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Novel steroidal 1,3,4-thiadiazines: Synthesis and biological evaluation in androgen receptor-positive prostate cancer 22Rv1 cells

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ABSTRACT

A flexible approach to previously unknown spirofused and linked 1,3,4-thiadiazine derivatives of steroids with selective control of heterocyclization patterns is disclosed. (*N*-Arylcarbamoyl)spiroandrostene-17,6′ [1,3,4] thiadiazines and (*N*-arylcarbamoyl)17-[1′,3′,4′]thiadiazine-substituted androstenes, novel types of heterosteroids, were prepared from 16*β*,17*β*-epoxypregnenolone and 21-bromopregna-5,16-dien-20-one in good to high yields by the treatment with oxamic acid thiohydrazides. The synthesized compounds were screened for antiproliferative activity against the human androgen receptor-positive prostate cancer cell line 22Rv1. Most of (*N*-arylcarbamoyl)17-[1′,3′,4′]thiadiazine-substituted androstenes exhibit better antiproliferative potency (IC_{50} = 2.1–6.6 μM) than the antiandrogen bicalutamide. Compounds **7d** with IC_{50} = 3.0 μM and **7j** with IC_{50} = 2.1 μM proved to be the most active in the series under study. Lead synthesized compound **7j** downregulates AR expression and activity in 22Rv1 cells. NF-κB activity is also blocked in **7j**-treated 22Rv1 cells. Apoptosis is considered as a possible mechanism of **7j**-induced cell death.

1. Introduction

Extranuclear heterosteroids have been considered over the years to be a privileged scaffold for drug discovery due to their outstanding biological activity, which is especially true for D-ring modified steroids [1–8]. Thus, the naturally occurring steroids cardenolides (i.e. digoxin, ouabain) and bufadienolides (i.e. bufalin, marinobufogenin) are inhibitors of the plasma membrane Na^+, K^+-ATPase and are of interest as potential drugs for cardiac failure and certain types of arrhythmias [9,10]. Spirostan saponins display hemolytic activity and high cytotoxicity [11,12]. Moreover, synthetic heterosteroids encompass a wide range of compounds with various biological activities, e.g., reductase inhibitors such as finasteride [13], high-affinity agonist ligands for the glucocorticoid receptor, e.g., cortivazol [14], neuromuscular junction blocking agents such as rocuronium and pipecuronium [15], the anti-inflammatory and immunosuppressive drug hydrocortamate [16], anticancer agents such as abiraterone [17].

Our interest in the preparation of D-ring heterosteroids [18,19] suggested a need for a facile flexible strategy, in which a common intermediate can be used in a conjunctive fashion to form an array of structurally diverse *N*-heterocycles attached or fused to a steroid core. In this regard, we studied oxamic acid thiohydrazides as simple versatile agents for the modification of steroids bearing a carbonyl group. In contrast to classical hydrazones, three major Lewis structural formulas provided by an extra *C*═*S* group contribute to the real structure of hydrazones of oxamic acid thiohydrazides, thereby efficiently extending the number of possible chemical transformations (Fig. 1). The Lewis structural formulas of thione **A** and thiol **B** are defined by the highly active competitive *NH*- and *SH*-nucleophilic sites, while the additional cyclic thiadiazoline structure **C** is characterized by facilitated *C*—*N* bond oxidation. Structure **B** also can be considered as 2,3-diazapolyene synthon.

In this context, thiohydrazides serve as synthetic building blocks for diverse five- and six-membered *O,N,S*-heterocycles, including such

Abbreviations: DHT, dihydrotestosterone; RPMI-1640, Roswell Park Memorial Institute medium 1640; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; AR, androgen receptor; ERK, extracellular signal-regulated kinase; ph, phosphorylated; PARP, poly (ADP-ribose) polymerase; IC_{50}, half maximal inhibitory concentration; ECL, enhanced chemiluminescence

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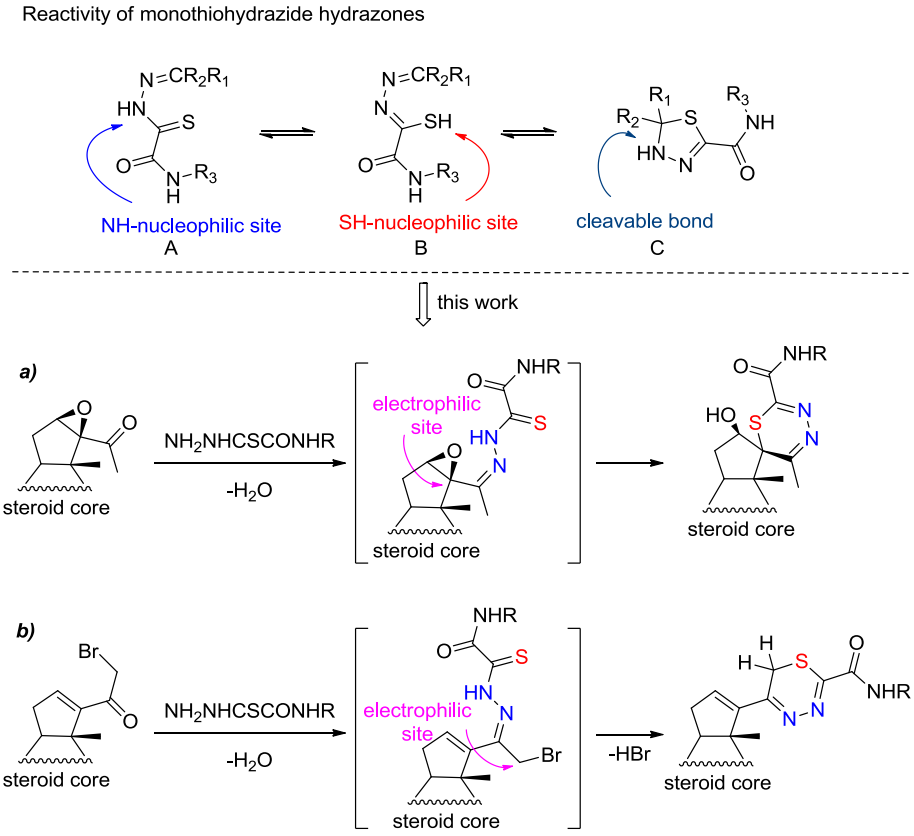


Fig. 1. Reactivity and application of monothiohydrazide hydrazones in the synthesis of extranuclear heterosteroids.

widely used pharmacophores as pyridazines [20], 1,3,4-thiadiazoles [21], and 4,5-dihydro-1,3,4-thiadiazoles [22]. In particular, monothiohydrazide hydrazones are employed in the synthesis of steroidal pyrazolines [23], pyridazines [19], and 1,3,4-thiadiazoles [24].

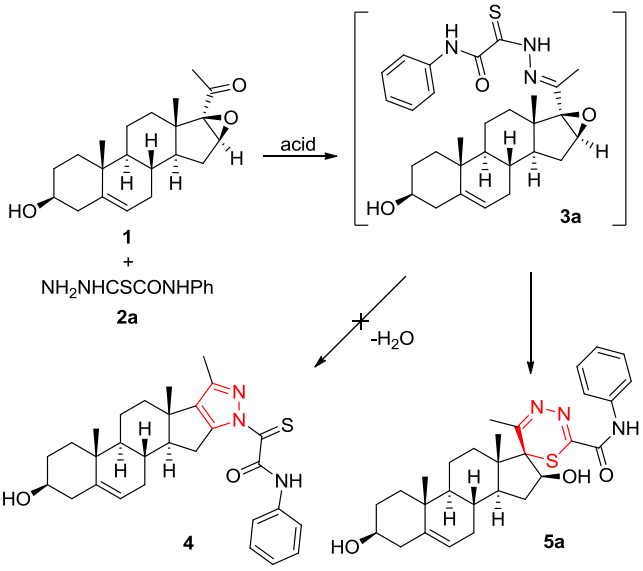
Herein, we report the synthesis of unique derivatives of androstene series containing a 1,3,4-thiadiazine motif using oxamic acid thiohydrazides (Fig. 1). The relative spatial arrangement of the electrophilic reaction site and the carbonyl moiety in the steroid structure defines the reaction product. Spiroandrostene-17,6′[1′,3′,4′]thiadiazines were exclusively synthesized employing 16*β*,17*β*-epoxypregnenolone (Fig. 1, line a). Using 21-bromopregna-5,16-dien-20-one as a substrate, 17-[1′,3′,4′]thiadiazine-substituted androstenes were prepared (Fig. 1, line b). Their inhibitory activity was evaluated against human androgen receptor-positive prostate cancer cells 22Rv1. Effects of two lead compounds on signaling pathways were analyzed.

2. Results and discussion

2.1. Chemistry

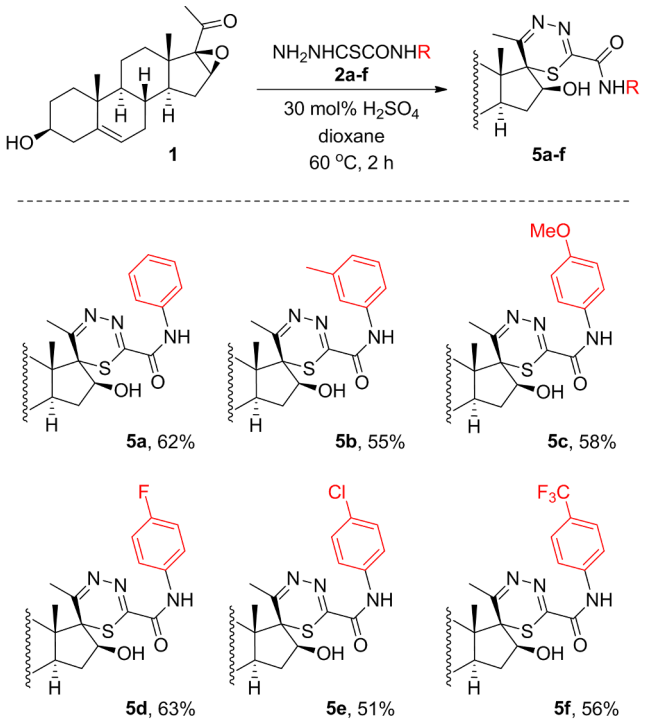
Based on our previous results [23] and the chemistry of hydrazones [25], we could envision the possibility of assembling new steroidal pyrazole derivative **4** from 16*β*,17*β*-epoxypregnenolone **1** by the classical nucleophilic oxirane ring opening with an *NH*-nucleophile followed by the dehydration driven by aromatization. However, by serendipity, we found that hydrazone **3a** generated from oxirane **1** and oxamic acid thiohydrazide **2a** chemoselectively react with a sulfur nucleophile, thus providing an approach to the enantiopure synthesis of previously unknown 16*β*-hydroxy-spiroandrostene-17,6′[1,3,4]thiadiazines **5a** (Scheme 1).

