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**14. ABSTRACT**

Aim 1 is to determine the precise molecular basis for NCoR binding to the RU486 liganded AR. Since the previous report we have used chromatin immunoprecipitation to demonstrate that RU486 enahnces AR NCoR recruitment to AR assembled on androgen regulated genes. We have also generated the additional AR and NCoR mutants to define the precise amino acids mediating the interaction. Aim 2 is to determine whether NCoR recruitment can suppress androgen independent expression of AR regulated genes and prostate cancer growth, and identify molecular markers that predict whether RU486 (or related drugs) will be effective in particular prostate cancers in vivo. We have now used chromatin immunoprecipitation to examine the functional effects of RU486 mediated NCoR recruitment, and find that NCoR is not mediating deacetylation and hence not suppressing gene expression. The reason for this is now under investigation. These results, in conjunction with our previous data, reflect further progress towards determining the structural basis for AR-NCoR interaction (Aim 1) and determining whether this interaction can be exploited to treat prostate cancer (Aim 2).

**15. SUBJECT TERMS**
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## INRODUCTION

The androgen receptor (AR) plays a central role in prostate cancer (PCa) and androgen ablation therapy is the standard systemic therapy for metastatic PCa, but most patients relapse with an aggressive stage of the disease termed hormone refractory or androgen independent PCa. The AR and androgen regulated genes are still expressed in androgen independent PCa, indicating that the AR remains as a therapeutic target for higher affinity pure antagonists. However, such drugs that can compete with dihydrotestosterone (DHT) for AR binding have not been developed. An alternative is the development of drugs that enhance AR recruitment of nuclear receptor corepressors (NCoR or SMRT), as such drugs could actively repress AR regulated genes. We have shown that the DHT liganded AR binds NCoR, and that this binding can be markedly enhanced by mifepristone (RU486), a steroidal antagonist of the progesterone and glucocorticoid receptors. **Our hypothesis is that enhancement of the AR-NCoR interaction is a therapeutic approach for the treatment of PCa, including advanced androgen independent PCa.** The RU486 data provide a "proof of principle" that the AR-NCoR interaction can be enhanced, and suggest a novel mechanism for antagonist binding that may be valuable in the further development of high affinity AR antagonists**.**

**Aim 1 is to determine the precise molecular basis for NCoR binding to the RU486 liganded AR.**

**Aim 2 is to test the hypothesis that NCoR recruitment can suppress androgen independent expression of AR regulated genes and prostate cancer growth, and identify molecular markers that predict whether RU486 (or related drugs) will be effective in particular prostate cancers *in vivo*.**

## BODY

The data presented from the previous reporting period showed that while RU486 could mediate NCoR recruitment to AR regulated genes, it had limited effectiveness in suppressing AR regulated genes. Based on this conclusion with respect to Aim 2, we have focused on Aim 1 as defining the molecular basis for NCoR recruitment is critical for the development of more potent antagonists. We present below our progress toward this specific aim that has occurred since the previous progress report. The initial sections focus on the role of NCoR in mediating the activity of AR agonists and antagonists (Figs. 1-6). The subsequent sections establish the molecular basis for NCoR binding to the RU486 liganded AR.

### AR INTERACTION WITH NCoR IN RESPONSE TO AGONIST VERSUS ANTAGONIST LIGANDS

**Partial Agonist Activities Of Weak Androgens And AR Antagonists Are Dependent On The AR N-C Terminal Interaction.** The physiological high affinity ligands for AR (testosterone and DHT) induce conformational changes in the LBD and a strong interaction with the AR N-terminus. In contrast, previous studies have found no detectable interaction between the bicalutamide liganded AR LBD and the N-terminus, which may account for bicalutamide's lack of agonist activity. However, AR transcriptional activity can be stimulated by other steroid hormones or drugs such as cyproterone acetate that do not mediate clearly detectable AR N-C terminal interactions, suggesting that the agonist activities of these drugs may not be dependent on the N-C interaction. The interaction between the AR LBD and N-terminus is mediated by a phenylalanine motif at amino acids 23-27 (FQNLF), which binds tightly to the LXXLL coactivator cleft in the AR LBD. Therefore, we examined whether deletion of this motif in AR(dFQNLF) impaired AR activity in response to DHT versus weak agonists or drugs.

Consistent with previous data, deletion of FQNLF markedly impaired AR transcriptional activity in response to DHT (Fig. 1A). Significantly, the FQNLF deletion also markedly impaired AR activity in response to a weak androgen (androstenedione) and to progesterone, both used at micromolar concentrations (Fig. 1B and C). Moreover, the deletion abrogated the partial agonist activity of the AR antagonist drug, cyproterone acetate (CPA) (Fig. 1D). In contrast, bicalutamide, did not stimulate the wild-type or mutant ARs (data not shown).

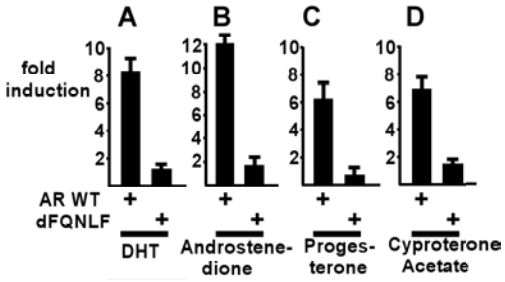



**Figure 1. AR partial agonist activities are dependent on the N-terminus phenylalanine motif. CV1 cells were cotransfected with full-length wild-type AR (pSVARo, AR WT) or full-length AR with the phenylalanine motif (FQNLF, aa 23-27) deleted (dFQNLF), $ARE_4$-luciferase reporter and a control CMV regulated *Renilla* luciferase plasmid (pRL-CMV). Cells were incubated for 24 hrs in steroid-hormone depleted medium (DMEM/ 5% CDS-FBS) treated with A, 10 nM DHT; B, 1 μM androstenedione; C, 1 μM progesterone; or D, 10 μM cyproterone acetate. Luciferase versus *Renilla* luciferase activities were determined from triplicate samples and presented as fold induction relative to the activity determined in the absence of ligand.**

These results suggested that the agonist activities of the above steroid hormones, and of CPA, were dependent on the AR N-C terminal interaction. Therefore, we next carried out mammalian one- and two-hybrid protein interaction assays to determine whether CPA could induce a detectable AR N-C terminal interaction. CV1 cells were cotransfected with expression vectors encoding an N-terminal domain deleted AR (AR-DBD/LBD), the AR N-terminal domain linked to the VP16 transactivation domain (VP16-AR-NTD), and an ARE regulated reporter gene ($ARE_4$-luciferase). As shown previously, DHT induced a strong interaction between the AR N-terminal domain and DBD/LBD fragment (Fig. 2A). Significantly, CPA also induced an interaction, although it was clearly weaker than the DHT stimulated response and required higher concentrations (1-10 nM for DHT versus 1-10 μM for CPA) (Fig. 2A). In contrast, there was no detectable interaction in response to bicalutamide. Consistent with previous data, the AR DBD/LBD fragment by itself had no detectable transcriptional activity in response to DHT or antagonists (data not shown).

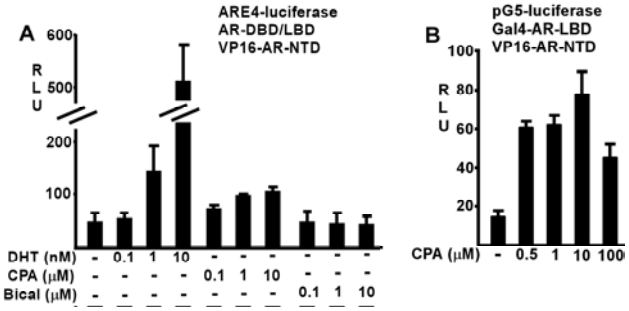



**Figure 2. Partial agonists mediate AR N/C interaction. A, CV1 cells were cotransfected with a VP16-AR N-terminus expression vector (VP16-AR-NTD) and an N-terminal domain deleted AR (AR-DBD/LBD) in the presence of $ARE_4$-Luc luciferase reporter vector and the pRL-CMV control. Cells were treated with the indicated ligands for 24 hrs in steroid depleted medium and luciferase versus *Renilla* luciferase activities were determined from triplicate samples. The data are expressed as relative light units (RLU) ± S.E. B, cells were transfected with a multimerized UAS-Luc reporter (pG5-Luc) and pRL-CMV reporter in the presence of Gal4-AR-LBD and VP16-AR-NTD, and treated with increasing concentrations of cyproterone acetate (CPA) as indicated. Luciferase versus *Renilla* luciferase activities were determined from triplicate samples (expressed as RLU ± S.E.).**

To confirm the interaction between the AR N-terminal domain and the CPA liganded AR LBD, we next carried out two-hybrid experiments using the AR LBD alone fused to the Gal4 DBD (Gal4-AR-LBD).

CV1 cells were cotransfected with the Gal4-AR-LBD and VP16-AR-NTD expression vectors, and the pG5-luciferase reporter (containing 5 tandem copies of the Gal4 binding element). Consistent with the above results, CPA induced an interaction between the N-terminal domain and the LBD (Fig. 2B). Taken together, these data indicate that the AR N-C terminal interaction is critical for AR transcriptional activity mediated by weak androgens and partial agonist drugs. Moreover, the failure of bicalutamide to mediate this interaction is consistent with this drug's lack of partial agonist activity.

**SRC-1 Coactivator Can Interact With Bicalutamide Liganded AR N-Terminus But Does Not Stimulate Transcriptional Activity.** Previous studies have shown that AR recruitment of p160 steroid receptor coactivator (SRC) proteins (in particular SRC-1) is mediated primarily by the AR N-terminal domain (NTD), with one proposed function for the N-C terminal interaction being to structure the NTD and thereby enhance coactivator binding. Consistent with this hypothesis and previous data, SRC-1 can coactivate both the DHT and CPA liganded AR, but not the bicalutamide liganded AR (Fig. 3A). To further assess whether SRC-1 fails to coactivate the bicalutamide liganded AR due to the lack of an N-C terminal interaction, we examined SRC-1 coactivation of an LBD deletion mutant (AR-NTD/DBD). Significantly, cotransfection of SRC-1 enhanced the transcriptional activity of AR-NTD/DBD on an $ARE_4$-luciferase reporter, showing that SRC-1 could associate with the AR NTD independently of the N-C terminal interaction (Fig. 3B).

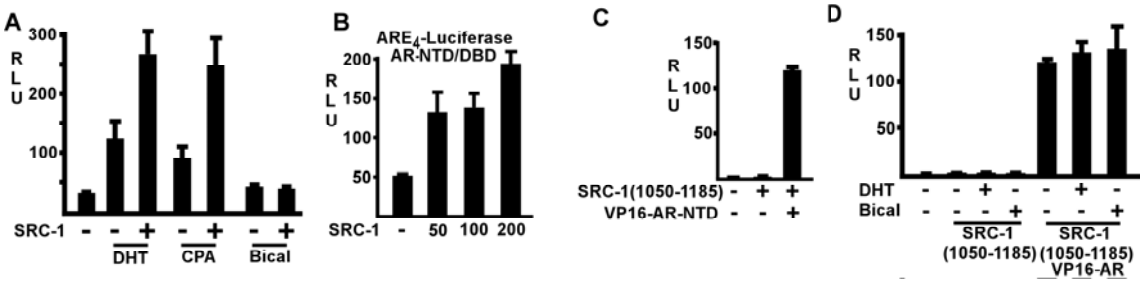



**Figure 3. SRC-1 coactivates AR N-terminus and not the bicalutamide liganded AR. A, CV1 cells were cotransfected with full-length AR, $ARE_4$-Luc luciferase reporter vector and the pRL-CMV control. In addition, cells were transfected with full-length SRC-1 (pSG5-SRC-1). Cells were treated for 24 hrs with DHT (10 nM), CPA (10 μM) or bicalutamide (10 μM) and luciferase versus *Renilla* luciferase activities were determined from triplicate samples (expressed as RLU ± S.E.). B, cells were transfected as in A with an expression vector for the AR N-terminus and DNA binding domain (AR-NTD/DBD), the $ARE_4$-Luc luciferase reporter vector and the pRL-CMV control, with increasing amounts of SRC-1. C, CV1 cells were cotransfected with the pG5-Luc luciferase reporter and expression plasmids for SRC-1 (aa 1050-1185) fused to the Gal4-DNA binding domain, and the AR-NTD fused to VP16, as indicated. D, CV1 cells were cotransfected with expression plasmids for the full length AR fused to VP16 (VP16-AR) and SRC-1 (aa 1050-1185) fused to the Gal4-DNA binding domain, the pG5-Luc luciferase vector, and pRL-CMV control. Cells were treated for 24 hrs with DHT (10 nM) or bicalutamide (10 μM). Luciferase versus *Renilla* luciferase activities were determined from triplicate samples (expressed as RLU ± S.E.).**

This result indicated that absence of an N-C terminal interaction in the bicalutamide liganded AR was not the basis for lack of SRC-1 mediated coactivation, and suggested that there may be an inhibitory interaction between the AR NTD and the bicalutamide liganded LBD that prevents SRC-1 binding. To test this hypothesis, we expressed the glutamine rich domain of SRC-1 (amino acids 1050-1185), which mediates SRC-1 binding to the AR NTD (but does not mediate transactivation), as a fusion protein with the Gal4 DBD. Significantly, the Gal4-SRC-1(1050-1185) fusion had no transcriptional activity on the pG5-luciferase reporter in transfected CV1 cells, but could be strongly activated by the cotransfected AR NTD, further demonstrating that the AR-SRC-1 interaction was not dependent on the AR LBD (Fig. 3C) (it should be noted that the AR constructs in these experiments are fused to the VP16 transactivation domain to provide an assessment of binding that is independent of intrinsic AR transcriptional activity).

