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Brief Article

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Antiandrogenic, Maspin Induction, and Antiprostate Cancer Activities of Tanshinone IIA and Its Novel Derivatives with Modification in Ring A

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Supporting Information

ABSTRACT: Expression of metastatic suppressor maspin is lost in advanced prostate cancer. Clinically relevant mutations in androgen receptor (AR) convert antiandrogens into AR agonists, promoting prostate tumor growth. We discovered tanshinone IIA (TS-IIA) is a potent antagonist of mutated ARs and induces maspin expression through AR. TS-IIA suppressed AR expression and induced apoptosis in LNCaP cells. Syntheses of TS-IIA derivatives (**1**–**9**) revealed that the 4,4-dimethyl group at ring A is important for TS-IIA's antiandrogenic and maspin induction activities.

INTRODUCTION

The growth and survival of prostate cancer cells are critically dependent on androgen receptor (AR) signaling. Current therapeutic strategy for metastatic prostate cancer is to suppress AR signaling by reducing level of androgens (via castration) and disrupting binding of androgens to the AR by antiandrogens such as bicalutamide and flutamide.[1] However, lethal "castration-resistant" prostate cancer (CRPC) arises and eventually causes cancer death. Emerging biological observations in prostate cancer and data from recent clinical trials of novel agents Abiraterone and MDV3100 revealed: (i) most CRPC cells are still dependent on AR signaling for proliferation and survival and (ii) in CRPC cells, AR is activated by multiple mechanisms that can not be effectively suppressed by castration and currently available antiandrogens.[2] In particular, accumulated studies underscore a role of AR mutations in aberrant activation of the AR in CRPC cells. To date, a series of AR mutations, such as T877A, W741C, and H874Y, were identified from tissue specimens of patients with advanced prostate cancer.[3,4] The T877A mutant is paradoxically activated by hydroxyflutamide, an active metabolite of flutamide.[4] The W741C mutant is activated by bicalutamide.[3] Identifying novel antiandrogens that are full antagonists of the wild type and multiple clinically relevant mutated ARs appears to be an attractive strategy for overcoming resistance to current antiandrogens.

Maspin has been shown to be involved in processes that are important to both tumor growth and metastasis such as cell invasion, angiogenesis, and apoptosis. Maspin is an important tumor suppressor and metastasis suppressor in prostate cancer.[5] Normal prostate cells express abundant level of maspin. At the critical transition from noninvasive to invasive prostate cancer, maspin expression was lost.[6] Maspin silencing involves transcriptional regulations and aberrant promoter methylation.[5] In particular, there is a negative hormone response element (HRE) in the maspin promoter that is recognized by AR.[7] Treatment with recombinant maspin inhibits invasion and motibility of LNCaP, DU145, and PC-3 prostate cancer cell lines in vitro.[8] In a rodent model using DU145 engineered to overexpress maspin, Cher et al. showed that maspin overexpression suppressed bone tumor growth and angiogenesis.[9] Thus, it is highly desirable to induce expression of endogenous maspin in prostate cancer cells.

Recently, we have identified several novel synthetic antiandrogens effective against the wild type and mutant ARs.[10] Because natural compounds are the major source for drug discovery, we turned our effort toward exploration of natural products for novel antiandrogens. In the present work, we discovered that tanshinone IIA (TS-IIA), a major chemical constituent of Danshen (roots of *Salvia miltiorrhiza* Bunge), is a potent antiandrogen and induces maspin expression in LNCaP cells. To investigate the structure–activity relationship, we have synthesized a series of derivatives of TS-IIA and found the 4,4-dimethyl group at ring A is important for antiandrogenic and maspin induction activities of TS-IIA.

RESULTS

Chemistry. The LNCaP prostate cancer cells are androgen-dependent and express functional AR that harbors the T877A mutation. We have screened ethanol extracts of a series of medicinal herbs by MTT assays in LNCaP cells. Ethanol extract of Danshen (roots of *S. miltiorrhiza*) showed potent antiproliferative activity against LNCaP cells (result not

