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Authors: Marijana B. Živković, Ivana Z. Matić, Marko V. Rodić, Irena T. Novaković, Ana M. Krivokuća, Dušan M. Sladić, Natalija M. Krstić



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**Anticancer potential of new steroidal thiazolidin-4-one derivatives.  
Mechanisms of cytotoxic action and effects on angiogenesis *in vitro***

Marijana B. Živković<sup>a</sup>, Ivana Z. Matić<sup>b</sup>, Marko V. Rodić<sup>c</sup>, Irena T. Novaković<sup>a</sup>, Ana M. Krivokuća<sup>b</sup>, Dušan M. Sladić<sup>d</sup>, Natalija M. Krstić<sup>a,1</sup>

<sup>a</sup>Center for Chemistry, Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Studentski trg 12-16, P. O. Box 473, 11001 Belgrade, Republic of Serbia; E-mail: marijana@chem.bg.ac.rs; irenan@chem.bg.ac.rs

<sup>b</sup>Institute of Oncology and Radiology of Serbia, Pasterova 14, 11000 Belgrade, Republic of Serbia; E-mail: ivanamatic@sbb.rs; krivokuca.ana@gmail.com

<sup>c</sup>Faculty of Sciences, University of Novi Sad, Trg D. Obradovića 3, 21000 Novi Sad, Republic of Serbia; E-mail: marko.rodic@dh.uns.ac.rs

<sup>d</sup>Faculty of Chemistry, University of Belgrade, Studentski trg 12-16, P. O. Box 158, 11001 Belgrade, Republic of Serbia; dsladic@chem.bg.ac.rs

### Highlights

- The synthesis of new steroidal mono- and bis(thiazolidin-4-ones) was performed
- Pro-apoptotic action of 4a and 5a on HeLa cells
- Apoptosis in HeLa cells through extrinsic and intrinsic signaling pathways
- New steroidal mono- and bis(thiazolidin-4-ones) showed ability to decrease angiogenesis *in vitro*.

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<sup>1</sup>Corresponding author: Natalija Krstić, Center for Chemistry, Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Studentski trg 12-16, P. O. Box. 473, 11001 Belgrade, Republic of Serbia; Tel.: +381-11-3336-666; Fax: +3811-11-2636-061; E-Mail: [nkrstic@chem.bg.ac.rs](mailto:nkrstic@chem.bg.ac.rs)

**Abstract**

The synthesis and cytotoxic activities determination of new steroidal mono- and bis(thiazolidin-4-ones) **4a–f** and **5a–f** have been performed. Their anticancer action was also evaluated in comparison to previously synthesized and reported corresponding steroidal thiosemicarbazones. All compounds were obtained as stereoisomeric mixtures with different configuration (*E* or *Z*) in the hydrazone moiety at the C-3 position. After several consecutive crystallizations diastereomerically pure major (*E*)-isomers of mono-thiazolidin-4-ones were isolated. The structure and stereochemistry of 2,4-thiazolidinedione,2-[(17-oxoandrost-4-en-3-ylidene)hydrazone] were confirmed by X-ray analysis. A pathway for the formation of thiazolidin-4-one ring was proposed. The steroid thiazolidinone derivatives examined in this study exerted selective concentration-dependent cytotoxic activities on six tested malignant cell lines. Ten out of twelve examined compounds exhibited strong cytotoxic effects on K562 cells (IC<sub>50</sub> values from 8.5 μM to 14.9 μM), eight on HeLa cells (IC<sub>50</sub> values ranging from 8.9 μM to 15.1 μM) while against MDA-MB-361 cells six compounds exerted similar or even higher cytotoxic action (IC<sub>50</sub> values from 12.7 μM to 25.6 μM) than cisplatin (21.5 μM) which served as a positive control. Eight of these ten compounds showed high selectivity in the cytotoxic action against HeLa and K562 cancer cell lines when compared with normal human fibroblasts MRC-5 and normal human PBMC. The study of mechanisms of the anticancer activity of the two selected compounds, mono- and bis(thiazolidin-4-one) derivatives of 19-norandrost-4-ene-3,17-dione **4a** and **5a**, revealed that both of these compounds induced apoptosis in HeLa cells through extrinsic and intrinsic signalling pathways.

Treatment of EA.hy926 cells with sub-toxic concentrations of these compounds led to the inhibition of cell connecting and sprouting, and tube formation. The synthesized compounds exhibited poor antioxidant activity.

**Keywords:** Steroidal thiosemicarbazones; 1,3-Thiazolidin-4-ones; Cytotoxic activity; Apoptosis; Anti-angiogenic effect.

## 1. Introduction

Steroids are an important group of natural compounds playing a crucial role in many physiological and reproductive functions in the human body. Also, different types of steroids have been developed as drugs and anticancer agents [1]. The ability of hydrophobic steroid core to interact with cell membrane can enable transport of various functional groups or heterocyclic structures through them. As concerns the steroidal ligand-receptor binding mechanism, not only hydrophobic interactions but also hydrogen-bonding in some regions of the binding pocket is involved. Therefore, semisynthetic modifications involving the apolar sterane skeleton or the polar functional groups at C-3 and C-17 in the natural hormones may exert a significant influence on the binding affinity of the molecule [2]. Accordingly, the modifications of steroid molecules, by the addition of new functional groups or heterocyclic rings, resulting in new biological activities of these molecules, have become one of the major goals of steroid chemistry today.

Recently we reported synthesis of new steroidal mono- and bis(thiosemicarbazones) and mono- and bis(1,3,4-thiadiazolines) obtained from several androstene derivatives **1a–f** (Fig. 1) and their cytotoxic activity *in vitro* [3]. Mono- and bis(thiosemicarbazones) were found to be a mixtures of (*E*)- and (*Z*)-isomers differing in configuration on the C(3)=N1 double bond. In all cases the main isomer adopted *E* configuration. The corresponding mono- and bis(1,3,4-thiadiazolines) were formed as single heterocyclic compounds. 3-Thiosemicarbazones and 3,17-bis(thiadiazolines) exhibited the best activity against all cancer cell lines used in this work [3]. The study of the mechanisms of anticancer activity revealed that these compounds induced apoptosis in HeLa cells, through extrinsic and intrinsic signalling pathways [3]. Additionally, these compounds showed a strong anti-angiogenic activity. The results from our research also, suggest that  $\alpha,\beta$ -unsaturated thiosemicarbazone moiety at C-3 as well as the spiro heterocyclic substituent at C-17 position enhanced the activity of investigated compounds [3].

Motivated by the results mentioned above and as a continuation of our work on the new heterocyclic steroid derivatives as biologically active molecules [3-5], we decided to prepare new steroidal heterocyclic compounds derived from previously synthesized mono- and bis(thiosemicarbazones) **2a–f** and **3a–f** (Fig. 1) [3] and to investigate their biological activities. It is well known that thiosemicarbazones have been used as intermediates for a great variety of heterocyclic products, such as thiazolidinones, thiohydantoines, and thioxopyrimidinediones [6]. Among them 4-thiazolidinone derivatives have attracted continuing attention due to their diverse biological activities [7]. In recent years, the synthesis of 4-thiazolidinone derivatives

with anticancer activity against leukemia, melanoma, lung, colon, CNS, ovarian, renal, prostate and breast cancers cell lines has become a promising area of research [8,9]. To the best of our knowledge, very few steroidal thiazolidinones have been prepared so far [10-14] and their biological evaluation has been reported only for antibacterial action [15,16].

Here we describe the synthesis and configurational study of novel steroidal mono- and bis(thiazolidin-4-ones), and their *in vitro* cytotoxic activity. Since one of the goals of this study was to compare the activity of new heterocyclic derivatives with those reported earlier [3], the new compounds were tested against the same human malignant cell lines as the previous ones: cervical adenocarcinoma (HeLa), chronic myelogenous leukemia (K562), breast carcinoma (MDA-MB-453), breast adenocarcinoma (MDA-MB-361) colon adenocarcinoma (LS174) and lung adenocarcinoma (A549) cells. Besides, cytotoxicity of these compounds was tested on normal, human lung fibroblasts (MRC-5). The selectivity in the cytotoxic action of the most active compounds was evaluated against human normal peripheral blood mononuclear cells (PBMC).

The specific objective of this study was to clarify the mechanisms of the cytotoxic actions of the tested compounds. Therefore, the morphological analysis by fluorescence microscopy and cell cycle analyses by flow cytometry were performed, as well as the effects on caspase-3, caspase-8 and caspase-9 activities of two selected derivatives. The anti-angiogenic potential of these compounds was also explored. Finally, the effects of two selected steroid derivatives on the gene expression levels of matrix metalloproteinases 2 and 9 (*MMP2* and *MMP9*), and vascular endothelial growth factor A (*VEGFA*) were examined.

## 2. Materials and methods

### 2.1. Chemistry

#### 2.1.1. General methods

All chemicals and solvents were reagent grade and purchased from commercial sources. Solvents were prepared according to the standard procedures prior to use. Starting steroid derivatives were recrystallized from a suitable solvent. Melting points (uncorrected) were determined on Digital melting point WRS-1B apparatus. IR spectra were recorded on a Perkin-Elmer spectrophotometer FT-IR 1725X:  $\nu$  in  $\text{cm}^{-1}$ . NMR spectra (1D and 2D,  $^1\text{H}$  and  $^{13}\text{C}$ , DEPT, COSY, NOESY, HSQC, HMBC) were recorded in DMSO (if not otherwise stated) with a Bruker Avance 500 spectrometer (500 MHz for  $^1\text{H}$ , 125 MHz for  $^{13}\text{C}$ ) or Varian Gemini-200 spectrometer (200 MHz for  $^1\text{H}$ , 50 MHz for  $^{13}\text{C}$ ) using TMS as internal standard. The spin multiplicities are indicated by the symbols, s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br (broad). Chemical shifts are expressed in ppm ( $\delta$ ) values and coupling constants ( $J$ ) in Hz. The high-resolution mass spectra (HRMS) were taken with Agilent 6210 LC ESI-TOF-MS or Thermo Scientific HESI-LTQ Orbitrap XL spectrometers. Reactions were monitored by thin-layer chromatography (TLC) performed on precoated Merck silica gel 60 F<sub>254</sub> plates using double development in various solvent systems. The chromatograms were visualized first with ultraviolet light (254 nm) and then TLC spots were detected by spraying with 50% aq.  $\text{H}_2\text{SO}_4$  followed by heating. Flash column chromatography (FCC) was performed on silica gel Merck 0.040-0.063 mm.

#### 2.1.2. Synthesis of thiosemicarbazones and bis(thiosemicarbazones)

Thiosemicarbazones **2a–f** and bis(thiosemicarbazones) **3a–f** were prepared as described earlier [3].

#### 2.1.3. Synthesis of thiazolidin-4-one derivatives

The preparation procedure of new steroidal thiazolidin-4-one derivatives was analogous to that described previously [17] with minor modifications. Into a solution of steroidal thiosemicarbazones (**2a–f** or **3a–f**, 1 mmol) and anhydrous sodium acetate (123 or 246 mg, 1.5 or 3 mmol) in absolute ethanol (30 or 50 mL), ethyl chloroacetate (122 or 244 mg, 1 or 2 mmol) was slowly added. The mixture was heated under reflux until the reaction was completed (for 24–45h, monitored by TLC), allowed to attain room temperature and then poured into water. The precipitate was separated by filtration, washed with cold water and cold ethanol and dried. The collected solid was recrystallized from methanol and the filtrate was further chromatographed by FCC (yield, eluent and the ratio mentioned in each experiment). The chemical and physical spectral characteristics of these products (**4a–f** and **5a–f**) are given below.

2.1.3.1. *2,4-Thiazolidinedione,2-[(17-oxoestr-4-en-3-ylidene)hydrazone]* (**4a**). After filtration the product was recrystallized from MeOH to give 86 mg (22.3%) of pure **4a-E**.  $R_f = 0.58$  (PhMe/EtOAc 6:4). Purification of the residue by flash chromatography (8:2 PhMe/EtOAc) gave *E/Z* (2:1) mixture of **4a** (129 mg, 33.5%), which could not be further separated. Mp > 215 °C (decomp.); IR (ATR/cm<sup>-1</sup>): 3130 (NH) 3082, 2941, 2857 (CH), 1731 (C=O), 1717 (C=O), 1616 (C=N), 1327, 1248, 886. ESI-TOF-MS ( $m/z$ ): [M+H]<sup>+</sup> for C<sub>21</sub>H<sub>27</sub>N<sub>3</sub>O<sub>2</sub>S: calcd 386.18967, found 386.18961.

(**4a-E**). <sup>1</sup>H-NMR (500 MHz, DMSO-*d*<sub>6</sub>): 0.75 (m, 1H, H-9), 0.84 (s, 3H, H<sub>3</sub>C-18), 0.99 (qd,  $J = 12.5, 3$  Hz, 1H, H $\alpha$ -7), 1.13–1.33 (m, 4H, H $\alpha$ -1, H $\alpha$ -11, H $\alpha$ -12, H-14), 1.40–1.56 (m, 2H, H-8, H $\beta$ -15), 1.64 (br.d.,  $J = 10.5$  Hz, 1H, H $\beta$ -12), 1.78–1.90 (m, 3H, H $\beta$ -7, H $\beta$ -11, H $\alpha$ -15), 1.97–2.11 (m, 4H, H $\beta$ -1, H $\beta$ -2, H-10, H $\alpha$ -16), 2.22 (td,  $J = 13.5, 3.0$  Hz, 1H, H $\alpha$ -6), 2.40 (dd,  $J = 19, 8.5$  Hz, 1H, H $\beta$ -16), 2.47 (dt,  $J = 14, 3.0$  Hz, 1H, H $\beta$ -6), 3.04 (dt,  $J = 16.5, 4$  Hz, 1H, H $\alpha$ -2), 3.79 (s, 2H, H<sub>2</sub>-5'), 5.95 (s, 1H, H-4), 11.72 (s, 1H, NH). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>): 219.6 (s, C-17), 173.8 (s, C-4'), 162.3 (s, C-3), 161.2 (s, C-2'), 153.6 (s, C-5), 121.9 (d, C-4), 49.4 (d, C-14), 49.1 (d, C-9), 47.1 (s, C-13), 41.5 (d, C-10), 39.2 (d, C-8), 35.3 (t, C-16), 34.4 (t, C-6), 32.7 (t, C-5'), (31.2 (t, C-12), 29.7 (t, C-7), 26.1 (t, C-1), 25.2 (t, C-11), 24.3 (t, C-2), 21.4 (t, C-15), 13.4 (q, C-18).

(**4a-Z**) from the mixture *E/Z*=2:1. <sup>1</sup>H-NMR (500 MHz, DMSO-*d*<sub>6</sub>): 0.75 (m, 1H, H-9), 0.84 (s, 3H, H<sub>3</sub>C-18), 0.99 (qd,  $J = 12.5, 3$  Hz, 1H, H $\alpha$ -7), 1.13–1.33 (m, 4H, H $\alpha$ -1, H $\alpha$ -11, H $\alpha$ -12, H-14), 1.40–1.56 (m, 2H, H-8, H $\beta$ -15), 1.64 (br.d., 1H,  $J = 10.5$  Hz, 1H, H $\beta$ -12), 1.78–1.90 (m, 3H, H $\beta$ -7, H $\beta$ -11, H $\alpha$ -15), 1.97–2.11 (m, 3H, H $\beta$ -1, H-10, H $\alpha$ -16), 2.17–2.27 (m, 2H, H $\beta$ -2, H $\alpha$ -6), 2.33–2.43 (m, 2H, H $\alpha$ -2, H $\beta$ -16), 2.48 (m, 1H, H $\beta$ -6), 3.78 (s, 2H, H<sub>2</sub>-5'), 6.60 (s, 1H, H-4), 11.72 (s, 1H, NH). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>): 219.6 (s, C-17), 174.0 (s, C-4'), 161.3 (s, C-3), 159.5 (s, C-2'), 155.4 (s, C-5), 111.8 (d, C-4), 49.4 (d, C-14), 49.1 (d, C-9), 47.1 (s, C-13), 42.2 (d, C-10), 39.2 (d, C-8), 35.3 (t, C-16), 35.1 (t, C-6), 32.6 (t, C-5'), (31.2 (t, C-12), 30.0 (t, C-2), 29.8 (t, C-7), 26.9 (t, C-1), 25.1 (t, C-11), 21.4 (t, C-15), 13.4 (q, C-18).