The Gal4-SRC-1(1050-1185) fusion protein was also strongly activated by the full length AR fused to VP16 (VP16-AR) in the absence of added ligand or in the presence of DHT (Fig. 3D). Importantly, this activation by VP16-AR was not diminished by bicalutamide (Fig. 3D). Taken together, these results show that the bicalutamide liganded AR LBD does not interfere with SRC-1 binding by the AR NTD, although full length SRC-1 does not coactivate the bicalutamide liganded full length AR on an ARE reporter gene.

**Bicalutamide Antagonist Activity is Independent of NCoR and SMRT Corepressors.** As the above results indicated that SRC-1 recruitment may not be directly blocked by the bicalutamide liganded AR LBD, we next considered other mechanisms by which bicalutamide may block coactivator recruitment or induction of transcriptional activity. One such possible mechanism is clearly corepressor recruitment, as previous studies indicate that bicalutamide can enhance NCoR recruitment by the AR, and that bicalutamide may function as an agonist in the absence of NCoR. A previous study also indicated that AR overexpression in PCa cells could by itself alter the response to bicalutamide and make it function as an agonist. The molecular basis for this latter effect of AR overexpression was not clear, but could possibly reflect a relative decrease in the levels of corepressors versus coactivators. Therefore, we first examined whether transfection of increasing amounts of AR into CV1 cells could reveal bicalutamide agonist activity. Significantly, increasing the amount of transfected AR enhanced DHT stimulated transcriptional activity, but did not reveal any clear bicalutamide agonist activity (Fig. 4A).

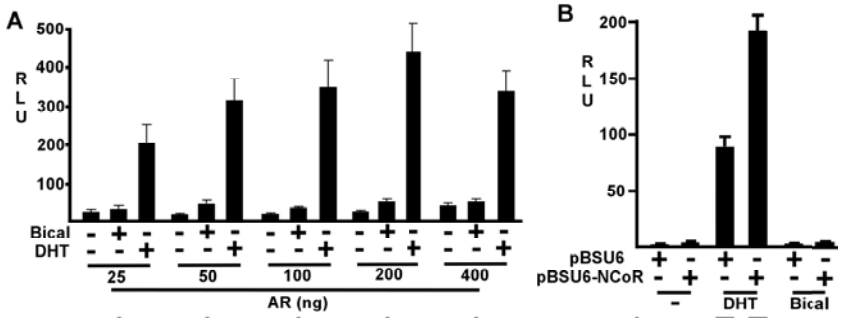



**Figure 4. AR overexpression and NCoR knockdown does not convert bicalutamide into an agonist in CV1 cells. A, cells were cotransfected with the $ARE_4$-Luc luciferase reporter vector, pRL-CMV control and increasing amounts of full-length AR (25-400 ng). Cells were treated for 24 hrs with DHT (10 nM) or bicalutamide (10 μM) and luciferase versus *Renilla* luciferase activities were determined from triplicate samples (expressed as RLU ± S.E.). B, cells were transfected with 400 ng of AR, and $ARE_4$-Luc reporter, 3 ng of control pCMV-βGal and 1 μg of a control (pBSU6) or NCoR shRNA expression vector (pBSU6-NCoR). Cells were stimulated for 24 hrs with DHT (10 nM) or bicalutamide (1 μM) and luciferase versus β-galactosidase activities were determined from triplicate samples (expressed as RLU ± S.E.).**

To more directly test the hypothesis that the agonist activity of bicalutamide is suppressed by NCoR recruitment, we next carried out shRNA experiments. CV1 cells were transfected with wild-type AR in conjunction with a control or NCoR shRNA expression vector. Consistent with previous results, NCoR shRNA enhanced the transcriptional activity of the DHT liganded AR, indicating that NCoR negatively regulates the agonist liganded AR (Fig. 4B). In contrast, NCoR shRNA did not reveal substantial bicalutamide agonist activity, although it may very weakly enhance both the unliganded and bicalutamide liganded AR (Fig 4B).

To further determine whether NCoR contributes physiologically to the antagonist activity of bicalutamide on the endogenous AR in PCa cells, we examined whether NCoR downregulation by siRNA would stimulate bicalutamide agonist activity on the endogenous AR regulated *PSA* gene in LNCaP PCa cells. It should be noted that LNCaP cells express a mutant AR (T877A) that is stimulated by the AR antagonist hydroxyflutamide, but is still repressed by bicalutamide. Transfection with 20 μM or 40 μM of an NCoR

siRNA pool (NCoR1 siRNA) substantially decreased NCoR protein expression in LNCaP cells relative to control siRNA, with no clear effect on SMRT (Fig. 5A). LNCaP cells cultured in steroid hormone depleted medium were then transfected with NCoR1 or control siRNA and stimulated with DHT or bicalutamide. Expression of the *PSA* gene was then assessed by real time RT-PCR Significantly, treatment with the NCoR siRNA did not stimulate *PSA* gene expression in response to bicalutamide (Fig. 5A). In contrast, DHT stimulated *PSA* gene expression by ~100-fold, although this DHT stimulated *PSA* expression was not further enhanced by the NCoR1 siRNA.

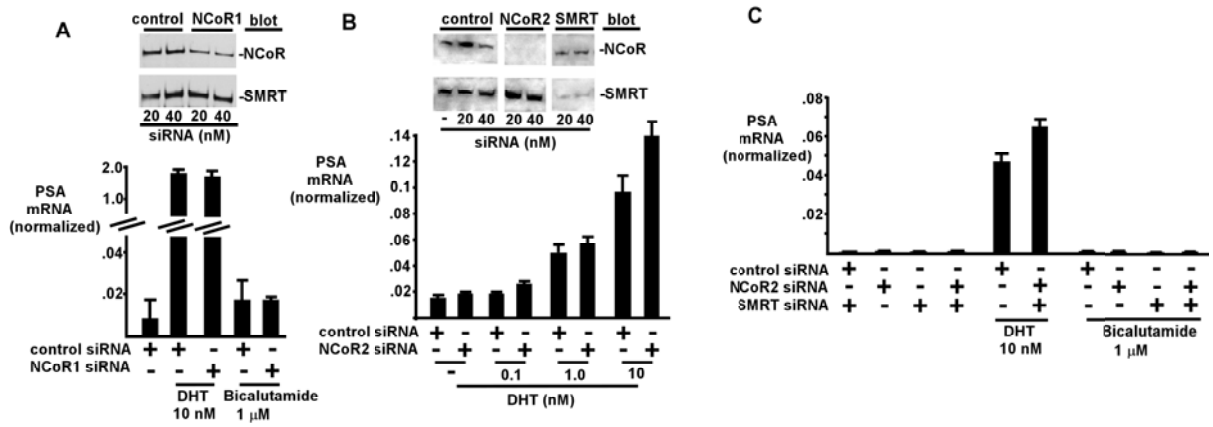



**Figure 5. Bicalutamide antagonist activity is independent of AR corepressors NCoR and SMRT. A, (above) LNCaP cells in steroid hormone depleted medium were transfected with NCoR1 siRNA or control siRNA for 24 hours, cultured for an additional 24 hours, and equal amounts of protein were then immunoblotted for NCoR and SMRT. A, (below) LNCaP cells in steroid hormone depleted medium were transfected with NCoR1 siRNA or control siRNA (both at 40 μM) for 24 hours, and then cultured for an additional 24 hours in medium supplemented with DHT or bicalutamide, as indicated. Equal amounts of RNA were then used to determine endogenous PSA mRNA levels by real time RT-PCR in triplicate samples, which were normalized to *cyclophilin*. B, (above) whole cell lysates were prepared from LNCaP cells transfected as above with 20 or 40 μM NCoR2, SMRT, or a control siRNA. Specific downregulation of NCoR and SMRT proteins was then determined by immunoblotting equal amounts of protein. B, (below) LNCaP cells transfected for 24 hours with NCoR2 or control siRNA (40 μM) were treated for a subsequent 24 hours with increasing concentrations of DHT, as indicated. Equal amounts of RNA were then used to measure endogenous *PSA* gene expression in triplicate samples by real time RT-PCR and normalized to *cyclophilin* mRNA. C, LNCaP cells were transfected with 40 μM control, NCoR2, SMRT or combined NCoR/SMRT siRNA for 24 hours, and then stimulated for 24 hours with DHT or bicalutamide. *PSA* gene expression from triplicate samples was evaluated as above.**

We next examined another NCoR siRNA pool (NCoR2 siRNA), which almost completely suppressed NCoR protein expression (Fig. 5B). Interestingly, SMRT protein expression was increased in response to this siRNA, possibly reflecting compensation by the cells for the marked decline in NCoR levels. Significantly, maximal *PSA* gene expression at higher DHT concentrations was enhanced by this NCoR2 siRNA (Fig. 5B). In contrast, bicalutamide agonist activity was still not stimulated by the NCoR2 siRNA or by SMRT siRNA, or by the combination of both siRNAs (Fig. 5C). Taken together, these results indicate any substantial partial agonist activity of bicalutamide is not being blocked through recruitment of the NCoR or SMRT corepressors. Based on these findings, we propose the model outlined in figure 6 for AR binding of coactivators and corperessors in response to agonist and antagonist ligands.

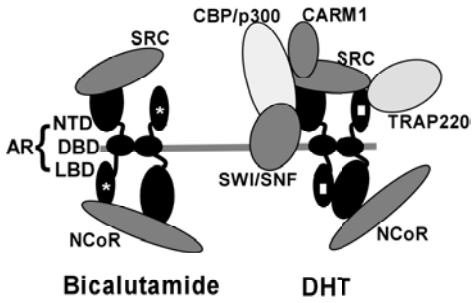



**Figure 6. Model of AR interactions with coactivators versus corepressors in response to DHT and bicalutamide. The AR domains (N-terminal domain, NTD; DNA binding domain, DBD; ligand binding domain, LBD) are shown and the AR homodimer bound to DNA is depicted in an anti-parallel conformation with an intermolecular N-C terminal interaction in response to DHT. In the presence of bicalutamide (asterix, left panel), there is no N-C interaction and NCoR can bind to both the AR N- and C-terminal domains. SRC-1 can also interact with the N-terminal domain of the bicalutamide liganded AR, but is unable to mediate recruitment of additional coactivators. In contrast, the DHT liganded AR (right panel) effectively recruits SRC proteins in conjunction with additional coactivator and chromatin remodelling complexes. These interactions may be mediated directly by the LBD and/or be dependent on conformational changes in the NTD as a result of the N-C terminal interaction. NCoR can still interact weakly with the AR NTD and repress transcriptional activity, but its binding is no longer stabilized**

## MOLEULCAR BASIS FOR NCoR BINDING TO AR

**NCoR interaction with the AR NTD is mediated by a region flanking the N2 CoRNR box.** Previous studies have shown that NCoR can interact with the agonist and antagonist liganded AR, that the AR antagonist RU486 (mifepristone) can enhance AR binding to NCoR, and that both the AR NTD and the LBD are required for this latter mifepristone enhanced NCoR-AR interaction. We have further shown that this NCoR interaction with the mifepristone liganded AR is mediated by a C-terminal fragment of NCoR containing the N2 and N1 CoRNR boxes. To further determine whether this region of NCoR interacts with the AR NTD independently of the LBD, we assessed coactivation of an AR fragment containing only the NTD and DBD (AR-NTD-DBD). Consistent with previous studies, the AR-NTD-DBD was constitutively active in the absence of ligand when assayed on an androgen responsive element regulated luciferase reporter gene ($ARE_4$-luciferase) (Fig. 7B). However, this activity could be increased by cotransfection of the NCoR(2005-2440) fragment fused to the VP16 transactivation domain, VP16-NCoR(2005-2440). This coactivation was decreased by deletion of the region immediately N-terminal to the N2 CoRNR box in VP16-NCoR(2043-2440) (Fig. 7C). Further deletion of the N2 CoRNR box in VP16-NCoR(2065-2440) completely abrogated coactivation. These findings indicated that a region encompassing the N2 CoRNR box (residues 2005-2065) mediated an interaction with the AR NTD that was independent of the LBD.

