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Antiproliferative and antimetastatic properties of modified estradiol analogs against gynecological cancer cell lines

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1. Introduction

In spite of the continuously developed new therapeutical opportunities, cancer is still an unsolved health problem and a leading cause of death globally. Based on the database of Globocan 2018, 18.1 million new cases and 9.6 million tumor-related deaths were estimated worldwide in 2018. Among women, breast malignancy was the most frequently diagnosed and correlating to this fact, it is the leading cause of cancer death in 103 countries. Another significant gynecological tumor is cervical cancer, since it takes the fourth position in incidence and mortality. The presence of secondary tumors can be connected with approximately 90% of cancer-related deaths. The assessment of results of randomized clinical trials covering a 30-year period could not show sustained evidence of survival improvement among women with metastatic breast cancer.

The triple-negative breast cancer (TNBC) is distinguished by the lack of estrogen receptor (ER), progesterone receptor, (PR) and human epidermal growth factor receptor 2 (HER2) amplification. Approximately 15-20% of the total diagnosed breast tumors are triple-negative. However, the fact that 25% of the total breast cancer mortality can be connected with the subtype shows its serious burden. Despite the promising results of drug candidates against TNBC in clinical trials, the specific treatment of TNBC remains an urgent unmet medical need. Cervical tumor development is featured by different factors, including the most significant human papillomavirus (HPV) infection and the required genetic and epigenetic alterations for the process. The main role of the persistent high-risk HPV type infection is well-established both epidemiologically and biologically; the presence of HPV was demonstrated in 99.7% of cervical cancers. HPV 16 and 18 are definied as the most dominant considering that 70-80% of all cervical cancers are affected.

Despite that 17β-estradiol promotes cell proliferation, the anticancer property of structurally modified estrogen derivatives was determined in several studies. The absence of estrogenic activity is an essential demand during the development of estrane-based antitumor drug candidates. The lack of hormonal properties can be reached via the definite alteration of the estrane skeleton, including the substitution at C-2 or C-3; furthermore, the inversion of configuration at C-13 of estradiol. The potential increase of the antiproliferative action of 3-benzyl ether moiety and triazol ring on the estrane core was demonstrated in previous studies. The loss of binding ability to estrogen receptor of possible isomers of 16-hydroxymethyl-3,17-estradiol was demonstrated through using receptor protein prepared from rabbit uteri for radioligand-binding assay.

In order to integrate the structural modifications responsible for an increase in the antiproliferative and a decrease in the hormonal characteristic, 16-triazoles and their precursors in the 13α-estradiol series; 3-benzyl ethers bearing electronically different substituent at the 4'-position and containing hydroxymethyl function at C-16; moreover, the four possible isomers of 16- hydroxymethyl-3-methoxy- and 16-hydroxymethyl-3-benzyloxy-13α-estradiol derivatives were synthesized.

2. Specific Aims

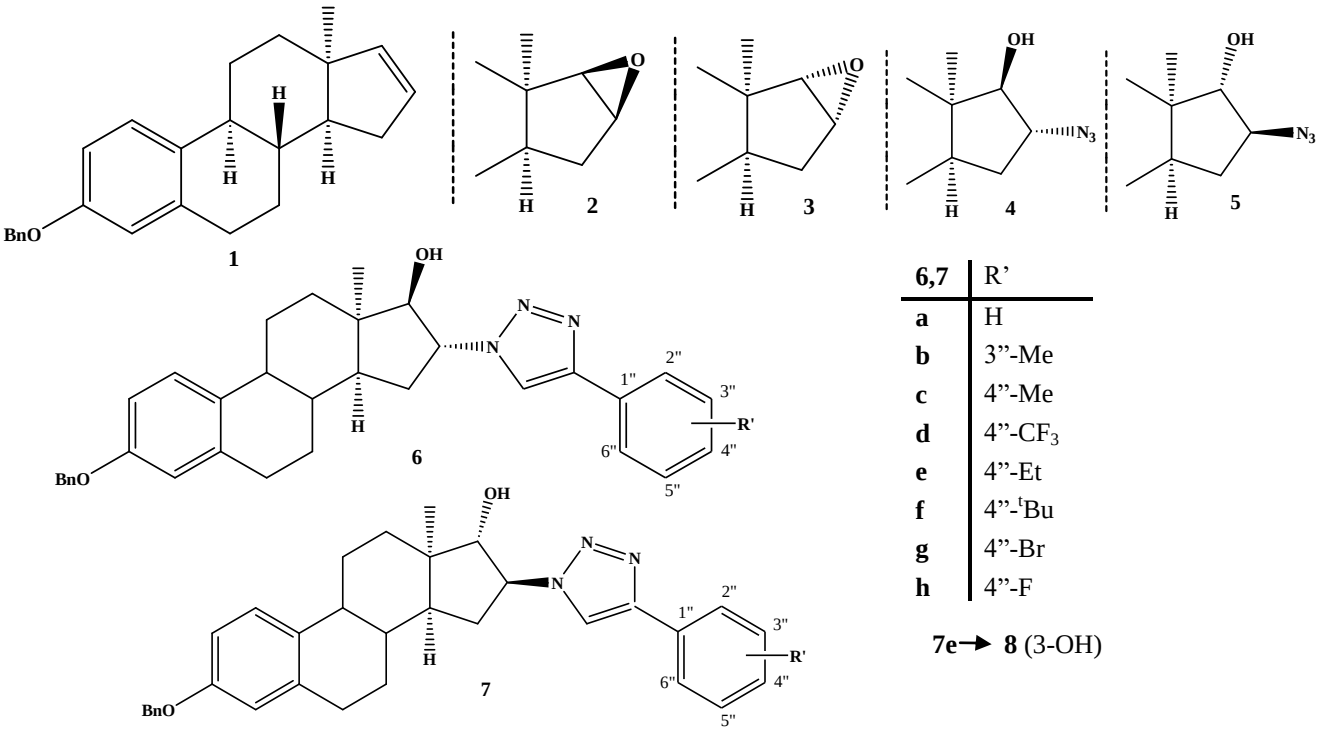
The current study aimed to determine the anticancer properties of newly synthesized modified estradiol analogs on a panel of gynecological cancer cell lines *in vitro*:

- Antiproliferative screening and the assessment of cancer-selectivity of the examined compounds by means of MTT assay. Comparing the activity of the most potent analogs on different gynecological tumor cell lines and/or against breast cancer cell lines with different receptor status.
- Investigating the antiproliferative activity of selected compounds by performing cell cycle analysis, morphological examination of the cells with Hoechst 33258 (HO) and propidium iodide (PI) staining, and determining the activity of key caspases.
- Examination of the antimetastatic ability of selected agents through their activities on cell migration and invasion. Performing gelatin zymography and Western Blot analysis to further assess the antimetastatic property of the agents. Considering the previous results of the compounds and the unmet medical need of TNBC, MDA-MB-231 TNBC cell line was chosen for the antimetastatic experiments.

