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Brief Article

Design and Synthesis of Androgen Receptor Antagonists with Bulky Side Chains for Overcoming Antiandrogen Resistance

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Incorporation of curcumin and β-ionone into one chemical entity led to identification of a novel antiandrogen with two bulky side chains, **6**, which is a pure antagonist of the wild-type and the T877A, W741C, and H874Y mutated androgen receptors (AR), showing no cross-reactivity with progesterone receptor and low micromolar cytotoxicity in LNCaP, PCa-2b, 22Rv1, and C4-2B prostate cancer cells. Molecular modeling indicates **6** adopts a "Y"-shape conformation and forms multiple hydrogen bonds with AR backbone.

Introduction

The androgen receptor (AR[a]) is a critical mediator of prostate cancer, even at the castration-resistant status.[1] Once prostate cancer becomes castration-resistant, hormonal therapy with current antiandrogens is not effective. Mutation in AR is an important mechanism that accounts for the development of resistance to the current clinically used antiandrogens such as flutamide and bicalutamide.[2] One particular mutation is the T877A in the ligand-binding domain (LBD) of AR, which actually results in paradoxical activation by hydroxyflutamide, an active metabolite of antiandrogen flutamide.[3] The W741C AR mutant is activated by antiandrogen bicalutamide.[4] Yoshida et al. have demonstrated the agonistic effect of bicalutamide to a xenograft with the clinically induced W741C mutated AR.[5] Thus, in laboratory setting, AR mutations, such as T877A and W741C, have turned the growth-inhibitory effect of the current clinically used antiandrogens into a growth-promoting effect. To develop novel pan-antiandrogens effective against the wild type (WT) and multiple clinically relevant mutant ARs represents an attractive strategy for circumventing antiandrogen resistance.

The helix-12 (H12) at the AR ligand-binding domain (AR-LBD) plays a critical role in AR transactivation. On binding of androgen, such as dihydrotestosterone (DHT), H12 is repositioned to cover the hormone-binding pocket, forming the activation function-2 surface. One way to antagonize AR function is to design compounds bearing an extending bulky arm to displace H12. Such a strategy has been successfully utilized for the design of estrogen receptor antagonists.[6] However, several DHT-derived molecules bearing one bulky chain surprisingly turned out to be potent agonists of the AR.[7]

In the present work, we report the design, synthesis, and biological characterization of a novel class of antiandrogens with two bulky side chains. Our computational study revealed that chemical compounds with two bulky side chains could be obtained by incorporating two dietary agents, β-ionone and curcumin, into one chemical entity. The β-ionone is a phytochemical present in many fruits, vegetables, and grains. It is found to exert in vitro anticarcinogenic and antitumor activities.[8] Curcumin, the major pigment in the dietary spice turmeric, is found to possess diverse pharmacological effects including anti-inflammatory, antioxidant, antiangiogenic, and anticancer activities.[9] Several curcumin analogues possess antiandrogenic activity.[10] We hypothesize that the hybrid molecule of β-ionone and curcumin could furnish a novel class of antiandrogens (Scheme 1). Eleven compounds (**1**–**11**) were synthesized. Among them, compound **6** shows low micromolar cytotoxicity in a panel of five prostate cancer cell lines and potently suppress DHT-induced transactivation of the WT and the T877A, W741C, and H874Y mutated ARs, showing no cross-reactivity with human progesterone receptor (PR). AR fluorescence polarization assays indicate compound **6** binds to the AR-LBD at the hormone-binding pocket. Molecular modeling indicated that compound **6** forms multiple hydrogen bonds with the backbone of AR and adopts a "Y" shape conformation.