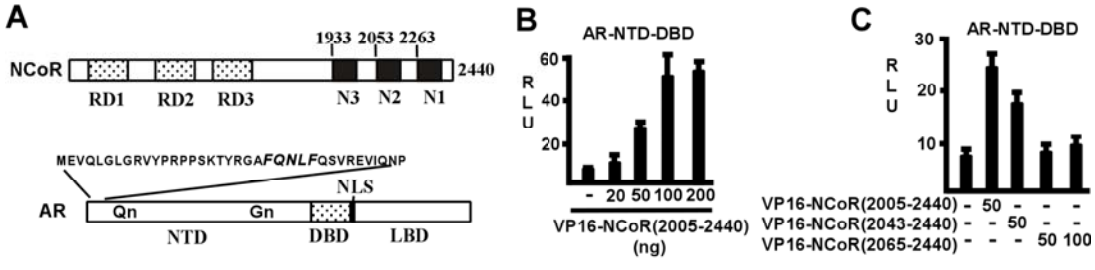



**Figure 7. NCoR interaction with AR NTD. A, outline of NCoR and AR structures. B, CV1 cells were transfected with AR-NTD-DBD, $ARE_4$-luciferase reporter, control pRL-CMV, and increasing amounts of VP16-NCoR(2005-2440) expression vector. C, CV1 were**

**transfected with AR-NTD-DBD, $ARE_4$-luciferase reporter, control pRL-CMV, and 50 or 100 ng of the indicated VP16-NCoR expression vectors. Firefly luciferase activity normalized for *Renilla* luciferase activity (relative light units, RLU) was determined from triplicate samples ( ± SE).**

**The extreme N-terminus of the AR NTD is required for NCoR binding to the mifepristone liganded AR.** We showed previously that deletion of the amino-terminal end of the AR NTD (residues 1-366) abrogated NCoR interaction with the mifepristone liganded AR. In contrast, mutation or deletion of the FQNLF peptide in the AR NTD (residues 23-27) (Fig. 7A), which binds strongly to the agonist liganded AR LBD, did not impair NCoR binding to the mifepristone liganded AR. To further map the region in the AR NTD that mediates binding to the NCoR C-terminus, we generated additional AR NTD mutants and cotransfected them with the C-terminal end of NCoR (residues 2005-2440, containing the N2 and N1 CoRNR boxes) fused to the VP16 transactivation domain. Consistent with previous results, mifepristone had minimal agonist activity relative to DHT, but could strongly stimulate recruitment of VP16-NCoR(2005-2440) (Fig. 8A). Deletion of the first 11 residues in the AR NTD did not impair NCoR recruitment, but NCoR recruitment was abrogated by deletion of residues 1-37 in AR(del 1-37) (Fig. 8A). A larger NCoR fragment containing all three CoRNR boxes, VP16-NCoR(1806-2440), was also strongly recruited by the mifepristone liganded wild-type AR, but similarly failed to coactivate the mifepristone liganded AR(del 1-37) (Fig. 8B and C).

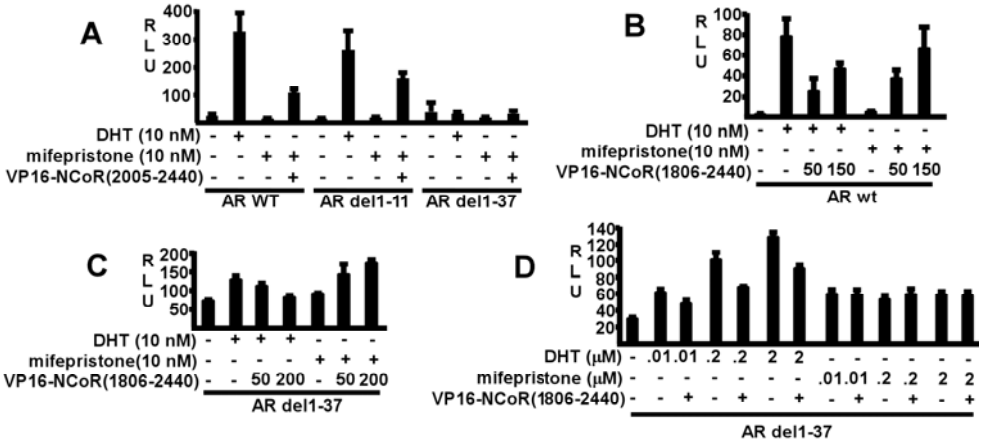



**Figure 8. RU486 mediated NCoR recruitment requires the androgen receptor N-terminus. A, CV1 cells were cotransfected with wild type AR or truncated del11-AR or del37-AR lacking the first 11 or 37 amino acids respectively, VP16-NCoR(2005-2440), $ARE_4$-luciferase reporter vector and a CMV regulated *Renilla* luciferase plasmid (pRL-CMV). Cells were treated with the indicated ligands for 24 hrs in steroid depleted medium (DMEM/10% charcoal dextran stripped fetal bovine serum) and luciferase versus *Renilla* luciferase activities were determined from triplicate samples. The data are expressed as RLU ± SE. B, CV1 cells were cotransfected and analyzed as in A, except for use of the longer NCoR vector, VP16-NCoR(1806-2440). C and D, CV1 cells were transfected with $ARE_4$-luciferase and control *Renilla* reporters, AR(del1-37), and VP16-NCoR(1806-2440) vectors and the indicated concentrations of ligands for 24 hours.**

Importantly, the AR(del 1-37) was only very weakly stimulated by 10 nM DHT, suggesting that the failure to recruit NCoR could reflect a gross defect in AR protein folding rather than loss a specific protein-protein interaction (Fig. 8C). However, the transcriptional activity of the AR(del 1-37) was partially restored at higher DHT concentrations (~4-fold induction at 2 μM DHT) (Fig. 8D), consistent with previous data showing that loss of the AR N-C terminal interaction (mediated by the FQNLF peptide) increases the off-rate for DHT binding to the LBD and thereby decreases affinity. In contrast,

there was no NCoR recruitment by the AR(del1-37) mutant even at higher mifepristone concentrations (Fig. 8D).

While these results suggested that NCoR was interacting with a region between residues 11-37 in the AR, we have not been able to detect a direct interaction using the AR(1-37) peptide in two-hybrid protein interaction assays (data not shown). Therefore, an alternative interpretation of these results was that this peptide was stabilizing NCoR binding through an interaction with the mifepristone liganded AR LBD, and that this interaction with the LBD was independent of the FQNLF peptide. To test this hypothesis, we next examined mutations in arginines 20 and 31, which flank the FQNLF peptide and have been shown to contact the agonist liganded AR LBD. However, these mutations did not impair AR activation by DHT or decrease NCoR recruitment in response to mifepristone (Fig. 9A). We also deleted a second short helical domain (VREVI, residues 30-34) and residues 36-7 (NP). These mutations similarly had no effect on NCoR recruitment in response to mifepristone (Fig. 9B). Taken together, these studies identify a region in the AR NTD that is critical for NCoR binding to the mifepristone liganded AR, either directly through weak interactions with NCoR or indirectly by stabilizing AR in a conformation that can bind NCoR.

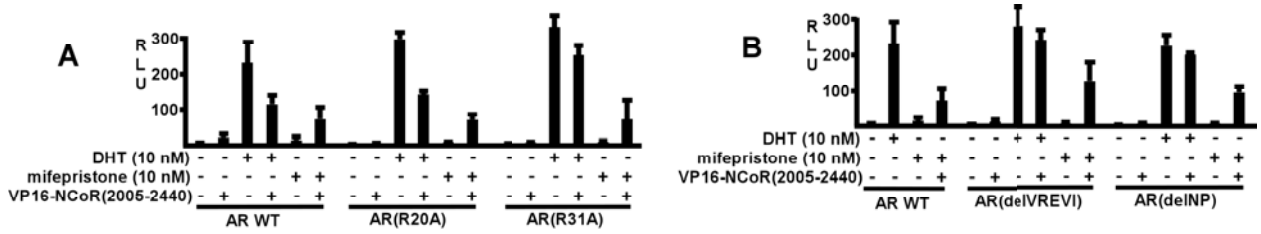



**Figure 9. Mutagenesis of sites in AR N-terminus that may mediate NCoR recruitment. A and B, CV1 cells were transfected for 24 hrs with VP16-NCoR(2005-2440), $ARE_4$-luciferase and *Renilla* control reporters, and wild-type (WT) or the indicated mutant AR expression vectors in steroid depleted medium. They were then stimulated for 24 hrs with DHT or mifepristone as indicated, and RLU were determined.**

**The N1 CoRNR box is critical for NCoR binding to the mifepristone liganded AR.** We showed previously that removal of the region containing the N1 CoRNR box abrogated NCoR binding to the mifepristone liganded AR. To further assess whether the N1 CoRNR box mediates the interaction with the mifepristone liganded AR LBD, we mutated a double isoleucine in N1 to alanines in the NCoR(1806-2440) fragment, which contains all three CoRNR boxes. We also mutated a double isoleucine in the N2 CoRNR box to alanines. These fragments were fused to the Gal4 DBD, and assessed for interaction with VP16-AR and pG5-luciferase reporter. There was a strong interaction between the mifepristone liganded VP16-AR and Gal4DBD-NCoR(1806-2440), and this interaction was not impaired by the N2(AA) mutation. However, the interaction was abrogated by the N1(AA) mutation, indicating that the N1 CoRNR box was critical for binding (Fig. 10A). As a further control to confirm that the N1(AA) mutation was not non-specifically altering the structure of the protein, we determined whether the interaction with unliganded TRβ (which is mediated by the N3 CoRNR box) was intact. As shown in figure 10B, the wild-type, N1(AA) and N2(AA) Gal4-NCoRc interacted with the VP16-TRβ (Fig. 10B).

To confirm that the N1 CoRNR box was critical for binding to the intact AR bound to an androgen responsive element, we cloned the N1(AA) mutation into the VP16-NCoR(1806-2440) vector. As shown in figure 10C, the N1(AA) mutation abrogated NCoR recruitment by the mifepristone liganded wild-type AR. The N1(AA) mutation cloned into the VP16-NCoR(2005-2440) vector (containing the N2 and N1 CoRNR boxes) similarly abrogated recruitment (Fig. 10D). To control for non-specific effects of the mutation in the NCoR(2005-2440) fragment, we showed that the mutation did not impair recruitment by the unliganded RARα fused to the Gal4 DNA binding domain (Fig. 10E).

