Fulvestrant (ICI 182,780) down-regulates androgen receptor expression and diminishes androgenic responses in LNCaP human prostate cancer cells

Rumi S. Bhattacharyya, Aruna V. Krishnan, Srilatha Swami, and David Feldman

Division of Endocrinology, Gerontology, and Metabolism, Department of Medicine, Stanford University School of Medicine, Stanford, California

Abstract

The androgen receptor (AR) plays a key role in the development and progression of prostate cancer. Targeting the AR for down-regulation would be a useful strategy for treating prostate cancer, especially hormone-refractory or androgen-independent prostate cancer. In the present study, we showed that the antiestrogen fulvestrant [ICI 182,780 (ICI)] effectively suppressed AR expression in several human prostate cancer cells, including androgen-independent cells. In LNCaP cells, ICI (10 μmol/L) treatment decreased AR mRNA expression by 43% after 24 hours and AR protein expression by ~50% after 48 hours. We further examined the mechanism of AR down-regulation by ICI in LNCaP cells. ICI did not bind to the T877A-mutant AR present in the LNCaP cells nor did it promote proteasomal degradation of the AR. ICI did not affect AR mRNA or protein half-life. However, ICI decreased the activity of an AR promoter-luciferase reporter plasmid transfected into LNCaP cells, suggesting a direct repression of AR gene transcription. As a result of AR down-regulation by ICI, androgen induction of prostate-specific antigen mRNA and protein expression were substantially attenuated. Importantly, LNCaP cell proliferation was significantly inhibited by ICI treatment. Following 6 days of ICI treatment, a 70% growth inhibition was seen in androgen-stimulated LNCaP cells. These data show that the antiestrogen ICI is a potent AR down-regulator that causes significant inhibition of prostate cancer cell growth. Our study suggests that AR down-regulation by ICI would be an effective strategy for the treatment of all prostate cancer, especially AR-dependent androgen-independent prostate cancer. [Mol Cancer Ther 2006;5(6):1539–49]

Introduction

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer death in men in the United States (1). For patients with localized prostate cancer, initial treatment includes surgery or radiation as a means of removing or destroying tumor cells localized within the prostate capsule (2, 3). For those men not cured by primary therapy, androgen deprivation therapy is often successful in causing cancer regression because these cancers almost always express androgen receptor (AR) and exhibit androgen-dependent growth (4). Unfortunately, most men eventually fail androgen deprivation therapy and their disease transforms from androgen-dependent to an androgen-independent prostate cancer (AIPC), progressing even in the presence of castrate levels of androgens (2–4). Currently, there is no therapy that successfully treats AIPC.

AR expression is retained in a significant proportion of AIPC (5, 6). Several mechanisms or pathways influence the development of AIPC, allowing the AR to stimulate proliferation even in the absence of androgens. These include the presence of (*a*) a ''hypersensitive'' AR often resulting from AR overexpression due to gene amplification or increased sensitivity to very low androgen levels, (*b*) a ''promiscuous'' AR harboring mutations in its ligand-binding domain that allow nonandrogen ligands to bind and activate the AR, and (*c*) an ''outlaw'' AR that is activated in a ligand-independent manner often through cross-talk with other signal transduction pathways (7). Regardless of the mechanisms underlying AIPC development, the AR seems to be a key protein involved in many cases of AR-dependent AIPC and is critical for promoting prostate cancer cell growth. Therefore, targeting the AR for down-regulation or degradation could be a useful approach for decreasing AR-dependent prostate cancer cell growth and for treating AIPC (8, 9).

Estrogens and androgens are known to exert opposing effects on each other's actions in many tissues (10–12). Androgens and estrogens have also been shown to regulate the expression of each other's receptors (12–15). We have shown in earlier studies that estrogens repress AR expression in human breast cancer cells (12). In the current study, we examined the possibility that estrogenic and antiestrogenic molecules down-regulate AR expression

Received 2/2/06; revised 4/18/06; accepted 4/28/06.

Grant support: NIH grants DK42482 and CA92238 (D. Feldman) and training grant T32 DK007217 and Department of the Army grant PC050349 (R.S. Bhattacharyya).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: David Feldman, Division of Endocrinology, Department of Medicine, Stanford University School of Medicine, 300 Pasteur Drive, Stanford University Medical Center S-025, Stanford, CA 94305-5103. Phone: 650-725-2910; Fax: 650-725-7085. E-mail: feldman@cmgm.stanford.edu

doi:10.1158/1535-7163.MCT-06-0065

in prostate cancer cells. Of the compounds examined, the antiestrogen fulvestrant, also known as ICI 182,780 (ICI) or Faslodex, exhibited the most potent AR down-regulatory effect. ICI caused significant suppression of AR mRNA and protein expression, AR-mediated functional responses, and cell proliferation in LNCaP prostate cancer cells. Further studies carried out to unravel the mechanism of the down-regulation suggested that the ICI effect was due to a direct transcriptional repression of the *AR* gene.

Materials and Methods

Materials

ICI was purchased from Tocris Cookson, Inc. (Ellisville, MO). Tritiated 5α-dihydrotestosterone ([^{3}H]DHT; specific activity 50 Ci/mmol) and tritiated 1α,25-dihydroxyvitamin D_3 [[^{3}H]-1,25$(OH)_2D_3$; specific activity 106 Ci/mmol] were purchased from Amersham Biosciences (Piscataway, NJ). The synthetic androgen methyltrienolone (R1881) was obtained from DuPont NEN Life Science Products (Boston, MA). Genistein, daidzein, raloxifene, tamoxifen, cycloheximide, and actinomycin D were purchased from Sigma-Aldrich (St. Louis, MO). 5α-DHT and 17β-estradiol (E_2) were purchased from Steraloids, Inc. (Wilton, NH). LNCaP, T47D, and MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA). LAPC-4 cells were a gift from Dr. Charles Sawyers (University of California at Los Angeles, Los Angeles, CA). LN95 and LN97 cells were a generous gift from Dr. Joel Nelson (Johns Hopkins University, Baltimore, MD). 22Rv1 cells were established from a CWR22R xenograft by Sramkoski et al. (16) and were kindly provided by Dr. Zijie Sun (Stanford University, Stanford, CA). PS-341 was a gift from Millennium Pharmaceuticals (Cambridge, MA). Tissue culture media were from Mediatech (Herndon, VA). Antibiotics and fetal bovine serum (FBS) were from Invitrogen/Life Technologies (Carlsbad, CA). Charcoal-stripped serum (CSS) was purchased from Fisher Scientific (Hampton, NH).

Cell Culture

LNCaP and T47D cells were cultured in RPMI 1640 supplemented with 5% FBS and penicillin (100 units/mL) and streptomycin (100 μg/mL). LAPC-4 cells were maintained in RPMI 1640 without phenol red supplemented with 10% FBS. MCF-7 cells were cultured in DMEM/F-12 medium supplemented with 10% FBS and penicillin (100 units/mL) and streptomycin (100 μg/mL). LN95 and LN97 cells were maintained in RPMI 1640 without phenol red supplemented with 10% CSS and penicillin (100 units/mL) and streptomycin (100 μg/mL). 22Rv1 cells were cultured in RPMI 1640 supplemented with 10% FBS, 10 mmol/L HEPES, 1.0 mmol/L sodium pyruvate, 4.5 g/L glucose, 100 units/mL penicillin, and 100 μg/mL streptomycin. All cells were routinely cultured in T-75 flasks at 37°C with 5% CO_2 in a humidified incubator. For most experiments, the growth medium was replaced with phenol red–free RPMI 1640 supplemented with 5% CSS. Stock solutions of all test compounds were made in 100% ethanol and added to the treatment medium. All controls received ethanol vehicle at a concentration equal to that in the hormone-treated cells (0.1%, v/v).

Ligand-Binding Assays

Radioligand-binding assays were done using [^{3}H]DHT (for measurement of AR) or [^{3}H]-1,25$(OH)_2D_3$ [for measurement of vitamin D receptor (VDR)] as the ligand. Semiconfluent cell cultures were treated for 24 to 72 hours with various estrogenic molecules or antiestrogens. Cells were harvested, washed, and pelleted. Cell extracts were prepared by sonication of the cell pellet in a high-salt buffer followed by high-speed centrifugation as described previously (17). Aliquots of cell extracts, which contained both nuclear and cytoplasmic proteins, were incubated overnight at 4°C with saturating concentrations of [^{3}H]DHT (10 nmol/L) or [^{3}H]-1,25$(OH)_2D_3$ (1 nmol/L). Nonspecific binding was assessed in parallel assays containing 250-fold excess radioinert DHT or 1,25$(OH)_2D_3$, respectively, and subtracted from total binding to yield specific binding. Specific binding is a quantitative assessment of functional AR or VDR. Protein concentrations were quantitated using the Bradford method (18). AR or VDR concentrations were expressed as femtomoles of ligand bound per milligram of protein.