3. Materials and methods

3.1 Chemicals

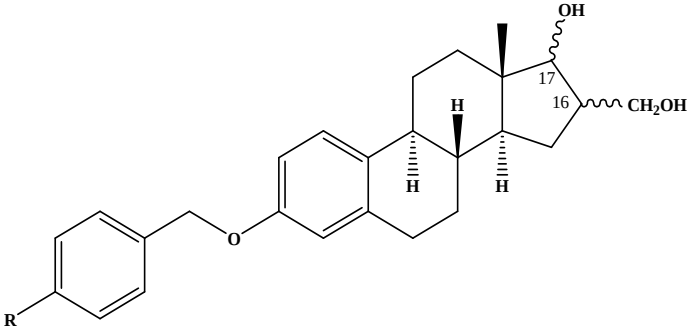
Novel 16-triazoles and their precursors in the 13α-estradiol series, the four possible stereoisomers of 3-benzyloxy-16-hydroxymethylene-estra-1,3,5(10)-trien-17-ol and three further analogs with a substituted benzyl function at C-3; moreover, the four possible isomers of 16- hydroxymethyl-3-methoxy- and 16-hydroxymethyl-3-benzyloxy-13α-estra-1,3,5(10)-trien-17-ols were designed and synthesized by the Department of Organic Chemistry, University of Szeged, Hungary. The structures of the examined molecules are summarized in **Figure 1-3**.



6,7	R’
a	H
b	3”-Me
c	4”-Me
d	4”-CF_3
e	4”-Et
f	4”-^{t}Bu
g	4”-Br
h	4”-F

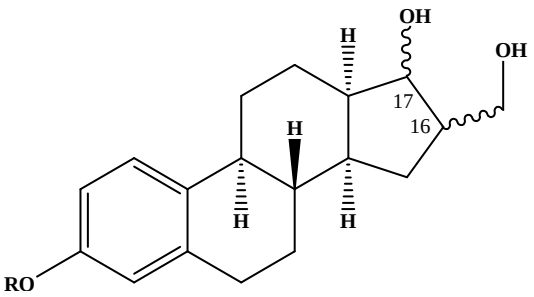
7e→ 8 (3-OH)

Figure 1. *Chemical structures of the tested compounds of the 16-triazolyl-13α-estradiol series.*



9	16β,17β
10	16α,17β
11	16β,17α
12	16α,17α
9a	16β,17β R=Cl
9b	16β,17β R=OMe
9c	16β,17β R=CN

Figure 2. *Chemical structures of the tested 3-benzyloxy-16-hydroxymethyl-estradiol analogs.*



13	16α,17β R=Me
14	16β,17α R=Me
15	16α,17β R= Bn
16	16β,17α R=Bn
17	16α,17α R=Me
18	16β,17β R=Me
19	16α,17α R=Bn
20	16β,17β R=Bn

Figure 3. *Chemical structures of the tested of 16- hydroxymethyl-3-methoxy- and 16-hydroxymethyl-3-benzyloxy-13α-estradiols.*

3.2 Cell cultures

Human cancer cell lines HeLa, A2780, and A431 derived from cervical tumor, ovarian tumor, and epidermal carcinoma, respectively, breast cancer cell lines, including MCF7,

T47D, MDA-MB-231, and MDA-MB-361, together with non-cancerous MRC-5 fibroblasts isolated from human fetal lung were utilized in the experiments. Minimal essential medium supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids and 1% antibiotic-antimycotic mixture was utilized to maintain each cell line. For the storage of the cells, humidified atmosphere containing 5% CO_2 and temperature of 37 °C were provided.

3.3 Determination of antiproliferative activities

To assess the antiproliferative property of the test compounds against the above mentioned cancer cell lines, an MTT assay was performed. Cells were seeded into 96-well plates and after an overnight growing period, the treatment with test compounds was performed. After 72 h, 5 mg/ml MTT solution was added into the plates for 4h. The precipitated formazan crystals were solubilized, and then absorbance was measured at 545 nm with a microplate reader. Control samples were determined as wells containing untreated cells.

3.4 Cell cycle analysis by flow cytometry

Flow cytometry was implemented to assess the DNA content of cells. HeLa and MDA-MB-231 cells were seeded into 6-well plates and allowed to grow overnight. Following 24 h, and in case of HeLa cells 48 h incubation with test compounds, cells were harvested and fixed in ethanol. The addition of dye solution was performed into the samples for 1 h. Partec CyFlow instrument was utilized to carry out cell cycle analysis. 20,000 cells from each well were recorded in all measurements. The percentages of cells in different cell cycle phases (subG1, G1, S and G2/M) were evaluated by ModFit LT software. The subG1 phase was regarded as apoptotic cell population.

3.5 Hoechst 33258 – propidium iodide double staining

HeLa cells were seeded into 96-well plates and incubated overnight. Cells were treated for 24 h with the test compound, and then Hoechst 33258 (HO) and propidium iodide (PI) were added into the medium of cells. Following incubation for 1 h with the dying mixture, cells were photographed by means of a fluorescence microscope provided with the appropriate optical blocks for HO and PI and a QCapture CCD camera. By means of the staining, intact, early-apoptotic, late-apoptotic, and necrotic cells could be identified based on the shaping of cell chromatin and damaging of cell membrane.

3.6 Investigation of the activity of key caspases

The activity of caspase-3, -8, and -9 enzyme was analyzed by using a commercially available colorimetric kit. HeLa cells were seeded into tissue culture flasks and incubated overnight. Cells were treated with the selected compound for 48 h. Equal amounts of protein content were utilized for the assay. Specific substrate of caspase-3, caspase-8 or caspase-9 was incubated with the samples for 2 hours. The absorbance of the released *p*-nitroaniline was measured at 405 nm by means of a microplate reader. The comparison of the absorbance of the control and treated cells determines the fold increase of activity of the examined caspase.

3.7 Wound healing assay

Wound healing assay was carried out to determine the action of selected test compounds on cell migration, by utilizing specific wound healing assay chambers. MDA-MB-231 cells were seeded into each chamber of inserts, and then following an overnight incubation inserts were removed and test compounds were added to the cells in a medium containing 2% FBS for 24 h. Visualization of cell migration into the wound area was performed by a phase-contrast inverted microscope.

3.8 Boyden chamber assay

Examination of the invasion ability of MDA-MB-231 cells was performed by means of a BD BioCoat Matrigel Invasion Chamber. Cells were suspended in serum-free medium and placed into the top insert before the tested compounds were added in sub-antiproliferative concentrations. A medium containing 10% FBS was used as chemoattractant in the bottom well. After 24 hours, the inserts were removed, fixed and then stained. The invading and stained cells were counted under a phase-contrast inverted microscope.