To study the scope of this approach, we performed the reaction of thiohydrazides **2a-f** containing both electron-donating and -withdrawing substituents on the aryl group with 16*β*,17*β*-epoxypregnenolone **1** (Scheme 2). The reaction of thiohydrazides **2** with oxirane **1** in 1,4-dioxane in the presence of a catalytic amount of H_2SO_4 at 60 °C gave, after 2 h, the corresponding spiro(androstene thiadiazines) **5** in good yield with virtually complete stereoselectivity. Other solvents, such as ethanol or acetic acid, at variable temperatures were not so effective (the reactions afforded complex mixtures). The use of 30 mol% TsOH, TFA, or BF_3*Et_2O did not lead to the target products in higher yields. Product **5a** derived from phenyl-substituted thiohydrazide was obtained in 62% yield.



Scheme 1. Synthesis of spiro[1,3,4]thiadiazine **5a**.

The investigation of the substrate scope by employing

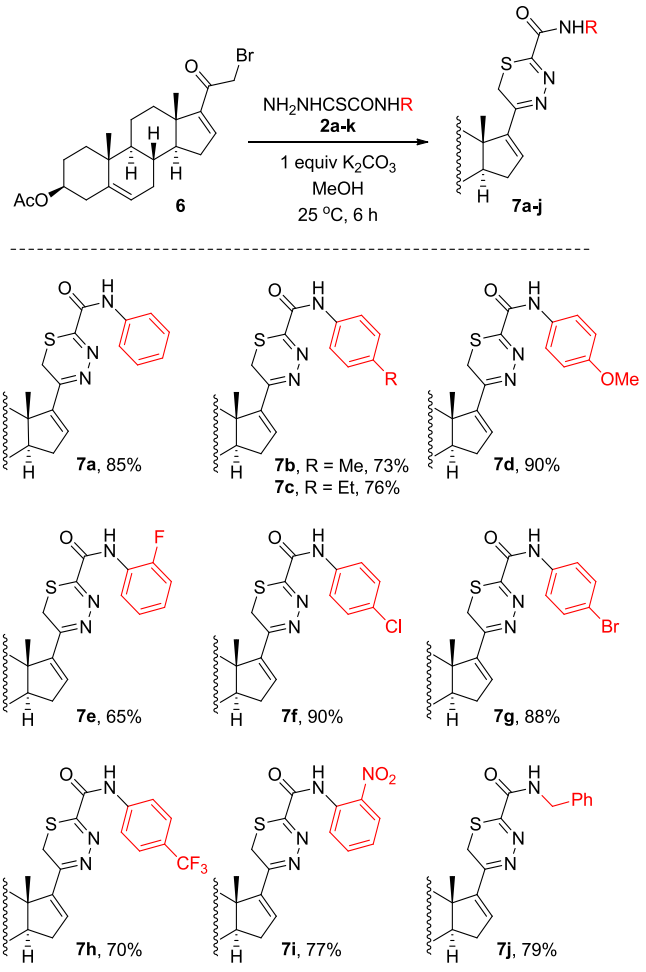


Scheme 2. Synthesis of 16*β*-hydroxyspiro-androsteno-17,6′[1,3,4]thiadiazines **5**.

thiohydrazides **2** with electron-donating and -withdrawing groups in the reaction showed that the electronic effects of substituents on the aryl ring are not pronounced. Methyl, methoxy, fluoride, chloride, and trifluoromethane derivatives **5b-f** were obtained in 51–63% yields, respectively.

The solution-state structures of compounds **5d-f** were derived by means of detailed NMR studies, such as 2D NOESY, ^{1}H–^{1}H COSY, ^{13}C–^{1}H HMBC, ^{13}C–^{1}H RSQC, ^{1}H–^{1}H TOCSY, and J-coupling constants (see the Supporting material).

Further, we made an attempt to generate novel D-ring androstene derivatives by treating 21-bromopregna-5,16-dien-20-one **6** with oxamic acid thiohydrazides **2** under mild basic conditions (Scheme 3). Having so far observed a seemingly general transformation pattern, we found that the reaction affords 17-(6′*H*-1′,3′,4′-thiadiazine-2′-carboxamide)androst-5,17-dienes **7** in virtually quantitative yield. The structural assignments were confirmed by 2D NMR (^{1}H–^{1}H COSY, ^{13}C–^{1}H HMBC, and ^{13}C–^{1}H HSQC) techniques and HRMS. We tested acid thiohydrazides **2** containing electron-donating and -withdrawing groups. The reaction showed high chemoselectivity and functional group tolerance. Thiohydrazides **2** bearing methyl, ethyl, methoxy, fluoride, chloride, bromide, trifluoromethane, and nitro groups provided pyridazines **7a-i** in high yields. Moreover, (*N*-benzylcarbamoyl)-17-[1′,3′,4′]thiadiazine-substituted androstene **7j** was obtained in 79% yield.



Scheme 3. Synthesis of 17-(6′*H*-1′,3′,4′-thiadiazine-2′-carboxamide)androst-5,17-dienes **7**.

2.2. Biology

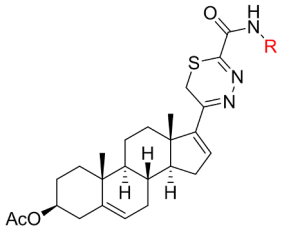
2.2.1. Cytotoxic effect against prostate cancer cells

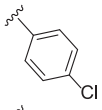
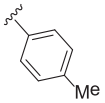
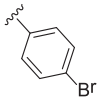
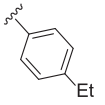
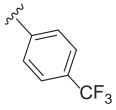
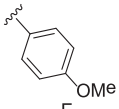
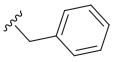
Inspired by novel steroidal drugs effective in the treatment of prostate cancer [26,27], we evaluated the antiproliferative activity of all synthesized compounds against the human androgen receptor-positive prostate cancer cell line 22Rv1 using the MTT assay.

In the late 90s, 22Rv1 prostate cancer cell line has been derived from a human prostatic carcinoma xenograft, CWR22R [28]. These cells are derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the androgen-dependent CWR22 xenograft. The AR mutation was detected subsequently in 22Rv1 cells, indicating their differences from CWR22 xenograft. 22Rv1 cell line displayed both androgen-responsive and androgen-insensitive features due, at least in part, to an insertional mutation of the AR as described by Dr. Clifford G. Tepper in the work [29]. Summary, 22Rv1 cell line represents very intriguing AR-positive prostate cancer model, demonstrating AR-dependent and AR-independent growth.

The hormonal drug bicalutamide was used as the reference compound. (*N*-Arylcarbamoyl)spiroandrostene-17,6′[1,3,4]thiadiazines **5a-f** showed weak activity with inhibitory concentrations IC_{50} (IC_{50} is the half maximal inhibitory concentration) ranging between 20 and 25 μM. Meanwhile, virtually all (*N*-arylcarbamoyl)17-[1′,3′,4′]thiadiazine-substituted androstenes **7** exhibited significant antiproliferative potency. The corresponding inhibitory concentrations of compounds **7** are enlisted in Table 1. All the tested compounds showed toxicity with IC_{50} lower than 10 μM; the only exception was compound **7g** with the IC_{50} value higher 25 μM. High toxicity was observed for compounds **7a** (IC_{50} = 3.1 μM) and **7d** (IC_{50} = 3.0 μM) with phenyl and 4-methoxyphenyl substituents. Compounds **7b,c,e,f,h** containing methyl, ethyl, fluorine, chlorine, or trifluoromethyl groups at the aryl moiety showed toxicity with IC_{50} ranging between 3.7 and 6.6 μM. Steroidal 1,3,4-thiadiazine **7j** bearing a benzyl substituent at the carboxamide moiety was the most active compound in the series of the tested compounds with IC_{50} = 2.1 μM (Table 1, entry 9). Nonsteroidal antiandrogen bicalutamide showed low antiproliferative activity with the IC_{50} value higher 25 μM, indicating partial AR-independent features of 22Rv1 cells [29].

Table 1
Antiproliferative activity of the synthesized steroidal 1,3,4-thiadiazines **7** and the reference drug against the human prostate cancer cell line 22Rv1.



Entry	Compound	R	IC_{50}, μM	Entry	Compound	R	IC_{50}, μM
1	**7a**		3.1 ± 0.3	6	**7f**		3.7 ± 0.5
2	**7b**		6.1 ± 0.7	7	**7 g**		> 25
3	**7c**		6.6 ± 0.7	8	**7 h**		6.2 ± 0.7
4	**7d**		3.0 ± 0.3	9	**7j**		2.1 ± 0.3
5	**7e**		6.6 ± 0.7	10	Bicalutamide		> 25

*2.2.2. Effects of compounds **7d** and **7j** on the signaling pathways in 22Rv1 cells*

Two most active compounds were selected for detailed studies. Androgen receptor is a well-known driver of the hormone-dependent prostate cancers. 22Rv1 cells harbor two AR forms, a full one and a C-terminally truncated, constitutively active form, both of which have been investigated in various works [30–34]. We analyzed expression of full AR isoform (110 kDA) by immunoblotting after 24-h treatment of 22Rv1 cells with compounds **7d** and **7j**. Compound **7d** did not cause significant changes in AR expression, whereas **7j** inhibited AR expression in a dose-dependent manner (Fig. 2A).

The NF-κB pathway actively supports the growth of tumor cells, including prostate cancer. Many studies have shown that steroidal molecules can significantly affect this signaling pathway. In the study [37] Mary Kaileh et al. showed that the leave extract of Withania somnifera, as well as its major constituent steroidal lactone withaferin A, potently inhibits NFκB pathway by preventing the tumor necrosis factor-induced activation of IκB kinase β *via* a thioalkylation-sensitive redox mechanism. IκBα is a key constitutive inhibitor of NFκB activity. It was described a pivotal role for glucocorticoids in inducing expression of IκBα to enhance the cytosolic retention of NF-κB [38]. Dexamethasone induces the synthesis of IκBα mRNA in GR-positive cells [39]. Corticosteroids can also partially inhibit transcriptional activation by NF-κB but not through an increase in IκBα protein alone [40]. Analysis of NF-κB p65 and its phosphorylated (ph) form using immunoblotting after 24-h treatment with compounds **7d** and **7j** revealed that **7j** inhibited phNF-κB p65 in dose-dependent manner, while **7d** had no effects on this protein. Both compounds did not block Akt phosphorylation and expression (Fig. 2A).