Competition Analysis

Competition-binding experiments to assess the ability of the test compounds to bind to the T877A-mutant AR present in LNCaP cells were conducted as described previously (19). Briefly, high-salt extracts from LNCaP cells were incubated with [^{3}H]DHT as the ligand and increasing concentrations (1–1,000 molar excess) of the test molecules as competitors.

Immunoblots

Aliquots of high-salt extracts prepared from cells treated with vehicle, estrogenic compounds, or antiestrogens (25–50 μg protein) were separated by NuPAGE gel electrophoresis (Invitrogen) and transferred to nitrocellulose membranes. Membranes were probed with specific primary antibodies against AR (N-20), estrogen receptor-α (ER-α; D-12), and actin (C-2, Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1,000 dilution or anti α-tubulin (clone DM1A, NeoMarkers, Fremont, CA) at a 1:5,000 dilution in 5% Blotto solution (Santa Cruz Biotechnology). Either an anti-rabbit or an anti-mouse secondary antibody was used at a 1:2,000 dilution (Cell Signaling Technology, Beverly, MA). Chemiluminescence reagents (Cell Signaling Technology) were used to visualize immunoreactive protein bands. The blots were simultaneously probed for the expression of actin or α-tubulin as a loading control.

RNA Isolation, Reverse Transcription, and Real-time PCR

RNA was isolated from control or treated cells using the Trizol reagent (Invitrogen), and total cellular RNA (5 μg) was reverse transcribed using the SuperScript III synthesis system for reverse transcription-PCR (RT-PCR; Invitrogen). An aliquot of the reverse transcription product was

amplified by real-time PCR using gene-specific primers and the DyNAmo SYBR Green PCR kit (New England Biolabs, Beverly, MA) using the Opticon 2 Real-time PCR Detection System (Bio-Rad Laboratories, Waltham, MA). Expression levels of mRNA for *AR*, *prostate-specific antigen* (*PSA*), *TATA box-binding protein*, and *glyceraldehyde-3-phosphate dehydrogenase* were measured using specific primers for each gene. The mRNA expression of TATA box-binding protein or glyceraldehyde-3-phosphate dehydrogenase was used as a control. AR primers were 5′-AGTCCCACTTGTGTCAAAAGC-3′ (forward) and 5′-ACTTCTGTTTCCCTTCAGCG-3′ (reverse). PSA primers were 5′-GCAGCATTGAACCAGAGGAG-3′ (forward) and 5′-CACCATTACAGACAAGTGGGC-3′ (reverse). TATA box-binding protein primers were 5′-TGCTGAGAAGAGTGTGCTGGAG-3′ (forward) and 5′-TCTGAATAGGCTGTGGGGTC-3′ (reverse). Glyceraldehyde-3-phosphate dehydrogenase primers were 5′-AAATCCCATCACCATCTTCC-3′ (forward) and 5′-TCTTGAGGCTGTTGTCATACTTC-3′ (reverse). Changes in gene expression were determined using the comparative $C_T(\Delta\Delta C_T)$ method as described (20).

PSA Assay

Conditioned media from control or treated LNCaP cells were collected and centrifuged at low speeds to remove cell debris. PSA concentrations in the conditioned media were determined using an ELISA kit (Diagnostic Systems Laboratories, Webster, TX) according to the manufacturer's instructions.

Cell Proliferation Assay

LNCaP cells were seeded in six-well plates at a density of 3×10^5 per well in RPMI 1640 plus 5% FBS. After 24 hours, the cultures were treated with various agents in phenol red–free RPMI 1640 supplemented with 5% CSS for the next 6 days. Media containing the treatments were replenished after 3 days. Cell proliferation was assessed by determining the DNA content at the end of the experiment (21).

Transient Transfections and Luciferase Assays

LNCaP cells were seeded into six-well plates at a cell density of 3×10^5 per well in RPMI 1640 plus 5% FBS without antibiotics. Cells were allowed to attach for 24 hours before transfection of a plasmid containing an ~6-kb AR promoter linked to a luciferase reporter in the pGL3-Basic vector (a generous gift from Dr. Donald Tindall, Mayo Clinic, Rochester, MN). Transient transfections of the AR promoter-luciferase plasmid or the pGL3-Basic vector plasmid were carried out using LipofectAMINE (Invitrogen) for 18 hours. Cells were cotransfected with a renilla luciferase plasmid (Promega, Madison, WI) as a control for transfection efficiency. Following transfections, cells were treated with vehicle (0.1% ethanol) or ICI in RPMI 1640 plus 5% CSS. After 24 hours, cells were harvested and reporter and renilla luciferase activities were determined by the Dual-Luciferase Assay System (Promega).

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism software (version 3.02) for Windows (GraphPad Software, San Diego, CA). Significance of results was determined by ANOVA and Newman-Keuls' post-test or Student's t test as appropriate. $P < 0.05$ was considered significant.

Results

AR Down-Regulation by ICI

We examined the effect of several estrogenic molecules and antiestrogens on AR expression in LNCaP human prostate cancer cells. These compounds included E_2, the phytoestrogens genistein and daidzein, selective ER modulators, such as tamoxifen and raloxifene, and the antiestrogen ICI. All of the compounds examined, with the exception of genistein, exhibited significant decreases in AR levels as measured by [^{3}H]DHT binding after a 48-hour treatment (Fig. 1A). The antiestrogen ICI (10 μmol/L) was the most effective in reducing AR expression in LNCaP cells, decreasing AR to 56% of control levels after 48 hours of treatment. Significant AR down-regulation was also seen after treatment with the phytoestrogen daidzein (67% of control) and the selective ER modulator raloxifene (66% of control). The effects of E_2 (77% of control) and tamoxifen (75% of control) were more modest. In subsequent experiments, we focused on ICI as the most effective down-regulator of AR expression.

Because the AR in LNCaP cells has a mutation in its ligand-binding domain, we determined whether ICI had any effects on cell lines harboring the wild-type AR, such as LAPC-4 human prostate cancer cells and T47D breast cancer cells. In both the LAPC-4 and T47D cells, ICI (10 μmol/L) elicited smaller but statistically significant decreases (15–25% inhibition) in [^{3}H]DHT binding (Fig. 1B), showing that ICI down-regulated the expression of both mutant and wild-type AR. We also examined the effects of ICI on AR expression in AIPC cells, such as 22Rv1, LN95, and LN97. The 22Rv1 cell line was established from the CWR22R human prostate cancer xenograft, which was serially propagated in mice after castration-induced regression and relapse of the parental androgen-dependent CWR22 xenograft (16). 22Rv1 cells represent an androgen-independent but androgen-responsive cell line (16, 22). We also used the LN95 and LN97 androgen-independent human prostate cancer cells. These cells are LNCaP sublines that were established by culturing LNCaP cells under androgen-deprived conditions for prolonged periods. These LNCaP sublines can readily form tumors in both castrate and intact male athymic *nu/nu* mice (23). In all three cell lines examined, ICI (10 μmol/L) decreased [^{3}H]DHT binding after 48 hours of treatment (20–25% inhibition; Fig. 1B). We also found ~30% decrease in AR mRNA levels in 22Rv1 cells following ICI treatment for 6 to 24 hours (data not shown). These results suggest that ICI decreases AR expression in both androgen-dependent and AIPC cells.

Because the antiestrogen ICI is known to down-regulate ER levels (24) and we showed down-regulation of AR, we wished to determine whether ICI modulated the expression

