherein AR activity significantly enhances the response to paclitaxel; however, this synergistic function of AR on paclitaxel-mediated cell death is exquisitely dependent on the mitogenic capacity of AR. A table summarizing the dose-dependent effects of AR agonists on the response to paclitaxel is shown in Fig. 2*D*.

Androgen-independent activation of AR synergizes with paclitaxel to reduce cell survival. The data herein suggest that AR ligands synergize with paclitaxel to reduce prostate cancer cell survival dependent on the cell cycle progression function of AR activation. Ligand-independent modes of AR activation may play a role in prostate cancer disease progression, as activation of AR via growth factor pathways results in cell cycle progression under androgen deprivation conditions (21). Therefore, we aimed to



D

Treatment	Fold Δ in Survival
CDT+EtOH	1
FBS	**0.42**
CHL 10^{-9}	0.88
CHL 10^{-6}	0.99
DHT 10^{-10}	**0.54**
DHT 10^{-7}	0.97
R1881 10^{-11}	**0.72**
R1881 10^{-7}	1.30
E2 10^{-9}	**0.41**
E2 10^{-6}	1.05

Figure 2. Mitogenic doses of AR ligand synergize with paclitaxel to reduce cell survival. *A,* 3.5×10^5 LNCaP cells (*dotted line*) were seeded into CDT medium supplemented with EtOH control, DHT (10^{-13}, 10^{-10}, 10^{-7}, and 10^{-5} mol/L), R1881 (10^{-13}, 10^{-11}, 10^{-10}, and 10^{-7} mol/L), or E2 (10^{-12}, 10^{-9}, 10^{-6}, and 10^{-5} mol/L). Viable cells were counted after 48 hours of culture. Experiments were done at least twice in triplicate. *B,* LNCaP cells were seeded into CDT medium ± cholesterol (*CHL*; 10^{-9} or 10^{-6} mol/L), DHT (10^{-10} or 10^{-7} mol/L), R1881 (10^{-11} or 10^{-7} mol/L), or E2 (10^{-9} or 10^{-6} mol/L). After 24 hours, 10^{-6} mol/L paclitaxel or DMSO control was added for 24 hours. Cell survival in paclitaxel was determined by comparing the number of viable cells exposed to paclitaxel relative to each culture condition in DMSO alone. ***, $P < 0.001$; *, $P < 0.05$. *C,* LNCaP cells were seeded as described above in CDT medium containing either 10^{-9} or 10^{-6} mol/L BPA. Cell survival following 10^{-6} mol/L paclitaxel challenge was determined as in (*B*). **, $P < 0.01$. All cell survival assays were done at least twice in triplicate. *D,* summary of fold changes in cell survival following paclitaxel challenge relative to androgen-depleted conditions (CDT).

determine the effect of a previously shown and clinically relevant androgen-independent growth stimulation on paclitaxel-mediated cell death. Androgen-independent proliferation of prostate cancer cells can occur through HRG activation of HER2-HER3 or HER2-HER4 heterodimers, which has been shown to induce AR transactivation and phosphorylation (22, 23) and also increase AR stability and DNA binding (24). LNCaP cells were seeded as described in Fig. 2*A* and supplemented with recombinant HRG (50 ng/mL) for 48 hours in the absence of hormone (CDT). Consistent with previous reports, treatment with HRG resulted in increased cell proliferation compared with vehicle control conditions (Fig. 3*A*; compare *white columns* with *black columns*). Parallel experiments were done in the presence of the direct AR antagonist, Casodex, wherein 10^{-6} mol/L Casodex was added before the supplementation with HRG (Fig. 3*A, stripped columns*). Casodex had no effect on the growth of LNCaP cells in hormone-depleted medium; however, blocking AR with Casodex resulted in partial inhibition of HRG-mediated growth. These data are consistent with previous observations, wherein HRG-induced AR activation is only partially sensitive to AR antagonist action (21, 23). To determine whether HRG activity is sufficient to synergize with paclitaxel, LNCaP cells were cultured as described for Fig. 3*A* but challenged with 10^{-6} mol/L paclitaxel for 24 hours before viability assessment. Consistent with Fig. 1*A*, LNCaP cells exposed to paclitaxel for 24 hours in the absence of hormone showed ~60% survival (Fig. 3*B*). Importantly, this survival was greatly attenuated by growth factor receptor activation, wherein LNCaP cells supplemented with 50 ng/mL HRG showed diminished cell survival following paclitaxel challenge (~50% cells remaining; Fig. 3*B*). These data show that, in addition to ligand-induced AR activation, the efficacy of taxanes can be enhanced by androgen-independent, AR-dependent proliferation.

