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SEARCH FOR NEW DRUGS

SYNTHESIS AND CYTOSTATIC ACTIVITY OF NEW MEPREGENOL 17-ACETATE DERIVATIVES WITH RESPECT TO HELA CANCER CELL CULTURE

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New mepregenol 17-acetate derivatives with potential cytotoxicity were synthesized. Mepregenol 17-acetate 3-acrylate (**III**) was shown to exhibit potent cytotoxicity although the ester of *N*-benzylpyrrolidine-3-carboxylic acid (**IV**) had weaker activity that was comparable with those of megestrol acetate and progesterone. The IC_{50} values for **III**, **IV**, progesterone, and megestrol acetate were 30, 200, 480, and 130 μM with respect to native HeLa culture and 9.1, 180, 5.6, and 115 μm for an estradiol-stimulated culture of HeLa, respectively. The cytotoxicities of steroids **III** and **IV** correlated with their DNA-damaging effect in a DNA comet assay. Thus, **III** was promising for further research as an antitumor compound with respect to estradiol-dependent tumors.

Keywords: pregnane steroids, gestagens, estradiol, HeLa, cervical cancer.

Esters of 17α-acetoxy-3β-hydroxy-6-methylpregna-4,6-dien-20-one with gestagenic and antiproliferative activities after peroral administration that exceed significantly those of progesterone were reported [1 – 3]. The main metabolic centers on C3, C6, and C17 are blocked in 17α-acetoxy-β-hydroxy-6-methylpregna-4,6-dien-20-one esters, which prolongs the *in vivo* residence time and the pharmacological effects [4]. However, the structure—activity relationship of 17α-acetoxy-β-hydroxy-6-methylprena-4,6-dien-20-one esters cannot be fully analyzed because of their limited number. The goals of the present work were to prepare and study the cytotoxicities of new 17α-acetoxy-β-hydroxy-6-methylpregna-4,6-dien-20-one esters as compared to megestrol acetate and progesterone using HeLa native human cervical cancer cell culture and an estradiol-stimulated culture (HeLa-ESC).

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EXPERIMENTAL CHEMICAL PART

Commercially available reagents and solvents were used in the syntheses. Melting points were determined in open capillaries and are uncorrected. PMR and ^{13}C NMR spectra were recorded in $CDCl_3$ and DMSO-d_6 solutions with residual solvent resonances as standards on Bruker AM-400 and Agilent 400 MR instruments at operating frequency 400 MHz and 293 – 303 K. Chemical shifts (δ) were given in ppm; SSCC (*J*), in Hz. Structural data for mepregenol 17-acetate 3-acrylate (**III**) were obtained using a STOE diffractometer, Pilatus 100K semiconducting detector, microfocused Cu Kα beam (1.54086 Å), and a multilayer thin-film focusing monochromator (M. V. Lomonosov MSU, Chemistry Department). X-ray data were processed using the STOE X-AREA 1.67 program suite (STOE & Cie GmbH, Darmstadt, Germany, 2013). Specific rotation was measured at 589 nm (Na *D*-line) on a PerkinElmer 341 MC polarimeter. Elemental analyses of the compounds were determined on a Vario microcube Elementar automated analyzer.

Mepregenol 17-acetate (II, AMOL). A solution of megestrol 17-acetate (**I**, 1.000 g) in THF (50 mL) was stirred

and treated at room temperature with $NaBH_4$ (0.100 g) and then distilled H_2O (5 mL). Additional $NaBH_4$ (0.030 g) was added after 2 h. The mixture was stirred for 1 h, treated with distilled H_2O (150 mL), and stirred for 2 h until a precipitate formed. The precipitate was filtered off, rinsed with distilled H_2O, and dried to constant mass to afford **II** (0.965 g, 97%) as a colorless solid, mp 195°C. PMR spectrum (400 MHz; $CDCl_3$), δ, ppm: 5.55 (s, 1H), 5.49 (s, 1H), 4.29 (br.s, 1H), 2.95 (dd, J 13.9, 11.3 Hz, 1H), 2.16 – 2.08 (m, 1H), 2.06 (s, 3H), 2.08 – 2.05 (m, 1H), 2.04 (s, 3H), 1.92 (dd, J 12.8, 4.1 Hz, 1H), 1.90 – 1.82 (m, 2H), 1.80 (s, 3H), 1.73 (s, 1H), 1.71 – 1.49 (m, 5H), 1.49 – 1.24 (m, 4H), 1.17 – 1.04 (m, 1H), 0.97 (s, 3H), 0.69 (s, 3H). ^{13}C NMR spectrum (100 MHz; $CDCl_3$), δ, ppm: 14.37, 18.08, 20.08, 20.31, 21.14, 23.29, 26.33, 28.67, 30.30, 31.24, 33.69, 35.01, 36.83, 47.55, 49.39, 51.06, 68.25, 96.60, 123.88, 128.32, 131.40, 145.16, 170.70, 204.20.

