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Pharmacological Exploitation of the Peroxisome Proliferator-Activated Receptor γ Agonist Ciglitazone to Develop a Novel Class of Androgen Receptor-Ablative Agents

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Abstract

Based on our finding that the peroxisome proliferator-activated receptor (PPAR) γ agonist ciglitazone at high doses was able to mediate PPAR γ -independent transcriptional repression of androgen receptor (AR) in a tumor cell-specific manner, we used Δ 2CG, a PPAR γ -inactive analogue of ciglitazone, to conduct lead optimization to develop a novel class of AR-ablative agents. Structure-activity analysis indicates a high degree of flexibility in realigning Δ 2CG's structural moieties without compromising potency in AR repression, as evidenced by the higher AR-ablative activity of the permuted isomer **9** [(Z)-5-(4-hydroxy-benzylidene)-3-(1-methyl-cyclohexylmethyl)-thiazolidine-2,4-dione]. Further modifications of **9** gave rise to **12** [(Z)-5-(4-hydroxy-3-trifluoromethyl-benzylidene)-3-(1-methyl-cyclohexylmethyl)-thiazolidine-2,4-dione] which completely inhibited AR expression in LNCaP cells at low μ M concentrations. This AR downregulation led to growth inhibition in LNCaP cells through apoptosis induction. Moreover, the role of AR repression in the antiproliferative effect of compound **12** was validated by the differential inhibition of cell viability between androgen-responsive and androgen-nonresponsive cells.

Introduction

Mounting evidence indicates that dysregulation of androgen receptor (AR) through gene amplification or mutations plays a key role in the development of androgen-refractory prostate cancer^{1–8}, a hallmark of incurable and lethal prostate cancer progression. These molecular changes enhance AR sensitivity or permit AR activation by antiestrogen, thus allowing prostate cancer cells to become resistant to androgen ablation-induced apoptosis^{9, 10}. From a clinical perspective, targeting AR expression represents an important strategy to improve the treatment of androgen-independent prostate cancer and ultimately to increase the survival of prostate cancer patients. A recent study indicates that knocking down the AR protein level by a small interfering RNA (siRNA) resulted in significant apoptotic cell death in LNCaP androgen-responsive prostate cancer cells, but not in the AR-null PC-3 cells¹¹. Moreover, in a LNCaP tumor xenograft model, short hairpin RNA (shRNA)-mediated AR knockdown was effective in blocking tumor growth and delaying tumor progression¹², which provides a proof-of-principle of this AR-targeted therapy.

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Supporting Information Available. Elemental analysis data are available free of charge via the Internet at <http://pubs.acs.org>.

Although a number of natural product-based, small-molecule agents exhibit the ability to suppress AR expression, including resveratrol¹³, vitamin E succinate¹⁴, genistein¹⁵, and curcumin⁶, their therapeutic use in humans is limited by unattainable therapeutic concentrations as a result of low potency. Thus, it is of urgency to develop potent AR-ablative agents in the pursuit of new strategies for prostate cancer treatment. During the course of our investigation of the effect of the thiazolidinedione family of peroxisome proliferator-activated receptor (PPAR) γ agonists on repressing prostate specific antigen (PSA)¹⁶, we demonstrated that troglitazone and ciglitazone at high doses mediated PPAR γ -independent transcriptional repression of AR in a tumor cell-specific manner¹⁷. This PPAR γ -independent suppression of AR expression might, in part, underlie the antiproliferative activity of troglitazone in prostate cancer cells, and is of translational value to the development of troglitazone and ciglitazone into potent AR-ablative agents. Our early optimization of troglitazone has generated STG28 (Fig. 1A) with moderate potency in suppressing AR expression in LNCaP prostate cancer cells¹⁷. In this study, the lead optimization of ciglitazone gave rise to compound **12**, which exhibited the ability to mediate AR ablation at low μ M concentrations.

Chemistry

The lead optimization of ciglitazone to develop compound **12** consisted of three stages (Fig. 1B). Stage 1 was to abrogate ciglitazone's PPAR γ agonist activity by introducing a double bond adjoining the terminal thiazolidinedione ring, leading to the PPAR γ inactive analogue Δ 2CG¹⁶. Stage 2 was to structurally modify Δ 2CG via three distinct strategies: (a) regioisomerization of the (1-methylcyclohexyl)-methyl moiety to yield compound **1**, (b) phenyl ring substitutions to give compounds **2** – **8**, and (c) permutational rearrangement of the terminal cyclohexyl moiety to generate compound **9**. In Stage 3, compound **9** underwent modifications at the terminal phenyl ring, generating two series of compounds, i.e., **10** and **11**, and **12** – **19**. These Δ 2CG derivatives were synthesized according to general procedures described in Fig. 1C, and their ability to suppress AR expression in LNCaP cells was assessed by the AR promoter-luciferase reporter gene assay followed by Western blot analysis.

Results

Dissociation of the PPAR γ activity does not affect the ability of Δ 2CG to inhibit AR expression at both mRNA and protein levels in LNCaP cells

Dose- and/or time-dependent effects of ciglitazone and Δ 2CG on suppressing AR expression were assessed in LNCaP cells in 10% fetal bovine serum (FBS) by Western blotting and reverse transcriptase-polymerase chain reaction (RT-PCR). These analyses indicate that Δ 2CG, albeit lacking PPAR γ agonist activity, exhibited modestly higher potency than ciglitazone in mediating transcriptional repression of AR. For example, the concentrations required for complete suppression of AR protein expression were approximately 30 μ M and 60 μ M for Δ 2CG and ciglitazone, respectively (Fig. 2A).

Furthermore, RT-PCR analysis indicates that the downregulation of AR expression occurred at the transcriptional level (Fig. 2B). Together, these findings confirmed the ability of ciglitazone to ablate AR independently of PPAR γ activation, which provided a molecular rationale to use Δ 2CG as a starting point for lead optimization to generate potent AR-ablative agents. To expedite the screening of AR-ablative agents, we used a luciferase reporter assay¹⁷ to analyze the effect of individual derivatives on suppressing AR transcription by using LNCaP cells transiently transfected with the AR promoter-linked luciferase reporter plasmid.