2.1.3.2. *2,4-Thiazolidinedione,2-[(17-oxoandrost-4-en-3-ylidene)hydrazone]* (**4b**). After filtration and purification of the crude product by FCC (8:2 PhMe/EtOAc) the *E/Z* (2:1) diastereoisomeric mixture (267 mg, 67%) was obtained. Crystallization from MeOH gave pure (*E*)- isomer (107 mg, 27 %).  $R_f = 0.61$  (PhMe/EtOAc 6:4). Mp. 230.0–236.4 °C. The rest was inseparable *E/Z* (2:3) mixture. IR (ATR/cm<sup>-1</sup>): 3082 (NH), 2924 (CH), 1721 (C=O), 1612 (C=N), 1332, 1245. ESI-TOF-MS:  $m/z$  for C<sub>22</sub>H<sub>29</sub>N<sub>3</sub>O<sub>2</sub>S [M+H]<sup>+</sup>: calcd 400.20532, found 400.20549.

(**4b-E**). <sup>1</sup>H-NMR (500 MHz, DMSO-*d*<sub>6</sub>): 0.83 (s, 3H, H<sub>3</sub>C-18), 0.86 (m, 1H, H-9), 0.97 (qd, *J* = 12.5, 4.5 Hz, 1H, H $\alpha$ -7), 1.06 (s, 3H, H<sub>3</sub>C-19), 1.16 (td, *J* = 13, 4 Hz, 1H, H $\alpha$ -12), 1.21–1.40 (m, 3H, H $\alpha$ -1, H $\alpha$ -11, H-14), 1.51 (dtd, *J* = 3, 3.5, 3 Hz, 1H, H $\beta$ -15), 1.56 (m, 1H, H $\beta$ -11), 1.62–1.70 (m, 2H, H-8, H $\beta$ -12), 1.81–1.92 (m, 3H, H $\beta$ -1, H $\beta$ -7, H $\alpha$ -15), 1.99 (dd, *J* = 9.5, 18 Hz, 1H, H $\alpha$ -16), 2.11 (td, *J* = 16, 4.5 Hz, 1H, H $\beta$ -2), 2.30 (dtd, *J* = 5.0 Hz, 1H, H $\alpha$ -6), 2.35–2.45 (m, 2H, H $\beta$ -6, H $\beta$ -16), 3.03 (dt, *J* = 16.5, 3.5 Hz, 1H, H $\alpha$ -2), 3.795 (s, 2H, H<sub>2</sub>-5'), 5.88 (s, 1H, H-4), 11.75 (s, 1H, NH). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>): 219.6 (s, C-17), 173.8 (s, C-4'), 161.8 (s, C-3), 161.3 (s, C-2'), 157.8 (s, C-5), 121.2 (d, C-4), 53.3 (d, C-9), 50.2 (d, C-14), 46.9 (s, C-13), 37.9 (s, C-10), 35.3 (t, C-16), 34.8 (t, C-1), 34.6 (d, C-8), 32.7 (t, C-5'), 31.7 (t, C-6), 31.1 (t, C-12), 30.7 (t, C-7), 22.0 (t, C-2), 21.4 (t, C-15), 20.2 (t, C-11), 17.4 (q, C-19), 13.4 (q, C-18).

(**4b-Z**) from the mixture *E/Z*=2:3. <sup>1</sup>H-NMR (200 MHz, DMSO-*d*<sub>6</sub>): 0.83 (s, 3H, H<sub>3</sub>C-18), 0.92 (m, 1H, H-9), 1.00 (m, 1H, H $\alpha$ -7), 1.11 (s, 3H, H<sub>3</sub>C-19), 3.788 (s, 2H, H<sub>2</sub>-5'), 6.52 (s, 1H, H-4), 11.73 (s, 1H, NH). <sup>13</sup>C NMR (125 MHz, DMSO): 219.5 (s, C-17), 173.8 (s, C-4'), 160.7 (s, C-2'), 159.7 (s, C-3), 159.5 (s, C-5), 113.8 (d, C-4), 53.3 (d, C-9), 50.1 (d, C-14), 46.9 (s, C-13), 38.5 (s, C-10), 35.8 (t, C-1), 35.3 (t, C-16), 34.5 (d, C-8), 32.6 (t, C-5'), 32.3 (t, C-6), 31.0 (t, C-12), 30.9 (t, C-7), 27.0 (t, C-2), 21.4 (t, C-15), 20.0 (t, C-11), 17.5 (q, C-19), 13.4 (q, C-18).

2.1.3.3. *2,4-Thiazolidinedione,2-[(17-oxoandrosta-4,9(11)-dien-3-ylidene)hydrazone]* (**4c**). After filtration the crude product was purified by flash chromatography (75:25 PhMe/EtOAc) to give 226 mg (57%) of **4c**. After crystallization from MeOH pure (*E*)-isomer was obtained (88 mg, 22%). *R*<sub>f</sub> = 0.63 (PhMe/EtOAc 6:4). Mp > 200 °C (decomp.). The rest was *E/Z* (1:1) mixture which could not be separated. IR (ATR/cm<sup>-1</sup>): 2965 (NH), 2953 (CH), 1741 (C=O), 1724 (C=O), 1625 (C=N), 1334, 1252. ESI-TOF-MS: *m/z* for C<sub>22</sub>H<sub>27</sub>N<sub>3</sub>O<sub>2</sub>S [M+H]<sup>+</sup>: calcd 398.18967, found 398.18997.

(**4c-E**). <sup>1</sup>H-NMR (500 MHz, DMSO-*d*<sub>6</sub>): 0.79 (s, 3H, H<sub>3</sub>C-18), 1.00 (qd, *J* = 13, 3.5 Hz, 1H, H $\alpha$ -7), 1.23 (s, 3H, H<sub>3</sub>C-19), 1.46 (m, H-14), 1.58 (dtd, *J* = 3, 2.5, 3 Hz, 1H, H $\beta$ -15), 1.71 (td, *J* = 13.5, 4 Hz, 1H, H $\beta$ -1), 1.92–2.14 (m, 6H, H $\alpha$ -1, H $\beta$ -7, H $\alpha$ -12, H $\beta$ -12, H $\alpha$ -15, H $\alpha$ -16), 2.26 (dtd, *J* = 5, 4, 5 Hz, 1H, H $\beta$ -2), 2.32–2.47 (m, 3H, H $\alpha$ -6, H-8, H $\beta$ -16), 2.54 (m, 1H, H $\beta$ -6), 3.08 (dt, *J* = 17, 3.5 Hz, 1H, H $\alpha$ -2), 3.80 (s, 2H, H<sub>2</sub>-5'), 5.48 (d, *J* = 5.5 Hz, 1H, H $\beta$ -11), 5.91 (s, 1H, H-4), 11.78 (s, 1H, NH). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>): 220.3 (s, C-17), 173.8 (s, C-4'), 161.8 (s, C-3), 161.6 (s, C-2'), 156.3 (s, C-5), 146.3 (s, C-9), 121.4 (d, C-4), 116.4 (d, C-11), 47.5 (d, C-14), 45.2 (s, C-13), 40.5 (s, C-10), 36.4 (d, C-8), 35.8 (t, C-16), 33.1 (t, C-12), 33.0



(t, C-1), 32.7 (t, C-5'), 31.6 (t, C-6), 31.2 (t, C-7), 26.2 (q, C-19), 22.3 (t, C-2), 21.2 (t, C-15), 13.6 (q, C-18).

(**4c-Z**) from the mixture *E/Z*=1:1. <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>): 0.87 (s, 3H, H<sub>3</sub>C-18), 1.11 (m, 1H, H $\alpha$ -7), 1.28 (s, 3H, H<sub>3</sub>C-19), 1.48 (m, H-14), 1.61 (dtd, *J* = 3, 2.5, 3 Hz, 1H, H $\beta$ -15), 1.91–2.02 (m, 2H, H $\alpha$ -1, H $\beta$ -1), 2.03–2.21 (m, 5H, H $\beta$ -7, H $\alpha$ -12, H $\beta$ -12, H $\alpha$ -15, H $\alpha$ -16), 2.29–2.45 (m, 2H, H $\alpha$ -6, H-8), 2.46–2.50 (m, H $\beta$ -6, H $\beta$ -16), 2.57–2.63 (m, 2H, H $\alpha$ -2, H $\beta$ -2), 3.77 (s, 2H, H<sub>2</sub>-5'), 5.51 (d, *J* = 5.5 Hz, 1H, H $\beta$ -11), 6.52 (s, 1H, H-4). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): 221.5 (s, C-17), 173.8 (s, C-4'), 161.7 (s, C-3), 159.8 (s, C-2'), 159.4 (s, C-5), 145.9 (s, C-9), 117.1 (d, C-11), 111.4 (d, C-4), 48.1 (d, C-14), 45.9 (s, C-13), 41.6 (s, C-10), 36.9 (d, C-8), 36.2 (t, C-16), 34.2 (t, C-1), 33.4 (t, C-12), 33.2 (t, C-5'), 32.8 (t, C-6), 31.4 (t, C-7), 27.8 (t, C-2), 26.8 (s, C-19), 22.7 (t, C-15), 13.9 (q, C-18). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>): 220.2 (s, C-17), 173.8 (s, C-4'), 159.5 (s, C-3), 161.5 (s, C-2'), 158.0 (s, C-5), 146.1 (s, C-9), 116.6 (d, C-11), 114.1 (d, C-4), 47.5 (d, C-14), 45.2 (s, C-13), 41.3 (s, C-10), 36.3 (d, C-8), 35.8 (t, C-16), 34.0 (t, C-1), 33.1 (t, C-12), 33.3 (t, C-6), 32.7 (t, C-5'), 31.2 (t, C-7), 27.4 (t, C-2), 26.4 (q, C-19), 22.2 (t, C-15), 13.6 (q, C-18).

2.1.3.4. *2,4-Thiazolidinedione,2-[[11 $\alpha$ ]-11-hydroxy-17-oxoandrost-4-en-3-ylidene]hydrazone* (**4d**). After filtration the crude product was purified by flash chromatography (100:3 CH<sub>2</sub>Cl<sub>2</sub>/MeOH) to give *E/Z* (3:1) mixture of **4d** (357 mg, 86%) which could not be further separated. *R*<sub>f</sub> = 0.34 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 20:1). Mp > 200 °C (decomp.); IR (ATR/cm<sup>-1</sup>): 3480, 2928 (NH), 1725 (C=O), 1620, 1558 (C=N), 1330, 1242. HESI-Orbitrap MS: *m/z* for C<sub>22</sub>H<sub>29</sub>N<sub>3</sub>O<sub>3</sub>S [M+H]<sup>+</sup>: calcd 416.20079, found 416.19876.

(**4d-E**) from the mixture *E/Z*=3:1. <sup>1</sup>H-NMR (500 MHz, DMSO-*d*<sub>6</sub>): 0.83 (s, 3H, H<sub>3</sub>C-18), 0.94–1.01 (m, 2H, H $\alpha$ -7, H-9), 1.16 (s, 3H, H<sub>3</sub>C-19), 1.18 (m, 1H, H $\alpha$ -12), 1.31 (m, 1H, H-14), 1.48 (dtd, *J* = 3, 2.5, 3 Hz, 1H, H $\beta$ -15), 1.56 (td, *J* = 14, 3.5 Hz, 1H, H $\alpha$ -1), 1.63 (qd, *J* = 11.5, 2.5 Hz, 1H, H-8), 1.79–1.90 (m, 3H, H $\beta$ -7, H $\beta$ -12, H $\alpha$ -15), 2.02 (dd, *J* = 19, 9.5 Hz, 1H, H $\alpha$ -16), 2.16 (m, 1H, H $\beta$ -2), 2.27–2.33 (m, 2H, H $\alpha$ -6, H $\beta$ -6), 2.40 (dd, *J* = 19, 10.5 Hz, 1H, H $\beta$ -16), 2.58 (dt, *J* = 14, 4 Hz, 1H, H $\beta$ -1), 2.875 (dt, *J* = 17, 4 Hz, 1H, H $\alpha$ -2), 3.787 (s, 2H, H<sub>2</sub>-5'), 3.81 (m, 1H, H $\beta$ -11), 4.36 (d, *J* = 6.6 Hz, 1H, OH-11), 5.87 (s, 1H, H-4), 11.73 (s, 1H, NH). <sup>13</sup>C NMR (125 MHz, DMSO): 218.6 (s, C-17), 173.8 (s, C-4'), 162.3 (s, C-3), 161.7 (s, C-2'), 158.7 (s, C-5), 121.7 (d, C-4), 67.3 (d, C-11), 58.7 (d, C-9), 49.6 (d, C-14), 47.4 (s, C-13), 42.4 (t, C-12), 39.5 (s, C-10), 36.4 (t, C-1), 35.4 (t, C-16), 34.1 (d, C-8), 32.7 (t, C-6), 32.5 (t, C-5'), 30.3 (t, C-7), 22.4 (t, C-2), 21.3 (t, C-15), 18.2 (q, C-19), 14.3 (q, C-18).

(**4d-Z**) from the mixture *E/Z*=3:1. <sup>1</sup>H-NMR (500 MHz, DMSO-*d*<sub>6</sub>): 0.83 (s, 3H, H<sub>3</sub>C-18), 0.94–1.01 (m, 2H, H $\alpha$ -7, H-9), 1.20 (s, 3H, H<sub>3</sub>C-19), 1.18 (m, 1H, H $\alpha$ -12), 1.31 (m, 1H, H-8), 1.476

(dtd,  $J = 3, 2.5, 3$  Hz, 1H, H $\beta$ -15), 1.482 (m, 1H, H-14), 1.72 (m, 1H, H $\alpha$ -1), 1.79–1.90 (m, 3H, H $\beta$ -7, H $\beta$ -12, H $\alpha$ -15), 2.02 (dd,  $J = 19, 9.5$  Hz, 1H, H $\alpha$ -16), 2.16 (m, 1H, H $\beta$ -6), 2.26 (m, 1H, H $\beta$ -2), 2.37 (m, 1H, H $\alpha$ -6), 2.39 (m, 1H, H $\alpha$ -2, overlapped with H $\beta$ -16), 2.40 (dd,  $J = 19, 10.5$  Hz, 1H, H $\beta$ -16), 2.53 (t,  $J = 4.5$  Hz, 1H, H $\beta$ -1), 3.784 (s, 2H, H $_2$ -5'), 3.81 (m, 1H, H $\beta$ -11), 4.36 (d,  $J = 6.6$  Hz, 1H, OH-11), 6.50 (s, 1H, H-4), 11.73 (s, 1H, NH).  $^{13}\text{C}$  NMR (125 MHz, DMSO- $d_6$ ): 218.6 (s, C-17), 173.8 (s, C-4'), 161.7 (s, C-2'), 160.3 (s, C-5), 160.1 (s, C-3), 114.5 (d, C-4), 67.2 (d, C-11), 58.8 (d, C-9), 49.5 (d, C-14), 47.4 (s, C-13), 42.4 (t, C-12), 40.2 (s, C-10), 37.5 (t, C-1), 35.4 (t, C-16), 33.9 (d, C-8), 33.3 (t, C-6), 32.5 (t, C-5'), 30.7 (t, C-7), 27.2 (t, C-2), 21.3 (t, C-15), 18.4 (q, C-19), 14.3 (q, C-18).

2.1.3.5. *2,4-Thiazolidinedione,2-[[[(11 $\alpha$ )-11-(acetyloxy)-17-oxoandrost-4-en-3-ylidene]hydrazone]* (**4e**). After filtration the crude product was purified by flash chromatography (100:1.5 CH $_2$ Cl $_2$ /MeOH) to afford *E/Z* (3:1) mixture of **4e** (352 mg, 77%).  $R_f = 0.59$  (CH $_2$ Cl $_2$ /MeOH 20:1). Mp > 200 °C (decomp.); IR (ATR/cm $^{-1}$ ): 2967, 2934 (NH), 1733 (C=O), 1628, 1576 (C=N), 1330, 1248. HESI-Orbitrap MS:  $m/z$  for C $_{24}$ H $_{31}$ N $_3$ O $_4$ S [M+H] $^+$ : calcd 458.21135, found 458.20976.