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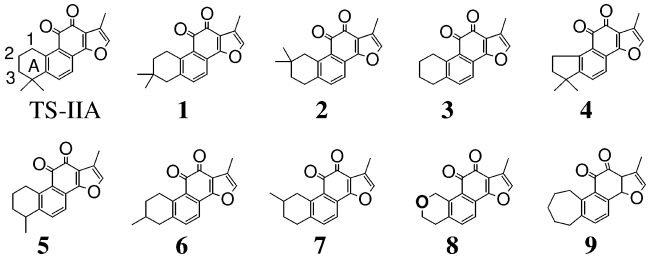
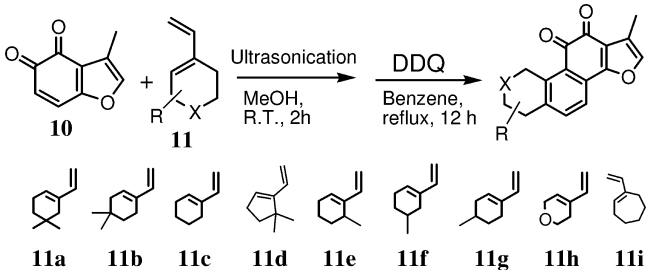
shown). By bioactivity-directed fractionations, from Danshen ethanol extract we have isolated one compound, which was identified as TS-IIA by comparison of NMR spectral data with published values (Table S1, Supporting Information (SI)).[11] After we discovered TS-IIA is a potent antiandrogen (see next section for details), we decided to explore structure−activity relationship with a view to optimizing the potency. As shown in Scheme 1, we have synthesized nine derivatives with

Scheme 1. TS-IIA and Its Derivatives with Modifications in Ring A



modification at ring A (**1**−**9**). The general procedure for the syntheses of compounds **1**−**9** was outlined in Scheme 2.

Scheme 2. Synthesis of TS-IIA Derivatives 1−9, Using Intermediates 11a−11i, Where R = H, CH_3, or two CH_3 and X = C or O



Intermediate **10** was synthesized according to literature (SI).[12] Vinylcyclohexene **11a** was synthesized according to Scheme S1 (SI), and **11b**, **11e**, **11f**, and **11g** were similarly prepared (SI).[12] Intermediates **11c**, **11d**, **11h**, and **11i** were synthesized according to Scheme S2 (SI).[12] Reaction of **10** with **11a** according to Scheme 2 offered **1**.

Biology. *TS-IIA Is a Potent Full Antagonist of the Wild Type and the T877A and W741C Mutant ARs.* To evaluate antiandrogenic activity of TS-IIA, we performed luciferase reporter assays by transfecting the wild type or the mutated full-length AR plasmid into PC-3 cells. TS-IIA dose-dependently suppressed DHT-induced transactivation of the wild type and the T877A and W741C mutated ARs, but it is almost inactive against the H874Y mutant (Figure 1). Importantly, tanshinone was a nonagonist in the wild type and the T877A, W741C, and H874Y mutants (white blocks, Figure 1). In contrast, bicalutamide significantly activated the W741C mutated AR in the absence of DHT, suggesting bicalutamide is an agonist of the W741C mutant (white block, Figure 1c). This is consistent with a previous finding.[3] Bicalutamide together with 0.1 nM DHT induced transactivation of the W741C mutant to a greater extent than 0.1 nM DHT alone (Figure 1c).

TS-IIA at 1 μM Has No Effect on Transactivation of AR-v7, PR, and GR. To test whether antiandrogenic activity of TS-IIA

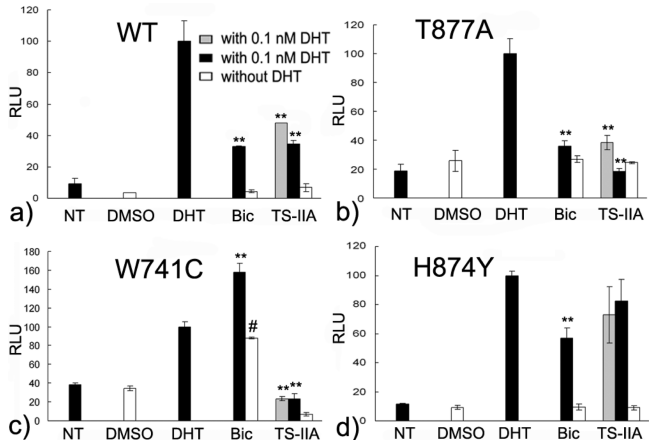


Figure 1. Effect of TS-IIA at 0.1 μM (gray bars) and 1 μM (black and white bars), and Bic at 1 μM (black and white bars) on DHT-induced transactivation of the wild type and the T877A, W741C, and H874Y mutated ARs. Experiments were in triplicate. $^{**}P < 0.005$ when compared with 0.1 nM DHT. $\#p < 0.0005$ when compared with DMSO vehicle. NT, cells were transfected with MMTV-luc and Renilla null luciferase plasmids and treated with 0.1 nM DHT. RLU, relative luciferase units.