Lead optimization of $\Delta 2\text{CG}$

As aforementioned, $\Delta 2\text{CG}$ underwent three types of structural modifications, leading to compounds **1** – **9**. Individual derivatives at 10 μM , compared to ciglitazone and $\Delta 2\text{CG}$, each at 20 μM , were evaluated in the luciferase reporter assay in the transiently transfected LNCaP cells. Relative to $\Delta 2\text{CG}$, these derivatives showed improved potency in suppressing the activity of the AR promoter, suggesting that a high degree of flexibility existed in the structure-activity relationship. This premise was borne out by the regioisomer **1** and the permuted isomer **9**, both of which showed enhanced AR-ablating activity despite substantial configuration changes (Fig. 3A, left panel). Moreover, examination of the IC_{50} values of individual derivatives in suppressing the viability of LNCaP cells after 48 h of treatment indicates a positive correlation between the ability to suppress AR mRNA transcription and that of inhibiting cell viability (Fig. 3A).

Of these derivatives, we chose compounds **1**, **6**, and **9** as representatives to conduct Western blot analysis. As shown in Fig. 3B, these three derivatives showed a dose-dependent effect on suppressing AR protein expression (Fig. 3B). In light of the unique structural feature of compound **9**, we used this permuted derivative to carry out further structural optimization, generating compounds **10** – **19**. The luciferase reporter analysis and cell viability assay indicated a subtle structure-activity relationship among these derivatives (Fig. 4A).

For example, moving the terminal *para*-OH function to the *ortho* or *meta* position (compounds **10** and **11**) abolished the ability to suppress AR promoter-luciferase activity and cell viability, indicating its important role in interacting with the target protein. Moreover, substitutions of the phenyl ring with CF_3 or Br led to substantially higher potency in AR repression, while those with NO_2 or electron-donating groups attenuated the activity (Fig. 4A). Of these derivatives, compounds **12** and **16** represented the optimal agents in inhibiting AR mRNA transcription and LNCaP cell viability.

To demonstrate that this drug-induced transcriptional repression of AR was independent of $\text{PPAR}\gamma$, we examined the ability of compounds **12** and **16** versus troglitazone, ciglitazone, and $\Delta 2\text{CG}$ to transactivate $\text{PPAR}\gamma$ by using the PPAR response element (PPRE)-luciferase reporter assay. In PC-3 cells transfected with a reporter construct (PPRE-x3-TK-Luc), troglitazone and ciglitazone at 10 μM exhibited a significant effect on increasing luciferase activity, ranging from 2.5-fold to 4-fold ($P < 0.05$). In contrast, compounds **12** and **16**, like their parent compounds $\Delta 2\text{CG}$, lacked appreciable activity in $\text{PPAR}\gamma$ activation.

Western blot analysis indicates that the IC_{50} values for suppressing AR expression by compounds **12** and **16** after 72-h exposure were approximately 2 μM and 4 μM , respectively (Fig. 4C). The ability of compound **12** to suppress AR expression was further demonstrated by immunocytochemical analysis (Fig. 4D). As shown, exposure of LNCaP cells to 5 μM compound **12** for 48 h led to a substantial decrease in AR levels in the nucleus.

Antitumor effects of compound **12** in prostate cancer cells

We assessed the antitumor effects of compound **12** in both LNCaP androgen-responsive and PC-3 androgen-nonresponsive prostate cancer cells via three different methods, including the MTT assay for cell viability, cell counting for cell proliferation, and flow cytometric analysis for cell cycle distribution. Due to lack of AR expression, PC-3 cells exhibited substantially lower sensitivity to the antiproliferative activities of compound **12** as compared to LNCaP cells. The IC_{50} values for suppressing cell viability were 8 μM and 3 μM , at 48 h and 72 h of drug treatment, respectively, in LNCaP cells, and 15 μM and 12 μM , respectively, in PC-3 cells (Fig. 5A).

This differential susceptibility was also manifest in the cell counting assay, in which compound **12** exhibited at least twofold higher potency in inhibiting the proliferation of LNCaP cells as compared to PC-3 cells (Fig. 5B). Moreover, cell cycle analysis was carried out after exposing LNCaP cells to different doses of compound **12** for 72 h (Fig. 5C). As shown, compound **12** caused a dose-dependent increase in the sub-G₁ population, accompanied by decreases in the G₂/M phase (Fig. 5C). Furthermore, the ability of compounds **12** and **16** to induce apoptotic death in LNCaP cells was demonstrated by their dose-dependent effects on modulating various apoptosis-related biomarkers, including PARP cleavage, and the proteolytic activation of caspase 3 and caspase 7 (Fig. 5D).

Our earlier study indicates that thiazolidinediones mediated the transcriptional repression of AR by facilitating the degradation of the transcription factor Sp1¹⁷. However, it was reported that compounds containing a 5-arylidene-3-aryl-2,4-thiazolidinedione substructure underwent conjugation addition with *p*-thiocresol in the presence of piperidine upon heating¹⁸. This report raised a possibility that compound **12** and other derivatives might act as “Michael acceptors” by covalently modify the target enzyme/protein upon binding. Here, we obtained two lines of evidence to refute this possibility. First, compound **20**, a saturated counterpart of compound **12**, retained the ability to suppress AR expression and cell viability, though with slightly lower potency, in LNCaP cells (Fig. 6A and B). Second, the expression level of AR in drug-treated LNCaP cells would be rapidly restored once compound **12** was removed (or washed out) from the medium (Fig. 6C). This rapid restoration of the AR expression suggests a reversible nature of this ligand-protein interaction.