(**4e-E**) from the mixture *E/Z*=3:1.  $^1\text{H}$ -NMR (500 MHz, CDCl $_3$ ): 0.97 (s, 3H, H $_3$ C-18), 1.14 (qd,  $J = 12.5, 5$  Hz, 1H, H $\alpha$ -7), 1.18 (s, 3H, H $_3$ C-19), 1.26 (m, 1H, H $\alpha$ -12), 1.36–1.45 (m, 2H, H-9, H-14), 1.57 (dtd,  $J = 3, 2.5, 3$  Hz, 1H, H $\beta$ -15), 1.69 (td,  $J = 13.5, 4.5$  Hz, 1H, H $\alpha$ -1), 1.73–1.86 (m, 2H, H $\beta$ -1, H-8), 1.97 (m, 1H, H $\beta$ -7), 2.01 (s, 3H, OCOCH $_3$ ), 2.013 (m, 1H, H $\alpha$ -15), 2.13 (dd,  $J = 19, 9.5$  Hz, 1H, H $\alpha$ -16), 2.20 (m, 1H, H $\beta$ -12), 2.30 (m, 1H, H $\beta$ -2), 2.34–2.40 (m, 2H, H $\alpha$ -6, H $\beta$ -6), 2.49 (dd,  $J = 19, 10.5$  Hz, 1H, H $\beta$ -16), 2.96 (dt,  $J = 17, 4$  Hz, 1H, H $\alpha$ -2), 3.767 (s, 2H, H $_2$ -5'), 5.24 (m, 1H, H $\beta$ -11), 6.05 (s, 1H, H-4).  $^{13}\text{C}$  NMR (125 MHz, CDCl $_3$ ): 218.2 (s, C-17), 173.6 (s, C-4'), 169.9 (c, OCOCH $_3$ ), 163.5 (s, C-3), 161.4 (s, C-2'), 157.3 (s, C-5), 122.6 (d, C-4), 70.4 (d, C-11), 55.7 (d, C-9), 49.6 (d, C-14), 47.4 (s, C-13), 39.5 (s, C-10), 38.2 (t, C-12), 35.9 (t, C-1), 35.6 (t, C-16), 34.7 (d, C-8), 33.2 (t, C-5'), 32.9 (t, C-6), 30.6 (t, C-7), 22.4 (t, C-2), 21.7 (q, OCOCH $_3$ ), 21.6 (t, C-15), 18.4 (q, C-19), 14.2 (q, C-18).

(**4e-Z**) from the mixture *E/Z*=3:1.  $^1\text{H}$ -NMR (500 MHz, CDCl $_3$ ): 0.97 (s, 3H, H $_3$ C-18), 1.14 (qd,  $J = 12.5, 5$  Hz, 1H, H $\alpha$ -7), 1.22 (s, 3H, H $_3$ C-19), 1.26 (m, 1H, H $\alpha$ -12), 1.39 (m, 1H, H-8), 1.49 (m, 1H, H-9) 1.52–1.63 (m, 2H, H-14, H $\beta$ -15), 1.73–1.86 (m, 2H, H $\alpha$ -1, H $\beta$ -2), 2.01 (m, 1H, H $\alpha$ -15), 2.02 (s, 3H, OCOCH $_3$ ), 2.13 (dd,  $J = 19, 9.5$  Hz, 1H, H $\alpha$ -16), 2.18 (m, 1H, H $\beta$ -7), 2.20 (m, 1H, H $\beta$ -12), 2.29–2.32 (m, 2H, H $\alpha$ -6, H $\beta$ -6), 2.43–2.47 (m, 2H, H $\beta$ -1, H $\alpha$ -2), 2.49 (dd,  $J = 19, 10.5$  Hz, 1H, H $\beta$ -16), 3.774 (s, 2H, H $_2$ -5'), 5.24 (m, 1H, H $\beta$ -11), 6.55 (s, 1H, H-4).  $^{13}\text{C}$  NMR (125 MHz, CDCl $_3$ ): 218.2 (s, C-17), 173.6 (s, C-4'), 169.9 (c, OCOCH $_3$ ), 161.5 (s, C-3), 161.4 (s, C-2'), 159.2 (s, C-5), 115.2 (d, C-4), 70.2 (d, C-11), 55.8 (d, C-9), 49.5 (d, C-14), 47.4 (s, C-

13), 30.2 (s, C-10), 38.2 (t, C-12), 36.9 (t, C-1), 35.6 (t, C-16), 34.6 (d, C-8), 33.3 (t, C-6), 33.1 (t, C-5'), 30.8 (t, C-7), 27.6 (t, C-2), 21.7 (q, OCOCH<sub>3</sub>), 21.6 (t, C-15), 18.6 (q, C-19), 14.2 (q, C-18).

2.1.3.6 *2,4-Thiazolidinedione,2-[(20-oxopregn-4-en-3-ylidene)hydrazone]* (**4f**). After filtration and crystallization from MeOH the *E/Z* mixture (9:1) of **4f** (264 mg, 62%) was obtained. *R*<sub>f</sub> = 0.65 (PhMe/EtOAc 6:4). Mp > 170 °C (decomp.); IR (ATR/cm<sup>-1</sup>): 2932 (NH), 2876 (CH), 1718, 1700 (C=O), 1618, 1576 (C=N), 1330, 1242. ESI-TOF-MS: *m/z* for C<sub>24</sub>H<sub>33</sub>N<sub>3</sub>O<sub>2</sub>S [M+H]<sup>+</sup>: calcd 428.23662, found 428.23653.

(**4f-E**). <sup>1</sup>H-NMR (500 MHz, DMSO-*d*<sub>6</sub>): 0.56 (s, 3H, H<sub>3</sub>C-18), 0.85 (td, *J* = 12, 3 Hz, 1H, H-9), 0.93 (qd, *J* = 12, 3.5 Hz, 1H, H $\alpha$ -7), 1.04 (s, 3H, H<sub>3</sub>C-19), 1.10–1.21 (m, 2H, H-14, H $\beta$ -15), 1.28–1.42 (m, 3H, H $\alpha$ -1, H $\alpha$ -11, H $\alpha$ -12), 1.46 (qd, *J* = 11, 2.5 Hz, 1H, H-8), 1.51–1.59 (m, 2H, H $\beta$ -11, H $\beta$ -16), 1.62 (m, 1H, H $\alpha$ -15), 1.74 (d, *J* = 12.5 Hz, 1H, H $\beta$ -7), 1.88 (m, 1H, H $\beta$ -1), 1.96–2.04 (m, 2H, H $\beta$ -12, H $\alpha$ -16), 2.05 (s, 3H, H<sub>3</sub>C-21), 2.10 (dtd, *J* = 4.5, 5, 4.5 Hz, 1H, H $\beta$ -2), 2.21–2.34 (m, 2H, H $\alpha$ -6, H $\beta$ -6), 2.56 (t, *J* = 9 Hz, 1H, H-17), 3.04 (dt, *J* = 17, 3.5 Hz, 1H, H $\alpha$ -2), 3.795 (s, 2H, H<sub>2</sub>-5'), 5.86 (s, 1H, H-4), 11.75 (s, 1H, NH). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>): 208.5 (s, C-20), 173.8 (s, C-4'), 161.9 (s, C-3), 161.4 (s, C-2'), 158.0 (s, C-5), 121.1 (d, C-4), 62.5 (d, C-17), 55.4 (d, C-14), 53.1 (d, C-9), 43.3 (s, C-13), 37.9 (t, C-12), 37.8 (s, C-10), 35.1 (d, C-8), 34.8 (t, C-1), 32.6 (t, C-5'), 31.9 (t, C-7), 31.8 (t, C-6), 31.1 (q, C-21), 23.9 (t, C-15), 22.2 (t, C-16), 22.0 (t, C-2), 20.9 (t, C-11), 17.3 (q, C-19), 13.0 (q, C-18).

(**4f-Z**). <sup>1</sup>H-NMR (500 MHz, DMSO-*d*<sub>6</sub>): 0.56 (s, 3H, H<sub>3</sub>C-18), 0.85 (m, 1H, H-9), 0.91 (m, 1H, H $\alpha$ -7), 1.07 (s, 3H, H<sub>3</sub>C-19), 1.10–1.21 (m, 2H, H-14, H $\beta$ -15), 1.28–1.42 (m, 3H, H $\alpha$ -1, H $\alpha$ -11, H $\alpha$ -12), 1.51–1.59 (m, 3H, H-8, H $\beta$ -11, H $\beta$ -16), 1.62 (m, 1H, H $\alpha$ -15), 1.77 (m, 1H, H $\beta$ -7), 1.89 (m, 1H, H $\beta$ -1), 1.96–2.04 (m, 2H, H $\beta$ -12, H $\alpha$ -16), 2.07 (s, 3H, H<sub>3</sub>C-21), 2.12 (m, 1H, H $\beta$ -6), 2.30 (m, 1H, H $\beta$ -2), 2.36 (m, 1H, H $\alpha$ -6), 2.43 (td, *J* = 14.4, 4 Hz, 1H, H $\alpha$ -2), 2.56 (t, *J* = 9 Hz, 1H, H-17), 3.789 (s, 2H, H<sub>2</sub>-5'), 6.50 (s, 1H, H-4), 11.75 (s, 1H, NH). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>): 208.5 (s, C-20), 173.8 (s, C-4'), 161.4 (s, C-2'), 159.7 (s, C-5), 159.5 (s, C-3), 113.8 (d, C-4), 62.5 (d, C-17), 55.4 (d, C-14), 53.4 (d, C-9), 43.3 (s, C-13), 37.9 (t, C-12), 38.6 (s, C-10), 35.9 (t, C-1), 35.0 (d, C-8), 32.5 (t, C-5'), 32.6 (t, C-6), 32.2 (t, C-7), 31.1 (q, C-21), 27.1 (t, C-2), 23.9 (t, C-15), 22.2 (t, C-16), 20.8 (t, C-11), 17.5 (q, C-19), 13.0 (q, C-18).

2.1.3.7. *Estr-4-ene-3,17-dione,3,17-bis[2-(4,5-dihydro-4-oxo-2-thiazolyl)hydrazone]* (**5a**). After filtration the crude product was purified by flash chromatography (1:1 PhMe/EtOAc) to give inseparable *E/Z* (2:1) mixture of **5a** (264 mg, 53%).. *R*<sub>f</sub> = 0.28 (PhMe/EtOAc, 6:4). Mp > 245 °C (decomp.); IR (ATR/cm<sup>-1</sup>): 2921 (NH), 2859 (CH), 1716 (C=O), 1620 (C=N), 1325,

1239. ESI-TOF-MS:  $m/z$  for  $C_{24}H_{30}N_6O_2S_2$   $[M+2H]^{2+}$ : calcd 250.10086, found 250.10125;  $m/z$  for  $C_{24}H_{30}N_6O_2S_2$   $[M+H]^+$ : calcd 499.19444, found 499.11940.

(**5a-E**).  $^1H$ -NMR (500 MHz, DMSO- $d_6$ ): 0.77 (m, 1H, H-9), 0.89 (s, 3H, H<sub>3</sub>C-18), 0.99 (qd,  $J = 12.5, 3.5$  Hz, 1H, H $\alpha$ -7), 1.12–1.19 (m, 2H, H $\alpha$ -1, H-14), 1.27–1.43 (m, 4H, H-8, H $\alpha$ -11, H $\alpha$ -12, H $\beta$ -15), 1.72–1.81 (m, 2H, H $\beta$ -7, H $\alpha$ -15), 1.82–1.89 (m, 2H, H $\beta$ -11, H $\beta$ -12), 1.96–2.13 (m, 3H, H $\beta$ -1, H $\beta$ -2, H-10), 2.23 (m, 1H, H $\alpha$ -6), 2.35 (m, 1H, H $\alpha$ -16), 2.43 (m, 1H, H $\beta$ -6), 2.47 (m, 1H, H $\beta$ -16), 3.04 (dt,  $J = 16.5, 3.5$  Hz, 1H, H $\alpha$ -2), 3.74 (s, 2H, H<sub>2</sub>-5''), 3.79 (s, 2H, H<sub>2</sub>-5'), 5.95 (s, 1H, H-4), 11.72 (s, 2H, 2NH).  $^{13}C$  NMR (125 MHz, DMSO- $d_6$ ): 178.6 (s, C-17), 174.0 (s, C-4''), 173.8 (s, C-4'), 162.4 (s, C-3), 161.8 (s, C-2''), 161.3 (s, C-2'), 153.6 (s, C-5), 121.9 (d, C-4), 51.7 (d, C-14), 49.3 (d, C-9), 44.2 (s, C-13), 41.6 (d, C-10), 39.28 (d, C-8), 34.5 (t, C-6), 33.8 (t, C-12), 32.7 (t, C(5')), 32.6 (t, C-5''), 30.4 (t, C-7), 27.9 (t, C-16), 26.1 (t, C-1), 25.6 (t, C-11), 24.4 (t, C-2), 22.8 (t, C-15), 16.9 (q, C-18).

(**5a-Z**).  $^1H$ -NMR (500 MHz, DMSO- $d_6$ ): 0.77 (m, 1H, H-9), 0.89 (s, 3H, H<sub>3</sub>C-18), 0.99 (qd,  $J = 12.5, 3.5$  Hz, 1H, H $\alpha$ -7), 1.25 (m, 1H, H $\alpha$ -1), 1.27–1.43 (m, 5H, H-8, H $\alpha$ -11, H $\alpha$ -12, H-14, H $\beta$ -15), 1.72–1.81 (m, 2H, H $\beta$ -7, H $\alpha$ -15), 1.82–1.89 (m, 2H, H $\beta$ -11, H $\beta$ -12), 2.06–2.13 (m, 2H, H $\beta$ -1, H-10), 2.23 (m, 1H, H $\alpha$ -6), 2.27 (m, 1H, H $\beta$ -2), 2.30–2.41 (m, 3H, H $\alpha$ -2, H $\beta$ -6, H $\alpha$ -16), 2.47 (m, 1H, H $\beta$ -16), 3.74 (s, 2H, H<sub>2</sub>-5''), 3.78 (s, 2H, H<sub>2</sub>-5'), 6.57 (s, 1H, H-4), 11.75 (s, 2H, 2NH).  $^{13}C$  NMR (125 MHz, DMSO- $d_6$ ): 178.6 (s, C-17), 174.0 (s, C-4''), 173.9 (s, C-4'), 160.2 (s, C-3), 161.8 (s, C-2''), 160.6 (s, C-2'), 155.5 (s, C-5), 121.7 (d, C-4), 51.6 (d, C-14), 49.5 (d, C-9), 44.2 (s, C-13), 42.4 (d, C-10), 39.33 (d, C-8), 35.2 (t, C-6), 33.8 (t, C-12), 32.7 (t, C(5')), 32.6 (t, C-5''), 30.6 (t, C-7), 27.9 (t, C-16), 26.9 (t, C-1), 25.5 (t, C-11), 29.9 (t, C-2), 22.8 (t, C-15), 16.9 (q, C-18).

2.1.3.8. *Androst-4-ene-3,17-dione,3,17-bis[2-(4,5-dihydro-4-oxo-2-thiazolyl)hydrazone]* (**5b**).

After filtration and crystallization from CHCl<sub>3</sub>/MeOH a pure (*E*)- isomer of **5b** was obtained (282 mg, 55%). The rest was complex mixture which could not be further separated.  $R_f = 0.25$  (PhMe/EtOAc, 6:4). Mp > 200 °C (decomp.); IR (ATR/cm<sup>-1</sup>): 3124, 2930 (NH), 2859 (CH), 1715 (C=O), 1655, 1618 (C=N), 1338, 1243. ESI-TOF-MS:  $m/z$  for  $C_{25}H_{32}N_6O_2S_2$   $[M+2H]^{2+}$ : calcd 257.10868, found 257.10937;  $m/z$  for  $C_{25}H_{32}N_6O_2S_2$   $[M+H]^+$ : calcd 513.21009, found 513.21057.

(**5b-E**).  $^1H$ -NMR (500 MHz, DMSO- $d_6$ ): 0.88 (s, 3H, H<sub>3</sub>C-18), 0.89 (m, 1H, H-9, overlapped with H<sub>3</sub>C-18), 0.97 (qd,  $J = 12.5, 3.5$  Hz, 1H, H $\alpha$ -7), 1.07 (s, 3H, H<sub>3</sub>C-19), 1.13 (m, 1H, H-14), 1.26–1.47 (m, 4H, H $\alpha$ -1, H $\alpha$ -11, H $\alpha$ -12, H $\beta$ -15), 1.55–1.67 (m, 2H, H-8, H $\beta$ -11), 1.72–1.92 (m, 4H, H $\beta$ -1, H $\beta$ -7, H $\beta$ -12, H $\alpha$ -15), 2.11 (dtd,  $J = 5, 2, 5$  Hz, 1H, H $\beta$ -2), 2.27 (m, 1H, H $\alpha$ -6), 2.35 (m, 2H, H $\beta$ -6, H $\beta$ -16), 2.47 (m, 1H, H $\alpha$ -16), 3.04 (dt,  $J = 17, 3.5$  Hz, 1H, H $\alpha$ -2), 3.74 (s,

2H, H<sub>2</sub>-5''), 3.79 (s, 2H, H<sub>2</sub>-5'), 5.87 (s, 1H, H-4), 11.72 (s, 2H, 2NH). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>): 178.5 (s, C-17), 173.8 (s, C-4' and C-4''), 162.0 (s, C-3), 161.4 (s, C-2' and C-2''), 157.9 (s, C-5), 121.1 (d, C-4), 53.5 (d, C-9), 52.4 (d, C-14), 43.9 (s, C-13), 37.9 (d, C-10), 34.8 (t, C-1), 34.7 (d, C-8), 33.8 (t, C-12), 32.6 (t, C-5' and C-5''), 31.7 (t, C-6), 31.3 (t, C-7), 27.8 (t, C-16), 22.9 (t, C-15), 22.0 (t, C-2), 20.4 (t, C-11), 17.4 (q, C-19), 16.8 (q, C-18).

