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Apoptosis induction in HeLa cervical cancer cell line by steroidal 16,17-seco-16,17*a*-dinitriles

LIDIJA D. ALEKSIĆ[1], ANDREA R. NIKOLIĆ[2#], VESNA V. KOJIĆ[1], MARIJA N. SAKAČ[2] and SUZANA S. JOVANOVIĆ-ŠANTA[2*#]

[1]*Oncology Institute of Vojvodina, Faculty of Medicine, University of Novi Sad, Put Doktora Goldmana 4, 21204 Sremska Kamenica, Serbia and* [2]*University of Novi Sad, Faculty of Sciences, Department of Chemistry, Biochemistry and Environmental Protection, Trg Dositeja Obradovica 3, Novi Sad, Serbia*

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Abstract: Steroids are good candidates for drug development, thanks to their low general toxicity and possibility for structure modifications connected with change of their activity. Several 16,17-secoandrost-4-ene-16,17*a*-dinitrile compounds were synthesized and screened for anticancer effect previously, including 6-oxo and 6-hydroxyimino compounds. This research is continued with the attempts for different synthetic strategy and evaluation of anticancer effect mechanism. Synthesis of 3-hydroxyimino compounds was successful, but inseparable mix of isomers was excluded from biological tests. Tested secodinitriles expressed cytotoxic effect on HeLa cervix cancer cells as a model system, with submicromolar to molar IC_{50} values, where 6-substituted derivatives were more effective. After 72 h treatment with equitoxic concentrations equal IC_{50} values of test compounds the mechanism of this effect was studied using flow cytometry and specific fluorescent dyes. Modest change in both G0/G1 and G2/M resting phases and change in mitochondrial membrane potential were noticed, while the most pronounced effect was apoptosis induction. Total apoptosis was in range 50.72–58.31 % in all cell samples treated with secodinitriles, compared to 7.44 % in control samples. Total percent of dead cells, including both apoptotic and necrotic, ranged from 55.24 to 65.34 %, compared to 10.68 % in control. Selectivity towards cancer cells is very important feature of these compounds indicating their potential use as lead compounds in the drug development for the treatment of cancers of steroid hormone-dependent tissues.

Keywords: secosteroids; anticancer effect; cell cycle; mitochondrial membrane potential; proapoptotic effect; flow cytometry.

* Corresponding author. E-mail: suzana.jovanovic-santa@dh.uns.ac.rs
# Serbian Chemical Society member.
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INTRODUCTION

Cancer incidence and mortality are rapidly growing worldwide. Cervical cancer, with an estimated 570,000 cases and 311,000 deaths in 2018 worldwide, ranks as fourth most frequently diagnosed cancer, fourth leading cause of cancer death in women and second in incidence and mortality, behind breast cancer. It is the most commonly diagnosed cancer in 28 countries and the leading cause of cancer death in 42 countries.[1] Such scary data are very stimulating for many researchers to participate actively in the discovery of new, more active anticancer drugs. In the last period, increasing advances in cancer research have enhanced the understanding of cancer biology and mechanism, so it is established that anticancer effects of cytotoxic drugs or compounds under test can be exhibited through selective cytotoxicity, anti-proliferative action, induction of apoptosis or other changes in the cancer cells, which would lead to cancer cells death.[2]

The design and development of effective anticancer drugs with low or no side effects are the main topic of research in many scientific groups worldwide.[3] Steroids are among the substances of interest, thanks to their huge structural diversity and, accordingly, huge ability of modified steroids to interact with various biological targets, as well as to take roles in signalling pathways. Many synthetic steroids are pharmacologically active compounds. They can be used either for hormonal therapy, one of the main approaches for the medical treatment of hormone-dependent cancers, including inhibition of specific steroid-converting enzymes or steroid hormone receptors signaling,[4] or for treating of cancer thanks to their cytotoxic effects through non-hormonal targets or pathways.