Discussion

In light of the pivotal role of PPAR γ in prostate cell proliferation and differentiation, the chemopreventive activity of thiazolidinediones in prostate cancer has been attributed to their ability to activate PPAR γ signaling, leading to the terminal differentiation and growth arrest of tumor cells^{19–21}. However, mounting evidence suggests that the antiproliferative ability of these agents is independent of their PPAR γ agonist activity^{16, 17, 22–31}. In our laboratory, we have identified several “off-target” mechanisms that might underlie the antitumor effects of thiazolidinediones, including Bcl-2/Bcl-xL inhibition, proteasomal degradation of cyclin D1²⁵, β -catenin³², and Sp1¹⁷, and transcriptional repression of PSA¹⁶ and AR¹⁷. Separation of these pharmacological effects from PPAR γ activation provides a mechanistic rationale for using thiazolidinediones as a scaffold to develop potent molecularly targeted agents. Considering the importance of AR in prostate tumorigenesis and tumor progression, we carried out lead optimization of ciglitazone and its PPAR γ -inactive derivative Δ 2CG to develop potent AR-ablative agents.

There existed a high degree of tolerance for the substructural rearrangement of Δ 2CG without compromising the AR-ablative activity, as evidenced by the improved potency of compounds **1** and **9**. In contrast, modifications of the phenyl ring exhibited a subtle effect on the AR-ablative potency. For example, changing the orientation of the terminal hydroxyl function of compound **9** completely abrogated the ability of the resulting compounds **10** and **11** to suppress AR expression, while the CF₃- or di-Br-substitution led to enhanced potency. Together, these findings suggest that the benzylidene-thiazolidinedione substructure played a crucial role in interacting with the target protein.

Among all derivatives examined, compound **12** represented a structurally optimized derivative with an order-of-magnitude higher potency than ciglitazone in suppressing AR expression. This AR downregulation led to growth inhibition in LNCaP cells through apoptosis induction, as evidenced by flow cytometry, PARP cleavage and caspase

activation. The role of AR repression in the antiproliferative effect of compound **12** was supported by the differential inhibition of cell viability between LNCaP androgen-responsive and PC-3 androgen-nonresponsive cells. Because thiazolidinediones mediate AR repression through downregulation of Sp1¹⁷, compound **12** also suppresses the transcription of many Sp1-targeted genes (data not shown), which accounts for the ability of compound **12** to inhibit PC-3 cell viability.

Relative to many natural product-based agents that suppress AR expression/function, such as resveratrol¹³, vitamin E succinate¹⁴, genistein¹⁵, and curcumin⁶, compound **12** is substantially more effective in downregulating AR expression. Thus, this AR-ablative agent has translational potential to foster new therapeutic strategies for prostate cancer treatment as a single agent or in combination with other molecularly targeted agents.

Conclusion

The *in vivo* efficacy of targeting AR expression to block tumor growth and delaying tumor progression has recently been demonstrated in a LNCaP tumor xenograft model by using shRNA-mediated AR knockdown¹². This finding provides a proof-of-principle that inhibition of AR expression represents a therapeutically relevant strategy for prostate cancer treatment. Relative to shRNAs, small-molecule agents prove to be advantageous in many aspects of cancer therapy. Consequently, *in vivo* testing of compound **12** to suppress prostate tumor growth is currently underway.

Experimental Section

Chemical reagents and organic solvents were purchased from Sigma-Aldrich unless otherwise mentioned. Nuclear magnetic resonance spectra (¹H NMR) were measured on a Bruker DPX 300 model spectrometer. Chemical shifts (δ) were reported in parts per million (ppm) relative to the TMS peak. Electrospray ionization mass spectrometry analyses were performed with a Micromass Q-ToF II High-Resolution electrospray mass spectrometer. Elemental analyses were performed by the Atlantic Microlab, Inc. (Norcross, GA), and were reported within 0.4% of calculated values. Flash column chromatography was performed with silica gel (230–400 mesh). Δ 2CG and the two series of compounds, **1** – **8** and **9** – **19**, were synthesized according to the general methods described in Figure 1B, which are illustrated by the synthesis of compounds **1** and **9** as examples.

(Z)-5-[3-(1-Methyl-cyclohexylmethoxy)-benzylidene]-thiazolidine-2,4-dione (**1**)

Step a: To a stirring solution of LiAlH₄ (20 mmol) in anhydrous THF (10 mL) at 4°C was added 1-methyl-cyclohexanecarboxylic acid (**i**, 7.0 mmol) in 50 mL of THF dropwise over a period of 1 hour. The solution was stirred at refluxing temperature under N₂ for 6 hours. The solution was cooled to 4°C by ice bath, and 1 mL of 1 N NaOH (1 mL) followed by H₂O (2 mL) was slowly added to the solution to quench the reaction. The solution was stirred at 23 °C for 1 hour and then filtered to remove solid material. The solution was concentrated. Purification by flash silica gel chromatography (ethyl acetate/hexanes, 1:2) gave the product, (1-methyl-cyclohexyl)-methanol (**ii**), in 82% yield.

Step b: A solution of compound **ii** (1 mmol) in dry CH₂Cl₂ (5 mL) was cooled to 4°C, to which was added pyridine (1.1 mmol) and triflate anhydride (1.1 mmol). After stirring at 4°C for 2 h, the solution was concentrated, and the residue was purified by flash silica gel column chromatography (ethyl acetate/hexanes, 1:10) to afford trifluoro-methanesulfonic acid 1-methyl-cyclohexylmethyl ester (**iii**) in 35% yield.

Step c: A mixture of compound **iii** (0.5 mmol), 3-hydroxy-benzaldehyde (**iv**, 0.6 mmol) and K_2CO_3 (0.7 mmol) were dissolved in DMF (3 mL). The solution was heated to 80°C for 4 hr. The solution was poured into water, extracted with ethyl acetate (10 mL) three times, and concentrated. The residue was purified by chromatography and resulted in 0.22 mmol of 3-(1-methyl-cyclohexylmethoxy)-benzaldehyde (**v**) with a 44% yield.

