



Antiproliferative effect of normal and 13-*epi*-D-homoestrone and their 3-methyl ethers on human reproductive cancer cell lines

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ABSTRACT

The possibility of the therapeutic use of estrogens emerged following the recognition that certain estradiol analogs, and particularly metabolites (e.g. the A-ring metabolite 2-hydroxyestrone, etc.) inhibit the differentiation of diverse tumor cell lines. Until recently, despite the investigation of numerous synthetic D-ring-substituted estrone derivatives, no analysis had been published on the effects of D-ring expansion of estrone on its tumor-suppressing activity. The aim of the present study was to characterize the antiproliferative effects of normal and 13-*epi*-D-homoestrone and their 3-methyl ethers (1–4) on human reproductive cancer cell lines.

The antitumor activities of the two epimer pairs on HeLa, MCF-7 and Ishikawa cells were determined. Normal D-homoestrone exerted the greatest cytostatic effect on HeLa cells ($IC_{50} = 5.5 \mu M$) and was subjected to further investigations to elucidate its mechanism of action on apoptosis induction. Morphological changes detected by Hoechst 33258–propidium iodide double staining, the cell cycle arrest at phase G2/M and the subsequent increase in the proportion of the subG1 fraction determined by flow cytometric analysis and the significant increase in the activity of caspase-3 confirmed the induction of apoptosis in HeLa cells treated with D-homoestrone. D-Homoestrone was also tested on a non-cancerous human lung fibroblast cell line (MRC-5) to determine its selective toxicity. The concentration in which it inhibited cell proliferation by 50% was at least six times higher for the fibroblast cells than for cervical cancer cells. No significant *in vivo* estrogenic activity was observed as concerns the uterus weight of gonadectomized rats after a 7-day treatment with normal D-homoestrone.

These results led to the conclusion that normal D-homoestrone is a novel antitumor compound with a similar activity on HeLa cells as that of the reference agent cisplatin, but its selectivity toward non-cancerous cells is significantly higher than that of cisplatin. It may be considered to be a basic lead molecule for the preclinical development of potential anticancer agents.

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1. Introduction

Although 17 β -estradiol has been described as a proliferation-inducing compound [1], recent experimental results indicated that certain of its analogs can exert potent antiproliferative effects without appreciable estrogenic activity. One of the most widely investigated compounds is an A-ring-modified metabolite of estrone, 2-methoxyestradiol [2], which exerts potent inhibitory effects on the proliferation of ovarian [3,4], cervical [5–7], endometrial [8] and breast cancer cell lines [3,9,10], among others. 2-Methoxyestradiol has a complex mechanism of action, because

various pathways have been proved to be involved, e.g. the inhibition of neoangiogenesis [9,11,12], microtubule disruption [3,13], cell cycle arrest [8,14] and upregulation of the extrinsic and intrinsic apoptotic pathways [7,15,16].

A tumor-suppressing effect has been observed in *in vivo* animal studies (e.g. on methylnitrosourea-induced mammary carcinoma in rats [17]), when no toxic side effects were registered. Clinical studies of the possible antitumor potency of 2-methoxyestradiol revealed good tolerability, but limited oral bioavailability and metabolic instability [18,19]. To overcome this difficulty, the carbamate analog of 2-methoxyestradiol was synthesized, which possesses the beneficial features of 2-methoxyestradiol, but is metabolically more stable. However, this compound too may suffer limitations in therapeutic use: in a phase I study, neutropenia was observed at a dose of 550 mg/m²/day [20].

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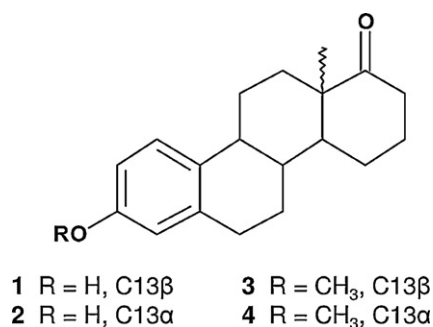


Fig. 1. Chemical structures of normal D-homoestrone (**1**), 13-*epi*-D-homoestrone (**2**) and their 3-methyl ethers (**3** and **4**, respectively).

The metabolism of estrone can continue by another pathway, hydroxylation of the D ring, resulting in the formation of 16 α -hydroxyestrone and estriol. Similarly to A-ring derivatives, these have minimal estrogen receptor binding affinity [21,22]. Various types of D-ring-modified estrone analogs have been synthesized and tested for possible inhibitory effects on cell proliferation [23–25], but these modifications were restricted to substitution with different functional groups at position 17 and dehydration at positions 14, 15 and 16.

Until recently, no experimental data had been published in connection with the effect of D-ring expansion and substitution on the antiproliferative activity of the estrone derivatives, although they might be expected not to exhibit appreciable estrogen receptor binding affinity and therefore no estrogenic activity *in vivo*. Wölfling et al. [26] reported the synthesis of normal and 13-*epi*-D-homoestrone and their 3-methyl ethers, and results on their estrogen and progesterone receptor binding abilities. There is a difference of at least three orders of magnitude between the K_i values of the D-ring-modified estrone analogs and the reference molecule, 17 β -estradiol, which raised our interest concerning their possible antiproliferative effects. The epimer pairs of the compounds are available for investigation of the influence of the stereoselectivity of the molecules on their antitumor activity, thereby furnishing detailed data concerning the structure–activity relationships of the tested compounds. As concerns 17 β -estradiol derivatives with no *in vitro* estrogenic activity, a question that frequently emerges is whether they can be transformed by liver metabolizing enzymes into new molecules able to bind to and transactivate the estrogen receptor, which might even lead to a reduction of the antiproliferative effect exerted by the parental compound.

