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**Androgen receptor amplification is reflected in the transcriptional responses of Vertebral-Cancer of the Prostate cells**

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**Key words**

Androgen receptor; VCaP; prostate cancer cell; antiandrogen; gene expression; chromatin immunoprecipitation (ChIP)

**Abstract**

Androgen receptor (AR) is overexpressed in a majority of castration-resistant prostate cancers, but most of the cell model studies addressing AR function have been conducted in LNCaP prostate cancer cells expressing unamplified AR levels. Here, we have compared the responses of various types of AR ligands towards a pattern of AR target genes and chromatin binding sites in Vertebral-Cancer of the Prostate (VCaP) cells and LNCaP cells. In keeping with the AR gene amplification in VCaP cells, our analyses show that these cells contain  $\geq 10$ -fold receptor mRNA and protein than LNCaP cells. Loading of the agonist-occupied AR onto chromatin regulatory sites and expression of several AR target genes, including their basal expression, were stronger in VCaP cells than LNCaP cells. Bicalutamide displayed a trend towards agonism in VCaP cells. Bicalutamide also evoked AR-chromatin interaction, whereas diarylthiohydantoin antiandrogen RD162 was inert with this respect both in VCaP and LNCaP cells. These results support the notion that the AR protein level translates into augmented occupancy of AR-regulated enhancers and target gene activity in prostate cancer cells.

## 1. Introduction

Androgen signaling is essential for both normal and malignant growth of prostate (Heinlein and Chang, 2004). Proliferation of prostate cancer cells can initially be restricted by androgen ablation therapy. However, for reasons that are currently elusive, the therapy eventually fails and prostate cancer turns into a hormone-refractory, *i.e.* castration-resistant or androgen-depletion-independent, state (Bonkhoff and Berges, 2010). The situation has been proposed to result from mutations in AR that liberate its hormone-specificity, altered expression of AR or its coregulator proteins, up-regulation of intraprostatic androgen synthesis during androgen withdrawal, and gene fusions resulting in abnormal androgen regulation of oncogenic transcription factors (Han et al., 2005; Culig et al., 2004; Gregory et al., 2001; Shi et al., 2008; Montgomery et al., 2008). Despite of the apparent hormone-refractory state of the disease, AR remains a critical factor for the growth and survival of the majority of the hormone-refractory tumors. Most of these tumors overexpress AR, and a significant portion of hormone refractory prostate cancers carry an *AR* gene amplification (Koivisto et al., 1997; Linja et al., 2001; Chen et al., 2004). Overexpression of AR has been suggested to hypersensitize the cancer cells to minuscule levels of androgens and thereby allow them grow in the apparent absence of androgens (Linja et al., 2001; Li et al., 2004; Henshall et al., 2001; Rosner et al., 2007; Waltering et al. 2009).

The AR is a hormone-controlled DNA-binding transcription factor that regulates genes and gene networks in response to androgen exposure (Heemers and Tindall, 2007). Androgen-occupied AR binds to androgen response elements (AREs) in the regulatory regions of its target genes and interacts with transcriptional coregulatory proteins, which results in remodeling of chromatin structure and activation of RNA polymerase II function on the target gene promoter (Heemers and Tindall, 2007; Makkonen et al., 2009). Synthetic antiandrogens, such as nonsteroidal bicalutamide, that directly target the AR are often used for the treatment of advanced prostate cancer (Steward et al., 2005). The currently available antiandrogens act as competitive inhibitors for natural androgens, but they do not support the formation of productive AR transcription complexes. Unfortunately, overexpression of AR may convert the bicalutamide (that is under normal cells considered a pure antagonist) to an agonist (Chen et al., 2004).

Established cell lines resembling the situation in castration-resistant prostate cancer are instrumental models in improving our understanding of the molecular mechanism of AR action and developing strategies for targeting AR in prostate cancer. However, the majority of single gene and large-scale or genome-wide expression analyses of AR target genes have been performed in LNCaP cells, the most popular cell model for prostate cancer research (Dehm and Tindall, 2006; Ngan et al., 2009; Lamont and Tindall, 2010). The LNCaP cells correspond to a lymph node metastasis, containing a ligand-binding domain-mutated version of the receptor (Veldscholte et al., 1990). The *AR* gene locus is not amplified in the LNCaP cells, and the receptor protein is normally expressed in these cells. VCaP (Vertebral-Cancer of the Prostate) cells in turn are derived from a hormone-refractory cancer, possessing amplified *AR* gene locus encoding otherwise normal AR protein (Korenchuk et al., 2001; van Bokhoven et al., 2003; Liu et al., 2008). Additionally, this interesting cell line contains the chromosomal rearrangement fusing the androgen-regulated gene *TMPRSS2* to the oncogenic *ERG* transcription factor found in about half of the prostate cancers (Liu et al., 2008; Tomlins et al., 2005). Therefore, the VCaP cells might better than the LNCaP cells represent the state of androgen signaling in the castration-resistant prostate cancer. To investigate whether the amplification of *AR* in VCaP cells is reflected in the transcriptional responses of AR target genes, we have in this work compared the regulation and the function of AR between the

VCaP and the LNCaP cells. We quantified the expression of AR and that of a pattern of AR target genes in response to various types of AR ligands and compared the loading of AR to a pattern of specific gene loci by using quantitative chromatin immunoprecipitation (ChIP) assays. Our results reveal interesting differences in the AR-target chromatin interaction and the AR target gene expression between these two cell lines.

## 2. Materials and methods

### 2.1. Cell culture

VCaP and LNCaP cells were from American Type Culture Collection (ATCC). The cells were maintained as described (Makkonen et al., 2009).