3.9 Gelatin zymography

In order to assess how the test compounds influence the activity of matrix metalloprotease-2 (MMP-2) and -9 (MMP-9), the gelatin zymography assay was performed. In experimental dishes, MDA-MB-231 cells were seeded and incubated overnight, and then cells were treated with increasing concentration of test compounds in serum-free medium for 24 h. The supernatant comprising the secreted MMP-2 and MMP-9 enzymes was gathered from above the cells. Equal amounts of the samples were separated by SDS-PAGE containing 0.1% gelatin via electrophoresis. The gel was treated with the reaction solution for 24 h, and then it was stained and captured by a Luminescent Image Analyzer System.

3.10 Western Blot assay

A Western Blot assay was carried out with the aim of assessing the influence of the tested compounds on the expression of focal adhesion kinase (FAK) and its phosphorylated form (pFAK) in MDA-MB-231 cells. Cells were seeded into experimental dishes, and following an overnight incubation, the examined compounds were added into the dishes for 24 hours. Equal amounts of whole-cell extracts were separated via electrophoresis and transferred from gels to nitrocellulose membranes. After blocking of the samples, incubation with a primary antibody followed by appropriate secondary antibody was executed. Visualization of the blots was performed by means of enhanced chemiluminescence.

4. Results

4.1 Antiproliferative activity of the tested compounds

Several examined estradiol analogs have shown significant antiproliferative property determined by MTT-assay. At first, screening of 16-triazolyl-13α-estradiol derivatives and their precursors on cell lines derived from different organs was performed (**Table 1**.). Compounds from the series of 16β,17α isomers (**7c-g**) that displayed the most potent cell-growth inhibition were selected for testing against a panel of breast cancer cell lines (**Table 2**.). Since **7e** did not influence the proliferation of MRC-5 cells significantly at 30 μM, and its calculated IC_{50} values on cancer cell lines were 2.4–6.5 μM, it can be declared as a cancer-selective agent. Based on the results, **7e** was selected for further examination.

The 3-benzyloxy-16-hydroxymethyl-estradiol derivatives demonstrated notable antiproliferative activity against a panel of breast cancer cell lines (**Table 3.**). Despite that the selectivity towards cancer could not be proved, the antiproliferative feature of test compounds was comparable to that of cisplatin. Based on the obtained data, the analog with the most potent IC_{50} value (**9a**) and its related, unsubstituted analog (**9**) were selected for additional examinations.

In the set of 16- hydroxymethyl-3-methoxy- and 16-hydroxymethyl-3-benzyloxy-13α-estradiols, the 3-benzyl ethers were demonstrated to be more potent compared to their 3-methyl ether counterparts (**Table 4.**). It was found, that the IC_{50} value of **20** was notably lower on cancer cells, than that on non-cancerous MRC-5 fibroblast cells [26.01 μM]. Regarding **15,** based on its calculated IC_{50} value against MRC-5 cells [15.65 μM] in comparison with the results of A2780 and HeLa cells, a modest cancer selectivity could be assessed.

Table 1. *Antiproliferative properties of 16-triazolyl-13α-estradiol derivatives and their precursors*

		Inhibition (%) ± SEM [calculated IC_{50} value[1] (μM)]			
Comp.	Conc. (μM)	**HeLa**	**MCF7**	**A2780**	**A431**
1	10	–[2]	–	–	–
	30	–	27.3 ± 1.6	24.6 ± 0.4	–
2	10	–	–	–	–
	30	28.9 ± 0.8	48.3 ± 1.2	36.3 ± 1.0	46.7 ± 1.1
3	10	–	–	–	–
	30	39.9 ± 1.4	48.8 ± 1.3	44.8 ± 0.6	68.8 ± 1.2
4	10	21.5 ± 0.7	36.2 ± 1.4	33.3 ±0.8	–
	30	98.3 ± 0.1	90.3 ± 0.4	96.4 ±0.2	97.7 ± 0.1
5	10	20.4 ± 0.5	34.6 ± 1.4	39.0 ±1.0	–
	30	96.0 ± 0.4	77.3 ± 1.3	96.0 ±0.4	82.3 ± 1.1
6a	10	64.7 ± 1.7	–	46.5 ±1.1	44.9 ± 0.9
	30	80.3 ± 0.4	–	50.7 ±2.0	36.1 ± 1.3
6b	10	23.9 ± 0.9	28.3 ± 1.0	38.9 ±0.2	–
	30	27.4 ± 1.0	30.0 ± 0.5	38.6 ±0.4	–
6c	10	57.3 ± 2.4	35.2 ± 2.0	46.9 ±0.8	–
	30	55.5 ± 1.0	37.6 ± 2.0	56.5 ±0.9	36.6 ± 0.4
6d	10	58.3 ± 1.0	42.9 ± 1.2	53.5 ±1.5	25.5 ± 2.6
	30	56.7 ± 1.2	43.6 ± 0.4	55.4 ±1.1	19.0 ± 4.4
6e	10	28.1 ± 1.4	30.4 ± 0.6	41.4 ±0.5	–
	30	24.1 ± 1.2	28.7 ± 2.3	40.5 ±0.3	–
6f	10	46.2 ± 2.4	36.4 ± 1.4	43.6 ±0.7	38.7 ± 0.4
	30	52.0 ± 2.4	39.6 ± 2.1	51.0 ±1.4	37.0 ± 1.1
6g	10	61.2 ± 2.7	22.9 ± 1.5	38.8 ± 0.98	18.5 ± 2.8
	30	65.1 ± 2.0 [2.0]	27.9 ± 1.0	39.8 ± 1.2	33.7 ± 2.1
6h	10	55.5 ± 1.0	22.3 ± 2.0	40.2 ± 2.2	13.1 ± 0.5
	30	62.8 ± 2.2	23.4 ± 0.8	36.8 ± 1.4	26.2 ± 2.8
7a	10	45.7 ± 2.7	–	33.3 ±1.6	–
	30	84.9 ± 0.5	74.7 ± 0.7	81.7 ±0.8	53.4 ± 0.4
7b	10	58.4 ± 0.1	41.5 ± 1.9	56.4 ±0.6	–
	30	79.3 ± 0.4	66.8 ± 1.5	82.9 ±1.0	–
7c	10	72.0 ± 2.0	82.0 ± 0.9	80.9 ±2.3	49.3 ± 1.3
	30	88.3 ± 1.3 [7.6]	93.6 ± 0.3 [6.0]	88.8 ±1.5 [6.0]	64.8 ± 2.4 [10.2]
7d	10	75.8 ± 1.0	67.7 ± 2.6	69.5 ±2.6	44.9 ± 0.5
	30	89.2 ± 0.9 [8.8]	94.3 ± 0.2 [9.3]	90.7 ±1.8 [7.5]	46.8 ± 1.1 –
7e	10	93.5 ± 0.2	83.3 ± 2.1	92.7 ±0.2	87.5 ± 0.7
	30	97.3 ± 0.1 [2.6]	89.5 ± 1.1 [2.4]	96.9 ±0.1 [2.6]	92.3 ± 0.6 [2.9]
7f	10	76.0 ± 0.8	73.5 ± 0.9	88.4 ±0.9	68.6 ± 0.6
	30	89.6 ± 0.5 [3.4]	84.8 ± 0.5 [2.9]	95.0 ±0.3 [2.9]	76.6 ± 0.3 [3.2]
7g	10	60.5 ± 1.1	35.1 ± 2.6	47.8 ± 1.0	31.2 ± 1.2
	30	86.3 ± 0.9 [8.5]	73.5 ± 1.2	58.5 ± 2.1	42.6 ± 1.4
7h	10	31.6 ± 2.4	18.4 ± 2.4	16.7 ± 2.4	4.3 ±1.4
	30	49.6 ± 0.4	34.2 ± 2.3	38.1 ± 1.7	12.8 ± 2.8
8	10	24.3 ± 1.6	8.8 ± 1.0	9.9 ± 0.9	4.6 ± 1.1
	30	24.5 ± 1.9	57.9 ± 0.9	39.0 ± 1.0	30.0 ± 1.3
Cisplatin	10	42.6 ± 2.3	53.0 ± 2.3	83.6 ± 1.2	88.6 ± 0.5
	30	99.9 ± 0.3 [12.4]	86.9 ± 1.3 [9.6]	95.0 ± 0.3 [1.3]	90.2 ± 1.8 [2.8]