The synergistic effect of paclitaxel and AR activation is mediated through caspase-dependent apoptosis and p53 activation. These data indicate that AR-mediated mitogenic action in prostate cancer cells enhances sensitivity to paclitaxel-mediated cytotoxicity; therefore, the effect of AR function on the apoptotic index was monitored. The precise cytotoxic mechanisms of paclitaxel-mediated cell death in prostate cancer have not been completely defined, and paclitaxel action is heavily dependent on cellular context and genetic background (16). To assess the influence of AR on paclitaxel-induced cell death, the appearance of micronuclei was monitored. Cells develop multiple micronuclei after paclitaxel exposure due to nuclear membrane reformation around unsegregated chromosomes, and this process leads to apoptosis (25). LNCaP cells were treated as in Fig. 2*B*, and the percentage of micronucleated cells in each hormone condition was analyzed via nuclear Hoechst stain (Fig. 4*A*). As expected, cells cultured in a steroid-depleted condition showed no micronucleated cells in the absence of paclitaxel challenge. Identical results were observed on hormone stimulation (FBS or DHT). However, following 24 hours of 10^{-6} mol/L paclitaxel challenge, micronuclei were readily discerned and counted. In the steroid-depleted condition (CDT plus EtOH vehicle), ~20% of the population showed signs of micronucleation (Fig. 4*A, right*). This apoptotic index was markedly increased in the presence of AR agonists (FBS or DHT), wherein the micronuclear index was raised to 55% ($P < 0.01$) and 43% ($P < 0.05$), respectively. The observed increase in micronucleated cells is consistent with the hypothesis that AR activation enhances cell death in response to paclitaxel.

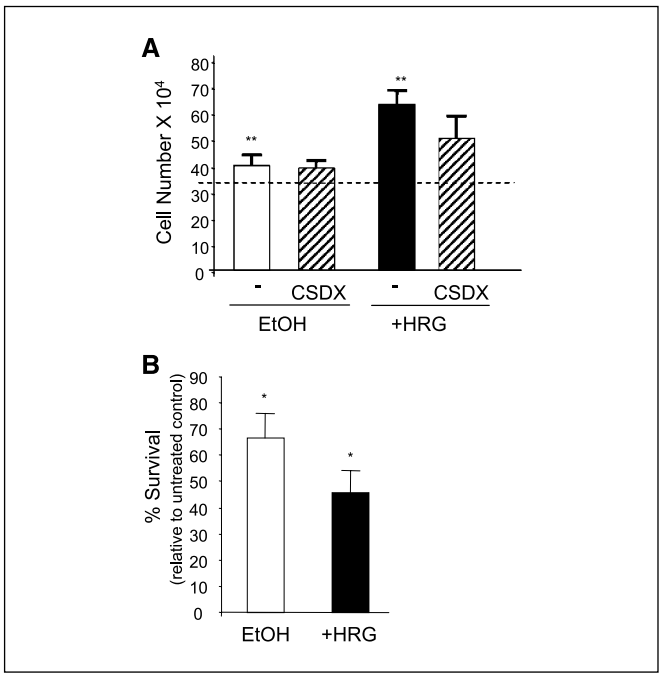


Figure 3. Androgen-independent AR activation synergizes with paclitaxel to reduce cell survival. *A,* 3.5×10^5 LNCaP cells (*dotted line*) were seeded into CDT medium supplemented with DMSO control or 50 μg/mL recombinant HRG. Viable cells were counted after 48 hours of culture. ***, $P < 0.001$. *B,* LNCaP cells were seeded into CDT medium ± 50 μg/mL HRG. After 24 hours, 10^{-6} mol/L paclitaxel or DMSO control was added and culture was continued for an additional 24 hours. Cell survival in paclitaxel was determined by comparing the number of viable cells exposed to paclitaxel relative to each culture condition in DMSO alone. *, $P < 0.05$. Experiments were done at least twice in triplicate.

To directly monitor induction of apoptosis, the abundance of cleaved PARP in each hormonal environment was determined in the presence or absence of paclitaxel treatment. PARP is enzymatically cleaved by active caspases as a final step in cellular apoptosis, and the relative amount of cleaved PARP to total PARP reflects the proportion of the population undergoing apoptosis (26). Paclitaxel challenge has been shown to induce cleavage and activation of caspases (25). As expected, no PARP cleavage was observed in the absence of paclitaxel challenge, regardless of hormonal environment (Fig. 4*B, lanes 1, 3,* and *5*). Cells cultured in androgen-depleted conditions and treated with paclitaxel exhibited only a marginal increase in cleaved PARP fragment as evident by the appearance of the 89-kDa band. However, more pronounced induction of the cleaved PARP band was observed in conditions of AR activation (FBS or DHT; Fig. 4*B*, compare *lanes 1* and *2* with *lanes 3* and *4* and *lanes 5* and *6*) when normalized to the loading control (CDK4). Combined, these data indicate that AR agonists synergize with paclitaxel to induce an apoptotic response. Furthermore, these data indicate that this synergistic effect is manifest through caspase-dependent mechanisms.

To validate the concept that AR activation enhances caspase-dependent apoptosis in response to paclitaxel, cell survival assays were done in the presence of a caspase inhibitor. Specifically, cells were treated as in Fig. 2*B* but were pretreated for 1 hour before paclitaxel administration with 50 μmol/L Ac-VAD-CHO, a pan-caspase inhibitor (Fig. 4*C*). Paclitaxel-mediated reduction in cell survival was not affected by Ac-VAD-CHO in cells pretreated in androgen-depleted conditions (CDT plus EtOH; ~65%). However,

Ac-VAD-CHO effectively nullified the synergistic effect of androgen on paclitaxel-mediated cell death. As shown, pretreatment in FBS before paclitaxel exposure reduced cell survival to 25%, but Ac-VAD-CHO restored cell survival to levels comparable with the no-hormone condition (60%). Similar effects were observed with DHT, wherein cell survival was restored to 65% from 35% on Ac-VAD-CHO administration. These data indicate that caspase activation is required for the ability of AR agonists to enhance paclitaxel-mediated cell death.