The extracellular signal-regulated kinase (ERK) signaling pathway plays a crucial role in cell growth and death. Depending on stimulus, ERK activity mediates different antiproliferative events, such as apoptosis and autophagy [41–43]. Chemotherapeutic agents and DNA damage stimuli, including etoposide, doxorubicin, ionizing irradiation, and ultraviolet irradiation (UV) activate ERK1/2 protein kinase in various cells [41,44]. TNFα plays interesting role in balance between proliferation, survival and apoptosis. It was found out, that TNFα induces apoptosis or supports survival through the FLIP-L-dependent activation of the ERK pathway [45–49]. Opposite and diverse effects of TNF-α and ERKs in malignant and normal cells are reported in various works, showing intricate mechanisms underlying cell death [49,50]. Moreover, compounds with hormonal activity, including estradiol, tamoxifen, apigenin, and quercetin also influence on cell death via ERK1/2 modulation [41,43]. In our study ERK1/2 activation was analyzed in 22Rv1 cells using immunoblotting. Both compounds showed high ERK1/2 phosphorylation (Fig. 2A).

Interestingly, ERK1 was maximally activated by compound **7j** at 2.5 μM concentration, whereas at higher concentrations **7j** was less active. Compound **7d** caused increase in phERK1/2 expression in a dose-dependent manner. Thus, compound **7j** has effects on ERKs activity together with impact on AR and phNF-κB p65 expression, while only ERKs are involved in the cell response to compound **7d**.

To prove the effects of compound **7j** on AR and NF-κB pathways additional experiments were performed using gene reporter analysis. The luciferase reporter assay is commonly used as a tool to study activity of transcription factors. In our study we carried out experiments with plasmids containing luciferase gene under AR- or NF-kB-dependent promoter. To activate AR signaling 10 nM DHT was used. As shown in Fig. 3A, compound **7j** significantly inhibited DHT-induced AR activity. Moreover, NF-kB activity was also blocked by compound **7j** at 2 μM concentration (Fig. 3B). Targeting NF-κB signaling may restore sensitivity of castrate-resistant prostate cancer cells to antiandrogens. Artemisinin derivatives, semisynthetic compounds derived from Artemisia annua, are approved first-line antimalarial drugs. Dr. Jessica J.

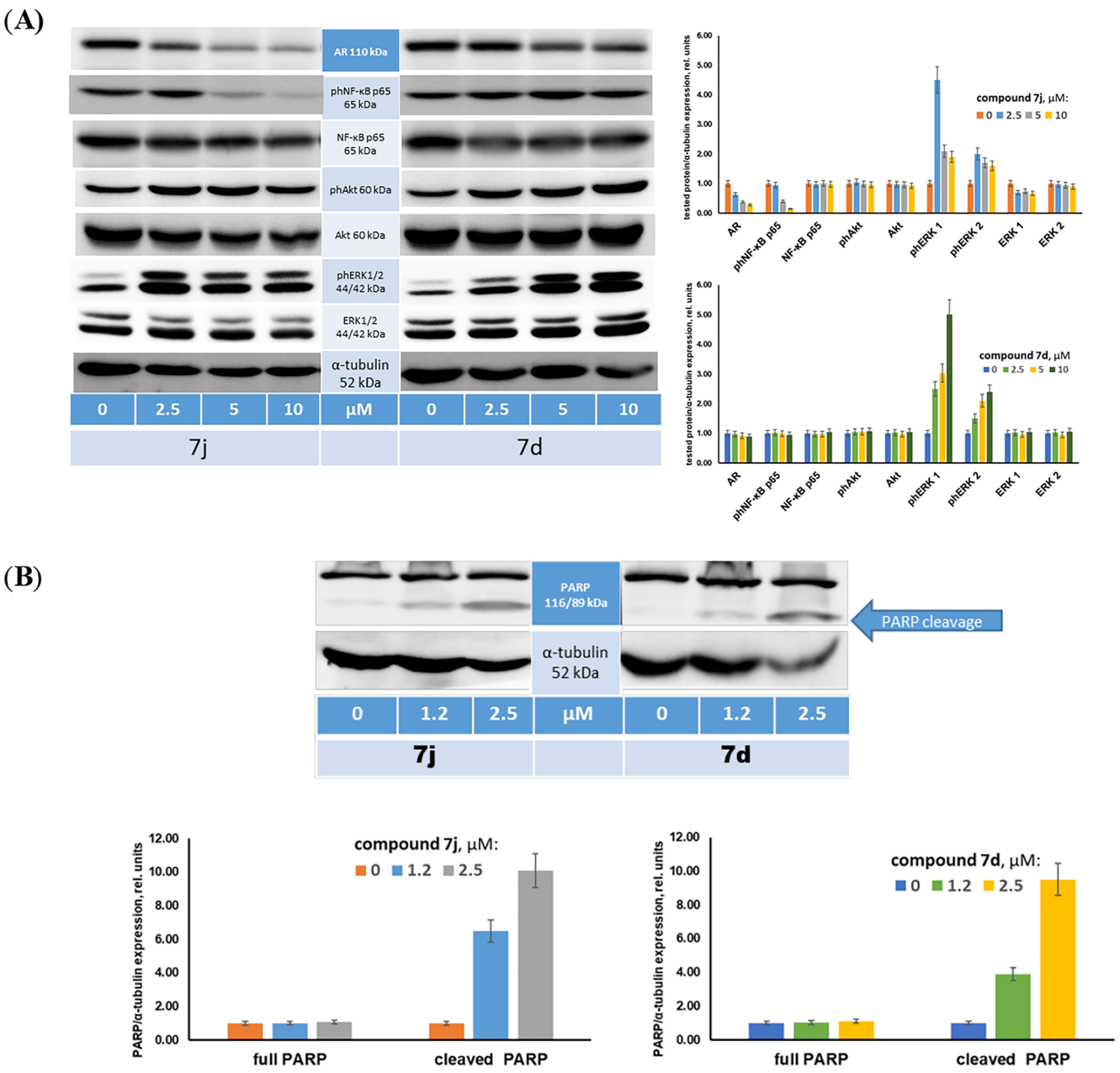


Fig. 2. Immunoblotting for AR, PARP, α-tubulin, phosphorylated (ph) and unphosphorylated NF-κB p65, Akt, ERK1/2 using lysates from 22Rv1 cells treated with the indicated concentrations of compounds **7j** and **7d**. (**A**) 24-h treatment, (**B**) 48-h treatment. Densitometry for immunoreactive proteins/α-tubulin (right (**A**) and bottom (**B**) diagrams) ratio was carried out using ImageJ software (Wayne Rasband, NIH) with the densitometry protocol provided by The University of Queensland and the recommendations from Refs. [35,36]. Density of the probes treated with vehicle control was taken as one relative unit.

Nunes and co-authors showed, that artesunate, clinically important artemisinin derivative, in combination with bicalutamide inhibits NF-κB signaling and decreases AR and/or mutant AR expression *via* ubiquitin-mediated proteasomal degradation [51].

Compound **7j** exhibits influence on AR and NF-κB activity, as well as on NF-κB p65 phosphorylation and AR expression. Thus, targeting NF-κB signaling holds great promise for therapies of hormone-dependent or castrate-resistant prostate cancers.

Cleaved PARP is an apoptotic marker, which can be detected by immunoblotting. PARP cleavage was analyzed after 48-h incubation with compounds **7j,d**. As shown in Fig. 2B, both compounds induced PARP cleavage at 2.5 μM concentration. Thus, compounds **7j,d** elicit apoptosis in the AR-positive prostate cancer 22Rv1 cells.

3. Conclusion

This study demonstrates the utility of the modified strategy of hydrazone cyclization for providing easy access to a variety of steroidal products containing functionalized D-ring starting from 16,17-epoxy/21-bromo-20-keto steroids and oxamic acid thiohydrazides as readily available precursors. Due to operational simplicity, these protocols may be useful in pharmacological research for chemoselective transformations of other complex substrates bearing a simple carbonyl group. The evaluation of cytotoxic activity of the newly synthesized compounds against the human androgen receptor-positive prostate cancer cells 22Rv1 showed that 17-[1′,3′,4′]thiadiazine-substituted androstenes display higher cytotoxicity than the spiroandrostene-17,6′[1,3,4]thiadiazines and the reference drug. Compounds **7d** with IC_{50} = 3.0 μM and **7j** with IC_{50} = 2.1 μM proved to be the most active in the series under

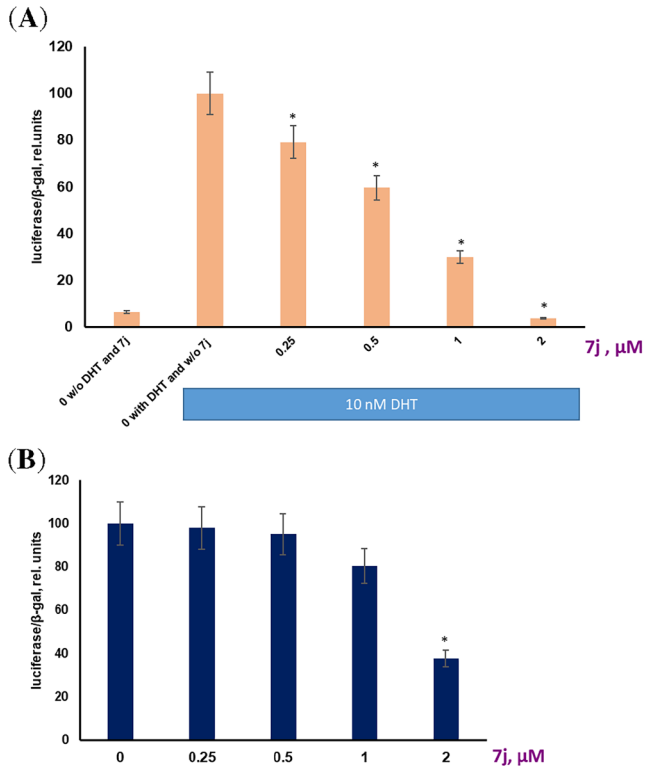


Fig. 3. Gene reporter analysis. Plasmids containing luciferase gene under AR- or NF-κB-dependent promoter were used. (**A**) AR-mediated luciferase activity induced by DHT treatment was assessed after incubation 22Rv1 cells with the indicated doses of compound **7j**, *p < 0.05 – versus 22Rv1 cells treated with 10 nM DHT alone. (**B**) NF-κB-mediated luciferase activity was analyzed without additional inducer, *p < 0.05 – versus 22Rv1 cells treated with vehicle control.

study. Our data suggest that the modulation of the AR, NF-κB, ERK1/2, PARP pathways without decreasing Akt phosphorylation is involved in compound **7j**-induced 22Rv1 cells death. Targeting AR and NF-κB by novel steroidal 1,3,4-thiadiazines are very promising but future preclinical and clinical trials should confirm initial experimental observations. This work provides evidence that steroidal 1,3,4-thiadiazines are lead compounds for developing novel and highly effective anticancer drugs for treatment of prostate cancer.

