



## Methoxychalcone inhibitors of androgen receptor translocation and function

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### ABSTRACT

Androgen receptor activity drives incurable castrate-resistant prostate cancer. All approved antiandrogens inhibit androgen receptor-driven transcription, and in addition the second-generation antiandrogen MDV3100 inhibits ligand-activated androgen receptor nuclear translocation, via an unknown mechanism. Here, we report methoxychalcones that lock the heat shock protein 90-androgen receptor complex in the cytoplasm in an androgen-non-responsive state, thus demonstrating a novel chemical scaffold for antiandrogen development and a unique mechanism of antiandrogen activity.

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Prostate cancer is one of the most common cancers in men and the second leading cause of cancer death in men in the United States. Although hormonal therapy is typically effective initially, resistance often develops and results in a state known as castration-resistant prostate cancer (CRPC).<sup>1</sup> Recently it has been discovered that even in patients with castrate levels of androgen, prostate cancer remains dependent on androgen receptor (AR). Several AR reactivation mechanisms have been reported including overexpression of AR or AR coactivators, mutation of AR, indirect activation of AR by growth factors or cytokines, and aberrant phosphorylation of AR.<sup>1–5</sup>

Targeting AR signaling is a rational approach to the treatment of CRPC. The AR consists of an N-terminal regulatory domain (NTD), a DNA-binding domain (DBD), a hinge region and a C-terminal ligand-binding domain (LBD).<sup>4,5</sup> In the absence of androgen, AR is held in the cytoplasm by heat shock protein 90 (Hsp90). Androgen binding to the LBD of AR results in a change of AR conformation and release of AR from Hsp90. AR translocates to the nucleus, binds to androgen response elements and AR target gene expression is activated.<sup>1,5</sup> Prostate-specific antigen (PSA) and transmembrane protease serine 2 (TMPRSS2) are representative AR target genes. Several strategies have been reported to target AR in either a direct

or indirect manner.<sup>6–13</sup> These approaches include AR antagonists for reducing AR target gene expression,<sup>6–8</sup> cytochrome P-450 CYP17A1 inhibitors for blocking androgen synthesis,<sup>14,15</sup> and Hsp90 inhibitors for degrading AR protein.<sup>16</sup> Recently, the antiandrogen MDV3100 has shown promising results in preclinical and clinical trials.<sup>17</sup> MDV3100 inhibits AR translocation to the nucleus and binding of AR to androgen response elements. **Chart 1** shows structures of androgen receptor agonists and antiandrogens.

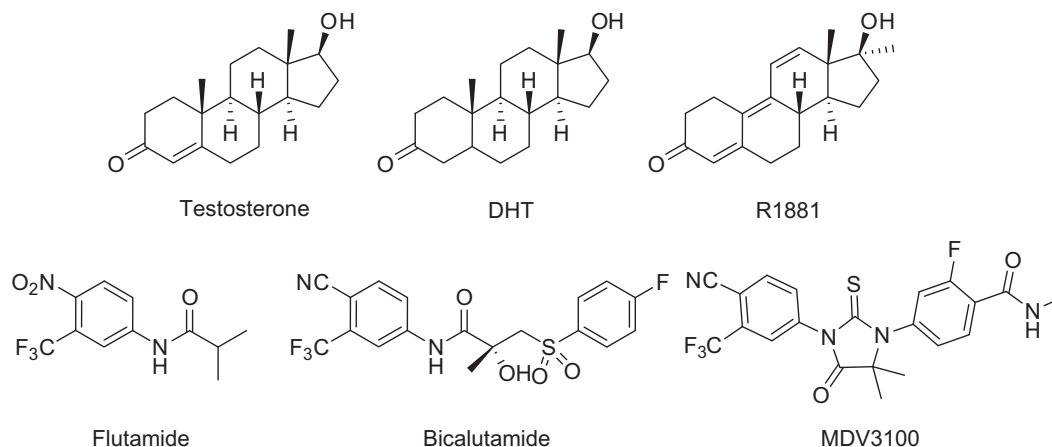
Chalcones (*trans*-1,3-diphenyl-2-propene-1-ones) are  $\alpha,\beta$ -unsaturated carbonyl compounds with two aromatic rings. They are intermediates in the biosyntheses of flavonoids and isoflavonoids and are abundant in plants. They are known to exhibit several biological activities such as anti-oxidant, anti-inflammatory, antiviral, antibacterial, antimalarial and anticancer activities.<sup>18–21</sup> We describe here a novel mechanism of action of chalcone derivatives in the AR signaling pathway for the treatment of prostate cancer.