[1] Mean value from two independent determinations with five parallel wells, standard deviation less than 15%.
[2] Inhibition values <20% are not presented.

Table 2. *Antiproliferative properties of **7c–g**.*

		Inhibition (%) ± SEM [calculated IC_{50} value[3] (μM)]		
Comp.	Conc. (μM)	**T47D**	**MDA-MB-231**	**MDA-MB-361**
7c	10	85.9 ± 1.1	84.5 ± 1.01	83.6 ±_ 0.6
	30	92.0 ± 0.4	94.5 ± 0.5	89.2 ± 0.5
		[5.9]	[6.5]	[5.3]
7d	10	58.4 ± 0.9	41.8 ± 2.5	57.2 ± 1.8
	30	90.9 ± 0.5	82.2 ± 0.7	74.1 ± 1.2
		[9.7]	[10.3]	[9.7]
7e	10	85.6 ± 0.6	85.3 ± 1.8	74.1 ± 0.9
	30	92.0 ± 0.4	92.4 ± 0.9	83.6 ± 1.6
		[5.9]	[6.5]	[5.4]
7f	10	65.4 ± 0.8	57.0 ± 1.3	61.4 ± 0.3
	30	87.7 ± 0.6	84.5 ± 0.7	74.3 ± 1.2
		[6.2]	[8.3]	[6.6]
7g	10	56.5 ± 1.0	48.5 ± 1.5	56.0 ± 2.0
	30	81.1 ± 1.4	76.9 ± 1.9	73.7 ± 1.3
		[9.0]	[10.3]	[8.8]
Cisplatin	10	51.0 ± 2.0	20.08 ± 0.8	67.5 ± 1.0
	30	57.9 ± 1.4	71.7 ± 1.2	87.7 ± 1.1
		[9.8]	[19.1]	[3.7]

[3] Mean value from two independent determinations with five parallel wells; standard deviation less than 15%.

Table 3. *Antiproliferative properties of 3-benzyloxy-16-hydroxymethyl-estradiol derivatives*

		Inhibition (%) ± SEM [calculated IC_{50} value[4] (μM)]				
Comp.	Conc. (μM)	**MDA-MB-231**	**MCF7**	**T47D**	**MDA-MB-361**	**MRC-5**
9	10	31.5 ± 1.8	60.2 ± 1.6	54.5 ± 0.3	93.1 ± 0.5	78.0 ± 2.6
	30	94.9 ± 0.5	97.2 ± 0.4	90.0 ± 04	93.3 ± 1.1	96.5 ± 0.2
		[12.7]	[8.2]	[8.4]	[4.6]	[6.7]
10	10	93.8 ± 0.5	91.5 ± 0.6	82.7 ± 1.6	93.8 ± 0.7	94.8 ± 0.5
	30	96.2 ± 0.3	97.8 ± 0.2	82.1 ± 0.5	94.0± 0.3	96.9 ± 0.2
		[3.9]	[5.1]	[4.6]	[3.8]	[5.5]
11	10	76.4 ± 0.7	27.0 ± 1.3	38.8 ± 2.0	62.7 ± 2.2	39.1 ± 3.2
	30	92.2 ± 0.6	88.6 ± 1.2	90.3 ± 0.6	86.2 ± 0.9	92.6 ± 0.2
		[5.9]	[13.6]	[15.7]	[7.2]	[10.2]
12	10	84.3 ± 1.2	56.6 ± 2.2	47.5 ± 2.4	79.1 ± 2.7	53.7 ± 3.4
	30	92.8 ± 0.3	86.6 ± 0.9	94.6 ± 0.7	89.9 ± 1.0	76.8 ± 2.2
		[5.5]	[9.1]	[10.4]	[5.4]	[7.3]
9a	10	89.5 ± 0.8	96.5 ± 0.7	95.6 ± 0.3	81.1 ± 1.8	76.5 ± 2.3
	30	87.7 ± 0.3	96.7 ± 0.2	95.3 ± 0.9	87.0 ± 1.3	79.8 ± 1.0
		[4.6]	[3.5]	[2.8]	[1.3]	[6.7]
9b	10	61.1 ± 2.4	72.7 ± 0.8	80.2 ± 2.1	76.8 ± 1.2	61.0 ± 2.8
	30	82.8 ± 0.9	89.1 ± 0.9	87.0 ± 1.5	81.1 ± 1.7	78.1 ± 1.8
		[7.5]	[4.2]	[3.0]	[4.7]	[1.5]
9c	10	30.9 ± 2.4	39.3 ± 0.7	34.7 ± 1.4		
	30	68.8 ± 1.7	57.7 ± 0.8	52.7 ± 3.1	-[5]	-
		-	-	-		
Cisplatin	10	20.8 ± 0.8	53.0 ± 2.3	51.0 ± 2.0	67.5 ± 1.0	60.3 ± 3.3
	30	74.5 ± 1.2	87.0 ± 1.2	58.0 ± 1.5	87.8 ± 1.1	61.9 ± 1.0
		[19.1]	[5.8]	[9.8]	[3.7]	[6.2]

[4] IC_{50} values were calculated from two experiments with 5 parallel wells if the growth inhibition of the compound was> 75% at 30 μM.

[5] Not determined.

