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ANTICANCER EFFECTS OF ESTRONE DERIVATIVES AND NONSTEROIDAL 17β-HYDROXYSTEROID DEHYDROGENASE INHIBITORS

PhD Thesis

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2013

SCIENTIFIC PUBLICATIONS RELATED TO THE SUBJECTS OF THE THESIS

I. Berényi Á, Minorics R, Iványi Z, Ocsovszki I, Ducza E**,** Thole H, Messinger J, Wölfling J, Mótyán G, Mernyák E, Frank É, Schneider G, Zupkó I: Synthesis and investigation of the anticancer effects of estrone-16-oxime ethers *in vitro*. *Steroids* **78:** 69–78 (2013) IF_{2011}: 2.829

II. Berényi Á, Frotscher M, Marchais-Oberwinkler S, Hartmann RW, Minorics R, Ocsovszki I, Falkay G, Zupkó I: Direct antiproliferative effect of nonsteroidal 17β-hydroxysteroid dehydrogenase type 1 inhibitors *in vitro*. *J Enzyme Inhib Med Chem* **in press** (2012) IF_{2011}: 1.617

III. Ekholm FS, Berényi Á, Lagerquist L, Saloranta T, Zupkó I, Schneider G, Wölfling J, Leino R: Cytotoxic activity of some glycoconjugates including saponins and anthracyclines. *Carbohydr Res* **356:** 295–8 (2012) IF_{2011}: 2.332

ADDITIONAL PUBLICATIONS

I. Fürst R, Zupkó I, Berényi Á, Ecker GF, Rinner U: Synthesis and antitumor-evaluation of cyclopropyl-containing combretastatin analogs. *Bioorg Med Chem Lett* **19:** 6948–51 (2009) IF_{2009}: 2.650

II. Csapi B, Hajdú Zs, Zupkó I, Berényi Á, Forgo P, Szabó P, Hohmann J: Bioactivity-guided isolation of antiproliferative compounds from Centaurea arenaria. *Phytother Res* **24:** 1664–69 (2010) IF_{2010}: 1.878

III. Vasas A, Sulyok E, Rédei D, Forgo P, Szabó P, Zupkó I, Berényi Á, Molnár J, Hohmann J: Jatrophane diterpenes from Euphorbia esula as antiproliferative agents and potent chemosensitizers to overcome multidrug resistance. *J Nat Prod* **74:** 1453–61 (2011) IF_{2011}: 3.128

IV. Ndonsta BL, Tatsimo JSN, Csupor D, Forgo P, Berkecz R, Berényi Á, Tene M, Molnár J, Zupkó I, Hohmann J, Tane P: Alkylbenzoquinones with antiproliferative effect against human cancer cell lines from stem of Ardisia kivuensis. *Phytochem Lett* **4:** 227–30 (2011) IF_{2011}: 1.222

V. Sarikaya BB, Zencir S, Somer NU, Kaya GI, Onur MA, Bastida J, Berényi Á, Zupkó I, Topcu Z: The effects of arolycoricidine and narciprimine on tumor cell killing and topoisomerase activity. *Rec Nat Prod* **4:** 381–5 (2012) IF_{2011}: 1.508

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LIST OF ABBREVIATION

Ac-DEVD-*p*NA	acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide
ANOVA	analysis of variance
Bp	base pair
BrdU	5-Bromo-2'-deoxyuridine
CDK2	cyclin-dependent kinase 2
CDK4	cyclin-dependent kinase 4
CDK6	cyclin-dependent kinase 6
cDNA	copy deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
FBS	foetal bovine serum
17β-HSD1	17β-hydroxysteroid dehydrogenase type 1
hGAPDH	human glyceraldehydes-3-phosphate dehydrogenase
HO	Hoechst 33258
kDa	kilodalton
MMLV-RT	Moloney murine leukaemia virus-reverse transcriptase
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	phosphate-buffered saline
PI	propidium iodide
***p*NA**	*p*-nitroaniline
Rb	retinoblastoma protein
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
SEM	standard error of the mean
SERM	selective estrogen receptor modulator

1 INTRODUCTION

Cancer belongs to one of the major public health problems and became a leading disease worldwide. In 2012 the numbers of new cancer cases and deaths in the United States were estimated by American Cancer Society. According to the survey, a total of 1,638,910 new cancer cases and 577,190 deaths from cancer are predicted in the United States in 2012. Besides, death rates continue to decline for all four most commonly diagnosed cancer sites (lung, colorectum, breast, and prostate), with lung cancer accounting for almost 40% of the total decline in men and breast cancer accounting for 34% of the total decline in women [1].

Cancer can be characterized with dedifferentiation and uncontrolled cellular proliferation [2]. Tumor growth is a very complex and multifactorial process. Both genetic and epigenetic lesions contribute to heritable changes in gene expression in cancer cells. The genetic instability and a progressive loss of differentiation are consequences of mutations of critical genes, including suppressor genes, oncogenes and genes involved in deoxyribonucleic acid (DNA) repair. The transformation of intact cells into a tumor cells by mutations or errors of genetic sequences in protooncogene and tumor suppressor genes is evidenced and generally accepted [3]. In summary, an uncontrolled cell proliferation based on genetic instability is initiated which leads finally to tumor formation.

Several hallmark features of the cancer cell phenotype have been identified such as disregard of signals to stop proliferating and to differentiate; a capacity for sustained proliferation; evasion of apoptosis; and invasion and angiogenesis [4] as well as cancer-related inflammation [5] and a shift in cellular metabolism [6].

Current possible therapeutic approaches are determined by an interacting set of factors including the status of the patient, the behavior of the disorder and the cancer burden of the body. The most well-established treatment modalities for cancer elimination are surgery, radiotherapy and chemotherapy. While the usage of surgery and radiotherapy is dependent on the progression of the disorder, presence of metastases and additional crucial factors chemotherapy is utilized in a substantially wider clinical spectrum. Traditional chemotherapeutic agents exhibit proven efficacy with inefficient cancer selectivity which explains their well characterized side effects on relatively fast growing normal cells (vomitus, fatigue, loss of hair). Based on the recent advent of more selective anticancer agents it can be

speculated that all tumor type possesses different suitable points for intervention and therefore a unique agent could be developed for the treatment of each cancer types.

1.1 Overview of anticancer properties of estrone-related compounds (estrone oxime derivatives, steroid aglycones and glycosteroids)

Natural products with steroid or related skeleton structure could be considered a basic source of innovative drugs. Therefore, the steroidal skeleton could be a beneficial backbone for design and synthesis of original drug candidates with a wide range of pharmacological activities. Several plant steroids are published to possess inotropic, antihypercholesterolemic, anti-inflammatory and antioxidant properties [7][11]. Natural steroid products and their synthetic analogs are intensively investigated in order to describe and exploit their anticancer capacity. Diosgenin, belongs to the most intensively studied saponins and it exerts antiproliferative effects against several human cancer cell lines, such as 1547 osteosarcoma, HER2-overexpressing breast cancer, M4Beu melanoma, K562 leukemia, HEL, and HT-29 colon [12][17]. An appreciable inhibition of human HT-29 and HepG2 liver cancer cell proliferation have been reported by steroidal glycoalkaloids, including α-tomatine and solanine [18]. Solanine and α-tomatine capacities were greater than those of the aglycones tomatidine and solanidine, respectively, indicating that the steroidal backbone is not an exclusive factor determining the overall cytostatic activities of these natural products.

Besides those of the active components isolated from plants, the outstanding anticancer and cardioprotective properties of endogenous estrogen hormone metabolites have been described [19]. 2-Methoxyestradiol, an intensively investigated metabolite synthesized by catechol-*O*-methyltransferase, exerts a pronounced anticancer effect on a broad variety of human malignant cell lines of reproductive origin [20][22].

A set of oxime-containing flavone and isoflavone derivatives have been synthesized and tested against three human adherent cell lines, and the effective agents were further evaluated against the full panel of 60 human tumor cell lines derived from nine cancer types. Flavon-6-yl oximes proved to exhibit the most pronounced cytostatic effects, with the lowest mean GI_{50} value as low as 0.08 μM. The apoptosis induction of the most effective compound was evidenced by flow cytometry. Introduction of an oxime function into an appropriate skeleton is a reasonable approach to the preparation of potent cytotoxic agents.

The 5-nitrofuran-2-yl-vinyl quinoline derivative exerted pronounced antiproliferative activity when an oxo function was substituted with a hydrogen-bond-donating oxime group. The derivative containing hydrogen-bond-accepting methyloxime was less favorable against cancer cells of reproductive origin [23].

Since glycosylation may fundamentally alter the physic-chemical character of natural products and their synthetic analogues and have a crucial impact on their biological activities. Therefore, the aim of the 1st part of the thesis was the *in vitro* investigation of some synthetic estrogens. Compounds with reasonable action on the viability of human adherent cancer cells were selected for an additional set of experiments in order to describe the mechanism of the detected antiproliferative action.