To determine the structure–activity relationship, 100 chalcone derivatives **1a–10j** were synthesized in this study by condensation between substituted benzaldehyde and substituted acetophenone in the presence of LiOH in ethanol (**Scheme 1**). Once the synthesis of desired chalcones was achieved, their antiandrogen activity was screened using real-time reverse transcription-polymerase chain reaction (RT-PCR). The prostate cancer cell line LNCaP was treated with the test compounds at 2.5  $\mu$ M for 20 h and PSA mRNA levels were measured by real-time RT-PCR. The AR of LNCaP cells has a T877A point mutation in the ligand binding domain. This AR mutation, which has been observed in primary prostate cancer tissue of

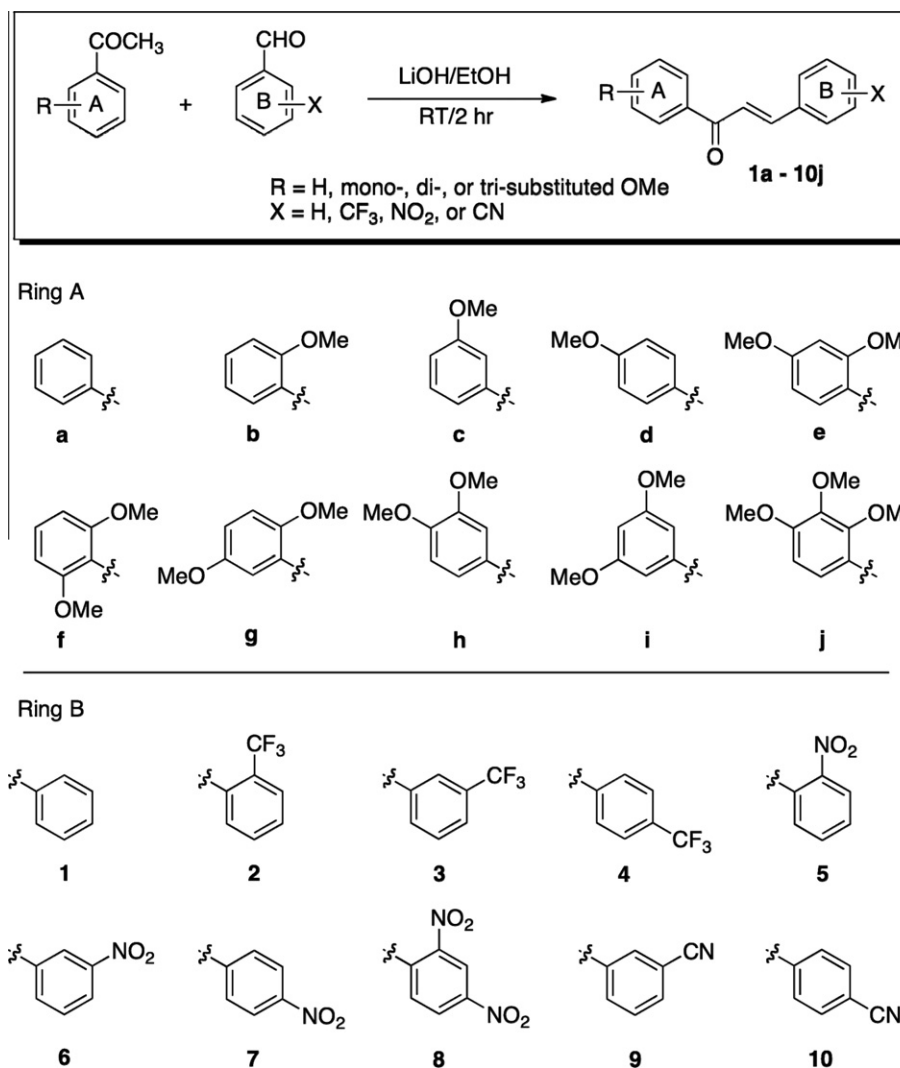
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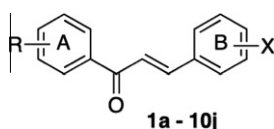
**Chart 1.** Structures of androgen receptor modulators.



