Bicalutamide Functions as an Androgen Receptor Antagonist by Assembly of a Transcriptionally Inactive Receptor*

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The majority of prostate cancers (PCa)1 are androgen-dependent and respond to androgen deprivation therapies, which include orchietomy or administration of leutinizing hormone-releasing hormone agonists to suppress testicular androgen production (1). The effects of androgens on the normal prostate include orchiectomy or administration of leutinizing hormone-pendent and respond to androgen deprivation therapies, which Stabilize AR association with cytosolic heat shock protein complexes. This study found nuclear AR expression in bicalutamide-treated androgen-independent PCa and found that bicalutamide could stimulate AR nuclear translocation. Moreover, specific DNA binding by the bicalutamide-liganded AR was demonstrated in vivo using a VP16-AR fusion protein and was confirmed by chromatin immunoprecipitation showing binding to the prostate-specific antigen enhancer in LNCaP PCa cells. Nonetheless, bicalutamide could not stimulate interactions between the AR N and C termini or recruitment of steroid receptor coactivator proteins (SRC-1 or -2), although SRC transfection augmented AR activity in the presence of dihydrotestosterone and inhibitory concentrations of bicalutamide. These results demonstrate that bicalutamide stimulates the assembly of a transcriptionally inactive AR on DNA and support altered coactivator (or corepressor) expression as a mechanism of bicalutamide-resistant androgen-independent PCa.

Prostate cancers (PCa) that relapse after androgen deprivation therapy invariably express high levels of androgen receptor (AR) and AR-regulated genes. Most do not respond to secondary hormonal therapies, including AR antagonists, and the mechanisms of AR activation in these clinically androgen-independent tumors are unclear. Bicalutamide, the most widely used AR antagonist, is a competitive antagonist shown previously to stabilize AR association with cytosolic heat shock protein complexes. This study found nuclear AR expression in bicalutamide-treated androgen-independent PCa and found that bicalutamide could stimulate AR nuclear translocation. Moreover, specific DNA binding by the bicalutamide-liganded AR was demonstrated in vivo using a VP16-AR fusion protein and was confirmed by chromatin immunoprecipitation showing binding to the prostate-specific antigen enhancer in LNCaP PCa cells. Nonetheless, bicalutamide could not stimulate interactions between the AR N and C termini or recruitment of steroid receptor coactivator proteins (SRC-1 or -2), although SRC transfection augmented AR activity in the presence of dihydrotestosterone and inhibitory concentrations of bicalutamide. These results demonstrate that bicalutamide stimulates the assembly of a transcriptionally inactive AR on DNA and support altered coactivator (or corepressor) expression as a mechanism of bicalutamide-resistant androgen-independent PCa.

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‡ The abbreviations used are: PCa, prostate cancer(s); AR, androgen receptor; hAR, human AR; ARE, androgen-responsive element; ARN, N-terminal AR; SRC, steroid receptor coactivator; PSA, prostate-specific antigen; CS-FCS, charcoal-dextran-stripped fetal calf serum; DHT, dihydrotestosterone; PBS, phosphate-buffered saline; Luc, luciferase; receptor superfamily (2). Unfortunately, patients receiving androgen deprivation therapies invariably relapse with PCa that tends to be aggressive. In some cases, these relapsed tumors respond to secondary hormonal therapy or chemotherapy, but these responses are generally partial and transient, and no current therapies have been shown to prolong survival at this stage. Therefore, these prostate cancers that relapse after androgen deprivation therapy have been termed hormone refractory or androgen-independent and represent a major clinical challenge in PCa (3–5).

Although these relapsed cancers are clinically androgen-independent, many lines of evidence indicate that the AR remains active and may contribute to their androgen-independent growth. Immunohistochemical data show that the AR is highly expressed by most androgen-independent PCa (6, 7). Moreover, the AR appears to be transcriptionally active as most androgen-independent tumors express AR-regulated genes such as prostate-specific antigen (PSA). Consistent with these observations, AR mRNA appears to be increased in androgen-independent PCa, and the AR gene is amplified in ~30% of cases (8, 9). These findings have suggested that AR expression is increased in response to androgen deprivation therapy to enhance activation by residual androgens, in particular weak adrenal-derived androgens that can be converted to testosterone and dihydrotestosterone (DHT). Consistent with this hypothesis, some androgen-independent PCa patients respond to secondary hormonal agents that antagonize adrenal hormone production or to AR antagonists that directly block androgen binding to the AR (10, 11). However, these responses to further AR blockade in androgen-independent PCa occur in a minority of patients and are generally transient.