4. Experimental section

4.1. Chemistry

4.1.1. General Information

NMR spectra were acquired on Bruker Avance 600, 500, 300 spectrometers at room temperature; the chemical shifts δ were measured in ppm relative to the solvent (^{1}H: $CDCl_3$, δ = 7.27 ppm, DMSO-d_6, δ = 2.50 ppm; ^{13}C: $CDCl_3$, δ = 77.00 ppm, DMSO-d_6, δ = 39.50 ppm). Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, double doublet; ddd, double double doublet. The coupling constants (*J*) are in Hertz. The structures of all compounds were established using 1D NMR (^{1}H, ^{13}C, JMOD) and 2D NMR (^{1}H–^{1}H COSY, ^{1}H–^{1}H TOCSY, ^{1}H–^{1}H ROESY, ^{13}C–^{1}H HMBC, ^{13}C–^{1}H HSQC, NOESY) spectroscopy. High-resolution and accurate mass spectra were obtained on BrukermicrOTOF-QTM ESI-TOF (Electrospray Ionization/Time of Flight) and Thermo Scientific* LTQ Orbitrap mass spectrometers. Melting points (mp) are uncorrected and were measured on a Boetius capillary melting point apparatus. Analytical thin layer chromatography (TLC) was carried out on silica gel plates (silica gel 60 F254 aluminum supported plates); the visualization was accomplished with an UV lamp (365 nm) and using chemical staining with [$Ce(SO_4)_2$/H_2SO_4]. Column chromatography was performed on silica gel 60 (230–400 mesh, Merck). Synthesis of 16*β*,17*β*-epoxy-17-isopregn-5-en-3*β*-ol-20-one (**1**), 3*β*-acetoxy-21-bromopregn-5,16-dien-20-one (**6**), and oxamic acids thiohydrazides **2** described in Supporting Information. All reactions were carried out using freshly distilled and dry solvents.

4.1.2. General procedure for the preparation of 5′-methyl-[2′-(N-arylcarbamoyl)]-3β,16β-dihydroxy-spiroandrost-5-ene-17,6′[1,3,4] thiadiazines **5a-f**

Sulfuric acid (20 μL, 30 mol%) was added to a solution of 16*β*,17*β*-epoxy-17-isopregn-5-en-3*β*-ol-20-one (330 mg, 1.0 mmol) and oxamic acid thiohydrazide (1.2 mmol) in dry dioxane (15 mL). The reaction mixture was heated at 60 °C for 2 h, cooled to room temperature, and quenched with water (60 mL). The organic layer was separated. The aqueous layer was extracted with chloroform (3 × 20 mL) and washed sequentially with a 5% Na_2CO_3 solution (3 × 25 mL) and water (25 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by column chromatography using petroleum ether/ethyl acetate (3:1).

4.1.2.1. 5′-Methyl-[2′-(N-Phenylcarbamoyl)]-3β,16β-dihydroxy-spiroandrost-5-ene-17,6′[1,3,4]-thiadiazine (***5a***). Colorless solid; mp 188–190 °C; yield 62% (315 mg). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.79 (ddd, *J* = 4.94, 12.41, 15.37 Hz, 1H, 9-CH), 0.85 (s, 3H, 18-CH_3), 0.87 (s, 3H, 19-CH_3), 0.89–1.07 (m, 2H, 12-CH_2), 0.98–1.03 (m, 2H, 1-CH_2), 1.21–1.34 (m, 2H, 11-CH_2), 1.28–1.59 (m, 2H, 2-CH_2), 1.34–1.37 (m, 1H, 8-CH), 1.49–1.51 (m, 1H, 15-CH_2), 1.54–1.92 (m, 2H, 7-CH_2), 1.78 (ddd, *J* = 2.30, 10.43, 11.18 Hz, 1H, 14-CH), 1.88 (dd, *J* = 9.42, 11.67 Hz, 1H, 15-CH_2), 2.05 (dd, *J* = 13.31 Hz, 1H, 4-CH_2), 2.12 (dd, *J* = 4.92, 13.31 Hz, 1H, 4-CH_2), 2.47 (s, 3H, 21-CH_3), 3.19–3.20 (m, 1H, 3-CH), 4.55 (d, *J* = 5.14 Hz, 1H, OH), 5.22–5.25 (m, 1H, 6-CH), 5.23–5.25 (m, 1H, 16-CH), 5.67 (d, *J* = 4.11 Hz, 1H, OH), 7.12 (dd, *J* = 7.20 Hz, 1H, 6′-CH), 7.34 (dd, *J* = 7.20, 8.05 Hz, 2H, 5′-CH + 7′-CH), 7.86 (d, *J* = 8.05 Hz, 2H, 4′-CH + 8′-CH), 10.46 (br.s, 1H, NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 14.8 (18-CH_3), 19.0 (19-CH_3), 20.0 (11-CH_2), 23.5 (21-CH_3), 31.1 (8-CH), 31.2 (7-CH_2), 31.3 (2-CH_2), 33.7 (15-CH_2), 34.7 (12-CH_2), 36.0 (10-C), 36.6 (1-CH_2), 42.0 (4-CH_2), 46.0 (14-CH), 48.9 (9-CH), 51.2 (13-C), 62.2 (17-C), 69.9 (3-CH), 72.5 (16-CH), 120.0 (6-CH), 120.4 (4′-CH + C-8′-CH), 124.1 (6′-CH), 128.6 (5′-CH + 7′-CH), 137.9 (3′-C), 141.2 (5-C), 152.9 (2′-C), 154.0 (20-C), 160.0 (1′-C). HRMS (ESI) for $C_{29}H_{38}N_3O_3S$ ($[M+H]^+$): calcd 508.2628, found 508.2626.

4.1.2.2. 5′-Methyl-[2′-(N-(3-methylphenylcarbamoyl)]-3β,16β-di-hydroxyspiroandrost-5-ene-17,6′[1,3,4]thiadiazine (***5b***). Colorless solid; mp 190–195 °C; yield 55% (300 mg). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.89–0.91 (m, 1H, 9-CH), 0.93 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 0.97–1.07 (m, 2H, 1-CH_2), 1.01–1.12 (m, 2H, 12-CH_2), 1.24–1.43 (m, 2H, 11-CH_2), 1.40–1.42 (m, 1H, 8-CH), 1.45–1.79 (m, 2H, 2-CH_2), 1.62–1.96 (m, 2H, 7-CH_2), 1.67–1.70 (m, 1H, 15-CH_2), 1.97 (dd, *J* = 9.25, 13.36 Hz, 1H, 15-CH_2), 2.05–2.08 (m, 1H, 14-CH), 2.18 (dd, *J* = 12.94 Hz, 1H, 4-CH_2), 2.28 (dd, *J* = 4.00, 12.94 Hz, 1H, 4-CH_2), 2.38 (s, 3H, Me), 2.57 (s, 3H, 21-CH_3), 3.49–3.51 (m, 1H, 3-CH), 5.30–5.32 (m, 1H, 6-CH), 5.34 (dd, *J* = 3.30, 9.84 Hz, 1H, 16-CH), 6.98 (d, *J* = 7.80 Hz, 1H, 6′-CH), 7.26 (t, *J* = 7.80 Hz, 1H, 1H, 7′-CH), 7.50 (d, *J* = 7.80 Hz, 1H, 8′-CH), 7.60 (s, 1H, 4′-CH), 9.23 (br. s, 1H, NH), signals of OH groups were not observed. ^{13}C NMR (125 MHz, DMSO-d_6): δ 15.2 (18-CH_3), 19.2 (19-CH_3), 20.3 (11-CH_2), 21.5 (Me), 23.9 (21-CH_3), 31.4 (2-CH_2), 31.5 (7-CH_2), 31.6 (8-CH), 34.2 (15-CH_2), 34.8 (12-CH_2), 36.3 (1-CH_2), 36.8 (10-C), 42.1 (4-CH_2), 46.0 (14-CH), 48.7 (9-CH), 52.2 (13-C), 62.4 (17-C), 71.6 (3-CH), 74.3 (16-CH), 116.0 (8′-CH), 120.3 (6-CH), 120.9 (4′-CH), 125.6 (6′-CH), 129.0 (7′-CH), 137.0 (5′-C), 139.2 (3′-C), 140.8 (5-C), 153.7 (2′-C), 154.3 (20-C), 158.8 (1′-C). HRMS (ESI) for $C_{30}H_{39}N_3O_3SNa$ ($[M + Na]^+$): calcd

544.2604, found 544.2598.

*4.1.2.3. 5′-Methyl-[2′-(N-(4-methoxyphenylcarbamoyl)-3β,16β-dihydroxyspiroandrost-5-ene-17,6′[1,3,4]thiadiazine (**5c**)*. Colorless solid; mp 205–207 °C; yield 58% (312 mg). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.79 (ddd, J = 5.14, 11.31, 12.34 Hz, 1H, 9-CH), 0.84 (s, 3H, 18-CH_3), 0.87 (s, 3H, 19-CH_3), 0.89–1.07 (m, 2H, 12-CH_2), 0.91–1.03 (m, 2H, 1-CH_2), 1.21–1.34 (m, 2H, 11-CH_2), 1.28–1.59 (m, 2H, 2-CH_2), 1.33–1.37 (m, 1H, 8-CH), 1.47–1.51 (m, 1H, 15-CH_2), 1.52–1.91 (m, 2H, 7-CH_2), 1.78 (ddd, J = 1.83, 10.28, 11.30 Hz, 1H, 14-CH), 1.86 (dd, J = 10.28, 13.36 Hz, 1H, 15-CH_2), 2.05 (dd, J = 12.34 Hz, 1H, 4-CH_2), 2.12 (dd, J = 4.11, 12.34 Hz, 1H, 4-CH_2), 2.46 (s, 3H, 21-CH_3), 3.18–3.21 (m, 1H, 3-CH), 3.73 (s, 3H, OMe), 5.20–5.25 (m, 1H, 6-CH), 5.24 (m, 1H, 16-CH), 6.91 (d, J = 9.01 Hz, 2H, 5′-CH + 7′-CH), 7.77 (d, J = 9.01 Hz, 2H, 4′-CH + 8′-CH), 10.42 (br. s, 1H, NH), signals of OH groups were not observed. ^{13}C NMR (125 MHz, DMSO-d_6): δ 14.8 (18-CH_3), 19.0 (19-CH_3), 20.0 (11-CH_2), 23.5 (21-CH_3), 31.2 (8-CH), 31.3 (2-CH_2), 31.4 (7-CH_2), 33.7 (15-CH_2), 34.6 (12-CH_2), 36.0 (10-C), 36.6 (1-CH_2), 42.1 (4-CH_2), 46.0 (14-CH), 48.9 (9-CH), 51.2 (13-C), 55.2 (OMe), 62.2 (17-C), 69.9 (3-CH), 72.5 (16-CH), 113.8 (5′-CH + 7′-CH), 120.0 (6-CH), 121.8 (4′-CH + 8′-CH), 131.1 (3′-C), 141.2 (5-C), 153.0 (2′-CH_2), 153.8 (20-C), 155.8 (6′-C), 159.6 (1′-C). HRMS (ESI) for $C_{30}H_{40}N_3O_4S$ ($M+H]^+$): calcd 538.2734, found 538.2747.