Results

Chemistry. Eleven β-ionone derivatives **1**–**11** (Tables 1 and 2) were synthesized as outlined in Scheme 2. Condensation of (*E*)-6-(2,6,6-trimethylcyclohex-1-enyl)hex-5-ene-2,4-dione with aromatic aldehyde furnished compounds **1**–**5** (Table 1).[11] Compounds **6**–**11** (Table 2) were synthesized via a two-step procedure. Condensation of the second aromatic

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[a] Abbreviations: AR, androgen receptor; Bic, bicalutamide; CS-FBS, charcoal-stripped fetal bovine serum; DHT, dihydrotestosterone; FP, fluorescence polarization; H12, helix-12; LBD, ligand-binding domain; OHF, hydroxyflutamide; PR, progesterone receptor; WT, wild type.

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aldehyde with the middle methylene (−CO-CH_2-CO−) was accomplished by Knoevenagel condensation in methanol with catalytic amount of piperidine (Scheme 2). Purifications of the crude products were achieved by silica gel CC (elutant: *n*-hexane and ethyl acetate). Some of the products were further purified by preparative TLC.

We have tried to synthesize **6**−**9** by a one-pot reaction starting from (*E*)-6-(2,6,6-trimethylcyclohex-1-enyl)hex-5-ene-2,4-dione, with molar ratio of the dione and aromatic aldehyde as 1:2.3, using a method similar to that for curcumin derivatives but B_2O_3 was taken out.[11] This one-pot reaction afforded small amount of the 1:2 condensation product. Eventually, we have employed a two-step procedure for the syntheses of **6**−**11**, which involves condensation with the first aromatic aldehyde to obtain the corresponding diketone and condensation of the diketone with the second aromatic aldehyde (Scheme 2). This two-step procedure allows us to synthesize compounds bearing two different R groups, such as **10** and **11**.

The NMR analysis revealed **6** is a mixture of the cis and trans isomers that interconvert at room temperature (Scheme 3). For the cis isomer, the proton H20 has NOE with H12, and for the trans isomer, the H20 has NOE with H8. The ratio of the two isomers is about 3:2, with the cis isomer more favorable. The substituted C7═C8 and C12═C13 double bonds are both trans in the two isomers (Tables S1 and S2, Supporting Information (SI)). There is only one peak in the HPLC analysis of **6** (Table S3, SI).

Scheme 1. Design of the Hybrid of *β*-Ionone and Curcumin

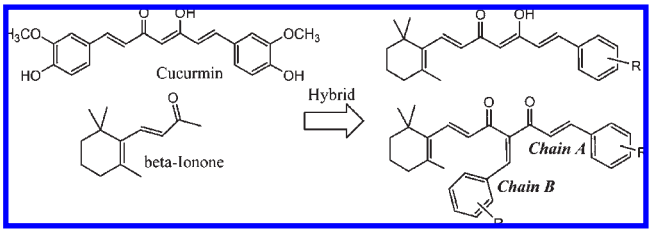
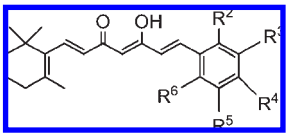
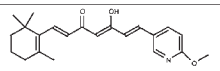


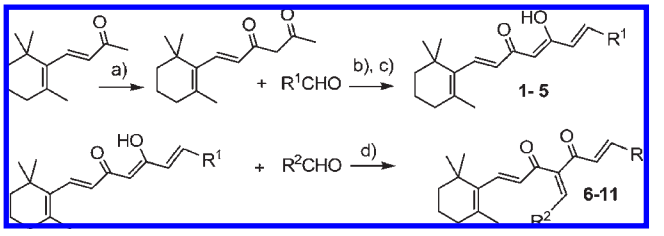
Table 1. Cytotoxicity of Compounds **1**−**5** in LNCaP and PC-3 Cells



Compound	Substituents					IC_{50} (μM)[a]			
	R^2	R^3	R^4	R^5	R^6	LNCaP	22Rv1	C4-2B	PC-3
1	H	OCH_3	OH	H	H	20.6	10.6	44.3	15.8
2	H	OH	OCH_3	H	H	25.9	N.D.[b]	23.1	38.3
3	H	OCH_3	OH	OCH_3	H	6.2	12.2	9.8	26.7
4	H	OCH_3	OCH_3	OCH_3	H	21.4	17.1	11.9	> 50
5						25.1	N.D.	N.D.	52.2

[a] IC_{50} is the concentration of compounds which causes a 50% inhibition as compared to the vehicle control (0.5% DMSO); [b] N.D., not determined.