Step d: A mixture consisting of compound **v** (0.5 mmol), 2, 4-thiazolidinedione (0.6 mmol) and catalytic amounts of piperidine was refluxed in EtOH (5 mL) for 24 h and then concentrated. The oily product was dissolved in ethyl acetate, poured into water and acidified with AcOH. The solution was extracted with ethyl acetate, dried and concentrated. The residue was purified by silica gel chromatography, providing compound **1** in 67% yield. 1H NMR (300 MHz, $CDCl_3$) δ 1.04 (s, 3H), 1.46–1.56 (m, 10H), 3.69 (s, 2H), 6.78–7.28 (m, 2H), 7.08 (d, $J = 8.40$ Hz, 1H), 7.39 (dt, $J = 2.10, 8.40$ Hz, 1H), 7.84 (s, 1H), 8.21–8.78 (br, 1H); HRMS exact mass of $(M + Na)^+$, 354.1140 amu; observed mass of $(M + Na)^+$, 354.113 amu.

(Z)-5-[3-Bromo-4-(1-methyl-cyclohexylmethoxy)-benzylidene]-thiazolidine-2,4-dione (2)— 1H NMR (300 MHz, $CDCl_3$) δ 1.11 (s, 3H), 1.40–1.61 (m, 10H), 3.77 (s, 2H), 6.95 (d, $J = 8.42$ Hz, 1H), 7.41 (dd, $J = 2.10, 8.42$ Hz, 1H), 7.69 (d, 1H, $J = 2.10$), 7.74 (s, 1H), 8.38 (s, 1H); HRMS exact mass of $(M + Na)^+$, 432.0245 amu; observed mass of $(M + Na)^+$, 432.0247 amu.

(Z)-5-[4-(1-Methyl-cyclohexylmethoxy)-3-nitro-benzylidene]-thiazolidine-2,4-dione (3)— 1H NMR (300 MHz, $CDCl_3$) δ 1.08 (s, 3H), 1.42–1.59 (m, 10H), 3.79 (s, 2H), 7.23 (d, $J = 8.40$ Hz, 1H), 7.85 (d, $J = 8.42$ Hz, 1H), 7.92 (s, 1H), 8.10 (s, 1H), 8.33 (s, 1H); HRMS exact mass of $(M + Na)^+$, 399.0991 amu; observed mass of $(M + Na)^+$, 399.0995 amu.

(Z)-5-[4-(1-Methyl-cyclohexylmethoxy)-3-trifluoromethyl-benzylidene]-thiazolidine-2,4-dione (4)— 1H NMR (300 MHz, $CDCl_3$) δ 1.08 (s, 3H), 1.42–1.59 (m, 10H), 3.79 (s, 2H), 7.10 (d, $J = 8.40$ Hz, 1H), 7.63 (d, $J = 8.42$ Hz, 1H), 7.71 (s, 1H), 7.80 (s, 1H), 8.09–8.12 (br, 1H), HRMS exact mass of $(M + Na)^+$, 422.1014 amu; observed mass of $(M + Na)^+$, 422.1019 amu.

(Z)-5-[3-Methoxy-4-(1-methyl-cyclohexylmethoxy)-benzylidene]-thiazolidine-2,4-dione (5)— 1H NMR (300 MHz, $CDCl_3$) δ 1.09 (s, 3H), 1.40–1.58 (m, 10H), 3.75 (s, 2H), 3.96 (s, 3H), 6.96 (d, $J = 8.40$ Hz, 1H), 7.00 (s, 1H), 7.11 (d, $J = 8.42$ Hz, 1H), 7.79 (s, 1H), 8.55 (s, 1H); HRMS exact mass of $(M + Na)^+$, 384.1245 amu; observed mass of $(M + Na)^+$, 384.1239 amu.

(Z)-5-[3-Ethoxy-4-(1-methyl-cyclohexylmethoxy)-benzylidene]-thiazolidine-2,4-dione (6)— 1H NMR (300 MHz, $CDCl_3$) δ 1.08 (s, 3H), 1.40–1.58 (m, 13H), 3.74 (s, 2H), 4.10 (q, $J = 6.9$ Hz, 2H), 6.95 (d, $J = 8.40$ Hz, 1H), 7.01 (d, $J = 2.10$ Hz, 1H), 7.11 (dd, $J = 8.40, 2.10$ Hz, 1H), 7.79 (s, 1H), 8.42 (s, 1H); HRMS exact mass of $(M + Na)^+$, 389.1402 amu; observed mass of $(M + Na)^+$, 389.1402 amu.

(Z)-5-[3,5-Dimethyl-4-(1-methyl-cyclohexylmethoxy)-benzylidene]-thiazolidine-2,4-dione (7)— 1H NMR (300 MHz, $CDCl_3$) δ 1.13 (s, 3H), 1.32–1.59 (m, 10H), 2.42 (s, 6H), 3.48 (s, 2H), 7.17 (s, 2H), 7.76 (s, 1H), 8.26 (s, 1H); HRMS exact mass of $(M + Na)^+$, 382.1453 amu; observed mass of $(M + Na)^+$, 382.1448 amu.

(Z)-5-[4-(1-Methyl-cyclohexylmethoxy)-naphthalen-1-ylmethylene]-thiazolidine-2,4-dione (8)—¹H NMR (300 MHz, CDCl₃) δ 1.18 (s, 3H), 1.51–1.59 (m, 10H), 3.91 (s, 2H), 6.915 (d, *J* = 8.70 Hz, 1H), 7.55–7.69 (m, 3H), 8.12 (d, *J* = 8.70, 1H), 8.39 (d, *J* = 8.40, 1H), 8.59 (s, 1H); HRMS exact mass of (M + Na)⁺, 404.1296 amu; observed mass of (M + Na)⁺, 404.1299 amu.