The aim of the present study was to characterize the mechanism of antiproliferative action of these four D-ring-modified estrone analogs on various human reproductive cancer cell lines. The compound found most effective in the cell-growth inhibition assay was subjected to Hoechst dye 33258 (HO)–propidium iodide (PI) double staining, flow cytometry analysis and caspase-3 activity measurement. Its selective cytotoxic activity was determined on the MRC-5 human fetal lung fibroblast cell line. The lack of its *in vivo* estrogenic activity was confirmed by means of *in vivo* uterotrophic assays performed on gonadectomized female rats.

2. Materials and methods

2.1. Chemicals

Normal (**1**) and 13-*epi*-D-homoestrone (**2**) and their 3-methyl ethers (**3** and **4**, respectively) were synthesized as described previously [26]. Their chemical structures are presented in Fig. 1. All other chemicals and kits, if otherwise not specified, were purchased from Sigma–Aldrich Ltd. (Budapest, Hungary).

2.2. Cell cultures

The HeLa human cervix epitheloid carcinoma, MCF-7 human breast adenocarcinoma, Ishikawa human endometrial adenocarcinoma and MRC-5 human lung fibroblast cell lines were purchased from ECACC (European Collection of Cell Cultures, Salisbury, UK). HeLa, MCF-7 and MRC-5 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% non-essential amino acids and 1% penicillin–streptomycin in a humidified atmosphere containing 5% CO₂ at 37 °C. However, Ishikawa cells were grown in DMEM medium supplemented with 5% FCS, 1% non-essential amino acids, 5% 200 mM glutamine and 1% penicillin–streptomycin in a humidified atmosphere containing 5% CO₂ at 37 °C. For culturing in steroid-depleted media, MCF-7 cells were maintained in phenol red-free DMEM containing 10% charcoal dextran-treated FCS with the addition of 1% non-essential amino acids, 5% 200 mM glutamine and 1% penicillin–streptomycin. All media and supplements were obtained from Life Technologies (Paisley, Scotland, UK). Cell counts were determined with a Z1 Coulter Particle Counter (Beckman Coulter Hungary Ltd., Budapest, Hungary). Cells in the near-confluent phase of growth were used in all the studies described below.

2.3. Antiproliferative (MTT) assay

The growth-inhibitory effects of the test compounds were determined on HeLa, MCF-7 and Ishikawa cells. All cell types were seeded into 96-well plates at a density of 5000 cells/well and incubated with increasing concentrations (0.3–30 μ M) of the four compounds at 37 °C under cell culturing conditions. After a 72-h incubation, cells were treated with 5.0 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution for 4 h, the precipitated formazan crystals were dissolved in dimethyl sulfoxide, and the absorbance was read at 545 nm with a microplate reader; wells with untreated cells were utilized as controls [27]. Sigmoidal dose–response curves were fitted to the measured points, and the IC₅₀ values were calculated by means of GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Cisplatin was used as a positive control in the same concentration range as the test compounds.

The subsequent analyses were carried out only with the most effective compound and the most sensitive cell type.

To determine the selective cytotoxic effect and the antiproliferative effect in steroid-depleted medium, **1** was subjected to the MTT assay with intact, non-cancerous MRC-5 cells and MCF-7 cells growing in steroid-free medium, respectively, under the same experimental conditions.

2.4. Hoechst 33258–propidium iodide (HOPI) double staining

HOPI staining was performed as described earlier [28]. HeLa cells at a density of 3000–5000 cells/well were seeded in 96-well plates and exposed to increasing concentrations of **1** in quadruplicate for 24, 48 or 72 h. HO and PI were added directly to the cells to give final concentrations of 5 and 2 μ g/ml, respectively. After incubation for 60 min at 37 °C, cells were examined under a Nikon ECLIPSE TS100 fluorescence microscope (Nikon Instruments Europe B.V., Amstelveen, The Netherlands) equipped with appropriate filters for HO (excitation: 360/40 nm bandpass filter, emission: 460/50 nm bandpass filter and 400 nm dichromatic mirror) and PI (excitation: 500/20 nm bandpass filter, emission: 520 nm longpass filter and 515 nm dichromatic mirror). This method allows a distinction between early apoptosis and secondary necrosis. Cells were assessed on the basis of their morphology and the integrity of their membranes, which can readily be seen after PI staining. Cells were