Steroidal glycosides exert crucial effect in heart failure and potent against several cancer cell lines [24]. Aglycones play role in cytotoxic activities which properties could be enhanced by glycosylation as in digitoxin analogues [25]. The antiproliferative effects of estrogen aglycones and their glycosylated counterparts were previously investigated [26].

1.2 Overview of anticancer properties of 17β-HSD1-related compounds

The most relevant estrogen, 17β-estradiol, is involved in many hormone-dependent proliferative disorders in humans, including cancers of gynecological origin and endometriosis. The suppression of estrogen exposure at the targeted tissue is therefore a part of the rational therapy. Such estrogen suppression may be implemented directly at the receptor level by the administration of selective estrogen receptor modulators (SERMs) or antagonists. The best-studied SERM, tamoxifen, proved to act as an agonist in the uterus, resulting in a higher incidence of endometrial cancer [27]. The second-generation SERMs (e.g. raloxifene or arzoxifene) do not exert a pronounced uterotropic effect, and the favorable safety profile therefore renders these agents suitable for the long-lasting treatment of osteoporosis besides breast cancer.

Alternatively, a pharmacological intervention influencing the synthesis of endogenous estrogens at any point of the pituitary-hypothalamus-ovary axis offers a considerable therapeutic modality. Gonadotropin releasing hormone analogs are suitable for the radical inhibition of sexual steroid production, and therefore have a role in both estrogen- and androgen-dependent disorders. Since estrogens are the only group of hormones with an

aromatic ring in the steroid skeleton, the inhibition of aromatase is another possibility via which to decrease the estrogen output selectively. Drugs acting by means of this mechanism (e.g. letrozole or anastrozole) are routinely utilized to treat breast cancers.

Since hormone-dependent cancers may have the capacity to produce steroids, the local endocrine milieu seems to be more relevant than the circulating level as a descriptor of the progression. Breast cancer tissue can utilize the directly ineffective blood-borne estrone sulfate for 17β-estradiol synthesis [28]. In the first step of local activation, steroid sulfatase converts estrone sulfate into estrone, which exerts limited activity. The most advanced irreversible steroid sulfatase inhibitor STX64 (irosustat) is currently undergoing phase II clinical trials for hormone-dependent cancers [29].

17β-Hydroxysteroid dehydrogenase type 1 (17β-HSD1) is responsible for the conversion of the much less potent estrogen estrone into 17β-estradiol, while 17β-HSD2 catalyzes the opposite reaction. In postmenopausal hormone-dependent breast cancer patients, the balance between the expression of these two isoenzymes is shifted in favor of 17β-HSD1, which results in an enhanced local 17β-estradiol concentration [30]. Clinical studies have revealed that a high intratumoral expression of this enzyme is associated with a significantly shorter disease-free period and a generally poor prognosis [31].

Besides the estrone → 17β-estradiol conversion, 17β-HSD1 catalyzes the transformation of dehydroepiandrosterone into 5α-androstene-3β,17β-diol, which is a potent estrogen in spite of the lack of an aromatic ring in the steroid skeleton [32]. This step comprises an estrogen-producing pathway, which can not be blocked by aromatase or steroid sulfatase inhibitors.

In view of all these findings, interaction with 17β-HSD1 recently emerged as a further possibility through which to induce estrogen suppression and hence to modulate the function of estrogen-dependent tissues. Although no inhibitor has been tested in a clinical setting, several potent and selective steroidal and nonsteroidal structures have been characterized *in vitro*. Since most of the targeted hormone-dependent disorders share a proliferative character, it is conceivable that a direct antiproliferative action of these enzyme inhibitors could be beneficial.

Therefore, the aim of the 2nd part of the study was to characterize the direct antiproliferative action of a set of previously synthesized nonsteroidal 17β-HSD1 inhibitors on human adherent cell lines of gynecological origin (HeLa, MCF7 and A2780).

1.3 Overview of G1-S transition

The G1-S transition is governed by an orchestrated interaction of a set of regulating factors, including retinoblastoma protein (Rb), cyclin-dependent kinase 2,4 and 6 (CDK2, CDK4/6), p16, p21 and p53. Entry into the S phase, and therefore cell proliferation, is inhibited as long as Rb remains unphosphorylated by a complex containing cyclin E - CDK2 and cyclin D - CDK4/6. Phosphorylated Rb dissociates from a heterodimeric complex of E2F allowing the transcription of S-phase-specific genes [33]. The principal regulator of the cyclin E - CDK2 complex is the tumor suppressor p21, which is transcriptionally activated by p53 encoded by the TP53 gene. The importance of this pathway is illustrated by the finding that nearly all cancers have a mutation in the TP53 gene or in some components of its downstream events [34]. The hypofunction of p16 protein (also referred to as CDK4 inhibitor) which is regulator of cyclin D - CDK4/6 complex has been associated with several malignancies and its expression correlates with the chemotherapy response in patients with solid tumors [35]. Therefore, inhibition of G1-S transition could be a point for pharmacological intervention in order to inhibit the deregulated cell proliferation.

2 SPECIFIC AIMS

The aim of the present study was the investigation of the antiproliferative properties of synthetic compounds selected on the base of the previously described theoretical considerations:

- Investigation of antiproliferative action of newly synthesized estrone-16-oxime ethers, *in vitro* using human adherent cancer cell lines. The most potent compounds were selected for a further set of experiments in order to describe the possible mechanism of the action.

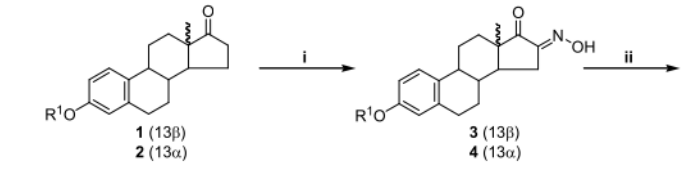
- A set of estradiol-derived steroid glycosides and aglycone were additionally screened for their antiproliferative effects.

- The determination of the antiproliferative effects of a set of previously designed and synthesized nonsteroidal 17β-hydroxysteroid dehydrogenase type 1 inhibitors, *in vitro*. Further *in vitro* assays including cycle analysis, HOPI double staining, BrdU incorporation assay, Caspase-3 activity assay, RT-PCR technique and Western blot studies are used to characterize the mechanism of the most potent agents.

3 MATERIALS AND METHODS

3.1 Chemical structures of estrone-related compounds (estrone oxime derivatives, steroid aglycone and glycosteroids) and 17β-hydroxysteroid dehydrogenase type 1 inhibitors

The first set of the investigated compounds were estrone-16-oxime ethers and related compounds designed and synthetized by the staff of the Department of Organic Chemistry, University of Szeged [36] (**Fig. 1**). All of the tested agents possessed the same estrone-based skeleton in which rings A and D were substituted with different functions at position 3 and 16.

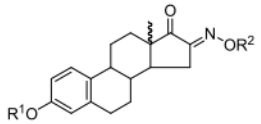


1 (13β)	R^1
a	H
b	Me
c	Bn
d	*p*-(MeO)$C_6H_4CH_2$
e	SO_2NH_2

2 (13α)	R^1
a	H
b	Me
c	Bn
d	*p*-(MeO)$C_6H_4CH_2$

3 (13β)	R^1
a	H
b	Me
c	Bn
d	*p*-(MeO)$C_6H_4CH_2$
e	SO_2NH_2

4 (13α)	R^1
a	H
b	Me
c	Bn
d	*p*-(MeO)$C_6H_4CH_2$



5 (13β)	R^1	R^2
a	H	Me
b	H	Et
c	H	*i*Pr
d	H	Bu

6 (13β)	R^1	R^2
a	SO_2NH_2	Et
b	SO_2NH_2	*i*Pr
c	SO_2NH_2	Pr

7 (13β)	R^1	R^2
a	Me	Me
b	Me	Et
c	Me	*i*Pr
d	Me	Pr
e	Me	Bu
f	Me	*p*-(CN)$C_6H_4CH_2$
g	Me	*o*-(CN)$C_6H_4CH_2$
h	Me	Ac
i	Me	$COCH_2CH_3$

8 (13α)	R^1	R^2
a	Me	Me
b	Me	Et
c	Me	*i*Pr
d	Me	Ac
e	Me	$COCH_2CH_3$

9 (13β)	R^1	R^2
a	Bn	Me
b	Bn	Et
c	Bn	*i*Pr
d	Bn	Bu
e	Bn	Bn
f	Bn	*p*-(CN)$C_6H_4CH_2$
g	Bn	*p*-(NO_2)$C_6H_4CH_2$
h	Bn	Ac
i	Bn	$COCH_2CH_3$

10 (13α)	R^1	R^2
a	Bn	Me
b	Bn	Et
c	Bn	*i*Pr
d	Bn	Pr
e	Bn	Bn
f	Bn	*p*-(NO_2)$C_6H_4CH_2$
g	Bn	Ac
h	Bn	$COCH_2CH_3$

11 (13β)	R^1	R^2
a	*p*-(MeO)$C_6H_4CH_2$	Me
b	*p*-(MeO)$C_6H_4CH_2$	Pr
c	*p*-(MeO)$C_6H_4CH_2$	Bn
d	*p*-(MeO)$C_6H_4CH_2$	*p*-(CN)$C_6H_4CH_2$
e	*p*-(MeO)$C_6H_4CH_2$	*p*-(NO_2)$C_6H_4CH_2$

12 (13α)	R^1	R^2
a	*p*-(MeO)$C_6H_4CH_2$	Bn
b	*p*-(MeO)$C_6H_4CH_2$	*p*-(CN)$C_6H_4CH_2$
c	*p*-(MeO)$C_6H_4CH_2$	*p*-(NO_2)$C_6H_4CH_2$

Figure 1. Chemical structures of the synthetized and investigated estrone-16-oxime ethers.