(Z)-5-(4-Hydroxy-benzylidene)-3-(1-methyl-cyclohexylmethyl)-thiazolidine-2,4-dione (9)

Step e: A mixture of p-hydroxybenzaldehyde (**vi**; 0.5 mmol), 2,4-thiazolidinedione, (0.6 mmol), and catalytic amounts of piperidine and AcOH was refluxed in toluene (5 mL) for 24 h. The precipitated product was filtered, washed with toluene (3 × 10 mL), and dried *in vacuo* at 60°C overnight, yielding 5-(4-hydroxybenzylidene)-thiazolidine-2,4-dione (**vii**) in a 85% yield.

Step f: A solution of compound **vii** (0.5 mmol), compound **iii** (0.6 mmol) and K₂CO₃ (0.65 mmol) were stirred in DMF (3 mL) at 80°C for 4 hr, poured into water, extracted with ethyl acetate (3 × 10 mL), dried and concentrated. The residue was purified by chromatography, affording compound **9** in 42% yield. ¹H NMR (300 MHz, CDCl₃) δ 0.94 (s, 3H), 1.14–1.86 (m, 10H), 3.63 (s, 2H), 5.69 (s, 1H), 6.94(d, *J* = 8.40 Hz, 2H), 7.43(d, *J* = 8.40 Hz, 2H), 7.83 (s, 1H); HRMS exact mass of (M + Na)⁺, 354.1140 amu; observed mass of (M + Na)⁺, 354.1141 amu.

(Z)-5-(3-Hydroxy-benzylidene)-3-(1-methyl-cyclohexylmethyl)-thiazolidine-2,4-dione (10)—¹H NMR (300 MHz, CDCl₃) δ 0.96 (s, 3H), 1.24–1.67 (m, 10H), 3.65 (s, 2H), 5.24 (s, 1H), 6.70 (d, *J* = 1.5 Hz, 1H), 6.93(dd, *J* = 8.10, 1.5 Hz, 1H), 7.10(d, *J* = 7.80 Hz, 1H), 7.36 (dd, *J* = 7.80, 7.50 Hz, 1H), 7.84 (s, 1H); HRMS exact mass of (M + Na)⁺, 354.1140 amu; observed mass of (M + Na)⁺, 354.1143 amu.

(Z)-5-(2-Hydroxy-benzylidene)-3-(1-methyl-cyclohexylmethyl)-thiazolidine-2,4-dione (11)—¹H NMR (300 MHz, CDCl₃) δ 0.95 (s, 3H), 1.22–1.65 (m, 10H), 3.66 (s, 2H), 6.44 (d, *J* = 0.9 Hz, 1H), 6.91 (dd, *J* = 8.10, 0.9 Hz, 1H), 7.04 (td, *J* = 7.2, 0.6 Hz, 1H), 7.32 (tdd, *J* = 7.5, 1.5, 0.6Hz, 1H), 7.46 (dd, *J* = 7.80, 1.5Hz, 1H), 8.42 (s, 1H); HRMS exact mass of (M + Na)⁺, 354.1140 amu; observed mass of (M + Na)⁺, 354.1145 amu.

(Z)-5-(4-Hydroxy-3-trifluoromethyl-benzylidene)-3-(1-methyl-cyclohexylmethyl)-thiazolidine-2,4-dione (12)—¹H NMR (300 MHz, CDCl₃) δ 0.95 (s, 3H), 1.46–1.56 (m, 10H), 3.64 (s, 2H), 6.08–6.38 (br, 1H), 7.09 (d, *J* = 8.40 Hz, 1H), 7.59 (d, *J* = 8.40 Hz, 1H), 7.69 (s, 1H), 7.83 (s, 1H); HRMS exact mass of (M + Na)⁺, 422.1014 amu; observed mass of (M + Na)⁺, 422.1012 amu. Anal. (C₁₉H₂₀F₃NO₃S) C, H, N, S, O, F.

(Z)-5-(4-Hydroxy-3-nitro-benzylidene)-3-(1-methyl-cyclohexylmethyl)-thiazolidine-2,4-dione (13)—¹H NMR (300 MHz, CDCl₃) δ 0.96 (s, 3H), 1.23–1.57 (m, 10H), 3.68 (s, 2H), 7.31(d, *J* = 8.40 Hz, 1H), 7.74 (dd, *J* = 8.40, 2.1Hz, 1H), 7.81 (s, 1H), 8.29 (d, *J* = 2.1 Hz, 1H); HRMS exact mass of (M + Na)⁺, 399.0991 amu; observed mass of (M + Na)⁺, 399.0991 amu.

(Z)-5-(3-Bromo-4-hydroxy-benzylidene)-3-(1-methyl-cyclohexylmethyl)-thiazolidine-2,4-dione (14)—¹H NMR (300 MHz, CDCl₃) δ 0.79 (s, 3H), 1.17–1.46 (m, 10H), 3.36 (s, 2H), 7.01 (d, *J* = 8.40 Hz, 1H), 7.37 (d, *J* = 8.40 Hz, 1H), 7.73 (s, 2H); HRMS exact mass of (M + Na)⁺, 432.0245 amu; observed mass of (M + Na)⁺, 432.0245 amu. Anal. (C₁₈H₂₀BrNO₃S) C, H, N, O

(Z)-5-(4-Hydroxy-3-methoxy-benzylidene)-3-(1-methyl-cyclohexylmethyl)-thiazolidine-2,4-dione (15)—¹H NMR (300 MHz, CDCl₃) δ 0.92 (s, 3H), 1.21–1.58 (m, 10H), 3.62 (s, 2H), 3.97 (s, 3H), 5.95 (br, 1H), 6.90–7.03 (m, 2H), 7.10 (d, *J* = 7.80 Hz, 1H), 7.82 (s, 1H), HRMS exact mass of (M + Na)⁺, 384.1245 amu; observed mass of (M + Na)⁺, 384.1245 amu. Anal. (C₁₉H₂₃NO₄S) C, H, N, O.