*4.1.2.4. 5′-Methyl-[2′-(N-(4-fluorophenylcarbamoyl)]-3β,16β-di-hydroxyspiroandrost-5-ene-17,6′[1,3,4]thiadiazine (**5d**)*. Colorless solid; mp 211–214 °C; yield 63% (345 mg). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.79 (ddd, J = 5.13, 8.30, 12.33 Hz, 1H, 9-CH), 0.85 (s, 3H, 18-CH_3), 0.88 (s, 3H, 19-CH_3), 0.92–1.05 (m, 2H, 12-CH_2), 0.93–1.06 (m, 2H, 1-CH_2), 1.22–1.37 (m, 2H, 11-CH_2), 1.28–1.63 (m, 2H, 2-CH_2), 1.36–1.39 (m, 1H, 8-CH), 1.49–1.52 (m, 1H, 15-CH_2), 1.52–1.93 (m, 2H, 7-CH_2), 1.78 (ddd, J = 1.85, 11.31, 11.64 Hz, 1H, 14-CH), 1.85 (dd, J = 10.20, 10.34 Hz, 1H, 15-CH_2), 2.04–2.07 (dd, J = 12.56 Hz, 1H, 4-CH_2), 2.10–2.14 (dd, J = 3.96, 12.56 Hz, 1H, 4-CH_2), 2.47 (s, 3H, 21-CH_3), 3.19–3.21 (m, 1H, 3-CH), 5.20–5.24 (m, 1H, 16-CH), 5.23–5.26 (m, 1H, 6-CH), 5.66 (br. s, 1H, OH), 7.18 (dd, J = 8.20 Hz, 2H, 5′-CH + 7′-CH), 7.88 (dd, J = 5.14, 8.20 Hz, 2H, 4′-CH + 8′-CH), 10.58 (br. s, 1H, NH), signal of one OH group was not observed. ^{13}C NMR (125 MHz, DMSO-d_6): δ 14.8 (18-CH_3), 19.0 (19-CH_3), 20.0 (11-CH_2), 23.6 (21-CH_3), 31.1 (8-CH), 31.2 (7-CH_2), 31.3 (2-CH_2), 33.7 (15-CH_2), 34.6 (12-CH_2), 35.8 (10-C), 36.6 (1-CH_2), 42.1 (4-CH_2), 46.0 (14-CH), 48.8 (9-CH), 51.2 (13-C), 62.2 (17-C), 69.9 (3-CH), 72.5 (16-CH), 115.1 (d, $J_{C\text{-}F}$ = 23.0 Hz, 5′-CH + 7′-CH), 120.0 (6-CH), 122.3 (d, $J_{C\text{-}F}$ = 6.9 Hz, 4′-CH + 8′-CH), 134.4 (3′-C), 141.2 (5-C), 152.8 (2′-C), 154.0 (20-C), 158.5 (d, $J_{C\text{-}F}$ = 240.9 Hz, 6′-CH), 160.0 (1′-C). HRMS (ESI) for $C_{29}H_{36}FN_3O_3SNa$ ($[M+Na]^+$): calcd 548.2354, found 548.2345.

*4.1.2.5. 5′-Methyl-[2′-(N-(4-chlorophenylcarbamoyl)]-3β,16β-di-hydroxyspiroandrost-5-ene-17,6′[1,3,4]thiadiazine (**5e**)*. Colorless solid; mp 215–217 °C; yield 51% (276 mg). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.89–0.92 (m, 1H, 9-CH), 0.94 (s, 3H, 18-CH_3), 0.95 (s, 3H, 19-CH_3), 1.03–1.11 (m, 2H, 12-CH_2), 1.27–1.43 (m, 2H, 11-CH_2), 1.40–1.42 (m, 1H, 8-CH), 1.43–1.80 (m, 2H, 2-CH_2), 1.62–1.93 (m, 2H, 7-CH_2), 1.67–1.98 (m, 2H, 1-CH_2), 1.69–1.96 (m, 2H, 15-CH_2), 2.05–2.08 (m, 1H, 14-CH), 2.20–2.30 (m, 2H, 4-CH_2), 2.57 (s, 3H, 21-CH_3), 3.49–3.51 (m, 1H, 3-CH), 5.30–5.33 (m, 1H, 6-CH), 5.34 (dd, J = 3.38, 9.49 Hz, 1H, 16-CH), 7.34 (d, J = 9.25 Hz, 2H, 5′-CH + 7′-CH), 7.67 (d, J = 9.25 Hz, 2H, 4′-CH + 8′-CH), 9.28 (br. s, 1H, NH), signals of OH groups were not observed. ^{13}C NMR (125 MHz, DMSO-d_6): δ 15.3 (18-CH_3), 19.3 (19-CH_3), 20.4 (11-CH_2), 23.8 (21-CH_3), 31.5 (7-CH_2), 31.6 (2-CH_2), 31.8 (8-CH), 34.4 (15-CH_2), 34.9 (12-CH_2), 36.8 (10-C), 37.0 (1-CH_2), 42.2 (4-CH_2), 46.2 (14-CH), 48.9 (9-CH), 52.2 (13-C), 62.2 (17-C), 71.6 (3-CH), 74.4 (16-CH), 119.7 (6-CH), 120.9 (4′-CH + 8′-CH), 129.3 (5′-CH + 7′-CH), 129.4 (6′-C), 135.8 (3′-C), 140.9 (5-C), 153.1 (2′-C), 153.9 (20-C), 158.9 (1′-C). HRMS (ESI) for $C_{29}H_{37}ClN_3O_3S$ ($M+H]^+$): calcd 542.2239, found 542.2246.

*4.1.2.6. 5′-Methyl-[2′-(N-(4-(trifluoromethyl-phenyl)carba-moyl)]-3β,16β-dihydroxyspiro-androst-5-ene-17,6′[1,3,4]thiadiazine (**5f**)*. Colorless solid; mp 218–220 °C; yield 56% (322 mg). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.78 (ddd, J = 4.11, 11.30, 12.93 Hz, 1H, 9-CH), 0.84 (s, 3H, 18-CH_3), 0.87 (s, 3H, 19-CH_3), 0.89–1.06 (m, 2H, 12-CH_2), 0.90–1.03 (m, 2H, 1-CH_2), 1.19–1.34 (m, 2H, 11-CH_2), 1.27–1.60 (m, 2H, 2-CH_2), 1.35–1.38 (m, 1H, 8-CH), 1.49–1.53 (m, 1H, 15-CH_2), 1.53–1.92 (m, 2H, 7-CH_2), 1.78 (ddd, J = 1.84, 9.24, 11.30 Hz, 1H, 14-CH), 1.88 (dd, J = 10.16, 12.01 Hz, 1H, 15-CH_2), 2.04 (dd, J = 11.30 Hz, 1H, 4-CH_2), 2.12 (dd, J = 3.92, 11.30 Hz, 1H, 4-CH_2), 2.47 (s, 3H, 21-CH_3), 3.19–3.23 (m, 1H, 3-CH), 4.55 (d, J = 4.11 Hz, 1H, OH), 5.20–5.25 (m, 1H, 6-CH), 5.24 (m, 1H, 16-CH), 5.69 (d, J = 5.13 Hz, 1H, OH), 7.71 (d, J = 8.80 Hz, 2H, 5′-CH + 7′-CH), 8.10 (d, J = 8.80 Hz, 2H, 4′-CH + 8′-CH), 10.86 (br. s, 1H, NH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 14.7 (18-CH_3), 18.9 (19-CH_3), 19.9 (11-CH_2), 23.4 (21-CH_3), 31.1 (7-CH_2), 31.2 (8-CH), 31.3 (2-CH_2), 33.7 (15-CH_2), 34.6 (12-CH_2), 35.9 (10-C), 36.6 (1-CH_2), 42.0 (4-CH_2), 46.0 (14-CH), 48.8 (9-CH), 51.1 (13-C), 62.2 (17-C), 69.8 (3-CH), 72.5 (16-CH), 119.8 (6-CH), 120.4 (4′-CH + 8′-CH), 124.1 (6′-C), 124.2 (CF_3), 125.7 (5′-CH + 7′-CH), 141.3 (3′-C), 141.7 (5-C), 152.5 (2′-C), 154.0 (20-C), 160.5 (1′-C). HRMS (ESI) for $C_{30}H_{37}F_3N_3O_3S$ ($[M+H]^+$): calcd 576.2502, found 576.2509.

*4.1.3. General procedure for the preparation of 17-(N-aryl-6′H-1′,3′,4′-thiadiazine-2′-carboxamide)-3β-acetoxy-androst-5,17-dienes **7a-j***

Oxamic acid thiohydrazide (0.30 mmol) and potassium carbonate (39 mg, 0.28 mmol) were added to a solution of 3β-acetoxy-21-bromopregn-5,16-dien-20-one (122 mg, 0.28 mmol) in methanol (1.5 mL). The reaction mixture was stirred at r.t. for 6 h and concentrated at reduced pressure. The crude product was purified by column chromatography using petroleum ether/ethyl acetate (5:1).