An alternative mechanism proposed to explain AR activity in androgen-independent PCa is AR mutations that result in constitutive activity or enhanced responses to other hormones. Several groups have found such mutant ARs with altered ligand responses, but their frequency in patients treated with androgen ablation monotherapy appears to be low (8, 12–14). In contrast, AR mutations were found in ~4% of patients who relapsed after initial combined therapy with the AR antagonist flutamide (15). Moreover, these mutations resulted in mutant ARs that were strongly activated by flutamide and other steroid hormones, indicating selective pressure to maintain AR activity. This flutamide-treated patient population also had an increased response rate to secondary hormonal therapy with bicalutamide, an AR antagonist that remains effective against identified AR mutants (11). However, responses were generally partial and transient, further indicating a limited overall role for AR mutations as a mechanism for AR activity in androgen-independent PCa.

Based on these observations, mechanisms proposed to account for AR activity in androgen-independent PCa should be...
consistent with and account for resistance to AR antagonists. The most widely used AR antagonists have been the steroidal drug cyproterone acetate and the nonsteroidal drugs flutamide and bicalutamide, which are all competitive antagonists of androgen binding (16). Cyproterone acetate has significant AR agonist activity, whereas weak agonist activity has been shown for hydroxyflutamide, the active metabolite of flutamide (17, 18). In contrast, previous studies have shown bicalutamide to be a pure antagonist of wild type and identified mutant ARs (18, 19). The unliganded AR associates with a heat shock protein 90 (HSP90) chaperone complex that facilitates AR binding with subsequent conformational changes resulting in AR homodimerization, nuclear translocation, DNA binding, and transcriptional activation (20). Previous studies in cell lines have indicated that the bicalutamide-ligated AR remains cytoplasmic- and HSP90-associated, which is not consistent with transcriptional activity (21). This study further examined the effects of bicalutamide on the AR to identify mechanisms that may contribute to androgen-independent and bicalutamide-resistant AR activity in androgen-independent PCAs.

EXPERIMENTAL PROCEDURES

Plasmids—A human AR expression vector, pSVARo, was from A. Brinkmann (22). pRL-CMV, pG5-Luc, pBind, and pACT vectors were from Promega (Madison, WI). VP16 activation domain-AR expression vectors were generated in pACT containing the full-length AR (pACT-hAR) or the N-terminal AR (amino acids 1–505, pACT-ARN) (23). The Gal4 DNA binding domain was fused to the AR ligand binding domain (amino acids 661–919) to generate pBind-ARLBD. The ARE4-Luc reporter contained four tandem androgen-responsive elements cloned into pGL3 (23). Expression vectors for human SRC1 (pSG5-SRC1) and murine GRIP1 (pCMV-GRIP1) were from M. Brown (Dana-Farber Cancer Institute, Boston, MA) (24, 25).

Immunohistochemistry and Immunofluorescence—Bone biopsies from patients with androgen-independent PCAs being treated with bicalutamide were obtained as described (15) and were immunostained using a rabbit anti-AR N-terminal antibody (PG-21, Upstate Biotechnology), 2 mg of sheared salmon sperm DNA, 20 μl of nonimmune sera, and 20 μl of a 50% slurry of protein A-Sepharose. Immunoprecipitations were with 500 ng of AR N-terminal rabbit polyclonal antibody (PG-21, Upstate Biotechnology), 2 μl of sheared salmon sperm DNA, and 20 μl of a 50% slurry of protein A-Sepharose. Control immunoprecipitations were with 500 ng of a rabbit anti-platelet-derived growth factor receptor antibody (Upstate Biotechnology). Precipitates were washed three times with 300 mM NaCl, 50 mM Tris, pH 8.0, 2.7 mM KCl, 0.05% Tween 20, and 1% deoxycholate. Three additional washes with 10 mM Tris, 1 mM EDTA were performed, and beads were then eluted three times with 35 μl of 1% SDS and 100 mM NaHCO3 at 37°C for 10 min each. Eluates were pooled and incubated at 65°C overnight to reverse cross-links. Products were then purified with QIAquick PCR purification spin resin (Qiagen, Valencia, CA), and 10% of the eluate was subjected to 50 cycles of PCR amplification with steps of 95, 55, and 72°C for 1 min each. The primers were TGAGAAACT-GAGATTAGGA and ATCTCTCTCAGATCCAGGCT, corresponding to nucleotides 4270 to 4250 and 4065 to 4045, respectively, in the PSA enhancer, which flank the major androgen-responsive element (ARE) regulating this gene (27–29). PCR products were analyzed by gel electrophoresis and ethidium bromide staining in 5% agarose gels. Inputs represent 5% of the DNA used for the AR immunoprecipitations, which was similarly treated to reverse cross-links and then PCR-amplified using the same PSA primers.