### 2.2. Isolation of RNA and RT-quantitative PCR (RT-qPCR)

VCaP and LNCaP cells were seeded onto 6-well plates ( $8 \times 10^5$  and  $3.3 \times 10^5$  cells/well, respectively) and grown for 72 h in transfection medium (VCaP: DMEM containing 2.5% charcoal-stripped FBS (CCS-FBS), LNCaP: RPMI 1640, 10% CCS-FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 25 mM glucose) devoid of steroids. In Fig. 2, the VCaP cells were simultaneously transfected either with negative control siRNA (siSCR, 5'-UUCUCCGAACGUGUCACGUGdTdT-3') or siRNA against AR (siAR, 5'-GGAGUUGUGUAAGGCAGUGdTdT-3') using TransIT-siQUEST<sup>®</sup> transfection reagent (Mirus Bio Corporation, Madison, WI, USA) according to the manufacturer's instructions. Subsequently, cells were treated with or without R1881 (17 $\beta$ -17-hydroxy-17-methyl-estra-4,9,11-trien-3-one, also known as methyltrienolone or metribolone, Perkin Elmer Inc., Waltham, MA, USA), cyproterone acetate (6-chloro-1 $\beta$ ,2 $\beta$ -dihydro-17-hydroxy-3'H-cyclopropa[1,2]pregna-4,6-diene-3,20-dione, Sigma-Aldrich, St. Louis, MO, USA), bicalutamide (*N*-[4-cyano-3-(trifluoromethyl)phenyl]-3-[(4-fluorophenyl)sulfonyl]-2-hydroxy-2-methylpropanamide, Bidragon Pharmservice LLC, Burlingame, CA, USA), or novel diarylthiohydantoin antiandrogen RD162 (4-[7-(4-cyano-3-trifluoromethyl-phenyl)-8-oxo-6-thioxo-5,7-diaza-spiro[3.4]oct-5-yl]-2-fluoro-N-methyl-benzamide, Helix&Bond Pharmaceuticals Inc., Shanghai, China) as indicated. Total RNA was extracted using TRIzol<sup>®</sup> Reagent (Invitrogen Lifetechnologies, Carlsbad, CA, USA) and converted to cDNA using Transcriptor First Strand cDNA synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) following manufacturer's instructions. cDNA was used as a template in RT-qPCR, which was carried out using Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA), FastStart SYBR Green Master (Roche) and specific primers for indicated genes (Supplementary Table S1). Analyzed GAPDH mRNA levels were used to normalize the amounts of total RNA between the samples. Fold changes (ligand inductions) in Fig. 6 were calculated using the formula  $2^{-(\Delta\Delta Ct)}$ , where  $\Delta\Delta Ct$  is  $\Delta Ct_{(ligand)} - \Delta Ct_{(EtOH)}$ ,  $\Delta Ct$  is  $Ct_{(gene X)} - Ct_{(GAPDH)}$  and  $Ct$  is the cycle at which the threshold is crossed. Relative mRNA levels were calculated in Fig. 2 using the formula  $2^{-(\Delta\Delta Ct)}$ , where  $\Delta\Delta Ct$  is  $\Delta Ct_{(ligand)} - \Delta Ct_{(siSCR EtOH)}$  and in Figures 3 and 4 using the formula  $2^{-(\Delta\Delta Ct)}$ , where  $\Delta\Delta Ct$  is  $\Delta Ct_{(ligand)} - \Delta Ct_{(LNCaP EtOH)}$ .

### 2.3. Immunoblotting

VCaP and LNCaP cells were seeded and grown as for RT-qPCR. The cell monolayers were washed with ice-cold Tris-buffered saline (TBS) and harvested in TBS containing 1:100 protease inhibitor cocktail (PIC) (Sigma-Aldrich, St. Louis, MO, USA). Cell pellets were

suspended in SDS-PAGE sample buffer containing 1:100 PIC and lysed by sonication (2 x 10 s). In Fig. 2B, proteins were extracted simultaneously with RNA using TRIzol® reagent following manufacturer's instructions. Samples were heated for 5 min at 95°C and separated on 10% SDS-PAGE, proteins were transferred onto nitrocellulose membranes and detected by a rabbit antiserum against AR (Karvonen et al., 1997) or anti-GAPDH (sc-25778, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The bands were visualized by horseradish peroxidase-conjugated anti-rabbit secondary antibody (Zymax™ Goat Anti-Rabbit IgG (H+L) HRP conjugate (81-6120), Zymed, San Francisco, CA, USA) using the enhanced chemiluminescence detection reagents according to the manufacturer's instructions (Pierce® ECL Western Blotting Substrate, Thermo Scientific, Rockford, IL, USA). For quantification of AR band intensities, goat anti-rabbit IgG (H&L) DyLight™ 800-conjugated secondary antibody (Pierce) was used with Li-COR Odyssey Infrared Imaging System according to manufacturer's instructions (LI-COR Inc., Lincoln, NE, USA). The amount of AR was normalized by the amount of GAPDH in the sample.

#### 2.4. Chromatin immunoprecipitation (ChIP)

VCaP and LNCaP cells were seeded at ~70% confluence onto 75-cm<sup>2</sup> bottles and allowed to grow in steroid-depleted transfection medium for 72 h prior to ChIP. The experiments were performed essentially as previously described (Makkonen et al., 2009) using the same rabbit antiserum against AR that was used for immunoblotting. Normal rabbit serum (NRS) (Millipore, Billerica, MA, USA) was used as a negative control antibody (IgG). Specific primers for different regions are listed in Supplementary Table S2. Quantitative PCR analyses were carried out with FastStart SYBR Green Master and Mx3000P Real-Time PCR System. In Fig. 5, results were calculated using the formula  $(E^{-(\Delta Ct_{(AR)})} * 10) / (E^{-(\Delta Ct_{(IgG)})} * 10)$ , where E (efficiency of target amplification) is a coefficient of DNA amplification by one PCR cycle for a particular primer pair and  $\Delta Ct$  is  $Ct_{(ChIP-template)} - Ct_{(Input)}$ . In Figure 7, results are presented as ligand fold inductions over the value of ethanol treated sample of given cells.

### 3. Results and Discussion

#### 3.1. AR is overexpressed, but down-regulated by androgens, in VCaP cells in comparison to LNCaP cells

We first compared the AR protein levels in LNCaP and VCaP cells grown in absence and presence of synthetic non-aromatizable ligands methyltrienolone (R1881, pure agonist), cyproterone acetate (CTA, steroidal antiandrogen), bicalutamide (BIC, nonsteroidal antiandrogen) and RD162 (novel arylthiohydantoin antiandrogen). We used saturating ligand concentrations that were adjusted to compensate for differences in their AR binding affinities (Gao et al., 2005; Tran et al., 2009). Immunoblotting of the AR bands showed that the VCaP cells contain markedly more receptor protein than the LNCaP cells, with the difference being dependent on the ligand exposure and time (Fig. 1A). Quantification of the AR bands revealed that the difference is 15-fold in the absence of ligand. A short-term (2 h) treatment with R1881, CTA or BIC, but not with RD162, stabilized the receptor protein similarly in both cell lines. Interestingly, a longer (18 h) R1881 or CTA treatment further stabilized the AR in LNCaP cells, but contrastingly reduced the receptor amount in VCaP cells, thus decreasing the difference in the receptor amount between these prostate cancer cell lines to ~4-5.5-fold.

To reveal whether the above differences in the AR protein levels mirror differences in the AR gene expression, AR mRNA levels from VCaP and LNCaP cells exposed to different

ligands were quantified by RT-qPCR (Fig. 1B). In accordance with the amplification of the *AR* gene locus in VCaP cells, the cells expressed ~11-fold more AR mRNA than LNCaP cells. Interestingly, the agonist R1881 significantly down-regulated the AR mRNA level in VCaP cells, resulting in ~85% decrease in the mRNA, whereas in LNCaP cells, the AR mRNA levels were not markedly altered by the agonist exposure. However, longer treatments ( $\geq 40$  h) with agonists have been reported to result in down-regulation of AR mRNA also in LNCaP cells (Krongrad et al., 1991). The effects of the other ligands on the AR mRNA expression in VCaP cells were smaller than that of R1881, decreasing in the order of CTA > RD162 > BIC (Fig. 1B). Despite of the agonist-mediated down-regulation, the AR mRNA level of VCaP cells exceeded that of LNCaP cells. VCaP cells have been recently reported to contain at least five extra copies of the *AR* gene (Liu et al., 2008). Thus, the *AR* gene dose is by and large translated into the expression of AR protein in these cells. Androgen-dependent down-regulation of AR expression in VCaP cells resembles the down-regulation that occurs in the normal rodent prostate *in vivo* (Shan et al., 1990).