The tested dihomoestradiol analogs (**Fig. 2**) were designed any synthetized in the Laboratory of Organic Chemistry, Abo Akademi University, Finland in cooperation with the Department of Organic Chemistry, University of Szeged [26].

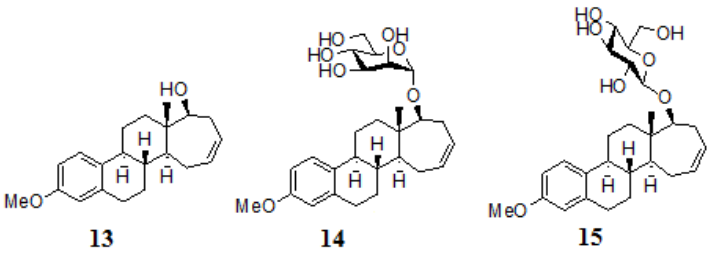
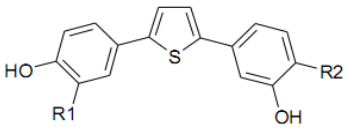
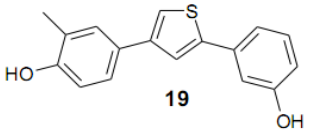


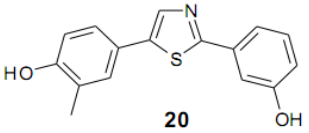
Figure 2. Chemical structures of the estradiol derived steroid aglycone and glycosteroids

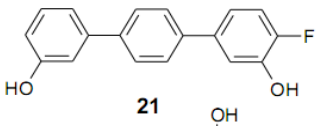
The design and synthesis of the investigated nonsteroidal 17β-HSD inhibitors was performed in the Pharmaceutical and Medicinal Chemistry, Saarland University, Germany [37][39]. The common properties of these agents were the aromatic core with two or more phenolic substituents. Five of the molecules contained thiophene (**16**–**19**) or thiazol (**20**) as central part. Compound **21** was a substituted *para*-terphenyl while agents **22**–**25** were diphenylnaphthols (**Fig. 3**).

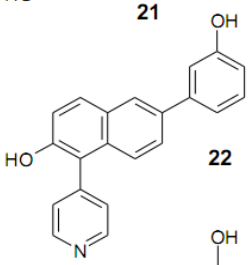


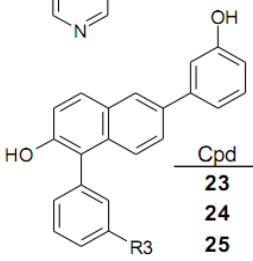
Cpd	R1	R2
16	CF_3	H
17	F	F
18	F	H











Cpd	R3
23	$NH_2SO_2CH_3$
24	H
25	OH

Figure 3. Chemical structures of the 17β-hydroxysteroid dehydrogenase type 1 inhibitors

10 mM stock solutions of the tested compounds were prepared with dimethyl sulfoxide (DMSO). The highest DMSO concentration of the medium (0.3%) did not have any substantial effect on the determined cellular functions. All the chemicals, if otherwise not specified, were purchased from Sigma-Aldrich Ltd. (Budapest, Hungary).

3.2 Tumor cell lines and cell culture

Human cancer cell lines HeLa (cervix adenocarcinoma; ECACC; Cat. No 93021013), MCF7 (breast adenocarcinoma; ECACC; Cat. No 86012803) and A431 (skin epidermoid carcinoma; ECACC; Cat. No 85090402) as well as noncancerous MRC-5 human lung fibroblasts (ECACC; Cat. No 05011802) were maintained in minimal essential medium supplemented with 10% foetal bovine serum (FBS) and 1% non-essential amino acids (NEAA) and an antibiotic-antimycotic mixture (AAM). A2780 cells (isolated from ovarian cancer; ECACC; Cat. No 93112519) were maintained in RPMI medium supplemented with 10% FBS, 1% AAM and 1% L-glutamine. All cell lines were purchased from the European Collection of Cell Cultures (Salisbury, UK).

3.3 MTT assay

The effects on the viability of cells were determined *in vitro* by using MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay. The cells were grown in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were seeded onto 96-well plates at a density of 5000 cells/well and allowed to stand overnight, after which the medium containing the tested compound was added. After a 72-hours incubation period, viability was determined by the addition of 20 µL MTT solution (5 mg/mL). The precipitated formazan crystals were solubilized in DMSO and the absorbance was read at 545 nm by an ELISA reader. Two independent experiments were performed with five parallel wells and cisplatin, an agent clinically used in some gynecological malignancies, was used as positive controls. Sigmoidal concentration-response curves were fitted to the measured points, and the IC_{50} values were calculated by means of GraphPad Prism 4.0 (GraphPad Software; San Diego, CA, USA) [40].

3.4 Analysis of cell cycle by flow cytometry

Flow cytometric analysis was performed in order to characterize the cellular DNA content of treated HeLa cells. After treatment for 24 and 48 hours cells (200,000/condition)

were trypsinized by Tryple express (Gibco BRL, Paisley, U.K.), washed with phosphate-buffered saline (PBS) and fixed in 1.0 mL ice-cold 70% ethanol for 30 min. After two washing steps in cold PBS, DNA was stained with propidium iodide (PI) (10 μg/mL) in the presence of RNA-ase (50 μg/mL). The samples were then analyzed by FACStar (Becton-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 calculated by using winMDI2.9. The sub G1 fractions were regarded as the apoptotic cell population [41].

3.5 Hoechst 33258 - propidium iodide double staining

Near confluent HeLa cells were seeded into a 96-well plate (5000 cells/well). After incubation for 24 hours with the test compound, Hoechst 33258 and PI were added to the culture medium to give final concentrations of 5 μg/mL and 2 μg/mL, respectively. The cells were incubated for 1 hour at 37 °C with the staining mixture and were then photographed by means of a Nikon Eclipse microscope equipped with an epifluorescence attachment containing the appropriate optical blocks and a QCapture CCD camera. The staining allowed the identification of live, early-apoptotic, late-apoptotic and necrotic cells. Hoechst 33258 permeates all the cells and makes the nuclei appear blue. Apoptosis was revealed by nuclear changes such as chromatin condensation and nuclear fragmentation. The necrotic and the late-apoptotic cells were identified as cell with PI uptake, which indicates loss of membrane integrity, the cell nuclei being stained red [9].

3.6 BrdU incorporation

5-Bromo-2’-deoxyuridine (BrdU) incorporation into the cellular DNA was determined by BrdU Labeling and Detection Kit I and III (Roche Diagnostic, Mannheim, Germany). The incorporation of BrdU in place of thymidine was monitored as a parameter for DNA synthesis. In accordance with the manufacturer's instructions, HeLa cells were labeled with BrdU for 2 hours and 1 hour, followed by fixation. The cellular DNA was partially digested by nuclease treatment and peroxidase labeled antibody was added for colorimetric measurement (Kit I). The absorbance was measured with a microplate reader at 405 nm with a reference wavelength at 492 nm. Two independent experiments were performed with 4 parallel wells to test the action of 17β-HSD 1 inhibitors. Alternatively, mouse monoclonal

antibody and fluorescein-conjugated anti-mouse antibody was added before fluorescent microscopy (Kit III, ex: 465-495 nm, em: 515-555 nm, dichromatic mirror: 505 nm). At least 400 cells were counted from four parallel wells for the expression of the BrdU-positive cells treated with estrone-based compounds.

3.7 Caspase-3 activity

The activity of caspase-3 from treated cells was determined in triplicate by means of a commercially available colorimetric kit in accordance with the instructions of the provider (Sigma-Aldrich, Budapest, Hungary). Briefly, HeLa cells (16 million per condition) were exposed to the test item for 48 hours and than scraped, counted and resuspended in lysis buffer (10 μL for 1 million of cells). The caspase-3 activity was measured by the addition of substrate (Ac-DEVD-*p*NA) and the amount of product (*p*NA) was measured at 405 nm after incubation for 17 hours. Results on treated cells are given as fold-increase by direct comparison with the untreated control results.