Table 4. *Antiproliferative properties of 16-hydroxymethyl-3-methoxy- and 16-hydroxymethyl-3-benzyloxy-13α-estradiol derivatives*

		Inhibition (%) ± SEM [calculated IC_{50} value[6] μM]					
Comp.	Conc. (μM)	**MDA-MB-231**	**MCF7**	**T47D**	**MDA-MB-361**	**HeLa**	**A2780**
13	10	<10	<10	20.2 ± 1.7	22.9 ± 2.6	17.4 ± 2.0	23.6 ± 1.3
	30	32.1 ± 2.5	21.6 ± 2.3	36.7 ± 0.8	31.2 ± 2.9	58.6 ± 1.7	54.4 ± 2.0
		-[7]	-	-	-	[24.7]	[23.2]
14	10	10.1 ± 1.7	11.9 ± 2.1	31.7 ± 1.2	<10	55.4 ± 2.1	38.7 ± 0.7
	30	40.4 ± 1.2	43.2 ± 1.6	57.0 ± 1.8	26.0 ± 1.3	66.0 ± 0.9	71.4 ± 0.6
		-	-	[22.5]	-	[8.4]	[13.3]
15	10	18.2 ± 2.7	40.4 ± 1.7	46.3 ± 1.3	11.1 ± 1.9	50.9 ± 2.4	50.8 ± 0.8
	30	89.0 ± 1.5	96.7 ± 0.2	93.7 ± 0.3	96.5 ± 0.5	97.5 ± 0.1	94.9 ± 0.7
		[17.4]	[10.7]	[10.6]	[17.4]	[8.8]	[9.3]
16	10	20.6 ± 0.9	26.4 ± 1.6	29.7 ± 0.7	15.7 ± 2.9	76.7 ± 0.7	51.9 ± 1.4
	30	94.2 ± 0.6	93.6 ± 0.7	46.8 ± 1.4	75.2 ± 1.1	89.6 ± 0.5	75.3 ± 0.7
		[16.0]	[14.6]	-	[13.5]	[3.4]	[7.7]
17	10	27.1 ± 3.0	15.0 ± 2.2	28.0 ± 2.0	17.1 ± 1.3	56.5 ± 1.4	47.9 ± 1.9
	30	42.8 ± 2.1	31.3 ± 1.4	44.3 ± 2.1	39.3 ± 2.5	56.4 ± 1.5	63.7 ± 1.3
		-	-	-	-	[4.8]	[10.7]
18	10	<10	<10	16.7 ± 1.4	<10	47.5 ± 1.1	36.1 ± 0.9
	30	30.1 ± 1.0	26.8 ± 1.8	38.0 ± 0.7	17.8 ± 1.7	58.4 ± 1.5	55.2 ± 2.3
		-	-	-	-	[11.0]	[16.2]
19	10	22.3 ± 2.2	27.1 ± 2.0	44.3 ± 1.0	20.3 ± 2.8	55.3 ± 2.4	54.9 ± 1.9
	30	94.4 ± 0.7	95.4 ± 0.2	94.1 ± 0.9	88.1 ± 0.9	96.8 ± 0.5	93.7 ± 0.5
		[11.6]	[14.6]	[11.2]	[12.3]	[7.0]	[8.7]
20	10	24.4 ± 2.9	37.3 ± 1.5	44.3 ± 1.2	15.2 ± 2.3	70.5 ± 1.3	54.6 ± 1.3
	30	88.3 ± 1.4	97.4 ± 0.3	93.5 ± 0.5	93.7 ± 0.3	98.1 ± 0.2	94.5 ± 0.8
		[11.5]	[12.0]	[10.3]	[13.2]	[4.9]	[7.9]

[6] IC_{50} values were calculated from two experiments with 5 parallel wells if the growth inhibition of the compound at 30 μM was> 75%.

[7] Not determined.

4.2 Cell cycle analysis by flow cytometry

Flow cytometry was performed to specify the activity of the selected compounds on the cell cycle phase distributions. The analysis of **7e** was carried out after 24 and 48 h incubation against HeLa cervical cancer cell line. The reduction of cell population in the G1 phase along with the increase in the S and G2/M phase was found to be significant after treatment with 10 μM for 24 h. A similar alteration was assessed after 48 h; however, the hypodiploid subG1 population was significantly elevated (**Fig. 4.**).

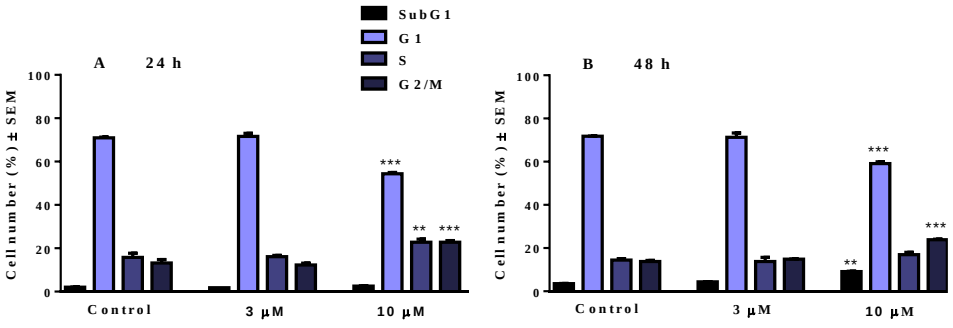


Figure 4. *Effects of **7e** on the cell cycle of HeLa cells after incubation for 24 or 48 h. Results are mean values ± SEM from two measurements. ** and *** indicate $p < 0.01$ and $p < 0.001$, respectively, as compared with the control cells.*

Compound **9** and **9a** induced a significant elevation of the cell population in G1 phase, with concomitant decrease in the G2/M phase. A remarkable effect on the cell cycle was observed by **9** even at 5 μM in spite of its relatively high IC_{50} value. The pro-apoptotic property of both compounds was demonstrated based on the significant but modest increase in the subG1 population, which was induced in all tested concentrations (**Fig. 5.**).