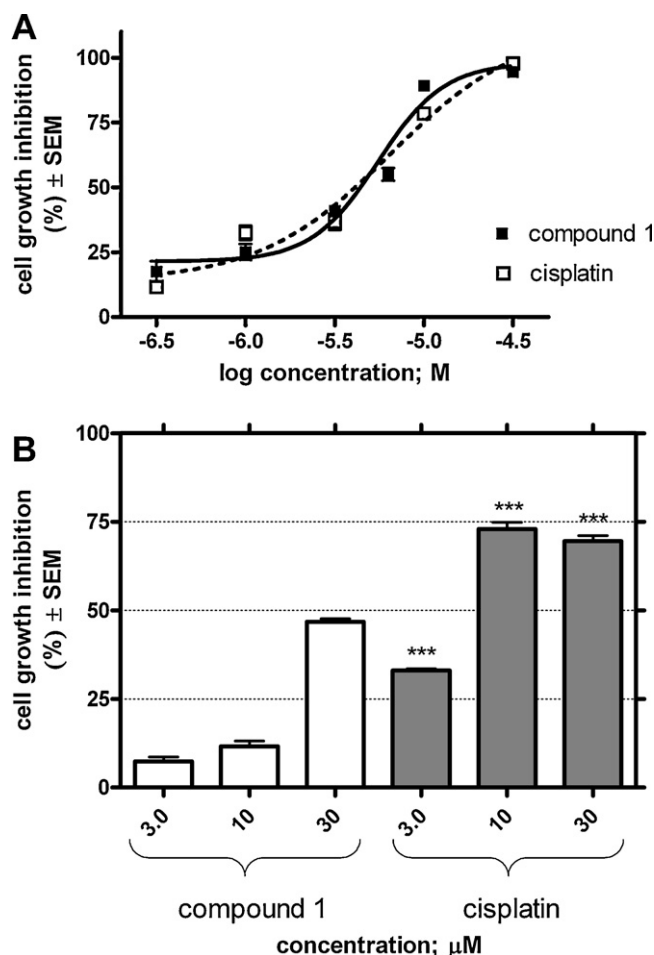


Fig. 2. The dose–response curves of compound **1** and cisplatin on HeLa cells, determined in the 72-h antiproliferative assay described in Section 2 (A). Proliferation-inhibitory effects of compound **1** and cisplatin on non-cancerous MRC-5 cells, determined in the 72-h antiproliferative assay described in the Materials and methods (B). Each bar denotes the mean $\pm$ SEM, $n = 5$. *** indicates $p < 0.001$ for the comparisons of the corresponding concentration pairs of compound **1** and cisplatin.

counted under the microscope (two fields of vision were captured in each well; 100–250 cells were counted in each capture) and the percentages of intact, early apoptotic and secondary necrotic cells were calculated. For statistical evaluation, data were analyzed by one-way ANOVA with the Neumann–Keuls post test, using Prism 4 software.

2.5. Analysis of apoptosis and cell cycle by flow cytometry

For the measurement of cellular DNA content, flow cytometric analysis was performed: 24, 48 and 72 h after treatment, HeLa cells (180,000–250,000 cells/condition) were washed twice with cold phosphate-buffered saline (PBS), harvested with trypsin, centrifuged at 1500 rpm for 10 min, washed in ice-cold PBS, and fixed in 1.0 ml -20°C 70% ethanol. Samples were stained with 1.0 ml dye solution containing 0.02 mg/ml RNase A, 1.0 mg/ml PI, 0.003 $\mu\text{l/ml}$ Triton-X and 1.0 mg/ml sodium citrate in distilled water, and the mixture was incubated for 60 min at room temperature. After an additional washing step with PBS, cells were analyzed on a FAC-Star (Beckton–Dickinson, Mountain View, CA, USA). In each analysis, 20,000 events were recorded, and the percentages of the cells in the different cell cycle phases (subG1, G1, S and G2/M) were determined by using winMDI2.9. The subG1 fraction was regarded as the apoptotic cell population [29]. For statistical evaluation, data were

analyzed by one-way ANOVA with the Neumann–Keuls post test, using Prism 4 software.

2.6. Determination of *in situ* caspase-3 activity

To analyze the effect of **1** on the activity of caspase-3, one of the executioner enzymes of apoptosis, a commercially available colorimetric assay (Cat. No.: CASP-3-C) was performed. Briefly, near-confluent HeLa cells were seeded in tissue culture flasks (10^7 cells/flask) and grown overnight under standard cell culturing conditions. The cells were then incubated with increasing concentrations (from 1.25 μM to 5.0 μM or 10 μM) of the test compound for 48 and 72 h. After incubation, the cells were counted, centrifuged and washed with PBS. Aliquots containing 10 million cells were suspended in 100 μl kit lysis buffer and incubated on ice for 20 min. The lysed cells were subsequently centrifuged and the supernatants were used for the measurement. In accordance with the manufacturer's protocol, 5.0 μl portions of treated and untreated supernatants were incubated with 10 μl acetyl-Asp-Glu-Val-Asp *p*-nitroaniline, a selective caspase-3 substrate, in a final volume of 100 μl in assay buffer. As a control experiment, 5.0 μl of each sample was incubated with 10 μl caspase-3 substrate and 10 μl acetyl-Asp-Glu-Val-Asp-aldehyde, a selective caspase-3 inhibitor, in a final volume of 100 μl in assay buffer. Each sample was prepared in five parallels. A standard solution of caspase-3 was used as positive control in parallel with its inhibitor-treated samples. After an overnight incubation at 37°C , the absorbance of the *p*-nitroaniline released was measured at 405 nm. The concentration of *p*-nitroaniline was calculated from a calibration curve previously prepared with *p*-nitroaniline solutions with known concentrations. The *in situ* caspase-3 activity was expressed in $\mu\text{mol p-nitroaniline/min/ml}$. For statistical evaluation, data were analyzed by one-way ANOVA with the Neumann–Keuls post test, using Prism 4 software.