There are many steroids, natural[5] or synthetic, expressing antiproliferative effect against cancer cells.[6] 6-Hydroxyimino cholestane compounds, isolated from marine sponges, expressed cytotoxicity against several types of cancer cells.[7] Based on that, many 6- or 3-hydroxyiminosteroid derivatives, mostly cholestane-based, were synthesized as analogs of natural steroids, where some of them expressed biomedical potential, including cytotoxic or antiviral activity.[8–11]

Based on these facts, as well as on our previous results, here we present an attempt to synthesize 3-hydroxyimino and 6-hydroxyimino-D-secoandrostane derivatives through modified synthetic steps. Besides, the present study aimed to investigate the mechanism of anticancer effect of a group of steroidal 16,17-seco-dinitrile against HeLa human cervical cancer cell line, as a model system for steroid hormone-dependent cancer of reproductive tissue. The proportion of the apoptotic and necrotic cells, cell cycle distribution and change in mitochondrial membrane potential ($\Delta\Psi m$) of HeLa cells after treatment with selected secosteroids, recognized previously to express antiproliferative effect,[12,13] was evaluated using flow cytometry (FC), powerful technique in drug discovery and medicine.

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EXPERIMENTAL

General

Melting points are reported as uncorrected (Electrothermal apparatus 9100). Infrared (IR) spectra were recorded on a spectrometer (Nexus 670), with wave numbers in cm^{-1}. Nuclear magnetic resonance (NMR) spectra were obtained using a spectrometer (Bruker AC 250E) operating at 250 (^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 were recorded using time-of-flight liquid chromatography–mass spectrometry (6210; Agilent Technologies), operated in positive electrospray ionization mode. Experimental conditions, yields and spectral characterisation of novel compounds are given in Supplementary material to this paper.

Test compounds. Steroidal 16,17-seco-16,17*a*-dinitriles (**5**–**8**), for which antiproliferative effect and/or potential for induction of apoptosis of HeLa cells were noticed after 48 h treatment, were synthesized as previously described,[12,13] or *via* alternative pathways, and purified to analytical grade. Reference compounds used in study – exemestane (Exe), cisplatin (Cis) and doxorubicin (Dox) – were dissolved in DMSO and diluted with PBS to prepare working solutions.

Cell-based experiments

Cell lines and treatment. Antiprolifertive activity of the steroidal compounds was tested against cervix adenocarcinoma HeLa cell line (ATCC CCL2), as well as against normal fetal lung fibroblasts, MRC-5 cell line (ATCC CCL 171), used for testing selectivity of cytotoxicity. Cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 4.5 % glucose, 10 % of fetal calf serum (Sigma-Aldrich) and antibiotic/antimycotic solution (Sigma–Aldrich). Cell lines were cultured in flasks (Costar, 25 cm^2) or 6-well plates at 37 °C in high humidity and 5 % CO_2 atmosphere. Only viable cells were used in the assay, while viability was measured by DET with trypan blue.[14]

Antiproliferative assay. Antiproliferative activity of selected modified steroids was evaluated by MTT assay,[15,16] where number of viable cells was measured after 72 h exposure to test compounds in concentration range from 0.01 to 100 μM (0.01; 0.1; 1; 10 and 100 μM). Reference compounds used in this assay were cisplatin (Cis) and doxorubicin (Dox), as non-selective anticancer agents,[17,18] and steroidal aromatase inhibitor, exemestane (Exe), used to test general steroidal toxicity (Fig. S-1, Supplementary material).[19] Cells were seeded into 96--well plates at a density of 5000 cells/well, stood overnight in complete medium at 37 °C, after which the solutions containing the test compounds were added (10 μL/well) in all wells except controls. After incubation during 72 h, 10 μL of MTT solution (5 mg/mL) and acidified 2-propanol were added to each well. Absorbance was read after few minutes incubation at room temperature on a spectrophotometric plate reader (Multiscan MCC340, Labsystems) at 540/690 nm. Wells without cells containing complete medium only were used as a blank.

Flow cytometry analysis. HeLa cells were seeded into 6-well plates at a densities from 3×10^5 to 4×10^5 cells/well. Treatment of HeLa cells for all flow cytometry analyses (cell cycle analysis, detection of apoptosis and necrosis and quantification of mitochondrial membrane potential change) was performed with equitoxic concentration of test compounds, corresponding IC_{50} values of each compound after 72 h treatment.[20] For each analysis treated cells were stained with specific dye (or dyes) and prepared for the analysis in specific manner. Flow cytometry was performed using FACSCalibur Becton Dickinson (BD) Bioscience Immunocytometry System, while results were analysed using CellQuest software;[21] in each analysis

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10000 (apoptosis detection) or 20000 (cell cycle and MMP change) events were recorded from samples containing 10^6 of cells, and percentage of cells in each population was determined.