*4.1.3.1. 17-(N-Phenyl-6′H-1′,3′,4′-thiadiazine-2′-carboxamide)-3β-acetoxy-androst-5,17-diene (**7a**)*. Yellow solid; mp $_{(\text{with dec})}$ 198–200 °C; yield 85% (126 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.81–0.97 (m, 2H, 1-CH_2, 9-CH), 1.11 (s, 3H, 19-CH_3), 1.12 (s, 3H, 18-CH_3), 1.49–1.77 (m, 7H, 1-CH_2, 2-CH_2, 8-CH, 11-CH_2, 14-CH), 1.81–1.96 (m, 2H, 7-CH_2), 2.06 (s, 3H, OAc), 2.09–2.24 (m, 2H, 4-CH_2), 2.29–2.44 (m, 2H, 12-CH_2), 2.44–2.50 (m, 1H, 15-CH_2), 2.61–2.71 (m, 1H, 15-CH_2), 3.18 (AB-system, J = 13.68 Hz, 1H, 2′-CH_2), 3.24 (AB-system, J = 13.68 Hz, 1H, 2′-CH_2), 4.57–4.71 (m, 1H, 3-CH), 5.38–5.47 (m, 1H, 6-CH), 6.62 (s, 1H, 16-CH), 7.18 (dd, J = 7.35 Hz, 1H, Ph), 7.39 (dd, J = 7.35 Hz, 2H, Ph), 7.70 (d, J = 7.35 Hz, 2H, Ph), 9.29 (br.s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 15.8 (18-CH_3), 19.4 (19-CH_3), 20.8 (2′-CH_2), 20.8 (11-CH_2), 21.5 (CO$\underline{CH_3}$), 27.8 (2-CH_2), 30.5 (8-CH), 31.6 (7-CH_2), 32.7 (12-CH_2), 35.4 (15-CH_2), 36.9 (10-C), 36.9 (1-CH_2), 38.3 (4-CH_2), 47.3 (13-C), 50.5 (9-CH), 57.1 (14-CH), 73.9 (3-CH), 119.8 (2 × CH, Ph), 122.1 (CH, Ph), 124.9 (6-C), 129.3 (2 × CH, Ph), 137.1 (16-CH), 140.4 (5-C), 141.9 (C, Ph), 146.0 (17-C), 150.9 (C), 152.4 (C), 158.3 (CO), 170.6 (CO). IR (KBr), ν/cm^{-1}: 3355 (NH), 2932, 2894, 2866 (CH), 1728 (CO), 1690 (CO), 1519, 1446, 1242, 1036, 751. HRMS (ESI) for $C_{31}H_{37}N_3O_3SNa$ ($[M+Na]^+$): calcd 554.2446, found 554.2448.

*4.1.3.2. 17-(N-(p-Tolyl)-6′H-1′,3′,4′-thiadiazine-2′-carboxamide)-3β-acetoxy-androst-5,17-diene (**7b**)*. Yellow solid; mp $_{(\text{with dec})}$ 186–188 °C; yield 73% (111 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.11 (s, 3H, 19-CH_3), 1.13 (s, 3H, 18-CH_3), 1.14–1.30 (m, 2H, 1-CH_2, 9-CH), 1.50–1.83 (m, 7H, 1-CH_2, 2-CH_2, 8-CH, 11-CH_2, 14-CH), 1.87–1.94 (m, 2H, 7-CH_2), 2.06 (s, 3H, OAc), 2.09–2.21 (m, 2H, 4-CH_2), 2.36 (s, 3H, Me), 2.36–2.42 (m, 2H, 12-CH_2), 2.41–2.48 (m, 1H, 15-CH_2), 2.64–2.69 (m, 1H, 15-CH_2), 3.18 (AB-system, J = 13.87 Hz, 1H, 2′-CH_2), 3.23 (AB-system, J = 13.87 Hz, 1H, 2′-CH_2), 4.60–4.68 (m, 1H, 3-CH), 5.41–5.45 (m, 1H, 6-CH), 6.61 (s, 1H, 16-CH), 7.19 (d, J = 8.20 Hz, 2H, Ar), 7.58 (d, J = 8.20 Hz, 2H, Ar), 9.24 (br.s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 15.7 (18-CH_3), 19.2 (19-CH_3), 20.7 (CH_3), 20.7 (2′-CH_2), 20.9 (11-CH_2), 21.4 (CO$\underline{CH_3}$), 27.7 (2-CH_2), 30.3 (8-CH), 31.5 (7-

CH_2), 32.5 (12-CH_2), 35.3 (15-CH_2), 36.8 (10-C), 36.9 (1-CH_2), 38.1 (4-CH_2), 47.2 (13-C), 50.3 (9-CH), 56.9 (14-CH), 73.9 (3-CH), 119.7 (2 × CH, Ar), 121.9 (6-CH), 129.7 (2 × CH, Ar), 134.5 (C, Ar), 134.5 (C, Ar), 140.3 (16-CH), 141.7 (5-C), 146.9 (17-C), 150.9 (C), 152.4 (C), 158.0 (CO), 170.5 (CO). IR (KBr), ν/cm^{-1}: 3355 (NH), 2932, 2894, 2866 (CH), 1728 (CO), 1690 (CO), 1519, 1446, 1242, 1036, 751. HRMS (ESI) for $C_{32}H_{40}N_3O_3S$ $(M+H]^+$): calcd 546.2786, found 546.2785.

*4.1.3.3. 17-(N-(4′-Ethylphenyl)-6′H-1′,3′,4′-thiadiazine-2′-carboxamide)-3β-acetoxy-androst-5,17-diene (**7c**)*. Yellow solid; mp (with dec) 159–161 °C; yield 76% (120 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.05–1.28 (m, 2H, 1-CH_2, 9-CH), 1.10 (s, 3H, 19-CH_3), 1.12 (s, 3H, 18-CH_3), 1.25 (t, J = 7.56 Hz, 3H, $CH_2\underline{CH_3}$), 1.48–1.83 (m, 7H, 1-CH_2, 2-CH_2, 8-CH, 11-CH_2, 14-CH), 1.85–1.95 (m, 2H, 7-CH_2), 2.05 (s, 3H, OAc), 2.07–2.21 (m, 2H, 4-CH_2), 2.30–2.39 (m, 2H, 12-CH_2), 2.39–2.47 (m, 1H, 15-CH_2), 2.61–2.70 (m, 1H, 15-CH_2), 2.65 (q, J = 7.56 Hz, 2H, CH_2CH_3), 3.17 (AB-system, J = 13.88 Hz, 1H, 2′-CH_2), 3.22 (AB-system, J = 13.88 Hz, 1H, 2′-CH_2), 4.59–4.68 (m, 1H, 3-CH), 5.38–5.45 (m, 1H, 6-CH), 6.60–6.61 (m, 1H, 16-CH), 7.21 (d, J = 8.20 Hz, 2H, Ar), 7.58 (d, J = 8.20 Hz, 2H, Ar), 9.25 (br.s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 15.6 ($CH_2\underline{CH_3}$), 15.7 (18-CH_3), 19.2 (19-CH_3), 20.6 (2′-CH_2), 20.7 (11-CH_2), 21.4 (CO$\underline{CH_3}$), 27.7 (2-CH_2), 28.4 ($\underline{CH_2}CH_3$), 30.3 (8-CH), 31.5 (7-CH_2), 32.5 (12-CH_2), 35.3 (15-CH_2), 36.8 (10-C), 36.9 (1-CH_2), 38.1 (4-CH_2), 47.1 (13-C), 50.3 (9-CH), 56.9 (14-CH), 73.9 (3-CH), 119.8 (2 × CH, Ar), 121.9 (6-CH), 128.5 (2 × CH, Ar), 134.6 (C, Ar), 140.3 (16-CH), 140.9 (5-C), 141.7 (C, Ar), 145.9 (17-C), 150.8 (C), 152.4 (C), 158.0 (CO), 170.5 (CO). IR (KBr), ν/cm^{-1}: 3339 (NH), 2963, 2935, 2897, 2858 (CH), 1734 (CO), 1674 (CO), 1522, 1374, 1248, 1034, 827, 670. HRMS (ESI) for $C_{33}H_{41}N_3O_3SK$ $([M+K]^+$): calcd 598.2499, found 598.2500.

*4.1.3.4. 17-(N-(4′-Methoxyphenyl)-6′H-1′,3′,4′-thiadiazine-2′-carboxamide)-3β-acetoxy-androst-5,17-diene (**7d**)*. Yellow solid; mp (with dec) 207–209 °C; yield 90% (140 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.81–0.97 (m, 2H, 1-CH_2, 9-CH), 1.11 (s, 3H, 19-CH_3), 1.12 (s, 3H, 18-CH_3), 1.51–1.72 (m, 7H, 1-CH_2, 2-CH_2, 8-CH, 11-CH_2, 14-CH), 1.84–1.96 (m, 2H, 7-CH_2), 2.06 (s, 3H, OAc), 2.09–2.23 (m, 2H, 4-CH_2), 2.28–2.44 (m, 2H, 12-CH_2), 2.44–2.50 (m, 1H, 15-CH_2), 2.61–2.71 (m, 1H, 15-CH_2), 3.17–3.23 (m, 2H, 2′-CH_2), 3.83 (s, 3H, OCH_3), 4.57–4.70 (m, 1H, 3-CH), 5.38–5.47 (m, 1H, 6-CH), 6.60–6.61 (m, 1H, 16-CH), 6.92 (d, J = 8.80 Hz, 2H, Ar), 7.62 (d, J = 8.80 Hz, 2H, Ar), 9.20 (br.s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 15.8 (18-CH_3), 19.3 (19-CH_3), 19.4 (2′-CH_2), 20.8 (11-CH_2), 21.5 (CO$\underline{CH_3}$), 27.8 (2-CH_2), 29.8 (8-CH), 30.5 (7-CH_2), 31.2 (12-CH_2), 36.6 (15-CH_2), 36.9 (1-CH_2), 37.0 (10-C), 38.2 (4-CH_2), 47.8 (13-C), 50.5 (9-CH), 55.6 (OCH_3), 57.1 (14-CH), 73.9 (3-CH), 114.3 (2 × CH, Ar), 120.4 (6-C), 121.5 (2 × CH, Ar), 130.0 (17-C), 138.3 (C, Ar), 140.2 (16-CH), 141.9 (5-C), 150.5 (C), 152.0 (C), 156.4 (C, Ar), 159.3 (CO), 170.7 (CO). IR (KBr), ν/cm^{-1}: 3363 (NH), 2965, 2931, 2864, 2820 (CH), 1724 (CO), 1678 (CO), 1519, 1371, 1245, 1037, 827. HRMS (ESI) for $C_{32}H_{39}N_3O_4SNa$ $([M+Na]^+$): calcd 584.2553, found 584.2556.

