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Heterocyclic androstane and estrane D-ring modified steroids: Microwave-assisted synthesis, steroid-converting enzyme inhibition, apoptosis induction, and effects on genes encoding estrogen inactivating enzymes

Ágnes Erika Kulmány [a], Bianka Edina Herman [b], István Zupkó [a], Masa Sinreih [c], Tea Lanišnik Rižner [c], Marina Savić [d], Aleksandar Oklješa [d], Andrea Nikolić [d], Viktória Nagy [a], Imre Ocsovszki [e], Mihály Szécsi [b], Suzana Jovanović-Šanta [d,*]

[a] Department of Pharmacodynamics and Biopharmacy, Faculty of Pharmacy, University of Szeged, H-6720 Szeged, Hungary
[b] Department of Medicine, University of Szeged, H-6720 Szeged, Hungary
[c] Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia
[d] Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad, 21000 Novi Sad, Serbia
[e] Department of Biochemistry, Faculty of Medicine, University of Szeged, H-6720 Szeged, Hungary

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ABSTRACT

D-ring–fused and D-homo lactone compounds in estratriene and androstane series were synthesized using microwave-assisted reaction conditions. Microwave-irradiated synthesis methods were convenient and effective, and provided high yields with short reaction times. Their inhibition of $C_{17,20}$-lyase and 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) activities were studied in *in vitro* enzyme assays. D-ring–fused triazolyl estrone analog **24** showed potent inhibition of NADH-complexed 17β-HSD1, with a binding affinity similar to that of the substrate estrone; its inhibition against NADPH-complexed 17β-HSD1 was markedly weaker. Compound **24** also significantly and selectively reduced proliferation of cancer cell lines of gynecological origin. This estrane triazole changed the cell cycle and induced apoptosis of HeLa, SiHa, and MDA-MB-231 cancer cells, measured by both increased subG1 fraction of cells and activation of caspase-independent signaling pathways. A third mode of anti-estrogenic action of **24** saw increased mRNA expression of the *SULT1E1* gene in HeLa cells; in contrast, its 3-benzyloxy analog **23** increased mRNA expression of the *HSD17B2* gene, thus showing pronounced pro-drug anti-estrogenic activity. Estradiol-derived D-ring triazole compound **24** thus acts at the enzyme, gene expression and cellular levels to decrease the production of active estrogen hormones, demonstrating its pharmacological potential.

1. Introduction

There are different strategies in the biology-driven medicinal chemistry of anticancer drug development for the treatment of hormone-dependent cancers: (1) development of compounds that inhibit enzymes that are essential for biosynthesis of steroid hormones (*e.g.*, aromatase); (2) development of compounds that compete with the appropriate receptor proteins (*e.g.*, progesterone, androgen or estrogen receptor antagonists, such as tamoxifen); and (3) development of compounds that induce cancer-cell death (*e.g.*, induction of apoptosis or other morphological changes, such as taxanes).

Proliferation of steroid-hormone-dependent cells is often associated with steroid-converting enzyme activities or expression. Accordingly, one of the therapeutic strategies in the treatment of steroid-hormone-dependent diseases is inhibition of steroid-converting enzyme activities, which are important in disease development and progression; instead, targeting their expression is a less common approach. The key enzymes as targets in the treatment of cancers of the female reproductive tissues (*e.g.*, breast cancer) are aromatase, steroid sulfatase, and 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) [1–6].

* Corresponding author at: University of Novi Sad, Faculty of Sciences, Department of Chemistry, Biochemistry and Environmental Protection, Trg Dositeja Obradovića 3, 21000 Novi Sad, Serbia.
E-mail address: suzana.jovanovic-santa@dh.uns.ac.rs (S. Jovanović-Šanta).

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Alternatively, for the treatment of benign prostate hyperplasia or male-occurring cancers (*e.g.*, prostate cancer), 17α-hydroxylase-$C_{17,20}$-lyase ($P450_{17\alpha}$) and 5α-reductase are important targets [7,8] (Fig. 1).

Steroid hormones such as estrogens, progesterone and androgens promote a plethora of important biological effects in the human body. Steroid hormones have crucial roles in proliferation of both healthy and cancerous tissues, so their structural modification might result in effective new antiproliferative agents without hormonal effects. Previous studies have shown that the most successful structural modifications include expansion of the D-ring or fusion of the heterocyclic ring to the D-ring [9–13]. These synthetic drug candidates can show pronounced antiproliferative effects on different gynecological cancer cell lines (*e.g.*, HeLa, MCF-7, T47D, A2780 cells); in addition, several of these derivatives have been reported to inhibit 17β-HSD1, 17β-hydroxysteroid dehydrogenase type 2 (17β-HSD2), aromatase, and steroid sulfatase [14,15].

Design and synthesis of new potential drugs are of great importance in medicinal chemistry. The use of environmentally friendly synthetic approaches are on the rise, as these can provide very significant opportunities for the synthesis of desired compounds with minimal pollution and energy consumption. Microwave-assisted reactions are the focus of the present study, as an alternative to conventional procedures and methods. Research involving the synthesis and modification of steroidal compounds using this relatively harmless (or 'green') procedure has recently gained increasing importance. Furthermore, microwave-assisted synthesis is ascending, because it is cleaner (especially when solvent-free), faster, and usually more efficient. Thus, today, many steroidal compounds are synthesized by microwave irradiation [16–22].

As a continuation of our previous synthetic studies [21–24], we present here the microwave-assisted synthesis of steroidal heterocyclic compounds, along with their anticancer, gene expression, and enzyme inhibition properties: D-ring–fused steroidal triazoles and tetrazoles, and D-homo lactones. The aim was to determine the *in vitro* inhibitory effects of these compounds on two major steroid-converting enzymes: $C_{17,20}$-lyase and 17β-HSD1. As estrogens can be formed in peripheral tissues *via* the aromatase or sulfatase pathways, we examined the effects of these compounds to determine the most effective on gynecological cancer cell lines. We targeted the expression of genes that encode enzymes involved in estrogens biosynthesis, as *CYP19A1*, *STS*, *HSD17B1*, *HSD17B7*, and *HSD17B12*, and enzymes involved in estrogens inactivation, as SULT1E1, HSD17B2, HSD17B4, HSD17B8, and HSD17B14 (Fig. 1). This study was based on the knowledge that introduction of a heteroatom or a heterocycle into the structure of a parent steroid often results in alterations of its biological and/or pharmacological activities [25,26].

2. Experimental

2.1. Chemical synthesis

2.1.1. General

The microwave-assisted synthesis of steroidal heterocyclic compounds defined here was specific for each type of precursor. In general, the planned syntheses were achieved by microwave irradiation of the reaction mixtures of the steroidal precursor and the appropriate reagents.

The microwave reactor was a monomode system (Discover Bench Mate microwave synthesis system; CEM Corporation) for focused waves in a closed-vessel system. It consisted of a continuous focused microwave power delivery system with operator-selectable power output from 0 W to 300 W. Melting points are reported as uncorrected (Electrothermal apparatus 9100). Flash chromatography was performed on silica gel columns (Kieselgel 60; 0.04–0.063 mm; Merck) or using basic aluminum oxide (Brockmann I). Organic solutions were dried over Na_2SO_4 and evaporated using a rotary evaporator under reduced pressure. Infrared (IR) spectra were recorded on a spectrometer (Nexus 670) using KBr pellets, with wave numbers in cm^{-1}. Nuclear magnetic resonance (NMR) spectra were obtained using a spectrometer (Bruker AC 250E) operating at 250 MHz (^1H) and 62.5 MHz (^{13}C), and are reported in ppm (δ-scale) downfield from the tetramethylsilane internal standard, with coupling constants (*J*) given in Hz. High resolution mass spectra