Cell cycle analysis. Distribution of cells in various phases of cell cycle was analysed by measuring cellular DNA content after 72 h exposure to modified steroids, when cells were harvested with trypsin and centrifuged at 1500 rpm for 10 min, then washed twice with cold phosphate-buffered saline (PBS). Cells were fixed with 70 % ethanol for 1 h at 20 °C, washed with PBS, re-suspended in a solution of PI (50 µl/mL, Invitrogen) and RNase A (250 µg/mL, Thermo Fisher Scientific) at a density of 10^6 cells /mL and incubated in the dark for 30 min at room temperature.[22] Such ethanol-fixed cells were analysed by flow cytometry. After recording 20000 events, the content of the cells in the different cell cycle phases (subG1, G0/G1, S and G2/M) were determined by using CellQuest software.[21] The subG1 fraction was regarded as the apoptotic cell population.

Detection of apoptosis and necrosis. Apoptosis induction of HeLa cells was measured by flow cytometry, using annexin V-fluorescein isothiocyanate propidium iodide (FITC/PI; BD Pharmingen) apoptosis detection assay. In brief, HeLa cells were exposed to test substances during 72 h at 37 °C, then harvested, washed twice with ice-cold PBS and re-suspended in annexin V binding buffer at a density of 10^6 cells /mL. Subsequently, 100 µL of cell suspension was transferred into a flow cytometry tube, and 5 µl of annexin V-FITC and 10 µl of PI were added to the cell suspension. The sample was gently mixed by vortex. The stained samples were incubated for 15 min at room temperature in the dark. An additional 400 µl annexin V binding buffer was added to each tube. Samples with 10^6 of cells were analysed within 1 h on flow cytometer. The percentages of the cells in the different populations (normal, early apoptotic, late apoptotic and necrotic cells) were determined in each analysis by using CellQuest software.

Quantification of mitochondrial transmembrane potential. Mitochondrial transmembrane potential ($\Delta\psi m$) was measured using a cationic fuorochrome Rhodamine 123 (Rh123; Sigma–Aldrich), as described previously.[23] Briefly, 10^6 cells, resuspended in 200 µL of PBS, were stained with Rh123 (2.5 µg/mL) for 30 min at 37 °C. After washing with PBS, samples were analysed by flow cytometry using Cell Quest software (Becton Dickinson, Heidelberg, Germany). M1 region represents cells with intact mytochondrial membrane integrity, while M2 region includes cells with membrane integrity loss.

Data analysis. All experiments in this study were performed twice. In the antiproliferative assay two independent experiments were conducted in quadruplicate for each concentration of test compound. Antiproliferative activity was determined using the MTT assay after exposure to 0.01, 0.1, 1, 10 and 100 µM test compound for 48 or 72 h. Mean values and standard deviations (*SD*) were calculated for each concentration, and IC_{50} values were determined by median effect analysis. All statistical processing were calculated in Microsoft Office Excel program.

RESULTS AND DISCUSSION

Steroidal compounds are known for their specific physiological effects. On the other hand, modified steroids can express effects more or less different from their natural analogues, despite large similarity in their structures. Design of novel compounds can be based on structural features of known physiologically or pharmacologically active compounds. In such chemistry-driven medicinal chem-