**Scheme 1.** Molecular structures of chalcones. Chalcones were prepared by the condensation of a substituted acetophenone and a substituted benzaldehyde in the presence of LiOH at room temperature.

prostate cancer patients treated with the antiandrogen flutamide, has been shown to be associated with conversion of flutamide from an antagonist to an agonist.<sup>22,23</sup> PSA is an androgen receptor target gene and inhibition of AR results in decreased PSA mRNA expres-

sion. We identified 24 potent compounds, which inhibited PSA mRNA expression over 80% at 2.5  $\mu$ M (Table 1). Unsubstituted chalcones **1a–j** were found to be least active with maximum inhibition of only 63% in case of **1b**. Also, the chalcone derivatives with

**Table 1**PSA mRNA level of LNCaP cells treated with the corresponding chalcone<sup>a</sup>

No.	1	2	3	4	5	6	7	8	9	10
a	0.78	0.13	0.41	0.44	0.14	0.24	0.21	0.64	0.67	0.61
b	0.37	0.05	0.09	0.52	0.06	0.10	0.08	0.09	0.31	0.26
c	0.43	0.32	0.67	0.59	0.30	0.21	0.21	0.38	0.58	0.36
d	0.68	0.46	0.56	0.71	0.22	0.35	0.32	0.43	0.71	0.71
e	0.63	0.35	0.36	0.51	0.44	0.33	0.30	0.39	0.34	0.29
f	0.81	0.13	0.29	0.27	0.29	0.15	0.10	0.19	0.32	0.35
g	0.47	0.23	0.48	0.44	0.16	0.11	0.33	0.24	0.24	0.18
h	0.74	0.40	0.20	0.20	0.31	0.16	0.06	0.75	0.81	0.15
i	0.52	0.31	0.48	1.61	0.26	0.80	0.20	0.40	0.60	0.51
j	0.50	0.48	0.19	0.62	0.23	0.16	0.16	0.36	0.35	0.30