3.8 Reverse transcriptase PCR studies

The effects of the tested compounds on the mRNA expression pattern of retinoblastoma protein (Rb), cyclin-dependent kinase 2,4 and 6 (CDK2, CDK4 and CDK6), p16, p21, p27 and p53 regulator factors, which play a crucial role in the transition from the G1 to S phase, were determined by RT-PCR in HeLa cells. After a 24 hours incubation period, the total RNA was isolated from the cells ($5x10^5$) using TRIzol Reagent in accordance with the instructions of the manufacturer (Molecular Research Center, Cincinnati, OH, USA) [42]. The pellet was resuspended in 100 μL DNase- and RNase-free distilled water. The RNA concentrations of the samples were determined from their absorbances at 260 nm. The RNA (0.5 μg) was mixed with DNase- and RNase-free distilled water and 20 μM oligo(dT) (Invitrogen, Carlsbad, CA, USA), in a final reaction volume of 10 μL, which was incubated at 70 ºC for 5 min. After the mixture had been cooled to 4 ºC, 20 U RNase inhibitor (Promega, Madison, USA), 20 U MMLV reverse transcriptase (Promega, Madison, USA), 200 μM dNTP (Sigma-Aldrich; Budapest, Hungary) in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 5 mM $MgCl_2$ in a final reaction volume of 10 μL were added. The mixture was incubated at 37°C for 60 min. The PCR was carried out with 5 μL cDNA, 25 μL ReadyMix Taq PCR

reaction mix, 2 μL 20 pM sense and antisense primer of Rb, CDK2, CDK4, CDK6, p16, p21, p27 or p53 and 16 μL DNase- and RNase-free distilled water [43][45]. Human glyceraldehydes-3-phosphate dehydrogenase (hGAPDH) primers were used as internal control in all samples. The PCR was performed with an ESCO SWIFT MAXI thermal cycler (Esco Technologies, Inc. Philadelphia, USA) and the products were separated on 2% agarose gels, stained with ethidium bromide and photographed under a UV transilluminator. Semiquantitative analysis was performed by densitometric scanning of the gel with Kodak IMAGE STATION 2000R (Csertex Ltd; Budapest, Hungary).

3.9 Western blotting studies

HeLa cells were treated with investigated compounds. Whole-cell extracts were prepared from the cells with PBS and suspending them in lysis buffer [46]. 50 μg of protein per well was subjected to electrophoresis on NuPAGE Bis-Tris Gel in XCell SureLock Mini-Cell Units (Invitrogen, Carlsbad, CA, USA). Proteins were transferred from gels to nitrocellulose membranes, using the iBlot Gel Transfer System (Invitrogen, Carlsbad, CA, USA). Antibody binding was detected with the WesternBreeze Chromogenic Western blot immunodetection kit (Invitrogen, Carlsbad, CA, USA). The blots were incubated on a shaker with Rb, phosphorylated Rb (pRb) and β-actin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:200 in the blocking buffer.

3.10 Statistical analysis

Statistical analysis was carried out by analysis of variance (ANOVA), followed by the Dunnet post-tests. RT-PCR data were analyzed by ANOVA, followed by the Neuman-Keuls post-tests. All statistical analysis of the data performed with GraphPad Prism 4.0 (GraphPad Software; San Diego, CA, USA).

4 RESULTS

4.1 Anticancer properties of estrone analogs

4.1.1 Antiproliferative effects of estrone analogs (MTT assay)

Estrone (**1a**) exhibited pronounced action on HeLa cells, limited action on MCF7 cells and no relevant action on the A431 cell line (Supplementary table 2 of [36]). None of the estrone ethers (**1b**–**d**) proved more potent and the 13α epimers (**2a**–**d**) were also less effective. Introduction of an oxime function at position 16 of the estrane skeleton resulted in a general increase in cytostatic action. **3a** and **3e** were highly effective against HeLa cells. Estrone analogs with a substituted oxime function (**5a**–**d**) were completely ineffective and their 3-sulfamoyloxy relatives (**6a**–**c**) likewise exhibited only limited actions. Estrone methyl ethers with a substituted oxime group at position 16 (**7a**–**i** and **8a**–**e**) were similarly minimally active, without any marked effect depending on the configuration of the methyl function at position 13. Substantially more marked effects were detected when the aromatic hydroxy group was etherified with benzyl or *p*-methoxybenzyl alcohol (**9a**–**i**, **10a**–**j**, **11a**–**e**, **12a**–**c**). Among these more effective compounds, 13β-methyl generally seemed to be preferred, though with some exceptions (**9i**–**10h**, **9h**–**10g**). In the light of these results, four oximes were selected for further *in vitro* investigations (**3a**, **3e**, **10h** and **11a**). All of these estrone oximes affected the proliferation of HeLa cells comparably to the reference agent cisplatin, while MCF7, A413 and A2780 cells were less sensitive. The viability of noncancerous fibroblast cell line MRC-5 was affected only by **11a**, with a higher calculated IC_{50} value than that of cisplatin. Agents with an unsubstituted oxime function (**3a** and **3e**) could be regarded as selective for HeLa cells, with some modest action against ovarian cancer (A2780) cell line, while **10h** and **11a** displayed a broader spectrum of activities. The antiproliferative effects of the estrone-16-oxime ethers are summarized in **Table 1** [36].

Table 1. Calculated IC_{50} values (μM) of the tested estrone analogs

Compounds	IC_{50} values (μM)[a]				
	HeLa cells	**MCF7 cells**	**A431 cells**	**A2780 cells**	**MRC-5 cells**
3a	4.41	–[b]	–	18.28	–
3e	4.04	–	–	11.96	–
10h	3.52	4.13	–	4.61	–
11a	5.63	–	13.25	12.62	6.94
13	15.68	19.91	15.20	10.45	n.d[c]
14	10.82	12.40	10.39	–	n.d
15	–	27.45	–	19.88	n.d
Cisplatin	5.66	7.99	8.81	0.86	4.13

[a] Mean value from two independent determinations with five parallel wells, standard deviation less than 15%.
[b] Mean value above 30 μM. [c] n.d: not dermined

The tested dihomoestradiol analogs (**13**, **14** and **15**) exerted moderate antiproliferative effect against the utilized cancer cell lines. Compounds **13** and **14** exhibited reasonable growth inhibitory activity against most cancerous cell lines, while compound **15** provided substantially weaker effect (**Table 1)**.

4.1.2 Morphological studies and cell cycle distribution

Staining with Hoechst 33258 and PI allowed to visualize apoptosis or necrosis according to cell morphology and membrane integrity. HeLa cells were incubated with compounds **3a** and **3e** at 3 μM, 10 μM and 30 μM and with **10h** and **11a** at 10 μM and 30 μM for 24 hours. Separate photos were recorded, in which Hoechst 33258 and PI fluorescence served as morphological markers (**Fig. 4**). Concentration-dependent increases in nuclear condensation and fragmentation and increase in membrane permeability were generally observed. The most markedly perturbed membrane integrity was seen in the case of **3e**, while treatment with **10h** resulted in pronounced nuclear condensation with poor staining, even at 30 μM, indicating apoptotic cell death.

Treatments with these selected agents resulted in significant changes in the cell cycle distribution of HeLa cells (**Fig. 5**). A 24 hours incubation with unsubstituted oximes (**3a** and **3e**) caused a pronounced decrease in the synthetic (S) phase and an increase in the G1 phase. At the highest concentration applied (30 μM), an increase in the subdiploid (subG1) population was detected. Although **11a** caused a decrease in the G1 phase, the actions of

agents with substituted oximes were less obvious. After a longer incubation (48 hours), **3a** and **3e** resulted in a concentration-dependent increase in the subG1 cells, but the S population was reduced. This induction in subdiploid ratio was characteristic for **10h** too, whereas the action of **11a** was limited to a slight S phase decrease at 10 μM.