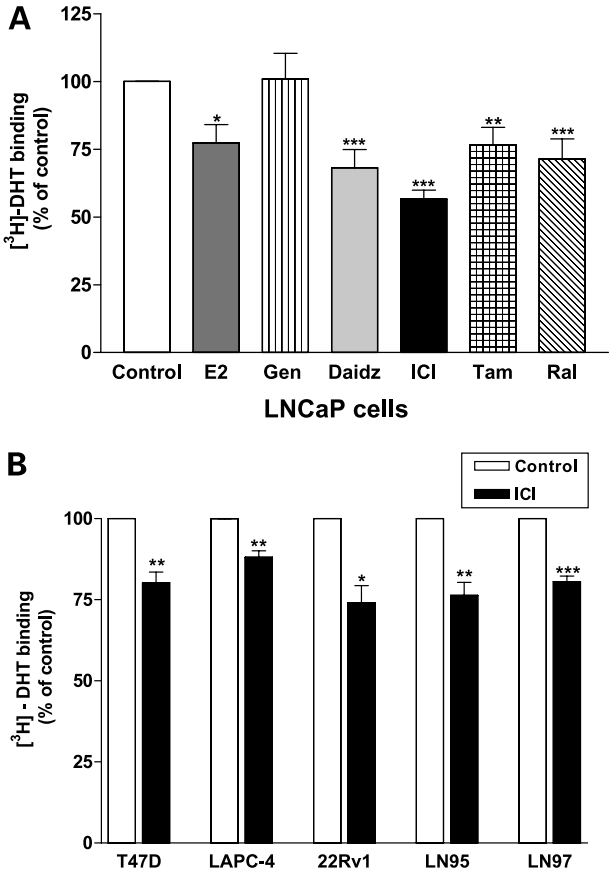

Figure 1. AR down-regulation by ICI. **A**, LNCaP cells were treated for 48 h with 0.1% ethanol vehicle (control) or the following estrogenic compounds: 10 nmol/L E_2, 10 μmol/L genistein (*Gen*), 10 μmol/L daidzein (*Daidz*), 10 μmol/L ICI, 10 μmol/L tamoxifen (*Tam*), or 10 μmol/L raloxifene (*Ral*). AR levels were determined by measuring [^{3}H]DHT binding in high-salt cellular extracts. Data are represented as % of specific binding in control cells set at 100%, which corresponded to 194 ± 70 fmol/mg. **B**, T47D, LAPC-4, 22Rv1, LN95, and LN97 cells were treated for 48 h with 0.1% ethanol vehicle (control) or 10 μmol/L ICI. AR levels were determined by measuring [^{3}H]DHT binding as described above. Specific binding in control cells (set at 100%) was equivalent to 77 ± 8 fmol/mg in T47D cells, 237 ± 50 fmol/mg in LAPC-4 cells, 30 ± 3 fmol/mg in 22Rv1 cells, 209 ± 12 fmol/mg in LN95 cells, and 240 ± 28 fmol/mg in LN97 cells. *Columns,* mean from 2 to 10 determinations; *bars,* SE. *, $P < 0.05$ versus control; **, $P < 0.01$ versus control; ***, $P < 0.001$ versus control.

of other nuclear receptors as well. We examined the ICI effect on VDR levels in LNCaP cells. ICI treatment of LNCaP cells for 48 hours did not significantly change VDR levels as measured by [^{3}H]-1,25$(OH)_2D_3$ binding (control VDR levels, 9.87 ± 2.9 fmol/mg; ICI-treated VDR levels, 9.98 ± 1.7 fmol/mg). These data suggest that the ICI-mediated down-regulation is not a general effect on all nuclear receptors.

Time Course and Dose Response of AR Protein Down-Regulation

The effect of ICI on AR protein expression was determined by both [^{3}H]DHT binding and Western blot analysis using an anti-AR antibody (Santa Cruz Biotechnology). Time course experiments in LNCaP cells showed that ICI (10 μmol/L) decreased [^{3}H]DHT binding by 41% after 24 hours of treatment (Fig. 2A). Maximal inhibition (52%) was seen at 48 hours, and the inhibitory effect persisted up to 72 hours. Western blot analysis revealed similar decreases in AR immunoreactive bands at 24, 48, and 72 hours following ICI treatment (Fig. 2B). Densitometric analysis of the Western blot shown in Fig. 2B showed that the decreases in AR protein seen at the various time points agreed with the results of the ligand-binding data (an ~50% decrease in AR/actin expression compared with control at each time point). Dose-response studies showed that ICI at 5 and 10 μmol/L significantly decreased [^{3}H]DHT binding by 20% and 50% of control, respectively (Fig. 2C). Higher concentrations of ICI (100 μmol/L) did not further down-regulate AR levels. We also examined the effects of ICI on AR protein expression under androgen-replete conditions in the presence of medium supplemented with FBS. After 48 hours, ICI (10 μmol/L) inhibited [^{3}H]DHT binding by 55% of control (Fig. 2D). These responses are similar to the effects of ICI observed under androgen-depleted conditions (CSS; Fig. 2A).

Lack of ICI Effect on AR Protein Degradation

Previous studies on the effects of ICI on ER have shown that ICI binds to the ER and prevents estrogen signaling, thereby acting as an antiestrogen (24). At the same time, by binding to the ER, ICI decreases the stability of the ER-α protein and promotes ER-α degradation by the proteasome (25, 26). The AR expressed in LNCaP cells contains a point mutation (T877A) in its ligand-binding domain that relaxes its specificity and allows nonandrogenic ligands to bind to and activate the receptor (27). Several nonandrogenic steroids, such as progestins and glucocorticoids, as well as various estrogenic molecules have been shown to bind to the T877A-mutant AR (28, 29). Therefore, we examined whether ICI could bind to the T877A-mutant AR in LNCaP cells and thereby promote AR protein degradation in a manner similar to its effects on the ER. Competition-binding assays were done in nuclear extracts of LNCaP cells using [^{3}H]DHT as the ligand and increasing concentrations of radioinert DHT, E_2, and ICI as competitors. Unlike radioinert DHT and E_2, increasing concentrations of ICI up to a 1,000-fold excess of [^{3}H]DHT did not displace [^{3}H]DHT bound to the AR, showing that ICI did not bind to the mutant AR (Fig. 3A).

We examined the effect of the proteasomal inhibitor PS-341 (bortezomib, Velcade) on AR down-regulation by ICI (30). The ICI effect on ER-α protein degradation in MCF-7 breast cancer cells was assessed in parallel assays as a positive control. LNCaP or MCF-7 cells were treated with ICI (10 μmol/L) in the presence or absence of PS-341 (100 nmol/L) for 24 to 48 hours. High-salt cellular extracts were made, and AR and ER-α protein expression were determined by Western blot analysis. Immunoreactive ER-α protein was decreased after 24 hours of ICI treatment in MCF-7 cells (Fig. 3B). As expected in the presence of the proteasomal inhibitor PS-341, the down-regulation of ER-α by ICI was abolished. PS-341 treatment by itself caused a

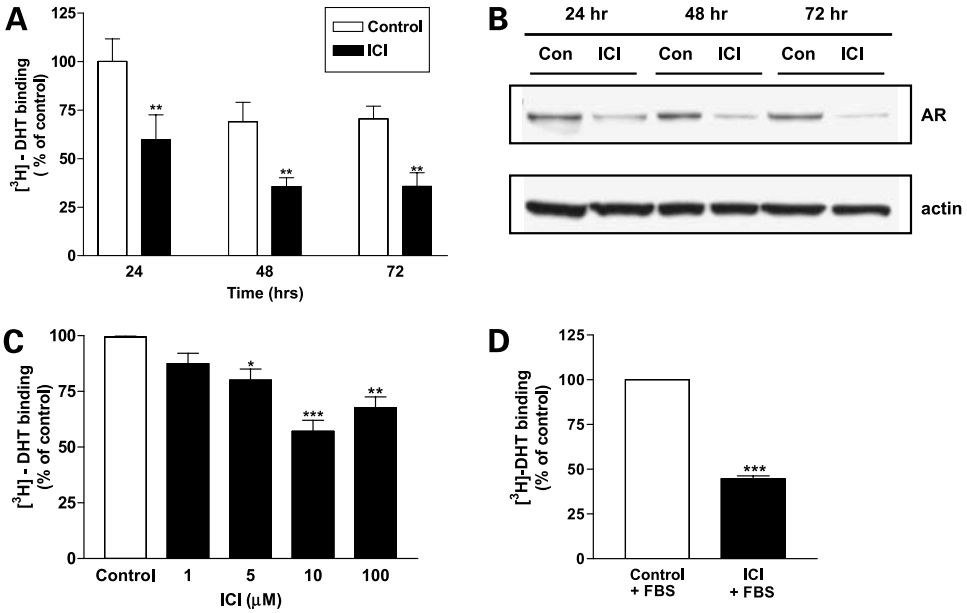

Figure 2. Time course and dose response of AR protein down-regulation. LNCaP cells were treated for 24 to 72 h with 0.1% ethanol vehicle (control) or 10 μmol/L ICI. **A,** AR levels were determined by measuring [^{3}H]DHT binding in high-salt cellular extracts. Data are represented as % of specific binding in 24-h control cells set at 100%, which was equivalent to 238 ± 28 fmol/mg protein. **B,** AR levels were determined by Western blot analysis with the AR N-20 monoclonal antibody (Santa Cruz Biotechnology). Equal amounts of protein were loaded into each lane (50 μg), and immunoreactive actin was used as a control. The experiment was carried out thrice. One representative blot. **C,** LNCaP cells were treated for 48 h with 0.1% ethanol vehicle (control) or 1 to 100 μmol/L ICI. AR levels were determined by measuring [^{3}H]DHT binding in high-salt cellular extracts. Data are represented as % of specific binding in control cells set at 100%, which was equivalent to 169 ± 60 fmol/mg protein. **D,** LNCaP cells were treated for 48 h with 0.1% ethanol vehicle (control) or 10 μmol/L ICI in RPMI 1640 supplemented with 5% FBS. AR levels were determined by measuring [^{3}H]DHT binding in high-salt cellular extracts. Data are represented as % of specific binding in control cells set at 100%, which was equivalent to 220 ± 26 fmol/mg protein. *Columns,* mean from two to five determinations; *bars,* SE. *, $P < 0.05$ versus control; **, $P < 0.01$ versus control; ***, $P < 0.001$ versus control.

slight decrease in AR protein in LNCaP cells (Fig. 3B). Cotreatment with PS-341 did not prevent the decrease in AR protein levels elicited by ICI, suggesting the lack of involvement of the proteasomal pathway in AR down-regulation by ICI.