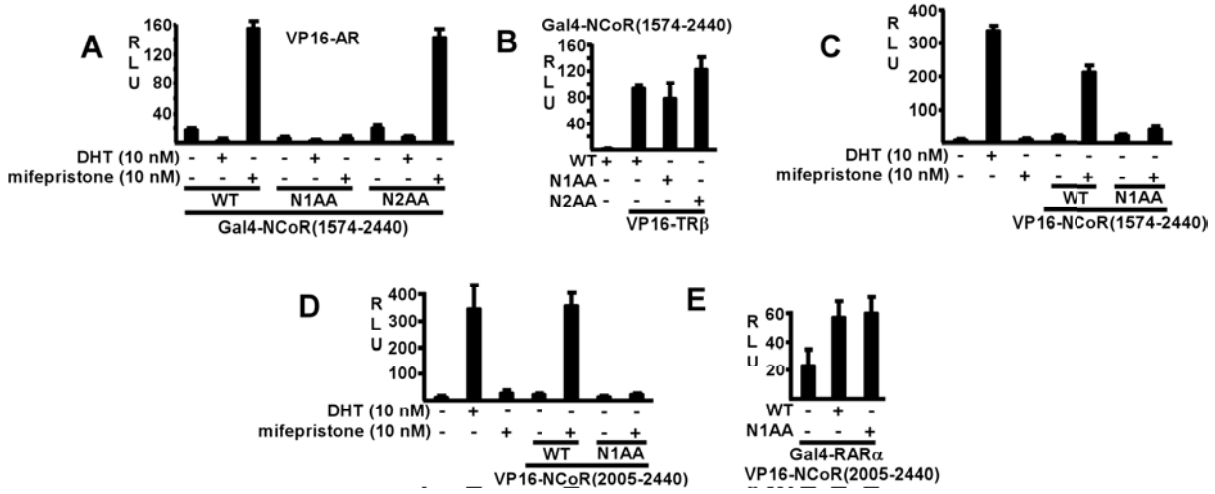
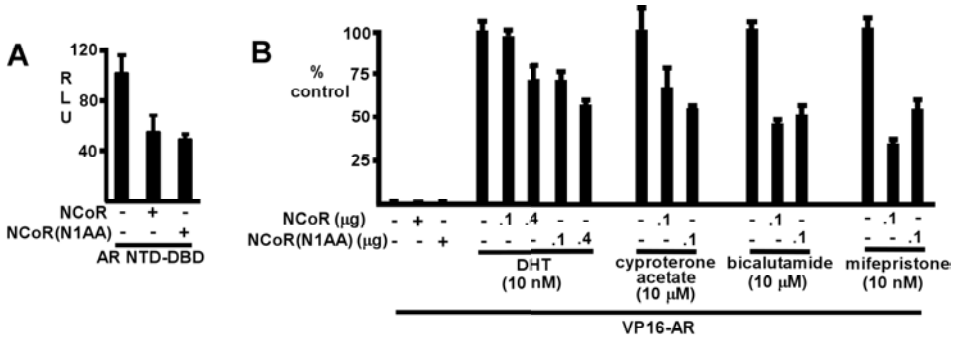



**Figure 10. Mutation of NCoR N1 CoRNR box prevents NCoR C-terminal recruitment to RU486 liganded AR. A, CV1 cells were cotransfected with Gal4-NCoR(1806-2440) wild-type or with double alanine substitution at CoRNR boxes N1 or N2, VP16-AR, UAS-luciferase reporter vector (pG5-Luc) and a CMV regulated *Renilla* luciferase plasmid (pRL-CMV). Cells were treated with the indicated ligands for 24 hrs in steroid depleted medium and luciferase versus *Renilla* luciferase activities were determined from triplicate samples. The data in this experiment and below are expressed as RLU ± SE. B, Cells were transfected as in A with an expression vector for full-length VP16-TRβ1, the pG5-Luc luciferase reporter and the pRL-CMV control in the absence of ligand. C, cells were transfected with full-length AR, $ARE_4$-luciferase reporter and the pRL-CMV control, VP16-NCoR(1806-2440) wild-type or N1AA mutant. Cells were treated with the indicated ligands for 24 hrs and luciferase versus *Renilla* luciferase activities were determined from triplicate samples. D, cells were transfected as in C, but using wild-type or N1AA mutant VP16-NCoR(2005-2440). E, CV1 cells were transfected with Gal4-RARα, VP16-NCoR(2005-2440) wild-type or N1AA mutant, pG5-luciferase and *Renilla* control reporters, and luciferase versus *Renilla* luciferase activities were determined from triplicate samples.**

Finally, we cloned the N1(AA) mutation into the intact full length NCoR. As expected, both the wild-type and mutant NCoR could suppress the constitutive activity of the AR NTD (Fig. 11A). We then assessed inhibition of the DHT versus mifepristone liganded AR. As the latter does not have substantial transcriptional activity, we carried out these cotransfections with a VP16-AR fusion protein. Significantly, both the wild-type and N1(AA) mutant NCoR suppressed the DHT liganded VP16-AR, with the N1(AA) mutant being more effective (Fig. 11B). The N1AA was also more effective at repressing VP16-AR transactivation by a partial agonist (cyproterone acetate), while its effect on a pure antagonist (bicalutamide) were comparable to the wild-type NCoR. In contrast, the N1AA was less active at repressing the mifepristone liganded VP16-AR, consistent with the N1 CoRNR box enhancing the interaction. It should be noted that while the N1(AA) mutation abrogates interaction with NCoR C-terminal fragments, it does not do so in the context of full length NCoR. This likely reflects additional contacts between NCoR and the AR NTD mediated by N-terminal regions of NCoR.

**Figure 11 (below). NCoR N1 CoRNR box contributes to binding of full length NCoR to the mifepristone liganded AR. A, CV1 cells were transfected with AR NTD-DBD, $ARE_4$-luciferase and pRL-CMV control reporters, full length wild-type NCoR (NCoR) or N1AA mutant NCoR, NCoR(N1AA). Firefly versus *Renilla* luciferase activities were determined from triplicate samples, and the data are expressed as RLU ± SE. B, CV1 cells were transfected with VP16-AR, full length wild-type or N1AA mutant NCoR, $ARE_4$-luciferase and pRL-CMV control reporters for 24 hrs in steroid hormone depleted medium. Cells were**

**then treated for 24 hrs with the indicated ligands, and luciferase versus *Renilla* luciferase activities were determined from triplicate samples.**




**Charged residues common to N1 CoRNR boxes in NCoR and SMRT are critical for binding.** A previous study found that the interaction between AR and SMRT was dependent on the SMRT N1 CoRNR box. An alignment of the SMRT and NCoR CoRNR boxes shows that the N1 CoRNR boxes are almost identical (Fig. 12A). Moreover, they are distinct from the other CoRNR boxes in having a charged residue (arginine) at position 6. They also share a glutamate at position 2, which is aspartate in the N3 CoRNR box of NCoR and alanine in the other CoRNR boxes. Therefore, we carried out further mutagenesis to determine whether these charged residues common to the NCoR and SMRT CoRNR boxes contribute to NCoR binding to the mifepristone liganded AR. Significantly, mutations at either site markedly impaired NCoR recruitment by the mifepristone liganded AR (Fig. 12B).

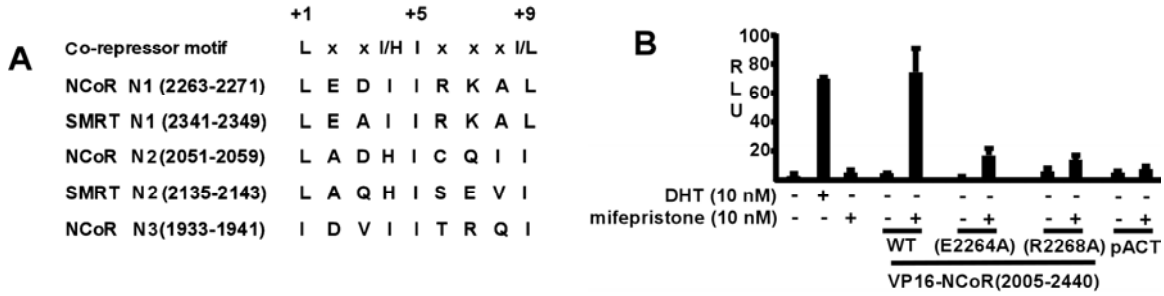



**Figure 12. Charged residues in NCoR N1 CoRNR box contribute to binding to mifepristone liganded AR. A, sequence alignment of NCoR and SMRT CoRNR boxes. B, CV1 cells were cotransfected for 24 hrs with AR, $ARE_4$-luciferase and pRL-CMV control reporters, and VP16-NCoR(2005-2440) wild-type or the indicated mutants at position 2 (E2264A) or 6 (R2268A) of the N1 CoRNR box. Cells were then treated with DHT or mifepristone for 24 hrs and assayed for luciferase activity.**

**N1 is interacting with the AR coactivator binding site.** To further assess the molecular basis for N1 CoRNR box binding to the AR, and in particular to test the hypothesis the N1 CoRNR box was binding to the coactivator site, we compared the available crystal structures of the agonist liganded AR binding to a FQNLF peptide and the antagonist liganded PPARγ binding to a SMRT N1 CoRNR box peptide. A conserved lysine at the C-terminal end of helix 3 in AR and PPARγ anchors both peptides by forming hydrogen bonds with the C-terminal phenylalanine or leucine residues, respectively. The FQNLF peptide forms 2 helical turns and is anchored at its N-terminus by helix 12 in the AR. In contrast, displacement of this helix in the antagonist conformation allows the site to accommodate a third helical turn in the CoRNR box, with leucines at position 1 and 9, and isoleucine at position 5, forming a hydrophobic face that binds to helix 3. Another face of the CoRNR box helix is formed by glutamic acid at position 2 and arginine at position 5, which interact with K310 and N303 in helix 4 of PPARγ. Significantly, these residues in PPARγ correspond to Q738 and D731 in helix 4 of the AR, suggesting that strong interactions

between these acidic and basic residues may stabilize CoRNR box binding to the mifepristone liganded AR (Fig. 13A).

To determine whether K720, D731, and Q738 do contribute to NCoR binding by mifepristone liganded AR, we next examined site directed mutants. A K720A mutation in the AR only moderately decreased DHT stimulated activity, possibly due to strong hydrophobic interactions mediated by the phenylalanines in the FQNLF peptide (Fig. 13B). In contrast, the K720A mutation markedly impaired recruitment of VP16-NCoR(1806-2440) in response to mifepristone. The mutations in helix 4 (D731A and Q738A) had substantial effects on DHT stimulated AR activity, but again more markedly impaired the response to mifepristone (Fig. 13C). Taken together, the NCoR mutagenesis data above and these AR mutagenesis data strongly support the conclusion that the N1 CoRNR box is binding to the coactivator/corepressor binding site in the AR LBD.

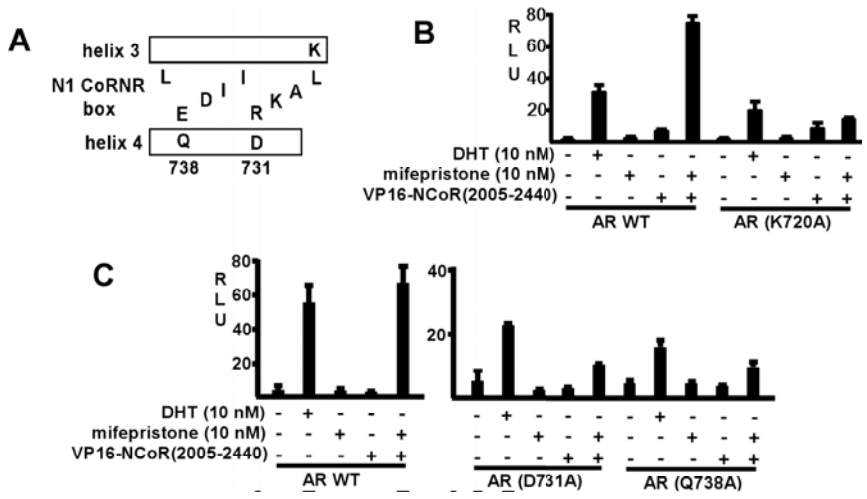



**Figure 13. Charged residues in NCoR N1 CoRNR box contribute to binding to mifepristone liganded AR. A, predicted interactions between the NCoR N1 CoRNR box and AR helices 3 and 4. B, CV1 cells were cotransfected with wild-type or K720A mutant AR, VP16-NCoR(2005-2440), $ARE_4$-luciferase and pRL-CMV control reporters for 24 hrs in steroid depleted medium. Cells were then treated for 24 hrs with the indicated amounts of DHT or mifepristone and assayed for luciferase activity. B, CV1 cells were transfected as in B with the wild-type or indicated mutant ARs.**

Based on the data in this study, a model for AR-NCoR binding is outlined in figure 14. We suggest that NCoR interaction with the agonist liganded AR is mediated primarily by the AR NTD. Significantly, multiple domains on NCoR may mediate this AR NTD interaction, including a region flanking the N2 CoRNR box and a domain in the middle of NCoR containing RD3. Mifepristone induces an alternative conformation of the LBD that increases its affinity for the N1 CoRNR box, and this interaction further stabilizes NCoR binding through the AR NTD (which may be to the same or distinct ARs in the homodimer). We propose that N1 CoRNR box binding may be further stabilized by other ligands, and currents efforts are focused on screening for ligands with this property, which we predict will be more potent AR antagonists.