### 3.2. Differential sensitivity of target genes to AR depletion in VCaP cells

Since the AR function has not been widely studied in VCaP cells, we first confirmed the role of AR in the regulation of selected genes that have been previously identified as AR targets in other prostate cancer models, mainly in LNCaP cells. To that end, we set up AR RNAi that silenced  $\geq 80\%$  of the AR mRNA, resulting in an efficient depletion of the AR protein (Fig. 2A,B). We next compared the expression of nine AR targets; *FKBP51*, *C6orf81*, *TMPRSS2*, *PSA*, *S100P*, *ChGn*, *SLC45A3*, *SPOCK1*, and *NKX3.1* (Amler et al., 2000; Bolton et al., 2007; Lin et al., 1999; Young et al., 1991; Averboukh et al., 1996; Tomlins et al., 2007; Nitsche et al., 1996; Bieberich et al., 1996), in control siRNA (siSCR)- and siAR-transfected VCaP cells that were treated with vehicle or R1881 by measuring their mRNAs by RT-qPCR. As shown in Fig. 2C, all of the nine candidate target gene mRNAs were induced in response to androgen exposure in VCaP cells. Depletion of AR blunted or abolished the androgen induction of five target genes; *FKBP51*, *C6orf81*, *S100P*, *ChGn*, and *SPOCK1*. In the case of four AR targets, *TMPRSS2*, *SLC45A3*, *PSA*, and *NKX3.1*, the AR depletion markedly reduced their expression also in vehicle-exposed cells, and therefore their androgen (fold-increase) inductions were not affected. Conversely, the basal expression of *C6orf81*, *S100P* and *ChGn* were interestingly increased upon the AR depletion. All in all, these results indicate the active role of the AR protein in the regulation of these nine genes. However, these genes display interesting differences in their sensitivity towards AR silencing in VCaP cells.

### 3.3. Increased basal expression of several AR target genes in VCaP cells

We next investigated whether the observed difference in the amount of AR between VCaP and LNCaP cells (*cf.* 3.1.) translates into differences in AR target gene expression. Quantification of the mRNAs for the above-mentioned AR targets in relation to the GAPDH mRNA levels revealed that, except for *PSA* and *S100P*, their expression levels in vehicle-treated cells were markedly higher in VCaP cells than LNCaP cells (Fig. 3A). The fold difference ranged from 3- (*C6orf81*) to 660-fold (*SPOCK1*). R1881 treatment (0.01, 0.1, 1 and 10 nM) for 12 h dose-dependently increased the accumulation of these mRNAs in both cell lines (Fig. 3A). The maximum fold-increases in response to the androgen varied less; *FKBP51* (36- vs. 26-fold), *C6orf81* (7- vs. 3-fold), *S100P* (12- vs. 2-fold), *TMPRSS2* (9- vs. 14-fold), *PSA* (3- vs. 6-fold), *ChGn* (4- vs. 34-fold), *SLC45A3* (2- vs. 17-fold), *NKX3.1* (2- vs. 18-fold), and *SPOCK1* (3- vs. 7-fold), being often higher in LNCaP cells. This is probably

derived from the generally lower basal expression of these mRNAs in LNCaP cells. The *PSA* that has historically been employed as a canonical model for AR target genes contrastingly showed a higher expression in LNCaP cells. However, *PSA* mRNA does not seem to be a very sensitive marker of AR action, as the *PSA* mRNA levels were not significantly suppressed in prostatectomy patients in response to androgen deprivation therapy (Mostaghel et al., 2007). In addition to the AR, FoxA1 transcription factor plays a central role in the up-regulation of the *PSA* (Gao et al., 2003; Wang et al., 2007). Compared to the *PSA* mRNA, *FKBP51* mRNA has been shown to better reflect the androgen activity in human prostate (Mostaghel et al., 2007). Interestingly, both the basal and the androgen-induced expression of *FKBP51* were clearly higher in VCaP than LNCaP cells. These findings imply that the increased AR dose in VCaP cells is translated into a higher expression level of several AR target genes. However, since we were also comparing two distinct cell types, not only AR levels, it is likely that also disparities in the cellular milieu, such as overexpression of ERG due to *TMPRSS2-ERG* gene fusion in VCaP cells (Tomlins et al., 2005), contribute to the above differences. ERG was recently reported to occupy AR target genes and repress androgen signaling in VCaP cells (Yu et al., 2010). Even then, depletion of ERG in VCaP cells resulted in only a small increase (<25%) in the androgen-induced expression of genes such as *FKBP51*, *SLC45A3* and *TMPRSS2* (Yu et al., 2010). Increased expression of androgen-regulated genes has been previously reported in androgen-independent prostate cancer cells (Gregory et al., 1998; Oosterhoff et al., 2005; Dehm and Tindall, 2006).

### 3.4. Cyproterone acetate shows gene-selective AR modulator properties both in VCaP and LNCaP cells

Cyproterone acetate (CTA) acts as a near-full agonist in reporter gene assays with the T877-to-A-mutated AR originally found from the LNCaP cells (Kuil and Mulder, 1996; Bohl et al., 2007). However, in VCaP cells expressing wild-type AR, the activity of CTA has not been previously reported. We compared the effect of increasing CTA concentrations (0.01, 0.1, 1 and 10  $\mu$ M) on the expression of the above pattern of AR target genes in VCaP and LNCaP cells. Interestingly, the agonistic activity of CTA varied more between the nine AR target genes than between the two cell lines (Fig. 4). Even in the LNCaP cells and at the highest CTA concentration used, the agonistic activity of CTA on *FKBP51*, *C6orf81*, *S100P*, *TMPRSS2*, *PSA*, *ChGn*, and *NKX3.1* however remained clearly below the maximal induction obtained with R1881, showing on the average ca. 55% of the maximal R1881 induction. On the other hand, the CTA acted as a surprisingly potent agonist on *SPOCK1* and *SLC45A3* expression, equaling the maximum induction obtained with R1881 in both cell lines. In contrast, the agonistic effect of CTA was not evident on *S100P* and it was poorer than the average on *ChGn* in both cell lines and on *FKBP51* in VCaP cells. These data indicate the CTA acts a partial agonist-antagonist also in the VCaP cells. Moreover, our results imply that the CTA possesses a certain degree of gene-selective activity both in the VCaP and the LNCaP cells.

### 3.5. R1881 and cyproterone acetate dose-dependently trigger loading of AR onto chromatin

We next analyzed the binding of AR to ARE-containing chromatin regions in VCaP and LNCaP cells exposed to increasing R1881 or CTA concentrations by using quantitative chromatin immunoprecipitation (ChIP) assays. The following six distinct AR-binding chromatin regions were examined: an upstream enhancer for *TMPRSS2*, *C6orf81* and *PSA*, a proximal regulatory region for *S100P*, an intronic enhancer for *SPOCK1* and *FKBP51* (Wang

et al., 2007; Paakinaho et al., 2010; Schuur et al., 1996; Makkonen et al., unpublished data; Makkonen et al., 2009). As shown in Fig. 5A, generally more AR occupied these enhancer regions in VCaP than LNCaP cells, and addition of the R1881 brought about dose-dependent increase in the occupancy of AR at these enhancers. Also *PSA* and *S100P* enhancers harbored more agonist-bound AR in VCaP cells, although their genes displayed higher expression levels in LNCaP cells. The R1881 concentration responses of AR chromatin binding closely mirrored those of mRNA induction studies, indicating that the availability of the hormone governs the binding of the AR to its cognate chromatin binding sites.