RESULTS

Bicalutamide Does Not Prevent AR Nuclear Localization in Prostate Cells—Previous biochemical studies showed that bicalutamide can maintain the AR in a cytoplasmic complex with HSP90 (21). Immunohistochemical studies of the AR in androgen-independent PCAs have generally shown strong nuclear expression, but AR in bicalutamide-treated patients with androgen-independent PCAs has not been specifically examined. Therefore, tumor-containing bone marrow biopsies obtained from bicalutamide-treated patients with androgen-independent PCAs were examined for AR expression. The results demonstrated strong nuclear AR expression in each of four cases examined (Fig. 1, A–C, and data not shown).

To determine whether nuclear localization was inhibited by
bicalutamide in normal prostate epithelium, mice were treated with bicalutamide (1 mg in 0.1 ml of PBS by intraperitoneal injection every other day for 2 weeks), and their prostates were examined. The seminal vesicles in the treated mice were markedly shrunken, indicative of AR inhibition (not shown). However, immunohistochemistry showed strong nuclear AR expression in the prostate epithelium of both untreated and bicalutamide-treated mice (Fig. 1, A and D). These results demonstrated that bicalutamide did not prevent AR nuclear translocation in normal or malignant prostate epithelial cells.

To assess the effect of bicalutamide on AR cellular localization in PCa cells in vitro, the LNCaP prostate cancer cell line was examined. LNCaP expresses a mutant AR that is activated by 8-hydroxyflutamide (the active metabolite of flutamide) but is still blocked by bicalutamide (19, 30). Moreover, bicalutamide can inhibit LNCaP growth and PSA production. Consistent with previous studies, immunofluorescence showed that the AR in LNCaP cells grown in steroid hormone-depleted medium was diffusely distributed in the cytoplasm and nucleus (Fig. 2A). Treatment with DHT resulted in rapid nuclear accumulation with the AR being almost completely nuclear at 2 h (Fig. 2, B and C). Treatment with bicalutamide also resulted in rapid nuclear translocation (Fig. 2, D and E). Similar results have been obtained using transfected wild type AR in HeLa cells (data not shown). Taken together, these findings indicated that bicalutamide, in addition to not blocking nuclear expression of AR in prostate epithelium, had agonist activity with respect to AR nuclear translocation.

**Bicalutamide Can Stimulate AR DNA Binding**—Despite nuclear AR expression, bicalutamide can completely block DHT-stimulated AR transactivation and has no detectable partial agonist activity on the full-length AR (18). However, transcriptional activation by the full-length AR is a multistep process involving DNA binding, homodimerization, interaction between the AR N and C termini, and association with coactivator proteins. Therefore, to assess bicalutamide effects on DNA binding independent of these latter protein-protein interactions, the heterologous VP16 transactivation domain was fused to the N terminus of full-length AR. The VP16-AR (2–919) fusion protein was inactive in the absence of androgen but could be strongly stimulated by DHT (Fig. 3A, left panel). Bicalutamide similarly induced substantial activation of the VP16-AR (2–919) fusion (Fig. 3A, right panel). Importantly, maximal activity was seen in the micromolar concentration range, well within the bicalutamide levels in patients treated with this drug (31). Therefore, this result indicated that bicalutamide had partial agonist activity with respect to stimulating AR binding to DNA.

Chromatin immunoprecipitation was used to further assess whether bicalutamide stimulated AR binding to an endogenous androgen-regulated gene. LNCaP or DU145 (AR-negative) PCa cells grown in steroid hormone-depleted medium were treated with DHT or bicalutamide. They were then examined by chromatin immunoprecipitation for AR binding to the PSA enhancer (~4270 to ~4045 from the start site), which contains the major ARE regulating this gene and multiple weak AREs (27–29). Immunoprecipitates with an irrelevant antibody (anti-platelet-derived growth factor receptor) and AR immunoprecipitates from the DU145 cells or the untreated LNCaP cells contained little or no detectable PSA enhancer DNA (Fig. 3B). In contrast, both DHT and bicalutamide treatments stimulated AR association with the enhancer, further supporting the conclusion that bicalutamide stimulates AR binding to AREs.