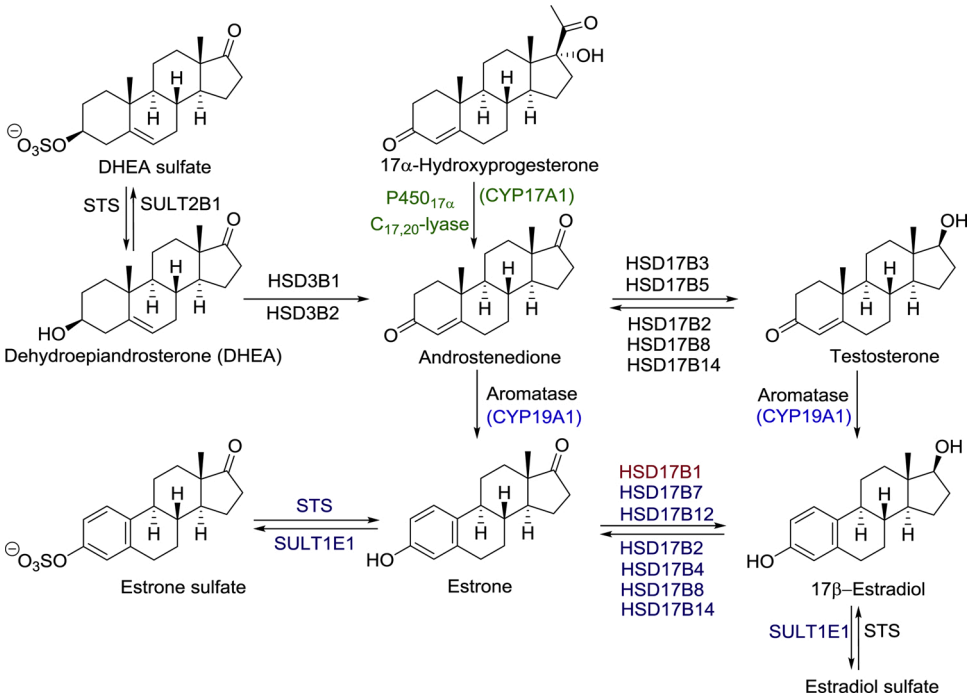


Fig. 1. Key steroid-converting enzymes (and appropriate encoding genes) in the physiology and pathology of reproductive tissues: steroid sulfatase (STS), sulfotransferases (SULT1), 17α-hydroxylase-$C_{17,20}$-lyase ($P450_{17\alpha}$, CYP17A1), aromatase (CYP19A1), 3β-hydroxysteroid dehydrogenases (HSD3), and 17β-hydroxysteroid dehydrogenases (HSD17, different types). This study examined the effects of steroidal compounds on (iso)enzymes (green), or genes (blue), or both (red).

were recorded on a 6210 Time-of-Flight LC/MS Agilent Technologies instrument, operated in positive electrospray ionization mode.

2.1.2. D-homo lactone compounds

*2.1.2.1. Microwave-assisted synthesis of 3β-hydroxy-17-oxa-17a-homo-androst-5-en-16-one (**2**).* A mixture of 3β,17β-dihydroxyandrost-5-en-16-one 16*E*-oxime (**1**; 0.0633 g; 0.20 mmol) and potassium hydroxide (0.06 g; 1.052 mmol) in ethylene glycol (2 mL) was exposed to microwave irradiation for 10 min at 170 °C. The resulting mixture was poured into water (10 mL), acidified with HCl (1:1) to pH 1, and extracted with dichloromethane (4 × 5 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated, to give the crude product. After recrystallization from hexane/ethyl acetate, pure compound **2** was obtained as a brown powder (0.06 g; 93 %; mp. 207 °C; Ref. [27] mp. 206−207 °C; details are given in the Supplement).

*2.1.2.2. Microwave-assisted synthesis of 3β-hydroxy-isoxazolo [5′,4′,3′:4,5,6]-17-oxa-17a-homo-androst-4-en-16-one (**4**).* (6*E*)-Hydroximino-17a-homo-17-oxaandrost-4-en-16-on-3β-yl acetate (**3**; 0.031 g, 0.08 mmol) was dissolved in a mixture of tetrahydrofuran (1.5 mL) and water (0.5 mL), and then iodine (0.024 g, 0.09 mmol), potassium iodide (0.04 g, 0.23 mmol), and sodium hydrogen carbonate (0.028 g, 0.32 mmol) were added. This reaction mixture was exposed to microwave irradiation for 10 min at 140 °C. The resulting mixture was poured into water (5 mL), acidified with HCl (1:1) to pH 1, and extracted with dichloromethane (4 × 3 mL). The combined organic extracts were washed with 5 % solution of sodium thiosulfate and brine, dried and evaporated, giving the crude product. After recrystallization from hexane/ ethyl acetate, pure 3β-hydroxy-isoxazolo[5′,4′,3′:4,5,6]-17a-homo-17-oxaandrost-4-en-16-one (compound **4**) was obtained as white crystals (0.015 g; 55 %; mp. 150−152 °C; Ref. [28] mp. 150–152 °C).

Estrane lactone compounds **6** and **7** were prepared according a previously reported procedure [29].

2.1.3. D-ring–fused tetrazoles

*2.1.3.1. General procedure for preparation of compounds **10** and **11**.* A solution of the corresponding steroidal compound **8** or **9** (0.11 mmol) and sodium azide (0.87 mmol) in hexamethylphosphoramide (HMPA) (2.3 mL) was exposed to microwave irradiation for 1 h at 135 °C. The reaction mixture was poured into water (90 mL) and extracted with dichloromethane (7 × 15 mL) for compound **8**, or it was poured into brine (90 mL), acidified with HCl (6 M aqueous), and extracted with benzene (7 × 15 mL) for compound **9**. The combined organic layers were dried over Na_2SO_4, filtered and evaporated to dryness. The crude solid was purified by flash chromatography (aluminum oxide, basic, hexane/ ethyl acetate [10:7] for **10**, or hexane/acetone [3:1] for **11**), giving pure compounds **10** or **11** as white powders (93 %, mp. 218−220 °C; Ref. [23] mp. 218−220 °C; 84 %, mp. 218 °C; Ref. [23] mp. 218 °C; respectively).

*2.1.3.2. General procedure for preparation of compounds **14**-**17**.* A solution of corresponding steroidal mesylate **12** or **13** (0.18 mmol) and sodium azide (0.37 mmol) in HMPA (2.3 mL) was exposed to microwave irradiation for 30 min at 130 °C, with an additional 40 min at 140 °C for compound **13**. The resulting mixture was poured into brine (90 mL), acidified with HCl (6 M aqueous), and extracted with benzene (7 × 15 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated. The residual oil was purified by flash chromatography (aluminum oxide, basic, benzene/ ethyl acetate [4:1] for **14** and **16**; and aluminum oxide, basic, benzene/ ethyl acetate [7:1], followed by a second colum chromatography using silica gel and hexane/acetone [9:1] for **15** and **17**), giving the pure tetrazole compounds **14** and **15** as colorless crystals (40 %, mp. 163 °C; Ref. [23] mp. 163 °C; 30 %, mp. 225−228 °C; Ref. [23]. mp. 225−228 °C; respectively), and olefins **16** and **17** as colorless crystals (54 %, mp. 129 °C; Ref. [23] mp. 129 °C; 43 %, mp 105 °C; Ref. [23]. mp. 105 °C; respectively).

2.1.4. D-ring–fused triazoles

*2.1.4.1. General procedure for the preparation of compounds **20** and **21**.* A mixture of the corresponding steroidal compound **18** or **19** (0.7 mmol), *p*-toluenesulfonyl hydrazide (1.5 mmol) and ethanol (EtOH; 3 mL) was microwave-irradiated at 85 °C, for 4 min for **18**, or 6 min for **19**. The mixture was poured into water (100 mL). The precipitated solid product of **20** (87 %) was recrystallized from benzene (mp. 190 °C). The crude product of **21** was purified by flash chromatography (toluene/ ethyl acetate [6:1]) for pure compound **21** (37 %, mp. 161 °C, after recrystallization from methanol/ methylene chloride).

*2.1.4.2. 3β-Hydroxy-17-oxo-16,17-secoandrost-5-ene-16-nitrile p-toluenesulfonyl hydrazone (**20**).* IR (KBr, cm^{-1}): 3438, 2245, 1630, 1363, 1168, 673. ^{1}H NMR (acetone-d_6, ppm): 1.01 (s, 3H, H-18); 1.04 (s, 3H, H-19); 2.42 (s, 3H, CH_3 from Ts); 3.40 (m, 1H, H-3); 3.80 (bs, 1H, OH); 5.38 (m, 1H, H-6); 7.09 (s, 1H, H-17); 7.42 (d, 2H, J = 8.2 Hz, CH_3 from Ts); 7.78 (d, 2H, J = 8.2 Hz, Ts); 9.62 (s, 1H, NH). ^{13}C NMR (acetone-d_6, ppm): 15.7 (CH_3); 16.7 (C-15); 19.6 (CH_3); 20.7 (C-11); 21.4 (CH_3); 32.3; 32.7; 37.3; 37.4; 37.7; 42.7; 42.9; 44.9; 49.6; 71.5 (C-3); 119.8 (CN); 120.5 (C-6); 128.6 (Ts); 130.3 (Ts); 141.9 (C-5); 144.6 (Ts); 158.9 (C-17). MS *m/z*: 343 (M^+ - TsNNH). Anal. Calcd for $C_{26}H_{35}N_3O_3S$ × ½H_2O (478.65): C, 65.24; H, 7.58; N, 8.77; S, 6.70. Found: C, 65.12; H, 7.61; N, 8.75; S, 6.70.