We also determined whether ICI treatment altered AR protein half-life ($t_{1/2}$). After 24 hours of 0.1% ethanol vehicle (control) or ICI (10 μmol/L) treatment, LNCaP cells were treated with the protein synthesis inhibitor cycloheximide at 2.5 μg/mL. Cells were harvested at 2-hour intervals following cycloheximide treatment, and AR protein expression was examined by Western blotting analysis (Fig. 3C). ICI treatment did not significantly change AR protein $t_{1/2}$, suggesting that ICI does not have any post-translational effects on AR down-regulation.

Inhibition of AR mRNA Expression and AR Transcription by ICI

We examined the effects of ICI on AR mRNA expression by using real-time RT-PCR analysis. Time course experiments showed that AR mRNA expression in LNCaP cells was decreased by 34% as early as 4 hours after ICI (10 μmol/L) treatment (Fig. 4A). These inhibitory effects persisted after 6 and 24 hours of ICI treatment, producing a 40% to 45% decrease in AR mRNA expression. By 48 hours of ICI treatment, the down-regulatory effects of ICI subsided. In parallel, we measured VDR mRNA expression after ICI treatment and no significant changes in VDR mRNA were apparent (data not shown), suggesting the selectivity of the down-regulatory effects of ICI on AR mRNA.

We carried out the following experiments to distinguish between transcriptional and post-transcriptional effects of ICI. LNCaP cells were treated with 10 μmol/L ICI in the presence and absence of various concentrations (up to 1 μg/mL) of the protein synthesis inhibitor cycloheximide, and AR mRNA levels were measured. Figure 4B illustrates the lack of an effect of 0.25 μg/mL cycloheximide on ICI down-regulation of AR mRNA, showing that new protein synthesis was not required for the suppression of AR mRNA by ICI.

Figure 4C shows the effect of ICI on AR mRNA $t_{1/2}$. LNCaP cells were treated with vehicle or ICI (10 μmol/L). After 24 hours of ICI treatment, the transcriptional inhibitor actinomycin D (4 μmol/L) was added to the cultures. Cells were harvested at 2-hour intervals (following the addition of actinomycin D), and AR mRNA levels were measured by real-time RT-PCR. Our results suggest that ICI did not significantly change AR mRNA $t_{1/2}$.

The time course of AR mRNA repression by ICI showed that the effect was seen as early as 4 hours (Fig. 4A). This fact, coupled with the lack of change in mRNA $t_{1/2}$, suggests that ICI may be directly repressing the *AR* gene at

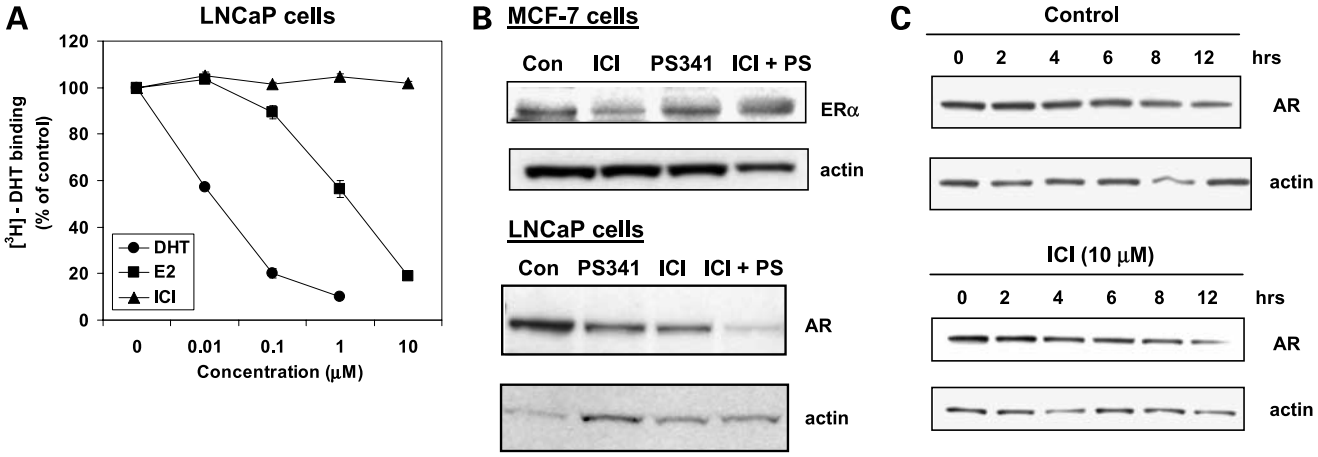

Figure 3. Lack of ICI effect on AR protein degradation. **A**, high-salt extracts from LNCaP cells were incubated with increasing concentrations of unlabeled DHT, E_2, and ICI in the presence of [^{3}H]DHT (10 nmol/L). Data are represented as % of specific binding in the absence of competitors set at 100%, which was equivalent to 286 ± 92 fmol/mg protein. *Points,* mean of two determinations; *bars,* SE. **B,** MCF-7 or LNCaP cells were treated with 0.1% ethanol vehicle [control (*Con*)], 100 nmol/L PS-341, 10 μmol/L ICI, or ICI + PS-341 for 24 (MCF-7) or 48 (LNCaP) h. Protein expression of ER-α and AR was determined by Western blot analysis using the ER-α D-12 and the AR N-20 monoclonal antibody, respectively. Actin expression was used as a control. **C,** LNCaP cells were treated with 0.1% ethanol vehicle (control) or 10 μmol/L ICI for 24 h. After 24 h, cycloheximide (2.5 μg/mL) was added to all cultures and cells were harvested at 2-h intervals (0–12 h). AR protein expression was examined by Western blot analysis as described in **B. B** and **C,** experiments were carried out thrice. Representative blots.

the transcriptional level. To determine whether ICI acted directly on the AR promoter to inhibit AR gene transcription, we examined the effect of ICI on the activity of an AR promoter-reporter construct transfected into LNCaP cells. LNCaP cells were transiently transfected with a plasmid containing an ~6-kb AR promoter fragment linked to a luciferase reporter plasmid. A renilla luciferase plasmid (pRL) was used as a control for transfection efficiency. Transfected cells were treated with either vehicle or ICI (10 μmol/L) for 24 hours, and reporter and renilla luciferase activities were measured. ICI treatment significantly decreased AR promoter-luciferase activity compared with vehicle treatment (Fig. 4D).

Inhibition of AR-Mediated Functional Responses by ICI

We next assessed whether the decrease in AR levels due to ICI treatment would result in the attenuation of AR-mediated functional responses in LNCaP cells. The effect of ICI on androgen-induced PSA mRNA expression and PSA secretion was determined. LNCaP cells were treated with ICI (10 μmol/L) in the presence or absence of the synthetic androgen R1881. R1881 (0.1–10 nmol/L) substantially induced PSA mRNA expression in LNCaP cells (Fig. 5A). Cotreatment with ICI totally abolished the induction of PSA mRNA seen at 0.1 nmol/L R1881 and restored the values to control levels. ICI partially reversed the induction seen at higher concentrations (1 and 10 nmol/L) of R1881 (~20% inhibition). ICI treatment alone seemed to slightly decrease basal PSA mRNA expression.

We also examined the effect of ICI on basal as well as androgen-stimulated secretion of PSA protein (Fig. 5B). LNCaP cells were treated for 6 days with 0.1% ethanol vehicle or 0.1 nmol/L R1881 with and without ICI (1–10 μmol/L) cotreatment. Conditioned media from treated cells were collected during the last 3 days of treatment, and PSA concentrations were determined by an ELISA. Treatment with 0.1 nmol/L R1881 resulted in an ~3-fold increase in the levels of secreted PSA. ICI dose dependently inhibited androgen-stimulated PSA secretion. The lowest concentration of ICI (1 μmol/L) elicited a 55% inhibition in the R1881-stimulated PSA secretion, and the highest concentration of ICI examined (10 μmol/L) decreased PSA secretion by 90% (Fig. 5B). As shown in Fig. 5C, ICI treatment alone decreased basal PSA secretion to almost undetectable levels. A higher concentration of R1881 (10 nmol/L) caused a much more substantial increase (~25-fold over control) in secreted PSA, which was significantly decreased (to ~5-fold over control) by ICI cotreatment (Fig. 5C).