Even though the CTA was weaker than the R1881 in evoking induction of *FKBP51*, *TMPRSS2* and *PSA* transcription in VCaP cells, the maximum AR loading to these genes' major AR-binding sites did not differ between the CTA- and the R1881-treated cells (Fig. 5B). Moreover, despite of the lack of CTA-induction of *S100P*, the CTA was interestingly capable of promoting some chromatin loading of the receptor at the two highest CTA concentrations in VCaP cells. In LNCaP cells, the CTA- and R1881-occupied AR showed practically equal loading onto all of the six AR-binding chromatin regions. Collectively, these results imply that although the CTA-bound AR is competent with chromatin binding both in VCaP and LNCaP cells, it fails to fully support the formation of AR transcription complexes in these cells. The CTA-bound AR seems to concomitantly recruit both coactivators and corepressor, which may result in the formation of mixed coactivator/corepressor assemblies (Karvonen et al., 2002, 2006).

### 3.6. RD162 is more potent than bicalutamide in inhibiting AR target gene expression both in VCaP and LNCaP cells

We next compared the gene responses to nonsteroidal antiandrogens bicalutamide (BIC) and RD162, a novel arylthiohydantoin, in VCaP and LNCaP cells. One of the main differences in the behavior of BIC between these two cell lines was that in LNCaP cells, but not in VCaP cells, BIC (10  $\mu$ M) inhibited the expression of *SPOCK1* ( $P < 0.001$ ), *C6orf81* ( $P < 0.05$ ) and *TMPRSS2* ( $P < 0.05$ ) to a transcript level that was significantly below the control (vehicle-treated) level. In the VCaP cells, the antiandrogen showed a trend towards a weak agonism, which was significant on the *FKBP51* ( $P < 0.001$ ) and the *TMPRSS2* ( $P < 0.01$ ) (Fig. 6).

We also examined the capability of BIC (10  $\mu$ M) to block the action of R1881 (1 nM) on the AR target gene expression. Interestingly, the inhibitory effect of BIC varied considerably between different target genes. For example, BIC abolished  $\geq 80\%$  of the agonist activity on *FKBP51* and *S100P* in both cell lines as well as that on *TMPRSS2*, *ChGn* and *SPOCK1* in LNCaP cells, whereas it acted as a relatively poor inhibitor ( $< 40\%$  inhibition) of the androgen induction of *PSA* in both cells lines as well as that of *TMPRSS2*, *SLC45A3* and *SPOCK1* in VCaP cells.

Two diarylthiohydantoins RD162 and MDV3100 were recently reported retain their antagonistic activity under increased AR expression (Tran et al., 2009). The RD162 proved to be more antagonistic than BIC also in our AR target gene expression assays (Fig. 6). Notably, there was no evidence for a residual agonism in VCaP cells exposed to RD162 (10  $\mu$ M) alone (Fig. 6). The compound was also in VCaP cells capable of repressing the target gene expression to a transcript level that was below the control level. With the curious exception of *S100P* in LNCaP cells and that of *SLC45A3* in VCaP cells, the RD162 also abolished the effect of R1881 on AR target gene expression in both cell types (Fig. 6). These results indicate that, despite of the overexpression of AR, BIC on its own possesses very limited agonistic activity in VCaP cells. Amplified expression level of AR in VCaP cells is thus not sufficient to convert BIC to an efficient agonist, as has been reported for LNCaP and LAPC4 prostate cancer cell models in which about three-fold augmented expression of AR protein

was brought about by infecting the cells with a recombinant retrovirus encoding AR cDNA (Chen et al., 2004). However, our results suggest that the AR level contributes to the efficacy of BIC, as it was less efficient in curbing the activity of agonist-bound AR in VCaP cells than in LNCaP cells.

### 3.7. RD162 does not provoke AR-chromatin interaction in LNCaP or VCaP cells as bicalutamide does

We also tested the effect of the nonsteroidal antagonists BIC and RD162 on the AR-chromatin interaction at the six chromatin regions studied (*cf.* 3.5.) by using quantitative ChIP assays. As shown in Fig. 7, BIC promoted a considerable loading of the AR onto the chromatin, especially in VCaP cells. The ability of BIC (10  $\mu$ M) to promote the loading was dependent on the gene enhancer, approaching in VCaP cells the level of R1881-bound AR on *C6orf81*, *TMPRSS2* and *PSA* enhancer, but being < 80% of the agonist-promoted levels on *S100P* and *SPOCK1* enhancer. Thus, binding of the AR to the regulatory region of a given gene *in vivo* by no means indicates that the receptor is competent to activate the gene (*cf.* 3.6.). In contrast to BIC, RD162 (10  $\mu$ M) was inert in promoting loading to any of the six AR chromatin binding sites studied in VCaP or LNCaP cells. In line with the latter finding and agreement with the data in 3.6., the RD162 was also more efficient than BIC in curbing the agonist-exposed AR from interacting with the chromatin. The compound prevented most of the agonist-occupied receptor from interacting with the six AR-regulated enhancers in LNCaP cells, but it still (at 10,000-fold molar excess) allowed a considerable amount of agonist-exposed AR to reside on *C6orf81*, *TMPRSS2* and *PSA* enhancer in VCaP cells, suggesting that the AR level can modulate the efficiency of antiandrogens in prostate cancer cells in an AR target locus-selective fashion. Our results thus emphasize the importance of investigating AR function, in addition to the popular LNCaP cell model, in prostate cancer cell models, such VCaP cells, showing elevated levels of AR protein.

According to Tran and co-workers, one of the key differences in the molecular properties between the BIC and the RD162 is that the novel compound in contrast to the BIC is inert in inducing the nuclear translocation of AR (Tran et al., 2009). Our current ChIP data and unpublished immunohistochemical analyses in VCaP cells are in agreement with the latter notion (Rytinki M., unpublished observations). Based on these data, compounds that prevent the nuclear translocation of the AR should block the amplified AR activity in prostate cancer cells more efficiently than the currently widely used antiandrogen BIC.

### Contributors

This study was designed by HM and JP; the experimental procedures were performed by HM with assistance from MK and TJ.

### Conflicts of interest

The authors declare no conflict of interest.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found in the online version.

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## Figure legends

**Fig. 1.** Androgen receptor (AR) expression is differently regulated by androgens in VCaP and LNCaP cells. (A) Immunoblotting analysis of AR protein in VCaP and LNCaP cells grown for 2 h or 18 h in the presence vehicle (EtOH, -), 10 nM R1881, 10  $\mu$ M cyproterone acetate (CTA), 10  $\mu$ M bicalutamide (BIC), or 10  $\mu$ M RD162 as indicated. AR and GAPDH were detected by respective specific antibodies as described in Materials and methods. The intensities of the AR bands of duplicate blots were measured by Li-COR Odyssey infrared imaging system, and the numbers above the immunoblots depict the amount of AR in relation to AR in vehicle-treated LNCaP cells (=1). The immunoblots are representative examples of three experiments. (B) RT-qPCR analysis of AR mRNA expression in VCaP and LNCaP cells. Cells were treated with indicated ligands for 12 h, and the AR mRNA was quantified as described in Materials and methods. The value of vehicle-treated LNCaP sample was set as 1. Columns represent the mean  $\pm$  SD of three independent experiments.