AR agonists modulate the expression of selected apoptotic proteins (14, 27–30). In addition, paclitaxel treatment of cells can also alter expression of apoptotic factors dependent on cellular context (31, 32). Therefore, protein levels of suggested AR and paclitaxel effectors were assessed under each hormone condition. The *p53* tumor suppressor gene has been implicated as a mediator of cellular sensitivity to paclitaxel, as p53 is a known modulator of cell death following DNA damage (33). However, paclitaxel does not activate p53 in all cell types (34). To assess the effect of paclitaxel on p53 status, in the LNCaP model system, cells were treated as in Fig. 2*B* and harvested and protein was analyzed by immunoblot. As shown in Fig. 4*D, left*, basal levels of p53 were identical in the absence of paclitaxel, regardless of hormonal status (Fig. 4*D*, compare *lanes 1, 3,* and *5*). Although after paclitaxel challenge, p53 levels were induced in each condition (Fig. 4*D, lanes 2, 4,* and *6*); interestingly, this induction was modestly enhanced by the presence of AR agonists (FBS and DHT). A similar profile was observed when phosphorylation-specific antisera against Ser^{15} of p53 were used to identify active p53 (Fig. 4*D*, *second row*, compare *lane 2* with *lanes 4* and *6*). Fold increase of active p53 (phosphorylated Ser^{15}) for each hormone condition challenged with paclitaxel was determined by signal intensity relative to no-paclitaxel treatment control. In androgen-depleted conditions, p53 was activated 1.67-fold, whereas under mitogenic conditions (FBS and DHT) p53 was activated 18-fold and 10-fold, respectively. Equal loading was confirmed using CDK4 as a control (Fig. 4*D, bottom row*). These data indicate that paclitaxel challenge activated p53 under all hormonal conditions but that p53 activation is enhanced by the presence of AR agonists.

In specific cell types, the bcl-2 family of apoptotic proteins has also been suggested to be modified by both AR and paclitaxel