2.7. *In vivo* uterotrophic assay

The *in vivo* effect of compound **1**, the most effective antiproliferative agent, on the uterus weight was measured in gonadectomized female rats.

The estrogenic effect was determined as reviewed by Sandow [30] with some modifications. Mature female Sprague–Dawley rats (180–200 g) under general anesthesia induced by isoflurane inhalation were ovariectomized. After laparotomy, the oviducts and the arteries supplying the ovaries were ligated on both sides, and the ovaries were excised. The abdomen and the skin were closed with surgical sutures, and the animals were then observed until awakening. Three weeks postoperatively, the test animals (5 animals/group) were injected s.c. daily for 7 days with **1** dissolved in olive oil at 10, 30 or 50 $\mu\text{g}/0.1$ ml. The negative and positive control groups received pure olive oil or 10 $\mu\text{g}/0.1$ ml 17β -estradiol, respectively. On day 8, the animals were sacrificed, and the uteri were removed and weighed.

The uterus weight was calculated per 100 g body weight, and the data were statistically analyzed by one-way ANOVA with the Neuman–Keuls post test, using Prism 4 software. The experimental animal protocol was in full accordance with the Guidelines for Animal Experimentation approved by the Animal Experimentation Committee at the University of Szeged.

3. Results

3.1. Antiproliferative (MTT) assay

In the MTT assay, **2**, **3** and **4** at concentrations from 0.3 μM to 30.0 μM did not exhibit substantial cell growth inhibition on any

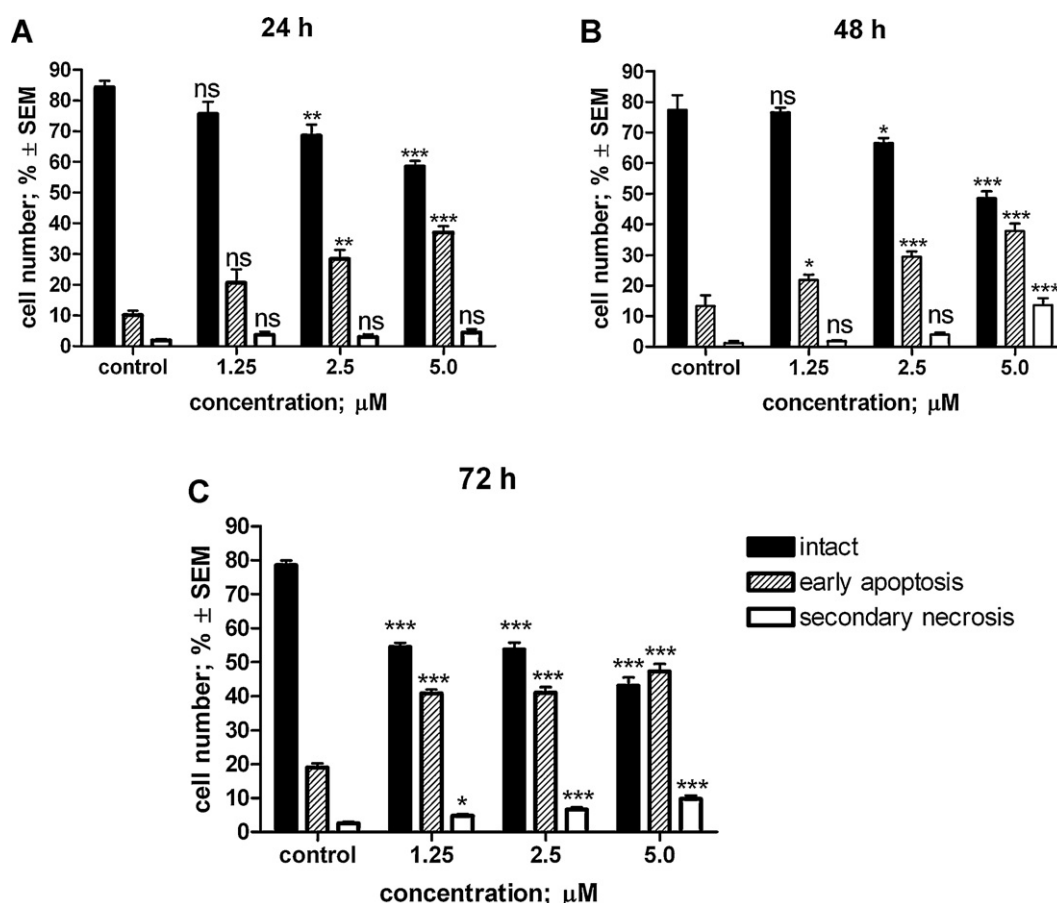


Fig. 3. Effects of compound **1** on the induction of apoptosis and necrosis of HeLa cells, using Hoechst dye 33258–propidium iodide double staining after incubation for 24, 48 or 72 h. Cells were counted under a fluorescence microscope and the numbers of intact, early apoptotic or secondary necrotic cells are given as percentages. Each bar denotes the mean $\pm$ SEM, $n = 3$. ns indicates $p > 0.05$, * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$ for the comparisons with the control cell numbers.