2.1.3.9. *Androsta-4,9(11)-diene-3,17-dione,3,17-bis[2-(4,5-dihydro-4-oxo-2-thiazolyl)hydrazone]* (**5c**). After filtration the crude product was purified by flash chromatography (6:4 PhMe/EtOAc) to give *E/Z* (2:1) mixture of **5c** (260 mg, 51%) which could not be further separated. *R*<sub>f</sub> = 0.25 (PhMe/EtOAc, 6:4). Mp > 200 °C (decomp.); IR (ATR/cm<sup>-1</sup>): 2959 (NH), 2925 (CH), 1726 (C=O), 1660, 1626 (C=N), 1324, 1240. ESI-TOF-MS: *m/z* for C<sub>25</sub>H<sub>30</sub>N<sub>6</sub>O<sub>2</sub>S<sub>2</sub> [M+2H]<sup>2+</sup>: calcd 256.10086, found 256.10172; *m/z* for C<sub>25</sub>H<sub>30</sub>N<sub>6</sub>O<sub>2</sub>S<sub>2</sub> [M+H]<sup>+</sup>: calcd 511.19444, found 511.19440.

(**5c-E**). <sup>1</sup>H-NMR (500 MHz, DMSO-*d*<sub>6</sub>): 0.85 (s, 3H, H<sub>3</sub>C-18), 1.00 (qd, *J* = 13, 3.5 Hz, 1H, H<sub>α</sub>-7), 1.22 (s, 3H, H<sub>3</sub>C-19), 1.33 (m, 1H, H-14), 1.43 (dbr.sd, *J* = 3 Hz, 1H, H<sub>β</sub>-15), 1.72 (td, *J* = 14, 5 Hz, 1H, H<sub>β</sub>-1), 1.92 (m, 1H, H<sub>α</sub>-15), 1.95–2.05 (m, 2H, H<sub>α</sub>-1, H<sub>β</sub>-7), 2.05–2.18 (m, 2H, H<sub>α</sub>-12, H<sub>β</sub>-12), 2.26 (m, 1H, H<sub>β</sub>-2), 2.29 (m, 1H, H-8), 2.35 (m, 1H, H<sub>α</sub>-6), 2.39 (m, 1H, H<sub>α</sub>-16), 2.52–2.60 (m, 2H, H<sub>β</sub>-6, H<sub>β</sub>-16), 3.08 (dt, *J* = 17, 3.5 Hz, 1H, H<sub>α</sub>-2), 3.76 (s, 2H, H<sub>2</sub>-5''), 3.80 (s, 2H, H<sub>2</sub>-5'), 5.52 (d, *J* = 4.5 Hz, 1H, H<sub>β</sub>-11), 5.90 (s, 1H, H-4), 11.73 (s, 2H, 2NH). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>): 178.5 (s, C-17), 173.9 (s, C-4''), 173.8 (s, C-4'), 161.8 (s, C-3), 161.6 (s, C-2'), 161.5 (C-2''), 156.4 (s, C-5), 146.1 (s, C-9), 121.3 (d, C-4), 117.1 (d, C-11), 49.6 (d, C-14), 42.4 (s, C-13), 40.5 (d, C-10), 36.3 (d, C-8), 36.1 (t, C-12), 32.9 (t, C-1), 32.7 (s, C-5'), 32.6 (t, C-5''), 31.7 (t, C-6), 31.7 (t, C-7), 28.4 (t, C-16), 26.2 (q, C-19), 23.8 (t, C-15), 22.4 (t, C-2), 17.1 (q, C-18).

(**5c-Z**). <sup>1</sup>H-NMR (500 MHz, DMSO-*d*<sub>6</sub>): 0.85 (s, 3H, H<sub>3</sub>C-18), 1.00 (qd, *J* = 13, 3.5 Hz, 1H, H<sub>α</sub>-7), 1.26 (s, 3H, H<sub>3</sub>C-19), 1.43 (m, 1H, H-14, overlapped with H<sub>β</sub>-15), 1.43 (dbr.sd, *J* = 3 Hz, 1H, H<sub>β</sub>-15), 1.83 (td, *J* = 13.5, 4 Hz, 1H, H<sub>β</sub>-1), 1.92 (m, 1H, H<sub>α</sub>-15), 1.95–2.05 (m, 2H, H<sub>α</sub>-1, H<sub>β</sub>-7), 2.05–2.18 (m, 2H, H<sub>α</sub>-12, H<sub>β</sub>-12), 2.23 (m, 1H, H<sub>α</sub>-6), 2.29 (m, 1H, H-8), 2.39 (m, 1H, H<sub>α</sub>-16), 2.42 (m, 1H, H<sub>β</sub>-2), 2.52–2.60 (m, 3H, H<sub>α</sub>-2, H<sub>β</sub>-6, H<sub>β</sub>-16), 3.76 (s, 2H, H<sub>2</sub>-5''), 3.79 (s, 2H, H<sub>2</sub>-5'), 5.52 (d, *J* = 4.5 Hz, 1H, H<sub>β</sub>-11), 6.52 (s, 1H, H-4), 11.73 (s, 2H, 2NH). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>): 178.5 (s, C-17), 173.9 (2s, C-4' and C-4''), 161.5 (2s, C-2' and C-2''), 159.5 (s, C-3), 158.2 (s, C-5), 146.0 (s, C-9), 117.2 (d, C-11), 114.1 (d, C-4), 49.6 (d, C-14), 42.4 (s, C-13), 41.2(d, C-10), 36.3 (d, C-8), 36.1 (t, C-12), 34.0 (t, C-1), 32.7 (s, C-5'), 32.6 (t, C-5''), 32.4 (t, C-6), 31.7 (t, C-7), 28.4 (t, C-16), 27.4 (t, C-2), 26.4 (q, C-19), 23.8 (t, C-15), 17.1 (q, C-18).

2.1.3.10. (*11 $\alpha$* )-11-Hydroxyandrost-4-ene-3,17-dione,3,17-bis[2-(4,5-dihydro-4-oxo-2-thiazolyl)hydrazone] (**5d**). After filtration the crude product was purified by flash chromatography (100:5 CH<sub>2</sub>Cl<sub>2</sub>/MeOH) to give *E/Z* (4:1) mixture of **5d** (320 mg, 61%) which could not be further separated. *R<sub>f</sub>* = 0.30 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 20:1). Mp > 200 °C (decomp.). IR (ATR/cm<sup>-1</sup>): 3480, 2927 (NH), 1711 (C=O), 1615 (C=O), 1560 (C=N), 1331, 1240. HESI-Orbitrap MS: *m/z* for C<sub>25</sub>H<sub>32</sub>N<sub>6</sub>O<sub>3</sub>S<sub>2</sub> [M+H]<sup>+</sup>: calcd 529.20556, found 529.20381.

(**5d-E**). <sup>1</sup>H-NMR (500 MHz, DMSO-*d*<sub>6</sub>): 0.88 (s, 3H, H<sub>3</sub>C-18), 0.94–1.04 (m, 2H, H $\alpha$ -7, H-9), 1.17 (s, 3H, H<sub>3</sub>C-19), 1.20 (m, 1H, H-14, overlapped with H<sub>3</sub>C-19), 1.27–1.39 (m, 2H, H $\alpha$ -12, H $\beta$ -15), 1.52–1.63 (m, 2H, H $\alpha$ -1, H-8), 1.68–1.85 (m, 2H, H $\beta$ -7, H $\alpha$ -15), 2.08 (dd, *J* = 5, 12 Hz, 1H, H $\beta$ -12), 2.16 (dtd, *J* = 4.5, 4, 4.5 Hz 1H, H $\beta$ -2), 2.22–2.30 (m, 2H, H $\alpha$ -6, H $\beta$ -6), 2.39 (dd, *J* = 19, 9 Hz, 1H, H $\alpha$ -16), 2.50 (dd, *J* = 18.5, 9 Hz, 1H, H $\beta$ -16), 2.58 (dt, *J* = 14, 4 Hz, 1H, H $\beta$ -1), 2.88 (dt, *J* = 17, 3.5 Hz, 1H, H $\alpha$ -2), 3.76 (s, 2H, H<sub>2</sub>-5''), 3.79 (s, 2H, H<sub>2</sub>-5'), 3.85 (m, 1H, H $\beta$ -11), 4.32 (d, *J* = 6.5 Hz, 1H, OH-11), 5.86 (s, 1H, H-4), 11.72 (s, 1H, NH). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>): 177.9 (s, C-17), 173.9 (s, C-4'), 174.0 (s, C-4''), 162.4 (s, C-3), 161.8 (s, C-2''), 161.4 (s, C-2'), 158.9 (s, C-5), 121.7 (d, C-4), 67.4 (d, C-11), 58.4 (d, C-9), 51.9 (d, C-14), 45.1 (t, C-12), 44.4 (s, C-13), 39.5 (s, C-10), 36.4 (t, C-1), 34.2 (d, C-8), 32.8 (t, C-6), 32.7 (t, C-5''), 32.6 (t, C-5'), 31.0 (t, C-7), 28.0 (t, C-16), 22.9 (t, C-15), 22.4 (t, C-2), 18.3 (q, C-19), 17.8 (q, C-18).

(**5d-Z**). <sup>1</sup>H-NMR (500 MHz, DMSO-*d*<sub>6</sub>): 0.88 (s, 3H, H<sub>3</sub>C-18), 0.94–1.04 (m, 2H, H $\alpha$ -7, H-9), 1.21 (s, 3H, H<sub>3</sub>C-19), 1.27–1.39 (m, 3H, H $\alpha$ -12, H-14, H $\beta$ -15), 1.55 (m, 1H, H-8), 1.68–1.85 (m, 3H, H $\alpha$ -1, H $\beta$ -7, H $\alpha$ -15), 2.08 (dd, *J* = 5, 12 Hz, 1H, H $\beta$ -12), 2.10 (m, H $\beta$ -6), 2.21 (m, 1H, H $\beta$ -2), 2.39 (dd, *J* = 19, 9 Hz, 1H, H $\alpha$ -16), 2.50 (dd, *J* = 18.5, 9 Hz, 1H, H $\beta$ -16), 2.49 (m, 1H, H $\beta$ -1), 3.76 (s, 2H, H<sub>2</sub>-5''), 3.79 (s, 2H, H<sub>2</sub>-5'), 3.85 (m, 1H, H $\beta$ -11), 4.32 (d, *J* = 6.5 Hz, 1H, OH-11), 6.49 (s, 1H, H-4), 11.72 (br.s, 2H, 2NH). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>): 177.9 (s, C-17), 173.9 (s, C-4'), 174.0 (s, C-4''), 161.8 (s, C-2''), 161.4 (s, C-2'), 160.4 (s, C-3), 160.2 (s, C-5), 114.4 (d, C-4), 67.4 (d, C-11), 59.0 (d, C-9), 51.9 (d, C-14), 45.1 (t, C-12), 44.4 (s, C-13), 39.8 (s, C-10), 37.6 (t, C-1), 34.1 (d, C-8), 33.4 (t, C-6), 32.7 (t, C-5''), 32.6 (t, C-5'), 31.4 (t, C-7), 28.0 (t, C-16), 27.3 (t, C-2), 22.9 (t, C-15), 18.5 (q, C-19), 17.8 (q, C-18).

2.1.3.11. (*11 $\alpha$* )-11-(Acetyloxy)-androst-4-ene-3,17dione,3,17-bis[2-(4,5-dihydro-4-oxo-2-thiazolyl)hydrazone] (**5e**). After filtration the crude product was purified by flash chromatography (100:2 CH<sub>2</sub>Cl<sub>2</sub>/MeOH) to give inseparable *E/Z* (3:1) mixture of **5e** (410 mg, 72%). *R<sub>f</sub>* = 0.48 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 20:1). Mp > 200 °C (decomp.). IR (ATR/cm<sup>-1</sup>): 2967, 2934 (NH), 1719 (C=O), 1656, 1616, 1562 (C=N), 1332, 1244. HESI-Orbitrap MS: *m/z* for C<sub>27</sub>H<sub>34</sub>N<sub>6</sub>O<sub>4</sub>S<sub>2</sub> [M+H]<sup>+</sup>: calcd 571.21612, found 571.21406.

(**5e-E**). <sup>1</sup>H-NMR (500 MHz, DMSO-*d*<sub>6</sub>): 0.91 (s, 3H, H<sub>3</sub>C-18), 1.06 (qd, *J* = 13.5, 4.5 Hz, 1H, H $\alpha$ -7), 1.12 (s, 3H, H<sub>3</sub>C-19), 1.26–1.43 (m, 4H, H-9, H $\alpha$ -12, H-14, H $\beta$ -15), 1.56 (td, *J* = 13.5, 4 Hz, 1H, H $\alpha$ -1), 1.62–1.88 (m, 4H, H $\beta$ -1, H $\beta$ -7, H-8, H $\alpha$ -15), 2.01 (s, 3H, OCOCH<sub>3</sub>), 2.07–2.21 (m, 2H, H $\beta$ -2, H $\beta$ -12), 2.26–2.34 (m, 1H, H $\alpha$ -6), 2.36–2.45 (m, 2H, H $\beta$ -6, H $\alpha$ -16), 2.50 (m, 1H, H $\beta$ -16, overlapped with DMSO), 2.93 (dt, *J* = 17, 3.5 Hz, 1H, H $\alpha$ -2), 3.75 (s, 2H, H<sub>2</sub>-5''), 3.795 (s, 2H, H<sub>2</sub>-5'), 5.15 (m, 1H, H $\beta$ -11), 5.91 (s, 1H, H-4), 11.70 (br.s., 2H, 2NH). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>): 174.05 (s, C-4''), 173.95 (s, C-4'), 173.3 (s, C-17), 169.9 (c, OCOCH<sub>3</sub>), 162.2 (s, C-3), 162.2 (s, C-2''), 161.6 (s, C-2'), 157.1 (s, C-5), 122.2 (d, C-4), 70.5 (d, C-11), 55.4 (d, C-9), 51.1 (d, C-14), 44.1 (s, C-13), 39.5 (s, C-10), 40.6 (t, C-12), 35.8 (t, C-1), 34.3 (d, C-8), 32.8 (t, C-5'), 32.6 (t, C-5''), 32.4 (t, C-6), 31.0 (t, C-7), 28.0 (t, C-16), 22.9 (t, C-15), 22.3 (t, C-2), 21.6 (q, OCOCH<sub>3</sub>), 18.3 (q, C-19), 17.5 (q, C-18).

(**5e-Z**). <sup>1</sup>H-NMR (500 MHz, DMSO-*d*<sub>6</sub>): 0.91 (s, 3H, H<sub>3</sub>C-18), 1.06 (qd, *J* = 13.5, 4.5 Hz, 1H, H $\alpha$ -7), 1.17 (s, 3H, H<sub>3</sub>C-19), 1.26–1.43 (m, 4H, H-8, H $\alpha$ -12, H-14, H $\beta$ -15), 1.62–1.88 (m, 4H, H $\alpha$ -1, H $\beta$ -7, H-9, H $\alpha$ -15), 2.02 (s, 3H, OCOCH<sub>3</sub>), 2.07–2.21 (m, 3H, H $\alpha$ -6, H $\beta$ -6, H $\beta$ -12), 2.26–2.34 (m, 1H, H $\beta$ -2), 2.36–2.45 (m, 2H, H $\beta$ -1, H $\alpha$ -16), 2.50 (m, 1H, H $\beta$ -16, overlapped with DMSO), 2.93 (dt, *J* = 17, 3.5 Hz, 1H, H $\alpha$ -2), 3.75 (s, 2H, H<sub>2</sub>-5''), 3.787 (s, 2H, H<sub>2</sub>-5'), 5.15 (m, 1H, H $\beta$ -11), 6.53 (s, 1H, H-4), 11.70 (br.s., 2H, 2NH). <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>): 174.05 (s, C-4''), 173.95 (s, C-4'), 173.3 (s, C-17), 169.9 (c, OCOCH<sub>3</sub>), 162.2 (s, C-2''), 161.6 (s, C-2'), 159.6 (s, C-3), 158.6 (s, C-5), 115.0 (d, C-4), 70.3 (d, C-11), 55.6 (d, C-9), 51.0 (d, C-14), 44.1 (s, C-13), 39.7 (s, C-10), 41.5 (t, C-12), 36.9 (t, C-1), 34.2 (d, C-8), 33.1 (t, C-6), 32.8 (t, C-5'), 32.6 (t, C-5''), 31.3 (t, C-7), 28.0 (t, C-16), 27.2 (t, C-2), 22.9 (t, C-15), 21.1 (q, OCOCH<sub>3</sub>), 18.3 (q, C-19), 17.5 (q, C-18).