*2.1.4.3. 3-Benzyloxy-17-oxo-16,17-secoestra-1,3,5(10)-triene-16-nitrile p-toluenesulfonyl hydrazone (**21**).* IR (KBr, cm^{-1}): 2243, 1607, 1362, 1168, 674.^{1}H NMR ($CDCl_3$, ppm): 1.08 (s, 3H, H-18); 2.48 (s, 3H, CH_3 fromTs); 5.04 (s, 2H, $CH_2C_6H_5$); 6.73–7.88 (12H, group of signals, aromatic protons); 8.12 (s, 1H, H-17). ^{13}C NMR ($CDCl_3$, ppm): 15.5 (CH_3); 16.5 (C-15); 21.6 (CH_3); 25.3; 26.9; 29.7; 36.9; 39.1; 42.4; 42.5; 69.9 (**C**$H_2C_6H_5$); 112.7 (CH); 114.4 (CH); 119.3 (CN); 126.3; 127.4; 127.9; 128.0; 128.5; 129.7; 130.9; 131.3; 134.6; 137.0; 137.3; 144.6; 156.9; 158.2 (C-17). HRMS (TOF) *m/z*: $C_{32}H_{35}N_3O_3S$ $[M+H]^+$ calculated: 542.24719. Found: 542.24708.

*2.1.4.4. General procedure for the preparation of compounds **22** and **23**; procedure for **24**.* To a solution of compound **20** or **21** (0.2 mmol) in EtOH (2 mL), $NaBH_4$ (0.68 mmol) was added, and the reaction mixture was microwave-irradiated at 80 °C for 20 min for **20**, or for 105 min for **21**. The resulting mixture was poured into water (100 mL) and acidified (6 M HCl) to pH = 1. The precipitated solid product of **22** or **23** was recrystallized from methanol for **22** (65 %, mp. 299 °C; Ref. [24] mp. 299 °C), or aqueous methanol (water was added to the methanol solution until cloudiness developed) for **23** (89 %, mp. 188 °C; Ref. [23] mp. 188 °C).

To a solution of **23** (103.2 mg, 0.27 mmol) in a mixture of methanol (3 mL) and dichloromethane (1 mL), 10 % Pd/C (30 mg) was added, and the reaction mixture was stirred at room temperature under a hydrogen atmosphere for 45 min. The catalyst was then filtered off, and the filtrate was evaporated. The pure product **24** was obtained after recrystallization from benzene/acetone (acetone was added to the benzene solution until cloudiness developed, 65 %, mp. 265−268 °C; Ref. [24] mp. 268 °C).

2.2. Determination of $C_{17,20}$-lyase and 17β-HSD1 activities, and inhibition by heterocyclic steroids

Inhibition of the $C_{17,20}$-lyase and 17β-HSD1 activities was investigated using *in vitro* radiosubstrate assays, following our previous methods [30–33], They are described briefly here, and the key

parameters are listed in Table 1.

2.2.1. Incubation procedures

As detailed in Table 1, the tissue preparations for enzyme sources were incubated in the assay buffer (100 mM HEPES, pH 7.3, 1 mM EDTA, 1 mM dithiothreitol) with 1 μM [^{3}H]-labeled substrate steroids in the presence of 0.1 mM coenzymes at 37 °C. The appropriate substrate was added as 20 μL of 25 % (v/v) propylene glycol in HEPES buffer. The test compounds were applied at the required concentrations in 10 μL of DMSO. These organic solvent contents in the 200 μL final assay volume did not have any significant effects on the enzyme activities.

2.2.2. Isolation of enzyme products

After the $C_{17,20}$-lyase and 17β-HSD1 incubations, the reactions were stopped by addition of 200 μL ethyl acetate combined with cooling. Unlabeled carriers of the substrate and the product steroids were added, and the samples were extracted. The substrates and products extracted using ethyl acetate were separated by thin-layer chromatography (Kieselgel-G plates; 0.25 mm thick; Merck Si 254 F) with the solvent system of dichloromethane/ diisopropyl ether/ ethyl acetate (75:15:10; v/v). UV light was used to reveal the separated steroids. The relevant spots were cut out, and the radioactivity associated with the products formed and of the substrates remaining were determined by liquid scintillation counting.

2.2.3. Inhibition assays

To define the enzyme inhibition of the test compounds, they were added to the assays as 0 (control), 10 μM, and 50 μM, for every series prepared. The relative conversions of substrate to product were defined, as compared to the non-inhibited controls (100 %). For the more potent inhibitors, the IC_{50} values were also determined, based on five or six concentrations of the test compounds from 0.1 μM to 50 μM. The IC_{50} values of the substrates were also determined, and were used as reference (Table 1).

2.3. Determination of the effects of the heterocyclic steroids against cancer cell lines

2.3.1. Chemicals and cell culture

The tumor cell lines used for the antiproliferative and proapoptotic assays were originally isolated from breast (MCF-7, T47D, MB-MDA-231 cells), cervix (HeLa, SiHa, C33A cells), and ovary (A2780 cells), with a non-cancerous fibroblast cell line also included (NIH/3T3 cells). Most of the cell lines were obtained from European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK), except for the SiHa and C33A cells (LGC Standards GmbH, Wesel, Germany). All of the cell lines were maintained in minimal essential medium supplemented with 10 % fetal bovine serum, 1 % non-essential amino acids, and 1 % penicillin–streptomycin–amphotericin B mixture, in an incubator with humidified air containing 5 % CO_2 at 37 °C. All of the chemicals and materials used for the assays were purchased from Sigma–Aldrich Ltd. (Budapest, Hungary), unless otherwise specified.

Table 1
Description of the radiosubstrate assay conditions used for the enzyme activities.

Condition	Enzyme	
	$C_{17,20}$-Lyase	17β-HSD1
Enzyme source	Rat testicular homogenate	Human placental cytosol
Substrate	17α-Hydroxyprogesterone	Estrone
Product	Androst-4-ene-3,17-dione	17β-Estradiol
Coenzyme	NADPH	NADPH or NADH
Incubation time	20 min	2.5 min
Reference IC_{50}	17α-Hydroxyprogesterone 1.6 μM	Estrone with NADPH 0.63 μM Estrone with NADH 2.0 μM

2.3.2. MTT assay

The *in vitro* antiproliferative properties of the test compounds were determined using the standard MTT assay [34]. The human breast, cervical and ovarian cancer cell lines and the mouse embryo fibroblasts were seeded in 96-well plates and treated with various concentrations of the test compounds (0.1–30 μM) for 72 h. After the treatments, 44 μL of 5 mg/mL 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the samples, which were then incubated at 37 °C for 4 h. The medium was removed, and the blue formazan crystals formed were dissolved using dimethylsulfoxide. The absorbance was measured at 545 nm using a microplate reader (Stat Fax-2100; Awareness Technologies Inc., Palm City, FL, USA).

2.3.3. Cell cycle analysis

For the analysis of the cell cycle, the HeLa, SiHa, and MDA-MB-231 cells were seeded into six-well plates at 300,000 cells/well for the controls, and 400,000 cells/well for the treated samples. The cells were treated with the test compounds at 3 μM and 10 μM for 24 h. The cells in the samples were harvested using trypsin, and with centrifugation at 264× *g* for 15 min at 4 °C. After an additional washing step with phosphate-buffered saline (PBS), the samples were fixed in 1 mL ice-cold 70 % ethanol. The fixed cells were stained with propidium iodide and analyzed by flow cytometry (CyFlow; Partec GmbH, Münster, Germany), and the proportions of the cells in the various phases of the cell cycle were calculated using the ModFit LT 3.3.11 software (Verity Software House, Topsham, ME, USA). At least 20,000 events were recorded for each sample.

2.3.4. Caspase-3 activity

The induction of caspase-3 activity of the cells by the test compound was determined in a colorimetric assay (Caspase 3 assay kits). The HeLa, SiHa, and MDA-MB-231 cells were seeded in cell culture flasks at 10^7 cells for the controls and 1.2×10^7 cells for the treated samples. The cells were treated with the test compounds at 3 μM and 10 μM for 24 h. The samples were then washed with PBS and removed from the flasks using a cell scraper. After additional centrifugation, the cells were lysed in lysis buffer on ice, and incubated with the different reagents, as recommended by the manufacturer protocol, for 90 min at 37 °C. The absorbances were determined at 405 nm using a microplate reader (Stat Fax-2100; Awareness Technologies Inc., Palm City, FL, USA).

2.3.5. RNA isolation

The isolation of the RNA from HeLa cells was carried out using NucleoSpin kits (Izinta Ltd., Budapest, Hungary). The cells were maintained under the culture conditions described above, and collected by trypsinization. Five million cells per sample were centrifuged at 206 × *g* for 15 min at 4 °C, washed twice with cold PBS, and lysed using β-mercaptoethanol. The lysates were filtered, treated with 70 % ethanol, and loaded onto the NucleoSpin RNA columns. The membranes of the columns were desalted using Membrane Desalting Buffer, and the DNA contents of the samples were digested using DNase reaction mixture, for 15 min at room temperature. Finally, the remaining pure RNA was washed three times using different buffers, as recommended by the manufacturer protocol, and was then eluted in RNase-free water.