(3*S*,8*R*,9*S*,10*R*,13*S*,14*S*,17*R*)-17-Acetoxy-17-acetyl-6,10,13-trimethyl-2,3,8,9,10,11,12,13,14,15,16,17-dodecahydro-1*H*-cyclopenta[*a*]phenanthrenyl-3-yl acrylate (III). Mepregenol 17-acetate (**II**, 9.60 g) in CH_2Cl_2 (150 mL) was cooled to –5°C, treated with Et_3N (3.50 mL), stirred under Ar, treated in portions with a solution of acryloyl chloride (2.70 g) in CH_2Cl_2 (30 mL) without allowing the temperature of the reaction mixture to rise, stirred at room temperature for 12 h after the addition of acryloyl chloride was finished, and washed with H_2O and saturated NaCl solution. The organic layer was dried over anhydrous Na_2SO_4. The solvent was removed at reduced pressure in a rotary evaporator. The solid was purified by column chromatography over silica gel with elution by petroleum-ether—EtOAc (5:1) to afford **III** (7.50 g, 73%) as a colorless solid, mp 184 – 185°C. $[\alpha]_D^{20}$ – 79.7 (*c* 0.98, CH_2Cl_2). Calc, %, $C_{27}H_{36}O_5$: C 73.61; H 8.24. Found, %: C 73.72; H 8.29. PMR spectrum (400 MHz; $CDCl_3$), δ, ppm: 6.41 (dd, J 17.3, 1.5 Hz, 1H), 6.13 (dd, J 17.3, 10.4 Hz, 1H), 5.82 (dd, J 10.4, 1.5 Hz, 1H), 5.49 (s, 1H), 5.46 (d, J 3.1 Hz, 1H), 2.96 (dd, J 15.2, 1.3 Hz, 1H), 2.93 (dd, J 15.8, 2.4 Hz, 1H), 2.15 – 2.07 (m, 2H), 2.06 (s, 3H), 2.02 (s, 3H), 1.94 (t.d, J 12.7, 4.2 Hz, 1H), 1.90 – 1.79 (m, 3H), 1.77 (dd, J 1.9, 1.3 Hz, 3H), 1.75 – 1.67 (m, 2H), 1.64 – 1.57 (m, 1H), 1.56 – 1.50 (m, 1H), 1.46 – 1.33 (m, 2H), 1.30 (ddd, J 16.5, 10.8, 3.7 Hz, 1H), 1.16 – 1.06 (m, 1H), 0.97 (s, 3H), 0.67 (s, 3H). ^{13}C NMR spectrum (100 MHz; $CDCl_3$), δ, ppm: 198.92, 165.51, 160.91, 141.72, 126.07, 125.40, 123.72, 123.63, 114.25, 66.04, 45.72, 44.19, 42.38, 31.65, 29.83, 28.25, 26.08, 25.15, 21.15, 19.16, 18.14, 15.99.