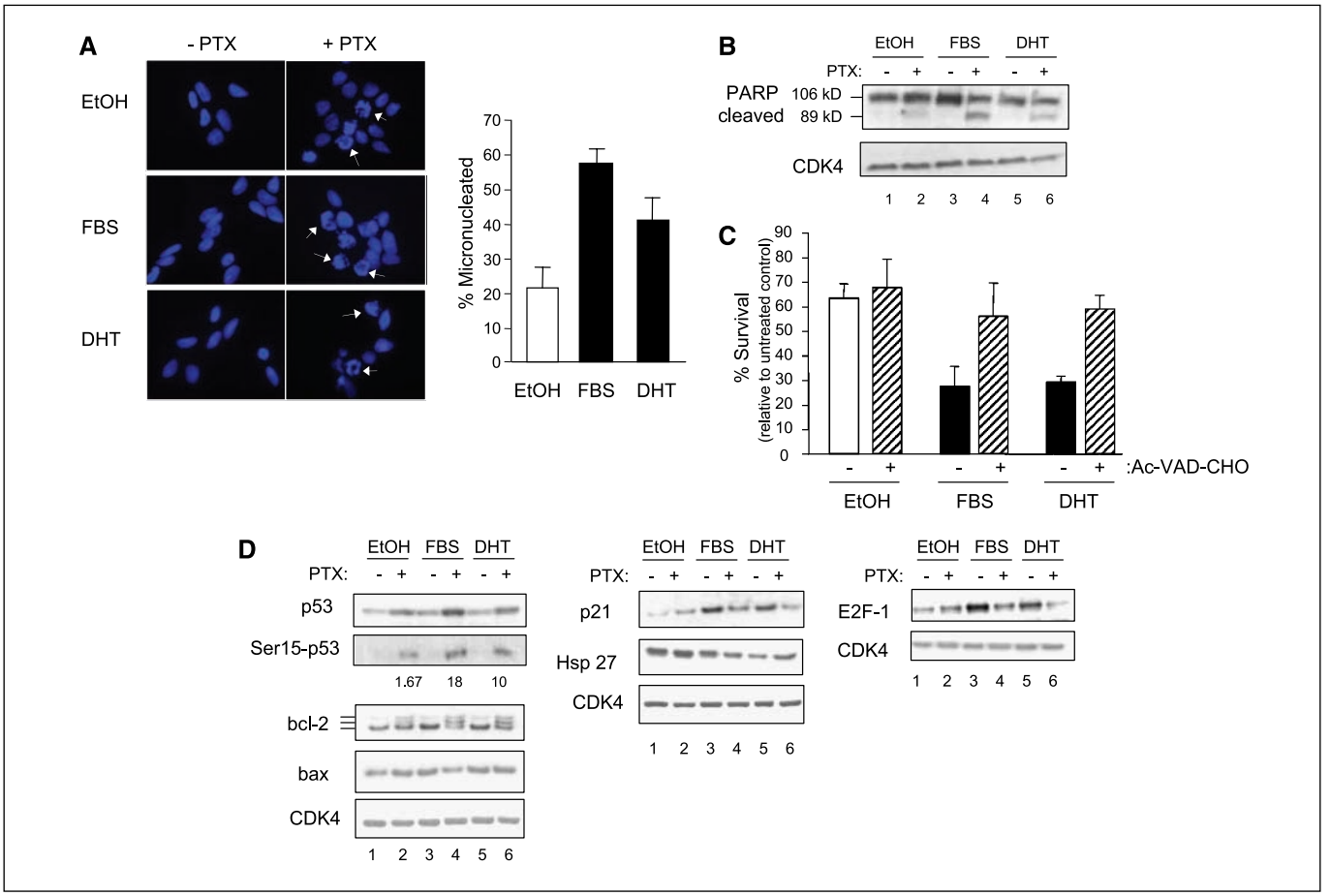


Figure 4. The synergistic effect of paclitaxel and AR activation is attributed to enhanced apoptosis. *A,* LNCaP cells were seeded on coverslips in CDT + EtOH, FBS, or CDT + 10^{-10} mol/L DHT. The cells were exposed to 10^{-6} mol/L paclitaxel (*PTX*) or DMSO control for 24 hours. Cells were fixed and nuclei were stained with Hoechst DNA dye. *Left,* representative images; *right,* percentage micronucleated nuclei from at least three experiments done in triplicate. *B,* LNCaP cells were seeded in CDT + EtOH, FBS, or CDT + 10^{-10} mol/L DHT, and adherent and floating cells were collected after 24 hours of 10^{-6} mol/L paclitaxel or DMSO control. Isolated protein was immunoblotted with an antibody specific for both full-length and cleaved PARP (106 and 89 kDa, respectively). CDK4 is the loading control. *C,* LNCaP cells were seeded as described. One hour before paclitaxel exposure, 50 μmol/L Ac-VAD-CHO pan-caspase inhibitor was added to the medium. Following 24 hours of 10^{-6} mol/L paclitaxel treatment in the presence of Ac-VAD-CHO, cell survival was determined as described previously. *D,* LNCaP cells were seeded, harvested, and collected as in (*B*). Isolated protein was immunoblotted with the following: (*left*) antisera against p53, Ser^{15} p53, bcl-2, and Bax (numbers correspond to fluorescence intensity of Ser^{15} p53 relative to no paclitaxel control in the same condition); (*middle*) antisera against $p21^{CIP1}$ and Hsp27; (*right*) antisera against E2F-1. CDK4 served as the loading control.

(10, 32). Therefore, expression levels of conventional apoptotic family members were monitored. Increased bcl-2 levels are correlated with disease progression in prostate cancer and androgen independence, suggesting a mechanism for resistance to apoptosis (35). As shown in Fig. 4*D* (*left*), bcl-2 was activated equally by paclitaxel in all conditions tested (as evident by the phosphorylated, slower mobility upper bands; *lanes 2, 4,* and *6*) and androgen treatment did not induce detectable changes of bcl-2 or its phosphorylated forms (compare *third row, lanes 1* and *2, 3* and *4*, and *5* and *6*). This finding matches similar results reported for this model system (27), wherein paclitaxel challenge phosphorylates bcl-2, resulting in decreased ability of bcl-2 to form heterodimers with Bax (27, 36). Bax is a proapoptotic factor that, on oligomerization, permeabilizes the mitochondria and induces cytochrome *c* release (37). No significant alteration in Bax was observed under any treatment condition examined (Fig. 4*D, left*). These data are congruent with previous observations that paclitaxel does not alter Bax levels in prostate cancer cells (32). Moreover, p53 is known to induce proapoptotic activity by both transcriptional and non-transcriptional mechanisms in prostate cancer cells (27).

In selected models, androgen has been suggested to modulate the expression of two genes implicated in cell survival: *p21* and *Hsp27* (13, 14, 38). Therefore, the relevance of these factors for androgen facilitated cell death after paclitaxel challenge was examined. As we and others have previously shown, androgens induce expression of the CDK2 inhibitor $p21^{CIP1}$ (13, 38). As expected, an increase in p21 under mitogenic conditions was seen, highlighting the AR-mediated cell cycle regulation of this protein (Fig. 4*D*, *middle*, compare *lane 1* with *lanes 3* and *5*). Although $p21^{CIP1}$ can also be induced by p53 (39), p21 levels actually decreased after paclitaxel challenge in FBS and DHT conditions (Fig. 4*D*, *middle*, compare *lanes 3* with *lanes 4* and *5* with *6*). This change in p21 on paclitaxel challenge is interesting, as p21 induction has been shown to protect LNCaP derivatives from paclitaxel-mediated apoptosis (40). Thus, observed deregulation of p21 may induce a permissive state for cell death that is enhanced by AR activity. It has been shown that androgen depletion induces up-regulation of survival proteins, such as Hsp27, whose expression correlates with increased survival in response to cytotoxic stimuli (14), and these observations were also recapitulated in this model (Fig. 4*D*, *middle*, compare *lanes 1* and *2* with *lanes 3* to *6*). However, Hsp27 was unaffected by paclitaxel exposure, suggesting that androgen deprivation conditions increase Hsp27 levels, thereby protecting the cells from cytotoxic insult, and that this protective response is impermeable to paclitaxel challenge. Collectively, these data indicate that the synergistic effect of AR activation with paclitaxel is dependent on mitogenic doses of AR ligand and the proapoptotic function of AR is likely dependent on cell cycle progression.