Scheme 2. Syntheses of Compounds **1**−**5** and **6**−**11**[a]



[a] Reagent and conditions: (a) CH_3ONa, ethyl acetate; (b) B_2O_3, $(nBuO)_3B$, $nBuNH_2$; (c) HCl; (d) piperidine, CH_3OH, room temp.

Scheme 3. Compound **6** Exists in Both the Cis and Trans Isomers

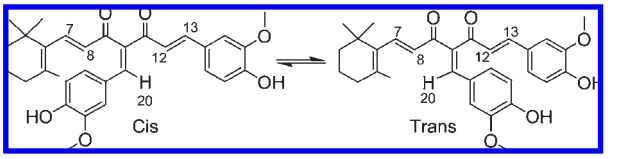
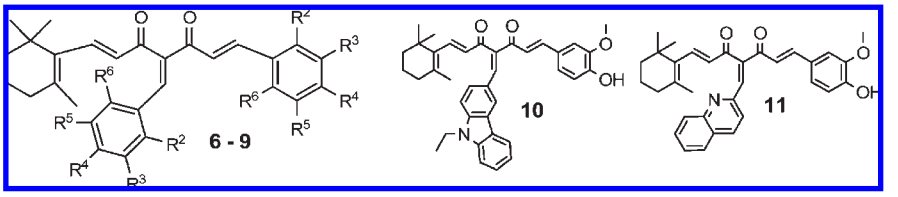


Table 2. Cytotoxicity of Compounds **6**−**11** in Prostate Cancer Cell Lines



Compound	substituents					cellular IC_{50} (μM)[a]				
	R^2	R^3	R^4	R^5	R^6	LNCaP	PCa 2b	22Rv1	C4−2B	PC-3
6	H	OCH_3	OH	H	H	1.3	2.5	2.2	1.6	4.2
7	H	OC_2H_5	OC_2H_5	H	H	14.9	6.8	9.3	4.5	14.1
8	H	H	OH	H	H	4.5	4.7	4.7	4.0	6.7
9	H	OCH_3	OH	OCH_3	H	8.7	15.3	17.6	8.5	12.5
10						2.3	8.1	2.4	2.3	7.0
11						8.5	ND[b]	5.1	5.3	15.2
curcumin[c]						8.5	29.5	18.3	6.1	16.8
β-ionone						151	ND	ND	ND	inactive[d]

[a] IC_{50} is the concentration of compounds which causes a 50% inhibition as compared to the vehicle control (0.5% DMSO). [b] ND, not determined. [c] Curcumin was synthesized according to the literature.[11] [d] Maximum concentration tested is 150 μM.

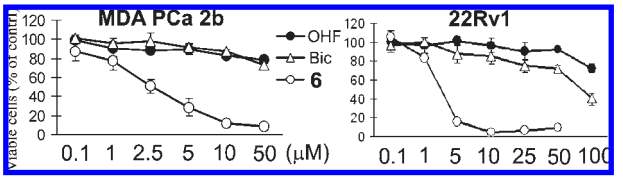


Figure 1. Growth inhibitory effects of compound **6**, hydroxyflutamide (OHF), and bicalutamide (Bic) in the PCa 2b and 22Rv1 cells.