(8*R*,10*R*,13*S*,14*S*,17*R*)-17-Acetoxy-17-acetyl-6,10,13-trimethyl-2,3,8,9,10,11,12,13,14,15,16,17-dodecahydro-1*H*-cyclopenta[*a*]phenanthren-3-yl-1-benzylpyrrolidine-3-carboxylate (IV). A solution of acrylate **III** (1.000 g) in toluene (100 mL) was stirred, cooled to 0°C under Ar, treated sequentially with *N*-methoxymethyl-*N*-(trimethylsilylmethyl)benzylamine (0.670 g) and CF_3CO_2H solution (0.1 mL, 1 M) in CH_2Cl_2, and stirred at room temperature for 12 h. The solvent was removed at reduced pressure in a rotary evaporator. The solid was purified by column chromatography over silica gel with elution by CH_2Cl_2—MeOH (100:1(50:1) to afford **IV** (1.140 g, 88%) as a colorless oil, $C_{36}H_{47}NO_5$. Calc, %: C 75.36; H 8.26; N 2.44. Found, %: C 75.48; H 8.29; N 2.51. $[\alpha]_D^{20}$ – 88.4 (*c* 1.18, MeOH). PMR spectrum (400 MHz; $CDCl_3$), δ, ppm: 7.41 – 7.17 (m, 5H), 5.51 (s, 1H), 5.41 (d, J 2.9 Hz, 1H), 3.64 (d, J 6.1 Hz, 1H), 3.13 – 2.85 (m, 3H), 2.67 (dd, J 14.1, 7.0 Hz, 1H), 2.56 (d, J 9.0 Hz, 1H), 2.06 (d, J 14.9, 8H), 2.22 – 2.01 (m, 4H), 1.96 (d, J 4.2 Hz, 1H), 1.92 – 1.63 (m, 10H), 1.57 (t, J 3.2 Hz, 1H), 1.54 (d, J 3.9 Hz, 1H), 1.50 – 1.22 (m, 4H), 1.12 (br.s, 1H), 0.98 (s, 3H), 0.69 (s, 3H). ^{13}C NMR spectrum (100 MHz; $CDCl_3$), δ, ppm: 204.14, 174.98, 170.71, 146.79, 131.26, 128.85, 128.69, 128.39, 128.22, 126.94, 119.52, 96.62, 71.18, 60.02, 56.78, 56.68, 53.80, 50.89, 49.38, 47.57, 47.41, 42.29, 36.83, 35.01, 33.43, 31.26, 30.34, 27.76, 27.59, 26.34, 24.32, 23.32, 21.18, 21.03, 20.27, 20.07, 17.93, 14.38.

EXPERIMENTAL BIOLOGICAL PART

HeLa cell culture (human epithelial cervical cancer) was obtained from the Russian Cell Culture Collection. Cells were cultivated under sterile conditions using an LB-V laminar flow hood and incubated at 37°C with 5% CO_2. Standard DMEM medium with added thermally inactivated fetal bovine serum (20%, PanEco, Russia), L-glutamine (100 μg/mL) and antibiotics gentamycin sulfate and streptomycin sulfate (40 μg/mL) was used for cell culture. HeLa-ESC cells were grown at 37°C in the presence of estradiol (10^{-8} M) (Sigma, USA) in DMEM medium containing fetal bovine serum (20%).

Cells were grown in 25-mL tubes, trypsinized after forming a monolayer, and placed (200 μL) in wells of a COSTAR flat-bottomed plate. Solutions of compounds in DMEM medium were added to final concentrations of 10^{-7} – 10^{-5} M. The plates were incubated for 72 h, after which the viability of the cultures was assayed using the standard MTT test [5]. Medium was removed from the plate wells after the cells were incubated with the compounds. DMEM/F12 medium (1:1, 200 μL) and starting MTT solution (10 mg/mL, 10 μL each) were placed into the wells. Cells were incubated at 37°C for 3 h in a moist atmosphere with 5% CO_2. When the incubation was finished, the medium was removed from the wells. DMSO (150 μL) was added to each well to dissolve the formed formazan crystals with shaking for 15 min at room temperature. The color was recorded by determining the optical density at 530 nm on a plate photometer (Uniplan AIFR-01, Russia). The ratio of optical densities of this compound concentration to the average for the control was taken as the fraction of surviving cells.

Apoptotic activity of progesterone, megestrol acetate, and the new mepregenol 17-acetate derivatives (**III** and **IV**) was analyzed using a DNA comet assay. For this, an HeLa