2.1.3.12. *Pregn-4-ene-3,20-dione,3,20-bis[2-(4,5-dihydro-4-oxo-2-thiazolyl)hydrazone]* (**5f**).

After filtration the crude product was purified by flash chromatography (6:4 PhMe/EtOAc) to afford *E/Z* (3:1) mixture of **5f** (366 mg, 68%) which could not be further separated. *R*<sub>f</sub> = 0.23 (toluene/EtOAc, 6:4). Mp = 275.0–278.3 °C. IR (ATR/cm<sup>-1</sup>): 2966 (NH), 2932 (CH), 1714 (C=O), 1632, 1616 (C=N), 1331, 1240, 1197. ESI-TOF-MS: *m/z* for C<sub>27</sub>H<sub>36</sub>N<sub>6</sub>O<sub>2</sub>S<sub>2</sub> [M+2H]<sup>2+</sup>: calcd 271.12433, found 271.12463; *m/z* for C<sub>24</sub>H<sub>30</sub>N<sub>6</sub>O<sub>2</sub>S<sub>2</sub> [M+H]<sup>+</sup>: calcd 541.24139, found 541.24142.

(**5f-E**). <sup>1</sup>H-NMR (500 MHz, DMSO-*d*<sub>6</sub>): 0.61 (s, 3H, H<sub>3</sub>C-18), 0.85 (td, *J* = 11, 3 Hz, 1H, H-9), 0.93 (qd, *J* = 13, 3.5 Hz, 1H, H $\alpha$ -7), 1.04 (s, 3H, H<sub>3</sub>C-19), 1.10–1.24 (m, 2H, H-14, H $\alpha$ -15), 1.25–1.35 (m, 3H, H $\alpha$ -1, H $\alpha$ -11, H $\alpha$ -12), 1.45–1.56 (m, 2H, H-8, H $\beta$ -11), 1.57–1.63 (m, 2H, H $\beta$ -15, H $\beta$ -16), 1.76 (m, 1H, H $\beta$ -7), 1.83–1.95 (m, 2H, H $\beta$ -1, H $\beta$ -12), 1.91 (s, 3H, H<sub>3</sub>C-21), 2.09 (m, 1H, H $\beta$ -2), 2.21–2.28 (m, 2H, H $\alpha$ -6, H $\alpha$ -16), 2.29–2.35 (m, 2H, H $\beta$ -6, H-17), 3.01 (dt,

$J = 17, 3.5$  Hz, 1H, H $\alpha$ -2), 3.76 (s, 2H, H $_2$ -5''), 3.793 (s, 2H, H $_2$ -5'), 5.86 (s, 1H, H-4), 11.71 (s, 2H, 2NH).  $^{13}\text{C}$  NMR (125 MHz, DMSO- $d_6$ ): 173.8 (2s, C-4' and C-4''), 165.3 (s, C-20), 162.0 (s, C-3), 161.4 (s, C-2''), 161.3 (s, C-2'), 158.1 (s, C-5), 121.1 (d, C-4), 58.1 (d, C-17), 55.4 (d, C-14), 53.4 (d, C-9), 43.3 (s, C-13), 38.2 (t, C-12), 37.8 (s, C-10), 35.4 (d, C-8), 34.9 (t, C-1), 32.7 (t, C-5'), 32.6 (t, C-5''), 31.9 (t, C-7), 31.8 (t, C-6), 23.8 (t, C-15), 23.1 (t, C-16), 22.0 (t, C-2), 20.9 (t, C-11), 19.0 (q, C-21), 17.4 (q, C-19), 13.0 (q, C-18).

(**5f-Z**).  $^1\text{H}$ -NMR (500 MHz, DMSO- $d_6$ ): 0.61 (s, 3H, H $_3$ C-18), 0.85 (td,  $J = 11, 3$  Hz, 1H, H-9), 0.93 (qd,  $J = 13, 3.5$  Hz, 1H, H $\alpha$ -7), 1.08 (s, 3H, H $_3$ C-19), 1.10–1.24 (m, 2H, H-14, H $\alpha$ -15), 1.25–1.35 (m, 2H, H $\alpha$ -11, H $\alpha$ -12), 1.40 (m, 1H, H $\alpha$ -1), 1.45–1.56 (m, 2H, H-8, H $\beta$ -11), 1.57–1.63 (m, 2H, H $\beta$ -15, H $\beta$ -16), 1.76 (m, 1H, H $\beta$ -7), 1.83–1.95 (m, 2H, H $\beta$ -1, H $\beta$ -12), 1.91 (s, 3H, H $_3$ C-21), 2.16 (m, 1H, H $\alpha$ -6), 2.24 (m, 1H, H $\alpha$ -16), 2.28–2.35 (m, 2H, H $\beta$ -2, H-17), 2.37 (m, 1H, H $\beta$ -6), 2.41 (qd,  $J = 14.5, 3.5$  Hz, 1H, H $\alpha$ -2), 3.76 (s, 2H, H $_2$ -5''), 3.787 (s, 2H, H $_2$ -5'), 6.50 (s, 1H, H-4), 11.70 (s, 2H, 2NH).  $^{13}\text{C}$  NMR (125 MHz, DMSO- $d_6$ ): 173.9 (s, C-4'), 173.8 (s, C-4''), 165.3 (s, C-20), 161.4 (s, C-2''), 161.3 (s, C-2'), 159.9 (s, C-5), 159.8 (s, C-3), 113.8 (d, C-4), 58.0 (d, C-17), 55.3 (d, C-14), 53.6 (d, C-9), 43.3 (s, C-13), 38.5 (s, C-10), 38.2 (t, C-12), 35.9 (t, C-1), 35.3 (d, C-8), 32.7 (t, C-5'), 32.6 (t, C-5''), 32.6 (t, C-6), 32.2 (t, C-7), 27.2 (t, C-2), 23.8 (t, C-15), 23.1 (t, C-16), 20.8 (t, C-11), 19.0 (q, C-21), 17.6 (q, C-19), 13.0 (q, C-18).

## 2.2. Single crystal X-ray crystallography

The crystal X-ray diffraction data for **4b-E** were collected on a Gemini S diffractometer (Oxford Diffraction) with Mo  $K\alpha$  radiation ( $\lambda = 0.71073$  Å) at room temperature. Data collection, reduction, and analytical absorption correction were performed with the *CRYCALISPRO* software [18]. Crystal structure was solved by using *SHELXT* [19] and refined by using *SHELXL-2014* [20], with *SHELXLE* [21] as a graphical interface.

All non-hydrogen atoms were refined anisotropically. The hydrogen atoms were placed at geometrically idealized positions with fixed bond lengths. Their isotropic displacement parameters were coupled to  $U_{\text{eq}}$  of the parent atoms. Absolute structure was assigned to match known absolute configuration of the steroid molecule. Occupational factor for water oxygen was refined to 0.475(2), and was fixed to 0.5 in the last refinement step. Hydrogen atoms belonging to the water molecule could not be located from difference density maps.

The final results of crystal structure determination are summarized as follows. Molecular formula: C $_{22}$ H $_{30}$ N $_3$ O $_{2.5}$ S;  $M_r = 408.55$ ; crystal system: orthorhombic; space group:  $P2_12_12_1$ ; unit cell parameters:  $a = 7.5223(3)$  Å,  $b = 9.7210(4)$ ,  $c = 28.8938(16)$  Å,  $V = 2112.84(18)$  Å $^3$ ;  $Z = 4$ ;  $\mu = 0.179$  mm $^{-1}$ ; 19050 reflections measured, 4949 unique reflections ( $R_{\text{int}} = 0.038$ ); 99.9% data completeness up to  $2\theta = 52.64^\circ$ , 91.1% data completeness up to  $2\theta_{\text{max}} = 58.04^\circ$ ; 3699



reflections with  $I > 2\sigma(I)$ ; number of refined parameters: 264;  $R_1 = 0.054$  for data with  $I > 2\sigma(I)$ ;  $wR_2 = 0.174$  for all data; goodness-of-fit  $S = 0.827$ ; Flack  $x = 0.02(5)$  determined by Parsons' quotient method [22].

Crystallographic data for **4b-E** have been deposited with the Cambridge Crystallographic Data Centre as Supplementary publication No. CCDC 1541508. A copy of these data can be obtained, free of charge, via [www.ccdc.cam.ac.uk/data\\_request/cif](http://www.ccdc.cam.ac.uk/data_request/cif), or by emailing [data\\_request@ccdc.cam.ac.uk](mailto:data_request@ccdc.cam.ac.uk).

### 2.3. Biology

#### 2.3.1. Cytotoxicity assay

The cytotoxic activity of the compounds was evaluated against six human malignant cell lines: cervical adenocarcinoma (HeLa), chronic myelogenous leukemia (K562), breast carcinoma (MDA-MB-453), breast adenocarcinoma (MDA-MB-361), colon adenocarcinoma (LS174), lung carcinoma (A549), and one normal human cell line, lung fibroblasts (MRC-5). The cytotoxicity assay procedure has been described elsewhere [3,23]. Stock solutions of compounds were prepared in DMSO at a concentration of 10 mM. For each experiment, the solutions were diluted with nutrient medium and applied to cells to various final concentrations ranging from 6.25  $\mu\text{M}$  to 100  $\mu\text{M}$  or 12.50  $\mu\text{M}$  to 200  $\mu\text{M}$ . The final concentration of DMSO did not cause any toxicity to the cells. Survival of cells was determined by MTT assay after 72 h of continuous action, according to the method of Mosmann [24], which was modified by Ohno and Abe [25] and described in detail in our previous studies [3-5, 23].

All tested cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Cisplatin served as a positive control.

The isolation and the treatment of human peripheral blood mononuclear cells (PBMC) were performed according to the previously reported procedure [26]. The applied concentrations of selected compounds ranged from 12.50  $\mu\text{M}$  to 200  $\mu\text{M}$ .

The experiments on PBMC isolated from healthy blood donors were approved by the Ethics Committee of the Institute of Oncology and Radiology of Serbia. All healthy donors completed and signed written informed consent.

#### 2.3.2. Cell cycle analysis

HeLa and K562 cells were treated with  $\text{IC}_{50}$  and  $2\text{IC}_{50}$  concentrations of compounds **4a** and **5a** for 24 h and 48 h. After incubation, the cells were collected, washed and fixed in 70% ethanol, according to the standard procedure [23,26,27]. Cell cycle phase distributions were determined using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The analysis of acquired data (10,000 events collected for each gated cell sample) was

performed using a CELLQuest software (BD Biosciences). Cell cycle distribution data are presented as mean±S.D. of three independent experiments. The statistical significance of differences between the control and treated cell samples was evaluated using one-way ANOVA with Dunnett's post test. *p* values below 0.05 were considered statistically significant.

### 2.3.3. Morphological analysis by fluorescence microscopy

To evaluate the mode of cell death induced by compounds **4a** and **5a**, morphological analysis by fluorescence microscopy of HeLa cells stained with a mixture of nucleic acid dyes acridine orange and ethidium bromide was performed, according to the previously described procedure [23,26]. The visualization was performed using Carl Zeiss PALM MicroBeam microscope with Axio Observer.Z1 and AxioCam MRm camera (filters: Alexa 488 and Alexa 568, 20x magnification).

### 2.3.4. Effects of specific caspase inhibitors

To explore the effects of specific caspase inhibitors on the pro-apoptotic activity of compounds **4a** and **5a** in HeLa cells, the percentages of cells within the subG1 phase were measured by flow cytometry, as previously described [23,26]. The specific caspase inhibitors applied at a concentration of 40 µM included: Z-DEVD-FMK, a caspase-3 inhibitor, Z-IETD-FMK, a caspase-8 inhibitor and Z-LEHD-FMK, a caspase-9 inhibitor (R&D Systems, Minneapolis, USA).

### 2.3.5. Tube formation assay

The potential antiangiogenic properties of compounds **4a** and **5a** were investigated on human umbilical vein EA.hy926 cell line using endothelial cell tube formation assay [28,29]. The *in vitro* angiogenesis assay was performed according to the previously described protocol [29]. The EA.hy926 cells seeded on Corning® Matrigel® basement membrane matrix (Corning: cat. number 356234) were treated with low sub-toxic IC<sub>20</sub> concentrations of compounds **4a** and **5a** (30 µM and 35 µM, respectively) for 20 h. Photomicrographs of EA.hy926 cells were captured under the inverted phase-contrast microscope using 6.3x magnification.

### 2.3.6. Gene expression analyses

HeLa cells were seeded in 75 cm<sup>2</sup> cell culture flasks (4×10<sup>6</sup> cells/flask). After 24 h, the nutrient medium was removed, and cells were treated with low sub-toxic IC<sub>20</sub> concentrations of compounds **4a** and **5a** (5 µM). Control cells were grown in nutrient medium. After a 24h incubation period, the cells were harvested by trypsinization and washed. The cell samples were then stored at -80°C until further analyses. For the purpose of real-time-quantitative PCR (RT-qPCR) analysis, total RNA was extracted from HeLa cells using TRI Reagent® (Sigma) according to the manufacturer's instructions. RNA RIN was determined by Agilent RNA 6000

Nano Kit (Agilent Technologies) on 2100 Bionalyzer and RNA concentration was determined spectrophotometrically (BioSpec Nano, Shimadzu). High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) was used to prepare primary complementary DNA (cDNA) with random primers by RT-PCR. 2 $\mu$ g total RNA was used as a template for MultiScribe™ Reverse Transcriptase.

#### Real Time quantitative PCR (RT-qPCR)

Measurement of mRNA expression levels was done by quantitative real time PCR (qPCR) and Taqman assays. TaqMan® Gene Expression Assays (*MMP2*- Hs01548727\_m1, *MMP9*- Hs00234579\_m1 and *VEGFA*- Hs00900055\_m1) consist of a 20X mix of unlabeled PCR primers and TaqMan® MGB probes (FAM™ dye-labeled). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for which data was obtained using TaqMan control reagents (Applied Biosystems- Hs02758991\_g1) was used as an endogenous control. PCR reactions were performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The PCR reaction conditions were described previously [30].

#### 2.3.7. Free-radical scavenging antioxidant assay (DPPH method)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined by the method of Blois [31]. Tested compounds were dissolved in pure DMSO.

Ascorbic acid (AA) was used as the reference compound (positive control) with concentrations from 50–500 mg mL<sup>-1</sup>.

### 3. Results and Discussion

#### 3.1. Chemistry

New steroidal mono- and bis(thiazolidin-4-ones) **4a–f** and **5a–f** were obtained in good yields (51-86%) as shown in Scheme 1 by intermolecular cyclocondensation reaction of thiosemicarbazones **2a–f** and **3a–f** [3] with ethyl chloroacetate in boiling absolute ethanol containing anhydrous sodium acetate [17]. The reaction conditions were optimized by varying amount of ethyl chloroacetate and anhydrous sodium acetate and duration of the reaction. The highest yields were obtained when reaction was carried out with small excess of anhydrous sodium acetate (1:1.5 and 1:3 equiv.) and with one and two equivalents of cyclization reagent for mono- and bis(1,3-thiazolidin-4-ones) **4a–f** and **5a–f** during 24 and 45 h, respectively.

As it was reported in our previous paper [3], thiosemicarbazones **2a–f** and **3a–f** were obtained as inseparable mixtures of isomers (*E/Z*) differing in configuration in the hydrazone moiety at the C-3 position. Since the cyclization reaction of these thiosemicarbazones does not affect the C(3)=N1 double bond, all mono- and bis(thiazolidin-4-ones) **4a–f** and **5a–f** were also obtained as stereoisomeric mixtures. This time, we were more fortunate and after several

consecutive crystallizations diastereomerically pure major (*E*)-isomers of **4a–f** were isolated. As all our attempts to get the pure minor isomers had failed, their structures were determined on the basis of the spectral data ( $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR) for the mixtures.