is dependent on the AR ligand-binding domain (LBD), we evaluated effect of TS-IIA on the transactivation of an AR splice variant called AR-v7, which lacks the LBD (Figure S1, SI).[13] AR-v7, MMTV-Luc, and pRL-TK plasmids were transiently transfected into PC-3 cells. Transfected cells were treated with DMSO vehicle, 0.1 nM DHT, bicalutamide, and TS-IIA at 1 μM, respectively. As expected, AR-v7 was constitutively activated and this was not affected by the LBD-targeting DHT and bicalutamide (Figure S1, SI). We found TS-IIA at 1 μM has no effect on transactivation of AR-v7, indicating its antiandrogenic activity against full-length AR is dependent on the AR LBD.

To be clinically useful, an antiandrogen should not interfere with signaling of other steroid receptors such as glucocorticoid receptor (GR) and progesterone receptor (PR). For example, a recent unsuccessful phase II clinical trial of antiandrogen mifepristone in CRPC patients revealed that inhibition of GR by mifepristone probably limited the efficacy of mifepristone in CRPC via an increase of adrenal androgens production and subsequent conversion to DHT.[14] To evaluate the possible cross-reactivity of TS-IIA with human PR and GR, we have performed luciferase-reporter assay in PC-3 cells. PC-3 cells expressed GR but did not express detectable PR. We therefore transfected PR-expressing plasmid and MMTV-luc reporter into PC-3 cells for PR reporter assays but only transfected the MMTV-luc reporter into PC-3 cells for GR reporter assay. TS-IIA at 1 μM was a nonagonist of PR and GR, and was inactive in suppressing transactivation of the PR and GR induced by 10 nM R5020 and dexamethasone (DEX), respectively (Figure S1, SI).

TS-IIA Induced Maspin Expression and Suppressed PSA Expression in LNCaP Cells. Expression of PSA and maspin are regulated by AR. To further investigate the antagonistic activity of TS-IIA, we evaluated effect of TS-IIA on mRNA expression of PSA and maspin in LNCaP cells by RT-PCR analysis (Figure 2a). TS-IIA dose-dependently suppressed PSA expression and induced maspin expression. In contrast, bicalutamide at 2.5 μM suppressed PSA expression but did not induce maspin expression (Figure 2a). TS-IIA at 2.5 μM has no effect on

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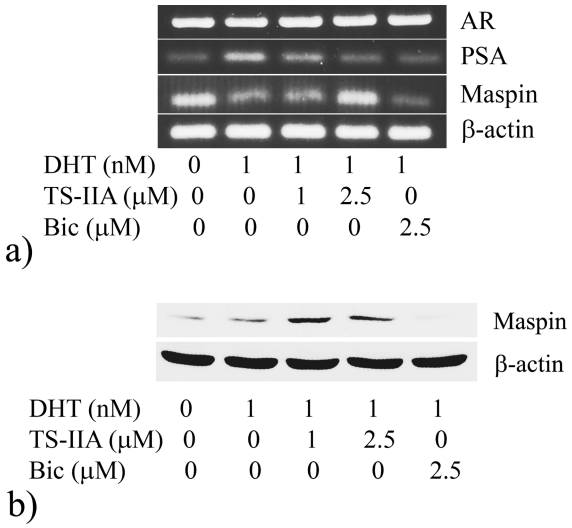

Figure 2. Effects of TS-IIA at 1 and 2.5 μM and Bic at 2.5 μM on (a) mRNA expression of the AR, PSA, and maspin and (b) maspin protein expression. LNCaP cells, cultured in phenol red-free RPMI 1640 with 10% CS-FBS for 48 h and were exposed to DMSO vehicle, 1 nM DHT, and test compounds in the presence of 1 nM DHT for another 24 h. β-action was included to monitor loading. Experiments were repeated twice.

AR expression at transcriptional level. Western blot analysis revealed TS-IIA induced maspin protein expression in LNCaP cells (Figure 2b).

The 4,4-Dimethyl Group at Ring A of TS-IIA Is Important for the Antiandrogenic and Maspin Induction and TS-IIA Induce Maspin Expression through AR. By reporter assays in LNCaP cells, we demonstrated that TS-IIA at 1 and 2.5 μM suppressed 1 nM DHT-induced PSA-luc reporter activity by 10- and 26-fold, respectively. In contrast, the fold of suppression was substantially smaller for compounds **1**−**3** (Figure 3a). In addition, we found TS-IIA significantly induced maspin promoter-dependent reporter activity, but this maspin induction activity was reduced when the 4,4-dimethyl group at ring A was removed or moved to 2- or 3-positions (compare TS-IIA and **1**−**3**, Figure 3b and Scheme 1). These results indicate the 4,4-dimethyl group at ring A is important for TS-IIA's antiandrogenic and maspin induction activities.