**Bicalutamide Does Not Support Association of the AR N and C Termini or Interaction with Steroid Receptor Coactivator Proteins**—Previous studies showed that a ligand-dependent interaction between the AR N and C termini made a major contribution to AR transcriptional activity and that this interaction was not stimulated by antagonists including cyproterone acetate or flutamide (32–34). A mammalian two-hybrid protein binding assay was similarly used to determine whether bicalutamide could stimulate this interaction. Cells were transfected with vectors encoding VP16-AR (2–505) and Gal4-AR (661–919) fusion proteins (corresponding to the AR N-terminal and ligand binding domains, respectively) in conjunction with a luciferase reporter gene regulated by tandem Gal4-responsive elements (pG5-Luc). There was no detectable interaction between these proteins in the absence of ligand, but the interaction could be strongly stimulated by DHT (Fig. 4, left panel). In contrast, bicalutamide did not stimulate this interaction.

The transcriptional activity of steroid hormone receptors is also mediated by ligand-dependent binding of coactivator proteins, in particular SRC-1 and -2 (35), and increased expression of these coactivators has been reported in androgen-independent PCa (36, 37). These coactivator proteins have leucine-X-leucine-leucine (LXXLL) motifs that mediate ligand-dependent binding to the ligand binding domain and contain a distinct site that binds to the N-terminal, with binding to the AR being mediated primarily by this latter N-terminal interaction (38, 39). To determine whether the bicalutamide-ligated AR could interact with SRC-1, cells were cotransfected with AR and SRC-1 expression vectors. Consistent with previous reports, SRC-1 could augment DHT-stimulated AR transcriptional activity (Fig. 5A). However, SRC-1 did not stimulate AR activity in the absence of ligand or in the presence of bicalutamide. SRC-2 transfection similarly enhanced DHT-stimulated transcriptional activity (although the effect was more modest) but not ligand-independent or bicalutamide-mediated activity (Fig. 5B). These results indicated that unliganded or bicalutamide-ligated AR could not associate with these coactivators, which did not support SRC-1 or SRC-2 overexpression as a mechanism of AR activity in androgen-independent PCa.

Although the SRC proteins did not stimulate activity of the unliganded or bicalutamide-ligated AR, castrated males still produce androgens that can be converted to DHT in prostate cells. Therefore, further experiments addressed whether SRC proteins could enhance AR responses to lower levels of DHT. Dose response studies showed that SRC-1 transfection enhanced DHT-stimulated AR activity over a broad range of DHT concentrations (Fig. 5, C and D). However, a marked left shift
in the dose response curve to lower DHT concentrations was not observed, suggesting that SRC-1 did not increase AR affinity for DHT.

Coactivator Expression Increases the Bicalutamide Concentrations Required to Antagonize AR Transcriptional Activity

It was next determined whether SRC-1 overexpression diminished the ability of bicalutamide, at concentrations obtained in vivo, to inhibit DHT-stimulated AR transcriptional activity. As shown in Fig. 6A, bicalutamide at 5 μM could completely block the AR transcriptional activity stimulated by 10 nM DHT. In contrast, SRC-1-transfected cells treated with 10 nM DHT and 5 μM bicalutamide had substantial AR activity with lower but still detectable activity at 25 μM bicalutamide. Cotransfection with SRC-2 similarly stimulated AR transcriptional activity in the presence of DHT and 1–5 μM bicalutamide (Fig. 6B). Further dose response studies showed that SRC-1 transfection did not markedly alter the IC50 for bicalutamide, which was between 0.1 and 1 μM (data not shown). In conjunction with the above DHT dose response studies, these results indicated that SRC-1 was functioning primarily by augmenting the transcriptional activity of agonist-bound AR rather than by decreasing the ability of bicalutamide to compete with DHT for AR binding.

Effects of Bicalutamide Dose Escalation on LNCaP Cells

A prediction based on the above data was that androgen-independent PCa cells overexpressing SRC proteins should respond to higher doses of bicalutamide or to more potent AR antagonists. Such AR-expressing cell lines derived from androgen-independent PCa are not available, but the LNCaP prostate cancer cell line has features of androgen-independent PCa. In particular, although LNCaP proliferation and PSA production are stimulated by DHT, they are only partially inhibited by bicalutamide at 5–10 μM. Therefore, the effects of higher bicalutamide concentrations on these cells were assessed.