*4.1.3.5. 17-(N-(2′-Fluorophenyl)-6′H-1′,3′,4′-thiadiazine-2′-carboxamide)-3β-acetoxy-androst-5,17-diene (**7e**)*. Yellow solid; mp (with dec) 198–200 °C; yield 65% (100 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.05–1.30 (m, 2H, 1-CH_2, 9-CH), 1.10 (s, 3H, 19-CH_3), 1.11 (s, 3H, 18-CH_3), 1.47–1.83 (m, 7H, 1-CH_2, 2-CH_2, 8-CH, 11-CH_2, 14-CH), 1.83–1.96 (m, 2H, 7-CH_2), 2.04 (s, 3H, OAc), 2.07–2.22 (m, 2H, 4-CH_2), 2.31–2.42 (m, 2H, 12-CH_2), 2.43–2.48 (m, 1H, 15-CH_2), 2.62–2.72 (m, 1H, 15-CH_2), 3.18–3.20 (m, 2H, 2′-CH_2), 4.56–4.69 (m, 1H, 3-CH), 5.37–5.46 (m, 1H, 6-CH), 6.60–6.61 (m, 1H, 16-CH), 7.08–7.22 (m, 3H, Ar), 8.38–8.47 (m, 1H, Ar), 9.50 (br.s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 15.6 (18-CH_3), 19.3 (19-CH_3), 20.2 (2′-CH_2), 20.8 (11-CH_2), 21.5 (CO$\underline{CH_3}$), 27.8 (2-CH_2), 30.3 (8-CH), 31.4 (7-CH_2), 32.5 (12-CH_2), 35.5 (15-CH_2), 36.7 (1-CH_2), 36.9 (10-C), 38.2 (4-CH_2), 46.9 (13-C), 50.3 (9-CH), 57.0 (14-CH), 74.0 (3-CH), 115.2 (d, $J_{C\text{-}F}$ = 18.91 Hz, CH, Ar), 121.8 (d, $J_{C\text{-}F}$ = 25.87 Hz, CH, Ar), 122.2 (6-CH), 124.7 (d, $J_{C\text{-}F}$ = 3.65 Hz, CH, Ar), 125.6 (d, $J_{C\text{-}F}$ = 10.36 Hz, CH, Ar), 137.8 (16-CH), 140.2 (5-C), 142.8 (C, Ar), 146.9 (17-C), 150.4 (C), 151.6 (C), 152.9 (d, $J_{C\text{-}F}$ = 245.76 Hz, C, Ar), 158.6 (CO), 170.6 (CO). IR (KBr), ν/cm^{-1}: 3361 (NH), 2966, 2932, 2897, 2861 (CH), 1721 (CO), 1690 (CO), 1535, 1523, 1458, 1365, 1249, 1105, 1035, 870, 755. HRMS (ESI) for $C_{31}H_{36}FN_3O_3SNa$ $([M+Na]^+$): calcd 572.2360, found 572.2354.

*4.1.3.6. 17-(N-(4′-Chlorophenyl)-6′H-1′,3′,4′-thiadiazine-2′-carboxamide)-3β-acetoxy-androst-5,17-diene (**7f**)*. Yellow solid; mp (with dec) 162–164 °C; yield 90% (142 mg). ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.76–1.13 (m, 2H, 1-CH_2, 9-CH), 1.02 (s, 3H, 19-CH_3), 1.04 (s, 3H, 18-CH_3), 1.37–1.73 (m, 7H, 1-CH_2, 2-CH_2, 8-CH, 11-CH_2, 14-CH), 1.76–1.87 (m, 2H, 7-CH_2), 1.99 (s, 3H, OAc), 2.00–2.16 (m, 2H, 4-CH_2), 2.26–2.32 (m, 2H, 12-CH_2), 2.33–2.41 (m, 1H, 15-CH_2), 2.58–2.66 (m, 1H, 15-CH_2), 3.37 (AB-system, J = 14.34 Hz, 1H, 2′-CH_2), 3.54 (AB-system, J = 14.34 Hz, 1H, 2′-CH_2), 4.41–4.51 (m, 1H, 3-CH), 5.37–5.41 (m, 1H, 6-CH), 6.88–6.89 (m, 1H, 16-CH), 7.41 (d, J = 8.85 Hz, 2H, Ar), 7.89 (d, J = 8.85 Hz, 2H, Ar), 10.82 (br.s, 1H, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ 15.7 (18-CH_3), 19.2 (19-CH_3), 20.6 (2′-CH_2), 20.8 (11-CH_2), 21.4 (CO$\underline{CH_3}$), 27.7 (2-CH_2), 30.3 (8-CH), 31.5 (7-CH_2), 32.6 (12-CH_2), 35.3 (15-CH_2), 36.8 (1-CH_2), 36.9 (10-C), 38.1 (4-CH_2), 47.1 (13-C), 50.3 (9-CH), 56.9 (14-CH), 73.9 (3-CH), 121.0 (2 × CH, Ar), 121.9 (6-CH), 129.2 (2 × CH, Ar), 135.6 (16-CH), 140.3 (5-C), 142.1 (C, Ar), 145.6 (17-C), 150.8 (C), 153.1 (C), 163.3 (CO), 170.6 (CO), signal of one C group was not observed. IR (KBr), ν/cm^{-1}: 3324 (NH), 2934, 2904, 2853 (CH), 1724 (CO), 1673 (CO), 1590, 1521, 1373, 1250, 1064, 1033, 826. HRMS (ESI) for $C_{31}H_{37}ClN_3O_3S$ $(M+H]^+$): calcd 566.2238, found 566.2239.

*4.1.3.7. 17-(N-(4′-Bromophenyl)-6′H-1′,3′,4′-thiadiazine-2′-carboxamide)-3β-acetoxy-androst-5,17-diene (**7g**)*. Yellow solid; mp (with dec) 191–193 °C; yield 88% (150 mg). ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.78–1.09 (m, 2H, 1-CH_2, 9-CH), 1.01 (s, 3H, 19-CH_3), 1.03 (s, 3H, 18-CH_3), 1.35–1.73 (m, 7H, 1-CH_2, 2-CH_2, 8-CH, 11-CH_2, 14-CH), 1.74–1.87 (m, 2H, 7-CH_2), 1.97 (s, 3H, OAc), 1.98–2.15 (m, 2H, 4-CH_2), 2.24–2.31 (m, 2H, 12-CH_2), 2.32–2.41 (m, 1H, 15-CH_2), 2.56–2.64 (m, 1H, 15-CH_2), 3.33 (AB-system, J = 13.94 Hz, 1H, 2′-CH_2), 3.48 (AB-system, J = 13.94 Hz, 1H, 2′-CH_2), 4.40–4.50 (m, 1H, 3-CH), 5.34–5.39 (m, 1H, 6-CH), 6.83–6.84 (m, 1H, 16-CH), 7.46 (d, J = 9.05 Hz, 2H, Ar), 7.82 (d, J = 9.05 Hz, 2H, Ar), 10.75 (br.s, 1H, NH). ^{13}C NMR (75 MHz, DMSO-d_6): δ 15.9 (18-CH_3), 19.3 (19-CH_3), 20.2 (2′-CH_2), 20.8 (11-CH_2), 21.5 (CO$\underline{CH_3}$), 27.8 (2-CH_2), 30.3 (8-CH), 31.4 (7-CH_2), 32.5 (12-CH_2), 35.5 (15-CH_2), 36.7 (1-CH_2), 36.9 (10-C), 38.2 (4-CH_2), 46.9 (13-C), 50.3 (9-CH), 56.8 (14-CH), 73.6 (3-CH), 116.5 (C, Ar), 122.2 (6-CH), 122.8 (2 × CH, Ar), 131.8 (2 × CH, Ar), 137.8 (16-CH), 140.3 (5-C), 142.8 (C, Ar), 146.9 (17-C), 150.4 (C), 151.6 (C), 159.8 (CO), 170.0 (CO). IR (KBr), ν/cm^{-1}: 3434 (NH), 2937, 2903, 2867 (CH), 1720 (CO), 1676 (CO), 1585, 1521, 1373, 1248, 1064, 1034, 822. HRMS (ESI) for $C_{31}H_{37}BrN_3O_3S$ $(M+H]^+$): calcd 612.1712, found 612.1715.

*4.1.3.8. 17-(N-(4′-(Trifluoromethyl)phenyl)-6′H-1′,3′,4′-thiadiazine-2′-carboxamide)-3β-acetoxy-androst-5,17-diene (**7h**)*. Yellow solid; mp (with dec) 186–188 °C; yield 70% (119 mg). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.72–0.86 (m, 2H, 1-CH_2, 9-CH), 1.00 (s, 3H, 19-CH_3), 1.01 (s, 3H, 18-CH_3), 1.35–1.73 (m, 7H, 1-CH_2, 2-CH_2, 8-CH, 11-CH_2, 14-CH_2), 1.74–1.85 (m, 2H, 7-CH_2), 1.96 (s, 3H, OAc), 1.98–2.14 (m, 2H, 4-CH_2), 2.23–2.30 (m, 2H, 12-CH_2), 2.32–2.38 (m, 1H, 15-CH_2), 2.56–2.62 (m, 1H, 15-CH_2), 3.37 (AB-system, J = 13.94 Hz, 1H, 2′-CH_2), 3.54 (AB-system, J = 13.94 Hz, 1H, 2′-CH_2), 4.39–4.47 (m, 1H, 3-CH), 5.34–5.40 (m, 1H, 6-CH), 6.87–6.88 (m, 1H, 16-CH), 7.70 (d, J = 8.44 Hz, 2H, Ar), 8.03 (d, J = 8.44 Hz, 2H, Ar), 11.00 (br.s, 1H, NH). ^{13}C NMR (150 MHz, DMSO-d_6): δ 16.3 (18-CH_3), 19.6 (19-CH_3), 20.3 (2′-CH_2), 21.0 (11-CH_2), 21.8 (CO$\underline{CH_3}$), 28.1 (2-CH_2), 30.6 (8-CH),

31.6 (7-CH_2), 32.8 (12-CH_2), 35.7 (15-CH_2), 36.9 (10-C), 37.1 (1-CH_2), 38.3 (4-CH_2), 47.2 (13-C), 50.5 (9-CH), 57.1 (14-CH), 73.9 (3-CH), 121.3 (2 × CH, Ar), 122.5 (6-CH), 126.7 (2 × CH, Ar), 136.0 (16-C), 140.6 (5-C), 143.5 (C, Ar), 147.5 (17-C), 150.6 (C), 151.7 (C), 160.5 (CO), 170.4 (CO), signals of two C were not observed. IR (KBr), ν/cm^{-1}: 3350 (NH), 2964, 2938, 2897, 2860 (CH), 1734 (CO), 1686 (CO), 1532, 1324 (CF_3), 1249, 1167, 1117, 1060, 839. HRMS (ESI) for $C_{32}H_{36}F_3N_3O_3SK$ ($[M+K]^+$): calcd 638.2061, found 638.2061.