of the cell lines ($IC_{50} > 20.0 \mu M$). The growth-inhibition curves of **1** and cisplatin and the IC_{50} values are presented in Fig. 2 and Table 1. **1** proved to be an effective inhibitor of the cell proliferation of HeLa cells ($IC_{50} = 5.5 \mu M$), but did not exert a significant antiproliferative effect on the other cell lines (Table 1). The IC_{50} of cisplatin on HeLa cells was $6.5 \mu M$. The IC_{50} of **1** on steroid-depleted MCF-7 cells was in the same range as that on normal MCF-7 cells (Table 1).

1 did not inhibit the proliferation of intact, non-cancerous MRC-5 cells to a significant extent ($IC_{50} > 30.0 \mu M$), whereas cisplatin displayed an IC_{50} value of $3.7 \mu M$ on MRC-5 cells (Fig. 2).

Table 1

Calculated IC_{50} values of D-homoestrone (**1**), 13-*epi*-D-homoestrone (**2**), their 3-methyl ethers (**3** and **4**, respectively) and cisplatin, measured in the 72-h antiproliferative assay described in the Materials and methods on the HeLa, MCF-7, steroid-depleted MCF-7, Ishikawa and MRC-5 cell lines. n.d. indicates not determined.

Compound	IC_{50} [μM]				
	HeLa	MCF-7	Steroid-depleted MCF-7	Ishikawa	MRC-5
1	5.5	>30	>30	21.2	>30
2	>30	>30	n.d.	>30	n.d.
3	>30	>30	n.d.	>30	n.d.
4	>30	>30	n.d.	>30	n.d.
Cisplatin	6.5	9.6	n.d.	3.5	3.7

3.2. Morphological changes in the cells

HeLa cells were incubated with increasing concentrations of **1** (1.25, 2.5 and $5.0 \mu M$) for 24, 48 or 72 h. The induction of early apoptosis or secondary necrosis was determined according to the nuclear morphology and membrane integrity. **1** at 2.5 and $5.0 \mu M$ induced significant early apoptosis (30.0–40.0%) even at 24 h, and this situation was not markedly changed at 48 h. The proportion of secondary necrotic cells was highest, at 15%, on exposure to $5.0 \mu M$ **1** for 48 h. At 72 h, the early apoptotic cell population accounted for almost half of the cells at all applied concentrations of **1** (Fig. 3), with significant differences from the control. The numbers of secondary necrotic cells in samples treated with **1** for 72 h were also significantly higher than the corresponding controls, but did not differ markedly from the data measured after incubation for 48 h.

3.3. Cell cycle distribution

For a quantitative characterization of the action of **1** on the cell cycle distribution, HeLa cells were treated with 1.25, 2.5 or $5.0 \mu M$ **1** for 72 h, with 2.5 or $5.0 \mu M$ **1** for 48 h and additionally with $10.0 \mu M$ or $20 \mu M$ **1** for 24 h, and the different cell populations were analyzed by flow cytometry after PI staining (Fig. 4). The number of cells in the subG1 phase (indicating apoptosis) did not differ significantly up to 48 h. However, the number of cells in the G2/M phase was already significantly higher after treatment with $20 \mu M$ **1** for 24 h, and the increase was even higher after treatment with $5.0 \mu M$ **1** for 48 h. At 72 h, a significant, dose-dependent increase