The hypothesis that enhanced cytotoxicity was a result of AR-induced proliferation was borne out in the examination of E2F-1 (Fig. 4*D, right*). As expected, E2F-1 was induced under conditions of AR activation (FBS and DHT; Fig. 4*D*, *right*, compare *lane 1* with *lanes 3* and *5*). These data are consistent with previous observations that androgen initiates RB inactivation and induction of E2F-dependent target genes (38) and suggest that the ability of androgen to stimulate E2F-1 and subsequent cellular proliferation likely underlies its ability to synergize with paclitaxel. E2F-1 expression is also known to be proapoptotic (41), and loss of E2F-1 can protect against chemotherapeutic-induced cell death in prostate cancer cells (42). Interestingly, E2F-1 was lost after paclitaxel stimulation (Fig. 4*D*, *right*, compare *lanes 2*, *4*, and *6*), similar to reports in head and neck cancer cells after effective docetaxel treatment (43). Combined, these data indicate that the AR-enhanced cell death is attributed to caspase-dependent apoptosis and associated with p53 activation. Consistent with the hypothesis that the mitogenic function of AR underlies enhanced cell death, AR-induced cells expressed higher levels of E2F-1 and were sensitized to paclitaxel.

The mitogenic action of AR is necessary for reduction of cell survival in the presence of paclitaxel. Based on the preceding observations, we speculated that the ability of AR to increase caspase-dependent cell death after paclitaxel challenge results from an increased percentage of the population passing through mitosis, therefore sensitizing those cells to paclitaxel action. To challenge this hypothesis, 22Rv1 cells were used. These cells express a functional endogenous AR, but androgen stimulation is dispensable for mitogenic progression (44). Androgen independence was verified by monitoring cellular proliferation in the presence or absence of androgen. As shown in Fig. 5*A*, and consistent with previous reports, 22Rv1 cells proliferated identically under conditions of no hormone, physiologic androgen stimulation, and even in the presence of high-dose androgen (45). However, AR is still activated by ligand in this cell line as validated by monitoring expression of an AR target gene, *PSA* (Fig. 5*B*). As can be seen, PSA mRNA levels were low under conditions of androgen depletion (Fig. 5*B, lane 1*) but induced on culture in FBS or CDT plus DHT (Fig. 5*B, lanes 2* and *3*). Therefore, although androgen is dispensable in 22Rv1 cells for proliferation, ligands induce AR target gene activation, including PSA. This model system was subsequently used to monitor the effect of androgen on paclitaxel-induced cytotoxicity. As shown in Fig. 5*C*, 22Rv1 cells were equally sensitive to paclitaxel treatment, regardless of androgen stimulation (compare EtOH with FBS or DHT; no statistical difference, $P > 0.05$). Moreover, this cell line expresses a promiscuous variant mutant receptor (AR-*H874Y*) that can arise during tumor progression and renders the cells sensitive to additional ligands, including estrogen (44). Similar to the results observed with androgen, estrogen stimulation had no effect on 22Rv1 proliferation (data not shown) or on the magnitude of paclitaxel-induced cell death (approximately 45–50% cell survival for all agents; Fig. 5*C*). To validate that AR activation in the androgen-independent cells does not alter the paclitaxel response, the effect of Casodex on paclitaxel-mediated cell death was monitored. As shown, addition of the AR antagonist Casodex had no effect on the cytotoxic effects of paclitaxel regardless of hormone environment (Fig. 5*D*). These data indicate that, although androgen-independent cells express active AR, activation of AR affords no enhancement of paclitaxel-mediated cell death. As such, these observations support the conclusion that AR enhances paclitaxel action through facilitating cell cycle progression.

Cell cycle progression is necessary for paclitaxel-mediated cell death. It has been hypothesized that the ability of paclitaxel to interfere with cytokinesis would make dividing cells more sensitive; however, there has been significant variation in cellular sensitivity to paclitaxel (46). The data herein suggest that the mitogenic action of AR enhances paclitaxel-induced apoptosis in prostate cancer cells and therefore implicate AR-mediated cell cycle progression as the underlying mechanism of enhanced cell death. Therefore, the necessity of cell cycle progression in prostate cancer cells for paclitaxel-mediated apoptosis was challenged using pharmacologic inhibitors of cell cycle progression. For these experiments, asynchronously proliferating LNCaP cells were validated to exhibit