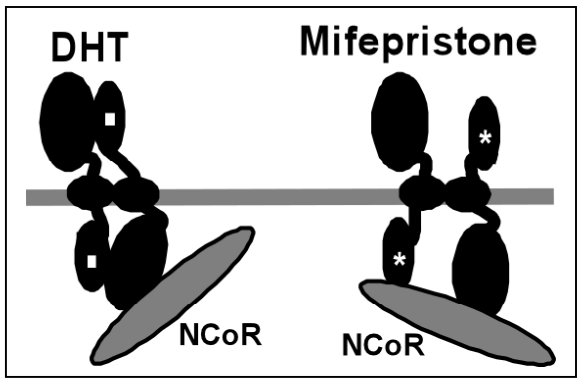



**Figure 14. Model for AR interaction with DHT versus mifepristone.**

## KEY RESEARCH ACCOMPLISHMENTS

1. Determined the role of corepressors in the antagonist activity of bicalutamide.
2. Elucidated the molecular basis for NCoR binding to the mifepristone liganded AR.

## REPORTABLE OUTCOMES

One new manuscript published (attached) (Hodgson et al., 2007). A second manuscript containing much of the above data is currently being submitted for publication.

## CONCLUSIONS

We have established the molecular basis for AR-NCoR interaction. Our data also show that the AR-NCoR interaction mediated by RU486 is strong compared to agonists, but the interaction with the LBD is still relatively weak. Importantly, we anticipate that our new insights into AR-NCoR binding will allow us to develop further more potent antagonists that effectively stimulate NCoR binding to the AR LBD and markedly suppress AR activity in PCa cells.

## REFERENCES

Hodgson,M.C., Astapova,I., Hollenberg, A.N., and Balk, S.P. (2007). Activity of Androgen Receptor Antagonist Bicalutamide in Prostate Cancer Cells is Independent of NCoR Cancer Res., 67, 8388-8395.

## APPENDICES

Manuscript attached (Hodgson et al., 2007).

# Activity of Androgen Receptor Antagonist Bicalutamide in Prostate Cancer Cells Is Independent of NCoR and SMRT Corepressors

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[1]Cancer Biology Program/Hematology-Oncology Division and [2]Endocrine Division, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts

## Abstract

**The mechanisms by which androgen receptor (AR) antagonists inhibit AR activity, and how their antagonist activity may be abrogated in prostate cancer that progresses after androgen deprivation therapy, are not clear. Recent studies show that AR antagonists (including the clinically used drug bicalutamide) can enhance AR recruitment of corepressor proteins [nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid receptors (SMRT)] and that loss of corepressors may enhance agonist activity and be a mechanism of antagonist failure. We first show that the agonist activities of weak androgens and an AR antagonist (cyproterone acetate) are still dependent on the AR $NH_2$/COOH-terminal interaction and are enhanced by steroid receptor coactivator (SRC)-1, whereas the bicalutamide-liganded AR did not undergo a detectable $NH_2$/COOH-terminal interaction and was not coactivated by SRC-1. However, both the isolated AR $NH_2$ terminus and the bicalutamide-liganded AR could interact with the SRC-1 glutamine-rich domain that mediates AR $NH_2$-terminal binding. To determine whether bicalutamide agonist activity was being suppressed by NCoR recruitment, we used small interfering RNA to deplete NCoR in CV1 cells and both NCoR and SMRT in LNCaP prostate cancer cells. Depletion of these corepressors enhanced dihydrotestosterone-stimulated AR activity on a reporter gene and on the endogenous AR-regulated *PSA* gene in LNCaP cells but did not reveal any detectable bicalutamide agonist activity. Taken together, these results indicate that bicalutamide lacks agonist activity and functions as an AR antagonist due to ineffective recruitment of coactivator proteins and that enhanced coactivator recruitment, rather than loss of corepressors, may be a mechanism contributing to bicalutamide resistance.** [Cancer Res 2007;67(17):8388–95]

## Introduction

The androgen receptor (AR) plays a central role in prostate cancer development and progression, and androgen deprivation therapy by suppression of testicular androgen production (surgical castration or administration of luteinizing hormone–releasing hormone superagonists), or by treatment with AR antagonists (flutamide or bicalutamide), is still the standard systemic treatment. The majority of patients have clinical and biochemical [decrease in serum prostate-specific antigen (PSA)] evidence of improvement but eventually relapse with a more aggressive form of prostate cancer that has been termed hormone-refractory, castration-resistant, or androgen-independent prostate cancer. However, the AR and AR-regulated genes are still expressed at high levels in androgen-independent prostate cancer, indicating that AR transcriptional activity is reactivated in these tumors and that AR remains as a potential therapeutic target (1–4). One mechanism that may contribute to AR reactivation is increased accumulation or synthesis of androgens by prostate cancer cells, and a subset of patients who relapse after castration or luteinizing hormone–releasing hormone agonist treatment will respond to secondary hormonal therapies with AR antagonists such as bicalutamide or to treatments that suppress residual adrenal androgen production such as ketoconozole. However, these responses are usually partial and transient, with AR activity becoming resistant to even high doses of the AR antagonist bicalutamide through unclear mechanisms (5).

Additional mechanisms that may contribute to AR reactivation after androgen deprivation therapy are increased AR expression, including *AR* gene amplification that occurs in approximately one third of patients, and AR mutations that can enhance responses to nonandrogen steroids and to antagonists (6–9). Increased AR expression can enhance the growth of prostate cancer xenografts in castrated mice and has been reported to enhance the agonist activity of the AR antagonist bicalutamide (10). Mutant ARs that are strongly stimulated by the AR antagonist flutamide have been found in approximately one third of patients who relapse after combination therapy with flutamide, and a distinct mutant AR that is strongly stimulated by bicalutamide has been found in long-term bicalutamide-treated patients, but such mutations are uncommon in patients treated with surgical or medical castration monotherapy (9, 11). Further mechanisms that can enhance AR activity and may contribute to AR activation and resistance to AR antagonists include increased expression of transcriptional coactivator proteins and activation of kinases and signal transduction pathways that can modulate AR function, including the protein kinase A, c-Src, cyclin-dependent kinase 1, Ras-Raf-mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase pathways (12–17). However, the extent to which these or other mechanisms can account for resistance to AR antagonists is unclear.

The physiologic high-affinity ligands for AR [testosterone and dihydrotestosterone (DHT)] induce conformational changes in the ligand binding domain (LBD) and a strong interaction with the AR $NH_2$ terminus (mediated by a phenylalanine motif, FQNLF) with subsequent recruitment of coactivator proteins and potent stimulation of transcriptional activity (18–20). We previously reported that the AR antagonist bicalutamide could mediate AR binding to DNA but failed to mediate the AR $NH_2$/COOH-terminal interaction or recruitment of SRC coactivators (21). More recent

**Note:** M.C. Hodgson and I. Astapova contributed equally to this work.

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data show that the agonist-liganded AR can also recruit corepressor proteins such as nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid receptors (SMRT); that recruitment of these corepressors can be enhanced by bicalutamide; and that bicalutamide may function as an agonist in the absence of these corepressors (22–31). The precise mechanisms by which AR antagonists function and how their activity is abrogated in prostate cancer that relapses after androgen deprivation are of central importance for the development of new therapies. Therefore, this study further examines the roles of AR $NH_2$/COOH-terminal interactions and recruitment of coactivators versus corepressors in mediating AR antagonist activity.

## Materials and Methods

**Plasmids and reagents.** Expression vectors for AR (pSVARo), VP16-AR, VP16-AR-NTD (amino acids 1–500), AR-DBD/LBD (amino acids 501–919), Gal4-AR-LBD (amino acids 661–919), AR-NTD/DBD, and SRC-1 (pSG5-SRC1) have previously been described (22, 29, 32). The Gal4-SRC-1 (amino acids 1,050–1,185) construct was kindly provided by Dr. Frank Claessens (Faculty of Medicine, University of Leuven, Leuven, Belgium; ref. 33). The mutant dFQNLF (deletion of amino acids 23–27) was generated from pSVARo using the QuickChange site-directed mutagenesis kit (Stratagene). The reporter construct $ARE_4$-luciferase, containing four tandem copies of a synthetic ARE, has been described (29). pG5-luciferase, regulated by five tandem Gal4 binding sites, and pRL-CMV, a cytomegalovirus (CMV) promoter regulated *Renilla* control, were from Promega. DHT, androstenedione, progesterone, and cyproterone acetate were from Sigma and bicalutamide was from Astra-Zeneca.

**Cell culture and transfection.** CV1 cells were maintained in DMEM supplemented with 5% fetal bovine serum (FBS; Hyclone). Cells in 48-well tissue culture plates in DMEM containing 5% charcoal dextran–stripped FBS (CDS-FBS; Hyclone) were cotransfected using LipofectAMINE 2000 (Invitrogen). Cells were transfected with 50 ng of reporter vector and AR and SRC-1 expression vectors, except AR-NTD/DBD was transfected at 10 ng/well and 1.25 ng of pRL-CMV *Renilla* vector was used for normalization. After 24 h, medium was replaced with fresh DMEM/5% CDS-FBS containing hormone or drugs at the indicated final concentrations. Following a further 24 h, firefly and *Renilla* luciferase activities were assayed with the dual-luciferase assay system from Promega as per supplier's instructions. All samples were in triplicate and firefly luciferase activities were normalized for cotransfected *Renilla* activity.

**RNA interference.** Vectors expressing NCoR small hairpin RNAs (shRNA) under the control of the U6 promoter were previously described (22). The target sequence for NCoR was: 5′-GGGCTTATGGAGGACCTATGA-3′. To assess AR transactivation in a reporter system, CV1 cells in six-well plates were cotransfected with 1 μg of this shRNA plasmid (pBSU6-NCoR) or a control plasmid (pBSU6), 400 ng of AR expression vector, 400 ng of $ARE_4$-luciferase reporter plasmid, and 3 ng of pCMV-βGal plasmid for normalization. After 24 h, medium was replaced with fresh DMEM/10% CDS-FBS containing either 10 nmol/L DHT or 1 μmol/L bicalutamide. After another 24 h, luciferase and β-galactosidase activities were measured. All samples were in triplicate and luciferase activities were normalized for cotransfected β-galactosidase activity.

To assess the role of corepressors in AR regulation of the endogenous *PSA* gene, a prostate cancer cell line (LNCaP) was used. LNCaP cells in six-well plates in 2 mL of RPMI 1640 containing 10% CDS-FBS (Hyclone) were transfected using LipofectAMINE 2000 (Invitrogen) with 40–80 pmol (20–40 nmol/L) NCoR1, NCoR2, SMRT, or negative control STEALTH small interfering RNA (siRNA; Invitrogen). After 24 h, the medium was replaced with fresh RPMI/10% CDS-FBS containing either 10 nmol/L DHT or 1 μmol/L bicalutamide. After 24-h incubation, RNA from the cells was isolated, and expression of target genes was determined by real-time quantitative reverse transcription-PCR (RT-PCR). Alternatively, to assess knockdown of specific proteins, whole-cell lysates were prepared. Proteins were separated on 4% to 12% NuPAGE gels (Invitrogen) and transferred onto nitrocellulose membranes. Blots were probed with a 1:500 dilution of an affinity-purified anti-NCoR antiserum or anti-SMRT antibody (BD Biosciences) in TBS containing 5% nonfat milk and 0.05% Tween 20, followed by horseradish peroxidase–conjugated antirabbit antibody (Amersham Biosciences) at 1:1,000 dilution. The blots were visualized with the use of ECL Plus Western blotting detection system (Amersham Biosciences).

**Real-time PCR.** Total RNA from LNCaP cells was extracted using STAT-60 reagent (Tel-Test), and 1 μg was reverse transcribed with random hexamer primers using Advantage RT-for-PCR kit (BD Biosciences). Quantitative PCR was done in MX3000P Real-time PCR System (Stratagene). TaqMan Gene Expression Assays for *PSA* and *cyclophilin* (endogenous control) were purchased from Applied Biosystems. The data presented are the mean of three biological replicates normalized by *cyclophilin* mRNA expression.