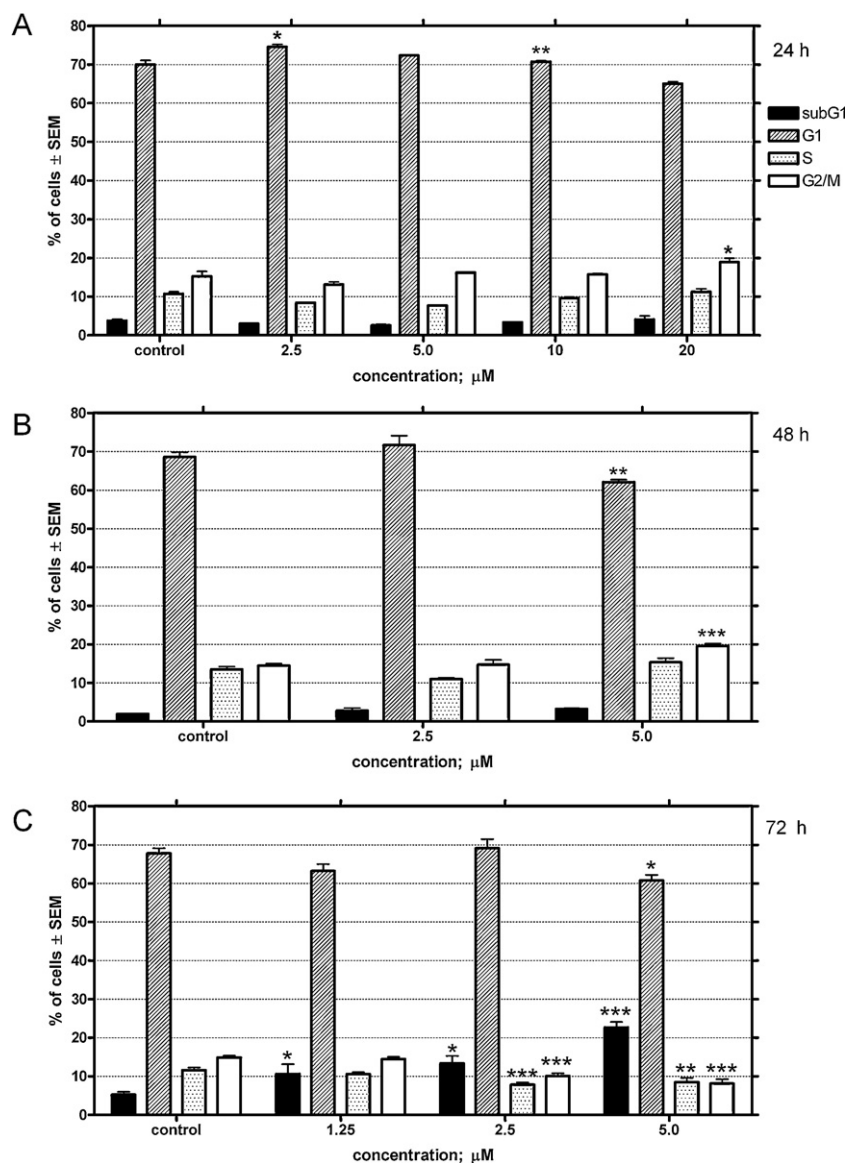


Fig. 4. Effects of compound **1** on the phase distribution of the HeLa cell cycle after incubation for 24 h (A), 48 h (B) or 72 h (C). The results are means $\pm$ SEM of the data on three separate measurements. * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$ for the comparisons with the untreated control cells. Non-significant changes are not indicated.

was detected in the number of cells in the subG1 phase, with concomitant decreases in the numbers of cells in the G1 and G2/M phases.

3.4. *In situ* caspase-3 activity

In view of the results of the cell cycle study, the caspase-3 activity on HeLa cells was determined after exposure to 1.25, 2.5 or 5.0 μ M **1** for 72 h. The caspase-3 activity was significantly increased by **1** under all of these conditions as compared with the vehicle-treated control samples, but in this concentration range there was no apparent dose-dependent relationship suggesting the detection of a maximally evoked effect. Following reduction of the length of the incubation period to 48 h and extension of the applied concentration range of **1** to 10 μ M, a significant induction of caspase-3 activity was likewise observed, indicating the development of apoptosis under all these experimental circumstances. After incubation for 48 h, the exerted induction in caspase-3 activity can be regarded as dose-dependent. The induction of caspase-3

activity by the different concentrations of **1** was inhibited by the competitive inhibitor of caspase-3 (Fig. 5).

3.5. *In vivo* uterotrophic assay

The weight of the rat uterus following treatment with **1** did not differ significantly from that following treatment with olive oil (vehicle control) in any applied dose up to 50 μ g/animal. In contrast, the administration of 10 μ g 17 β -estradiol induced a nearly 5-fold increase in uterus weight (Fig. 6).

4. Discussion

Cancer is still the leading cause of death in economically developed countries and the second leading cause of death in developing countries [31]. Among women, all types of tumors affecting the reproductive organs (the breasts, uterus, cervix and ovaries) were recently reported to be found in the list of the 10 most frequently diagnosed cancers [32]. Cervical cancer is the second most commonly diagnosed cancer affecting the reproductive system in