(Z)-5-(3,5-Dibromo-4-hydroxy-benzylidene)-3-(1-methyl-cyclohexylmethyl)-thiazolidine-2,4-dione (16)—¹H NMR (300 MHz, CDCl₃) δ 0.94 (s, 3H), 1.32–1.56 (m, 10H), 3.63 (s, 2H), 6.22 (s, 1H), 7.62 (s, 2H), 7.68 (s, 1H); HRMS exact mass of (M + Na)⁺, 511.9330 amu; observed mass of (M + Na)⁺, 511.9329 amu. Anal. (C₁₈H₁₉Br₂NO₃S) C, H, N, S, O, Br.

(Z)-5-(4-Hydroxy-3-iodo-5-methoxy-benzylidene)-3-(1-methyl-cyclohexylmethyl)-thiazolidine-2,4-dione (17)—¹H NMR (300 MHz, CDCl₃) δ 0.94 (s, 3H), 1.22–1.62 (m, 10H), 3.63 (s, 2H), 3.96 (s, 3H), 6.44 (s, 1H), 6.97 (s, 1H), 7.50 (s, 1H), 7.73 (s, 1H), HRMS exact mass of (M + Na)⁺, 510.0212 amu; observed mass of (M + Na)⁺, 510.0213 amu.

(Z)-5-(4-Hydroxy-3,5-dimethyl-benzylidene)-3-(1-methyl-cyclohexylmethyl)-thiazolidine-2,4-dione (18)—¹H NMR (300 MHz, CDCl₃) δ 0.94 (s, 3H), 1.22–1.66 (m, 10H), 2.30 (s, 6H), 3.62 (s, 2H), 5.06 (s, 1H), 7.17 (s, 2H), 7.78 (s, 1H); HRMS exact mass of (M + Na)⁺, 382.1453 amu; observed mass of (M + Na)⁺, 382.1454 amu.

(Z)-5-(4-Hydroxy-naphthalen-1-ylmethylene)-3-(1-methyl-cyclohexylmethyl)-thiazolidine-2,4-dione (19)—¹H NMR (300 MHz, CDCl₃) δ 0.98 (s, 3H), 1.20–1.66 (m, 10H), 3.67 (s, 2H), 5.91 (s, 1H), 6.91 (d, *J* = 7.80 Hz, 1H), 7.56–7.67 (m, 3H), 8.15 (d, *J* = 8.40 Hz, 1H), 8.29 (d, 1H, *J* = 7.20 Hz), 8.60 (s, 1H); HRMS exact mass of (M + Na)⁺, 404.1296 amu; observed mass of (M + Na)⁺, 404.1296 amu.

5-(4-Hydroxy-3-trifluoromethyl-benzyl)-3-(1-methyl-cyclohexylmethyl)-thiazolidine-2,4-dione (20)—A mixture of compound **12** (20 mg) and Pd-C (40 mg) in methanol (5 mL) was stirred under hydrogen (50 psi) overnight, filtered, and concentrated to dryness under vacuum. The residue was purified by silica gel flash chromatography and recrystallized with ethyl acetate-hexane (1:8), giving compound **20** (14 mg). ¹H NMR (250 MHz, CDCl₃) 0.81 (s, 3H), 1.16–1.59 (m, 10H), 3.13 (dd, 1H, *J* = 9.3 Hz, 8.7 Hz), 3.45 (s, 2H), 3.51 (dd, 1H, *J* = 9.3 Hz, 3.6 Hz), 4.44 (dd, 1H, *J* = 3.6 Hz, 8.7 Hz), 5.53 (s, 1H), 6.92 (d, 1H, *J* = 8.40 Hz), 7.33 (d, 1H, *J* = 8.40 Hz), 7.39 (s, 1H), HRMS exact mass of (M + Na)⁺, 422.1170 amu; observed mass of (M + Na)⁺, 422.1173 amu.

Cell Culture

LNCaP androgen-responsive (p53^{+/+}) and PC-3 androgen-nonresponsive (p53^{-/-}) prostate cancer cells were obtained from the American Type Culture Collection (Manassas, VA), and were maintained in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a humidified incubator containing 5% carbon dioxide.

Cell Counting and Cell Viability Assay

LNCaP or PC-3 cells were placed in six-well plates (2.5 × 10⁵ cells/well) in 10% FBS-supplemented RPMI 1640 for 24 h, and treated with various concentrations of compound **12** for additional 24, 48 and 72 h. Cells were then trypsinized and counted by using a Coulter counter (Model Z1 D/T, Beckman Coulter, Fullerton, CA). Cell viability was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay in

six replicates in 96-well plates. LNCaP or PC-3 cells were seeded at 6000 cells per well in 10% FBS-supplemented RPMI 1640 for 24 h, followed by treatments with various compounds in 5% FBS-supplemented RPMI 1640 at the indicated concentrations. Controls received DMSO at a concentration equal to that in drug-treated cells. After the end of incubation, MTT (0.5 mg/ml) in 10% FBS-supplemented RPMI 1640 was added to each well, and cells were incubated at 37°C for 2 hours. Medium was removed and the reduced MTT dye was solubilized in DMSO (200 μ l/well). Absorbance was determined at 570 nm by a 96-well plate reader.