Inhibition of Androgen-Stimulated LNCaP Cell Proliferation by ICI

We next examined the ability of ICI to decrease androgen stimulation of LNCaP cell proliferation. LNCaP cells were cultured for 6 days in CSS medium containing various concentrations of ICI (1–50 μmol/L). ICI at 1 μmol/L significantly decreased basal cell proliferation by 32% (Fig. 6A). This inhibitory effect of ICI was maximal at 10 μmol/L, producing a 48% inhibition in cell growth, an effect that was not further increased by 50 μmol/L ICI (42% inhibition). The ICI effect was also examined in LNCaP cells stimulated with R1881. LNCaP cells were cultured for 6 days in medium containing 5% CSS treated with R1881 (0.1 nmol/L) in the presence or absence of ICI (10 μmol/L; Fig. 6B). R1881 at a low concentration of 0.1 nmol/L stimulated the proliferation of LNCaP cells. ICI (10 μmol/L) alone significantly inhibited basal LNCaP cell proliferation. ICI also significantly

attenuated R1881-stimulated growth by ~70% (Fig. 6B). Western blotting analysis confirmed that AR expression remained suppressed following 6 days of ICI treatment. The blots were simultaneously probed with either actin or α-tubulin as the loading control. In this experiment shown in Figure 6B, the actin levels showed variations at the end of 6-day hormone treatments. Therefore, we used α-tubulin to normalize the data.

Additionally, we compared the growth-inhibitory effects of ICI with the antiandrogen Casodex (bicalutamide). LNCaP cells were treated for 6 days with ICI (10 μmol/L) or Casodex (1–10 μmol/L) in the presence or absence of 0.1 nmol/L R1881 (Fig. 6C). Both ICI and Casodex suppressed androgen stimulation of cell growth. The degree of inhibition of R1881-stimulated cell proliferation by ICI was comparable with that of 10 μmol/L Casodex at the end of 6 days of treatment. A lower concentration of Casodex (1 μmol/L) exhibited lesser inhibitory effects on androgen-stimulated cell proliferation. AR protein expression was again significantly decreased following ICI treatment for 6 days.

Discussion

A significant proportion of AIPC exhibits AR expression (31, 32), and some AIPC patients express up to 70-fold higher levels of AR mRNA and increased AR protein compared with primary prostate cancer patients (31, 33). In a recent study by Chen et al. (32), AR mRNA was found to be universally up-regulated in all of the hormone-refractory prostate cancer (AIPC) models that were examined. Because the AR is clearly a critical factor in prostate cancer and AIPC development, down-regulating or reducing the AR would be a very useful strategy for treating AR-dependent prostate cancer (8). Thus far, the techniques that have been used to down-regulate the AR include antisense oligonucleotides (34, 35), ribozyme treatments (36, 37), AR dominant negatives (38), and small interfering RNAs (39–41). Reducing AR levels by these various means results in the inhibition of prostate cancer cell growth and PSA expression, while the small interfering RNA knockdown of AR expression also leads to significant apoptotic cell death in androgen-sensitive and AIPC cells (39–41). Various studies have shown that estrogens have the ability

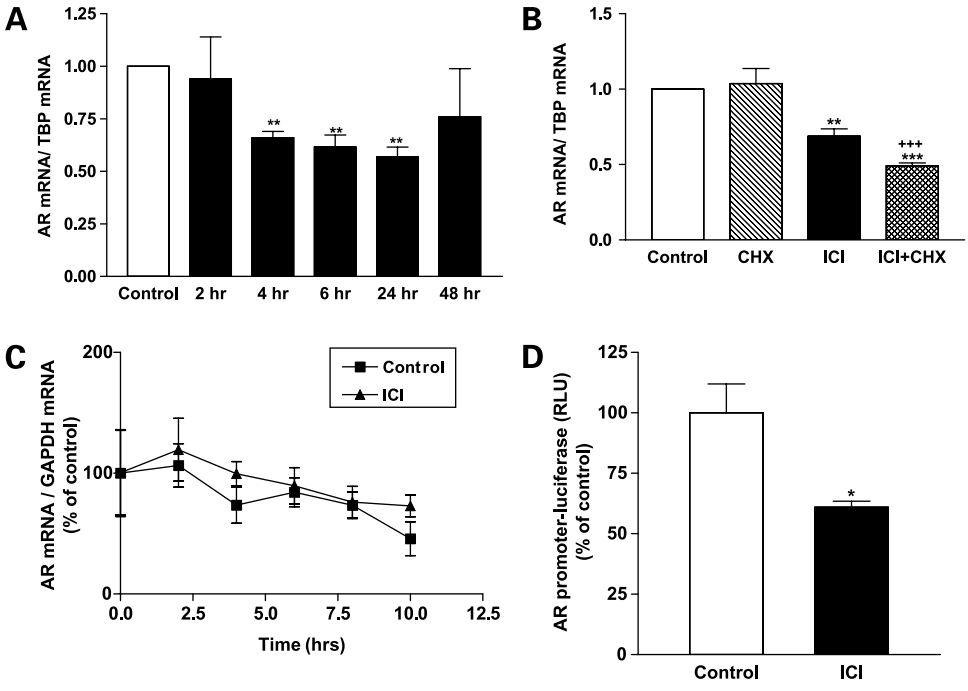

Figure 4. Inhibition of AR mRNA expression and AR transcription by ICI. **A,** LNCaP cells were treated for 2 to 48 h with 0.1% ethanol vehicle (control) or 10 μmol/L ICI. AR mRNA levels were determined by real-time RT-PCR analysis. Data are represented as fold change in gene expression relative to the 0-h control. **B,** LNCaP cells were treated for 24 h with 0.1% ethanol vehicle (control), 0.25 μg/mL cycloheximide (*CHX*), 10 μmol/L ICI, or both (ICI + cycloheximide). AR mRNA expression was measured by real-time RT-PCR analysis. Data are represented as fold change in gene expression relative to the control cells. **C,** LNCaP cells were treated with 0.1% ethanol vehicle (control) or ICI for 24 h. After 24 h, 4 μmol/L actinomycin was added to all cultures and cells were harvested at 2-h intervals (0–10 h). AR mRNA expression was measured by real-time RT-PCR analysis. Results were normalized to glyceraldehyde-3-phosphate dehydrogenase expression. Data are represented as % of control relative to AR/glyceraldehyde-3-phosphate dehydrogenase mRNA levels at time = 0 for both control and ICI-treated samples. *Points,* mean for triplicate samples; *bars,* SD. The individual experiment was conducted thrice. Representative experiment. **D,** LNCaP cells were transiently transfected with an ~6-kb AR promoter-luciferase reporter plasmid and renilla luciferase plasmid (pRL null). Eighteen h after transfection, cells were treated with 0.1% ethanol vehicle (control) or 10 μmol/L ICI for 24 h. Luciferase activity was measured using the Dual-Luciferase Reporter Assay kit. Relative luciferase activity (*RLU*) represents the ratio of AR promoter-luciferase activity to the corresponding renilla luciferase. Values are given as a % of relative luciferase activity in the control treatment (100%). **A** and **B,** results were normalized to expression of the *TATA box-binding protein* (*TBP*) gene. *Columns,* mean of two to eight determinations; *bars,* SE. *, $P < 0.05$ versus control; **, $P < 0.01$ versus control; ***, $P < 0.001$ versus control; $^{+++}$, $P < 0.001$ versus cycloheximide.