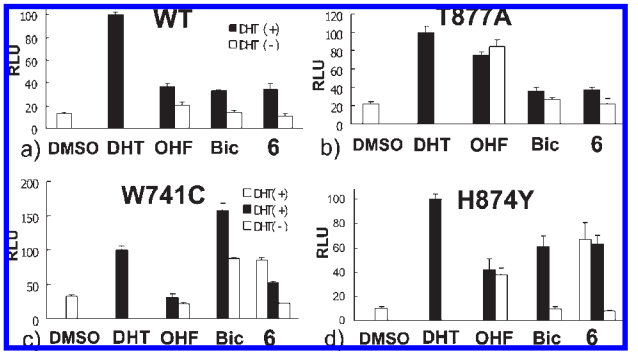


Figure 2. Effect of compound **6** at 0.1 μM (gray bar) and 1 μM (black bar), OHF at 1 μM, and Bic at 1 μM on the transactivation of WT and the T877A, W741C, and H874Y mutant ARs in the presence (black and gray bars) and absence (white bars) of 0.1 nM DHT. Plasmids expressing human ARs are transiently transfected in PC-3 cells. The results are reported as mean ± sd. Relative luciferase activity is standardized to the Renilla luciferase control and normalized to the 0.1 nM DHT RLU, relative luciferase units.

Antiproliferative Activity of 1−11. The in vitro cytotoxicity of **1−11** were evaluated by MTT assays in a panel of prostate cancer cell lines, including LNCaP, MDA PCa 2b, 22Rv1, C4-2B, and PC-3. The IC_{50} values were computed from cell survival curves (Tables 1 and 2). The effect of hydroxyflutamide, bicalutamide, and **6** in MDA PCa 2b and 22Rv1 cells are shown in Figure 1. Among **1−11**, compound **6** shows the most potent cytotoxicity in LNCaP cells and is substantially more potent than curcumin and β-ionone (Table 2). In LNCaP cells, the *para*-hydroxyl substituent appears to be critical for the cytotoxicity of **6** and removal of the *meta*-methoxy group reduces the activity (compare **6**, **7**, and **8**). Adding a second *meta*-methoxy to **6** does not improve the activity (compare **9** and **6**). Cytotoxic activity of **10** in LNCaP, 22Rv1, and C4-2B is comparable to that of **6**, indicating R group at chain B could be varied significantly (Table 2, Scheme 1).

Antiandrogenic Activity of Compound 6. To investigate antiandrogenic activity of compound **6**, AR-dependent reporter assays were performed in PC-3 cells using ARE-driven MMTV-luc reporter and AR-expressing plasmids (Figure 2). Antiandrogen hydroxyflutamide and bicalutamide were included as the control. We have tested the effect of compound **6** against the WT and the T877A, W741C, and H874Y mutated ARs. In accordance with previous studies,[3,4] our reporter assays revealed that in the absence of DHT, the T877A and W741C mutated ARs were activated by hydroxyflutamide and bicalutamide, respectively (white bars, Figure 2b,c). In contrast, compound **6** is a nonagonist for the WT and the W741C, T877A, and H874Y mutated ARs (white bars, Figure 2). Further, compound **6** at 1 μM demonstrates potent antiandrogenic activity in suppressing DHT-induced transactions of the WT and the T877A and W741C AR mutants, as well as modest antiandrogenic activity against the H874Y mutant (black bars, Figure 2).

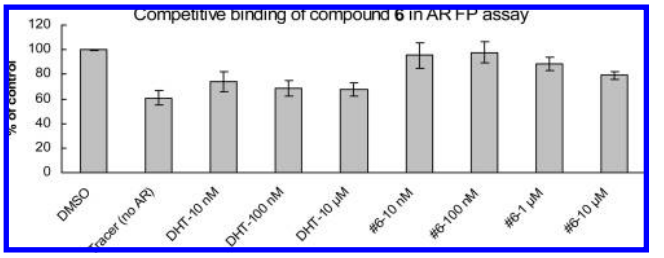


Figure 3. Competitive binding of compound **6** to the AR-LBD evaluated by AR fluorescence polarization (FP) assay, using PolarScreen AR competitor assay kit (P3018, Invitrogen). Fluorescence polarization was used as a read-out and normalized to that of DMSO vehicle. Results are reported as average ± sd of experiments performed in triplicate.