2.3.6. Sample concentration and quality, and transcription

The overall minimal RNA integrity number was 9.0. The total RNA was reverse transcribed using SuperScript VILOTM cDNA synthesis kits (Invitrogen, Carlsbad, CA, USA). The total RNA (2.0 μg in 40 μL) was converted into cDNA according to the manufacturer instructions. The cDNA was stored at −20 °C.

2.3.7. Quantitative real-time PCR

The expression of the 10 genes of interest and four selected reference genes (*PPIA*, *HPRT1*, *GAPDH*, *POLR2A*) was examined using real-time TaqMan PCR assays. The expression levels were determined with the

exon-spanning hydrolysis probes (labeled with the FAM dye) that are commercially available as 'Assay on Demand' (Applied Biosystems, Foster City, CA, USA), using optimized primer and probe concentrations. Quantification was accomplished using a real-time PCR system (ViiA 7; Applied Biosystems). All of the samples were run as triplicates, using 0.25 μL cDNA, and the reactions were performed in 386-well plates in a reaction volume of 5.0 μL. Gene stability was analyzed using the GeNorm software, with GAPDH and HPRT1 shown to provide the best reference combination. The gene expression normalization factor for each sample was calculated based on the geometric mean of these two selected reference genes. The PCR amplification efficiency was determined from the slope of the log-linear portion of the calibration curve for each gene investigated, and this was accounted for in the further calculations. The gene expression for each sample was calculated from the crossing-point value (Cq) as E-Cq, divided by the normalization factor, and multiplied by 1012.

2.3.8. Statistical analysis

Evaluation for the statistical significances of the experimental data were estimated by one-way analysis of variance (ANOVA), followed by the Dunnett post-tests, using the GraphPad Prism software, version 5.01 (GraphPad Software, San Diego, CA, USA). Data are given as means ± SEM, and*, **, and *** indicate significance levels of $p < 0.05$, $p < 0.01$, and $p < 0.001$ compared to the relevant control samples. Statistical analysis of PCR assays was performed using GraphPad Prism V8.3, with one-way ANOVA with multiple comparisons used, where $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Chemical synthesis

Synthesis of the steroidal heterocycles was successfully performed, where the starting steroids were converted into the target compounds using microwave irradiation, instead of the use of the conventional heating previously reported [23,24,27,28]. The reaction times for most of these microwave-assisted reaction procedures were shorter, and the yields were higher, compared to the conventional synthesis.

3.1.1. D-homo lactones

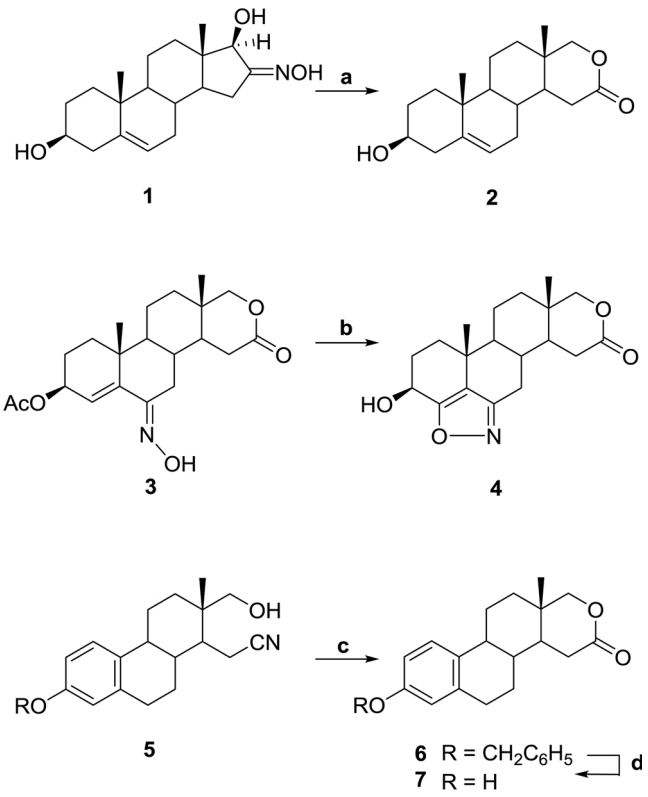
The treatment of starting hydroxyimino compound **1** (prepared as previously reported [35]) with potassium hydroxide in ethylene glycol, and its exposure to microwave irradiation enabled D-ring enlargement, and thus the formation of 3β-hydroxy-17-oxa-17a-homoandrost-5-en-16-one (**2**, Scheme 1). The microwave-assisted reaction was performed in a shorter time than the conventional reaction (reduced by a factor of 18) [27], while the yield of the product remained relatively higher (Table 2, 93 %).

3β-Hydroxy-isoxazolo[5′,4′,3′:4,5,6]-17a-homo-17-oxaandrost-4-en-16-one (**4**, Scheme 1) was synthesized by treatment of the 6-hydroxyimino parent compound **3** [28] with iodine, potassium iodide, and sodium hydrogen carbonate, in the mixture of tetrahydrofuran/ water, and with microwave irradiation conditions. This microwave-assisted reaction was more productive than the conventional one [28], with the reaction time reduced by a factor of 33, with an almost 9-fold higher yield (Table 2; 55 %).

The D-homo lactone compounds in the estrane series (**6** and **7**, Scheme 1) were synthesized by the conventional method [29], starting from known secocyanoalcohol **5** [36].

3.1.2. D-ring–fused tetrazoles

Tetrazoles with the androstane and estrane D-ring fused (*i.e.*, compounds **10**, **11**, **14** and **15**) were synthesized by click reactions from the appropriate mesylate parent compounds **8**, **9**, **12** and **13** [37,38], with azide in HMPA as the aprotic solvent. The reaction times for the microwave-assisted reactions were a little shorter than the relevant



Scheme 1. (**a**) I_2, KI, $NaHCO_3$, THF/H_2O (4:1), microwave 140 °C, 10 min. (**b**) $HOCH_2CH_2OH$, KOH, microwave 170 °C, 10 min. (**c**) *p*-TsOH, benzene, 4 h, reflux. (**d**) H_2, 10 % Pd/C, $CH_3OH + CH_2Cl_2$, rt.

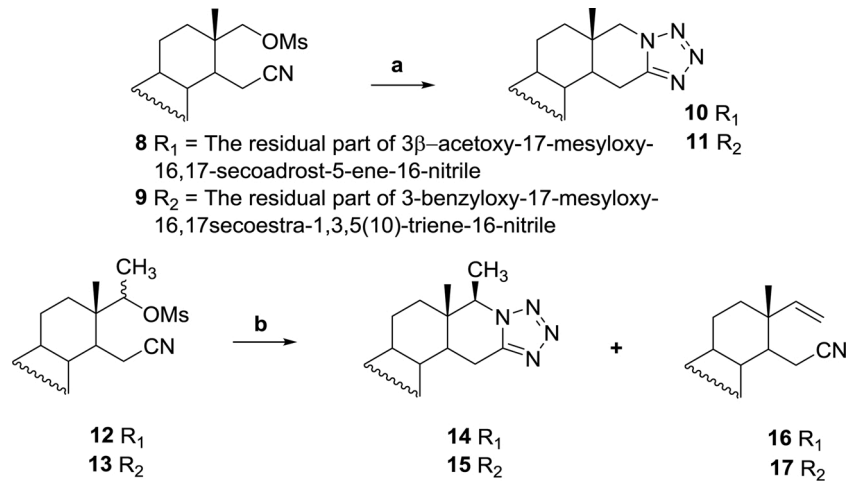
Table 2
Synthesis conditions and yields for the specific test compounds, under conventional and microwave-assisted heating.

Compound	Conventional heating			Microwave irradiation		
	Time (h)	Yield (%)	Reference	Temperature (°C)	Time (min)	Yield (%)
2	3.0	74	[27]	170	10	93
4	5.5	6.2	[28]	140	10	55
10	3.0	81	[23]	135	60	93
11	3.0	82	[23]	135	60	84
14	3.0	54	[23]	100	30	**14**: 40; **16**: 54
15	3.0	59	[23]	130/140	30/40	**15**: 30; **17**: 43
20	2.0	86	[24]	85	4	87
21	2.5	72	[24]	85	6	37
22	2.0	27	[24]	80	20	65
23	4.5	76	[24]	80	105	89

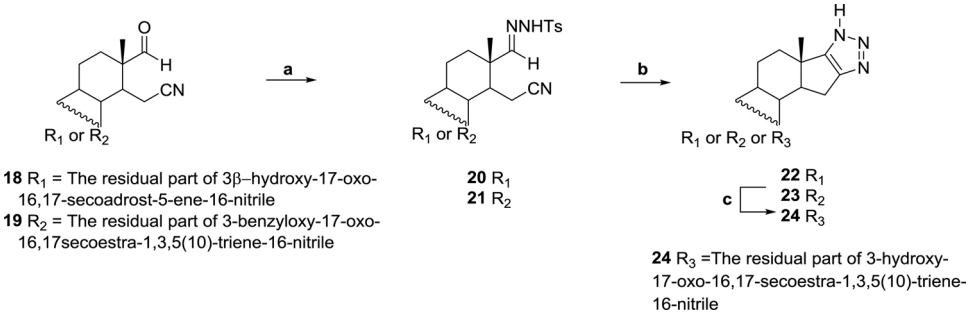
conventional ones [23], with similar yields obtained (Table 2). The most significant differences compared with the conventional synthesis were seen for the 17-methyl derivatives **12** and **13** as the parent compounds. Here, in addition to the expected tetrazole compounds **14** and **15**, the corresponding 17-olefines **16** and **17** were also obtained (Scheme 2).