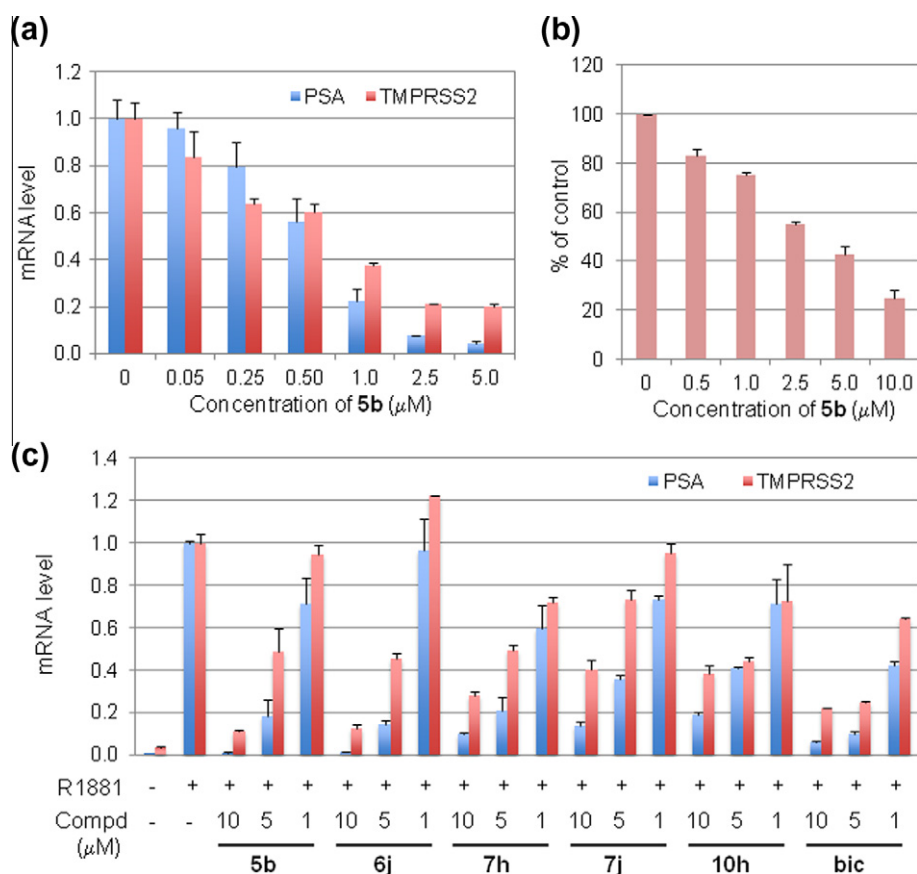
<sup>a</sup> PSA mRNA level was measured in LNCaP cells treated with the corresponding compound at 2.5  $\mu$ M for 20 h using real time RT-PCR. The PSA mRNA level is relative to DMSO-treated control cells in which PSA mRNA level is 1 (mean values,  $n = 3$ ).

cyano-substitution were inactive with the exception of compounds **10g** and **10h**, which showed 82 and 85% inhibition of PSA mRNA expression, respectively. As shown in Table 1, chalcone series b with an *o*-methoxy group, showed good activity when the B ring had a nitro- or trifluoromethyl-group. Among series b, compounds **2b** and **5b** showed the highest activity (over 94% inhibition of PSA mRNA expression) in which B ring had an *o*-trifluoromethyl or *o*-nitro group. It is also worth mentioning that the chalcone series f and j with two *o*-methoxy groups also showed significant inhibi-

tion, in particular, compounds **2f** (87%), **6f** (85%), **7f** (90%), **8f** (81%), **3j** (81%), **6j** (84%) and **7j** (84%). Thus, in general, structure-activity relationship (SAR) studies revealed that chalcones with an *o*-methoxy group on A ring were highly active, suggesting that the *o*-methoxy substituted ring is important to enhanced activity.

Further dose-dependent inhibition of AR target gene expression was achieved using compound **5b** in LNCaP cells (Fig. 1a). Chalcone **5b** inhibited PSA and TMPRSS2 mRNA expression 50% at 0.60 and 0.75  $\mu$ M, respectively. Importantly, no agonistic effect was observed in the range 5–0.05  $\mu$ M. Compound **5b** inhibited the growth of LNCaP cells after 3 days treatment with an IC<sub>50</sub> 3.4  $\mu$ M (Fig. 1b).