DHT Rescue of the Growth Suppression of LNCaP Cells by Compound 6. LNCaP cells in phenol red-free RPMI 1640 medium supplemented with 10% charcoal-stripped FBS (CS-FBS) were treated with DMSO vehicle, DHT, as well as compound **6** at designated concentrations in the presence of 0.1 or 1 nM DHT (Figure S1, SI). The 0.1 nM DHT-stimulated growth of LNCaP cells was suppressed by compound **6** in a dose-dependent manner (gray bars, Figure S1, SI). Importantly, the growth suppression of LNCaP cells by compound **6** at 0.1, 1, and 5 μM, but not 10 μM, was at least partially reversed by increasing the DHT concentration to 1 nM (Figure S1, SI). In contrast, this DHT rescue of growth suppression by compound **6** was not observed in the AR-negative PC-3 cells (Figure S1, SI). These results indicate that the cytotoxicity of compound **6** in LNCaP cells is at least in part attributable to its antiandrogenic activity.

Competitive Binding Assay and Cross-Reactivity with Other Steroid Receptors. The possible competitive binding of compound **6** to the hormone-binding pocket of the rat AR-LBD was evaluated by PolarScreen AR competitor assay kit (P3018, Invitrogen). If the test compound binds to AR-LBD, it will prevent the formation of the AR/tracer complex, and the tracer will be free in solution. When the tracer is free in solution, its rotational mobility is greater than when bound to the receptor, resulting in a low fluorescence polarization value. We have controlled the assay for minimal competition (DMSO vehicle), which has a maximum value of fluorescence polarization and for no receptor (tracer only), which represents the minimum value of the fluorescence polarization that can possibly be reached by a competitor. The DHT was included as a positive control (Figure 3). Compound **6** has reduced the fluorescence polarization value in a dose-dependent manner, indicating it binds to AR-LBD by competing with the tracer over the hormone-binding pocket (Figure 3).

To evaluate the cross-reactivity of **6** with PR, we have performed PR-dependent luciferase assays in PC-3 (Figure S2, SI). Compound **6** at 0.1 and 1 μM is inactive in suppressing 10 nM R5020-induced transactivation of the B form of human PR, indicating **6** is not the antagonist of PR. In the absence of R5020, compound **6** at 0.1 and 1 μM is incapable of activating PR, indicating **6** is a nonagonist of PR. The basal control revealed there is no detectable endogenous PR in PC-3 cells (Figure S2, SI).

Molecular Modeling. Despite the fact that the crystal structures of the T877A mutant in complex with hydroxyflutamide and the W741L mutant in complex with bicalutamide are available, the H12 of both of the two complexes are

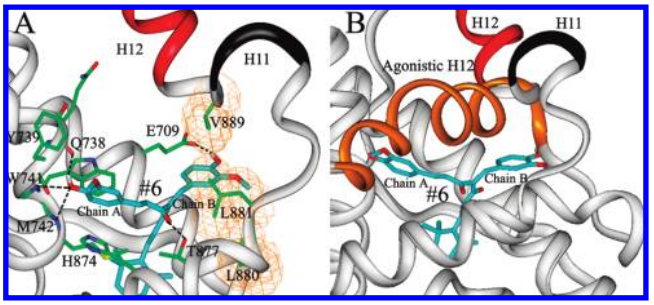


Figure 4. (A) Predicted binding mode of compound **6** in the structural model of AR-LBD at the antagonistic form; and (B) H12 at the agonistic form (orange ribbon), taken from the AR-LBD/DHT complex (PDB entry: 1t65), was merged into the structural model, showing steric clash of chains A and B with the agonistic H12. Compound **6** and side chains are colored according to the atomic-coloring scheme (O in red, N in blue, C in cyan for **6** but in green for side chains). The vdW surfaces of V889, L884, and L880 are in orange grid. Hydrogen bond is indicated by dash lines.

at the agonist form (PDB entries: 2ax6 and 1z95). To date, crystal structure of AR-LBD at the antagonist form has not been obtained. To investigate molecular basis for the finding that compound **6** remains as a pure antiandrogen in the WT and multiple mutated ARs, we have built a structural model of the WT AR-LBD with H12 at the antagonist form, using crystal structures of the AR-LBD/DHT and ER/antagonist complexes (PDB entries: 1t65 and 3ert) as templates (SI). Figure 4A demonstrates the predicted binding mode of compound **6** in the antagonistic model of WT AR-LBD. To investigate possible steric clash between the bulky antiandrogen **6** with the H12 at the agonist form, H12 from the crystal structure of the agonistic AR-LBD/DHT complex (PDB entry: 1t65) was merged with the antagonistic AR-LBD model and shown in Figure 4B.