3.1.3. D-ring–fused triazoles

Starting from the D-secoaldehydes **18** and **19** [35,36], *via* the appropriate *p*-toluene sulfonyl hydrazone derivatives (*i.e.*, **20** and **21**), synthesized by treating D-secoaldehydes with *p*-toluene sulfonyl hydrazide in ethanol, the D-ring–fused triazoles in both the androstane and estrane series (**22** and **23**) were synthesized. These compounds were then treated with $NaBH_4$, to yield the appropriate triazoles (**22** and **23**; Scheme 3). These reactions in both of the series were conducted under



Scheme 2. (**a**) NaN_3, HMPA, microwave 135 °C, 1 h. (**b**) NaN_3, HMPA, benzene, microwave 100 °C, 30 min for **12**, and microwave 130 °C, 30 min → microwave 140 °C, 40 min for **13**.



Scheme 3. Reagents and conditions: (**a**) $TsNHNH_2$, EtOH, 85 °C, microwave, 4 min for **18**, or 6 min for **19**. (**b**) $NaBH_4$, EtOH, 80 °C, microwave, 20 min for **20**, or 105 min for **21**. (**c**) H_2, Pd/C, rt, 46 h.

microwave irradiation. Compared to the conventional synthesis, the reaction times were shorter by a factor of 3–30, which depended on the reaction, while the yields obtained were in most cases improved a little (Table 2). Triazole **24** was obtained by hydrogenolysis of the appropriate 3-benzyloxy derivative **23** [24].

For easier comparisons of the reaction conditions and the product yields during both conventional and microwave-assisted reactions, these data are given in Table 2.

3.2. The effects of heterocyclic steroids on $C_{17,20}$-lyase and 17β-HSD1

$P450_{17\alpha}$ and 17β-HSD1 are key enzymes in the biosynthesis and activation of the sex hormones (Fig. 1). $P450_{17\alpha}$ is responsible for the supply of androgen and estrogen precursors. 17β-HSD1 activates the prehormones with the stereospecific reduction of the C17-oxo to 17β-hydroxy function. These steroidogenic processes take place primarily in the endocrine glands (*i.e.*, adrenals, ovaries, testes), while substantial steroid biotransformation occurs also in peripheral tissues of target organs. Blockade of the biosynthetic cascades with enzyme inhibitors can result in systemic and/or local androgen or estrogen ablation, which can be applied in the therapy of hormone-dependent diseases, such as prostatic cancer and breast cancer.

We investigated the inhibitory effects of the lactone compounds (**2**, **4**, **6** and **7**) and the D-ring–fused triazoles (**22**–**24**) and tetrazoles (**10**, **11**, **14** and **15**) on these two key steroid-converting enzyme activities *in vitro*. For $P450_{17\alpha}$, the inhibition of the rate-limiting $C_{17,20}$-lyase activity was tested. Molecular structural features defined the compounds according to certain enzyme assays. The inhibitory potentials of the estradiol analogs were investigated in the 17β-HSD1 enzyme assays. The $C_{17,20}$-lyase assays were performed with the androstane-derived compounds. These data are presented in Table 3.

Table 3
In-vitro inhibition of enzyme activities by the test compounds.

Compound		$C_{17,20}$-lyase inhibition		17β-HSD1 inhibition	
Group	No.	Rel. conversion (% ±SD)		Rel. conversion at 10 μM (% ±SD)	
		At 10 μM	At 50 μM	NADPH	NADH
Lactones	**2**	89 ± 1	74 ± 3	–	–
	4	93 ± 1	100 ± 1	–	–
	6	–	–	72 ± 7	63 ± 5
	7	–	–	90 ± 6	84 ± 8
Tetrazoles	**10**	107 ± 17	95 ± 9	–	–
	11	–	–	94 ± 9	89 ± 9
	14	100 ± 5	93 ± 2	–	–
	15	–	–	98 ± 0.3	91 ± 8
Triazoles	**22**	65 ± 6 [IC_{50}: 27 ± 7 μM]	41 ± 1	–	–
	23	–	–	110 ± 10	81 ± 5
	24	–	–	76 ± 16 [IC_{50}: 70 ± 15 μM]	27 ± 7 [IC_{50}: 2.6 ± 1.0 μM]

Rel. conversion, Relative conversions (control incubation, with no inhibition, 100 %) measured in the presence of 10 μM or 50 μM test compounds. Data are means ± standard deviation (SD) (n = 3).

3.2.1. Inhibition of $C_{17,20}$-lyase

Lactones **2** and **4**, and the 3-acetate tetrazoles **10** and **14**, did not show any inhibition in the $C_{17,20}$-lyase enzyme assays. The relative conversion was higher than 74 % even at the higher 50 μM test concentration, which indicated that the IC_{50} values were higher than 50 μM. Triazole **22** showed moderate inhibitory effects against rat testicular $C_{17,20}$-lyase, with an IC_{50} of 27 μM.

Series of potent $P450_{17\alpha}$ inhibitor androstene derivatives with imidazole, pyrazole, triazole, and tetrazole substituents on the D-ring can be found in the literature [39]. D-ring–fused derivatives have, however, been less investigated. In an earlier study, 16,17 D-ring–fused 3-hydroxy-androst-5-ene compounds were tested. Among these, a methyl-phenyl substituted methyl-pyrazole was more effective (IC_{50} = 5.8 μM), and its unsubstituted methylisoxazolidino counterpart showed a similar effect (IC_{50} = 27 μM) as **22** here [40].

3.2.2. Inhibition of 17β-HSD1

Triazole **24** showed weak inhibition of 17β-HSD1 in the presence of NADPH (IC_{50} = 70 μM). The inhibitory effect of **24** was more pronounced instead when the medium was supplemented with NADH, with an IC_{50} of 2.6 μM. This IC_{50} is close to that of the substrate estrone (IC_{50} = 2.0 μM), and thus indicates a similar affinity for 17β-HSD1. Benzylation of the 3-hydroxy group (**23**) resulted in the loss of this effect in the presence of both the phosphorylated and the unphosphorylated cofactor (relative conversions at 10 μM compound **23**: 110 % with NADPH, 85 % with NADH).

The two tetrazole D-ring–fused 3-benzyloxy estrane derivatives (**11** and **15**) and the lactone derivatives of both the estrone and its 3-benzyloxy counterpart (**6** and **7**) did not show any substantial 17β-HSD1 inhibition with both cofactors (relative conversions were higher than 63 % at 10 μM test concentration).

Numerous D-ring–fused, differently substituted *N*-heterocyclic estratriene compounds, have been reported as potent inhibitors of 17β-HSD1 [5,3,41]. The effects of the 16,17-fused pyrazolyl compounds have also been investigated in detail [12,42–45]. Molecular docking studies of a derivatized pyrazolyl estrone analog and a 17β-HSD1–NADPH complex indicated that the nitrogens of the pyrazole ring can bind the Pro-S hydrogen atom on the nicotinamide ring of the cofactor [45]. In another report, the binding mechanism of the pyrazolyl estrone analog was defined as the pyrazole N—H donating a H-bond to His210, with His213 adopting a tautomeric configuration to donate a H-bond to the pyrazole nitrogen atom (in the reference articles, these residues are numbered as His198 and His201, respectively [46,47]). It was also shown that the binding depends on the contributions of groups on the A and D rings, and compounds with phenolic hydroxyl function in the ring A show higher potential than the corresponding methyl ether derivatives [46].

The present triazole and tetrazole test compounds are close analogs of the pyrazole-fused inhibitors reported in previous studies. Accordingly, here also, the 3-hydroxy function was advantageous for binding to the 17β-HSD1. These 3-benzyloxy compounds do not inhibit 17β-HSD1 substantially. The 3-hydroxy triazolyl estrone analog **24** showed potent inhibition with NADH cofactor. This inhibition was selective towards the NADH-complexed enzyme, and the binding affinity was similar to that of the substrate estrone in this case. The mechanisms proposed earlier for the binding of the pyrazolyl estrone analog [45,46] can be regarded as plausible explanations for the binding of the present triazolyl compound. Determination of binding mechanisms of **24**, nevertheless, requires further structural investigations, *e.g.*, molecular docking studies.