**Fig. 2.** Effect of AR silencing on the expression of a selection of AR target genes in VCaP cells. The cells were transfected either with a control siRNA (siSCR) or an siRNA against AR (siAR) and subsequently treated with or without 1 nM R1881 for 12 h. (A,C) mRNAs of indicated AR target genes were analyzed by RT-qPCR, relative (rel.) mRNA levels were calculated as described in Materials and methods, and the value of vehicle-treated siSCR sample was set as 1. Columns represent the mean  $\pm$  SD of three independent experiments. (B) Immunoblotting analysis and AR and GAPDH in siSCR- and siAR-transfected cells grown in the absence (-) and the presence (+) of R1881.

**Fig. 3.** R1881 dose responses of AR target gene expression in VCaP and LNCaP cells. (A) Basal expression of a selection of AR target genes in VCaP cells vs. LNCaP cells. mRNAs of indicated AR target genes were analyzed by RT-qPCR. mRNA levels were calculated in relation to GAPDH mRNA levels in the samples, the values of LNCaP samples were set as 1, and the data were expressed as VCaP cell/LNCaP cell ratios. (B) Cells were treated with increasing concentrations of R1881 for 12 h and mRNAs of indicated AR target genes were analyzed by RT-qPCR. Relative (rel.) mRNA levels were calculated as described in Materials and methods, and the value of vehicle-treated LNCaP samples was set as 1. Dots and columns represent the mean  $\pm$  SD of three independent experiments.

**Fig. 4.** Cyproterone acetate as an inducer of AR target genes in VCaP and LNCaP cells. Cells were treated with increasing concentrations of cyproterone acetate (CTA) for 12 h and mRNAs of indicated AR target genes were analyzed by RT-qPCR. Relative (rel.) mRNA levels were calculated as described in Materials and methods, and the value of vehicle-treated LNCaP sample was set as 1. Columns represent the mean  $\pm$  SD of three independent experiments.

**Fig. 5.** Loading of AR onto chromatin sites in response to R1881 or cyproterone acetate in VCaP and LNCaP cells. VCaP and LNCaP cells were treated with increasing concentrations of R1881 (A) or cyproterone acetate (CTA) (B) for 2 h and ChIP assays were performed using an antiserum against AR or normal rabbit serum (normal IgG). ChIP samples were used as templates in quantitative PCR with primers specific for major AR-binding regions of *FKBP51*, *C6orf81*, *TMPRSS2*, *PSA*, *S100P* and *SPOCK1*. The results are shown as fold over the normal IgG and represent the mean  $\pm$  SD of three independent experiments.

**Fig. 6.** RD162 inhibits AR target gene expression more efficiently than bicalutamide in VCaP cells and LNCaP cells. Cells were treated with vehicle (EtOH), R1881 (1 nM), BIC (10  $\mu$ M), RD162 (10  $\mu$ M) or antagonists in combination with R1881 for 12 h as indicated and AR target gene mRNAs were analyzed by RT-qPCR and calculated as described in Materials and methods. The results are shown as induction by ligand over the vehicle-treated sample (set as 1) and represent the mean  $\pm$  SD of three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns, nonsignificant; for the difference between BIC- and RD162-treated samples.

**Fig. 7.** Bicalutamide, but not RD162, promotes AR-chromatin interaction in VCaP and LNCaP cells. Binding of AR to the same chromatin regions that were analyzed in Fig. 5 was studied by ChIP assays in LNCaP and VCaP cells that were exposed to vehicle (EtOH), R1881 (1 nM), BIC (10  $\mu$ M), RD162 (10  $\mu$ M) or antagonists in combination with R1881 for 2 h as depicted. The results are shown as ligand inductions over the vehicle-treated samples (set as 1) and represent the mean  $\pm$  SD of three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns, nonsignificant; for the difference between BIC- and RD162-treated samples.

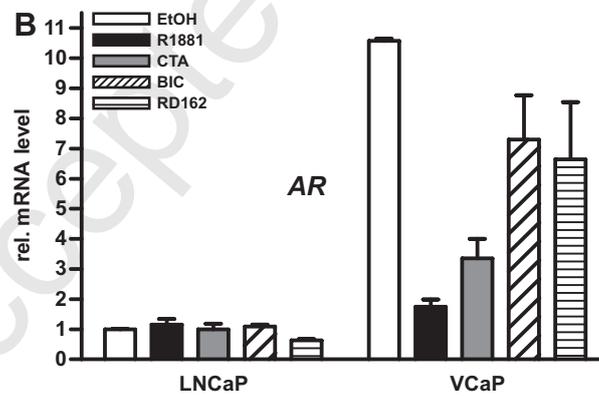
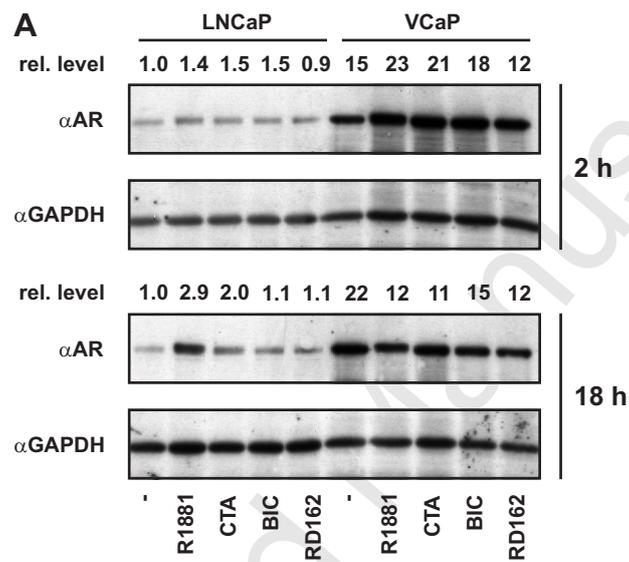