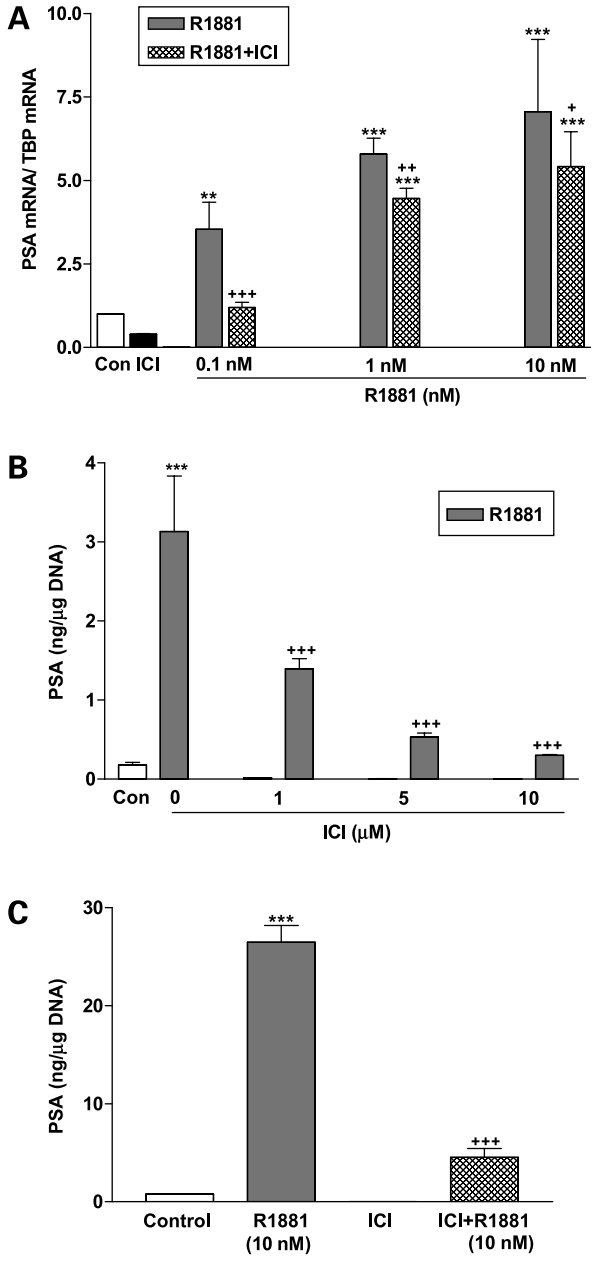

Figure 5. Inhibition of AR-mediated functional responses by ICI. **A,** LNCaP cells were treated with 0.1% ethanol vehicle (control), 0.1 to 10 nmol/L R1881, 10 μmol/L ICI, or both (R1881 + ICI) for 24 h. PSA mRNA levels were determined by real-time RT-PCR analysis. Data are represented as fold change in gene expression relative to the ethanol vehicle control. Results were normalized to expression of TATA box-binding protein. **B,** LNCaP cells were seeded in six-well plates at 3×10^5 per well in RPMI 1640 + 5% FBS. After 24 h, cells were treated with various concentrations of ICI in RPMI 1640 + 5% CSS in the presence of 0.1% ethanol (control; *white columns*) or 0.1 nmol/L R1881 (*black columns*). Fresh media and reagents were replenished on day 4. At the end of 6 d of treatment, PSA secretion in the medium was measured using an ELISA. **C,** LNCaP cells were cultured as in **B**. The treatments were control = 0.1% ethanol vehicle, 10 nmol/L R1881, 10 μmol/L ICI, or both (R1881 + ICI). Values are given as ng PSA secreted per μg DNA. *Columns,* mean of two to three determinations; *bars,* SE. **, $P < 0.01$ versus control; ***, $P < 0.001$ versus control; $^{+}$, $P < 0.05$ versus R1881; $^{++}$, $P < 0.01$ versus R1881; $^{+++}$, $P < 0.001$ versus R1881.

to down-regulate AR expression in different target cells (12, 14, 42). In particular, studies from our laboratory have shown that MCF-7 breast cancer cells treated with E_2 exhibited significantly lower levels of AR (12). The current study examined the effect of estrogenic compounds and antiestrogens on AR expression in prostate cancer cells. In our study, the order of potencies for AR down-regulation was ICI > daidzein > raloxifene > tamoxifen > E_2. The selective ER modulators tamoxifen and raloxifene have been shown to cause apoptosis of prostate cancer cells (43, 44). Zeng et al. (45) showed that raloxifene treatment of probasin/SV40 T antigen transgenic rats caused significant inhibition of prostate carcinogenesis and was associated with decreased AR expression in the ventral prostate. Although genistein has been shown to decrease AR levels in prostate cancer cells (46), we did not find an appreciable change in AR concentration following genistein treatment. Our study is the first to show the ICI effect to down-regulate AR in prostate cancer cells. In addition to LNCaP cells, ICI-mediated down-regulation was also seen in cells that express wild-type AR, such as the LAPC-4 prostate cancer and the T47D breast cancer cells. Furthermore, AIPC cells, such as the LNCaP sublines LN95 and LN97 as well as 22Rv1, also responded to ICI with AR down-regulation, suggesting that ICI might be therapeutically useful in AIPC. Importantly, we also showed that ICI significantly decreased both androgen-stimulated cell proliferation and PSA expression in LNCaP cells.

ICI is a potent antiestrogen that binds to the ER with high affinity (47) and impairs both ER dimerization (48) and nucleocytoplasmic shuttling (49). ICI binding to ER-α leads to decreased ER-α stability and increased turnover of the ER-α protein through enhanced proteasomal degradation (25, 26). Because the mutant AR in LNCaP cells binds to estrogenic compounds (27), it was possible that ICI could bind to the mutant AR and enhance its proteasomal degradation in a manner similar to its effect on ER-α. However, our data indicated that ICI did not bind to the mutant T877A AR (Fig. 3A), did not promote AR degradation through the proteasomal pathway (Fig. 3B), and did not change AR protein $t_{1/2}$ (Fig. 3C). The down-regulation of AR mRNA by ICI was not prevented by the addition of the protein synthesis inhibitor cycloheximide, indicating that new protein synthesis was not required (Fig. 4B). AR mRNA $t_{1/2}$ was also not altered by ICI treatment (Fig. 4C). Taken together, these data suggest that the ICI down-regulation of AR is not at a post-transcriptional level. We further showed that ICI directly suppressed the activity of an AR promoter-luciferase construct transfected into LNCaP cells (Fig. 4D). These data indicate that ICI down-regulation is due to a direct transcriptional repression of the *AR* gene.

The exact mechanism of the transcriptional repression of AR by ICI has yet to be defined. The effect of ICI on AR transcription might be mediated through the ER. LNCaP cells have been shown to express ER-β (50, 51). We have detected ER-β protein and mRNA expression in LNCaP cells, whereas ER-α protein was undetectable (data not

shown). It is therefore possible that ICI suppresses AR transcription by acting through ER-β via one or more estrogen response elements present in the AR promoter. An initial computer analysis of the AR promoter has revealed several potential estrogen response element sites. Although ICI down-regulates ER-α expression, ER-β protein is not always similarly degraded after ICI treatment (52, 53). Several studies have examined the role of ER-β in the regulation of AR and prostate cancer cell proliferation by other estrogenic molecules. Bektic et al. (46) have shown that AR down-regulation by genistein in LNCaP cells is mediated through ER-β. ER-β also plays an important role in the induction of LNCaP cell proliferation by 5α-DHT and E_2 (54). However, very recently, Taylor et al. (55) showed that estradiol down-regulates AR protein expression in the ventral prostate of both ER-α and ER-β knockout mice, arguing against a role for ER-α or ER-β in the down-regulation of AR. ICI is also capable of acting through progesterone response elements in the promoters of target genes (56). Further experiments need to be done to fully elucidate the mechanism of AR transcriptional repression by ICI.

Our data further showed that, as a consequence of down-regulating AR expression, ICI inhibited AR-mediated functional responses. Androgen stimulation of PSA mRNA expression and PSA protein secretion were both decreased by ICI in LNCaP cells (Fig. 5). Recent studies by Kawashima et al. (57) showed that ICI decreased DHT stimulation of the androgen-responsive mouse mammary tumor virus-luciferase reporter in LNCaP cells. ER ligands, including ICI, have been shown to inhibit DHT stimulation of PSA transcriptional activity in PC-3 and DU145 cells cotransfected with AR and ER-α and ER-β expression plasmids (58). Similarly, we have found that ICI inhibited R1881-mediated stimulation of a PSA-luciferase reporter plasmid transfected into LNCaP cells (data not shown). Importantly, ICI caused a significant inhibition of cell proliferation. Androgens exert a biphasic effect on LNCaP cell growth with low concentrations (<0.1 nmol/L) exhibiting growth stimulation, whereas higher concentrations inhibit growth (59). ICI completely blocked the growth stimulation seen with 0.1 nmol/L R1881 (Fig. 6B). The AR is critical for prostate cancer cell growth, and cell proliferation is significantly decreased in prostate cancer cells where AR expression has been decreased or disrupted (39–41). In our study, ICI also caused significant growth