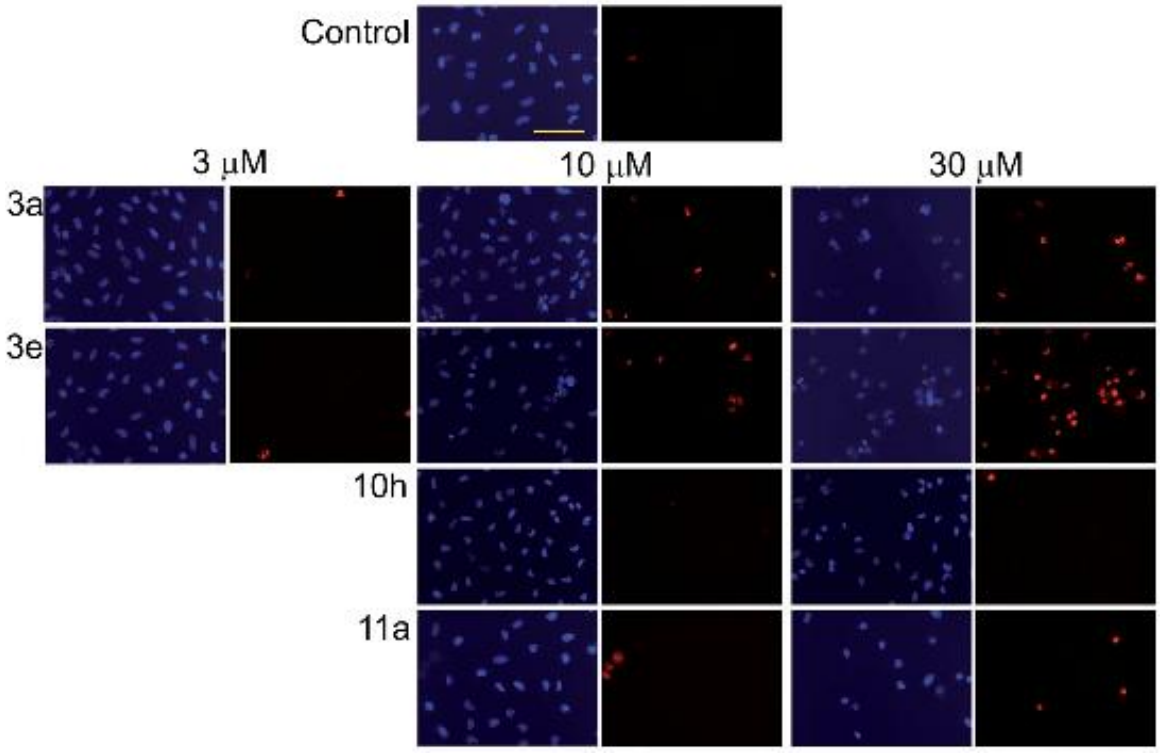


Figure 4. Fluorescence microscopy images of Hoechst 33258 - PI double staining. HeLa cells were treated with the vehicle (control), or with **3a**, **3e**, **10h** or **11a** at the given concentrations. The blue fluorescence indicates Hoechst 33258, and the red coloration is a result of cellular PI accumulation. The bar in the Hoechst 33258 control picture denotes 100 μm.

4.1.3 Brdu incorporation

BrdU is a synthetic thymidine analog which is useful for determination of the intensity of DNA synthesis. HeLa cells were treated with two or three concentrations of the investigated compounds (3 μM, 10 μM and 30 μM) for 24 hours. The incorporation of BrdU into the DNA was substantially and statistically significantly inhibited by all of the selected agents (**Fig. 6**). Compound **3e** was the most potent in inhibiting BrdU incorporation and, similarly to **3a**, exhibited a clear concentration dependence; their actions were comparable to that of cisplatin.

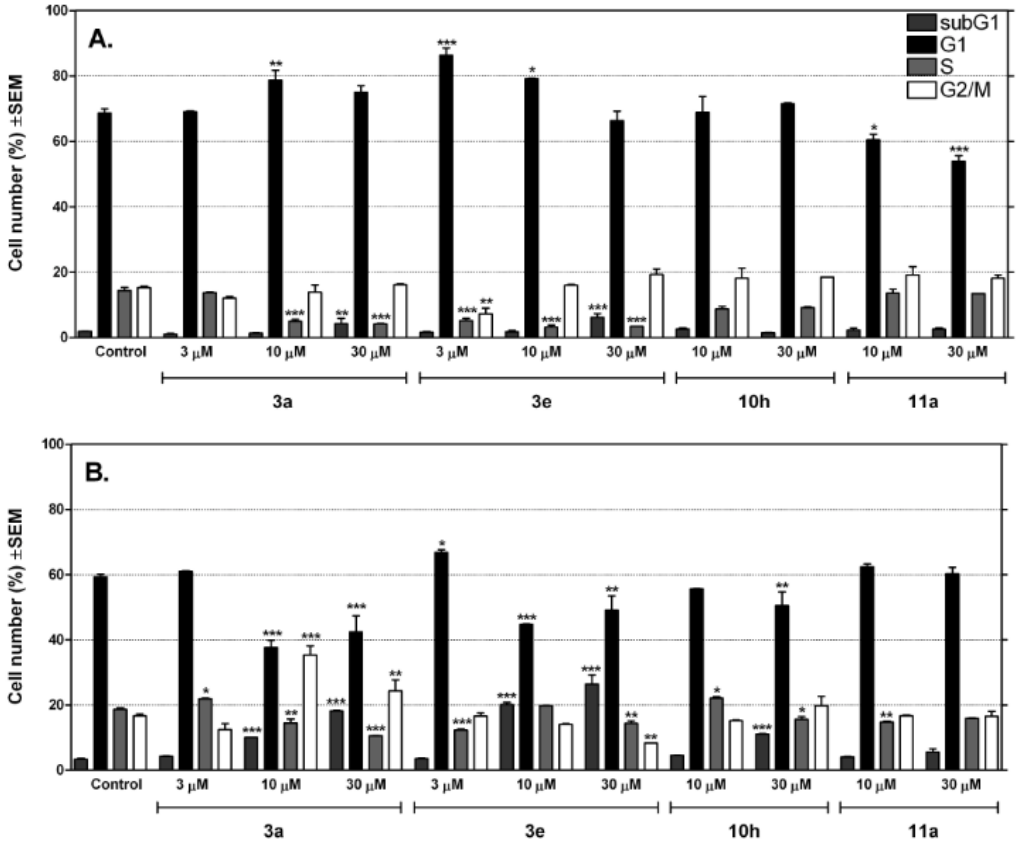


Figure 5. Effects of compounds **3a**, **3e**, **10h** and **11a** on the HeLa cell cycle distribution after incubation for 24 (panel A) or 48 hours (panel B). *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$, respectively, as compared with the control cells.

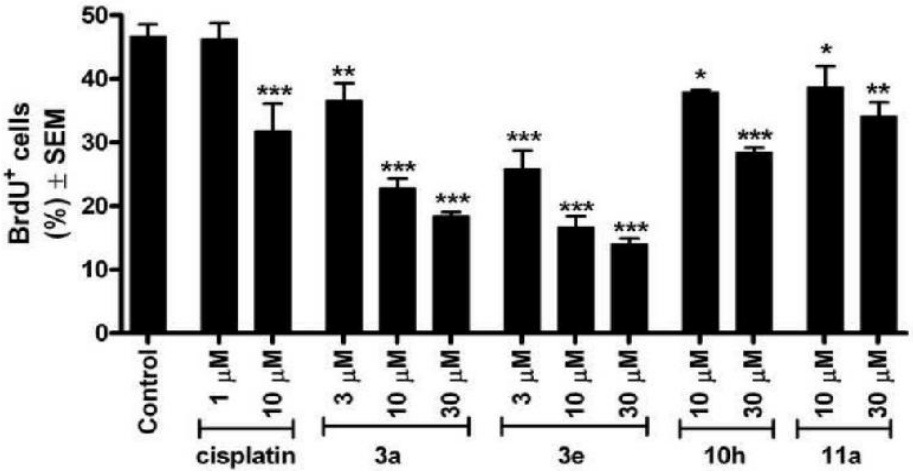


Figure 6. Incorporation of 5-bromo-2'-deoxyuridine into HeLa cells after incubation for 24 hours. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$, respectively, as compared with the control cells.

4.1.4 Caspase-3 activity

The results detailed above led to two of the investigated compounds (**3a** and **3e**) being selected for additional experiments, including determinations of the activity of caspase-3. The activity of this apoptosis-executing key enzyme was increased substantially and statistically significantly by **3a** and **3e** (**Fig. 7**). While **3e** resulted in a clear concentration-effect relationship, **3a** caused slightly lower activity at 30 µM than at 10 µM.

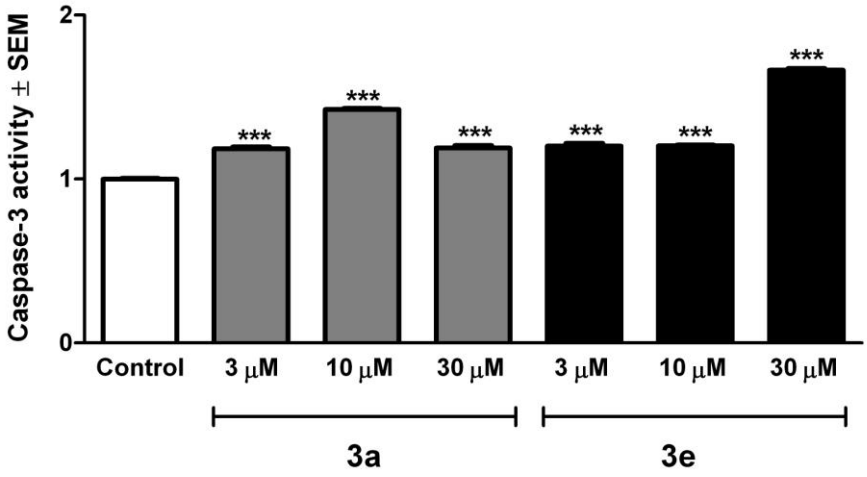


Figure 7. Induction of caspase-3 activity after incubation with compounds **3a** and **3e** for 48 hours. The activity of untreated cells was taken as one unit. *** indicates $p<0.001$ as compared with the control cells.