Figure 1

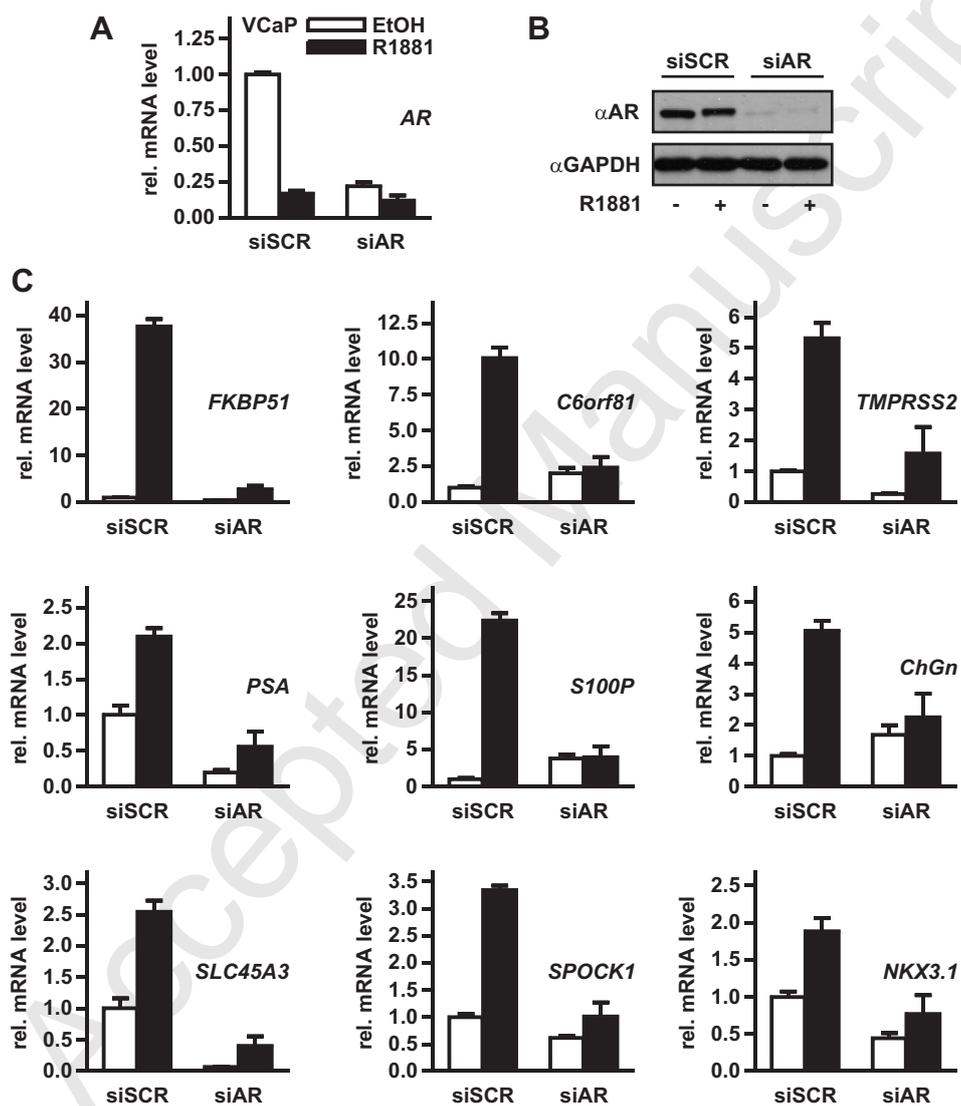


Figure 2

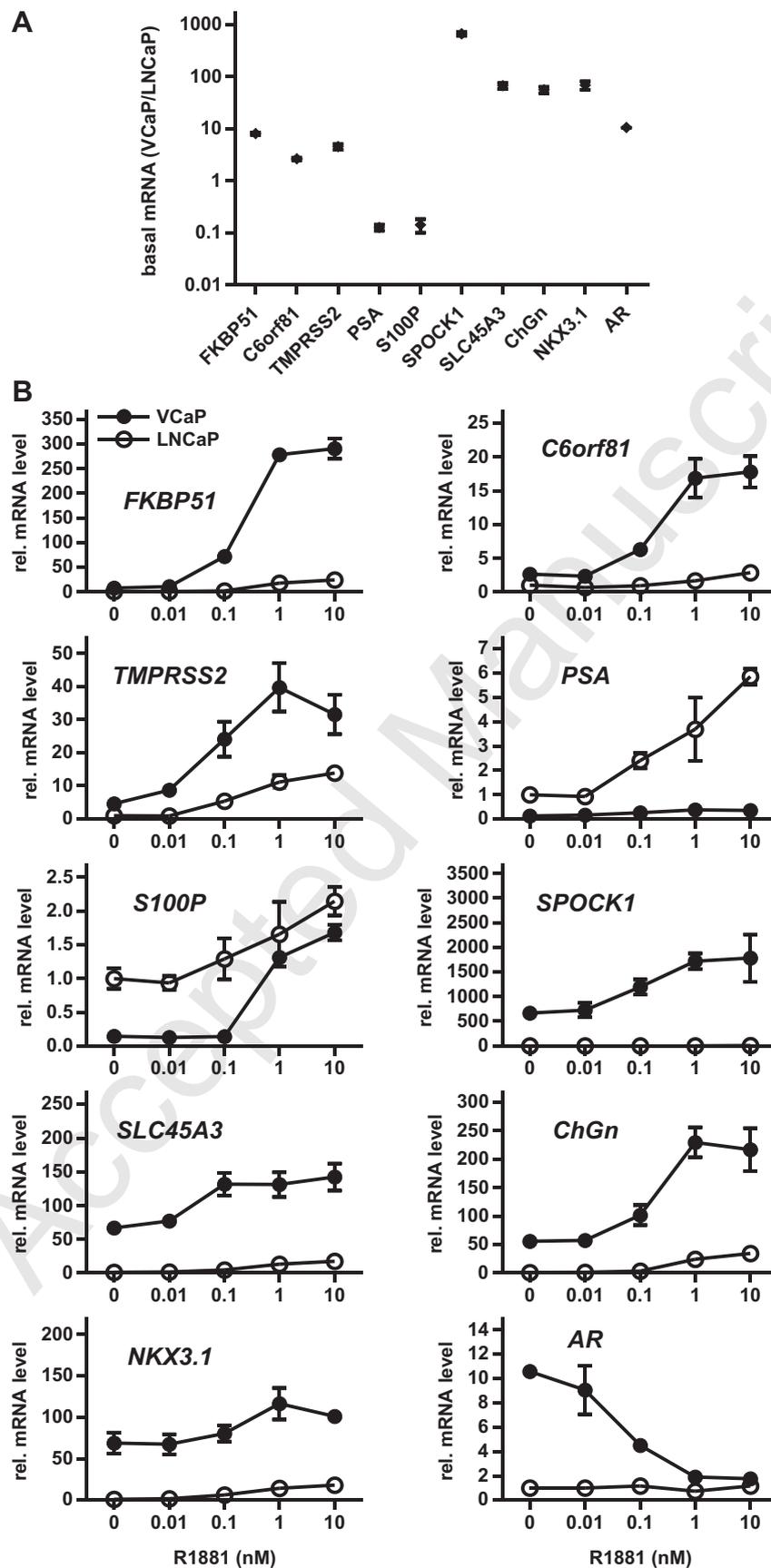