PSA production by LNCaP cells was inhibited progressively by bicalutamide concentrations up to 100 μM (Fig. 7A). Inhibition of LNCaP cell growth as assessed by the percentage of cells in S phase was also progressively inhibited at bicalutamide concentrations up to 40–80 μM (Fig. 7B). The inhibitory effects of bicalutamide were abrogated by added DHT, indicating that the inhibition reflected AR blockade and not nonspecific toxicity due to the high bicalutamide concentrations (Fig. 7C). These findings indicated that the AR in LNCaP cells remained active
DISCUSSION

Previous reports have suggested that bicalutamide functioned as a pure antagonist by preventing AR DNA binding (18–21). In contrast, the data reported here demonstrate that the bicalutamide-liganded AR localizes in the nucleus and, more importantly, that it can bind to AREs in episcopal reporter genes and in the endogenous PSA promoter. However, the bicalutamide-liganded AR is transcriptionally inactive. Consistent with this lack of transcriptional activity, bicalutamide did not stimulate binding of the AR N terminus to the C-terminal ligand binding domain. Moreover, cotransfection of bicalutamide-treated cells with SRC-1 or -2 did not stimulate AR activity, indicating that these coactivator proteins did not interact with the bicalutamide-liganded AR. Taken together, these findings indicate that the bicalutamide-liganded AR binds to DNA but in a transcriptionally inactive conformation.

The lack of AR N- and C-terminal interaction in the bicalutamide-liganded AR is not surprising as this is mediated by an LXXLL-like motif in the N terminus and is dependent on an agonist conformation in the ligand binding domain (40). However, SRC-1 binding to the AR is primarily or exclusively through the LXXLL motifs in SRC-1 do not bind to the AR ligand binding domain (39, 41). The precise site in the AR N terminus that mediates SRC-1 binding has not been identified, but the lack of SRC-1 interaction with the bicalutamide-liganded AR indicates that the N-terminal binding site may be affected by ligand binding, possibly reflecting a role for the AR N- and C-terminal interaction in positioning or exposing the N-terminal SRC-1 binding site. Alternatively, binding of a corepressor to the bicalutamide-liganded AR could block SRC-1 binding or inhibit its coactivator functions (see below).

Although SRC-1 and -2 did not stimulate transcriptional activation of the unliganded or bicalutamide-liganded AR, higher concentrations of bicalutamide were needed to block DHT-stimulated transcriptional activity in cells transfected with SRC-1 or -2. This did not appear to reflect an increase in the affinity for DHT relative to bicalutamide as indicated for 24 h and harvested. RLU, relative light units.

FIG. 5. SRC fails to stimulate bicalutamide (Bical)-liganded AR. In A and B, CV1 cells were cotransfected with AR (pSVARo), ARE-Luc, control pRL-CMV, and the indicated amounts of SRC1 (A) or SRC2 (B) expression vectors. After transfection, cells were treated with DHT or bicalutamide as indicated for 24 h and harvested. RLU, relative light units.

FIG. 6. SRC stimulation of AR activity in cells treated with DHT and bicalutamide (Bical). In A and B, CV1 cells were cotransfected with AR (pSVARo), ARE-Luc, control pRL-CMV, and the indicated amounts of SRC1 (A) or SRC2 (B) expression vectors and were then treated with hormones as indicated. RLU, relative light units.

FIG. 7. Inhibition of LNCaP PSA production and proliferation by bicalutamide (Bical). In A, LNCaP cells were grown in complete medium with 10% FCS and the indicated concentrations of bicalutamide for 72 h, and PSA secretion (ng/ml) into the culture supernatant was measured by enzyme-linked immunosorbent assay during the last 24 h. RLU, relative light units. In B and C, LNCaP cells were grown in complete medium with 10% FCS and treated for 48 h with bicalutamide and DHT as indicated. The percentage of S phase was then assessed by propidium iodide staining and flow cytometry.

even at 10 μM bicalutamide and could be antagonized by higher concentrations of the drug.
tion therapy (castration or luteinizing hormone releasing hormone agonist) in conjunction with bicalutamide. In this setting, in which there are still substantial levels of circulating androgens, increased expression of SRC proteins would diminish the efficacy of bicalutamide in blocking residual AR activity.

Recent reports demonstrate that the AR can also bind to corepressor proteins, including nuclear corepressor (NCoR), indicating that corepressor binding could further contribute to the in vivo antagonist activity of bicalutamide (42–44). This would suggest down-regulation of a corepressor as another mechanism for development of bicalutamide-resistant PCs. These data also suggest that bicalutamide may function as a selective AR modulator. However, in contrast to the selective estrogen receptor modulators tamoxifen and raloxifene, bicalutamide does not appear to activate the N-terminal activation function (AF-1) of the AR, and cell types or tissues in which bicalutamide functions as an AR agonist have not been identified.

In summary, these results demonstrate that bicalutamide does not function as an AR antagonist by preventing AR binding to DNA but instead stimulates the assembly of a transcriptionally inactive receptor on DNA. These findings support a continued role for the AR in androgen-independent proliferation in conjunction with bicalutamide (or more potent AR antagonists) may have efficacy in androgen-independent PCa.