4.1.5 RT-PCR studies

The expressions of four cell cycle-regulating factors (CDK4, CDK6, p16 and Rb) that play key roles in the early G1-S transition were additionally determined by means of a semiquantitative RT-PCR technique (**Fig. 8**). On the basis of the results of the cell cycle analyses and the BrdU incorporation assays, two compounds (**3a** and **3e**) were included at two concentrations (3 µM and 10 µM), with exposure for 24 hours. The expression of tumor suppressor gene p16 was substantially and statistically significantly increased at the mRNA level under all tested conditions. Treatment with these selected agents resulted in a concentration-dependent repression of CDK4, but not of CDK6. Retinoblastoma protein was significantly repressed by **3a** and **3e** at the above concentrations. **3e** seemed more potent than **3a** in this respect. Further assayed factors involved in the regulation of the cell cycle (CDK2,

p21, p53 and p27) did not exhibit statistically significant differences as compared with the control values (**Fig. 8**).

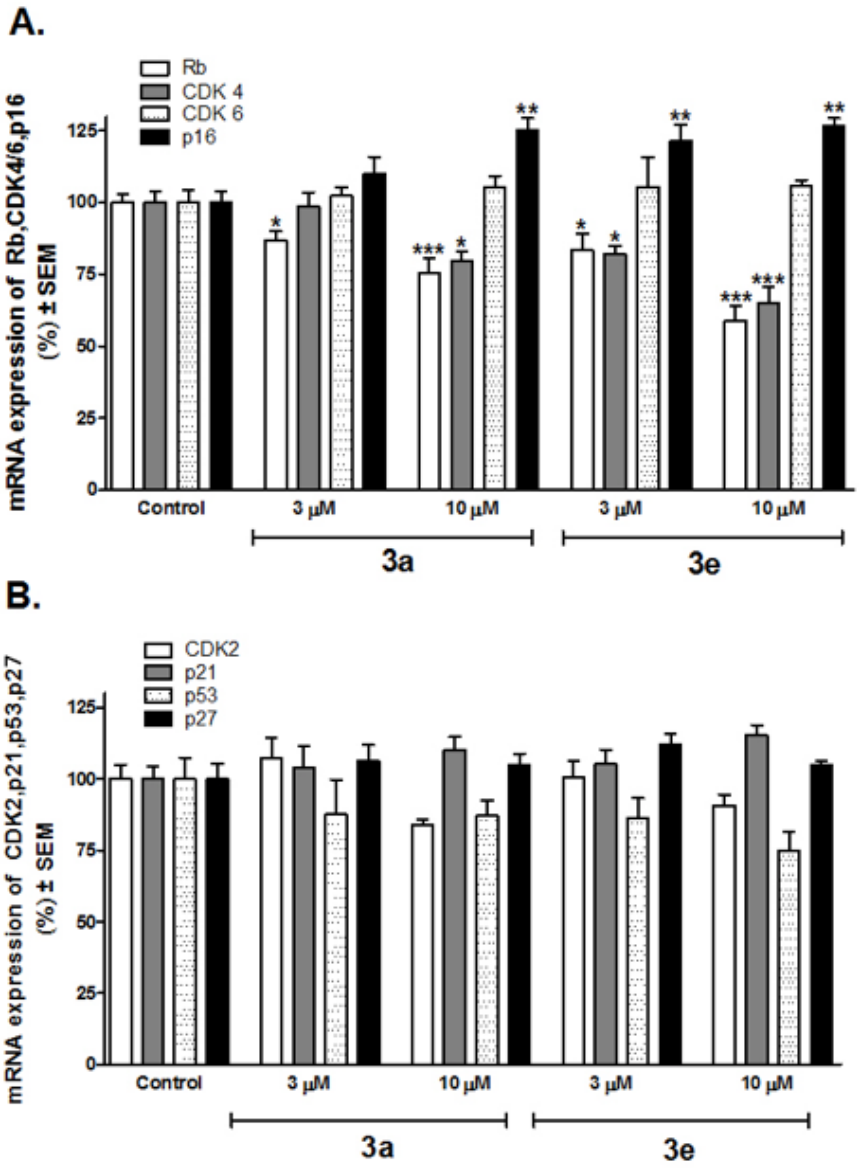


Figure 8. Expression of Rb, CDK4, CDK6 and p16 at the mRNA level after incubation with compounds **3a**, and **3e** for 24 hours. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$, respectively, as compared with the control condition (panel A). Expression of CDK2, p21, p53 and p27 at the mRNA level after incubation with compounds **3a** and **3e** for 24 hours (panel B).

4.1.6 Western blotting

Western blot analysis was performed to determine the expression of Rb and postsynthetically phosphorylated Rb at a protein level. Treatment with **3a** and **3e** at 3 μM and

10 µM reduced the expression of both forms of Rb relative to the untreated cells (**Fig. 9**). The relative expression of pRb, expressed as the density ratio Rb/pRb, was concentration-dependently decreased by the two tested steroids. This ratio in the control cells was 1.53, which was decreased to 0.59 and 0.46 by **3a**, and to 1.06 and 0.80 by **3e**, in concentrations of 3 µM and 10 µM, respectively. The expressions of this regulating factor at mRNA and protein levels were in good agreement.

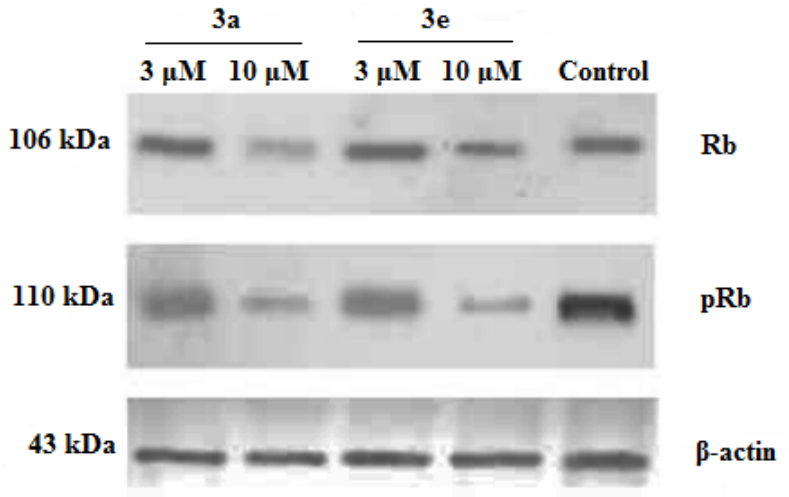


Figure 9. Expression of Rb and phosphorylated Rb at the protein level after incubation with compounds **3a**, and **3e** for 24 hours.

4.2 Anticancer properties of 17β-HSD 1 inhibitors

4.2.1 Antiproliferative effects of 17β-HSD 1 inhibitors (MTT assay)

The antiproliferative effects of the investigated 17β-HSD 1 inhibitors are presented in **Table 2**. Compounds **16**–**22** exhibited antiproliferative activities comparable to that of the reference agent cisplatin on the HeLa cells (IC_{50} for cisplatin: 5.66 µM), while the MCF7 and A2780 cells were generally less sensitive. When the assays of these agents were repeated in steroid-free milieu, the calculated IC_{50} values were not substantially different from those observed in the standard cell culture medium. Human fetal fibroblast MRC-5 cells were utilized for an additional set of experiment in order to characterize the selectivity of compounds **16**–**22**. None of these molecules exerted appreciable antiproliferative action against the noncancerous MRC-5 cells up to 30 µM. Compound **21** proved to be the most potent antiproliferative agent, exhibiting a lower IC_{50} than that of the reference compound

cisplatin. Test substances with a hydroxyphenylnaphthalene structure (compounds **23**–**25**) exhibited substantially weaker action against the cell lines used.

Table 2. Calculated IC_{50} values (μM) of the tested 17β-HSD1 inhibitors in standard and steroid–free cell culture medium.

Compounds	IC_{50} values (μM)[a]						17β-HSD1 activity (nM)[b]
	HeLa cells		MCF7 cells		A2780 cells	MRC-5 cells	
	Standard medium	Steroid-free medium	Standard medium	Steroid-free medium	Standard medium	Standard medium	
16	5.29	13.74	25.23	–[c]	–	–	38
17	6.84	23.76	13.88	–	–	–	17
18	3.81	11.13	16.11	29.37	–	–	8
19	14.53	–	–	–	–	–	64
20	5.66	6.05	–	–	–	–	143
21	1.41	3.21	–	–	–	–	51
22	5.30	12.19	25.51	–	9.71	–	64
23	–	n.d[d]	–	n.d	25.87	n.d	15
24	–	n.d	–	n.d	–	n.d	20
25	–	n.d	–	n.d	–	n.d	36
Cisplatin	5.66	n.d	7.99	n.d	0.86	4.13	n.d

[a] Mean value from two independent determinations with five parallel wells, standard deviation less than 15%.
[b] From references: [37][39].
[c] Mean value above 30 μM.
[d] n.d: not determined

4.2.2 Morphological studies and cell cycle distribution

HeLa cells were incubated with 3 μM and 10 μM of compounds **16**–**18** and **20**–**22** for 24 hours (**Fig. 10**). Concentration-dependent increases in nuclear condensation and in cell membrane permeability were generally detected. As nuclear condensation did not coincide with increased membrane permeability in the corresponding picture, an early apoptotic event is indicated. The morphological evidence demonstrated that compound **18** was the most potent apoptosis inducer: It elicited a pronounced condensation in the nuclei of the treated

HeLa cells even at 3 μM without affecting the membrane function to a substantial extent at 10 μM. Treatment with compound **20** resulted in the most pronounced deterioration in membrane integrity, as evidenced by intensive staining with PI.