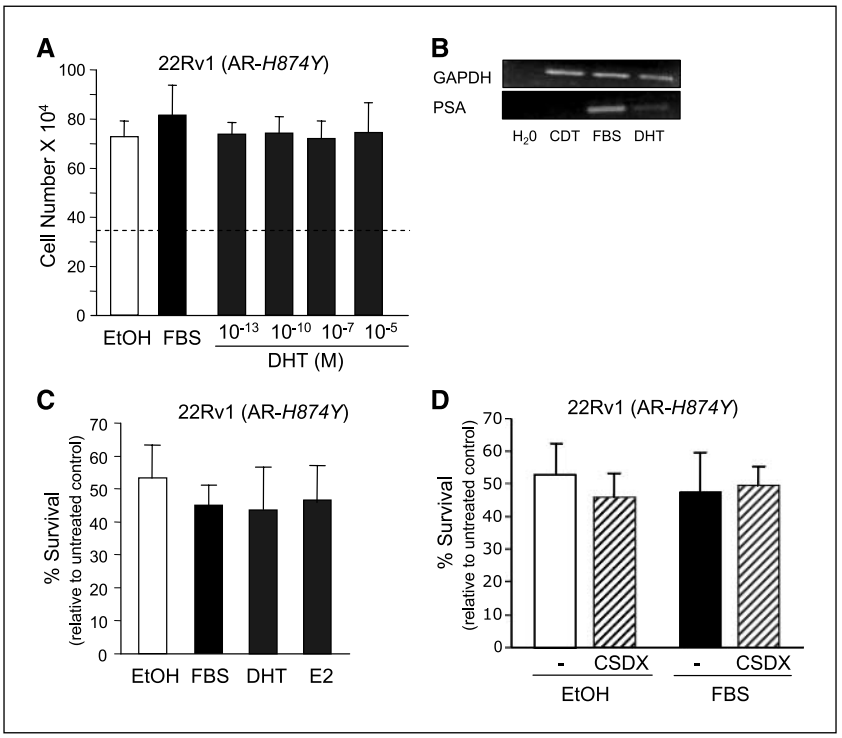


Figure 5. The mitogenic action of the AR is necessary for reduction of cell survival in the presence of paclitaxel. *A,* 3.5×10^5 22Rv1 cells were seeded (*dotted line*) in CDT + EtOH, FBS, or CDT supplemented with DHT (10^{-13}, 10^{-10}, 10^{-7}, and 10^{-5} mol/L). Viable cells were counted following 48 hours of culture. *B,* 22Rv1 cells were seeded in CDT, FBS, or CDT supplemented with DHT (10^{-10} mol/L). Total RNA was isolated and converted to cDNA and then subjected to PCR using primers specific to GAPDH (loading) or PSA. *C,* 22Rv1 cells were seeded in CDT + EtOH, FBS, CDT + 10^{-10} mol/L DHT, or CDT + 10^{-9} mol/L E2. After 24 hours of 10^{-6} mol/L paclitaxel treatment, percentage survival for each condition was determined by comparing cell viability with paclitaxel to the DMSO control in the same condition. *D,* 22Rv1 cells were seeded into CDT or FBS medium supplemented with 10^{-6} mol/L Casodex or DMSO control. Following 24 hours of 10^{-6} mol/L paclitaxel, cell survival was determined as in (*C*). All experiments were done at least twice in triplicate.

a normal cell cycle profile (FBS; Fig. 6*A*). Proliferating cells treated with the DNA polymerase inhibitor aphidicolin exhibited an early S phase (92.01% 2N), and similar effects were observed with the CDK inhibitor roscovitine (83.56% 2N: Fig. 6*A*), which arrests cells predominately in G_1 but also in S phase through its ability to inhibit CDK2 activity (47). These conditions were used to challenge the requirement of cell cycle progression on the cytotoxic response to paclitaxel (Fig. 6*B*). For these studies, LNCaP cells were pretreated in the presence of mitogenic AR agonists (FBS or DHT) and either 2 μg/mL aphidicolin or 5 μg/mL roscovitine. Subsequently, paclitaxel or DMSO control was administered, and after 24 hours of treatment, cell survival was assayed as described previously (Fig. 2*B*). In the presence of AR agonists (FBS or DHT), both aphidicolin and roscovitine markedly enhanced cell survival in the presence of paclitaxel. In both cases, forced G_1 arrest nullified the cytotoxic effect of paclitaxel. These observations validate a model in which the mitogenic capacity of AR is required to synergize with paclitaxel to sensitize prostate cancer cells to cell death.

Discussion

Prostate cancer growth and recurrence is dependent on AR activity, and ablation of AR activity is the first-line therapeutic intervention in disseminated disease. However, recent studies indicate that adjuvant, cytotoxic therapies could potentially extend patient survival (reviewed in ref. 10). Given the need to improve outcome for prostate cancer patients with metastatic disease, it is essential to discern the effect of androgen ablation therapy on cytotoxic regimens. Recent preclinical investigations assessing the effect of combinatorial and/or sequential use of androgen ablation and taxane administration on overall survival and tumor volume in xenograft models have led to divergent conclusions about androgen action. For example, Eigl et al. concluded that concurrent administration of the taxane paclitaxel and castration provided the highest efficiency (48), whereas Tang et al. showed that taxane (docetaxel) treatment was most efficacious as a neoadjuvant before castration (49). Given this discrepancy, it is imperative to define the effect of androgen action and AR activity on the response to taxane-based cytotoxic insult.