We then tested selected compounds for effect on AR target gene expression in the presence of the synthetic androgen R1881. LNCaP cells were incubated for 3 days in phenol red-free RPMI 1640 supplemented with 10% charcoal-stripped fetal bovine serum and 1% antimycotic–antibiotic solution, and then treated for 20 h as indicated. The concentration of R1881 was 0.5 nM and that of the corresponding compounds was 10, 5 or 1  $\mu$ M. As shown in Figure 1c, the AR target genes PSA and TMPRSS2 were induced about 500-fold and 30-fold, respectively, by R1881 compared with the DMSO control. R1881-induced gene expression was blocked by chalcones **5b**, **6j**, **7h**, **7j**, and **10h** in a dose-dependent manner. Compound **5b** (10  $\mu$ M) inhibited PSA and TMPRSS2 mRNA expression 99% and 89% in the presence of 0.5 nM R1881 (Fig. 1c). Although the activity of compound **6j** was similar to that of compound **5b** at high concentrations, it showed weak agonistic effect at 1  $\mu$ M, which is not desirable. Thus we performed further analysis of the impact of compound **5b** on androgen-regulated gene expression in LNCaP cells. We performed cDNA microarray analysis of LNCaP cells treated with 0.5 nM R1881 with/without 5  $\mu$ M **5b**



**Figure 1.** (a) Dose-dependent inhibition of PSA and TMPRSS2 mRNA expression by compound **5b** at 20 h. (b) Inhibition of LNCaP cell growth by compound **5b** at day 3. (c) Inhibition of PSA and TMPRSS2 mRNA expression by compounds **5b**, **6j**, **7h**, **7j**, **10h**, or **bic** in the presence of synthetic androgen R1881 at 20 h. Test compounds were evaluated at 10, 5, or 1  $\mu$ M and R1881 was used at 0.5 nM.

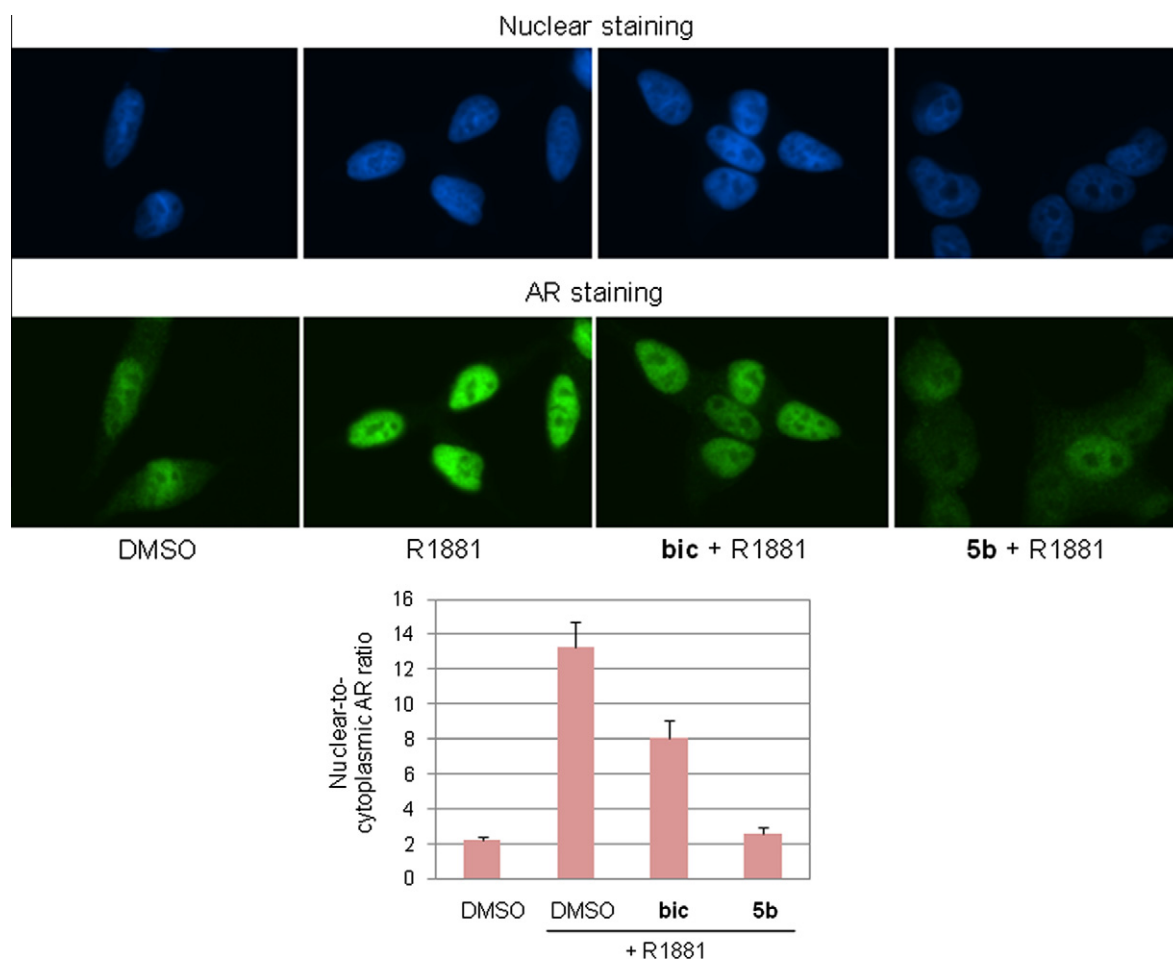
**Table 2**  
Changes of gene expression in LNCaP cells<sup>a</sup>