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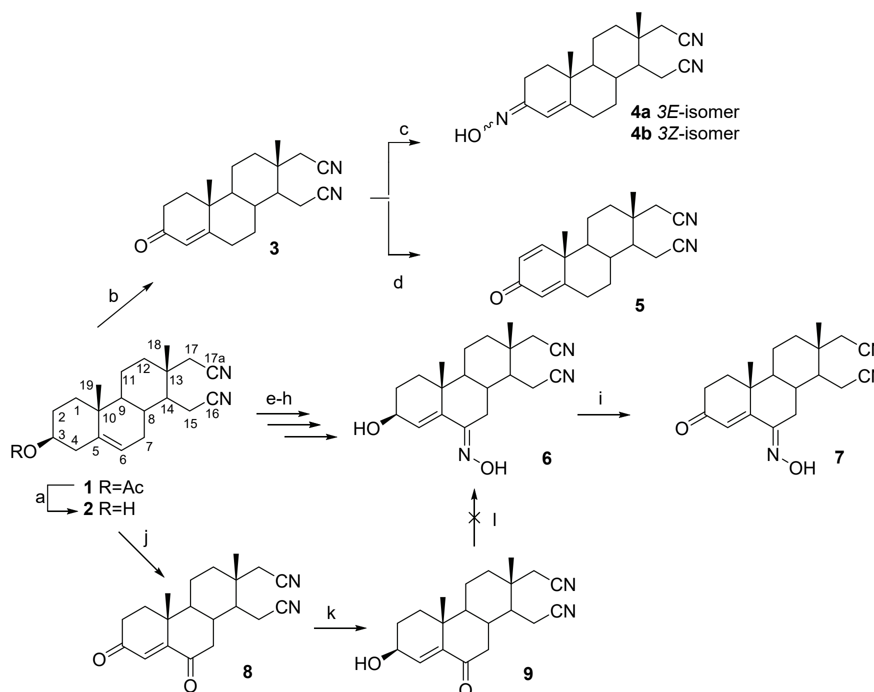
istry a series of steroidal 16,17-seco-16,17*a*-dinitriles were synthesized,[12,13] possessing both steroidal core, important for expressing some specific biological effects, and nitrile functions, present in structures of some drugs and responsible for their activity.[24]

Biomedical potential of hydroxyiminosteroids was recognized, while androstane-derived hydroxyimino compounds are among the topics in our research. Based on these facts and anticancer properties of our previously synthesized androstane-derived compounds, we synthesized two novel 3-hydroxyimino-16,17-secoandrost-4-en-16,17*a*-dinitrile compounds and attempted to apply alternative synthetic approach in the synthesis of (6*E*)-hydroxyimino derivative[12] in order to obtain substances for biomedical tests and potentially improve synthetic procedures (Scheme 1). Novel compounds and those, recognized earlier for their cytotoxic effect against cervical adenocarcinoma HeLa cell line, were planned to be tested for their pharmacological effect and an attempt was made to find the mechanism of activity against cancerous cell lines.



Scheme 1. a) EtONa, EtOH, 50–55 °C, 1 h; b) CrO_3, H_2SO_4, H_2O, acetone, 0 °C, 25 min; c) NH_2OH×HCl, EtOH, AcONa, 70 °C, 2 h; d) DDQ, toluene, TFA, BSTFA, reflux, 6 h; e) 1. *m*-CPBA, CH_2Cl_2, rt, 90 min; 2. CrO_3, H_2O, acetone, rt, 70 min; f) $SOCl_2$, pyridine, 0 °C, 90 min; g) NH_2OH×HCl, pyridine, rt, 24 h; h) EtONa, EtOH, rt, 1 h; i) CrO_3/Py, rt, 75 min; j) CrO_3, H_2SO_4, H_2O, acetone, 0 °C, 25 min; k) $NaBH_4$, $CoCl_2$×$6H_2O$, MeOH, rt, 15 min; l) NH_2OH×HCl, AcONa, EtOH, rt, 20 h.

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Starting compounds in these syntheses (**1** and **2**) were obtained by known procedure.[13] Oppenauer oxidation of compound **2** afforded enone **3**.[13] Oxyimination of compound **3** with hydroxylamine hydrochloride in ethanol in the presence of sodium-acetate afforded mixture of (3*E*) and (3*Z*) isomers, namely **4a** and **b**, in a total yield of 90 %. Isomers could not be separated by crystallization or column chromatography, so the mixture of isomers was characterized. The presence of both (3*E*) and (3*Z*) hydroxyimino derivatives in the obtained final product was confirmed by analysis of NMR spectra. In the ^{1}H-NMR spectrum in DMSO-d_6 two clearly differentiated singlets at 10.27 and 10.54 ppm were observed, corresponding to the protons of two hydroxyl groups of hydroxyimino functions of two isomers. Singlets on 5.73 and 6.36 ppm originate from H-4 atoms of two isomers. (3*E*) and (3*Z*) isomers were obtained in ratio 7.5:1, where (3*E*) isomer **4a** was identified as dominant isomer, based on NOE NMR experiment. Namely, by irradiation of H-4 proton (signal at 6.36 ppm), an increase of the signal intensity at 10.27 ppm was observed, suggesting distance less than 4 Å between H-4 and the hydrogen of hydroxyimino group. Compound **3** served also as starting compound in the synthesis of dienone **5**, synthesized by known procedure.[13]