Besides C(17)=O/C(20)=O carbonyl bands (at about 1730/1700  $\text{cm}^{-1}$ ) the IR spectra of **4a–f** showed the absorption bands for the new lactam C=O group at 1717–1725  $\text{cm}^{-1}$ . Also, all compounds showed stretches at 1612–1628  $\text{cm}^{-1}$  that were assigned to C=N vibrations, and intense bands at 1239–1252  $\text{cm}^{-1}$  attributed to the  $\nu(\text{CH}_2\text{-S})$  stretching vibration, all confirming the formation of thiazolidinone ring.

In the  $^1\text{H}$  NMR spectra the signals for the amino groups from the starting thiosemicarbazones **2a–f** were missing. Instead, the new signals for the methylene protons at C-5' of the thiazolidinone ring moiety appeared in the range of  $\delta$  3.787–3.795 ppm for (*E*)-isomers and at  $\delta$  3.770–3.789 ppm for (*Z*)-isomers of **4a–f**. Also, the singlets for H-4 proton at about  $\delta$  6.50 ppm for all (*Z*)-isomers of **4a–f** were situated downfield from the resonance for the corresponding proton in the starting (*Z*)-thiosemicarbazones **2a–f**, which was attributed to the C(4')=O carbonyl group anisotropy due to the position of the thiazolidinone ring. The signal for the same proton in (*E*)-isomers appeared at about  $\delta$  5.85 ppm. In addition, in all (*E*)-thiazolidinones, H $\alpha$ -2 protons resonate well-separated downfield from the other ring protons as a *dt* at about  $\delta$  3.00 ppm. The signals for NH appeared at about  $\delta$  11 ppm.

The  $^{13}\text{C}$  NMR spectra of **4a–f** displayed characteristic signals of the 4-thiazolidinone skeleton: at  $\delta$  173.8 ppm for C-4' carbonyl in both isomers, at  $\delta$  161.2–161.7 ppm for C(2')=N in (*E*)-isomers and at  $\delta$  159.5–161.4 ppm for C(2')=N in (*Z*)-isomers, and at  $\delta$  32.5–33.2 ppm for methylene C-5' carbons. The other characteristic signals were: at  $\delta$  121.2–122.4 ppm and 111.8–115.2 ppm (C-4), 161.8–163.5 ppm and 159.5–161.5 ppm (C-3), 153.6–158.7 ppm and 155.4–160.3 ppm (C-5) for **4a–f-E** and **4a–f-Z**, respectively.

Finally, the molecular formulas of **4a–f** were deduced from the HR mass spectra that have shown molecular weights of obtained compounds 40 Da higher compared to molecular weights of the parent thiosemicarbazones (**2a–f**).

In the absence of any NOESY correlations the X-ray analysis of **4b-E** was used to confirm the structure of the major isomer of **4a–f** and its geometry (Figure 2). The compound crystalize in non-centrosymmetric space group  $P2_12_12_1$ . The absolute structure has been unequivocally assigned by known chirality of steroidal carbon atoms, and is also in agreement with absolute structure parameter Flack  $x = 0.02(5)$ , determined by Parsons' quotient method [22]. The steroidal core adopts the expected conformation, *i.e.* rings B and C are in chair conformations  $^{\text{C}5}\text{C}_{\text{C}8}$  and  $^{\text{C}9}\text{C}_{\text{C}13}$ , respectively, while five-

membered ring D adopts envelope conformation  ${}^{C14}E$  [32]. As expected, due to  $sp^2$  hybridization of C3, C4, and C5 atoms, and presence of C4–C5 double bond (1.335(5) Å), the ring A is in half-chair conformation  ${}^{C2}H_{C1}$ . This can be quantified by asymmetry parameters [33], which have values  $C_2(C1-C2) = C_2(C4-C5) = 0.9(5)^\circ$ , and by torsion angle  $\tau(C3-C4-C5-C10) = -5.0(6)^\circ$ .

It is noteworthy to say that, up to now, there is only one structurally characterized compound with cyclohexene-3-ylidenehydrazono-thiazolidin-4-one fragment [34]. The geometry of thiazolidin-4-one ring is in accordance with structural data of compounds containing hydrazono-thiazolidin-4-one fragment, retrieved from the CSD [35]. The sulfur atom S1' is asymmetrically bonded with two carbon atoms in a way that bond with  $sp^2$  hybridized C2' is shorter (1.751(4) Å) than bond with  $sp^3$  hybridized C5' (1.812(5) Å). Also, the bond length C4'–O2 (1.216(5) Å) corresponds to localized double bond. The thiazolidin-4-one ring is roughly coplanar with C5–C4–C3–N1–N2 fragment of  $sp^2$  hybridized atoms. However, closer inspection shows that the ring is slightly puckered by small displacement of S1' atom from the ring plane. The nature and amount of puckering is evident through Cremer–Pople parameters  $Q_2 = 0.100(4)$  Å and  $\varphi_2 = 2(3)^\circ$  [36]. Based on these data and asymmetry parameters  $C_s(S1') = C_s(C4'-N3') = 1.5(5)^\circ$ , its conformation is best described as envelope  ${}^{S1'}E$ .

The bond lengths C3–N1 and C2'–N2 correspond to the localized double bonds between respective atoms. Structural data revealed *E*-configuration around C3–N1, and *Z*-configuration around C2'–N2 double bonds, which can be expressed through torsion angles  $\tau(C4-C3-N1-N2) = -179.9(3)^\circ$  and  $\tau(N1-N2-C2'-S1') = -2.7(5)^\circ$ , respectively. To the best of our knowledge, this is the first steroidal derivative containing C3-bonded hydrazono-thiazolidin-4-one fragment with known configuration of double bonds.

The dominant intermolecular interaction in the crystal structure is N3'–H3...O2<sup>i</sup> hydrogen bond, which connects molecules in chains along crystallographic axis *a* (Fig. S1), with graph set descriptor  $C_1^1(4)$  [37] (hydrogen bond data: N3'–H3 = 0.86 Å, H3...O2<sup>i</sup> = 2.05(4) Å, N3'...O2<sup>i</sup> = 2.903(5) Å,  $\angle(N-H\cdots O3^i) = 170.1^\circ$ ; symmetry code (i): 1/2+x, 1/2-y, 1-z).

Molecular formulas of bis(thiazolidin-4-ones) **5a–f** deduced from their HR mass spectra showed that the molecular weights of these compounds were 80 Da higher than ones of parent bis(thiosemicarbazones) **3a–f** and 113 Da higher than the molecular weights of **4a–f**. This indicates that compounds **5a–f** have one more thiazolidinone moiety. Also, in the IR spectra of **5a–f** all the characteristic absorption bands for thiazolidinone ring were present.

As in the case of parent bis(thiosemicarbazones) **3a–f**, the NMR spectral data of **5a–f** revealed the existence of two diastereoisomers differing only in the configuration around

C(3)=N1 double bond while at C-17/C-20 position only one isomer occurred. The  $^1\text{H}$  NMR spectra of **5a–f** showed singlets at  $\delta$  3.74, 3.74, 3.76, 3.76, 3.75 and 3.76 ppm, respectively, for the methylene protons at C-5'' (4-thiazolidinone ring at C-17/C-20) in both isomers. Also, the singlets for  $\text{CH}_3$ -18 protons appeared at the same value for both isomers, as well as the signals for  $\text{H}\alpha$ -16 and  $\text{H}\beta$ -16. Furthermore, the  $^{13}\text{C}$  NMR spectra showed single characteristic signals at  $\delta$  178.6, 178.5, 178.5, 177.9, 173.3 and 165.3 ppm for C-17, at  $\delta$  174.4, 173.8, 173.9, 174.0, 174.05, 173.8 ppm for C-4'' carbonyl and at 161.8, 161.4, 161.5, 161.8, 161.2 and 161.4 ppm for C-2'' in compounds **5a–f**, respectively. Regarding other signals, almost the same patterns of  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were observed as in the spectra for compounds **4a–f**.

The proposed mechanism of the formation of these new steroidal thiazolidin-4-one is as follows (Scheme 2). First step involves S-alkylation of thiosemicarbazones **2a–f** and **3a–f** in thiol form (due to the sodium acetate used). This step is followed by a nucleophilic attack by  $\text{NH}_2$  on carbon atom of carbonyl group and ethanol removal, resulting in formation of 4-thiazolidinones **4a–f** and **5a–f**. The excess of sodium acetate was used to scavenge the hydrogen chloride, whereas ethyl chloroacetate acts as the cyclizing agent.

### 3.2. Cytotoxic activity

The *in vitro* cytotoxic activity of all newly synthesized compounds **4a–f** and **5a–f** was tested against six human malignant cell lines (HeLa, K562, MDA-MB-453, MDA-MB-361, LS174 and LS174 ) and one normal human cell line (MRC-5). Cisplatin was employed as the positive control. The data summarized in Table 1 indicate that all given compounds exerted cytotoxic action at micromolar concentrations on investigated malignant cell lines, especially strong towards K562 cells, which showed the highest sensitivity to the cytotoxic action of these new steroid derivatives. Against this cell line compounds **4a–d**, **4f**, **5a–d** and **5f** showed a strong cytotoxic activity with  $\text{IC}_{50}$  values of 8.8, 8.7, 8.8, 11.3, 8.7, 8.9, 10.5, 9.6, 11.6 and 14.9  $\mu\text{M}$ , respectively, while the other two compounds **4e** and **5e** were slightly less active with  $\text{IC}_{50}$  values of 19.2 and 21.9  $\mu\text{M}$ , respectively. Compounds, **4a–c**, **4f**, **5a–c** and **5f** exhibited a strong cytotoxic action against HeLa cells with  $\text{IC}_{50}$  values of 8.9–15.1  $\mu\text{M}$ . This cell line demonstrated a moderate sensitivity ( $\text{IC}_{50}$  values from 28.0–51.4  $\mu\text{M}$ ) against all other compounds. Almost all new steroid derivatives, with the exception of **4d** and **5d**, showed good to moderate activity against both breast cancer cell lines, with  $\text{IC}_{50}$  values from 10.7–46.7  $\mu\text{M}$ . However, it must be noted that compounds **4a**, **4b**, **4c**, **4f**, **5c** and **5f** exerted similar or even better cytotoxic action ( $\text{IC}_{50}$  values of 12.7, 25.6, 19.6, 14.5, 24.6 and 20.6  $\mu\text{M}$ , respectively) against MDA-MB-361 cells compared to cisplatin (21.5  $\mu\text{M}$ ). A549 cells were sensitive to the cytotoxic actions of **4a**, **4f**, **5a–c** and **5f** which showed cytotoxic activity comparable to one of

cisplatin, with  $IC_{50}$  values in the range of 17.2–27.6  $\mu$ M. Moreover, the activity of these compounds was almost three times lower against lung fibroblasts MRC-5 cell line with  $IC_{50}$  values from 31.4–62.7  $\mu$ M. The colorectal adenocarcinoma LS174 cells were the least sensitive to all tested compounds, except towards **5b** and **5f** the activity of which was the same or even higher in comparison to cisplatin, with  $IC_{50}$  values of 21.8 and 11.0  $\mu$ M, respectively.

The results mentioned above demonstrate that mono-thiazolidinones **4a**, **4b**, **4c** and **4f** exhibited the highest cytotoxic activities against all cell lines tested, in some cases higher than corresponding starting thiosemicarbazones **2a–c** and **2f** [3]. The activity of bis-thiazolidinones **5a–f**, although slightly lower, was notably higher in comparison to corresponding bis-thiosemicarbazones **3a–f**, which were found to be practically inactive against almost all examined cancer cell lines [3]. Moreover, even the least active 11 $\alpha$ -substituted derivatives **4d**, **4e**, **5d** and **5e**, showed much more pronounced cytotoxicity than parent thiosemicarbazone derivatives. This pointed to the fact that this type of heterocyclization of thiosemicarbazone moiety at C-3 and especially at C-17 and/or C-20 position enhanced the anticancer activity of the investigated steroid compounds.

If we consider the type of steroid units it is clear that 3-mono-thiazolidinones **4a**, **4b**, **4c** and **4f** displayed similar anticancer actions against most of the cell lines used in this study. This which corroborates our previous finding that the absence of 19-methyl group, the degree of unsaturation or the substituent at C-17 (17-oxo or 17-COCH<sub>3</sub>) does not affect their cytotoxicity significantly [3]. Also, bis-thiazolidinones **5a**, **5b**, **5c** and **5f** were found to behave the same way. The exceptions were again the derivatives of 11 $\alpha$ -hydroxyandrost-4-ene-3,17-dione, **4d** and **5d**, which were the least active against malignant cell lines tested. Since the introduction of OCOCH<sub>3</sub> instead of OH group (compounds **4e** and **5e**) significantly improved the activity of these compounds, the data obtained in this study support our previous results [3] that the replacement of 11 $\alpha$ -hydrogen by the proton-donating substituent decreases the activity of tested steroid compounds.

Summarizing the results it can be concluded that  $\alpha,\beta$ -unsaturated substituent at C-3 and substituent at C-17 and/or C-20 position and their heterocyclic 4-thiazolidinone moieties are mostly responsible for the anticancer activity of the new synthesized steroid derivatives.

Since in our previous report [3] steroidal thiosemicarbazones **2a–f** and **3a–f** were obtained as inseparable stereoisomeric mixtures it could not be determined which geometric isomer had a greater cytotoxic action on malignant cells used in the study. Due to the fact that in this work, diastereomerically pure major, (*E*)-isomers, of most of the thiazolidinones (**4a–c** and **4f**) were successfully isolated, it was possible to compare the activity of pure (*E*)-isomers with the

activity of the mixtures. Examining the IC<sub>50</sub> values in Table 1, it can be concluded that there were no significant differences in cytotoxicity of (*E/Z*)-mixtures and pure (*E*)-isomers, indicating that the configuration around C(3)=N1 double bond had no influence on the activity of these compounds. For this reason, and also for better comparison of the activity of these compounds with the activity of previously synthesized thiosemicarbazones [3] and newly synthesized bis-thiazolidinones **5a-f** (obtained only as (*E/Z*) mixtures), all further experiments were carried out with the mixtures.

The compounds which exerted the most pronounced cytotoxic action were selected for further examination of cytotoxicity against normal, non-transformed human peripheral blood mononuclear cells (PBMC), both unstimulated and stimulated to proliferate by the mitogen phytohemagglutinin (PHA) (Table 2).

Each of the tested compounds (**4a-c**, **4f**, **5a-c** and **5f**) exhibited notably higher cytotoxic activities against all cell lines, especially against HeLa, K562, MDA-MB-453 and MDA-MB-361 cells in comparison with resting and PHA-stimulated PBMC. In all cases the action of tested compounds was less pronounced on unstimulated than upon stimulated PBMC.

The selectivity in the antitumor action of all investigated compounds was very high for both K562 and HeLa cell lines which were the most sensitive to their cytotoxic effects. It must be noted that compounds **4a**, **4b**, **5a**, **5c** and **5f** also displayed a very good selectivity in the anticancer effect against human breast carcinoma MDA-MB-453 and MDA-MB-361 cells and against A549 cells. In addition, compound **5f** showed much higher intensity of cytotoxic action against LS174 cells than against normal immunocompetent PBMC (Table 3).

Effective and selective killing of cancer cells, while exerting minimal toxicity to normal, non-transformed cells, and especially to immune system cells, involved in the antitumor immune response are highly desirable properties for development of novel anticancer compounds. Therefore, very good selectivity in the cytotoxic activity of the examined steroidal thiazolidin-4-one derivatives against cancer cells (especially high against K562 and HeLa cells) when compared with normal human PBMC suggests their promising anticancer potential. The most potent compounds, **4a** and **5a** were selected for a further biological evaluation.

The mechanisms of their anticancer activity were further examined by cell cycle analysis and fluorescence microscopy. Both tested compounds applied at IC<sub>50</sub> and 2IC<sub>50</sub> concentrations for 24 h and 48 h induced a significant increase of human cervical adenocarcinoma HeLa cells in the subG1 and G2/M cell cycle phases when compared with the control cell sample (Fig. 3). These alterations were accompanied with significantly decreased proportion of treated HeLa cells in G1 phase. In addition, the exposure to compound **4a** at 2IC<sub>50</sub> concentration for 24 h and



to  $IC_{50}$  concentration for 48 h led to significant accumulation of HeLa cells in the S phase, while the S phase arrest was observed in HeLa cells treated with compound **5a** at both tested concentrations after 24 h and 48 h exposure.

In human myelogenous leukemia K562 cells, treatment with  $IC_{50}$  and  $2IC_{50}$  concentrations of compound **4a** for 24 h and 48 h caused a statistically significant increase in the percentage of cells within the subG1 cell cycle phase in addition to significant G2/M cell cycle phase arrest in comparison with untreated cells (Fig. 4). The significantly decreased percentages of K562 cells in the G1 and S phases were observed as well. Compound **5a** at both tested concentrations induced a significant accumulation of K562 cells in the subG1 and G2/M cell cycle phases when compared to the control cell sample. The significantly decreased percentage of K562 cells in the G1 phase was demonstrated as well, while the reduction of cells in the S phase was statistically significant only after treatment that lasted 24 h.