Transfection and Luciferase assay

The 3.6-kilobase AR promoter-linked reporter plasmid p-3600ARCAT was kindly provided by Dr. Chawnshang Chang (University of Rochester Medical Center, Rochester, NY). The AR promoter gene (−3600 to +550) encompassing the transcription start site was isolated by using PCR to generate hAR-luc with the following primers: 5′-TACAGGTACCGGTATCTCGACCTGCAGGTC-3′ and 5′-TGTTAGATCTTGCTGAAGCCGCTCCCCAGT-3′. The fragment was subcloned into the *pGL3* luciferase reporter vector (Promega, Madison, WI) at KpnI and BglIII in the multiple cloning site. The PPRE-x3-TK-Luc reporter vector contains three copies of the PPAR-response element (PPRE) upstream of the thymidine kinase promoter-luciferase fusion gene and was kindly provided by Dr. Bruce Spiegelman (Harvard University, Cambridge, MA). The pCMVSp1 plasmid was purchased from Origene Technologies, Inc. (Rockville, MD). LNCaP or PC3 cells were transfected with 5 μ g of individual plasmids in an Amaxa Nucleofector using a cell line-specific nucleofector kit according to the manufacturer's protocol (Amaxa Biosystems, Cologne, Germany), and then seeded in 6-well plates at 5×10^5 cells per well for 48 h. The transfection efficiency was determined to be 70 – 80% by transfecting cells with 2 μ g of pmaxGFP plasmid, followed by fluorescence microscopy to measure GFP expression. For each transfection, herpes simplex virus thymidine kinase promoter-driven *Renilla reniformis* luciferase was used as an internal control for normalization.

For the reporter gene assay, after transfection, cells were cultured in 24-well plates in 10% FBS-supplemented RPMI 1640 medium for 48 h, subject to different treatments for the indicated times, collected, and lysed with passive lysis buffer (Promega). Fifty- μ l aliquots of the lysates were added to 96-well plates, and luciferase activity was monitored after adding 100 μ l of luciferase substrate (Promega) each well by using a MicroLumatPlus LB96V luminometer (Berthold Technologies, Oak Ridge, TN) with the WinGlow software package. All transfection experiments were carried out in six replicate.

Cell Cycle Analysis

LNCaP cells were seeded in 6-well plates (2.5×10^6 cells/well) and treated with different concentrations of compound **12** for 72 h. After extensive washing with PBS, cells were trypsinized followed by fixation in ice-cold 80% ethanol at 4°C overnight. Cells were then centrifuged for 5 min at $1500 \times g$ at room temperature, and stained with propidium iodide (50 μ g/ml) and RNase A (100 units/ml) in PBS. Cell cycle phase distributions were determined on a FACScort flow cytometer and analyzed by the ModFitLT V3.0 program.

RT-PCR and Immunoblotting

LNCaP cells were cultured in T25 flasks at an initial density of 1×10^6 cells/flask. After exposure to various compounds at the indicated conditions, cells were subject to total RNA isolation by using an RNeasy mini-kit (QIAGEN, Valencia, CA). RNA concentrations were determined by measuring absorption at 260 nm in a spectrophotometer. Aliquots of 6 μ g of total RNA from each sample were reverse-transcribed to cDNA using an Omniscript RT Kit

(QIAGEN) according to the manufacturer's instructions. The PCR primers used were as follows.

AR: 5'-ACACATTGAAGGCTATGAATGTC-3' (forward)

5'-TCACTGGGTGTGGAAATAGATGGG-3' (reverse)

β -actin: 5'-TCTACAATGAGCTGCGTGTG-3' (forward)

5'-GGTCAGGATCTTCATGAGGT-3' (reverse)

PCR reaction products were separated electrophoretically in 1.5% agarose gels. For immunoblotting, protein extracts were prepared by M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) with freshly added 1% phosphatase and protease inhibitor cocktails (Calbiochem) followed by centrifugation at $13,000 \times g$ for 10 min. Supernatant was collected and protein concentration was determined by protein assay reagent (Bio-Rad, CA). Protein extracts were then suspended in 2x SDS sample buffer, and subject to 10% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes using a semidry transfer cell. The transblotted membrane was washed twice with Tris-buffered saline containing 0.1% Tween 20 (TBST). After blocking with TBST containing 5% nonfat milk for 1 h, the membrane was incubated with mouse monoclonal anti-AR (Santa Cruz) or anti- β -actin (MP Biomedicals) antibodies (diluted 1:1000) in 1% TBST nonfat milk at 4°C overnight. After incubation with the primary antibody, the membrane was washed three times with TBST for a total of 30 min, followed by incubation with horseradish peroxidase conjugated goat anti-mouse IgG (diluted 1:2500) for 1 h at room temperature. After three thorough washes with TBST for a total of 30 min, the immunoblots were visualized by enhanced chemiluminescence.

Immunocytochemical Analysis

Cells were seeded onto coverslips in six-well plates (2.5×10^5 cells/well) for 24 h followed by exposure to 5 μ M compound **12** for an additional 48 h. After extensive washing with PBS, cells were fixed and permeabilized with PBS containing 0.1% Triton X-100 for 1 h, and then incubated with anti-AR (1:100 dilution) in PBS containing 0.1% Triton X-100, 0.2% bovine serum albumin, 0.5 mM PMSF, and 1 mM DTT at room temperature for 12 h followed by Alexa Fluor 488-conjugated goat anti-mouse IgG (1:100; Molecular Probes) for 2 h. Nuclear counterstaining was performed by mounting with 4,6-diamidino-2-phenylindole (DAPI)-containing medium. Images of immunocytochemically labeled samples were observed using a Nikon microscope (Eclipse TE300).

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Abbreviations

PPAR γ	peroxisome proliferator-activated receptor γ
AR	androgen receptor
shRNA	short hairpin RNA
PSA	prostate specific antigen
FBS	fetal bovine serum

RT-PCR	reverse transcriptase-polymerase chain reaction
PPRE	PPAR response element
TBST	Tris-buffered saline containing 0.1% Tween 20

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Figure 1. (A) Representative structures of troglitazone (TG), STG28, ciglitazone (CG), and derivatives. (B) Schematic representation of the course of structural optimization of ciglitazone to develop AR-ablative agents. (C) The general synthetic procedure for ciglitazone derivatives.

A. Western blotting

B. RT-PCR

Figure 2.