Figure 3

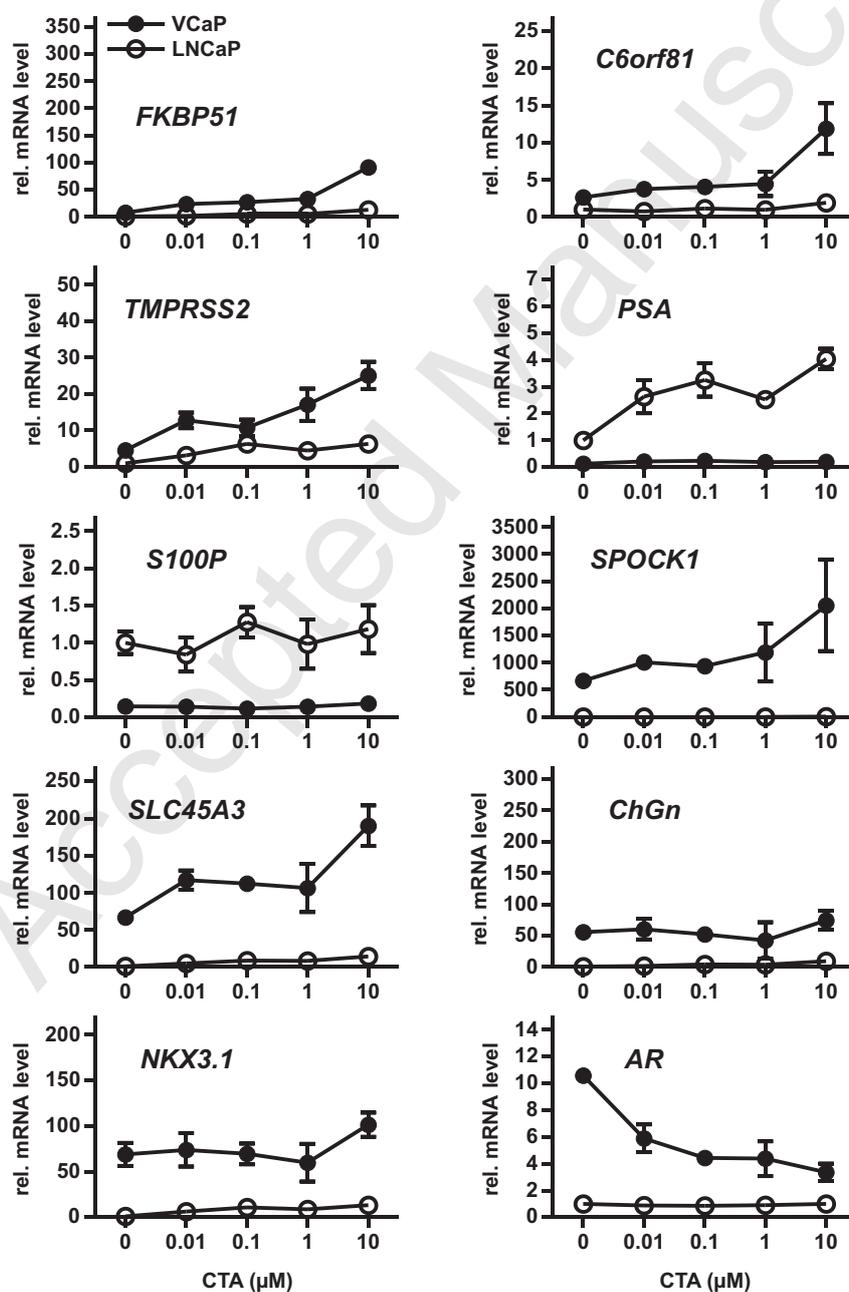


Figure 4

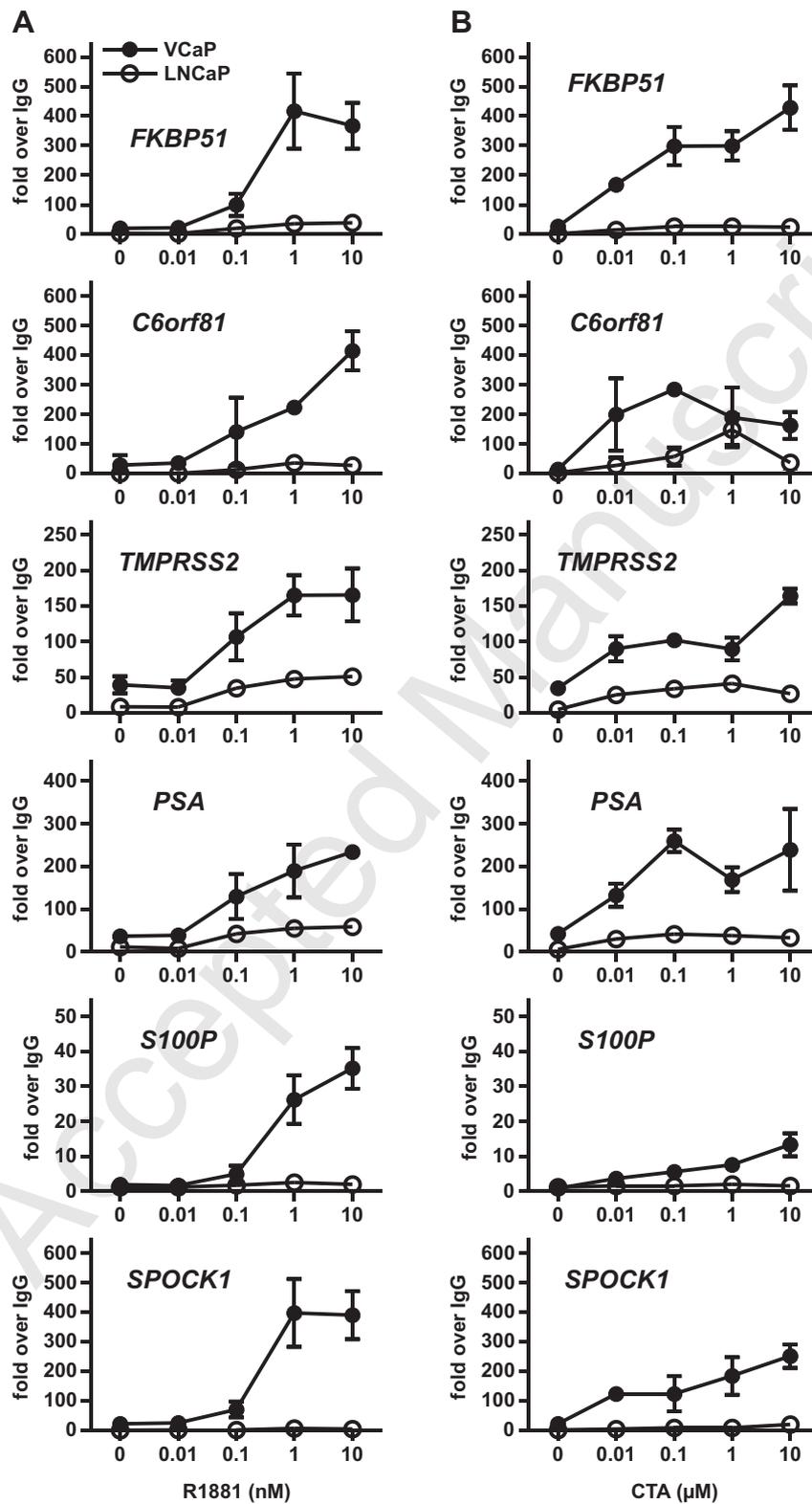


Figure 5