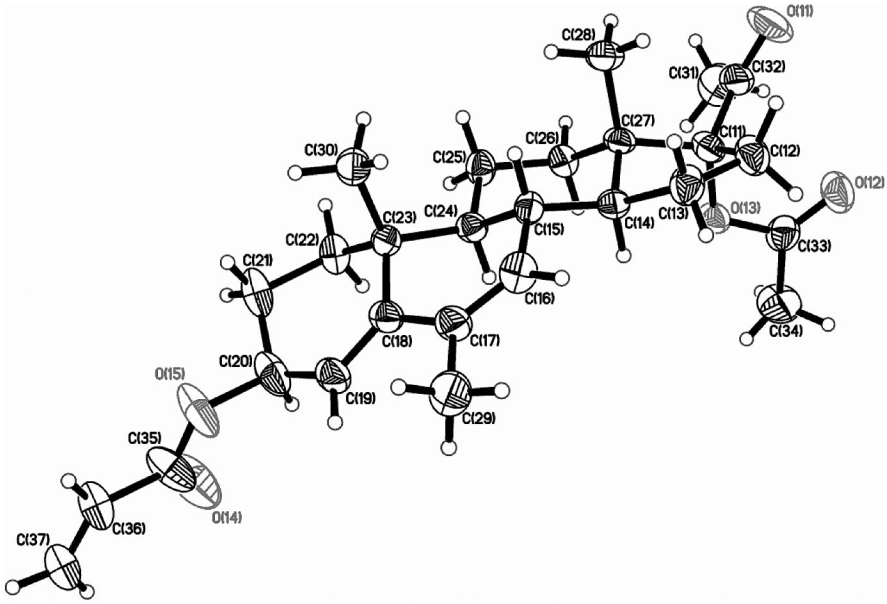


Fig. 1. Molecular structure of pregnane steroid **III** from an x-ray crystal structure analysis (CCDC 1909285).

cell suspension was prepared after incubation with the compounds. HeLa cells were incubated with the steroids at 5×10^{-5} M, which was equal to one half of IC_{50} for steroid **III** as the most active compound, and with cyclophosphamide (CPS) at 20 μM (Baxter, Germany) as a positive control. The incubation time was 20 h. The incubation medium was decanted. Each tube was rinsed with phosphate-buffered saline (PBS, 5 – 10 mL, pH 7.4; PanEco, Russia) and treated with trypsin solution (1 mL, 0.25%) while shaking the tube. The liquid was decanted. Cells were removed by DMEM medium (5 mL) with 10% FBS by tapping on the sides of the tube.

Preparation of gel slides. A solution (1%) of universal agarose was prepared on a water bath (<65°C) until fully liquified. A clean slide was held with forceps or by the sides. The first layer was deposited. Agarose (150 μL) was pipetted onto the edge of the glass opposite the rough or previously labeled edge. The gel was spread by the pipette tip over the whole smooth surface and 3 – 5 mm of the rough surface. The prepared glasses were placed on a hot plate to solidify the first agarose layer. The resulting gel-slides were stored in a dark dry place.

Preparation of microtubes. A solution of low-melting agarose (1%) was prepared in PBS on a water bath (<42°C). The resulting mixture was aliquoted (240 μL) into Eppendorf microcentrifuge tubes that were placed into a Gnom microthermostat (Russia) at 42°C to prevent solidification of the mixture. The microcentrifuge tubes with agarose gel were treated with previously prepared cell suspension (60 μL) and mixed 2 – 3 times with the pipette. The resulting mixture (60 μL) was placed onto the central part of a slide and immediately covered with a cover glass. The prepared slides were placed onto an ice-cold surface and left for 10 min to harden the new layer. Then, the cover glass was carefully removed using forceps along the edge.

Lysis and gel electrophoresis. Cover glasses with two agarose layers were placed into a 2-L flask, treated with previously cooled lysing buffer, capped with foil, and placed into a refrigerator (≥ 1 h but <24 h). Then, the glasses were removed. The liquid was allowed to drain off at an angle. The slides were placed on the surface of a horizontal electrophoresis chamber (Khelikon, Russia). The slides should lay perpendicular to the flask edge with the rough edge toward the cathode; the smooth, toward the anode. The chamber was filled with electrophoresis solution to 3 – 4 mm above the slides. Also, the slides should not change positions in the chamber. First, the slides were stored for 20 min in alkaline solution (300 mM NaOH, 1 mM Na_2EDTA, pH > 13) without switching on the electrophoresis apparatus. The electrophoresis lasted 20 min at field potential 1 V/cm and current 300 mA. When the procedure was finished, the slides were removed with forceps, allowed to drain at an angle, placed into a preliminary fixing solution for ≥ 15 min. The preparations were dried at room temperature for 1 – 2 h and stored in a dark dry place until analysis.