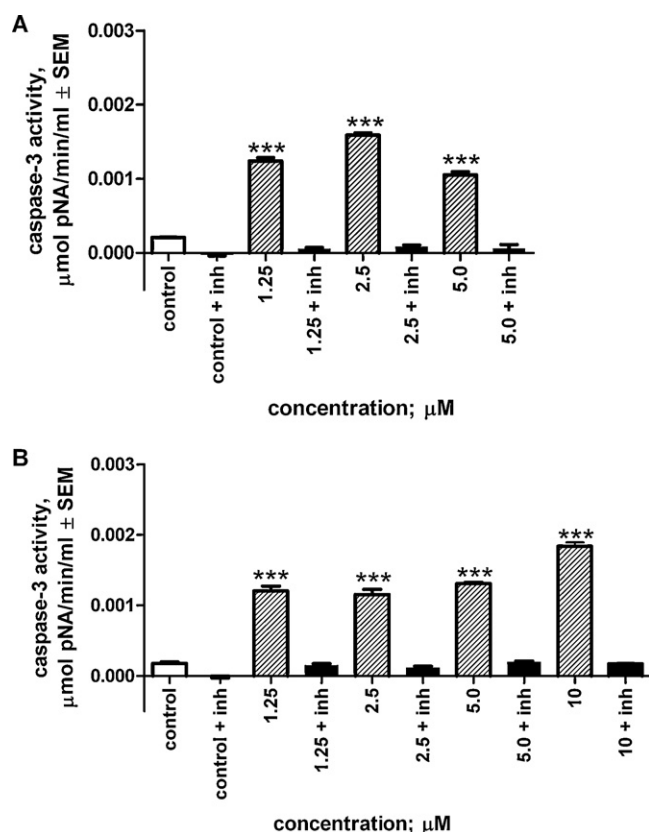


Fig. 5. *In situ* measurement of caspase-3 activity in HeLa cells after treatment with compound **1** for 72 h (A) and 48 h (B). The activity of caspase-3 is expressed as the concentration of *p*-nitroaniline (pNA) released by caspase-3 from its peptide substrate, acetyl-Asp-Glu-Val-Asp *p*-nitroaniline, during a given period of time. Data are means $\pm$ SEM, $n = 5$. *** indicates $p < 0.001$ as compared with the untreated control samples. The caspase-3 inhibitor (inh), acetyl-Asp-Glu-Val-Asp-aldehyde, suppressed the enzyme activity in all cases.

females worldwide. It accounted for 9% of the total new cancer cases and 8% of the total cancer deaths among women in 2008, with the highest incidence rates among the economically developed countries in Central and Eastern Europe. It is clear that the need for new,

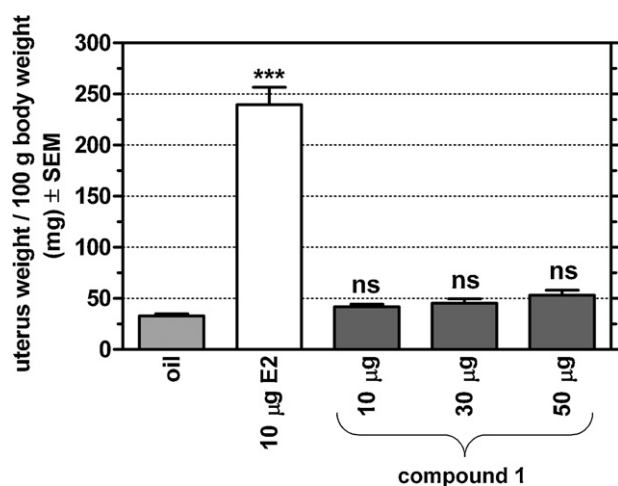


Fig. 6. *In vivo* effects of 7-day parenteral treatment with compound **1**, 17 β -estradiol (E2) or vehicle (olive oil) on the uterus weight in ovariectomized rats. The uterus weight gain is expressed as the tissue weight in mg per 100 g of living animal weight. Data are means $\pm$ SEM, $n = 5$. ns indicates $p > 0.05$ and *** indicates $p < 0.001$ as compared with the olive oil-treated samples.

more potent and better tolerated drugs for the therapy of this type of cancer has not declined.

The present paper provides the first convincing data concerning the cell proliferation-inhibiting effects of *D*-homoestrone, 13-*epi-D*-homoestrone and their 3-methyl ethers on human cancer cell lines of reproductive origin. *D*-Homoestrone proved to be the most effective antiproliferative compound on the human cervical cancer cell line (HeLa), with a potency under our experimental circumstances equivalent to that of cisplatin, one of the drugs used in the routine therapy of cervical carcinomas [33]. As *D*-homoestrone did not effectively inhibit the multiplication of other types of reproductive cancer cells (e.g. breast and endometrial cells), its antiproliferative effect appears to be specific to HeLa cells. HeLa cells possess a unique feature relative to the other two cell lines of reproductive origin involved in our investigations (MCF-7 and Ishikawa cells): human papilloma virus (HPV) 18 positivity [34]. The expression of HPV-encoded oncoprotein E6 results in the suppressed activity of the wild-type p53 transcription factor, which plays an important role in different steps of cell division [35]. Several compounds (e.g. roscovitine, cidofovir and 2-O- β -D-glucopyranosyl-L-ascorbic acid) have been reported to inhibit the proliferation of HeLa cells selectively. It has been demonstrated that most of these compounds inhibit the expression of the E6 oncoprotein; thus, they abrogate the suppression of p53, resulting in activation of the p53-dependent apoptotic pathway [36–38]. We postulate that the potential alteration of this signal transduction pathway on the incubation of HeLa cells with *D*-homoestrone might be the explanation of the specific antiproliferative effect of our test compound.

The results of the antiproliferative assays permit certain conclusions relating to the effects of structure modification. The 13-methyl group in the β position is essential for the significant antiproliferative effect, because the IC_{50} of 13-*epi-D*-homoestrone on HeLa cells is at least 6 times higher than that of *D*-homoestrone. On the other hand, the 3-methoxy derivative of *D*-homoestrone displayed a markedly weaker proliferation-inhibiting effect on HeLa cells than that of *D*-homoestrone itself. Thus, it can be concluded that the presence of a 3-hydroxy group on the *D*-homoestrone skeleton plays an important role in its cell proliferation-inhibiting effect. These data possibly also suggest that *D*-homoestrone exerts its action on a definite, but as yet undetermined target. Further investigations with variously substituted *D*-homoestrone analogs might promote further clarification of the structure–activity relationships between the ligand and the target.