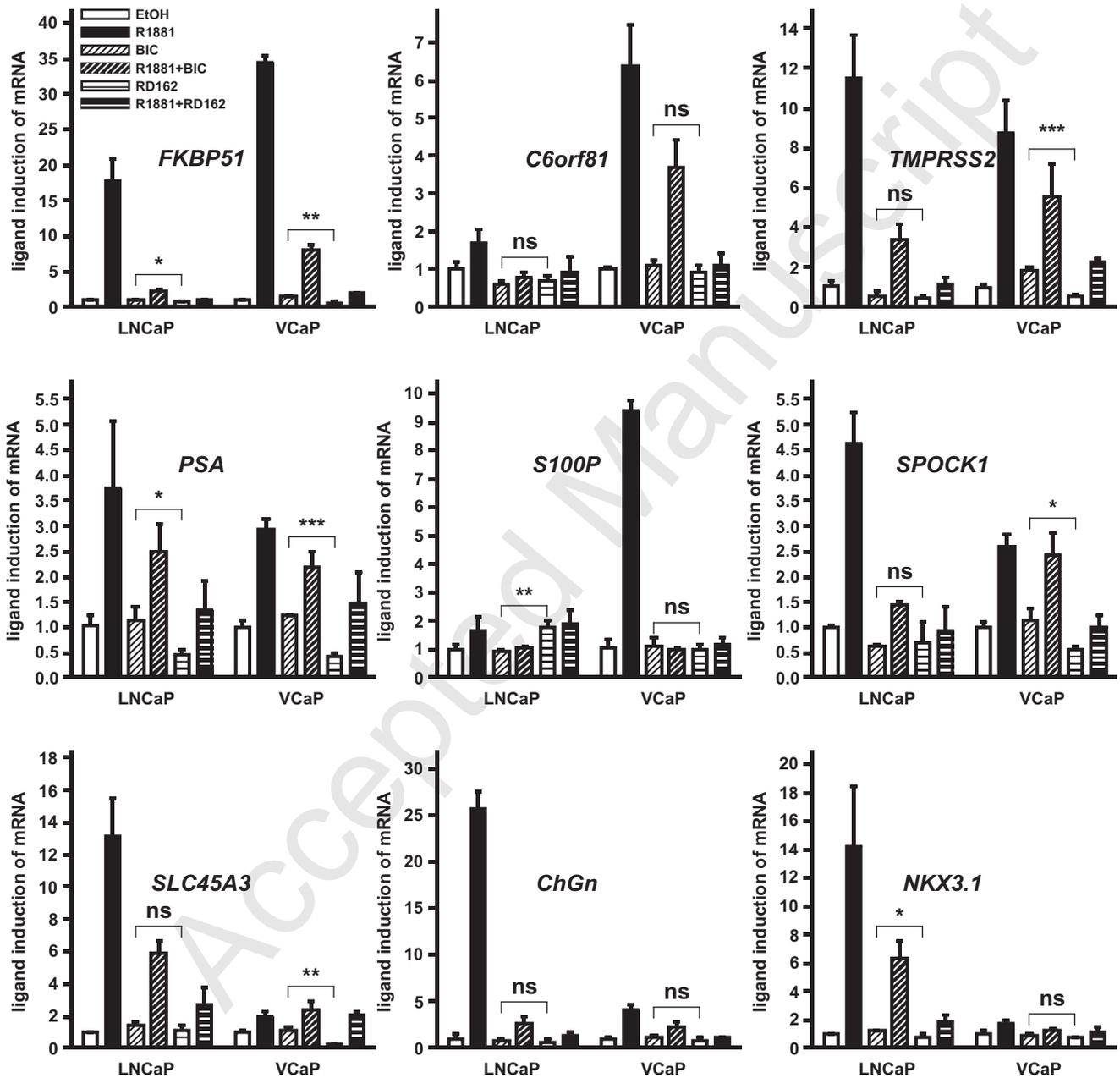


Figure 6

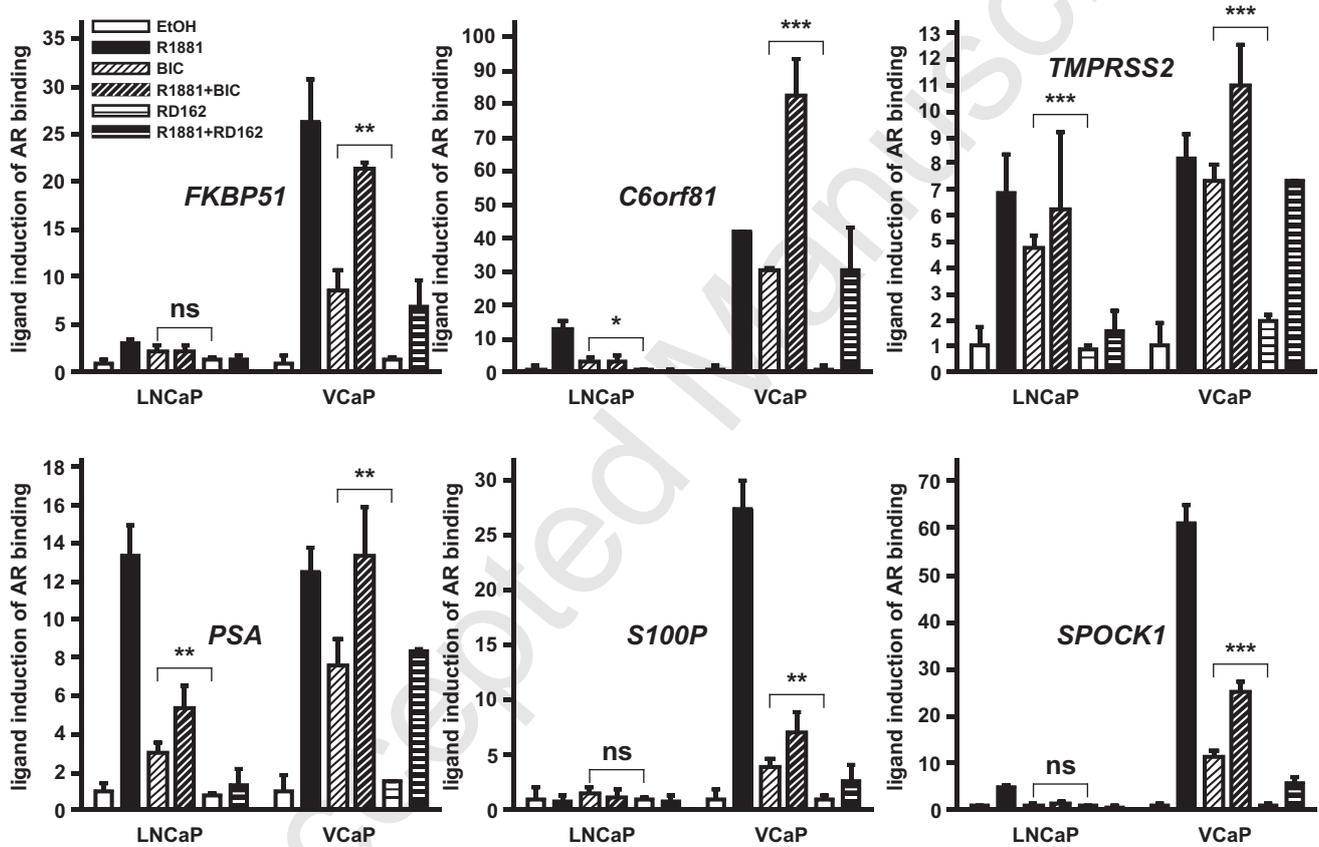


Figure 7