## Results

**Partial agonist activities of weak androgens and AR antagonists are dependent on the AR $NH_2$/COOH-terminal interaction.** The physiologic high-affinity ligands for AR (testosterone and DHT) induce conformational changes in the LBD and a strong interaction with the AR $NH_2$ terminus. In contrast, previous studies have found no detectable interaction between the bicalutamide-liganded AR LBD and the $NH_2$ terminus, which may account for the lack of agonist activity of bicalutamide (21). However, AR transcriptional activity can be stimulated by other steroid hormones or drugs such as cyproterone acetate that do not mediate clearly detectable AR $NH_2$/COOH-terminal interactions, suggesting that the agonist activities of these drugs may not be dependent on the $NH_2$/COOH-terminal interaction (19). The interaction between the AR LBD and $NH_2$ terminus is mediated by a phenylalanine motif at amino acids 23 to 27 (FQNLF), which binds tightly to the LXXLL coactivator cleft in the AR LBD (18). Therefore, we first examined whether deletion of this motif in AR (dFQNLF) impaired AR activity in response to DHT versus weak agonists or drugs.

Consistent with previous data, deletion of FQNLF markedly impaired AR transcriptional activity in response to DHT (Fig. 1*A*). We presume that this largely reflects loss of the $NH_2$/COOH-terminal interaction (although additional effects of the deletion cannot be ruled out) and that residual activity is independent of this interaction, is mediated by another site, or is due to bridging of the $NH_2$ and COOH termini by p160 coactivators (34). It should be noted that the fold induction can be further increased at higher DHT concentrations but that activity is similarly impaired by the deletion of FQNLF (data not shown). Significantly, the FQNLF deletion also markedly impaired AR activity in response to a weak androgen (androstenedione) and to progesterone, both used at micromolar concentrations (Fig. 1*B* and *C*). Moreover, the deletion abrogated the partial agonist activity of the AR antagonist drug cyproterone acetate (Fig. 1*D*). In contrast, bicalutamide did not stimulate the wild-type or mutant ARs (data not shown).

These results suggested that the agonist activities of the above steroid hormones and of cyproterone acetate were dependent on the AR $NH_2$/COOH-terminal interaction. Therefore, we next carried out mammalian one- and two-hybrid protein interaction assays to determine whether cyproterone acetate could induce a detectable AR $NH_2$/COOH-terminal interaction. CV1 cells were cotransfected with expression vectors encoding an $NH_2$-terminal domain (NTD)–deleted AR (AR-DBD/LBD), the AR NTD linked to the VP16 transactivation domain (VP16-AR-NTD), and an ARE regulated

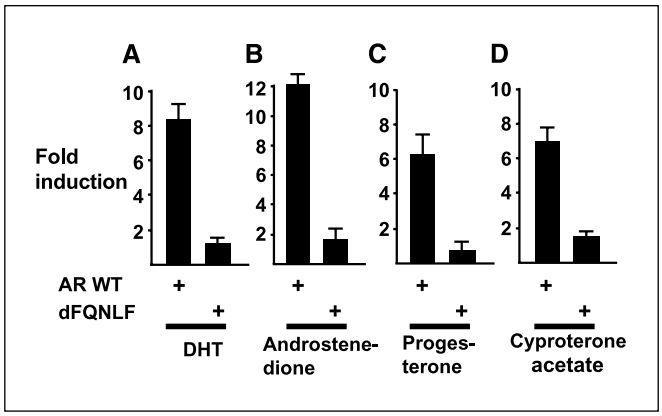


**Figure 1.** AR partial agonist activities are dependent on the $NH_2$ terminus phenylalanine motif. CV1 cells were cotransfected with full-length wild-type AR (pSVARo, AR WT) or full-length AR with the phenylalanine motif (FQNLF, amino acids 23–27) deleted (dFQNLF), $ARE_4$-luciferase reporter, and a control CMV regulated *Renilla* luciferase plasmid (pRL-CMV). Cells were incubated for 24 h in steroid hormone–depleted medium (DMEM/ 5% CDS-FBS) treated with 10 nmol/L DHT (*A*), 1 μmol/L androstenedione (*B*), 1 μmol/L progesterone (*C*), or 10 μmol/L cyproterone acetate (*D*). Firefly versus *Renilla* luciferase activities were determined from triplicate samples and presented as fold induction relative to the activity determined in the absence of ligand.

reporter gene ($ARE_4$-luciferase). As previously shown, DHT induced a strong interaction between the AR NTD and DNA binding domain (DBD)/LBD fragment (Fig. 2*A*). Significantly, cyproterone acetate also induced an interaction, although it was clearly weaker than the DHT-stimulated response and required higher concentrations (1–10 nmol/L for DHT versus 1–10 μmol/L for cyproterone acetate; Fig. 2*A*). In contrast, there was no detectable interaction in response to bicalutamide. Consistent with previous data, the AR DBD/LBD fragment by itself had no detectable transcriptional activity in response to DHT or antagonists (data not shown).

To confirm the interaction between the AR NTD and the cyproterone acetate–liganded AR LBD, we next carried out two-hybrid experiments using the AR LBD alone fused to the Gal4 DBD (Gal4-AR-LBD). CV1 cells were cotransfected with the Gal4-AR-LBD and VP16-AR-NTD expression vectors and the pG5-luciferase reporter (containing five tandem copies of the Gal4 binding element). Consistent with the above results, cyproterone acetate induced an interaction between the NTD and the LBD (Fig. 2*B*). Taken together, these data indicate that the AR $NH_2$/COOH-terminal interaction is critical for AR transcriptional activity mediated by weak androgens and partial agonist drugs. Moreover, the failure of bicalutamide to mediate this interaction is consistent with the lack of partial agonist activity of this drug.

**SRC-1 coactivator can interact with bicalutamide-liganded AR $NH_2$ terminus but does not stimulate transcriptional activity.** Previous studies have shown that AR recruitment of p160 SRC proteins (in particular, SRC-1) is mediated primarily by the AR NTD, with one proposed function for the $NH_2$/COOH-terminal interaction being to structure the NTD and thereby enhance coactivator binding (35–38). Consistent with this hypothesis and previous data, SRC-1 can coactivate both the DHT and cyproterone acetate–liganded AR but not the bicalutamide-liganded AR (Fig. 3*A*). To further assess whether SRC-1 fails to coactivate the bicalutamide-liganded AR due to the lack of an $NH_2$/COOH-terminal interaction, we examined SRC-1 coactivation of an LBD deletion mutant (AR-NTD/DBD). Significantly, cotransfection of SRC-1 enhanced the transcriptional activity of AR-NTD/DBD on an $ARE_4$-luciferase reporter, showing that SRC-1 could associate with the AR NTD independently of the $NH_2$/COOH-terminal interaction (Fig. 3*B*).

This result indicated that absence of an $NH_2$/COOH-terminal interaction in the bicalutamide-liganded AR was not the basis for lack of SRC-1 mediated coactivation, and suggested that there may be an inhibitory interaction between the AR NTD and the bicalutamide-liganded LBD that prevents SRC-1 binding. Indeed, previous studies have shown that an inhibitory interaction between the unliganded AR LBD and the NTD may abrogate the intrinsic transcriptional activity of the AR NTD (39). To test this hypothesis, we expressed the glutamine-rich domain of SRC-1 (amino acids 1,050–1,185), which mediates SRC-1 binding to the AR NTD (but does not mediate transactivation), as a fusion protein with the Gal4 DBD (33, 35). Significantly, the Gal4-SRC-1(1,050–1,185) fusion had no transcriptional activity on the pG5-luciferase reporter in transfected CV1 cells, but could be strongly activated by the cotransfected AR NTD, further showing that the AR/SRC-1 interaction was not dependent on the AR LBD (Fig. 3*C*; it should be noted that the AR constructs in these experiments are fused to the VP16 transactivation domain to provide an assessment of binding that is independent of intrinsic AR transcriptional activity).

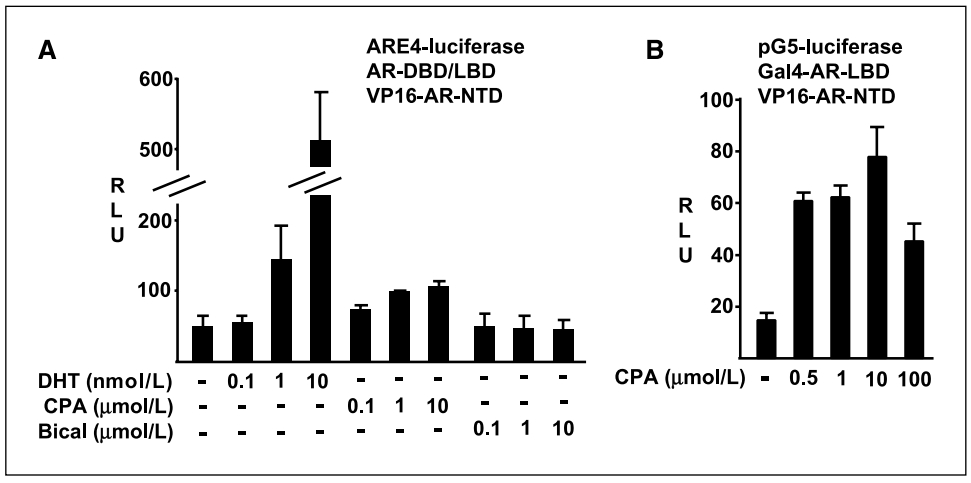


**Figure 2.** Partial agonists mediate AR $NH_2$/COOH-terminal interaction. *A,* CV1 cells were cotransfected with a VP16-AR $NH_2$ terminus expression vector (VP16-AR-NTD) and an NTD-deleted AR (AR-DBD/LBD) in the presence of $ARE_4$-luciferase reporter vector and the pRL-CMV control. Cells were treated with the indicated ligands for 24 h in steroid-depleted medium and firefly versus *Renilla* luciferase activities were determined from triplicate samples. *Columns,* mean relative light units (RLU); *bars,* SE. *Bical,* bicalutamide. *B,* cells were transfected with a multimerized UAS-Luc reporter (pG5-Luc) and pRL-CMV reporter in the presence of Gal4-AR-LBD and VP16-AR-NTD, and treated with increasing concentrations of cyproterone acetate (*CPA*) as indicated. Firefly versus *Renilla* luciferase activities were determined from triplicate samples (*columns,* mean RLU; *bars,* SE).