Treatment with the tested compounds resulted in the distribution of the cells in the cell cycle phases (subG1, G1, S, and G2/M phases) as illustrated in **Fig. 11**. HeLa cells were treated with the investigated compounds at 3 μM and 10 μM concentrations for 24 and 48 hours. After 24 hours, there was no essential change in the number of apoptotic cells. All the tested compounds decreased the population of the synthetic phase, resulting in an increase either of the G1 phase (compounds **16**–**18** and **20**) or the G2/M population (compounds **18**, **20** and **22**). After treatment for 48 hours, an unequivocal cumulation of hypodiploid (subG1) populations was detected for all items, accompanied by a decrease of the G1 population. The actions on later cell cycle phases were less conclusive, except for clear increases in the S and G2/M populations on the action of compounds **17** and **20**–**21**, respectively.

4.2.3 BrdU incorporation

The amount of thymidine analog BrdU incorporated was determined as a marker of DNA synthesis. HeLa cells were treated with two concentrations of investigated compounds (3 μM or 10 μM) for 24 hours. All of the selected compounds substantially and statistically significantly inhibited the incorporation of BrdU into the DNA in a concentration-dependent manner, a property comparable to that of cisplatin. Compound **21** was the most potent in this regard, exerting more than 50% inhibition even at 3 μM (**Fig. 12**). Theoretically, the marked inhibition of BrdU incorporation might be attributed to a substantial decrease of the viability of the treated cells independently of the mechanism of the intervention. In order to exclude this possibility, the antiproliferative assay was repeated under identical conditions. Although most of the selected compounds resulted in a statistically significant inhibition of cell proliferation, apart from agents **17** and **20**, the highest inhibition was ~30% exerted by 10 μM of compound **21**. A modest (0–20%) decrease in cell viability was generally detected after incubation for 24 hours at 10 μM.

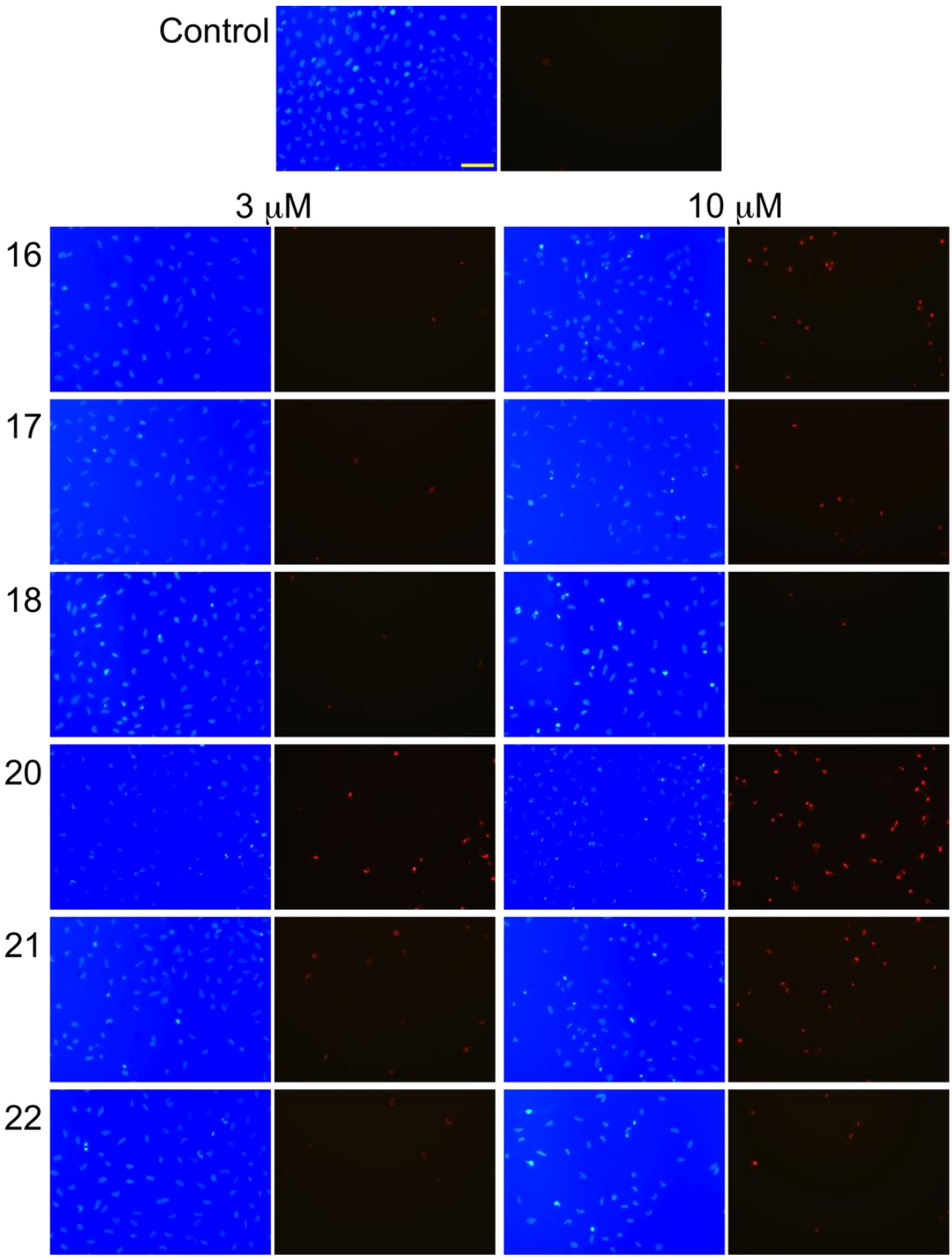


Figure 10. Fluorescence microscopy images of HOPI double staining. Two separate pictures from the same field have been taken for the two markers. HeLa cells were treated with vehicle (Control), **16**–**18** and **20**–**22** at 3 and 10 μM. Blue fluorescence indicates Hoechst 33258 and red coloration is a result of cellular PI accumulation. Bar on Control Hoechst 33258 picture indicates 100 μm.