Herein, we assessed the molecular and cellular consequence of AR activity on the response to taxanes, cytotoxic agents recently validated for use in patients with recurrent prostate cancer. Our data show that AR activity is a critical determinant of the cellular response to paclitaxel. We show that AR activation significantly enhances the response to paclitaxel in AR-proficient, androgen-dependent prostate cancer cells, whereas no effect of androgen was observed in AR-proficient, androgen-refractory cells or AR-deficient prostate cancer cells (Figs. 1 and 2). Additionally, ligand-independent AR activation was also sufficient to enhance the cytotoxic effects of paclitaxel (Fig. 3). AR-enhanced loss of survival was attributed to caspase-induced cell death and was associated with p53 activation (Fig. 4). Strikingly, the ability of AR to bolster cell death in response to paclitaxel precisely requires its mitogenic function and was not simply a consequence of AR-dependent transcriptional activity (Figs. 5 and 6). Combined, these data indicate that the mitogenic action of AR on cell cycle progression is requisite for efficient paclitaxel-induced cell death and lay the molecular foundation for design of efficacious therapeutic intervention.

The mechanism of AR-enhanced cell death revealed herein is inextricably linked to its mitogenic function. The concept that AR induces cell cycle progression is well validated (38, 50), and paclitaxel is known to enhance cell death, at least in part, through its ability to prevent chromosome segregation in mitosis (16, 46) and in prostate cancer cells to require G_2-M CDK activity (51). The present data show that the ability of AR to enhance cell death in response to paclitaxel requires its capability to induce cell cycle progression, as both ligand-dependent and ligand-independent

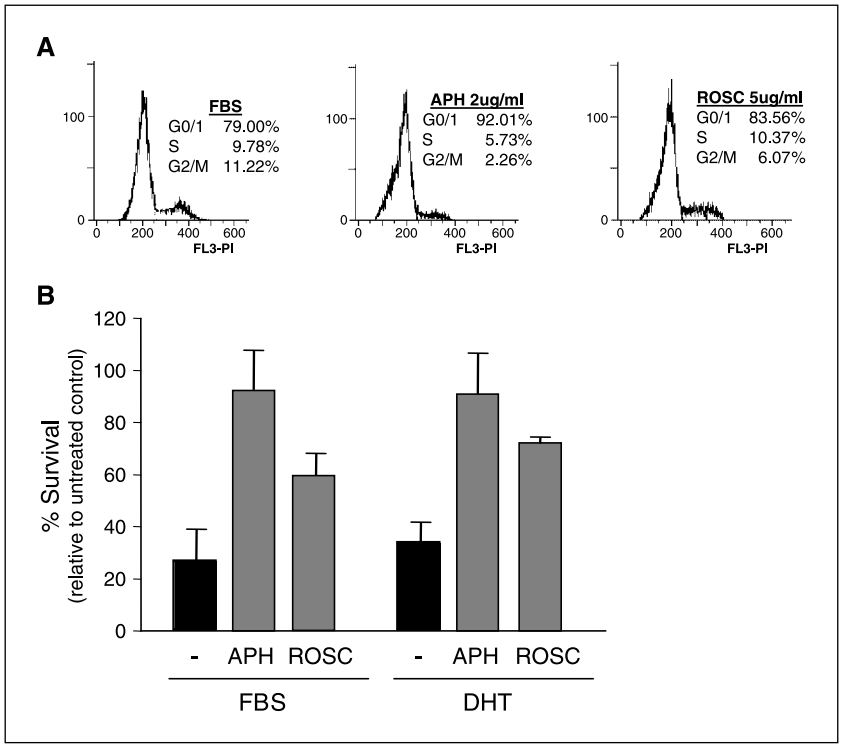


Figure 6. Cell cycle progression is necessary for enhancement of paclitaxel-mediated apoptosis, as cell cycle inhibitors reduce paclitaxel efficacy. *A,* representative cell cycle histograms of LNCaP cells either in CDT + EtOH, FBS, or FBS + 2 μg/mL aphidicolin (*APH*) or 5 μg/mL roscovitine (*ROSC*). Percentage of cells in each phase of cell cycle per treatment is noted. *B,* percentage survival of LNCaP cells seeded into FBS or CDT + 10^{-10} mol/L DHT with 2 μg/mL aphidicolin, 5 μg/mL roscovitine, or DMSO control. Following 24 hours of 10^{-6} mol/L paclitaxel treatment, cell survival was determined comparing paclitaxel-exposed cells with DMSO control in each culture condition. All experiments were done at least thrice in triplicate.

mechanisms induced AR activity and enhanced paclitaxel cytotoxicity. Additionally, high doses of androgen that strongly stimulate AR function but block cellular proliferation failed to bolster paclitaxel function. Moreover, the proapoptotic effect of androgen was nullified by AR antagonist administration or through forced cell cycle inhibition, thus validating the concept that the proproliferative function of AR underlies its ability to enhance paclitaxel action. Parallels have been observed in other systems, wherein advances in cell cycle progression can increase sensitivity to specific cytotoxic agents. For example, loss of the retinoblastoma tumor suppressor protein, RB, sensitizes cells to cell death induced by cisplatin and etoposide (52). These observations are relevant to the present study, as androgen induces cell cycle progression through induction of CDK-mediated RB phosphorylation and inactivation (38). RB inactivation is known to derepress E2F-1 function (reviewed in ref. 53), and indeed, E2F-1 expression was elevated in unchallenged cells under conditions of androgen-induced cell cycle progression (Fig. 4*D*). Interestingly, E2F-1 was specifically down-regulated in androgen-stimulated cells after paclitaxel administration; this result is consistent with observations of effective taxane treatment in head and neck cancers (43). Although the implications of this event have yet to be identified, it is intriguing that E2F-1 can regulate expression of multiple factors that control apoptosis, including Apaf-1, p73, PUMA, and Bim (54–57). In addition, the E2F-1 regulated gene *MDM2* has been implicated in controlling AR function (58); thus, potential cross-talk between these pathways is worthy of future study. Combined, these data indicate that the ability of AR to enhance paclitaxel-mediated cell death is a direct result of its mitogenic capacity.