Symbol	Gene description	R1881	R1881 + <b>5b</b>
TMPRSS2	Transmembrane protease, serine 2	22.7	7.0
FKBP5	FK506 binding protein 5	29.7	5.3
HPGD	Hydroxyprostaglandin dehydrogenase 15-(NAD)	70.8	13.9
KLK2	Kallikrein-related peptidase 2	423.8	159.3
NDRG1	N-myc downstream regulated 1	8.6	3.9
IGF1	Insulin-like growth factor 1	19.0	5.0
EAF2	ELL associated factor 2	20.5	1.6
CXCR4	Chemokine (C-X-C motif) receptor 4	7.1	2.7
CXCL5	Chemokine (C-X-C motif) ligand 5	15.1	2.6
CXCL9	Chemokine (C-X-C motif) ligand 9	18.7	3.4
KCNMA1	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	5.1	2.5
PHLDB2	Pleckstrin homology-like domain, family B, member 2	6.8	2.8
SOCS2	Suppressor of cytokine signaling 2	6.4	2.3
SRMS	Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites	10.3	0.5

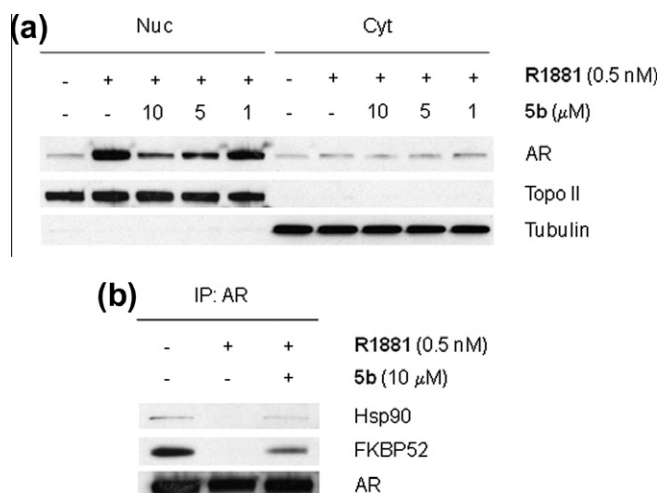
<sup>a</sup> LNCaP cells were treated with DMSO or **5b** (5  $\mu$ M) in the absence or presence of synthetic androgen R1881 (0.5 nM) for 20 h, cDNA microarray was performed using the GeneChip Human Genome U133 Plus 2.0 Array. The values shown represent fold-changes compared to DMSO-treated control cells.

for 20 h, and Table 2 shows the fold-changes of gene expression compared to DMSO-treated control cells. Compound **5b** inhibited expression of androgen-induced genes,<sup>24</sup> including TMPRSS2, FK506 binding protein 5 (FKBP5), kallikrein-related peptidase 2 (KLK2), insulin-like growth factor 1 (IGF1) and chemokine (C-X-C motif) receptor 4 (CXCR4). The real time RT-PCR and cDNA microarray data demonstrate that compound **5b** blocks AR target gene expression in LNCaP cells in the absence and presence of synthetic androgen R1881.