Staining preparations using intercalating stain SYBR Green I and analysis of the results. A working solution of SYBR Green I (140 μL; Syntol, Russia) in TE-buffer (1×, 10 mM Tris, 1 mM EDTA, pH 8.0; AppliChem, Spain) was placed onto half of a slide and stained for 20 min. The slides were analyzed on an epi-fluorescence microscope (Axiostar, Zeiss, Germany) at 200× magnification. The DNA content in the comet head (%) and in the comet tail (TDNA, Tail DNA, %); the tail length (μm); the tail moment (product of comet

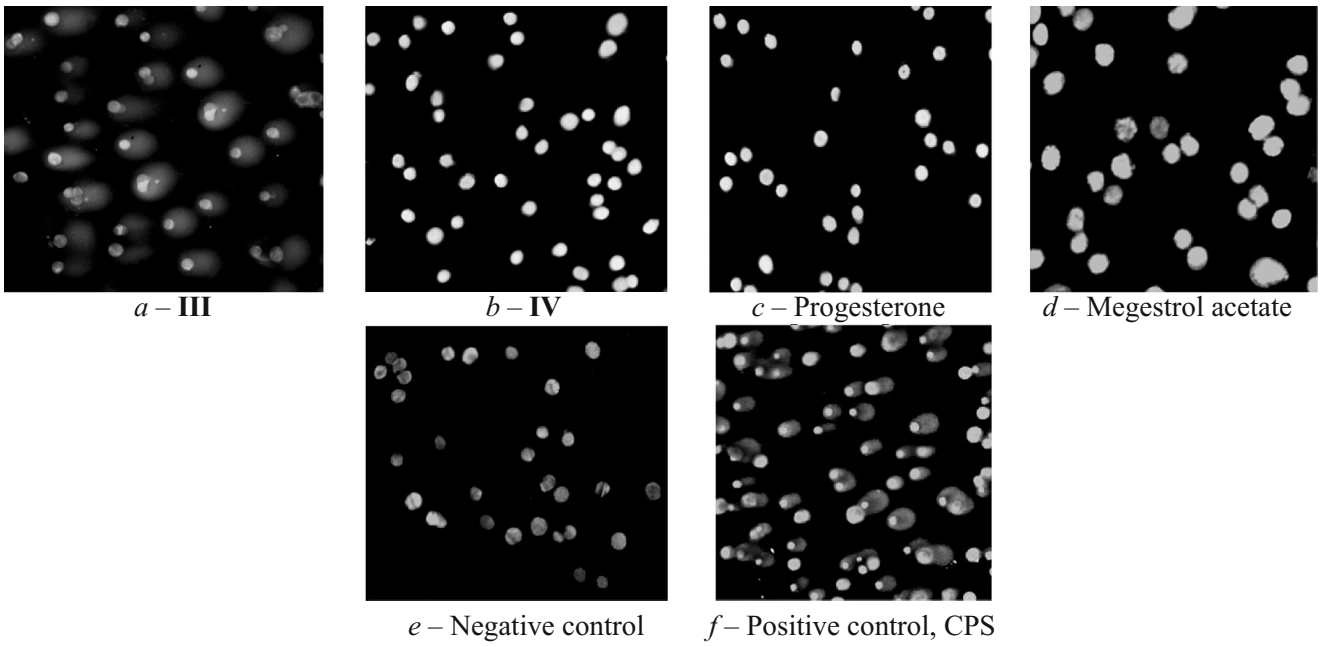


Fig. 2. Estimate of DNA damage at the single-cell level of HeLa-ESC after incubation with mepregenol derivatives at concentrations equal to their IC_{50} values for 20 h. Note: negative control, native cells; positive control, cells incubated with cyclophosphamide (CPS) (20 μM).

length and TDNA, %); Olive tail moment (OTM, product of TDNA in % and the distance between the center of the comet head and center of gravity of the comet tail). Slides were analyzed using the CASP 1.2.2 program. IC_{50} values were calculated using Excel software as before [6].