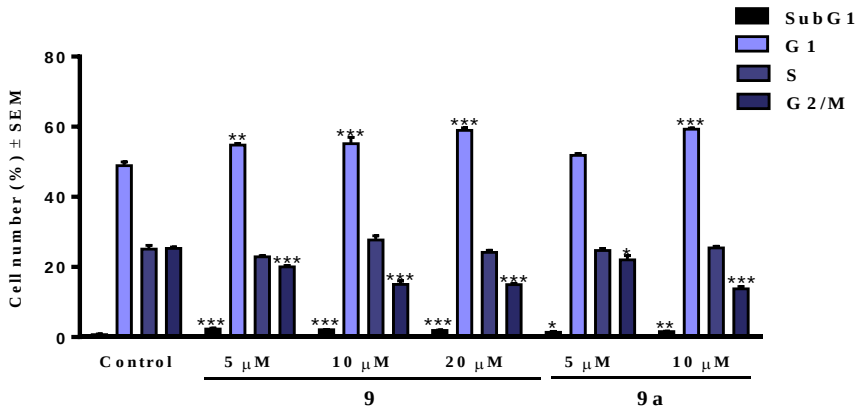


Figure 5. *Effects of **9** and **9a** on the MDA-MB-231 cell cycle after incubation for 24 h. Results are mean values ± SEM from three measurements. *, ** and *** indicates p <0.05, p <0.01 and p <0.001 as compared with the control cells, respectively.*

4.3 Hoechst 33258 – propidium iodide double staining

Hela cells were treated with **7e** for 24 or 48 h. Chromatin condensation and nuclear fragmentation of nuclei were used as indicators for apoptosis, while the loss of membrane integrity proved necrosis. Exposure to 10 μM led to the significant elevation of the ratio of early apoptotic and secondary necrotic cells after 24 h incubation. Treatment for 48 h resulted in similar, but more substantial alteration in the ratio of the cell population (**Fig. 6.**).

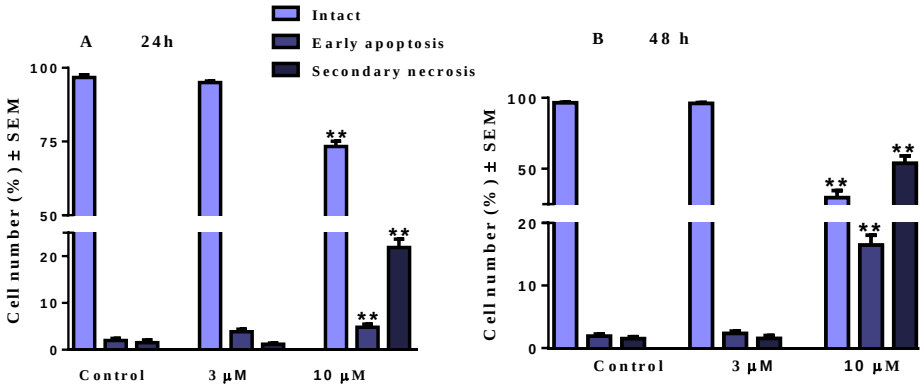


Figure 6. *Compound **7e** elicits apoptosis and necrosis in HeLa cells after incubation for 24 or 48 h. ** indicates p < 0.01 as compared with the control conditions.*

4.4 Determination of in situ caspase-3, caspase-8 and caspase-9 activity

The investigation of the activity of caspases supported the pro-apoptotic potency of **7e.** HeLa cells were treated with **7e** at 10 μM for 48 h, which resulted in a significant concentration-dependent increase of caspase-3. Significant elevation was observed in the activity of caspase-9 indicating that mitochondrial damage may play a role in the mechanism of action of **7e**, while the activity caspase-8 was not changed (**Fig. 7.**).

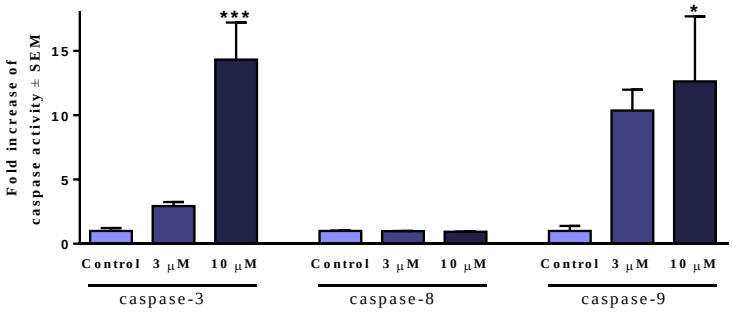


Figure 7. *Compound **7e** induces caspase-3 and caspase-9 but not caspase-8 activities after incubation for 48 h. The activities of the untreated cells were taken as one unit. * and *** indicate p <0.05 and p <0.001, respectively, as compared with the control cells.*

4.5 Wound healing assay

Wound healing assay was utilized to assess the migration ability of MDA-MB-231 cells after treatment for 24 h with elevating concentrations of **9** and **9a**. A significant, concentration-dependent decrease was observed in the migration ability of MDA-MB-231 cells (**Fig. 8.**).

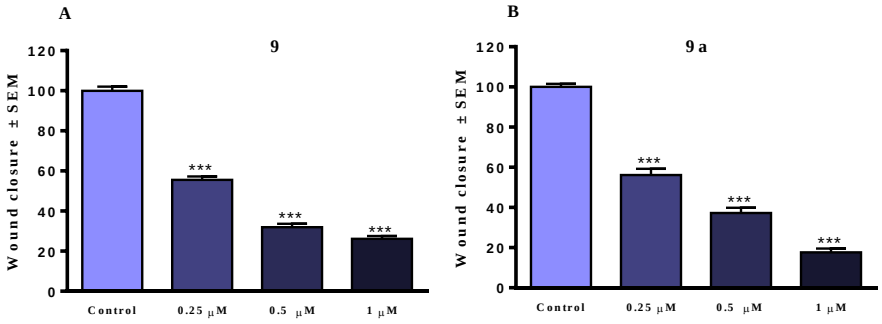


Figure 8. *Effect of **9 (A)** and **9a (B)** on the migration ability of MDA-MB-231 cells. The rates of the migration were evaluated after incubation for 24 h. Results are mean values ± SEM from three measurements. *** indicates p < 0.001 as compared with the untreated control samples.*

4.6 Boyden chamber assay

The influence of the tested compounds on the invasion ability of MDA-MB-231 cells was investigated by the means of Boyden chambers containing Matrigel Matrix-coated membrane. Invasive cells are capable of migrating through the membrane, while the crossing of non-invasive cells is blocked. After treatment for 24 h, the invasion of the examined cells was blocked by **9** and **9a** in a concentration-dependent manner (**Fig. 9.**).

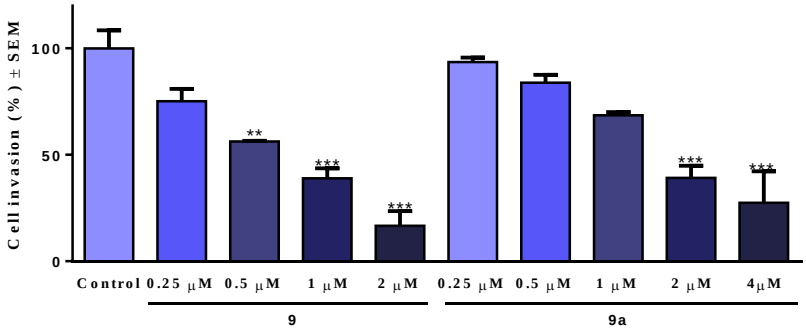


Figure 9. *Effects of **9** and **9a** on the cell-invasion capacity of MDA-MB-231 cells. Statistical analyses of the percentage of invasive cells after incubation for 24 h. Results are mean values ± SEM from three measurements. ** and *** indicates p<0.01 and p<0.001 as compared with the untreated control samples, respectively.*

4.7 Gelatin zymography assay

To characterize the mechanism of the antimetastatic feature of **9** and **9a**, the activities of MMP-2 and MMP-9 released by MDA-MB-231 cells were assessed by a gelatin zymography assay. As a result, no significant inhibition was observed by the examined compounds on the activity of the enzymes indicating that MMP-2 and MMP-9 may not be involved in the antimetastatic property of **9** and **9a** (**Fig. 10.**).