Inhibition of **24** was selective towards the NADH-complexed enzyme. A similar, highly cofactor-dependent 17β-HSD1 inhibition was already reported for EM-1745 by Fournier et al. [48]. The EM-1745 is a hybrid inhibitor, in which an unphosphorylated cofactor-mimicking moiety was coupled to the estradiol core, and which was planned to act on both the active centre and the cofactor binding site of the enzyme. Our compound **24** does not possess cofactor-mimicking moiety, but displays large difference in inhibitory potential measured in the presence of NADPH or NADH.

It is known that 17β-HSD1 changes its conformation upon cofactor binding, and that the presence or absence of the 2′-phosphate in the cofactors causes structural differences in the area of the substrate binding site of the NADPH- and NADH-bound holoenzymes [49,50,26]. These cofactor-dependent inhibitory effects of estrane triazolyl compound **24** indicate that the optimal conformation for binding only exists in the NADH-bound enzyme.

We previously reported cofactor-dependent 17β-HSD1 inhibition by D-seco-oxime and D-seco-alcohol estrane-derived compounds. We assumed then that the binding of NADPH or NADH resulted in different conformations of the enzyme residues involved in forming hydrogen bonds or hydrophilic interactions with polar functionalities of the inhibitor molecules [51].

Our present study identified another type of estrone analog, the D-ring–fused triazolyl compound **24**, which shows 17β-HSD1 inhibitory effects with marked cofactor dependence. These data demonstrate again that the *in vitro* potentials obtained with the two cofactors might differ substantially for certain inhibitors. NADPH appears to be the prevailing cofactor in the hormonal activation of estrone to 17β-estradiol *in vivo* [52,53]. It is, therefore, worth emphasizing that *in vitro* data measured in the presence of NADPH are more relevant for inhibitor optimization and for lead selection [51]. The data measured with the NADH-complexed enzyme, nevertheless, might be valuable for an understanding of the binding and inhibition mechanisms of 17β-HSD1. The present data concerning 17β-HSD1 inhibitory potential of these D-ring–fused triazolyl and tetrazolyl estrone analogs might contribute to the development of new drug candidatesas novel anti-estrogens that act at the enzyme level.

3.3. The effects of the heterocyclic steroids on the cancer cell lines

Several D-ring–modified heterocyclic steroidal drug candidates show promising anticancer properties *in vitro,* so the antiproliferative effects of four lactone (**2, 4, 6** and **7**), four tetrazole (**10**, **11**, **14** and **15**) and three triazole (**22**, **23** and **24**) modified steroids were determined using the standard MTT assay following 72 h of treatment with the test compounds at 10 μM and 30 μM. Furthermore, the IC_{50} values were investigated when a test compound showed >50 % growth inhibition at 30 μM. These growth inhibitions and IC_{50} values are presented in Table 4.

In all cases, the lactone and tetrazole derivatives showed little, if any, inhibition of proliferation of these cancer cells. However, the antiproliferative activities of the triazole compounds in the estrane series were high (**24**) or moderate (**23**, as the 3-benzyloxy derivative of **24**). Due to the favorable effects of compounds **23** and **24**, their tumor selectivity indices were also defined. For this, their IC_{50} values were also measured with the noncancerous fibroblast cell line (NIH 3T3 cells). The ratio of these values for the malignant and normal cells were then calculated (*i.e.*, IC_{50} [cancerous]/ IC_{50} [non-cancerous]) (Table 5).

If the selectivity index is >1.0, this means that the test compound shows stronger antiproliferative activity against the cancer cell line than the nontumoral cell line. In most of the cases here, compounds **23** and **24** indeed showed selectivity indices >1.0. For the HeLa cells, this estrane triazole derivative with the free OH function in position 3 (**24**) had a selectivity index of 5.263; so compound **24** was five-fold more effective against these cervical cancer cells than the control fibroblasts.

Estimates from the World Health Organization have indicated that two types of human papillomavirus (HPV-16, HPV-18) cause 70 % of cervical cancers and pre-cancerous cervical lesions. In addition, breast cancer is the most common type of cancer in the female population, and in 10 %–15 % of cases of the triple negative subtype, there is poor prognosis compared to other breast cancer subtypes. The low survival rate here is explained by the lack of expression of estrogen, progesterone, and HER2 receptors by the triple negative subtype. Therefore,

Table 4
Antiproliferative properties of the test compounds following 72 h treatments of the indicated cell types with two fixed concentrations (10, 30 μM) and as IC_{50} values (from normalized six-point dose-response curves, where relevant). Data are means ± SEM from two independent experiments (n = 5).

Compound			Growth inhibition according to cell type													
Group	No.		HeLa		SiHa		C33a		MCF-7		T-47D		MDA-MB-231		A2780	
			10 μM	30 μM	10 μM	30 μM	10 μM	30 μM	10 μM	30 μM	10 μM	30 μM	10 μM	30 μM	10 μM	30 μM
Lactones	**2**	% Inh	13.0	30.4	13.6 ±	20.4	12.3	20.2	<10	14.3	17.6	38.2	<10	16.2	<10	29.4
		±SEM	± 1.9	± 2.8	3.9	± 4.0	± 6.2	± 4.0		± 1.0	± 1.6	± 1.5		± 1.3		± 2.4
		IC_{50} (μM)	>30		>30		>30		>30		>30		>30		>30	
	4	% Inh	10.8	17.2	<10	12.9	<10	<10	<10	<10	21.2	29.4	13.5	17.5	<10	14.8
		±SEM	± 1.1	± 1.9		± 3.8					± 1.3	± 1.6	± 1.0	± 0.9		± 3.8
		IC_{50} (μM)	>30		>30		>30		>30		>30		>30		>30	
	6	% Inh	31.4	36.9	11.4 ±	29.6	25.1	39.3	<10	27.1	19.7	22.2	<10	20.4	10.3	31.1
		±SEM	± 1.5	± 2.6	4.5	± 2.1	± 2.2	± 2.0		± 1.0	± 1.3	± 4.2		± 1.6	± 1.4	± 1.4
		IC_{50} (μM)	>30		>30		>30		>30		>30		>30		>30	
	7	% Inh	13.0	26.4	<10	12.4	<10	12.1	<10	14.7	13.1	17.3	<10	15.0	<10	15.2
		±SEM)	± 0.8	± 2.2		± 2.7		± 4.5		± 0.3	± 1.3	± 1.3		± 1.8		± 1.8
		IC_{50} (μM)	>30		>30		>30		>30		>30		>30		>30	
Tetrazoles	**10**	% Inh	32.0	39.9	<10	<10	<10	30.7	15.6	48.3	27.9	43.2	<10	16.7	18.6	41.3
		±SEM	± 1.3	± 0.9				± 1.6	± 1.1	± 0.7	± 0.3	± 1.0		± 1.0	± 1.7	± 0.8
		IC_{50} (μM)	>30		>30		>30		>30		>30		>30		>30	
	11	% Inh	17.2	39.5	<10	22.8	<10	29.8	21.3	47.2	23.9	40.3	28.9	48.2	<10	26.5
		±SEM	± 1.2	± 2.1		± 1.4		± 2.0	± 1.1	± 1.8	± 1.7	± 0.6	± 2.1	± 1.7		± 0.9
		IC_{50} (μM)	>30		>30		>30		>30		>30		>30		>30	
	14	% Inh	27.2	33.2	<10	<10	<10	18.6	<10	40.7	28.4	30.9	14.9	20.7	<10	33.8
		±SEM	± 2.7	± 2.1				± 4.0		± 1.9	± 1.4	± 1.8	± 5.0	± 4.0		± 1.3
		IC_{50} (μM)	>30		>30		>30		>30		>30		>30		>30	
	15	% Inh	<10	47.2	<10	35.9	30.4	55.3	19.4	32.9	27.4	54.2	23.2	39.6	20.1	25.7
		±SEM		± 2.3		± 2.8	± 2.2	± 1.5	± 2.2	± 1.6	± 0.7	± 0.8	± 3.3	± 3.6	± 1.9	± 3.2
		IC_{50} (μM)	>30		>30		24.0 ± 1.0		>30		27.0 ± 1.9		>30		>30	
Triazoles	**22**	% Inh	<10	37.7	<10	<10	13.5	18.8	20.1	39.8	13.7	38.7	<10	44.4	<10	54.5
		±SEM		± 1.8			± 2.7	± 2.4	± 2.7	± 3.9	± 0.5	± 1.5		± 0.9		± 2.1
		IC_{50} (μM)	>30		>30		>30		>30		>30		>30		29.6 ± 0.3	
	23	% Inh	37.5	62.1	<10	64.1	28.9	79.7	10.7	84.0	15.9	75.9	<10	90.4	14.7	71.0
		±SEM	± 0.9	± 1.3		± 2.3	± 2.5	± 1.2	± 1.4	± 0.5	± 2.4	± 1.0		± 0.5	± 2.2	± 1.5
		IC_{50} (μM)	11.1 ± 1.2		28.1 ± 0.9		16.8 ± 1.3		26.1 ± 1.9		18.6 ± 1.3		17.9 ± 0.5		15.9 ± 2.0	
	24	% Inh	83.3	88.8	21.8 ±	48.7	49.2	68.7	39.7	63.1	52.8	62.7	42.2	53.9	60.4	80.9
		±SEM	± 1.4	± 1.1	3.8	± 0.7	± 0.4	± 1.3	± 0.5	± 2.3	± 0.4	± 0.7	± 1.4	± 2.3	± 1.1	± 0.8
		IC_{50} (μM)	5.7 ± 0.1		>30		13.3 ± 0.1		16.0 ± 1.5		11.5 ± 0.8		22.4 ± 2.2		11.2 ± 2.0	
Reference	Cisplatin	% Inh	32.2	93.7	60.10	89.0	77.2	97.5	54.1	95.5	40.8	60.0	42.7	86.4	64.6	94.8
		±SEM	± 1.2	± 0.8	± 0.9	± 0.5	± 1.1	± 0.1	± 1.2	± 0.3	± 1.8	± 0.7	± 2.7	± 0.4	± 0.5	± 0.4
		IC_{50} (μM)	12.14		4.29		5.85		8.34		17.48		10.17		5.27	