In order to improve total yield of hydroxyiminoenone **7**, synthesized by oxidation of C-3 hydroxyl group of **6** with chromium trioxide/pyridine complex and reported previously[13] (Scheme 1, e–i), an attempt to synthesize its precursor hydroxyiminoenol **6**, *via* alternative synthetic pathway, has been made (Scheme 1 a, j–l). Previously we synthesized compound **6** from **1** in four steps ("3-acetyloxy pathway"). Rapid one-pot procedure was used for the synthesis of 5*α*-hydroxy-6-oxo derivative starting from **1**. After dehydration of this compound, resulting 3*β*-acetoxy-4-en-6-one derivative was treated with hydroxylamine hydrochloride in pyridine to obtain 3*β*-acetoxy-4-en-6-hydroxyimino compound. The cleavage, of the acetate from C-3 with sodium ethoxide in ethanol, has occurred giving desired hydroxyiminoalcohol **6**.

Retro-synthetic analysis indicated that the (6*E*)-3*β*-hydroxy-hydroxyimino-16,17-secoandrost-4-ene-16,17*a*-dinitrile (**6**) could be obtained by oxyimination of 3*β*-hydroxy-6-oxo-16,17-secoandrost-4-ene-16,17*a*-dinitrile (**9**). This key intermediate (**9**) we synthesized by regioselective reduction of the oxo group at position C-3 of 4-ene-3,6-dione **8**. The reaction was performed with sodium borohydride in methanol, in the presence of cobalt (II) chloride hexahydrate, where after 15 min at room temperature product was obtained in satisfactory yield (64 %). The structure of the compound **9** was confirmed based on NMR spectral data. The presence of a hydroxyl group in the structure of compound **9** was determined based on the doublet at 5.03 ppm in the proton NMR spectrum, derived from the hydrogen atom of that group, while the presence of the oxo group was confirmed by ^{13}C-NMR spectral signal for the C-6 atom at 200.19

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ppm. In the mass spectrum dominant peak corresponds to $[M+H]^+$ signal. In a final step of the synthesis, oxyimination of compound **9** with hydroxylamine hydrochloride in ethanol at room temperature in the presence of sodium acetate, was attempted, but product **9** was not obtained even after 20 h.

All synthesized compounds were tested for their antiproliferative effects against the spectrum of cancer cell lines, except (3*E*)- and (3*Z*)-4-ene-3-oximes **4a** and **b**, whose mix could not be separated. Namely, separation and purification of compounds are of particular importance in drug development, where interactions of drug or drug candidate with receptors in the living cells are based on the compound's purity.[25] Some of tested secodinitriles previously were recognized as antiproliferative agents in *in vitro* experiments against cancer cell lines of reproductive tissues.[12,13] Since non-hormonal mechanism was proposed,[13] while apoptosis was visually noticed after 48 h treatment for couple of compounds,[12] more extensive research has been undertaken as an attempt to define the mechanism by which several of these compounds reduce the number of cancer cells, using HeLa cervix cancer cell line as a model system, which was the most sensitive cell line on these compounds.

In the previous studies antiproliferative effect of the series of androstane-derived secodinitriles after 48 h treatment was tested against the palette of carcinoma cell lines. Secodinitrile compounds which expressed significant cytotoxicity (with IC_{50} lower than 10 μM) against HeLa cervical adenocarcinoma cell lines (**5**–**8**) and novel compound **9** were included in this experiment, where their antiproliferative effects were measured against the same cell line after 72 h treatment. 3-hydroxyimino derivatives **4a** and **b** were excluded from this research, because they could not be separated and thus fully purified, while purity of compounds is crucial for their binding to the biological target molecules. Antiproliferative effects of the reference compounds against HeLa cell line were tested in the same conditions (Fig. S-1). All androstane-derived and reference compounds were also tested against normal fetal lung fibroblasts cell line MRC-5.