To investigate whether TS-IIA induces maspin expression through AR, maspin-luc with and without wild-type AR-expressing plasmids were transiently transfected into PC-3 cells. Transfected cells were exposed to DMSO vehicle, bicalutamide at 5 μM, TS-IIA, and compound **1** at 2.5 and 5 μM. Our study indicated TS-IIA and compound **1** need the presence of AR to exert its maspin induction activity (Figure 3c).

TS-IIA Depleted AR Protein Level and Induced Apoptosis in LNCaP Cells. To determine whether TS-IIA and compound **1** modulate AR protein expression and induce apoptosis, LNCaP cells in complete medium were exposed to DMSO vehicle control, TS-IIA, and compound **1** at 1, 2.5, and 5 μM for 24 h. Western blot analysis indicated TS-IIA and compound **1** potently suppressed AR protein expression in LNCaP cells in a dose-dependent manner (Figure 4a). We found TS-IIA and compound **1** dose-dependently induced caspase-3 activation and specific proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) (Figure 4b). This indicated that TS-IIA and compound **1** induced apoptosis in LNCaP cells. In addition,

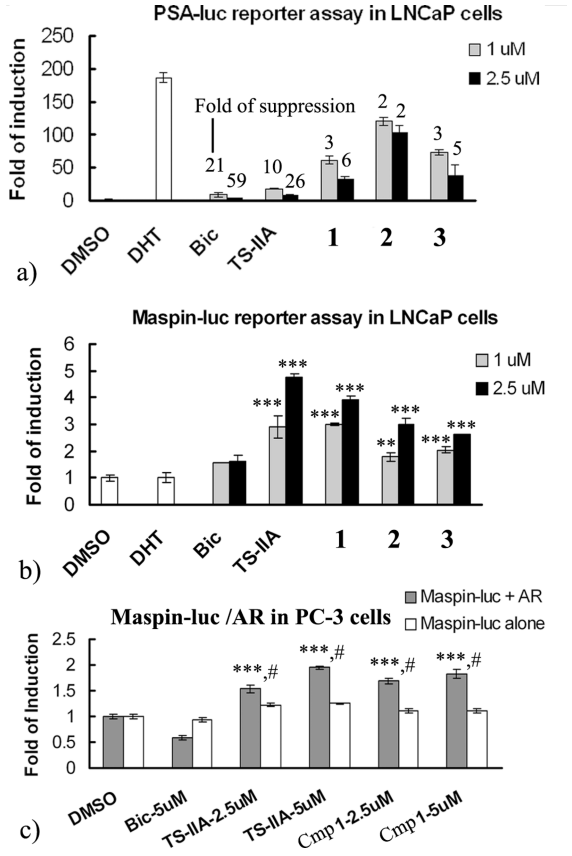

Figure 3. (a) TS-IIA and **1**−**3** dose-dependently suppressed 1 nM DHT-induced AR transactivation in LNCaP cells. Fold of suppression related to 1 nM DHT alone were indicated on top of the blocks. (b) TS-IIA and **1**−**3** dose-dependently induced maspin-luc activity in LNCaP cells. (c) In PC-3 cells, TS-IIA and **1** at 2.5 and 5 uM significantly induce maspin-luc activity only when maspin-luc was cotransfected with AR (gray blocks). ***p < 0.0005 when compared with DMSO vehicle or DHT alone. #p < 0.0005 when compared with white block (without AR) for same dose. Experiments were in triplicate.

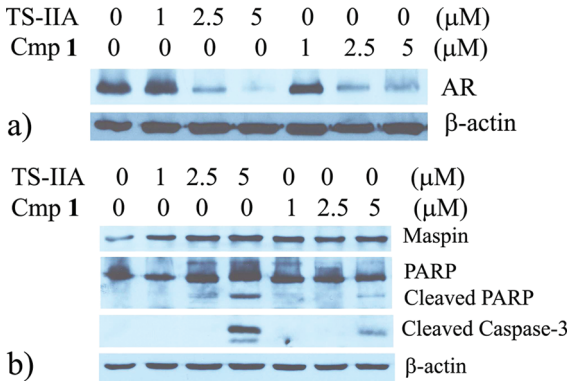

Figure 4. (a) Effect of TS-IIA on AR expression. (b) Effect of TS-IIA on maspin expression and apoptosis-related proteins, in LNCaP cells. LNCaP cells cultured in complete medium were exposed to TS-IIA or compound **1** for 24 h. β-actin was used as loading controls.