Table 5
Tumor selectivity indices of compounds **23** and **24** on cancer and non-cancerous cell lines, as calculated from their IC_{50} values (*i.e.*, IC_{50} [non-cancerous]/ IC_{50} [cancerous]).

Cell line	Tumor selectivity index	
	23	**24**
HeLa	1.811	5.263
SiHa	0.715	1.000
C33a	1.196	2.256
MCF-7	0.770	1.875
T-47D	1.081	2.609
MDA-MB-231	1.123	1.339
A2780	1.264	2.679

hormone therapy and drugs that target estrogen, progesterone or HER2 receptors are ineffective. On this basis, the cell lines chosen for further investigation here were: HeLa cells, as a cervical cancer cell line that contains the integrated genome of HPV-18; SiHa cells, as a HPV-16–positive cervical cancer cell line; and MDA-MB-231 cells, as a highly aggressive, invasive, and poorly differentiated triple negative breast cancer cell line.

The aim here was to characterize the mechanisms of action and the apoptosis-inducing properties of compound **24** on these three different gynecological cancer cell lines, with cell cycle analysis carried out by flow cytometry. As shown in Table 4, the antiproliferative IC_{50} values for compound **24** were 5.7 μM with HeLa cells, >30 μM with SiHa cells, and 22.4 μM with MDA-MB-231 cells. To allow for these large differences and for reasons of comparability, compound **24** was applied here at 3 μM and 10 μM, for 24 h. These treatments resulted in significant and concentration-dependent increases in hypodiploid subG1 phase cells for all three of these cell lines (Fig. 2). The relative proportions of this subG1 cell population compared to the control cells seen for the cells treated with 10 μM compound **24** was 2.5-fold for HeLa cells, 5-fold for MDA-MB-231 cells, and >14-fold for SiHa cells. Additionally, relative decreases were seen for S phase in all cases, with the greatest effects for the

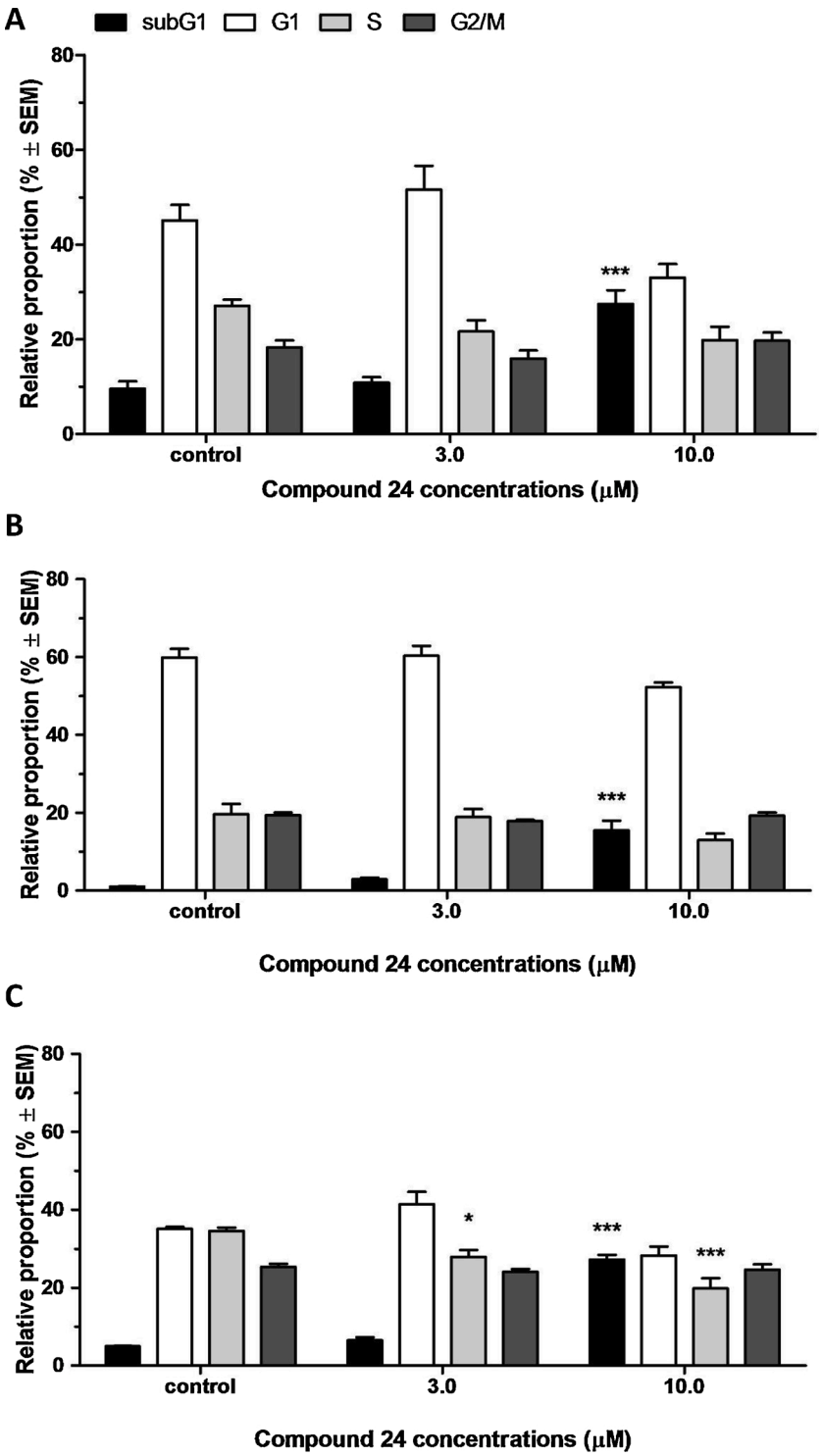


Fig. 2. Compound **24** increases the subG1 phase cell population in the HeLa (**A**), SiHa (**B**) and MDA-MB-231 (**C**) cell lines. The cell cycle distribution was examined after incubation with 3 μM or 10 μM compound **24** for 24 h. Data are means ± SEM. *, $p < 0.05$; ***, $p < 0.001$, *versus* relevant control cells.

triple negative breast cancer cell line; namely, the MDA-MB-231 cells. The significant increase in the fraction of cells with reduced DNA content (*i.e.*, subG1 phase) is considered as evidence of drug-induced programmed cell death (Fig. 2).

One of the main criteria for any new, effective antineoplastic agents is that the cancer cells are destroyed by programmed cell death. Programmed cell death is mainly identified as caspase-dependent apoptosis, although other caspase-independent pathways are involved in the manifestation of anticancer effects [54]. For compound **24**, significant increases in the activity of caspase-3 were seen in these HeLa, SiHa, and MDA-MB-231 cells at 3 μM compound **24**, with a large reduction then seen at 10 μM compound **24** after 24 h of treatment (Fig. 3). The apparent contradiction between the significant increase in the cell population with reduced DNA content that is measured here by the cell cycle analysis and the decrease in caspase-3 activity can be explained by activation of such caspase-independent signaling pathways. This activation might complement the caspase-dependent processes, as indicated by increased caspase-3 activity at the lower compound **24** concentration

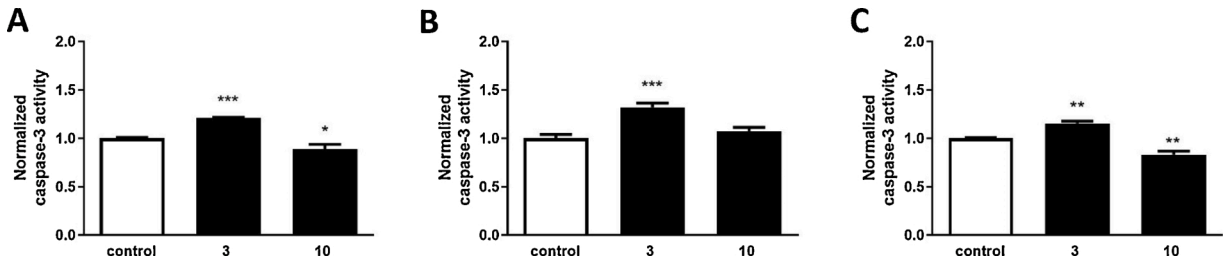


Fig. 3. Effects of 3 μM and 10 μM compound **24** for 24 h on caspase-3 activity in HeLa (**A**), SiHa (**B**), and MDA-MB-231 (**C**) cells. Data are means ± SEM *, $p < 0.05$; ***, $p < 0.001$, *versus* relevant control cells.