Fluorescence microscopy visualization of HeLa cells stained with a mixture of nucleic acid dyes acridine orange and ethidium bromide demonstrated pro-apoptotic effects of compounds **4a** and **5a** (Fig. 5). The exposure to  $2IC_{50}$  concentrations of compounds **4a** and **5a** for 24 h led to an appearance of typical morphological characteristics of apoptotic cell death such as green stained rounded HeLa cells with condensed chromatin and shrunken nuclei as well as orange-red stained late apoptotic cells. However, the rounded shape of treated HeLa cells could also be attributed to G2/M arrest induced by the tested compounds, as observed by cell cycle analysis.

To investigate the mechanisms of the pro-apoptotic activity of compounds **4a** and **5a**, the specific inhibitors of caspase-3, -8 and -9 were used for cell cycle analysis. Pretreatment with a caspase-3 inhibitor remarkably decreased the percentage of HeLa cells exposed to compounds **4a** and **5a** in sub G1 phase when compared to exposed cells that were not pretreated with an inhibitor, pointing to the ability of compounds to activate the effector caspase-3 in the apoptotic pathway (Fig. 6). Reduction of the percentage of HeLa cells in the subG1 phase, which were pre-exposed to caspase-8 or caspase-9 inhibitor before treatment with **4a** and **5a**, was found when compared with treated HeLa cells which were not pretreated with inhibitors. These results indicate that both compounds trigger extrinsic and intrinsic apoptotic signaling pathways in HeLa cells.

The endothelial cell tube formation assay demonstrated anti-angiogenic effects of compounds **4a** and **5a** applied at low sub-toxic concentrations as shown in Fig. 7. Both examined compounds were able to inhibit sprouting and connecting of EA.hy926 cells and formation of capillary-like tube structures, suggesting their anti-angiogenic potential.

To further explore the possible antimetastatic and anti-angiogenic properties of novel steroidal thiazolidin-4-one derivatives, the effects of compounds **4a** and **5a** on gene expression levels of *MMP2*, *MMP9* and *VEGFA* in HeLa cells were examined. Gene expression levels in treated HeLa cells were compared to the control cells (Fig. 8). The treatment with **4a** and **5a** barely affected *MMP2* and *MMP9* expression levels when compared to those levels in control HeLa cells. In addition, these compounds did not induce considerable alterations in gene expression profile of the pro-angiogenic growth factor *VEGFA* in HeLa cells.

To the best of our knowledge, the present research is the first to report the selective cytotoxic activities of novel steroidal thiazolidinone derivatives on human cancer cell lines, their ability to induce apoptosis and G2/M phase arrest in human cervical adenocarcinoma HeLa cells, in addition to inhibition of angiogenesis of EA.hy926 cells. This study represents significant contribution to the research efforts to elucidate the mechanisms of anticancer activity of compounds bearing 4-thiazolidinone bioactive scaffold.

The ability of investigated steroid derivatives to scavenge radicals was investigated by the DPPH test (Table 4). The results showed that compounds **4a**, **4e**, **4f**, **5a**, **5d** and **5e** have some antioxidant activity, although much weaker than ascorbic acid.

#### 4. Conclusion

New steroidal mono- and bis(thiazolidin-4-ones) **4a–f** and **5a–f** were efficiently synthesized by intermolecular cyclocondensation reaction of corresponding thiosemicarbazones **2a–f** and **3a–f** and ethyl chloroacetate as cyclizing reagent. All compounds were obtained as stereoisomeric mixtures with different configuration in the hydrazone moiety at the C-3 position. Diastereomerically pure major (*E*)-isomers of mono-thiazolidin-4-ones **4a–f** were successfully isolated. The structure and stereochemistry were confirmed by X-ray analysis of 2,4-thiazolidinedione,2-[(17-oxoandrost-4-en-3-ylidene)hydrazone] (**4b**). A pathway for the formation of thiazolidin-4-one ring was proposed.

The newly synthesized thiazolidinone derivatives exerted selective concentration-dependent cytotoxic action toward malignant cell lines tested. The introduction of heterocyclic 4-thiazolidinone moieties at C-3 and especially at C-17 and/or C-20 position enhanced the anticancer activity of the investigated steroid compounds. HeLa and K562 cells were the most sensitive to the cytotoxic effects of these compounds. Ten out of twelve examined compounds exhibited strong cytotoxic effects on K562 cells with  $IC_{50}$  values of 8.5–14.9  $\mu$ M, eight on HeLa cells with  $IC_{50}$  values of 8.9–15.1  $\mu$ M, while against MDA-MB-361 cells six compounds exerted similar or even higher cytotoxic action than cisplatin with  $IC_{50}$  values from 12.7  $\mu$ M to 25.6  $\mu$ M. On the contrary, a very low toxic effect upon both non-stimulated, and PHA

stimulated PBMCs has been detected. Moreover, eight of these ten compounds showed very high selectivity in the cytotoxic action, especially against HeLa and K562 cancer cell lines. The mechanisms of the anticancer activity of the two selected compounds, mono- and bis(thiazolidin-4-one) derivatives of 19-norandrost-4-ene-3,17-dione **4a** and **5a**, were further elucidated. Both examined compounds applied at  $2IC_{50}$  concentrations (17.8  $\mu$ M and 27.0  $\mu$ M, respectively) for 24 h induced a significant increase of HeLa cells in the subG1, S and G2/M cell cycle phases compared to the control cell sample. Morphological analysis by fluorescence microscopy of HeLa cells stained with a mixture of acridine orange and ethidium bromide revealed pro-apoptotic effects of these compounds. In addition, both compounds demonstrated the ability to trigger apoptosis in HeLa cells through extrinsic and intrinsic signaling pathways. Treatment of EA.hy926 cells, grown on the surface of matrigel, with sub-toxic concentrations of compounds **4a** and **5a** for 20 h, led to the inhibition of cell connecting and sprouting, and tube formation. The compounds **4a** and **5a** did not cause significant changes in *MMP2*, *MMP9* and *VEGFA* gene expression levels in HeLa cells.

Our results suggest the significant anticancer potential of newly synthesized steroid thiazolidinone derivatives due to their strong cytotoxicity against malignant cells, good selectivity and antiangiogenic effects.

#### **Acknowledgement**

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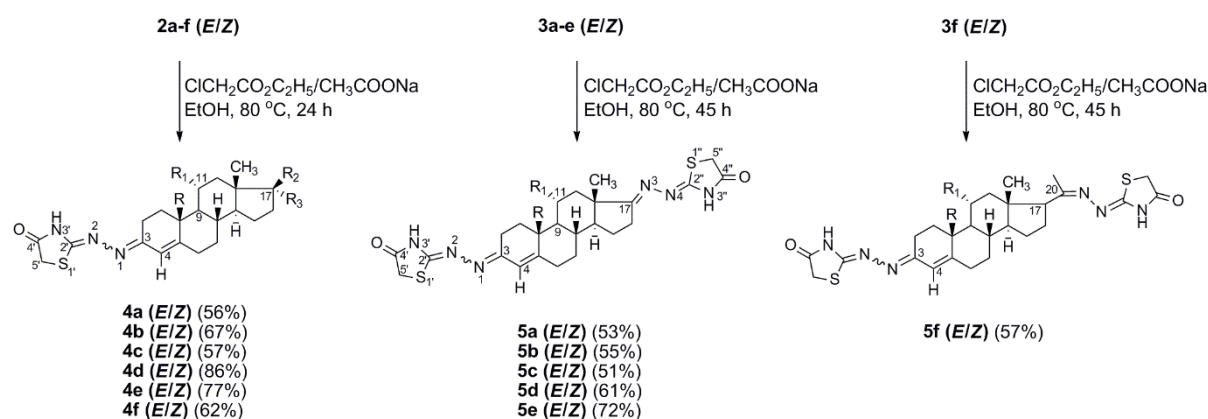
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## Figure captions

**Scheme 1.** Synthesis of new steroidal mono- and bis(thiazolidin-4-ones) **4a–f** and **5a–f**.



**Scheme 2.** The proposed mechanism of the formation 4-thiazolidinones **4a–f** and **5a–f**

**Figure 1.** Previously synthesized mono- and bis(thiosemicarbazones) **2a–f** and **3a–f** obtained from androstene derivatives **1a–f**.

**Figure 2.** Molecular structure of **4b-E** with atom numeration.

Anisotropic displacement ellipsoids are drawn at 50% probability level. Hydrogen atoms are depicted as spheres of arbitrary radius. Selected bond lengths (Å): S1'–C2' = 1.751(4), S1'–C5' = 1.812(5), O2–C4' = 1.216(5), N3'–C2' = 1.367(5), N3'–C4' = 1.369(6), C4'–C5' = 1.501(6), N2–C2' = 1.283(5), N1–N2 = 1.398(5), N1–C3 = 1.308(5), C3–C4 = 1.452(5), C4–C5 = 1.335(5). Selected torsion angles (°): C3–C4–C5–C10 = –5.0(6)°, C4–C3–N1–N2 = –179.9(3)°, N1–N2–C2'–S1' = –2.7(5)°.

**Figure 3.** Changes in the cell cycle phase distribution of HeLa cells

Changes in the cell cycle phase distribution of cervical adenocarcinoma HeLa cells, induced by the investigated compounds **4a** and **5a** after 24 h (A, B) and 48 h (C, D) treatment. Applied concentrations of tested compounds corresponded to IC<sub>50</sub> (A, C) and 2IC<sub>50</sub> (B, D) values determined for 72 h. The data shown represent the mean ± S.D. of three independent experiments. Significant differences between cell cycle distribution of control and treated cells are indicated by \* ( $p < 0.05$ )

**Fig. 4.** Changes in the cell cycle phase distribution of K562 cells

Changes in the cell cycle phase distribution of myelogenous leukemia K562 cells, induced by the investigated compounds **4a** and **5a** after 24 h (A, B) and 48 h (C, D) treatment. Applied concentrations of tested compounds corresponded to IC<sub>50</sub> (A, C) and 2IC<sub>50</sub> (B, D) values



determined for 72 h. The data shown represent the mean  $\pm$  S.D. of three independent experiments. Significant differences between cell cycle distribution of control and treated cells are indicated by \* ( $p < 0.05$ )

**Figure 5.** Induction of apoptosis in HeLa cells by compounds **4a** and **5a**

Photomicrographs of acridine orange/ethidium bromide-stained control HeLa cells and HeLa cells exposed to  $2IC_{50}$  concentrations of the compounds **4a** and **5a** for 24 h. Typical morphological characteristics of apoptotic cell death are green stained rounded HeLa cells with condensed chromatin and shrunken nuclei as well as orange-red stained late apoptotic cells. Magnification 20x.

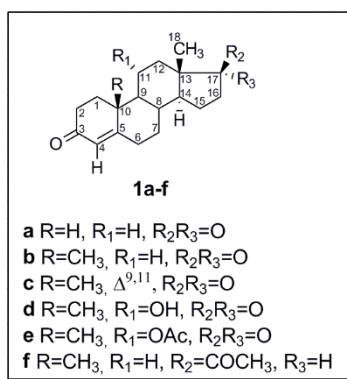
**Figure 6.** Identification of target caspases involved in the apoptotic signaling pathways induced by the compounds **4a** and **5a**

Effects of the specific caspase inhibitors (Z-DEVD-FMK - caspase-3 inhibitor; Z-IETD-FMK -caspase-8 inhibitor; Z-LEHD-FMK - caspase-9 inhibitor) on the percentages of subG1 HeLa cells treated with  $2IC_{50}$  concentrations of the compounds **4a** (A) and **5a** (B).

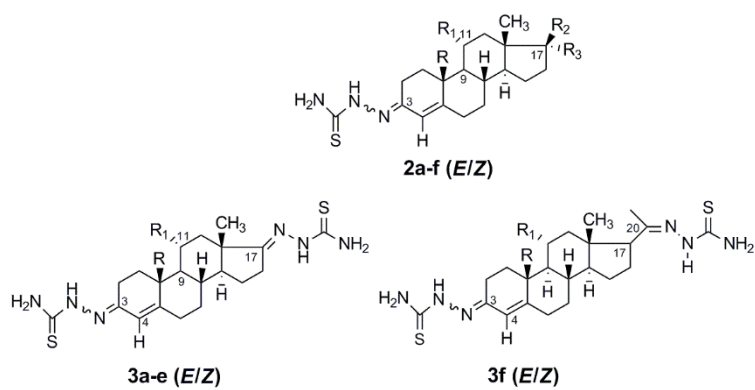
**Figure 7.** Anti-angiogenic effects of compounds **4a** and **5a**

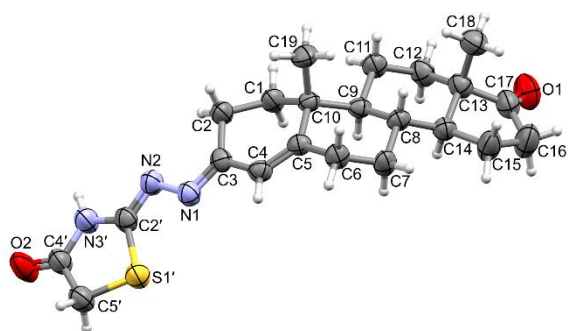
Photomicrographs of control EA.hy926 cells and EA.hy926 cells exposed to sub-toxic  $IC_{20}$  concentrations of the compounds **4a** and **5a** for 20 h. Both examined compounds were able to inhibit sprouting and connecting of EA.hy926 cells and formation of capillary-like tube structures. Photomicrographs were captured under the inverted phase-contrast microscope using 6.3x magnification.

**Figure 8.** Gene expression levels of *MMP2* (A), *MMP9* (B) and *VEGFA* (C) in control HeLa cells and HeLa cells exposed to sub-toxic  $IC_{20}$  concentrations of the compounds **4a** and **5a** for 24 h.