*4.1.3.9. 17-(N-(2′-Nitrophenyl)-6′H-1′,3′,4′-thiadiazine-2′-carboxamide)-3β-acetoxy-androst-5,17-diene (**7i**).* Yellow solid; mp (with dec) 136–138 °C; yield 77% (124 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.79–1.11 (m, 2H, 1-CH_2, 9-CH), 1.10 (s, 3H, 19-CH_3), 1.11 (s, 3H, 18-CH_3), 1.42–1.80 (m, 7H, 1-CH_2, 2-CH_2, 8-CH, 11-CH_2, 14-CH), 1.81–1.96 (m, 2H, 7-CH_2), 2.06 (s, 3H, OAc), 2.09–2.22 (m, 2H, 4-CH_2), 2.26–2.43 (m, 2H, 12-CH_2), 2.43–2.49 (m, 1H, 15-CH_2), 2.69–2.76 (m, 1H, 15-CH_2), 3.18–3.20 (m, 2H, 2′-CH_2), 4.59–4.69 (m, 1H, 3-CH), 5.36–5.47 (m, 1H, 6-CH), 6.59–6.60 (m, 1H, 16-CH), 7.23–7.28 (m, 1H, Ar), 7.67–7.76 (m, 1H, Ar), 8.25–8.33 (m, 1H, Ar), 8.84–8.91 (m, 1H, Ar), 12.09 (br.s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 15.7 (18-CH_3), 19.3 (19-CH_2), 20.6 (2′-CH_2), 20.7 (11-CH_2), 21.5 (CO$\underline{CH_3}$), 27.8 (2-CH_2), 30.4 (8-CH), 31.6 (7-CH_2), 32.6 (12-CH_2), 35.2 (15-CH_2), 36.8 (1-CH_2), 36.9 (10-C), 38.2 (4-CH_2), 47.2 (13-C), 50.4 (9-CH), 56.9 (14-CH), 73.9 (3-CH), 121.9 (6-CH), 122.3 (CH, Ar), 123.9 (CH, Ar), 126.0 (CH, Ar), 135.8 (CH, Ar), 137.2 (16-CH), 139.9 (5-C), 140.4 (C, Ar), 141.6 (C, Ar), 145.4 (17-C), 150.8 (C), 151.0 (C), 159.8 (CO), 170.6 (CO). IR (KBr), ν/cm^{-1}: 3286 (NH), 2937, 2903, 2863 (CH), 1728 (CO), 1696 (CO), 1583, 1502, 1436, 1341, 1247, 1033, 969, 861, 744. HRMS (ESI) for $C_{31}H_{37}N_4O_5S$ ($M+H]^+$): calcd 577.2470, found 577.2479.

*4.1.3.10. 17-(N-Benzyl-6′H-1′,3′,4′-thiadiazine-2′-carboxamide)-3β-acetoxy-androst-5,17-diene (**7j**).* Yellow solid; mp (with dec) 169–171 °C; yield 79% (120 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.08 (s, 3H, 19-CH_3), 1.09 (s, 3H, 18-CH_3), 1.11–1.20 (m, 2H, 1-CH_2, 9-CH), 1.43–1.81 (m, 8H, 1-CH_2, 2-CH_2, 8-CH, 11-CH_2, 12-CH_2, 14-CH), 1.85–1.93 (m, 2H, 7-CH_2), 2.05 (s, 3H, OAc), 2.09–2.18 (m, 2H, 4-CH_2), 2.30–2.38 (m, 1H, 12-CH_2), 2.38–2.45 (m, 1H, 15-CH_2), 2.58–2.64 (m, 1H, 15-CH_2), 3.13 (AB-system, J = 13.87 Hz, 1H, CH_2), 3.18 (AB-system, J = 13.87 Hz, 1H, CH_2), 4.57–4.64 (m, 1H, 3-CH), 4.60 (d, J = 5.99 Hz, 2H, CH_2), 5.38–5.45 (m, 1H, 6-CH), 6.56–6.57 (m, 1H, 16-CH), 7.30–7.37 (m, 5H, Ar), 7.73 (t, 1H, J = 5.99 Hz, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 15.7 (18-CH_3), 19.2 (19-CH_3), 20.5 (2′-CH_2), 20.6 (11-CH_2), 21.4 (CO$\underline{CH_3}$), 27.7 (2-CH_2), 30.3 (8-CH), 31.5 (7-CH_2), 32.5 (12-CH_2), 35.3 (15-CH_2), 36.8 (10-C), 36.9 (1-CH_2), 38.1 (4-CH_2), 43.8 ($\underline{CH_2}$Ph), 47.1 (13-C), 50.3 (9-CH), 56.9 (14-CH), 73.9 (3-CH), 121.9 (6-CH), 127.8 (CH, Ar), 128.1 (2 × CH, Ar), 128.8 (2 × CH, Ar), 137.2 (16-CH), 140.3 (5-C), 141.3 (C, Ar), 145.7 (17-C), 150.9 (C), 151.6 (C), 160.4 (CO), 170.5 (CO). IR (KBr), ν/cm^{-1}: 3350 (NH), 2937, 2904, 2868 (CH), 1733 (CO), 1685 (CO), 1524, 1373, 1245, 1072, 1033, 911, 811, 729. HRMS (ESI) for $C_{32}H_{40}N_3O_3S$ ($M+H]^+$): calcd 546.2783, found 546.2785.

4.2. Biology

4.2.1. Cell line and evaluation of antiproliferative activity

The human prostate cancer cell line 22Rv1 (CRL-2505) was purchased from the ATCC collection. Cells were cultured in standard RPMI-1640 medium (Gibco) supplemented with 10% fetal calf serum (FCS) (HyClone), RPMI-1640 Vitamins (PanEco) and 0.1 mg/ml sodium pyruvate (Santa Cruz) at 37 °C, 5% CO_2 and 80–85% humidity (NuAir CO_2 incubator). The cell growth was evaluated by the modified MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Applichem) test [52] as described in [19]. 22Rv1 cells were seeded at a density of 10^5 cells per well in 24-well plates (Corning) in 900 μL of the medium. The compounds were dissolved in DMSO (Applichem) to 5 mM before experiments and then were diluted in the medium to the required concentrations. The tested compounds with different concentrations in 100 μL of the medium were added 24 h after the seeding, and the cells were grown for 72 h. After incubation with the compounds, the medium was removed, and the MTT reagent dissolved in the medium was added to the final concentration of 0.2 mg/ml to each well and incubated for 3 h. The cell supernatants were removed and the MTT formazan purple crystals were dissolved in 100% DMSO (350 μL per well). Then the plates were gently shaken and the absorbance was measured at 571 nm with a MultiScan reader (ThermoFisher). The viability of the cells was assessed after subtraction of the blank value (the absorbance in the well w/o cells) from all wells. Dose-response curves were analyzed by regression analysis using sigmoidal curves (Log(concentration) vs normalized absorbance). The half maximal inhibitory concentrations (IC_{50}) were determined with GraphPad Prism.

4.2.2. AR and NF-κB activities

22Rv1 human prostate cancer cells were transfected with the NF-κB-LUC, ARE-LUC plasmids containing the luciferase reporter gene under the AR- or NF-κB-dependent promoters, and co-transfected with β-galactosidase plasmid. Cell transfection for reporter gene products was carried out as described earlier in [53]. Briefly, 22Rv1 cells were plated (5×10^5 cells/well) onto 24-well plate containing standard cell culture medium. After 24-h incubation, cells were co-transfected with plasmids containing luciferase and β-galactosidase genes. Phenol-free RPMI-1640 supplemented with 10% steroid-free serum were used for ARE-LUC assay. The plasmids used in this work were kindly provided by Prof. P. Hartig and Prof. A. Gasparian [54,55]. The transfection was carried out for 8 h at 37 °C using Lipofectamine 2000. Following transfection, medium was changed and cells were treated with synthesized compound or vehicle control. To induce AR transcriptional activity 22Rv1 cells were treated with 10 nM DHT.

The luciferase activity was measured according to a standard protocol (Promega) using a Tecan Infinite M200 Pro, β-galactosidase activity was analyzed by standard colorimetric assay using MultiScan FC (Thermo) as described in [8]. The luciferase/β-galactosidase activities were normalized by the internal control values and represented as the mean ± SD for the three independent experiments. NF-κB and AR activities calculated in arbitrary units as the ratio of luciferase/galactosidase activity.

4.2.3. Western blot analysis

22Rv1 cells were seeded on a 60 mm dishes (Corning). After 24-h growth the compounds were added in fresh medium as mentioned in the Fig. 2. To prepare cell extracts 22Rv1 cells were twice washed in phosphate buffer, and incubated for 10 min on ice in the modified lysis buffer containing 50 mM Tris-HCl, pH 7.5, 0.5% Igepal CA-630, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1 mM sodium orthovanadate and aprotinin, leupeptin, pepstatin (1 μg/ml each) as described earlier in [56]. The protein content was determined by the Bradford method [57].

Cell lysates (40 μg protein) were separated in 10% SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane (SantaCruz), and processed according to a standard protocol. To prevent nonspecific absorption, the membranes were treated with 5% nonfat milk solution in TBS buffer (20 mM Tris, 500 mM NaCl, pH 7.5) with 0.1% Tween-20 and then incubated with primary antibodies overnight at 4 °C.

AR, PARP, phosphorylated (ph) and unphosphorylated NF-κB p65, Akt and ERK1/2, antibodies were obtained from Cell Signaling Technology; the antibodies against α-tubulin (Cell Signaling Technology) were added to standardize loading. Goat anti-rabbit IgGs (Jackson ImmunoResearch) conjugated to horseradish peroxidase were used as secondary antibodies. Signals were detected using the ECL reagent as described in Mruk and Cheng's protocol [58] and an ImageQuant LAS4000 system (GE HealthCare). ImageJ software (NIH) was used for densitometry.

4.2.4. Statistical tests

Statistical analysis was performed using Microsoft Excel and GraphPad Prism. Each biology experiment was repeated three times and results were expressed as mean + S.D. (standard deviation value). Student's *t*-test was used to evaluate the significance of differences in comparisons. P value of < 0.05 was considered statistically significant.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103142.

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