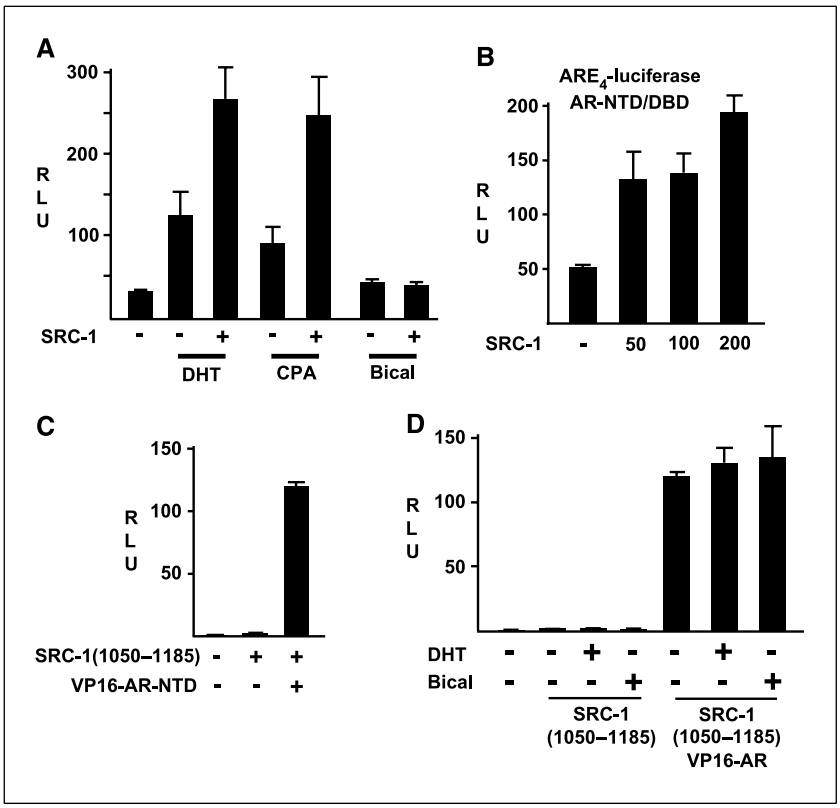


**Figure 3.** SRC-1 coactivates AR $NH_2$ terminus and not the bicalutamide-liganded AR. *A,* CV1 cells were cotransfected with full-length AR, $ARE_4$-luciferase reporter vector, and the pRL-CMV control. In addition, cells were transfected with full-length SRC-1 (pSG5-SRC-1). Cells were treated for 24 h with DHT (10 nmol/L), cyproterone acetate (10 μmol/L), or bicalutamide (10 μmol/L) and firefly versus *Renilla* luciferase activities were determined from triplicate samples (*columns,* mean RLU; *bars,* SE). *B,* cells were transfected as in (*A*) with an expression vector for the AR $NH_2$ terminus and DBD (AR-NTD/DBD), the $ARE_4$-luciferase reporter vector, and the pRL-CMV control, with increasing amounts of SRC-1. *C,* CV1 cells were cotransfected with the pG5-Luc luciferase reporter and expression plasmids for SRC-1 (amino acids 1,050–1,185) fused to the Gal4 DBD and the AR-NTD fused to VP16, as indicated. *D,* CV1 cells were cotransfected with expression plasmids for the full-length AR fused to VP16 (VP16-AR) and SRC-1 (amino acids 1,050–1,185) fused to the Gal4 DBD, the pG5-Luc luciferase vector, and pRL-CMV control. Cells were treated for 24 h with DHT (10 nmol/L) or bicalutamide (10 μmol/L). Firefly versus *Renilla* luciferase activities were determined from triplicate samples (*columns,* mean RLU; *bars,* SE).

The Gal4-SRC-1(1,050–1,185) fusion protein was also strongly activated by the full-length AR fused to VP16 (VP16-AR) in the absence of added ligand or in the presence of DHT (Fig. 3*D*). Importantly, this activation by VP16-AR was not diminished by bicalutamide (Fig. 3*D*). Taken together, these results show that the bicalutamide-liganded AR LBD does not interfere with SRC-1 binding by the AR NTD, although full-length SRC-1 does not coactivate the bicalutamide-liganded full-length AR on an ARE reporter gene.

**Bicalutamide antagonist activity is independent of NCoR and SMRT corepressors.** As the above results indicated that SRC-1 recruitment may not be directly blocked by the bicalutamide-liganded AR LBD, we next considered other mechanisms by which bicalutamide may block coactivator recruitment or induction of transcriptional activity. One such possible mechanism is clearly corepressor recruitment, as previous studies indicate that bicalutamide can enhance NCoR recruitment by the AR and that bicalutamide may function as an agonist in the absence of NCoR (23, 27, 28). A previous study also indicated that AR overexpression in prostate cancer cells could by itself alter the response to bicalutamide and make it function as an agonist (10). The molecular basis for this latter effect of AR overexpression was not clear but could possibly reflect a relative decrease in the levels of corepressors versus coactivators. Therefore, we first examined whether transfection of increasing amounts of AR into CV1 cells could reveal bicalutamide agonist activity. Significantly, increasing the amount of transfected AR enhanced DHT-stimulated transcriptional activity but did not reveal any clear bicalutamide agonist activity (Fig. 4*A*).

To more directly test the hypothesis that the agonist activity of bicalutamide is suppressed by NCoR recruitment, we next carried out shRNA experiments. CV1 cells were transfected with wild-type AR in conjunction with a control or NCoR shRNA expression vector. Consistent with previous results, NCoR shRNA enhanced the transcriptional activity of the DHT-liganded AR, indicating that NCoR negatively regulates the agonist-liganded AR (Fig. 4*B*; ref. 22). In contrast, NCoR shRNA did not reveal substantial bicalutamide agonist activity, although it may very weakly enhance both the unliganded and bicalutamide-liganded ARs (Fig. 4*B*).

To further determine whether NCoR contributes physiologically to the antagonist activity of bicalutamide on the endogenous AR in prostate cancer cells, we examined whether NCoR down-regulation by siRNA would stimulate bicalutamide agonist activity on the endogenous AR-regulated *PSA* gene in LNCaP prostate cancer cells. It should be noted that LNCaP cells express a mutant AR (T877A) that is stimulated by the AR antagonist hydroxyflutamide but is still repressed by bicalutamide. Transfection with 20 or 40 nmol/L of an NCoR siRNA duplex (NCoR1 siRNA) substantially decreased NCoR protein expression in LNCaP cells relative to control siRNA, with no clear effect on SMRT (Fig. 5*A*). LNCaP cells cultured in steroid hormone–depleted medium were then transfected with NCoR1 or control siRNA and stimulated with DHT or bicalutamide. Expression of the *PSA* gene was then assessed by real-time RT-PCR. Significantly, treatment with the NCoR siRNA did not stimulate *PSA* gene expression in response to bicalutamide (Fig. 5*A*). In contrast, DHT stimulated *PSA* gene expression by ~100-fold, although this DHT-stimulated *PSA* expression was not further enhanced by the NCoR1 siRNA.

We next examined another NCoR siRNA duplex (NCoR2 siRNA), which almost completely suppressed NCoR protein expression (Fig. 5*B*). Interestingly, SMRT protein expression was increased in response to this siRNA, possibly reflecting compensation by the

cells for the marked decline in NCoR levels. Significantly, maximal *PSA* gene expression at higher DHT concentrations was enhanced by this NCoR2 siRNA (Fig. 5*B*), confirming another recent report showing that NCoR negatively regulates the endogenous agonist–liganded AR in prostate cancer cells (24). In contrast, bicalutamide agonist activity was still not stimulated by the NCoR2 siRNA or by SMRT siRNA, or by the combination of both siRNAs (Fig. 5*C*). Taken together, these results indicate any substantial partial agonist activity of bicalutamide is not being blocked through recruitment of the NCoR or SMRT corepressors.

## Discussion

AR antagonists such as bicalutamide are effective at blocking AR activity and tumor growth in primary prostate cancer but are not effective at blocking the reactivated AR in prostate cancer that recurs after androgen deprivation therapy. Recent studies have indicated that the AR antagonist activity of bicalutamide may be mediated by NCoR corepressor recruitment and that bicalutamide can function as an AR agonist in response to high-level AR expression or removal of NCoR from the AR complex (10, 23, 25–28). However, the data presented here do not support these conclusions because NCoR and SMRT down-regulation enhanced DHT-stimulated AR activity but did not reveal any clear bicalutamide agonist activity. An alternative general mechanism of action for bicalutamide is that the bicalutamide-liganded AR LBD is unable to effectively recruit coactivators (21). The conformation of the AR LBD may influence coactivator recruitment directly through interactions with coactivators as well as indirectly through the $NH_2$/COOH-terminal interaction. This study shows that the AR $NH_2$/COOH-terminal interaction is critical for the agonist activity of even weak androgens and partial agonist drugs, indicating that bicalutamide may fail to recruit critical coactivators due to the absence of this interaction. This study further shows that the $NH_2$ terminus of the bicalutamide-liganded AR can still interact with SRC-1, indicating that ineffective recruitment of other coactivators or chromatin remodeling proteins is responsible for the antagonist activity of bicalutamide. Consistent with this conclusion, a recent study found that the AR $NH_2$/COOH-terminal interaction was required for AR binding to endogenous genes and likely for recruitment of the SWI/SNF complex (40).

The physiologic importance of NCoR and SMRT recruitment by the agonist-liganded AR is supported by results in previous studies that showed increased agonist-stimulated AR transcriptional activity in response to NCoR and SMRT down-regulation (22, 24, 29). A previous study further showed that NCoR and SMRT down-regulation by RNA interference in LNCaP cells could both enhance the DHT-stimulated expression of endogenous AR-regulated genes and decrease the AR antagonist activity of flutamide (24). This result is not inconsistent with the current study because flutamide has substantial agonist activity for the mutant AR (T877A) expressed in LNCaP cells. Therefore, the agonist activities of both DHT and flutamide in these cells are dependent on the balance between coactivators and corepressors, and on this basis, these ligands may be classified as selective AR modulators. In contrast, we conclude that bicalutamide lacks any substantial partial agonist activity, and its activity is therefore not dependent on corepressors or on coactivator-to-corepressor ratios. Importantly, these ratios may become significant if the bicalutamide-liganded AR in advanced prostate cancer cells can acquire the ability to more effectively recruit coactivators (through increased coactivator expression, AR or coactivator phosphorylation, or other modifications).

Chromatin immunoprecipitation studies have confirmed that NCoR is recruited to endogenous AREs by the agonist-liganded AR and showed that this recruitment can be enhanced by AR antagonists (22–26). Recent studies have further shown that NCoR and SMRT are recruited as part of a complex containing HDAC3, TBL1, TBLR1, and TAB2 (27, 41–44). Significantly, TAB2 phosphorylation by MAPK kinase kinase 1 (MEKK1) in response to inflammatory signals has been shown to result in the loss of NCoR and HDAC3 from this complex and has been reported to convert

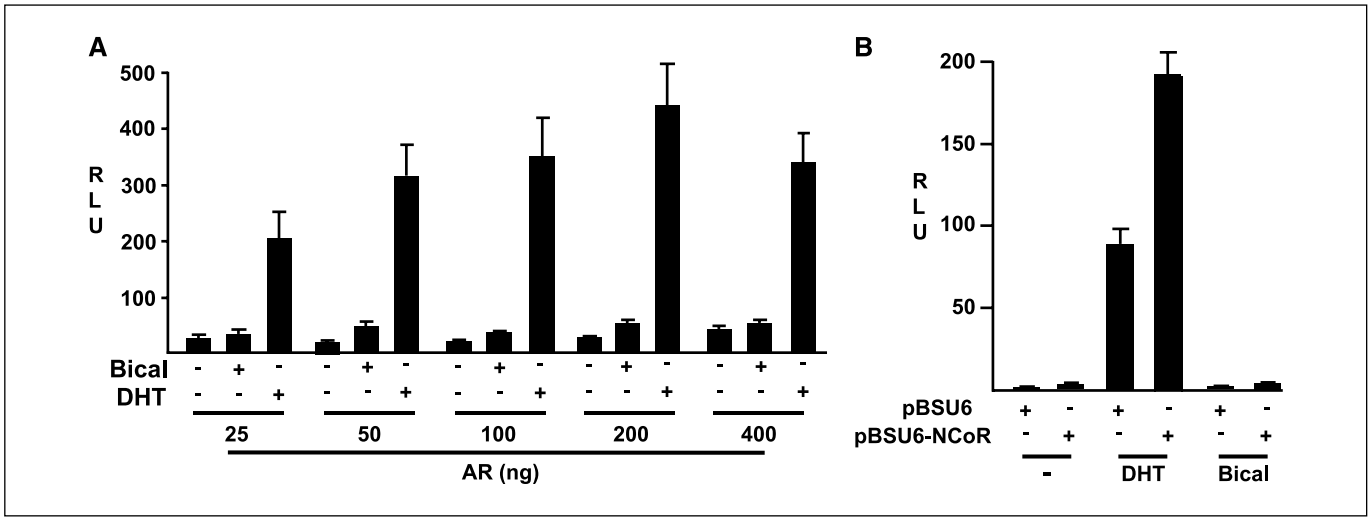


**Figure 4.** AR overexpression and NCoR knockdown do not convert bicalutamide into an agonist in CV1 cells. *A,* cells were cotransfected with the $ARE_4$-luciferase reporter vector, pRL-CMV control, and increasing amounts of full-length AR (25–400 ng). Cells were treated for 24 h with DHT (10 nmol/L) or bicalutamide (10 μmol/L) and firefly versus *Renilla* luciferase activities were determined from triplicate samples (*columns,* mean RLU; *bars,* SE). *B,* cells were transfected with 400 ng of AR and $ARE_4$-luciferase reporter, 3 ng of control pCMV-βGal, and 1 μg of a control (pBSU6) or NCoR shRNA expression vector (pBSU6-NCoR). Cells were stimulated for 24 h with DHT (10 nmol/L) or bicalutamide (1 μmol/L) and luciferase versus β-galactosidase activities were determined from triplicate samples (*columns,* mean RLU; *bars,* SE).