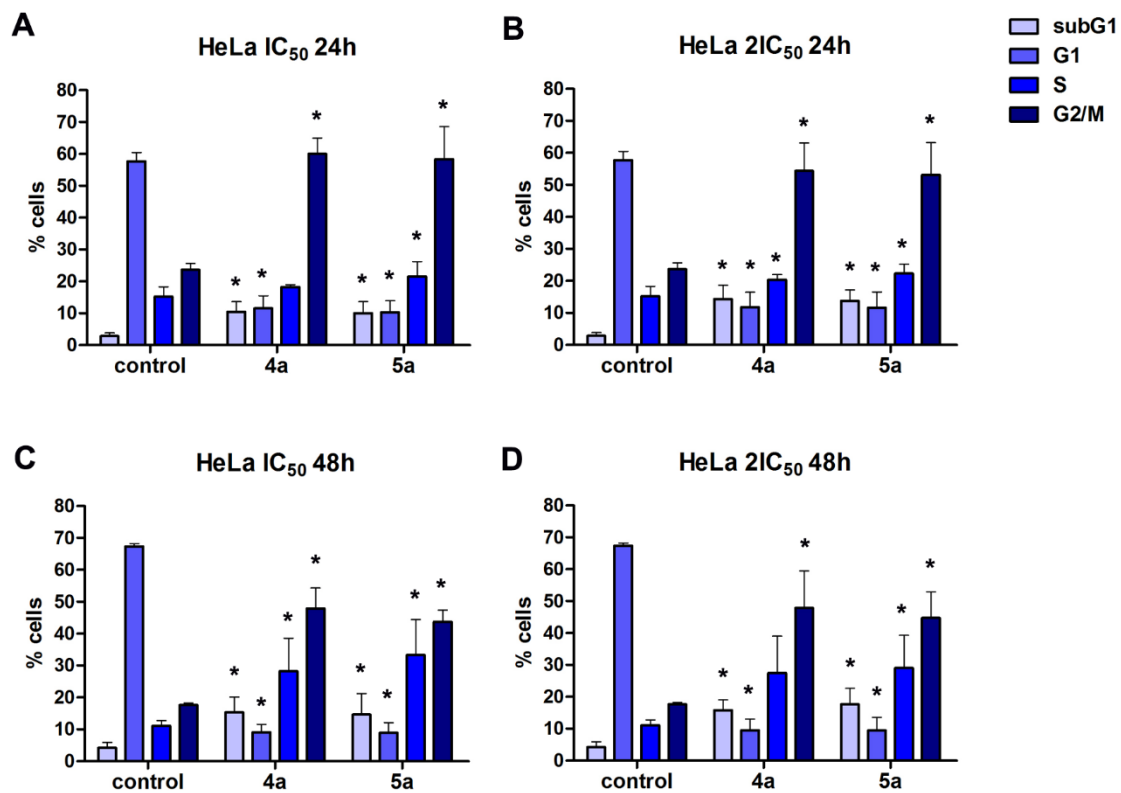


Figr-1





Figr-2



Figr-3

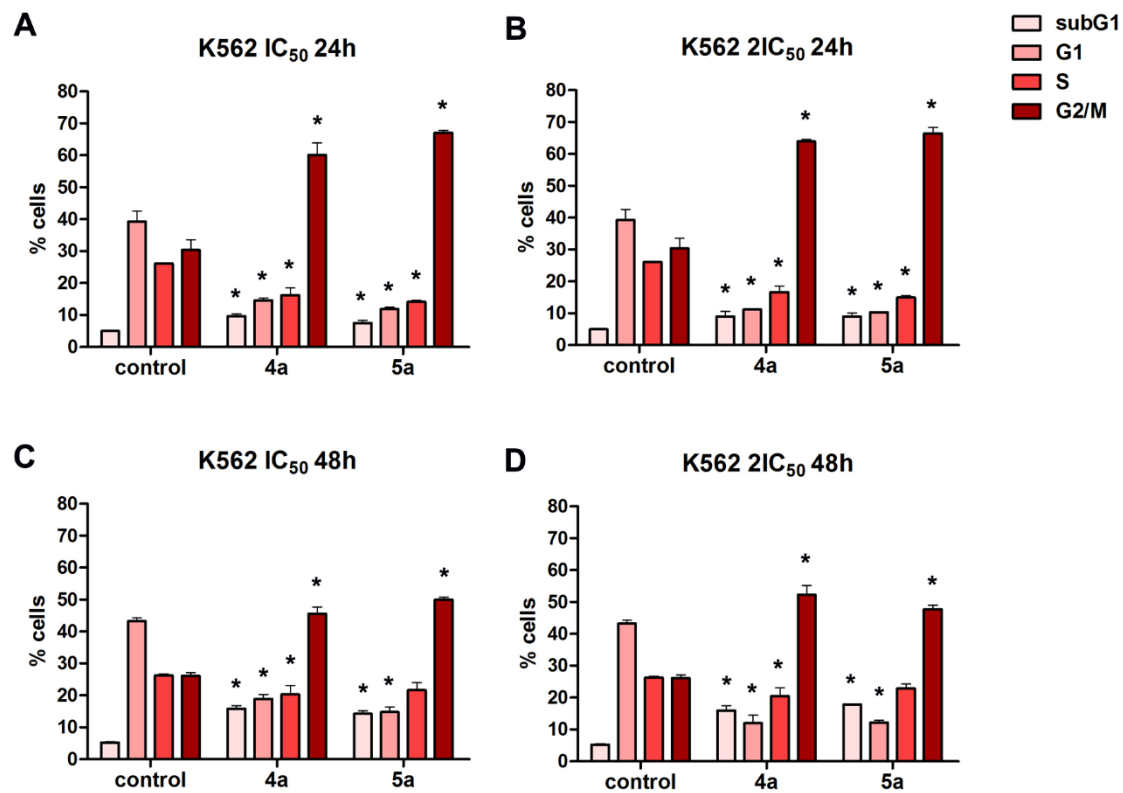
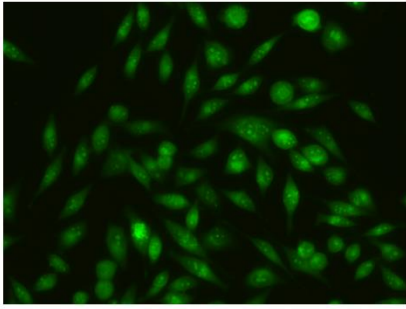
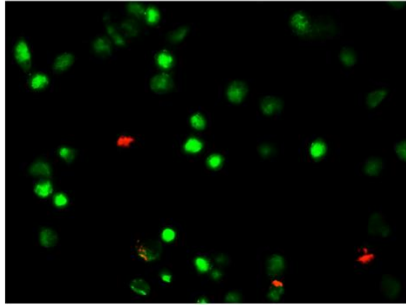


Fig-4

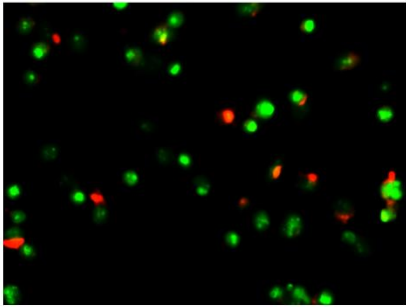
control



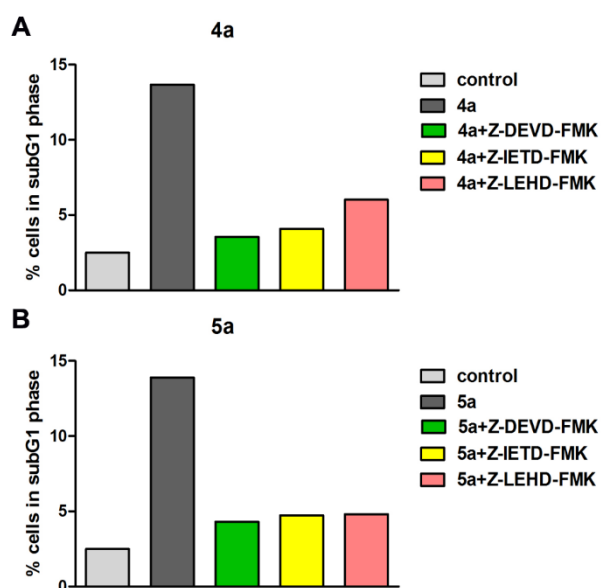
4a



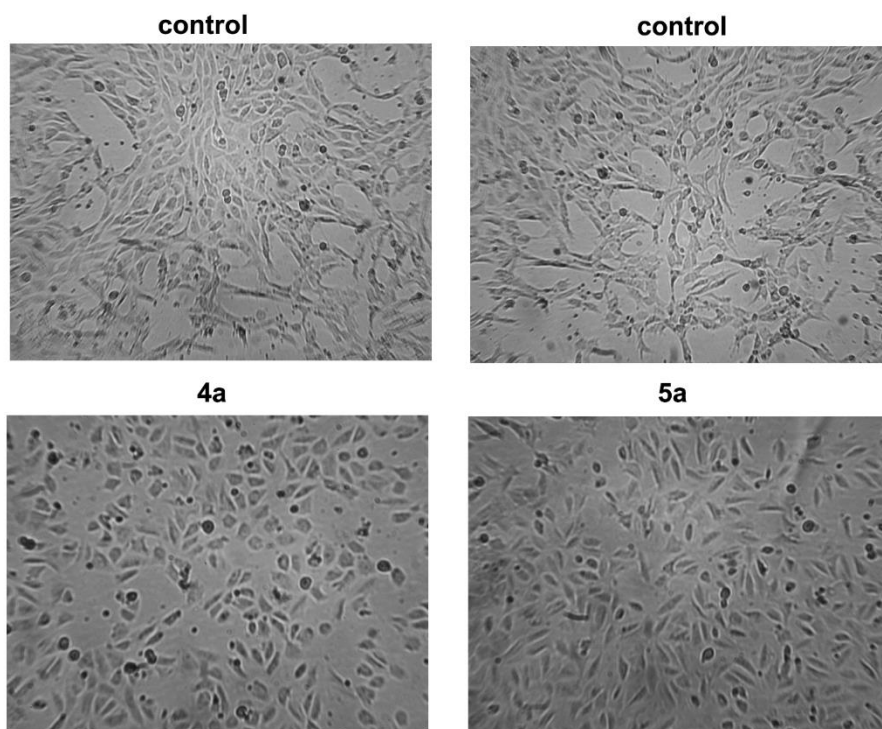
5a



Figr-5

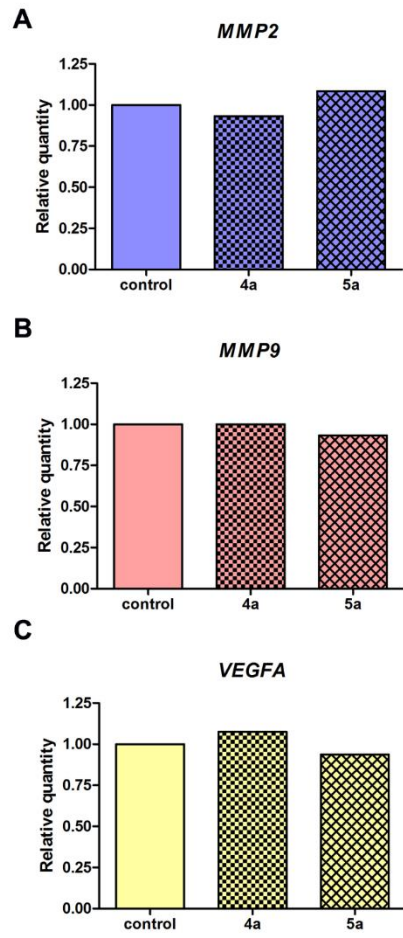


Figr-6



Figr-7





Figr-8

**Table 1.** Concentrations of investigated compounds **4a–f** and **5a–f** which induced 50% decrease (IC<sub>50</sub>) in malignant cell survival

Comp.	IC <sub>50</sub> ± SD (μM)						
	HeLa	K562	MDA-MB-453	MDA-MB-361	LS174	A549	MRC-5
<b>4a-E</b>	9.2 ± 2.3	8.5 ± 0.9	22.2 ± 2.4	10.7 ± 0.4	49.5 ± 6.7	22.0 ± 5.0	60.9 ± 6.8
<b>4a-E/Z</b>	8.9 ± 1.9	8.8 ± 1.1	23.7 ± 2.7	12.7 ± 2.0	48.2 ± 4.9	22.3 ± 4.7	62.7 ± 6.2
<b>4b-E</b>	10.8 ± 1.9	9.1 ± 1.3	24.9 ± 0.2	22.0 ± 0.8	48.2 ± 2.5	48.6 ± 1.9	55.7 ± 8.0
<b>4b-E/Z</b>	10.7 ± 2.5	8.7 ± 1.1	23.8 ± 1.7	25.6 ± 4.6	49.7 ± 0.5	49.5 ± 0.6	44.8 ± 7.3
<b>4c-E</b>	9.8 ± 1.1	9.4 ± 1.7	27.1 ± 0.1	19.1 ± 2.3	51.8 ± 9.0	42.1 ± 3.8	53.2 ± 7.0
<b>4c-E/Z</b>	9.5 ± 0.9	8.8 ± 1.2	26.2 ± 2.7	19.6 ± 2.2	51.3 ± 3.2	40.4 ± 7.5	49.9 ± 9.3
<b>4d-E/Z</b>	51.4 ± 10.5	11.3 ± 2.9	116.0 ± 11.8	134.0 ± 7.9	119.9 ± 25.9	176.1 ± 9.5	111.4 ± 20.1
<b>4e-E/Z</b>	28.0 ± 5.2	19.2 ± 0.4	29.4 ± 4.1	29.6 ± 1.6	104.5 ± 8.7	77.7 ± 26.8	88.4 ± 17.2
<b>4f-E</b>	8.9 ± 1.7	8.7 ± 0.3	21.5 ± 2.8	14.5 ± 5.3	49.1 ± 2.8	18.6 ± 6.6	42.8 ± 2.2
<b>5a-E/Z</b>	13.5 ± 0.1	8.9 ± 1.1	30.9 ± 6.2	36.0 ± 6.3	42.6 ± 6.4	25.3 ± 4.0	58.1 ± 2.6
<b>5b-E/Z</b>	12.9 ± 2.9	10.5 ± 1.3	37.3 ± 2.5	30.2 ± 7.3	21.8 ± 5.1	27.6 ± 7.2	53.4 ± 9.0
<b>5c-E/Z</b>	11.2 ± 1.6	9.6 ± 1.7	33.5 ± 6.3	24.6 ± 0.4	47.8 ± 3.9	24.9 ± 0.2	48.8 ± 1.6
<b>5d-E/Z</b>	41.2 ± 2.7	11.6 ± 2.1	77.1 ± 4.1	86.8 ± 2.6	73.0 ± 10.0	76.2 ± 4.0	91.1 ± 9.9
<b>5e-E/Z</b>	31.3 ± 3.8	21.9 ± 5.1	46.7 ± 9.0	39.5 ± 5.3	36.4 ± 5.6	39.8 ± 3.3	67.9 ± 9.5
<b>5f-E/Z</b>	15.1 ± 6.1	14.9 ± 3.8	23.9 ± 0.2	20.6 ± 1.0	11.0 ± 1.3	17.2 ± 4.6	31.4 ± 4.9
cisplatin	5.2 ± 0.5	5.7 ± 0.6	6.5 ± 0.5	21.5 ± 1.5	18.6 ± 2.0	17.4 ± 1.8	13.0 ± 1.7

**Table 2.** Concentrations of compounds **4a–c**, **4f**, **5a–c** and **5f** which induced 50% decrease (IC<sub>50</sub>) in PBMC and PBMC+ PHA cell survival

Comp.	IC <sub>50</sub> ± SD (μM)	
	PBMC <sup>a</sup>	PBMC+PHA <sup>a</sup>
<b>4a</b>	145.8 ± 2.5	92.1 ± 14.3
<b>4b</b>	>200	155.7 ± 13.3
<b>4c</b>	123.4 ± 4.4	64.4 ± 7.1
<b>4f</b>	88.6 ± 11.6	64.7 ± 8.0
<b>5a</b>	188.8 ± 15.9	88.1 ± 8.6
<b>5b</b>	87.1 ± 0.5	59.8 ± 14.0
<b>5c</b>	>200	188.7 ± 16.0
<b>5f</b>	123.8 ± 12.1	62.3 ± 7.6
cisplatin	71.3 ± 8.20	32.9 ± 3.7

<sup>a</sup>From two independent experiments (healthy blood donors)

**Table 3.** Coefficient of selectivity (Cs) in antitumor action of compounds **4a–c**, **4f**, **5a–c** and **5f** as a ratio of IC<sub>50</sub> values for PBMC (PBMC+PHA) and malignant cells

Comp.	Cs					
	PBMC (PBMC+PHA)/ HeLa	PBMC (PBMC+PHA)/ K562	PBMC (PBMC+PHA)/ MDA-MB-453	PBMC (PBMC+PHA)/ MDA-MB-361	PBMC (PBMC+PHA)/ LS174	PBMC (PBMC+PHA)/ A549
<b>4a</b>	16.3 (10.3)	16.6 (10.5)	6.2 (3.9)	11.5 (7.3)	3 (1.9)	6.5 (4.1)
<b>4b</b>	>18.6 (4.5)	>23 (17.9)	>8.4 (6.6)	>7.8 (6.1)	>4 (3.1)	>4 (3.1)
<b>4c</b>	13 (6.8)	14 (7.3)	4.7 (2.5)	6.3 (3.3)	2.4 (1.3)	3.1 (1.6)
<b>4f</b>	10 (7.3)	10.2 (7.5)	4.1 (3)	6.1 (4.5)	1.8 (1.3)	4.8 (3.5)
<b>5a</b>	14 (6.5)	21.3 (9.9)	6.1 (2.8)	5.2 (2.4)	4.4 (2.1)	7.5 (3.5)
<b>5b</b>	6.7 (4.6)	8.3 (5.7)	2.3 (1.6)	2.9 (2)	4 (2.7)	3.2 (2.2)
<b>5c</b>	>17.8 (6.8)	>20.8 (19.6)	>6 (5.6)	>8.1 (7.7)	>4.2 (3.9)	>8 (7.6)
<b>5f</b>	8.2 (4.1)	8.3 (4.2)	5.2 (2.6)	6 (3)	11.3 (5.7)	7.2 (3.6)
cisplatin	13.6 (6.3)	12.6 (5.8)	11.1 (5.1)	3.3 (1.5)	3.8 (1.8)	4.1 (1.9)

**Table 4.** Antioxidant activity (IC<sub>50</sub>/mM) of compounds **4a–f** and **5a–f**

Comp.	<b>4a</b>	<b>4b</b>	<b>4c</b>	<b>4d</b>	<b>4e</b>	<b>4f</b>	<b>5a</b>	<b>5b</b>	<b>5c</b>	<b>5d</b>	<b>5e</b>	<b>5f</b>	AA *
IC <sub>50</sub> (mM)	18.83	/	/	/	4.33	40.48	17.54	/	/	10.06	42.48	/	0.08

\*Ascorbic acid