Secodinitrile compounds expressed very good, submicromolar to micromolar cytotoxicity after 72 h treatment of HeLa cells (IC_{50} values 0.46–8.23 μM), similar to widely used cytotoxic drug cisplatin, though cisplatin was more toxic against healthy cells, MRC-5 cell line. Opposite, secosteroids were not toxic on healthy cells (Table I). Exemestane, aromatase inhibitor, the drug of choice in the treatment of estrogen-dependent diseases, decreased number of HeLa cells only after longer treatment (72 h). The most effective in reducing number of HeLa cells were 16,17-seco-16,17*a*-dinitriles with hydroxyimino function in position 6 (**6** and **7**), with submicromolar IC_{50} values, then 6-oxo derivative **8**, while secodinitrile with no substituent at C-6 (**5**) was less active. 16,17-Seco-16,17*a*-dinitrile compound, possessing A/B ring moiety similar as exemestane (6-methylene-2-en-3-one), was not effective in reducing numbers of HeLa cells, although sub-

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stitution at position 6 seems to be important for their biological effect, so this compound was not included in the further study.[13] Doxorubicin, very often used cytotoxic drug, expressed high toxicity, even equal, against both cervix cancer and normal cell lines.

TABLE I. Comparison of antiproliferative effect (IC_{50}±SD / µM) of the tested androst-5-ene-16,17-seco-16,17*a*-dinitriles and the reference compounds, exemestane (Exe), cisplatin (Cis) and doxorubicin (Dox) obtained for 48 and 72 h of action; N/A – IC_{50} value was not available due to very low effect

Compound	Cell line			
	HeLa		MRC-5[a]	
	48 h[13]	72 h	48 h[13]	72 h
5	12.49±1.23	8.23±2.04	>100	>100
6	0.48±0.007	0.46±0.11	>100	>100
7	2.64±0.51	0.79±0.36	>100	>100
8	4.31±0.96	2.83±0.06	>100	>100
9	>100	–	>100	–
Exe[b]	>100	18.18±1.25	>100	N/A
Cis[c]	1.77±0.11	2.10±0.78	0.48±0.08	0.24±0.01
Dox[c]	1.17±0.09	0.07±0.001	0.12±0.04	0.10±0.04

[a]Non-cancerous control; [b]control steroidal compound in clinical use against breast cancer; [c]control antiproliferative compound

Starting from the cytotoxicity data the mechanism of action of compounds, that showed to be good antiproliferative agents against treated HeLa cervical cancer cells (**5**–**8**), was studied.

Exemestane was used further as the reference compound, since active secosteroids are C-6 substituted. Cell cycle of HeLa cells was examined, after 72 h of treatment with secodinitrile compounds in equitoxic doses, equal IC_{50} values, where cells after treatment were stained with propidium iodide (PI). There was modest influence of secosteroids noticed in FC analysis (Figs. S-2 and S-3, and Table S-I of the Supplementary material): decrease in cell number in G0/G1 cell cycle phase arrest (about 10 % for all steroids, and the highest for compound **7**, 14.5 %) and increase of cell numbers in G2/M phase (about 10%, for all steroidal compounds, including reference exemestane), while number of cells in synthetic (S) phase was slightly higher when cells were treated with compound **7**. There was no significant change in subG1 fractions, referred hypodiploid cells.

Induction of apoptosis and necrosis was examined next. Cells treated for 72 h with secodinitrile compounds were stained with Annexin V-FITC and PI in order to distinguish and count early- and late-apoptotic, necrotic from normal cells using flow cytometry. Previously visually noticed apoptosis during shorter treatment[12] was now quantified after longer treatment, since cell number in early and late apoptosis was counted. Namely, annexin is dye that is binding to phospholipid constituents of cell membrane, indicating plazma membrane permeabil-

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ity, while PI is binding to DNA of cells with disturbed plazma membrane (cells in late apoptosis or necrotic cells). Combination of these two dyes gives information about integrity of the cells and availability of DNA binding. All steroids used in the treatment of HeLa cells induced apoptosis in high extent, where more cells were in late apoptosis than in early stage. Total apoptotic cell number ranged from 50.72 (**8**) to 58.31 % (**5**) in all samples of cells treated with secosteroids, while in case of exemestane this effect was a little bit less manifested, 42.16 %, compared to control samples with 7.44 % of total apoptosis*, i.e.*, spontaneous apoptosis. Necrosis was noticed in all samples as well, where in the highest extent was noticed in the samples with hydroxyimino compounds **6** and **7** (about 10 %). In all cases apoptosis was more common cause of cell deaths, while total percent of dead cells ranged from 55.24 (**8**) to 65.34 % (**5**), Figs. 1 and S-4, and Table S-2 of the Supplementary material).