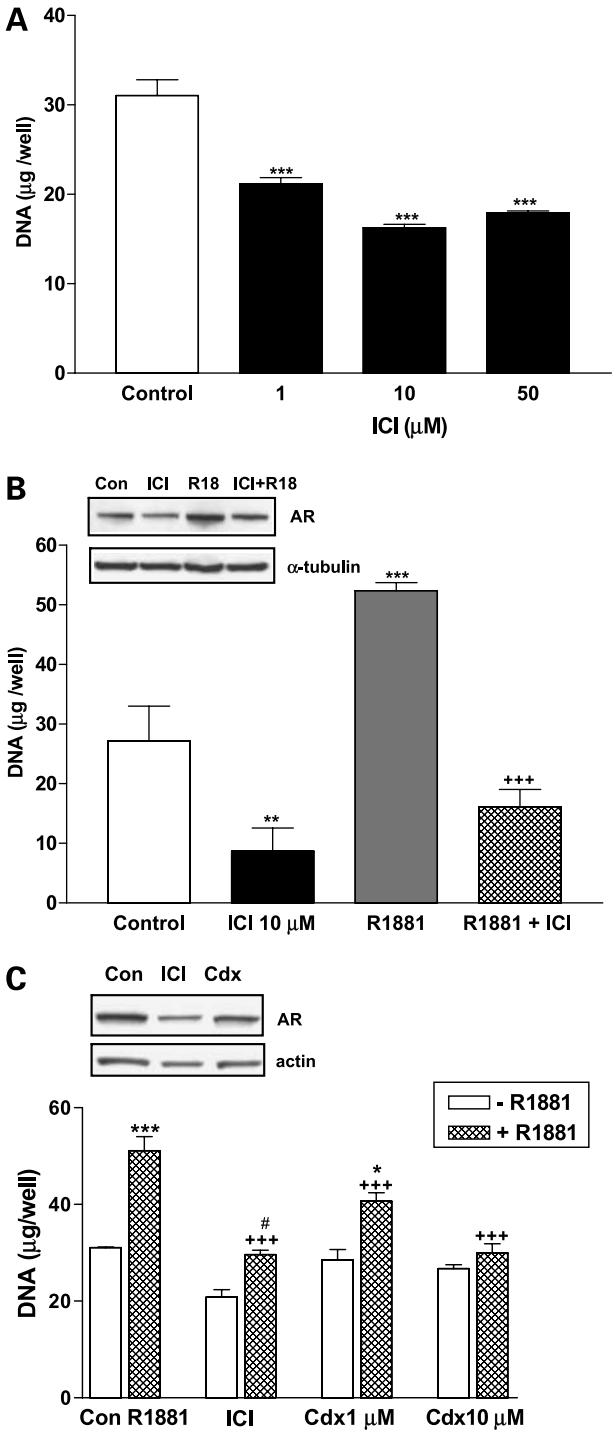

Figure 6. Inhibition of androgen-stimulated LNCaP cell proliferation by ICI. LNCaP cells were seeded in six-well plates at 3×10^5 per well in RPMI 1640 + 5% FBS. **A**, after 24 h, cells were treated with 0.1% ethanol (control) or various concentrations of ICI (1–50 μmol/L) in RPMI 1640 + 5% CSS. Fresh media and reagents were replenished on day 4. After 6 d of treatment, cell proliferation was measured by assaying the DNA content in the well. **B**, LNCaP cells were seeded and cultured as in **A**. The treatments were control = 0.1% ethanol vehicle, 10 μmol/L ICI, 0.1 nmol/L R1881 (*R18*), or both (R1881 + ICI). DNA content was measured after 6 d of treatment. *Inset,* AR and α-tubulin protein expression as determined by Western blot analysis (see Materials and Methods). **C**, LNCaP cells were seeded and cultured as in **A**. The treatments were control = 0.1% ethanol vehicle, 10 μmol/L ICI, and 1 and 10 μmol/L Casodex (*Cdx*), all in the presence (*black columns*) or absence (*white columns*) of 0.1 nmol/L R1881. DNA content was measured after 6 d of treatment. *Inset,* AR and actin protein expression as determined by Western blot analysis (see Materials and Methods). **B** and **C**, representative blots. *Columns,* mean of three determinations; *bars,* SE. *, $P < 0.05$ versus control; **, $P < 0.01$ versus control; ***, $P < 0.001$ versus control; $^{+++}$, $P < 0.001$ versus R1881; $^{\#}$, $P < 0.05$ versus Casodex (1 μmol/L) + R1881.

inhibition under basal conditions possibly due to a blockade of the stimulatory effect of residual androgens present in the CSS. However, other mechanisms, in addition to AR down-regulation, may also be contributing to the inhibition of cell growth by ICI. Lau et al. (50) showed growth-inhibitory effects of ICI on both PC-3 and DU145 prostate cancer cells that do not express AR. Their study concluded that the decrease in cell growth generated by ICI was mediated through ER-β. It is probable that multiple mechanisms are involved in the growth-inhibitory effects of ICI, including regulation of other signaling pathways (60). However, based on our data and that of others (34–41), we believe that the down-regulation of the AR plays an important role in the growth-inhibitory action of ICI.

AIPC is a lethal form of prostate cancer, and effective treatment options have yet to be established. We hypothesize that reducing AR concentration will be a useful therapeutic strategy in all cases of prostate cancer but especially in AIPC. ICI (fulvestrant) is a drug currently used to treat women with ER-positive metastatic breast cancer. ICI is relatively safe and well tolerated by women with advanced breast cancer. Our findings suggest that ICI may present a useful treatment option for patients with AR-dependent prostate cancer. Unlike the ribozyme, antisense, small interfering RNA, or dominant-negative techniques, ICI, as an already approved drug, can be rapidly moved to clinical trials in prostate cancer patients. A therapy that down-regulates the AR in AR-dependent AIPC would be particularly beneficial at a time in the course of prostate cancer, where effective therapies are currently not available.

Acknowledgments

We thank Dr. Donald Tindall for the AR promoter-luciferase reporter plasmid, Dr. Charles Sawyers for the LAPC-4 prostate cancer cells, Dr. Joel Nelson for the LN95 and LN97 cells, and Millennium Pharmaceuticals for the PS-341 (bortezomib, Velcade).

References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. CA Cancer J Clin 2006;56:106–30.

2. Taplin ME, Ho SM. Clinical review 134: the endocrinology of prostate cancer. J Clin Endocrinol Metab 2001;86:3467–77.

3. Nelson WG, De Marzo AM, Isaacs WB. Prostate cancer. N Engl J Med 2003;349:366–81.

4. Miyamoto H, Messing EM, Chang C. Androgen deprivation therapy for prostate cancer: current status and future prospects. Prostate 2004;61:332–53.

5. Santos AF, Huang H, Tindall DJ. The androgen receptor: a potential target for therapy of prostate cancer. Steroids 2004;69:79–85.

6. Ruizeveld de Winter JA, Janssen PJ, Sleddens HM, et al. Androgen receptor status in localized and locally progressive hormone refractory human prostate cancer. Am J Pathol 1994;144:735–46.

7. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nat Rev Cancer 2001;1:34–45.

8. Scher HI, Buchanan G, Gerald W, Butler LM, Tilley WD. Targeting the androgen receptor: improving outcomes for castration-resistant prostate cancer. Endocr Relat Cancer 2004;11:459–76.

9. Culig Z, Bartsch G. Androgen axis in prostate cancer. J Cell Biochem 2006.

10. Hung TT, Gibbons WE. Evaluation of androgen antagonism of estrogen effect by dihydrotestosterone. J Steroid Biochem 1983;19:1513–20.

11. Martikainen PM, Makela SI, Santti RS, Harkonen PL, Suominen JJ. Interaction of male and female sex hormones in cultured rat prostate. Prostate 1987;11:291–303.

12. Stover EP, Krishnan AV, Feldman D. Estrogen down-regulation of androgen receptors in cultured human mammary cancer cells (MCF-7). Endocrinology 1987;120:2597–603.

13. Poulin R, Simard J, Labrie C, et al. Down-regulation of estrogen receptors by androgens in the ZR-75-1 human breast cancer cell line. Endocrinology 1989;125:392–9.

14. Cardenas H, Pope WF. Attenuation of estrogenic effects by dihydrotestosterone in the pig uterus is associated with downregulation of the estrogen receptors. Biol Reprod 2004;70:297–302.

15. Woodham C, Birch L, Prins GS. Neonatal estrogen down-regulates prostatic androgen receptor through a proteosome-mediated protein degradation pathway. Endocrinology 2003;144:4841–50.

16. Sramkoski RM, Pretlow TG II, Giaconia JM, et al. A new human prostate carcinoma cell line, 22Rv1. In Vitro Cell Dev Biol Anim 1999;35:403–9.

17. Krishnan AV, Feldman D. Stimulation of 1,25-dihydroxyvitamin D_3 receptor gene expression in cultured cells by serum and growth factors. J Bone Miner Res 1991;6:1099–107.

18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

19. Krishnan AV, Zhao XY, Swami S, et al. A glucocorticoid-responsive mutant androgen receptor exhibits unique ligand specificity: therapeutic implications for androgen-independent prostate cancer. Endocrinology 2002;143:1889–900.

20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta C(T))$ method. Methods 2001;25:402–8.