The concept that AR enhances cell death is striking, as previous studies have implicated androgen as a survival factor under disparate conditions. For example, prostate cancer cells undergo apoptosis on androgen withdrawal *in vivo*, thus implicating AR requirement for prostate cancer maintenance (5). Moreover, androgen-dependent prostate cancer cells in culture exhibited enhanced survival in the presence of androgen after challenge with agents, such as tumor necrosis factor (TNF), Fas ligand, and okadaic acid (12). In the TNF and Fas ligand study, androgen attenuated proapoptotic Bax expression and prevented caspase-induced cell death (12). However, the present study failed to show any influence of androgen on Bax expression, and caspase-dependent cell death was enhanced in the presence of androgen after paclitaxel administration (Fig. 4). Similarly, although expression of the antiapoptotic protein bcl-2 is increased in androgen-independent prostate cancer and potentially contributes to therapeutic resistance (6, 59, 60), no alterations in bcl-2 expression or phosphorylation were discerned as a function of hormonal status (Fig. 4*D*). Together, the present data do not support a role for AR in altering paclitaxel-induced alterations in Bax or bcl-2 levels. Elevation in the expression of the cochaperone Hsp27 has also been documented as a prognostic for poor outcome and is associated with enhanced survival (14, 59). Although Hsp27 levels seemed higher in cells deprived of androgen, no significant alteration of basal Hsp27 levels was observed after paclitaxel administration under any hormonal condition (Fig. 4*D*). Thus, it is unlikely that Hsp27 contributes to the ability of AR activation to enhance paclitaxel-mediated cell death.

In contrast, several factors associated with the DNA damage response and cell cycle progression were significantly altered by androgen and paclitaxel. First, $p21^{CIP1}$ was induced by androgen (Fig. 4*D*), consistent with previous reports and consistent with the requisite ability of $p21^{CIP1}$ to activate CDK4/cyclin D1 activity in G_1 phase and promote cell cycle progression (61). Although $p21^{CIP1}$ can inhibit CDK2 activity in late G_1 in specific conditions, evidence has shown that $p21^{CIP1}$ induction correlates with enhanced cellular proliferation in prostate cancer cells (38) and is required for CDK4

function (61). Interestingly, when paclitaxel was administered in cycling (androgen stimulated) cells, $p21^{CIP1}$ was markedly reduced, perhaps reflecting the alteration in cell cycle position (G_2-M enrichment) and enhanced cell death on taxane exposure. Consistent with this idea, p53 activation was more pronounced in these same conditions as shown by increases in overall p53 levels and Ser^{15}-phosphorylated (activated) forms of the protein (Fig. 4*D*). In addition to cellular stress, p53 is induced in cells held in mitosis and is an indicator of "mitotic timing" (62). Our data are consistent with the hypothesis that increased p53 activation results from enhanced paclitaxel-mediated DNA damage and/or G_2-M accumulation in cycling (androgen stimulated) rather than arrested (androgen depleted) cells.

The fact that p53 and p21 expression levels were reciprocally induced was surprising, as *p21* is a target gene of p53 (39). However, this result is not without precedent, as the therapeutic agent and CDK inhibitor flavopiridol induces p53 activity with concurrent $p21^{CIP1}$ down-regulation in prostate cancer cells (63). Interestingly, $p21^{CIP1}$ has been shown to both protect against apoptosis [e.g., as induced by doxorubicin (64)] and enhance apoptosis (e.g., as induced by green tea polyphenols) in prostate cancer cells (65). Thus, although the mechanisms by which $p21^{CIP1}$ may affect the apoptotic response have not been elucidated, the present data indicate that induction of p53 and attenuated $p21^{CIP1}$ expression correlate with paclitaxel-induced cell killing in androgen-dependent cells. Thus, further studies should be directed at determining whether p53 and $p21^{CIP1}$ status alter hormonal influence on paclitaxel function.

In summary, although it is indisputable that androgen and the AR act as survival factors during the response to androgen ablation, our data show that the ability of AR activity to induce cellular proliferation can enhance cell death on genotoxic insult. The present data indicate that AR activation cooperates with paclitaxel to enhance cell death and that this function of AR is exquisitely dependent on its ability to promote cellular proliferation. Combined, these studies indicate that paclitaxel is likely to be most efficacious under conditions wherein AR exerts its mitogenic function, thus indicating that the response to cell cycle–dependent cytotoxic insult may be more pronounced before androgen deprivation or under specific molecular environments of heightened AR activity. Therefore, these studies provide the impetus for future investigations directed at delineating the effect of AR status on the response to cytotoxic insult.

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