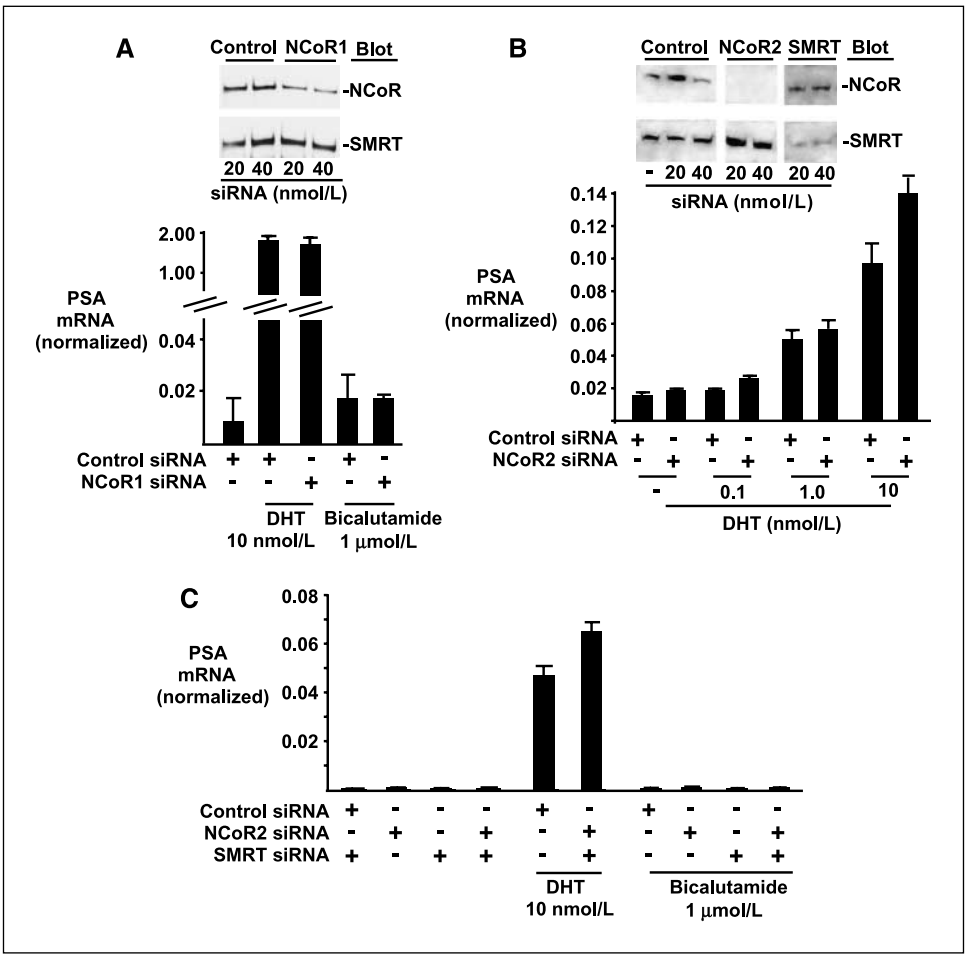


**Figure 5.** Bicalutamide antagonist activity is independent of AR corepressors NCoR and SMRT. *A, top,* LNCaP cells in steroid hormone–depleted medium were transfected with NCoR1 siRNA or control siRNA for 24 h, cultured for an additional 24 h, and equal amounts of protein were then immunoblotted for NCoR and SMRT. *Bottom,* LNCaP cells in steroid hormone–depleted medium were transfected with NCoR1 siRNA or control siRNA (both at 40 nmol/L) for 24 h, and then cultured for an additional 24 h in medium supplemented with DHT or bicalutamide, as indicated. Equal amounts of RNA were then used to determine endogenous *PSA* mRNA levels by real-time RT-PCR in triplicate samples, which were normalized to *cyclophilin. B, top,* whole-cell lysates were prepared from LNCaP cells transfected as above with 20 or 40 nmol/L NCoR2, SMRT, or control siRNA. Specific down-regulation of NCoR and SMRT proteins was then determined by immunoblotting equal amounts of protein. *Bottom,* LNCaP cells transfected for 24 h with NCoR2 or control siRNA (40 nmol/L) were treated for a subsequent 24 h with increasing concentrations of DHT, as indicated. Equal amounts of RNA were then used to measure endogenous *PSA* gene expression in triplicate samples by real-time RT-PCR and normalized to *cyclophilin* mRNA. *C,* LNCaP cells were transfected with 40 nmol/L control, NCoR2, SMRT, or combined NCoR/SMRT siRNA for 24 h, and then stimulated for 24 h with DHT or bicalutamide. *PSA* gene expression from triplicate samples was evaluated as above.

bicalutamide to an agonist (27, 28). In contrast to these previous results, we have failed to detect bicalutamide agonist activity in response to NCoR or SMRT down-regulation. One possible explanation for this difference could be that loss of NCoR via the MEKK1/TAB2 mechanism versus NCoR down-regulation by siRNA results in functionally distinct complexes, with MEKK1 having additional effects that do reveal substantial bicalutamide agonist activity. An alternative explanation may be that the qualitative reporter assay used to assess AR activity in the former MEKK1/TAB2 study (microinjection of a β-galactosidase reporter and visual counting of blue cells) is detecting small increases in agonist activity that do not seem to be significant in the current study relative to the much higher activity in response to DHT. In any case, it should be emphasized that even modest bicalutamide agonist activity could contribute to drug resistance *in vivo*; thus, it will be important to extend these studies into xenograft models to determine whether down-regulation of NCoR or SMRT by shRNA, MEKK1, or other mechanisms results in physiologically significant bicalutamide agonist activity.

Interestingly, whereas the above study detected bicalutamide agonist activity after interleukin-1–mediated MEKK1 activation (27), chromatin immunoprecipitation experiments showed that neither CREB binding protein/p300 nor CARM1 was associated with the bicalutamide-liganded AR on the *PSA* or *KLK2* genes (28). Moreover, the investigators found that distinct LXXLL helices in SRC-1 were required for coactivation of the DHT versus bicalutamide-liganded AR. The interpretation of these latter results is not entirely clear as previous studies indicate that the LXXLL helices in SRC-1 do not contribute significantly to coactivation of the agonist-liganded AR (35–38). Nonetheless, these data are consistent with our observation that SRC-1 can interact with the bicalutamide-liganded AR but does not stimulate transcriptional activity. Moreover, they suggest that impaired recruitment of CREB binding protein/p300 and CARM1, possibly secondary to altered SRC-1 recruitment, may be a mechanism contributing to bicalutamide antagonist activity. A model that incorporates features of these previous studies and our current data is outlined in Fig. 6, with important conclusions being that the bicalutamide-liganded AR interacts with NCoR (through both the NTD and the LBD), but even in the absence of NCoR is unable to effectively recruit coactivator proteins (which are directly or indirectly dependent on a permissive conformation of the LBD).

Another study showed that lentivirus-mediated AR overexpression could enhance the growth of prostate cancer xenografts in castrated mice, and that AR overexpression *in vitro* could alter the response to bicalutamide so that it functioned as an agonist (10). The molecular basis for bicalutamide agonist activity was not addressed in this study, and the study did not compare bicalutamide with a true agonist such as DHT. We have not observed any substantial bicalutamide agonist activity relative to DHT in transient transfections over a wide range of AR levels. However, stable AR overexpression in prostate cancer cells may have distinct

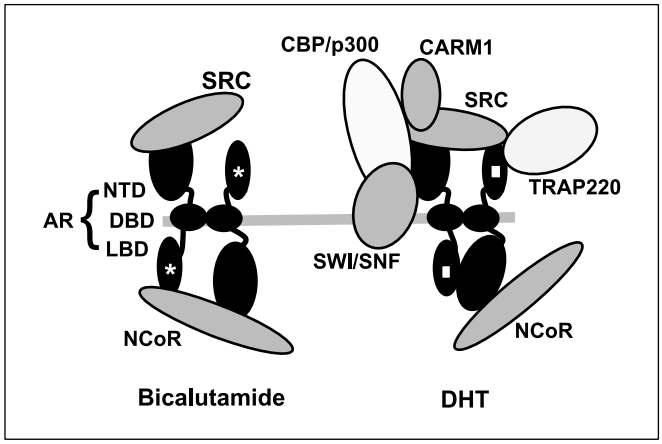


**Figure 6.** Model of AR interactions with coactivators versus corepressors in response to DHT and bicalutamide. The AR domains (NTD, DBD, and LBD) are shown and the AR homodimer bound to DNA is depicted in an antiparallel conformation with an intermolecular $NH_2$/COOH-terminal interaction in response to DHT. In the presence of bicalutamide (*left, asterisk*), there is no $NH_2$/COOH-terminal interaction and NCoR can bind to both the AR $NH_2$- and COOH-terminal domains. SRC-1 can also interact with the NTD of the bicalutamide-liganded AR but is unable to mediate recruitment of additional coactivators. In contrast, the DHT-liganded AR (*right*) effectively recruits SRC proteins in conjunction with additional coactivator and chromatin remodeling complexes. These interactions may be mediated directly by the LBD and/or be dependent on conformational changes in the NTD as a result of the $NH_2$/COOH-terminal interaction. NCoR can still interact weakly with the AR NTD and repress transcriptional activity, but its binding is no longer stabilized by the LBD.

effects on coactivators and corepressors that can account for bicalutamide agonist activity, particularly if this activity is only modest relative to a physiologic agonist. In any case, our NCoR and SMRT siRNA results in LNCaP cells indicate that loss of these corepressors is not the mechanism mediating any bicalutamide agonist activity in LNCaP cells overexpressing AR.

Previous studies have also shown that long-term growth of LNCaP cells in medium with bicalutamide can select for cells that are bicalutamide stimulated, and found that the AR in these cells had an additional mutation that is responsible for the agonist activity of bicalutamide (45, 46). This mutation in codon 741 has been found in patients treated previously with bicalutamide, but is rare overall in patients with relapsed androgen-independent prostate cancer (11). Finally, a recent study investigated bicalutamide resistance in C4-2 cells, which are derived from a LNCaP xenograft that relapsed after castration. Bicalutamide did not have AR agonist activity in these cells but it was unable to inhibit basal AF-2–independent AR transcriptional activity, suggesting that uncoupling of the $NH_2$-terminal AF-1 transactivation function from AF-2 (by unclear mechanisms) may contribute to bicalutamide resistance in androgen-independent prostate cancer (47).

Further studies comparing bicalutamide and DHT agonist activities in prostate cancer cells overexpressing AR and assessing other mechanisms that may mediate bicalutamide agonist activity are clearly warranted. However, it should also be emphasized that data supporting the hypothesis that bicalutamide functions as an agonist *in vivo* are based primarily on bicalutamide withdrawal responses, which occur in only a small fraction of patients who are treated with this drug as part of their initial androgen deprivation therapy. In contrast, bicalutamide has no effect or has a weak and transient inhibitory effect on the majority of tumors that recur after androgen deprivation by castration or luteinizing hormone–releasing hormone agonist treatment. Therefore, whereas bicalutamide may be an agonist in a small subset of tumors, this agonist activity is not clearly related to the intrinsic bicalutamide resistance observed in the majority of patients who relapse after castration.

In summary, this study further establishes the critical role of the AR $NH_2$/COOH-terminal interaction for AR transcriptional activity, and indicates that whereas NCoR and SMRT are recruited to the bicalutamide-liganded AR, they are not responsible for the antagonist versus agonist activity of this drug. Instead, we suggest that failure to recruit specific coactivators is primarily responsible for bicalutamide antagonist activity and that the molecular basis for any bicalutamide agonist activity that occurs *in vivo* (in the absence of AR mutation) may be enhanced activity of these coactivators (which may circumvent an AF-2 dependent function). Finally, it should be emphasized that bicalutamide resistance in androgen-independent prostate cancer may not be due to agonist activity but may instead reflect decreased bicalutamide binding to AR and hypersensitivity to low levels of endogenous agonist ligands. This is an important distinction, as the latter hypothesis would predict that bicalutamide-like drugs with higher affinity for AR would be effective whereas the former would indicate the need for drugs that block AR by distinct mechanisms.

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