Tumor specificity is one of the challenges facing new anticancer drug candidates. **1** did not appear to disturb the proliferation of MRC-5 non-cancerous, fetal lung fibroblast cells. In all applied concentrations, its proliferation-inhibiting effect on fibroblast cells was significantly weaker than that of cisplatin, an observation supported by the negative results on animal toxicity (*i.e.* no detectable symptoms) during the *in vivo* treatment of rats for 1 week.

On the basis of the data concerning the common apoptosis markers, **1** can be regarded as a new apoptosis-inducing antiproliferative agent. To investigate the mechanism of the antiproliferative effect of **1**, HOPI double staining, cell cycle analyses and caspase-3 activity measurements were performed. The apoptosis-inducing effect of **1** was clearly evidenced morphologically by the increased number of cells with condensed DNA that accumulated HO, regarded as one of the initiating steps in programmed cell death [39]. On increase of the incubation period, some of the early-apoptotic cells seemed to be converted to late-apoptotic or secondary necrotic cells by 48 h, as evidenced by a substantial cell population stained with PI. Besides the disrupted membrane integrity, the cells in this transient stage may not exhibit a decreased DNA content [29]. During longer incubation (72 h), these secondary necrotic cells may demonstrate the markers of substantial DNA elimination, as an increased subG1 population [40] detected by flow cytometry analysis. Further evidence

of the apoptosis-inducing effect of **1** was provided by the significant increases in the activity of caspase-3, the first executioner enzyme of the caspase family, after the treatment of HeLa cells with **1** in all three concentrations applied. The activity of caspase-3 was demonstrated to depend on the length of incubation with **1**; the gradual development of programmed cell death resulted in the maximal apoptotic signal after 72 h.

Flow cytometric analysis also revealed some details concerning the mechanism of action of **1**. The numbers of cells in the G2/M phase were significantly higher after the incubation of HeLa cells with 20 μ M or 5.0 μ M **1** for 24 or 48 h, respectively. When arrested in the G2/M phase, these cells may activate the intrinsic apoptotic signal. Significantly increased caspase-3 activity was already measured after the incubation of HeLa cells with **1** for 48 h. However, the accumulation of cells in the subG1 population was demonstrated only after 72 h. We hypothesized, that if cells arrested in the G2/M phase enter the process of apoptosis, the immediate appearance of the subG1 population can not be expected because these cells include double DNA content, the degradation of which may first result in particles with nearly simple DNA content, and particles with smaller DNA content may develop only during the next step, which requires several hours. Certain A-ring-substituted estrone derivatives have been demonstrated to exert a similar antiproliferative effect on MDA-MB-231 estrogen receptor-negative, metastatic breast cancer cells [41]. We earlier observed that the 4-methoxyphenylpyrazoline derivative of solanidine inhibits the cell cycle in the G2/M phase, and induces concomitant apoptosis on HL-60 leukemia cells [42]. This mechanism of action can be considered to be frequent among antiproliferative compounds with a sterane skeleton (e.g. diosgenin, ergone, 2-methoxyestradiol, etc.) [6,43,44].

As concerns steroid compounds with structures designed not to exert classical receptorial effects, their binding to and activation of any steroid receptor is an important and intriguing question in connection with their possible future therapeutic use. It has been reported that the estrogen receptor (ER) recognizes **1**, but its inhibition constant is three orders of magnitude lower than that of the reference molecule, 17 β -estradiol, measured by radioligand-binding assay on immature rabbit uterus cytosol [26]. **1** is not able to inhibit significantly the proliferation of ER-positive MCF-7 human breast carcinoma cells [45], growing in general medium, which suggests that it cannot bind and transactivate the human ER either. To test this assumption, the antiproliferative assay was repeated on MCF-7 cells maintained in steroid hormone-depleted medium [46]. Since there was no noteworthy difference between the cell proliferation inhibitory activity of 30 μ M **1** measured on MCF-7 cells maintained in media with or without steroid hormones, it can be concluded that **1** does not bind to and activate the human ER *in vitro*. Furthermore, it is widely accepted that cancer cell lines maintained for *in vitro* investigations can retain some metabolic capacity for the reactivation of estradiol metabolites [47]. However, most human cancer cell lines have no substantial xenobiotic-metabolizing enzyme activity (e.g. cytochrome enzymes) and therefore no action can be expected from an agent of prodrug type [48]. Thus, ovariectomized rats were treated with **1** in three different doses for 7 days in order to obtain information about the possibility of its *in vivo* conversion to an active metabolite with estrogenic activity. The result that there was no significant gain in uterus weight after the treatment with the test compound relative to the vehicle-treated animals indicates that **1** does not exert an estrogenic effect in an *in vivo* system. Additionally, the preliminary data on the toxic effect of **1** strongly suggested that it does not evoke macroscopic harmful reactions in laboratory rats [49].

In conclusion, our current findings provide the first evidence that **1** selectively suppresses HeLa cell proliferation and modulates

the cell cycle progression by promoting several steps of apoptosis and inducing the accumulation of cells in the G2/M phase. Its tumor specificity proved to be more beneficial than that of cisplatin, one of the drugs applied in the therapy of cervical carcinomas. Moreover, it is highly possible that neither **1** nor its metabolites possess estrogenic activity. Hence, **1** can be considered to be a new, noteworthy and promising lead compound among anticancer candidates.

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