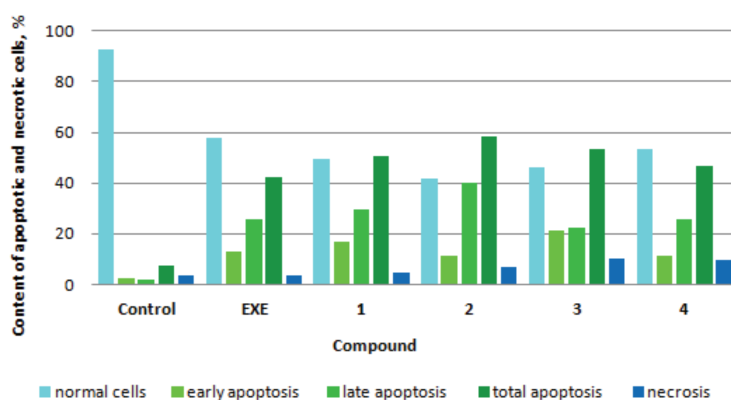

Fig. 1. Induction of apoptosis of HeLa cells after 72 h treatment with steroidal 16,17-seco-16,17*a*-dinitriles and exemestane.

A distinctive feature of apoptosis is the disruption of normal mitochondrial function, especially changes that affect the mitochondrial membrane potential (*MMP*). Mitochondrial membrane potential change ($\Delta\psi m$) is crucial for maintaining the physiological function of the respiratory chain to generate ATP, necessary for cell survival. One of the earliest changes that occurs in apoptosis is a change in the transmembrane potential, so $\Delta\psi m$ is usually associated with mitochondrial or intrinsic apoptosis, although it can cause increase in inner mytochondrial membrane permeability *via* death receptors or protein channels activation, associated with extrinsic apoptosis.[26] To test if change in mitochondrial membrane potential included in apoptosis of HeLa cells, after treatment with steroids cells were stained with rhodamine, which accumulates in mitochondrial matrix if permeability of inner mitochondrial membrane raised, and analysed by FC. As can be seen in Fig. S-6 (based on Fig. S-5 and Table S-3) of the Sup-

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plementary material, there is no big change in *MMP* of HeLa cells after treatment with secodinitriles or Exe. Percent of influenced cells (M2 fraction of cells), *i.e.*, cells with more permeable mytochondrial inner membrane, is quite small in all samples (about 8 % for secosteroids and 4 % for Exe).

Apoptosis can be induced with one or more events, which can start a series of further changes, leading to apoptotic death of the cell, *via* intrinsic or extrinsic pathway. In some cases, these pathways can be combined or crossed. Each signalling pathway consists of many components, so it is quite difficult to precise which compound is sensitive on certain treatment. For first screening, to test antiproliferative activity of compounds, viability test is used. For recognizing the mechanism underlying cytotoxic effect many methods are available, the most of them based on using fluorescent dyes, specific for tracking certain changes in the cells. The technique the most used and useful in tracking changes in the treated cells, using specific dyes, is flow cytometry. Flow cytometric analysis gives answers and/or directs further experiments, in order to find a way by which compounds express their biological or pharmacological effects. Based on all mentioned, namely generally accepted facts and obtained results, apoptosis induction in androstane compounds-treated HeLa cells is the main cause of cell death, with no significant evidence of disturbed cell cycle or change in inner mitochondrial membrane potential. Still, to have a complete insight into the mechanism and target molecules in the signalling pathways causing apoptosis, other methods should be included in the study.