Effect of ciglitazone (CG) and Δ 2CG on AR ablation in LNCaP cells. (A) Dose- and time-dependent effects of CG and Δ 2CG on suppressing AR protein expression levels. Cells were exposed to CG or Δ 2CG under the indicated conditions in 10% FBS-supplemented medium, and the lysates were subjected to Western blot analysis. (B) Time-dependent effect of CG (60 μ M) and Δ 2CG (30 μ M) on suppressing the mRNA levels of AR. Cells were treated with either agent in 10% FBS-supplemented medium for the indicated times. Total RNA was isolated and subjected to RT-PCR analysis as described in the Experimental Section.

Figure 3.

Differential effects of ciglitazone, Δ2CG, and compounds **1** – **9** on suppressing AR expression in LNCaP cells. (A) *Left panel*: analysis of the effects of individual compounds on the transcriptional repression of the AR gene by the AR promoter-luciferase reporter assay. LNCaP cells were transiently transfected with an AR promoter-linked luciferase reporter plasmid and exposed to DMSO vehicle (D), ciglitazone (CG, 20 μM), Δ2CG (Δ2, 20 μM), or compounds **1** – **9** (10 μM) in 10% FBS-supplemented RPMI 1640 medium for 48 h. Analysis of luciferase activity was carried out as described in the Experimental Section. *Columns*, mean (n = 3); *bars*, standard deviation (SD). *Right panel*: The IC₅₀ values of individual agents in inhibiting the cell viability of LNCaP cells. Cells were exposed to individual agents at various concentrations in 5% FBS-supplemented RPMI 1640 medium for 48 h, and cell viability was assessed by MTT assays. (B) Western blot analysis of the dose-dependent effect of compounds **1**, **6**, and **9** on reducing AR protein levels. Cells were exposed to individual agents at the indicated concentrations in 10% FBS-supplemented medium for 72 h, and the lysates were subjected to Western blot analysis.

Figure 4. Differential effect of compounds **10** – **19** on suppressing AR expression in LNCaP cells. (A) *Left panel:* analysis of the effects of DMSO vehicle (D) or individual compounds on the transcriptional repression of the AR gene by the AR promoter-luciferase reporter assay. LNCaP cells were transiently transfected with an AR promoter-linked luciferase reporter plasmid and exposed to compounds **10** – **19** (10 μM) in 10% FBS-supplemented RPMI 1640 medium for 48 h. Analysis of luciferase activity was carried out as described in the Experimental Section. *Columns*, mean (n = 3); *bars*, SD. *Right panel:* The IC₅₀ values of individual agents in inhibiting the cell viability of LNCaP cells. Cells were exposed to individual agents at various concentrations in 5% FBS-supplemented RPMI 1640 medium for 48 h, and cell viability was assessed by MTT assays. (B) Dose-dependent effect of

ciglitazone (CG), $\Delta 2$ CG, and compounds **12** and **16**, relative to that of 10 μ M troglitazone (TG), on PPAR γ activation in PC-3 cells. PC-3 cells were transiently transfected with PPRE-x3-TK-Luc reporter vector and then exposed to individual agents or DMSO vehicle (D) in 10% FBS-supplemented RPMI 1640 medium for 48 h. Analysis of luciferase activity was carried out as described in the Experimental Section. *Columns*, mean ($n = 6$); *bars*, SD. (C) Western blot analysis of the dose-dependent effect of compounds **12** and **16**, on reducing AR protein levels. Cells were exposed to individual agents at the indicated concentrations in 10% FBS-supplemented medium for 72 h, and the lysates were subjected to Western blot analysis. (D) Immunocytochemical analysis of the effect of 5 μ M compound **12** on suppressing AR expression after 24-h exposure. The nuclear counterstaining was achieved using a 4',6-diamino-2-phenylindole (DAPI)-containing mounting medium.

Figure 5.

Antitumor effects of compound **12** in LNCaP cells. (A) Differential dose-dependent effects of compound **12** on the inhibition of cell viability of LNCaP versus PC-3 cells at 48 h (inset) and 72 h of treatment. Cells were exposed to the indicated concentrations of compound **12** in 5% FBS-containing RPMI 1640 medium for 48 and 72 h, and cell viability was determined by MTT assays. Points, mean ($n = 6$); bars, SD. (B) Dose- and time-dependent antiproliferative effects of compound **12** in LNCaP (left panel) and PC-3 (right panel) cells. Cells were seeded into six-well plates (250,000 cells/well), incubated for 24 h, and exposed to compound **12** at the indicated concentrations in 5% FBS-supplemented medium for different time intervals. Cells were harvested, and counted using a Coulter counter. (C) Flow cytometric analysis of LNCaP cells after treatment with DMSO or the indicated concentrations of compound **12** for 72 h. Percentages of cell cycle distribution represent the

mean of two independent determinations. (D) Western blot analysis of the dose-dependent effects of compounds **12** and **16** on PARP cleavage, caspase 3 activation, and caspase 7 activation in LNCaP cells after 72 h of treatment.

Figure 6.

Evidence that the ability of compound **12** to inhibit AR expression in LNCaP cells is not mediated through an irreversible mechanism. (A) Structure and the dose-dependent effect of compound **20** on suppressing AR expression in LNCaP cells. Cells were exposed to compound **20** at the indicated concentrations in 10% FBS-supplemented medium for 72 h, and the lysates were subjected to Western blot analysis. (B) Dose-dependent effects of compound **12** versus compound **20** in suppressing the viability of LNCaP cells. Cells were exposed to individual agents at various concentrations in 5% FBS-supplemented RPMI 1640 medium for 48 h, and cell viability was assessed by MTT assays. (C) Restoration of AR expression in LNCaP cells after compound **12** was washed out. The effect of 5 μM compound **12** on AR repression in LNCaP cells was examined at different intervals throughout a 72 h-period in two different manners. Continuous exposure: cells in T-25 flasks were incubated in drug-containing, 10% FBS-supplemented medium for 72 h, and washout at 48 h of treatment: cells in T-25 flasks were exposed to the agent for 44 h, followed by incubation in drug-free medium for additional 24 h. AR levels in cell lysates were analyzed by Western blot analysis.