RESULTS AND DISCUSSION

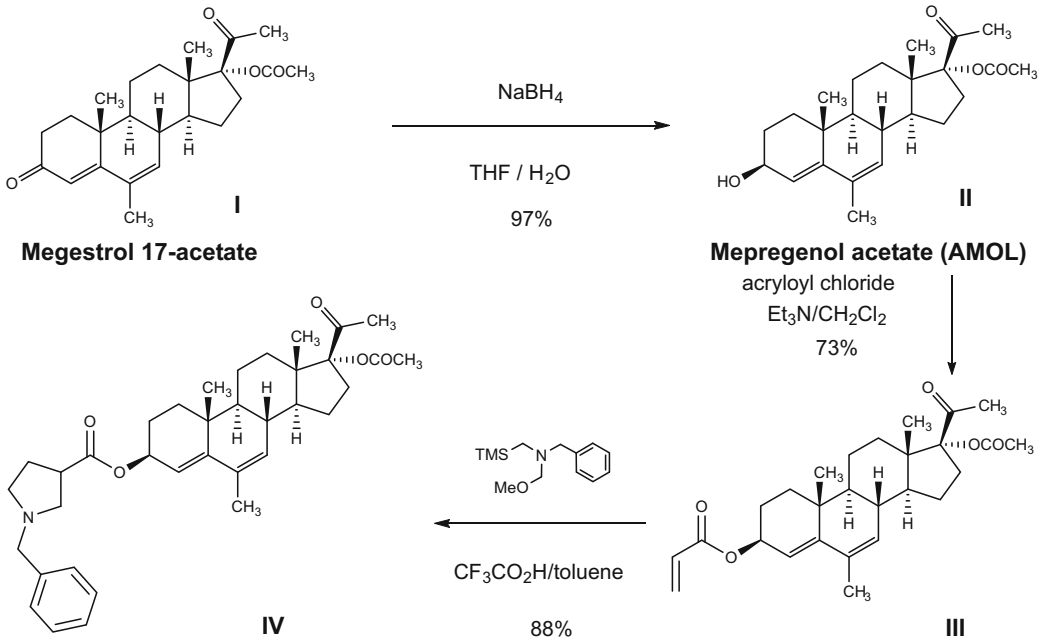
Scheme 1 illustrates the syntheses of the studied compounds. The starting compound was megestrol 17-acetate (**I**), which was selectively reduced at the pregnane backbone C3-position by $NaBH_4$ [7]. In contrast with methods reported in the scientific and patent literature for reducing pregnane steroid **I**, mepregenol 17-acetate (**II** or AMOL) was prepared in a medium without alcohol solvents, which produced spectrally and chromatographically pure product using precipitation from the reaction mixture by H_2O without additional purification. The C3-β-OH was acylated by carboxylic acid chlorides in the presence of a tertiary amine to produce mepregenol 17-acetate 3-acrylate (**III**) (Scheme 1). The molecular structure of the new ester of mepregenol 17-acetate (**III**) was established by an x-ray crystal structure analysis (Fig. 1). The complete crystallographic parameter set for **III** was deposited in the Cambridge Crystallographic Data Centre under identifier No. CCDC 1909285. Compound **III** could be further modified at the C3-position of the pregnane scaffold, primarily via cycloaddition, because of the activated double bond. Acrylate **III** underwent 1,3-dipolar cycloaddition with an azomethine ylide generated *in situ* from *N*-methoxymethyl-*N*-(trimethylsilylmethyl)benzylamine (Scheme 1) [8]. Oligopeptides constructed of monomeric fragments of functionalized pyrrolidine-3-carboxylic acids were found to have high antiproliferative activity with respect to hormone-resistant prostate cancer cell lines [9, 10]. As a result, the ester of *N*-benzylpyrrolidine-3-carboxylic acid (**IV**) was obtained in good yield.

The purities of the new mepregenol 17-acetate esters **III** and **IV** (>98%) were confirmed by elemental analyses.

HeLa cells were incubated for 14 d in the presence of estradiol (10^{-8} M) to produce HeLa-ESC. Survival of control tumor cells with estradiol stimulation was 22.5% greater ($p < 0.05$) than for the basal proliferation control. This indi-

TABLE 1. Half-maximum Steroid Concentrations IC_{50} (μM) Inhibiting by 50% Viability of HeLa and HeLa-ESC Cells After 72-h Incubation

Cell culture	Steroid			
	progesterone	megestrol acetate	III	IV
HeLa IC_{50}, μM	480 ± 20	130 ± 30	30 ± 1.03	200 ± 26
HeLa-ESC IC_{50}, μM	5.6 ± 0.73	115 ± 5	9.1 ± 0.8	180 ± 0.7



Scheme 1. Preparation of the new mepregenol 17-acetate esters.

cated that estradiol stimulated tumor cell growth and that the cell culture met the requirements for biotest systems for analyzing hormone activity.