21. Burton K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J 1956;62:315–23.

22. van Bokhoven A, Varella-Garcia M, Korch C, et al. Molecular characterization of human prostate carcinoma cell lines. Prostate 2003;57:205–25.

23. Pflug BR, Reiter RE, Nelson JB. Caveolin expression is decreased following androgen deprivation in human prostate cancer cell lines. Prostate 1999;40:269–73.

24. Osborne CK, Wakeling A, Nicholson RI. Fulvestrant: an oestrogen receptor antagonist with a novel mechanism of action. Br J Cancer 2004;90 Suppl 1:S2–6.

25. Preisler-Mashek MT, Solodin N, Stark BL, Tyriver MK, Alarid ET. Ligand-specific regulation of proteasome-mediated proteolysis of estrogen receptor-α. Am J Physiol Endocrinol Metab 2002;282:E891–8.

26. Stenoien DL, Patel K, Mancini MG, et al. FRAP reveals that mobility of oestrogen receptor-α is ligand- and proteasome-dependent. Nat Cell Biol 2001;3:15–23.

27. Veldscholte J, Ris-Stalpers C, Kuiper GG, et al. A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. Biochem Biophys Res Commun 1990;173:534–40.

28. Veldscholte J, Berrevoets CA, Ris-Stalpers C, et al. The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. J Steroid Biochem Mol Biol 1992;41:665–9.

29. Chang CY, Walther PJ, McDonnell DP. Glucocorticoids manifest androgenic activity in a cell line derived from a metastatic prostate cancer. Cancer Res 2001;61:8712–7.

30. Adams J, Behnke M, Chen S, et al. Potent and selective inhibitors of the proteasome: dipeptidyl boronic acids. Bioorg Med Chem Lett 1998;8:333–8.

31. Koivisto P, Kononen J, Palmberg C, et al. Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. Cancer Res 1997;57:314–9.

32. Chen CD, Welsbie DS, Tran C, et al. Molecular determinants of resistance to antiandrogen therapy. Nat Med 2004;10:33–9.

33. Balk SP. Androgen receptor as a target in androgen-independent prostate cancer. Urology 2002;60:132–8; discussion 8–9.

34. Eder IE, Culig Z, Ramoner R, et al. Inhibition of LncaP prostate cancer cells by means of androgen receptor antisense oligonucleotides. Cancer Gene Ther 2000;7:997 – 1007.

35. Ko YJ, Devi GR, London CA, et al. Androgen receptor down-regulation in prostate cancer with phosphorodiamidate morpholino antisense oligomers. J Urol 2004;172:1140 – 4.

36. Chen S, Song CS, Lavrovsky Y, et al. Catalytic cleavage of the androgen receptor messenger RNA and functional inhibition of androgen receptor activity by a hammerhead ribozyme. Mol Endocrinol 1998;12: 1558 – 66.

37. Zegarra-Moro OL, Schmidt LJ, Huang H, Tindall DJ. Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. Cancer Res 2002;62:1008 – 13.

38. Butler LM, Centenera MM, Neufing PJ, et al. Suppression of androgen receptor signaling in prostate cancer cells by an inhibitory receptor variant. Mol Endocrinol 2006. Epub citation pending.

39. Yang Q, Fung KM, Day WV, Kropp BP, Lin HK. Androgen receptor signaling is required for androgen-sensitive human prostate cancer cell proliferation and survival. Cancer Cell Int 2005;5:8.

40. Liao X, Tang S, Thrasher JB, Griebling TL, Li B. Small-interfering RNA-induced androgen receptor silencing leads to apoptotic cell death in prostate cancer. Mol Cancer Ther 2005;4:505 – 15.

41. Haag P, Bektic J, Bartsch G, Klocker H, Eder IE. Androgen receptor down regulation by small interference RNA induces cell growth inhibition in androgen sensitive as well as in androgen independent prostate cancer cells. J Steroid Biochem Mol Biol 2005;96:251 – 8.

42. Cardone A, Angelini F, Varriale B. Autoregulation of estrogen and androgen receptor mRNAs and downregulation of androgen receptor mRNA by estrogen in primary cultures of lizard testis cells. Gen Comp Endocrinol 1998;110:227 – 36.

43. Kim IY, Seong do H, Kim BC, et al. Raloxifene, a selective estrogen receptor modulator, induces apoptosis in androgen-responsive human prostate cancer cell line LNCaP through an androgen-independent pathway. Cancer Res 2002;62:3649 – 53.

44. El Etreby MF, Liang Y, Lewis RW. Induction of apoptosis by mifepristone and tamoxifen in human LNCaP prostate cancer cells in culture. Prostate 2000;43:31 – 42.

45. Zeng Y, Yokohira M, Saoo K, et al. Inhibition of prostate carcinogenesis in probasin/SV40 T antigen transgenic rats by raloxifene, an antiestrogen with anti-androgen action, but not nimesulide, a selective cyclooxygenase-2 inhibitor. Carcinogenesis 2005;26:1109 – 16.

46. Bektic J, Berger AP, Pfeil K, Dobler G, Bartsch G, Klocker H. Androgen receptor regulation by physiological concentrations of the isoflavonoid genistein in androgen-dependent LNCaP cells is mediated by estrogen receptor β. Eur Urol 2004;45:245 – 51.

47. Wakeling AE, Dukes M, Bowler J. A potent specific pure antiestrogen with clinical potential. Cancer Res 1991;51:3867 – 73.

48. Dauvois S, Danielian PS, White R, Parker MG. Antiestrogen ICI 164,384 reduces cellular estrogen receptor content by increasing its turnover. Proc Natl Acad Sci U S A 1992;89:4037 – 41.

49. Dauvois S, White R, Parker MG. The antiestrogen ICI 182780 disrupts estrogen receptor nucleocytoplasmic shuttling. J Cell Sci 1993;106: 1377 – 88.

50. Lau KM, LaSpina M, Long J, Ho SM. Expression of estrogen receptor (ER)-α and ER-β in normal and malignant prostatic epithelial cells: regulation by methylation and involvement in growth regulation. Cancer Res 2000;60:3175 – 82.

51. Linja MJ, Savinainen KJ, Tammela TL, Isola JJ, Visakorpi T. Expression of ERα and ERβ in prostate cancer. Prostate 2003;55:180 – 6.

52. Van Den Bemd GJ, Kuiper GG, Pols HA, Van Leeuwen JP. Distinct effects on the conformation of estrogen receptor α and β by both the antiestrogens ICI 164,384 and ICI 182,780 leading to opposite effects on receptor stability. Biochem Biophys Res Commun 1999;261:1 – 5.

53. Peekhaus NT, Chang T, Hayes EC, et al. Distinct effects of the antiestrogen Faslodex on the stability of estrogen receptors-α and -β in the breast cancer cell line MCF-7. J Mol Endocrinol 2004;32:987 – 95.

54. Maggiolini M, Recchia AG, Carpino A, et al. Oestrogen receptor β is required for androgen-stimulated proliferation of LNCaP prostate cancer cells. J Mol Endocrinol 2004;32:777 – 91.

55. Taylor RA, Cowin P, Couse JF, Korach KS, Risbridger GP. 17β-Estradiol induces apoptosis in the developing rodent prostate independently of ERα or ERβ. Endocrinology 2006;147:191 – 200.

56. Nawaz Z, Stancel GM, Hyder SM. The pure antiestrogen ICI 182,780 inhibits progestin-induced transcription. Cancer Res 1999;59:372 – 6.

57. Kawashima H, Tanaka T, Cheng JS, et al. Effect of anti-estrogens on the androgen receptor activity and cell proliferation in prostate cancer cells. Urol Res 2004;32:406 – 10.

58. Zhu YS, Cai LQ, Huang Y, et al. Receptor isoform and ligand-specific modulation of dihydrotestosterone-induced prostate specific antigen gene expression and prostate tumor cell growth by estrogens. J Androl 2005; 26:500 – 8; discussion 9 – 10.

59. de Launoit Y, Veilleux R, Dufour M, Simard J, Labrie F. Characteristics of the biphasic action of androgens and of the potent antiproliferative effects of the new pure antiestrogen EM-139 on cell cycle kinetic parameters in LNCaP human prostatic cancer cells. Cancer Res 1991; 51:5165 – 70.

60. Buck MB, Pfizenmaier K, Knabbe C. Antiestrogens induce growth inhibition by sequential activation of p38 mitogen-activated protein kinase and transforming growth factor-β pathways in human breast cancer cells. Mol Endocrinol 2004;18:1643 – 57.

Molecular Cancer Therapeutics

Fulvestrant (ICI 182,780) down-regulates androgen receptor expression and diminishes androgenic responses in LNCaP human prostate cancer cells

Rumi S. Bhattacharyya, Aruna V. Krishnan, Srilatha Swami, et al.

Mol Cancer Ther 2006;5:1539-1549.