CONCLUSION

Modified steroids can express similar or different, sometimes even opposite, biological or pharmacological effects, comparing to the endogen steroids, *i.e.*, their parent molecules. Strength of such compounds can be found during extensive biological tests. Accordingly, alternative or simplified routes for the synthesis of biologically effective compounds and synthetic strategies for the synthesis of novel derivatives are always needed. 16,17-Seco-16,17*a*-dinitriles, derivatives of androst-4-ene, were synthesized and recognized to express strong antiproliferative effect against HeLa cervix cancer cells, with submicromolar to molar IC_{50} values. An attempt to synthesize these active compounds *via* alternative synthetic pathway is performed, as well as synthesis of novel androstane-derived compounds. Apoptosis of HeLa cells treated with these compounds, observed in morphological study, initiated further research to discover the mechanism of cell toxicity of these compounds. Apoptosis was recognized as the most important cause cell number lowering, while certain cell cycle and mitochondrial membrane potential changes were detected, with no direct correlation with apoptosis. All changes were detected and quantified by using flow cytometry and dyes, specific for certain changes in the cells. Substitution in position 6 of stero-

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idal core seems to be important for the expressing of anticancer effect: polar 6-hydroxyimino derivatives were the most cytotoxic, with submicromolar IC_{50} values, while 6-methylene analogue or the compounds with no substituents at C-6 were less or no active. In the cells treatment with equitoxic doses, all tested compounds induced apoptosis in high extent (more than 47 % of the cells were apoptotic), while total percent of dead cells, both apoptotic and necrotic, was in the range of 55–65 %. Further experiments are needed to define target molecules in the apoptosis promoting, while novel compounds could be designed, based on the structural features of effective and selective anticancer agents, presented here.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/10993, or from the corresponding author on request.

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И З В О Д

ИНДУКЦИЈА АПОПТОЗЕ HeLa ЋЕЛИЈСКЕ ЛИНИЈЕ СТЕРОИДНИМ 16,17-СЕКО-16,17*а*-ДИНИТРИЛИМА

ЛИДИЈА АЛЕКСИЋ[1], АНДРЕА НИКОЛИЋ[2], ВЕСНА КОЈИЋ[1], МАРИЈА. Н. САКАЧ[2] и СУЗАНА ЈОВАНОВИЋ-ШАНТА[2]

[1]*Универзитет у Новом Саду, Медицински факултет, Институт за онкологију Војводине, Пут др Голдмана 4, 21204 Сремска Каменица и* [1]*Универзитет у Новом Саду, Природно–математички факултет, Департман за хемију, биохемију и заштиту животне средине, Трг Доситеја Обрадовића 3, 21000 Нови Сад*

Стероиди су добри кандидати за развој лекова, захваљујући њиховој ниској општој токсичности и могућим структурним модификацијама, повезаним са променом њихове активности. Раније је синтетисано више 16,17-секоандрост-4-ен-16,17*а*-динитрила, укључујући 6-оксо и 6-оксиимино деривате и прелиминарно је испитана њихова антиканцерска активност. Ова истраживања су настављена покушајима различите синтетичке стратегије и евалуације механизма антиканцерског деловања. Синтеза 3-хидроксиимино једињења је била успешна, али нераздвојива смеша изомера није укључена у биолошке тестове. Тестирани секодинитрили су испољили цитотоксични ефекат према HeLa ћелијама карцинома грлића материце, одабраном за модел систем, са субмикромоларним до моларним вредностима IC_{50}, при чему су 6-супституисани деривати били ефикаснији. После 72-часовног третмана ћелија еквитоксичним концентрацијама испитиваних једињења, једнаким IC_{50} вредностима испитиваних једињења, механизам овог деловања је проучаван применом проточне цитометрије и специфичних флуоресцентних боја. Уочене су извесне промене у G0/G1 и G2/M фазама мировања, као и промене у мембранском потенцијалу митохондрија, док је најизраженији ефекат био индукција апоптозе. Укупна апотоза била је у опсегу 50,72 до 58,31 % у свим узорцима третираним секостероидима, у поређењу са 7.44 % у контролном узорку. Проценат мртвих ћелија, укључујући апоптотичне и некротичне, био је у опсегу 55,24 до 65,34 %, у поређењу са 10,68 % у контролном узорку. Веома важна карактеристика ових једињења је селектив-

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ност према ћелијама канцера, без детектованог ефекта на здраве ћелије MRC-5 линије, што их сврстава у водећа једињења у развоју лекова против канцера ткива зависних од стероидних хормона.

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