Table 1 presents results for the cytostatic activities of the steroids.

Table 1 demonstrates that the mepregenol 17-acetate derivatives, in contrast to progesterone and megestrol acetate, inhibited the survival of native and HeLa-ESC cells whereas megestrol acetate and **IV** were significantly inferior to them in cytotoxicity with respect to HeLa-ESC. Compound **III** had the most potent cytotoxicity with respect to both cultures while megestrol acetate and **IV** had weak activity.

A preparation based on megestrol acetate, Megace, was indicated for palliative treatment of inoperable or metastatic breast cancer or endometrial cancer at a dose of 160 mg despite its moderate cytotoxicity [11].

The antitumor effect of Megace was related to inhibition of gonadotropin synthesis by the pituitary gland followed by reduced estrogen synthesis. This gestagen blocks estradiol from stimulating proliferation at the receptor level in hormone-dependent breast cancer cells and also has local effects on cancerous cells by transforming actively growing stroma into highly differentiated decidua cells [12].

The mechanism of cytotoxicity of **III** *in vitro* could be related to the DNA-damaging action of the steroid. As a rule, DNA in cells dying of apoptosis undergoes internucleosomal degradation, which is a characteristic marker of the process. Apoptotic cells appear as weakly fluorescing DNA-comets

TABLE 2. Quantitative Estimates of DNA-damaging Effects of Steroids on HeLa Cell Culture

Compound	DNA content, %		Comet length, μm	Tail moment (TM)	Olive tail moment (OTM)
	in comet head	in comet tail			
Progesterone, 5 μM	96.2 ± 2.1	3.8 ± 0.7	43.35 ± 4.63	0.04 ± 0.01	0.2 ± 0.06
Megestrol acetate, 5 μM	91.04 ± 4.32	8.96 ± 4.32	49.12 ± 4.1	0.06 ± 0.02	0.11 ± 0.03
Cyclophosphamide, 20 μM	49.15 ± 12.01*	50.85 ± 12.01*	174 ± 10.5**	60.5 ± 10.21*	45.2 ± 7.3*
III, 5 μM	58.3 ± 10.3**	41.7 ± 10.3**	84.57 ± 9.6**	16.3 ± 2.2*	11.8 ± 3.62*
IV, 5 μM	97.4 ± 2.44	2.6 ± 2.44	46.2 ± 1.3	1.05 ± 0.011	2.17 ± 0.04
Control	98.71 ± 1.36	1.06 ± 0.54	40.13 ± 2.75	0.03 ± 0.01	0.15 ± 0.04

* Parameter statistically significantly different from the negative control ($p < 0.05$) (DMEM medium with 0.1% DMSO); ** $p < 0.01$.