Inspection of the predicted binding mode indicated that **6** adopts a "Y" shape conformation, with the β-ionone core anchoring inside the hormone-binding pocket and chains A and B protruding toward the agonist form of H12 (orange ribbon, Figure 4B). The *para*-hydroxyl in chain A of compound **6** forms multiple hydrogen bonds with the backbones, but not the side chains, of residues Q738 (at carbonyl oxygen), W741 (at amide nitrogen), and M742 (at amide nitrogen), whereas chain B forms a hydrogen bond with E709 and interacts with a hydrophobic pocket formed by L880, L881, and V889 (Figure 4). This is in consistent with the finding that the *para*-hydroxyl in chain A is critical for the cytotoxic activity of **6** in LNCaP cells (Table 2). Compounds **6** and **10**, which demonstrate similar cytotoxicity in LNCaP cells are predicted to have similar binding mode in chain A (Figure S3, SI). In contrast, the para substituents of chain A in **7**, **9**, and **11** do not form multiple hydrogen bonds with the backbone of AR-LBD (Figure S3, SI). In addition, compound **6** is predicted to have similar binding modes in the WT and the T877A, W741C, and H874Y mutated ARs, forming hydrogen bonds with the backbones of Q738 and M742, as well as side chain of E709 (Figure 4 and SI Figure S4).

Discussion

A series of AR mutations, such as T877A, H874Y, and W741C, were identified from tissue specimens of patients with castration-resistant prostate cancer. The incidence of AR mutation in advanced prostate cancer is estimated to be in the range of 10−40%.[2,3,12] In particular, the T877A mutation has been found in patients who were treated with flutamide and eventually became refractory to the treatment.[2] The functional significance of the W741C mutation was demonstrated by the bicalutamide-stimulated tumor growth of a novel prostate xenograft model derived from a bicalutamide-treated patient.[5] It appears that aberrant AR activation due to AR mutations is an important mechanism that accounts for development of the resistance to the current clinically used antiandrogens. The pan-antagonistic property of compound **6** against multiple AR mutants (Figure 2) is of clinically significance as it would be harder for prostate tumor to acquire resistance to an antiandrogen effective against multiple mutated ARs. To date, reports of antiandrogens capable of antagonizing multiple mutant ARs are limited. McGinley et al. reported identification of bicalutamide derivatives that show potent antiandrogenic activity against the WT and the W741L and T877A mutated ARs.[13] We have recently identified two novel chalcones as potent pan-antiandrogens.[14,15]

Both LNCaP and MDA PCa 2b cell lines are androgen-dependent and express functional mutated ARs, with the T877A mutated AR in LNCaP cells and the T877A and L701H double mutated AR in PCa 2b cells. Both C4-2B and PC-3 cells are androgen-independent, but C4-2B cells express T877A mutated AR and PC-3 cells lack endogenous AR. Cellular growth of 22Rv1 cells, which express H874Y mutated AR, are stimulated by DHT and EGF. Therefore, these five prostate cancer cell lines constitute a panel of diverse cellular models for prostate cancer. Significantly, compound **6** shows low micromolar cytotoxicity in LNCaP, PCa 2b, 22Rv1, C4-2B, and PC-3 (Table 2). The following line of evidence suggests that the activity of compound **6** in AR-positive prostate cell lines is at least in part mediated via AR inhibition: the growth suppression of LNCaP cells by compound **6** at 0.1, 1, and 5 μM was at least partially reversed by increasing the DHT concentration to 1 nM, but at a higher dose of compound **6** (10 μM), the 1 nM DHT can not rescue the growth suppression (Figure S1, SI). In contrast, this DHT rescue of growth suppression by compound **6** was not observed in the AR-negative PC-3 cells (Figure S1, SI).