Immunocytochemistry was performed to determine if compound **5b** inhibits AR translocation to the nucleus. LNCaP cells were grown on cover slips and then were treated with DMSO, 10  $\mu$ M bicalutamide (**bic**) or 10  $\mu$ M **5b** for 3 h, followed by treatment with 0.5 nM R1881 for 3 h. After staining, AR protein distribution in the cells was identified by immunofluorescence and image analysis. Figure 2 shows the fluorescence images of LNCaP cells in which green and blue color represents AR and nuclear DNA, respectively. Before treatment with R1881, AR is located in both the cytoplasm



**Figure 2.** Inhibition of R1881-induced AR translocation to the nucleus. LNCaP cells were grown on cover slips and then were treated with DMSO, **bic** (10  $\mu$ M) or **5b** (10  $\mu$ M) for 3 h, followed by treatment with R1881 (0.5 nM) for 3 h. Fluorescence images show nuclear DNA (blue color) or AR protein (green color) in LNCaP cells. Nuclear-to-cytoplasmic AR ratio was measured using Openlab software.



**Figure 3.** (a) AR protein level in nuclear and cytoplasmic fractions. (b) Co-immunoprecipitation (IP) was performed with anti-AR antibody and western blots were performed with anti-Hsp90 and anti-FKBP52 antibodies.

and nucleus in LNCaP cells. After incubation with R1881, bright nuclear AR staining was observed, due to translocation of AR to the nucleus. The approved antiandrogen bicalutamide did not inhibit androgen-stimulated AR translocation to the nucleus, as shown in the fluorescence image with bright nuclear AR staining and very weak cytoplasmic AR staining, consistent with previous reports.<sup>17</sup> In contrast, compound **5b** inhibited AR translocation significantly, resulting in the presence of AR in the nucleus and cytoplasm (Fig. 2). In addition to the immunofluorescence technique, AR protein levels in the nucleus and cytoplasm were measured by subcellular fractionation, followed by western blot with anti-AR antibody. LNCaP cells were treated for 20 h in the presence of 0.5 nM R1881 with or without 10, 5 and 1 μM compound **5b**. Nuclear and cytoplasmic fractions were isolated and the AR protein level was measured by western blot. As shown in Figure 3a, compound **5b** inhibited R1881-induced AR nuclear translocation in a dose-dependent manner. The results are consistent with immunocytochemistry data and real time RT-PCR data showing inhibition of AR target gene expression in response to compound **5b**. To demonstrate the purity of the two subcellular fractions, topoisomerase II and tubulin were used as markers of the nucleus and the cytoplasm, respectively. For nuclear translocation to occur, AR must be released from association with cytoplasmic Hsp90.<sup>1,4</sup> Using co-immunoprecipitation (IP) analysis we found that R1881-induced release of AR from Hsp90 was inhibited by compound **5b** (Fig. 3b). Furthermore R1881-induced release of AR from the immunophilin FKBP52 was also inhibited, consistent with arrest of AR in a cytoplasmic Hsp90-AR complex,<sup>25</sup> a mechanism of anti-AR activity distinct from all approved antiandrogens.

In conclusion, we have synthesized, screened and identified a series of antiandrogen chalcones in LNCaP cells. As demonstrated by real time RT-PCR, cDNA microarray, western blot and co-IP data, lead

compound **5b**, (E)-1-(2-methoxyphenyl)-3-(2-nitrophenyl)prop-2-en-1-one, inhibits AR translocation to the nucleus as well as AR target gene expression by locking the AR-Hsp90 complex in the cell cytoplasm in an androgen-nonresponsive state.

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### Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.12.141.

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