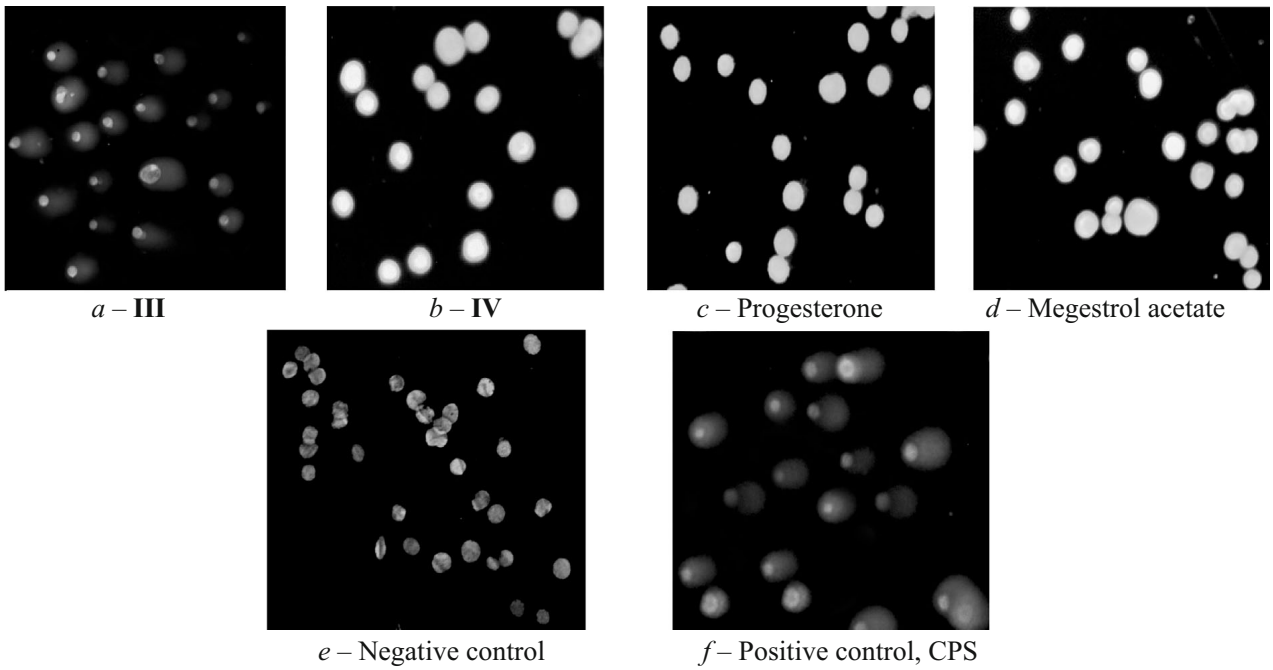


Fig. 3. Estimate of DNA damage at the single-cell level of HeLa-ESC after incubation with mepregenol derivatives at concentrations equal to their IC_{50} values for 20 h. Note: negative control, native cells; positive control, cells incubated with cyclophosphamide (CPS) (20 μM; Baxter, Germany).

with a broad diffuse tail and practically no head. The DNA comet assay is useful for detecting apoptotic cells in populations, especially when only a small amount of sample is available. The DNA comet assay produced information not only about the DNA content in apoptotic cells but also about the sizes of DNA fragments released from cells through the action of an electrical field [13].

The mechanism of the potent cytotoxicity of **III** with respect to HeLa and HeLa-ESC was related to apoptosis induction according to the increased percent contents of DNA in the comet tail in the presence of a steroid as compared to the control in the DNA comet assay (Figs. 2 and 3, Tables 2 and 3) [14].

Figures 2 and 3 demonstrate that **III** had the greatest DNA-damaging effects on both cultures whereas **IV** and megestrol acetate practically did not form comets. The DNA-damaging effects of the steroids (Tail moment, TM) for **III** and **IV** in the DNA comet assay correlated negatively with their IC_{50} values. For example, the correlation coefficient r for HeLa culture was 0.63; for HeLa-ESC, 0.5. Thus, **III** possessed potent cytotoxicity, the mechanism of which was related to a DNA-damaging apoptotic effect.

TABLE 3. Quantitative Estimates of DNA-damaging Effect of Steroids on HeLa Cell Culture Stimulated by Estradiol (HeLa-ESC)

Compound	DNA content, %		Comet length, μm	Tail moment (TM)	Olive tail moment (OTM)
	in comet head	in comet tail			
Progesterone, 5 μM	98.4 ± 1.6	1.6 ± 1.6	41.4 ± 2.2	0.06 ± 0.02	0.16 ± 0.08
Megestrol acetate, 5 μM	97.2 ± 0.5	2.8 ± 0.5	47.2 ± 5.2	0.055 ± 0.03	0.09 ± 0.02
Cyclophosphamide, 20 μM	45.5 ± 9.5*	54.5 ± 9.5*	180 ± 13.6**	62.5 ± 10.21*	50.5 ± 4.6*
III, 5 μM	54.2 ± 11.5**	45.8 ± 11.5**	86.64 ± 10.2**	18.5 ± 3.4*	13.6 ± 2.3*
IV, 5 μM	97.5 ± 1.5	2.5 ± 1.5	47.5 ± 3.2	0.99 ± 0.02	3.1 ± 0.05
Control	99.01 ± 0.4	0.99 ± 0.4	44.1 ± 1.5	0.05 ± 0.012	0.18 ± 0.03

* Parameter statistically significantly different from the negative control ($p < 0.05$) (DMEM medium with 0.1% DMSO); ** $p < 0.01$.

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