Molecular modeling suggested possible molecular basis for the pan-antagonistic property of compound **6** (Figure 4, SI Figures S3, S4). Analyses of the crystal structures of a series of mutant AR-LBDs (PDB entries: 1z95, 2ax6, 2q7k, 1gs4, etc.) indicates the backbone conformation of the mutated AR-LBD remains essentially the same with the WT receptor. Consequently, single point mutation can not easily break the hydrogen bonds between **6** and the backbone of the receptor (Figure 4, SI Figure S4) and compound **6** thus remains as an antagonist in the AR mutants (Figure 2). Indeed, the "backbone targeting strategy" has been successfully utilized in the development of inhibitors against HIV protease mutants.[16]

Conclusion

Compound **6** is a novel antiandrogen with two bulky side chains, which is a pure antagonist of the WT and the clinically relevant T877A, W741C, and H874Y mutated ARs and has no cross-reactivity with PR. Molecular modeling indicates the backbone-targeting characteristic and the "Y" shape conformation, bearing two bulky side chains, are important for the pure antagonistic activity of **6** in the multiple AR mutants.

Further, **6** shows low micromolar cytotoxicity in AR-positive LNCaP, PCa 2b, 22Rv1, and C4-2B cells. Its cytotoxicity in AR-negative PC-3 cells suggests **6** is a multitarget agent. The possible additional target(s) of **6** is not clear at this stage, and this is the subject of our further work. Taken together, **6** is a lead compound to be further optimized as a novel antiandrogen for advanced prostate cancer.

Experimental Section

General. All reagents for chemical syntheses were purchased from Sigma-Aldrich (Oakville, ON, Canada). Hydroxyflutamide, bicalutamide and DHT were purchased from Toronto Research Chemicals (North York, ON, Canada). R5020 was purchased from PerkinElmer Inc. (Woodbridge, ON, Canada). All the ^{1}H NMR spectra of compounds **1**−**5** and **7**−**11** were recorded on an Avance Bruker NMR spectrometer operating at 500 MHz on proton. The NMR spectra of compound **6** were recorded on an Avance Bruker NMR spectrometer operating at 600.17 MHz on proton and 150.93 MHz on carbon-13. Mass measurements were performed on a LC-MSD-TOF instrument from Agilent Technologies in positive electrospray mode. Purity was determined by HPLC (Waters Alliance 2695−2996) and purity of compound **1**−**11** was ≥95%.

General Procedure for the Synthesis of Compounds 6−11. Diketone **1** (0.25 mmol, 92 mg) and 4-hydroxy-3-methoxybenzaldehyde (0.3 mmol, 47 mg) were dissolved in 6 mL of methanol. After adding piperidine (25 μL), the mixture was stirred for 48 h at room temperature. The solvent was distilled off, and the crude product was purified by silica gel column chromatography (elutant: EtOAc/hexane, 1:2) to afford compound **6**. Yellow solid (26 mg, 21%). ESI-TOF MS m/z 503.24 $[M + H]^+$. The ^{1}H and ^{13}C NMR analyses for both cis and trans isomers of compound **6** were summarized in Tables S1 and S2 (SI). Compounds **7**−**11** were synthesized by the same procedure for compound **6**.

Biology. Details of antiproliferative assays in five prostate cancer cell lines, DHT rescue of the growth suppression of LNCaP cells by compound **6**, luciferase reporter assays, and AR fluorescence polarization assay are described in the Supporting Information.

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Supporting Information Available: Syntheses of (*E*)-6-(2,6,6-trimethylcyclohex-1-enyl)-hex-5-ene-2,4-dione and diketones **1**−**5**, the predicted binding modes of compounds **6**−**11**, NMR and MS analyses, biological assays details, and molecular modeling methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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