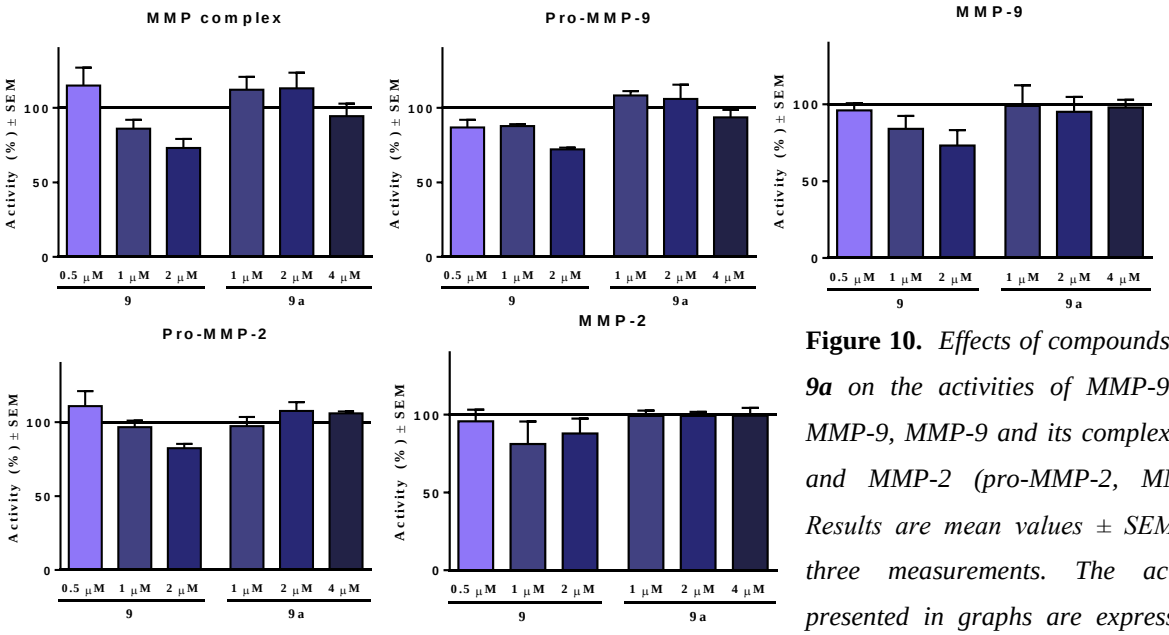


Figure 10. *Effects of compounds **9** and **9a** on the activities of MMP-9 (pro-MMP-9, MMP-9 and its complex form) and MMP-2 (pro-MMP-2, MMP-2). Results are mean values ± SEM from three measurements. The activities presented in graphs are expressed as the % of vehicle control group.*

4.8 Western Blot assay

Since focal adhesion kinase (FAK) and its phosphorylated form (pFAK) are possible targets for metastasis inhibition, the influence of the tested compounds on the expression of FAK and pFAK proteins was investigated. A significant concentration-dependent decrease was observed in the level of pFAK regarding both **9** and **9a**, while the total amount of FAK was not altered. These findings demonstrate that **9** and **9a** inhibited the phosphorylation of FAK protein (**Fig.11.**).

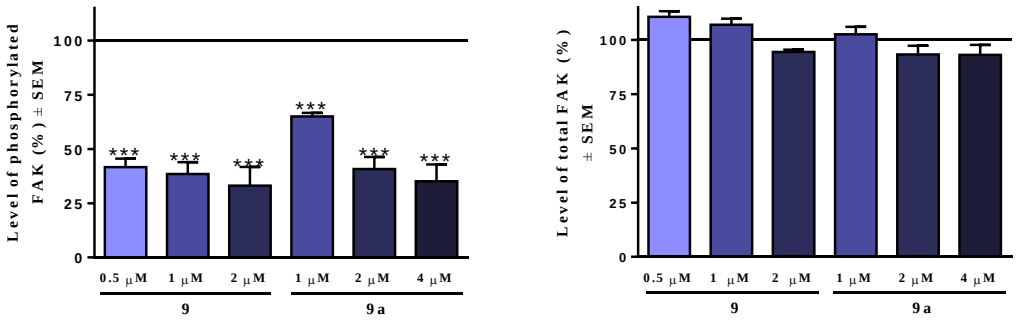


Figure 11. *Effects of compounds **9** and **9a** on the expression of phosphorylated and total FAK protein in MDA-MB-231 cells. Results are mean values ± SEM from three measurements. *** indicates p <0.001 as compared with the untreated control samples.*

5. Discussion

The first reported estrogen analog possessing considerable antiproliferative property with the lack of the binding ability to the ER was 2-methoxyestradiol (2-ME), an endogenous metabolite of 17β-estradiol. As a result of the extensive experiments on the anticancer activity, selectivity, and toxicity of 2-ME with promising findings, drug candidates with estrone skeleton became a new focus of the research and development of agents for the treatment of malignant diseases.

In the present study, we examined the anticancer property of three series of estradiol analogs. In the set of 16-triazolyl-13α-estradiols and their precursors, compounds containig triazolyl function displayed more robust antiproliferative activity. 16β,17α isomers generally showed more potent activity in comparison with their 16α,17β counterparts. The important role of benzyloxy function at C-3 was demonstrated, since compound **8** with a hydroxyl group at C-3 did not show any significant antiproliferative property. Compounds containing *p*-alkyl function, especially ethyl and *tert*-butyl on the triazolyl-phenyl substituent were demonstrated the most potent antitumor activities (**7e**, **7f**). Bearing alkyl group on the triazolyl-phenyl ring of the estradiol skeleton was reported previously to increase the antiproliferative effect, and our result correlates with this finding. Regarding the 3-benzyloxy-16-hydroxymethyl-estradiol derivatives, most compounds of the series generally induced notable inhibition of proliferation of breast tumor cells indicating no significant selectivity between the examined cell lines. Configuration of substituents at C-16 and C-17 displayed a limited affection on the antiproliferative potency. Certain *p*-substituents on the benzyl ether function at C-3 were shown to increase the antiproliferative potency (**9a**, **9b)**. The antiproliferative activity of 3-benzyloxy-13α-estradiols was found to be more pronounced compared to their 3-methyl ether counterparts considering the results of all examined cancer cell lines. One compound demonstrated notable cancer-selectivity (**20**).