(Fig. 3).

Yao et al. [55] reported that a steroidal compound 23-hydroxybetulinic acid (B4G2) causes depolarization of the mitochondrial membrane, and induces intrinsic apoptosis of HepG2 cells. This was not attenuated by Z-VAD-FMK, a pan-caspase inhibitor, so induction of mitochondrial apoptosis might indeed be independent of caspases. Moreover, corosolic acid induces caspase-independent cell death *via* lipid peroxidation in human malignant cell lines (*i.e.*, Caski, ACHN, A498, MDA-MB-231, SK-Hep1, Huh7 cells), which can be largely prevented by α-tocopherol [56]. Interestingly, in DS-sarcoma cells, 2-methoxyestradiol was shown to mediate its proapoptotic effects through a caspase-independent mechanism, *via* the release of apoptosis inducing factor (AIF) and endonuclease G from the mitochondria [57]. Due to similarities in the chemical structures and the experimental data seen here, it would appear that compound **24** might have a comparable mechanism of action. However, bearing in mind the diversity of the possible molecular interactions and signal transduction pathways involved here, in case of compound **24**, identification of the underlying mechanism of action requires additional investigations.

3.4. Expression of genes that encode enzymes of estrogen biosynthesis and inactivation

17β-Hydroxysteroid dehydrogenase type 2 (encoded by the *HSD17B2* gene) is responsible for rapid conversion of biologically potent estradiol to the weak estrogenic metabolite, estrone. 17β-HSD2 also mediates transformation of the active androgen hormone testosterone to androstenedione, and of androstendiol to dehydroepiandrosterone. Appropriate expression of this enzyme maintains the balance of endogen steroids, which helps to prevent the development of serious disorders linked to high estrogen levels, such as endometriosis or breast cancer [58,59]. Several potent steroidal lactone and spiro-γ-lactone derivatives have been reported to be inhibitors of 17β-HSD2 [60,61]. Compound **23** caused elevated mRNA expression of *HSD17B2* after 24 h of treatment in HeLa cells (Fig. 4). Investigations whether this was triggered by enhanced gene transcription or feedback of 17β-HSD1 inhibition and lack of activity in cells require further examination, as compound **23** is a benzylated analog of **24**.

One of the actions of estrogen sulfotransferase (encoded by *SULT1E1*) is catalysis of sulfate conjugation of estradiol and estrone, which inactivates them, and has a central role in estrogen homeostasis [62]. Overexpression of *SULT1E1* has been shown to inhibit cell proliferation, induce apoptosis, and suppress angiogenesis, invasion, and metastasis of breast cancer *in vitro* [63]. This estradiol-derived compound **24** significantly increased mRNA expression of *SULT1E1* in HeLa cells after 24 h exposure (Fig. 4), which indicated a further alternative mode of action for compound **24**.

No particular changes were seen for mRNA expression of the CYP19A1, HSD17B1, HSD17B4, HSD17B7, HSD17B8, HSD17B12, HSD17B14, and STS enzymes after 24 h treatment with these steroid triazole compounds **23** and **24** (Supplementary Materials).

4. Conclusions

Compared with conventional synthesis of organic compounds, the use of microwave reactors has been shown to be associated with several advantages. These can include shorter reaction times, less solvent and/or reagents used, and less energy spent, along with improved yields and/or the formation of different or additional products during microwave assisted reactions. Here we have reported on the synthesis of steroidal heterocycles using microwave-assisted reactions, with evaluation of the pharmacological properties of these compounds.

In vitro enzyme assays revealed that the D-ring–fused triazolyl estrone compound **24** showed substantial inhibition of the NADH-complexed 17β-HSD1 enzyme, with a binding affinity similar to that of the substrate estrone. The effects here were, however, markedly weaker against

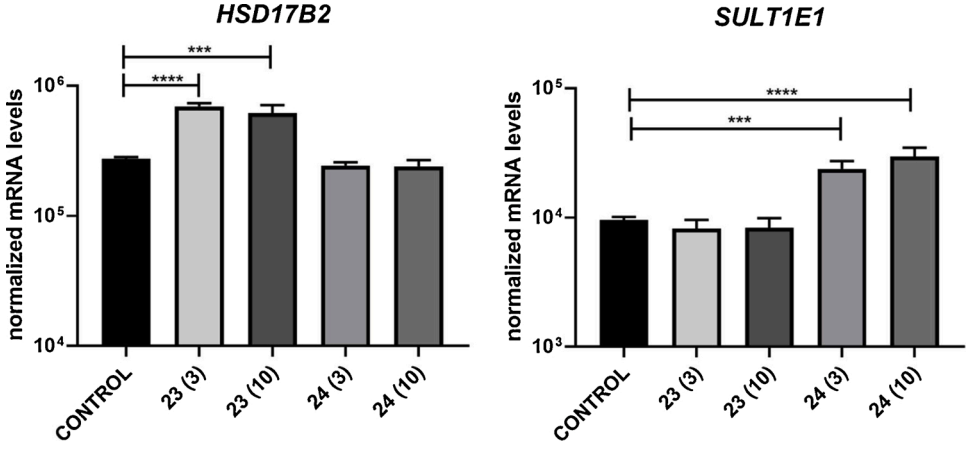


Fig. 4. Effects of 3 μM and 10 μM compounds **23** and **24** on expression of the HSD17B2 and SULT1E1 genes after 24 h treatment of HeLa cells. Data are means ± SEM. ***, $p < 0.0005$; ****, $p < 0.0001$, *versus* the relevant control.

the NADPH-bound enzyme complex. These data for compound **24** demonstrate again that the inhibitory potentials obtained with the two cofactors of 17β-HSD1 are not interchangeable. Comparative investigations on cofactor-dependent inhibition offer good experimental tools for studies of the mechanisms of inhibitor binding to 17β-HSD1, a key enzyme in the biosynthesis of 17β-estradiol.

In cell-based systems, both of these estrane triazole compounds (**23**, with a benzylated group; **24**, with a free 3-OH group) showed good antiproliferative effects, while compound **24** with free OH function proving more effective. Both of these compounds showed good selectivity towards cancer cells compared to non-cancerous cells. Compound **24** had a large influence on the cell cycle of the HeLa, SiHa, and MDA-MB-231 cancer cells, as cells of gynecological origin, where the subG1 fraction was especially pronounced, which is regarded as the apoptotic cell population. Induction of apoptosis in these cells treated with compound **24** was confirmed by the measures of the caspase-3 activity. This also indicated activation of caspase-independent signaling pathways in all three of these model cell lines.

Estrane triazole **23** promoted elevated mRNA expression of the *HSD17B2* gene after 24 h treatment in the HeLa cell model system, which is connected with the increased production of the less active estrone from estradiol. Having in mind the facts that compound **23** is benzylated analogue of compound **24**, and that eukaryotic etherase enzymes are present in liver cells, probably this compound can be hydrolyzed *in situ* [64]. However, hydrolysis of the ether-protected compounds does not occur or occurs slowly *in vitro*, which could explain weaker effect of compound **23** compared to **24** against cell lines. Benzylated estrane triazole **23** could serve as an excellent basis for the development of a prodrug of **24** in future studies, including parallel syntheses of substituted benzyl-ether analogues and their testing in benzyl-ether self-immolative strategy [65–67].

Further, compound **24** significantly increased mRNA expression of *SULT1E1* in HeLa cells after 24 h exposure, which indicates its activity in estrogen inactivation process. Bearing in mind all of these modes of action of this triazole **24**, which target the enzyme, gene expression and cell levels, compound **24** represents a potential drug candidate to target estrogen-dependent diseases.

Author contributions

Conceptualization and design: S. J-Š.; Synthesis and characterization of modified steroids: A.N., A. O., M. S. and S. J-Š.; Enzyme-based experiments and data analysis: B. E. H and M. Sz.; Cell-based experiments and data analysis: A. E. K., V. N., I. O. and I. Z.; Gene expressions experiments and data analysis: M. S. and T. L. R; Drafting and revising the article: S. J-Š., M.Sz, I. Z. and T. L. R.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2021.105997.

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