TS-IIA and compound **1** induced maspin protein expression in LNCaP cells in complete medium (Figure 4b).

To investigate in vitro antiprostate cancer activity, TS-IIA and its derivatives **1**−**9** were evaluated by MTT assays in a panel of prostate cancer cell lines, including LNCaP, 22Rv1,

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C4-2B, and PC-3 cells (Table 1). TS-IIA and **1**−**9** showed submicromolar or low micromolar cytotoxicity in LNCaP, C4-2B, and PC-3 cells. Expansion of ring A to a seven-membered ring reduced the cytotoxicity in LNCaP cells (compare **3** and **9**, Table 1 and Scheme 1). Reduction of ring A from a six-membered ring to a five-membered ring did not have marked effect on the cytotoxicity (compare **4** and TS-IIA, Table 1). While TS-IIA, **1**, **2**, **4**, and **7** showed modest cytotoxicity in 22Rv1 cells, compound **8** is approximately 4-fold more potent than TS-IIA in 22Rv1 cells.

Table 1. Cytotoxicity of TS-IIA and 1−9 in Prostate Cancer Cell Lines

	cellular IC_{50} (μM)			
compd	LNCaP	22Rv1	C4-2B	PC-3
TS-IIA	0.5	6.7	0.4	3.3
1	0.7	8.8	0.5	0.9
2	3.2	3.2	1.2	3.6
3	0.6	13.5	0.1	1.2
4	1.0	7.5	0.4	2.4
5	1.2	11.8	0.4	1.2
6	3.5	>20	0.7	2.5
7	1.7	8.2	0.3	1.9
8	2.4	1.5	0.1	2.1
9	3.6	>20	1.8	6.3

DISCUSSION

TS-IIA was previously shown to inhibit growth and induce apoptosis of the cancer cells of leukemia, breast cancer, colon adenocarcinoma, hepatocellular carcinoma, and prostate cancer.[15] In this study, we demonstrated that: (i) TS-IIA is a potent antiandrogen effective against the wild type and the T877A and W741C mutants, suppressing PSA expression. The antiandrogenic activity of TS-IIA is mediated by the AR-LBD and TS-IIA has no cross-reactivity with GR and PR. (ii) TS-IIA depleted AR expression and induced apoptosis in LNCaP cells. (iii) TS-IIA dose-dependently induced maspin mRNA and protein expression. (iv) TS-IIA induced maspin expression through AR. (v) Syntheses of TS-IIA derivatives **1**−**9** revealed that 4,4-dimethyl group at ring A is important for the antiandrogenic and maspin induction activities of TS-IIA. (vi) TS-IIA and **1**−**9** showed submicromolar or low micromolar cytotoxicity in LNCaP, C4-2B, and PC-3 cells and modest cytotoxicity in 22Rv1 cells.

Elevated level of AR protein and mutations in AR are two important mechanisms that may account for oncogenic AR reactivation in CRPC cells.[1] The incidence of AR mutation in advanced prostate cancer is estimated to be in the range of 10−40%.[1] The T877A and H874Y mutated ARs were reported to be activated by antiandrogen hydroxyflutamide and a series of other sex steroids.[4] The W741C mutation was derived from liver metastatic tissue of a patient treated with bicalutamide and died of CRPC.[3] The inhibitory activity of TS-IIA against the wild type and the T877A and W741C mutated ARs could be of clinically significant (Figure 1). In addition, TS-IIA at 2.5 μM had little effect on the AR expression at transcriptional level (Figure 2) but substantially reduced expression of AR protein (Figure 4a), suggesting TS-IIA may induce AR degradation.

As a general therapeutic strategy, re-expressing of the endogenous metastatic suppressor maspin in advanced prostate cancer by a chemical agent is appealing. Recent work by Hall et al. indicated that the role of maspin in prostate cancer metastasis may involve inhibiting homing to bone or seeding in a bone microenvironment.[16] TS-IIA potently induced maspin expression in LNCaP cells in a dose-dependent manner (Figures 2 and 4b). It is tempting to speculate that TS-IIA may be of value for inhibiting bone metastasis of prostate cancer. Our preliminary work indicated TS-IIA induces maspin expression through AR (Figure 3c). However, further work is needed to investigate precise mechanism by which TS-IIA induces maspin expression in prostate cancer cells.