In summary of structure-activity relationships of all three groups of the examined compounds, benzyl ether function at C-3 enhanced the antiproliferative potency, the configuration of substituents at C-16 and C-17 exerted a varying influence on the activity of the molecules; 16β,17β and 16β,17α isomers were proven to be the most potent estradiol analogs in general. The activity of the most potent compounds (**7e**, **7f**, **9a**, **20**, **15**) was comparable with cisplatin that is currently used in gyneological cancer therapy protocols, such as cervix and breast cancer protocols.

Treatment with **7e** generated changes in the cell cycle and elevated the subG1 population indicating a blockade at the G2/M phase and the induction of programmed cell death. G2/M phase arrest was also reported regarding other structurally modified estradiol analogs *i.e.* D-secoestrone-triazole and D-homoestrone. G1 cell population was significantly elevated together with the decrease of the ratio of cells in the G2/M phase after treatment with the increasing concentrations of compounds **9** and **9a** for 24h in MDA-MB-231 cancer cells. The increase of the percentage of subdiploid cells is a marker of apoptosis. These findings correlate with previously described G1 phase arrests induced by estradiol analogs, such as unsubstituted 16-oxime-estrone molecules and oxadiazole derivatives of estrone.

The result of morphological examinatation supports the apoptosis-inducing ability of **7e**. During the development of anticancer agents apoptosis-inducing molecules are preferred, since mainly there is no inflammatory reaction in connection with the apoptotic process or the removal of the apoptotic cells. The pro-apoptotic property of **7e** against HeLa cervical cancer cells was additionally supported by the findings of the investigation of the activity of caspase-3, the execution enzyme of the programmed cell death. The involvement of the intrinsic pathway of the apoptotic process was revealed through the increased activity of caspase-9, while the unaltered concentration of caspase-8 indicates that the extrinsic pathway does not play a role in the activity of **7e**.

Metastasis is the major cause of malignancy-related deaths; it can be connected with approximately 90% of the fatal cases. Triple-negative breast cancer is characterized by higher chance of recurrence, shorter median time to death and worse overall survival compared to other breast cancer subtypes. Compounds **9** and **9a** were additionally investigated regarding their activity on triple-negative MDA-MB-231 cell migration and invasion, the intitial steps of metastatic spread. Significant inhibiton of cell migration was observed at even 0.25 μM, indicating the potency of the compounds in sub-antiproliferative concentration. The inhibitory action of compound **9** on cell invasion capacity demonstrated to be more pronounced as evaluated by the Boyden chamber assay.

Matrix metalloproteinases participate in multiple steps of the processes of metastasis. We found that compound **9** and **9a** did not exhibit a notable influence on the level of MMP-2 and MPP-9, demonstrating that these enzymes do not play a crucial part in the mechanism of action of the examined estradiol analogs.

The role of focal adhesion kinase in the process of cell migration was reported in several studies; moreover, its elevated activity was demonstrated in several tumor types, such as breast cancer. A dose-dependent decrease was observed in the level of pFAK Y397 in

MDA-MB-231 cells after treatment with **9** and **9a** in sub-antiproliferative concentrations. The expression of total FAK was not influenced by the treatment indicating that the compounds inhibited the phosphorylation of the enzyme.

6. Summary

In summary, the present work revealed that numerous compounds from the set of 16-triazolyl-13α-estradiols, 3-benzyloxy-16-hydroxymethyl-estradiols, and 16-hydroxymethyl-3-benzyloxy-13α-estradiols are potent antiproliferative agents against gynecological cancer cell lines, especially breast and cervical tumors.

Apoptosis-induction was observed in cervical cancer cells bearing HPV 18 DNA by 16-triazolyl-13α-estradiol **7e** through G2/M phase blockage and involving the mitochondrial apoptotic pathway, while showing a beneficial cancer-selectivity. **9** and **9a** 3-benzyloxy-16-hydroxymethyl-estradiols caused programmed cell death and G1 phase increase in TNBC cells.

TNBC cell line was utilized for the investigation of the antimetastatic capacity of test compounds due to the unmet medical need of the subtype. **9** and **9a** exerted substantial dose-dependent inhibition on cell migration and invasion; furthermore, on the phosphorylation of focal adhesion kinase, an important target in antimetastatic research. The treatment proved to be potent in sub-antiproliferative concentrations that indicates the advantageous cancer selectivity of the compounds.

Our findings demonstrate that molecules with modified estradiol structure can be regarded as promising candidates in the development of new anticancer agents exerting antiproliferative and antimetastatic properties.

Scientific publications related to the subject of the thesis

I. Sinka, I; Kiss, A; Mernyák, E; Wölfling, J; Schneider, G; Ocsovszki, I; Kuo, C-Y; Wang, H-C; Zupkó, I:

Antiproliferative and antimetastatic properties of 3-benzyloxy-16-hydroxymethylene-estradiol analogs against breast cancer cell lines

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IF: 3.532

II. Kiss, A; Mernyák, E; Wölfling, J; Sinka, I; Zupkó, I; Schneider, Gy:

Stereoselective synthesis of the four 16-hydroxymethyl-3-methoxy- and 16-hydroxymethyl-3-benzyloxy-13α-estra-1,3,5 (10)-trien-17-ol isomers and their antiproliferative activities

STEROIDS 134 pp. 67-77., 11 p. (2018)

IF: 2.136

III. Mernyák, E; Kovács, I; Minorics, R; Sere, P; Czégány, D; Sinka, I; Wölfling, J; Schneider, Gy; Újfaludi, Zs; Boros, I; Ocsovszki, I; Varga, M; Zupkó, I:

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IF: 3.985

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I. Tastan, P; Hajdú, Zs; Kúsz, N; Zupkó, I; Sinka, I; Kivcak, B; Hohmann, J:

Sesquiterpene Lactones and Flavonoids from Psephellus pyrrhoblepharus with Antiproliferative Activity on Human Gynecological Cancer Cell Lines

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IF: 3.060

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Microwave-assisted synthesis of biologically relevant steroidal 17- exo -pyrazol-5'-ones from a norpregnene precursor by a side-chain elongation/heterocyclization sequence

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IF: 2.595

III. Vlaisavljević, S; Šibul, F; Sinka, I; Zupko, I; Ocsovszki, I; Jovanović-Šanta, S:

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IV. Baji, Á; Kovács, F; Mótyán, G; Schneider, G; Wölfling, J; Sinka, I; Zupkó, I; Ocsovszki, I; Frank, É:

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VII. Csábi, J; Martins, A; Sinka, I; Csorba, A; Molnár, J; Zupkó, I; Tóth, G; Tillekeratne, LMV; Hunyadi, A:

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IF: 2.608