PC-3 cells do not express endogenous AR, but TS-IIA and **1**−**9** showed considerable cytotoxicity in PC-3 cells. Antiandrogenic activities of **1** and **3** are weaker than TS-II by 3−5-fold, but they have IC_{50} values in LNCaP cells comparable to that of TS-IIA (Table 1). These results indicated there are additional targets for TS-IIA and its derivatives **1**−**9**.

CONCLUSION

Our study indicated that natural compound TS-IIA is a novel antagonist of the wild type and the T877A and W741C mutated ARs and has no cross-reactivity with human GR and PR. The antiandrogenic activity of TS-IIA is mediated by AR LBD. TS-IIA and **1** dose-dependently deplete AR expression and induce apoptosis in LNCaP cells. TS-IIA is a potent maspin expression inducer, and this induction is mediated by the AR. The 4,4-dimethyl group at ring A is important for the antiandrogenic and maspin induction activities of TS-IIA. The results collectively suggest that TS-IIA is a promising candidate 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). Bicalutamide and DHT were from Toronto Research Chemicals (North York, ON, Canada). R5020 was from PerkinElmer Inc. (Woodbridge, ON, Canada). All ^{1}H NMR spectra of compounds **1**−**9** were recorded on an Avance Bruker NMR spectrometer operating at 500 MHz on proton. The NMR spectra of TS-IIA 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-MS instrument from Agilent technologies in positive electrospray mode. Purity was determined by HPLC (Waters Alliance 2695−2996), and purity of TS-IIA and **1**−**9** were ≥95%.

Extraction and Isolation of TS-IIA. The EtOH extracts of *Salvia miltiorrhiza* Bunge (500 g) were evaporated under reduced pressure. Next, water (2 L) was added to the residues and the solution was extracted with EtOAc three times (300, 200, and 200 mL). The combined EtOAc solution was evaporated, affording 7.2 g of a dark-brown residue, which was subjected to a flash column chromatography (43 mm × 380 mm, 200−425 mesh, Silica Gel 320 g) and successively eluted with hexane−acetone gradient (25−55%). TS-IIA was obtained in crystals from the second fraction. The structure of TS-IIA was confirmed by ^{1}H, ^{13}C, HMBC, and NOESY NMR analyses (Table S1, SI). ESI-TOF-MS, m/z: 295.20 $[M + H]^+$.

General Procedure for the Syntheses of 1−9. A mixture of **10** (55 mg, 0.338 mmol) and **11a** (90 mg, 0.66 mmol) in anhydrous MeOH (0.30 mL) was subjected to ultrasonication for 2 h. DDQ (70 mg) and benzene (30 mL) were added to the mixture. The solution was kept refluxing for 12 h. The solvent was distilled off under reduced pressure. The residue was purified through chromatography on silica gel (eluent: Hex:EA = 10:1) to give **1** (red solid, 18 mg, 15% in yield). ^{1}H NMR (500 MHz, CD_3COCD_3): δ 7.61 (d, J = 8.0 Hz, 1H), 7.59 (s, 1H), 7.47 (d, J = 8.0 Hz, 1H), 3.07 (t, J = 6.5 Hz, 2H), 2.51 (s, 2H), 2.09 (s, 2H), 1.49 (t, J = 6.5 Hz, 2H), 0.87 (s, 6H). HRMS-ESI

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calcd for $[M + Na]^+$ 317.11482; found 317.11606. Compounds **2**–**9** were similarly prepared.

Biology. Details of reporter assays, RT-PCR, Western blot, and MTT assays were described in the SI. The *t* test was performed using GraphPad Prism 5 software (San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR analysis of TS-IIA, syntheses, NMR, and MS analyses of **1**–**10** and **11a**–**11i** as well as AR, AR-v7, GR, PR, and maspin-dependent reporter assays, RT-PCR and Western blot analyses, and MTT assays of TS-IIA and its derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

AR, androgen receptor; Bic, bicalutamide; CRPC, castration-resistant prostate cancer; CS-FBS, charcoal-stripped fetal bovine serum; DEX, dexamethasone; DHT, dihydrotestosterone; GR, glucocorticoid receptor; HRE, hormone responsive element; LBD, ligand-binding domain; PR, progesterone receptor; PSA, prostate-specific antigen; TS-IIA, tanshinone IIA

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