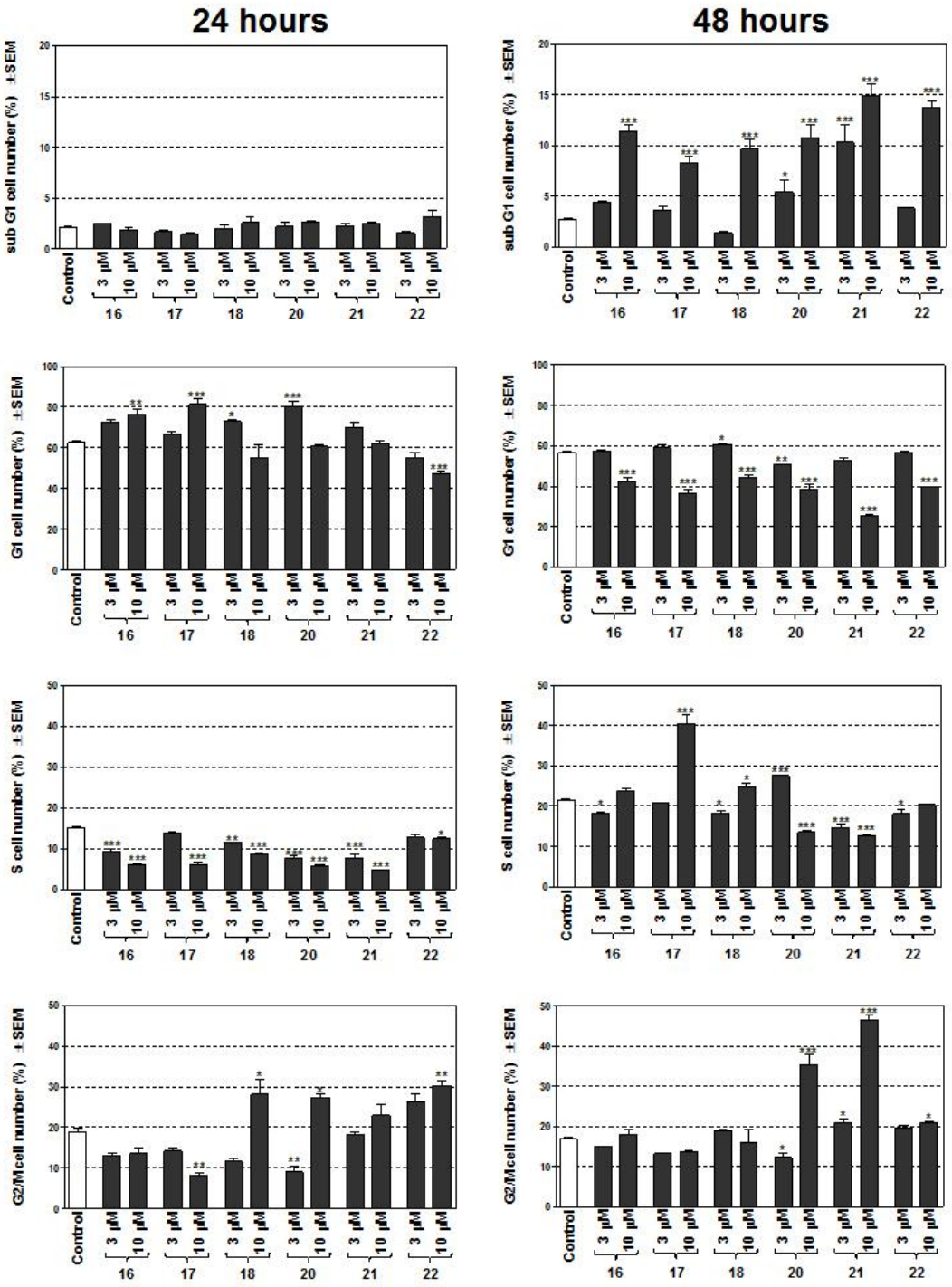


Figure 11. Effects of compounds **16**–**18** and **20**–**22** on HeLa cell cycle distribution after incubation for 24 (left panels) and 48 (right panels) hours. *****, ****** and ******* indicate $p<0.05$, $p<0.01$ and $p<0.001$, respectively, as compared with the control cells.

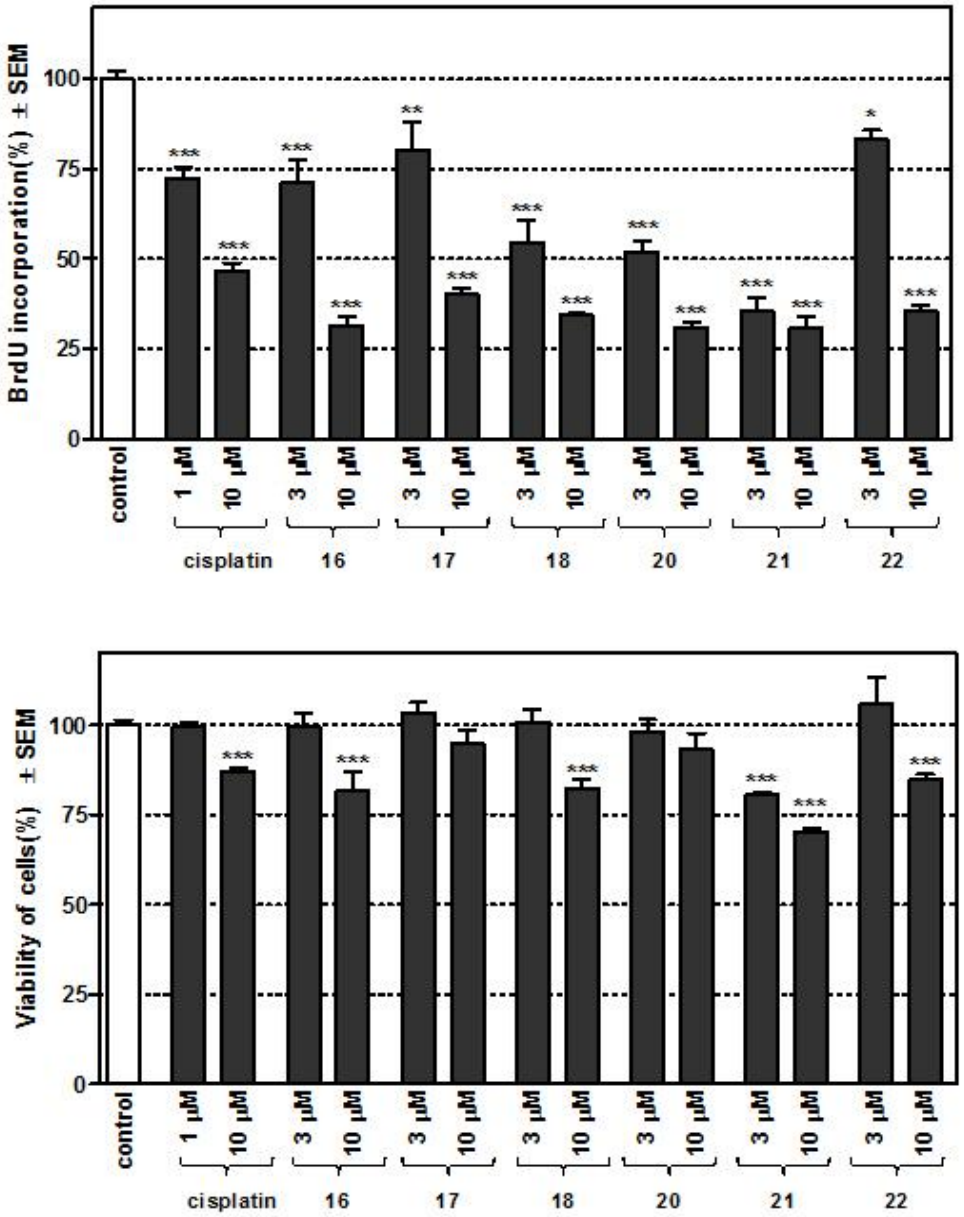


Figure 12. Incorporation of BrdU into HeLa cells (upper panel) and their viability determined by MTT assay (lower panel) after an incubation of 24 hours. *****, ****** and ******* indicate $p<0.05$, $p<0.01$ and $p<0.001$ as compared with the control cells, respectively.

4.2.4 Caspase-3 activity

On the basis of the cell cycle results, two compounds (**20** and **21**) were selected for additional experiments in order to determine their effects on the activity of caspase-3. Both of these agents induced a statistically significant and substantial increase in the activity of this

apoptosis-executing enzyme (**Fig. 13**). While **20** resulted in a clear concentration-effect relationship, **21** led to slightly lower activity at 10 μM than at 3 μM.

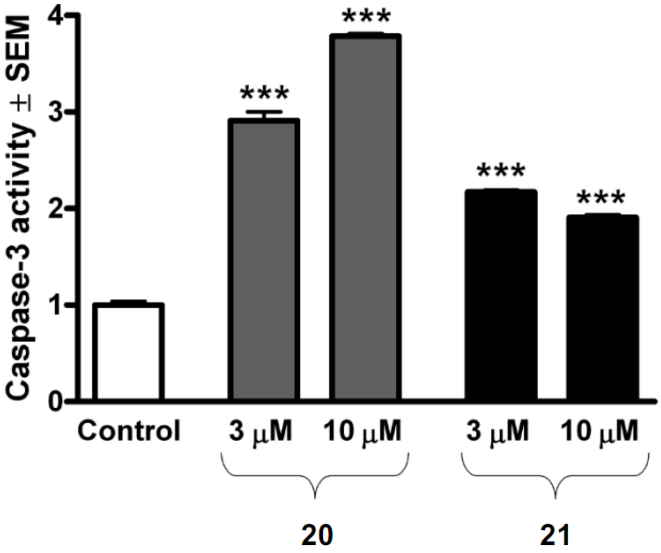


Figure 13. Induction of caspase-3 activity after 48 hours of incubation with compounds **20** and **21**. The activity of untreated cells was considered a unit. ******* indicates $p<0.001$ as compared with the control cells.

4.2.5 RT-PCR studies

The expressions of four cell cycle-regulating factors (CDK2, p21, p53 and Rb) that play key roles in the orchestration of the G1-S transition were additionally determined by means of a semiquantitative RT-PCR technique. In view of the results of the cell cycle analyses and the BrdU incorporation assays, three compounds (**16**, **17** and **21**) were included and two concentrations (3 μM and 10 μM) were used during exposure for 24 hours (**Fig. 14**). Tumor suppressor gene p53 was substantially and statistically significantly increased at an mRNA level under all the tested conditions. Although the concentration-response relationships were not always highly consequent, the ~2-fold induction by **16** and **21** proved to be the most pronounced effects. The other tumor suppressor p21 was also induced by **17** and **21** at a concentration of 3 μM, and by **16** at 10 μM. CDK2 is regarded as the main regulating factor promoting the transition from the G1 to the S phase. Treatment with the selected agents resulted in the concentration-dependent repression of CDK2 at the level of mRNA expression. Compound **16** seemed to be more potent than **17** and **21** in this respect. Rb was significantly repressed by **16** and **21**, but **17** did not exert any appreciable action at the concentrations used.

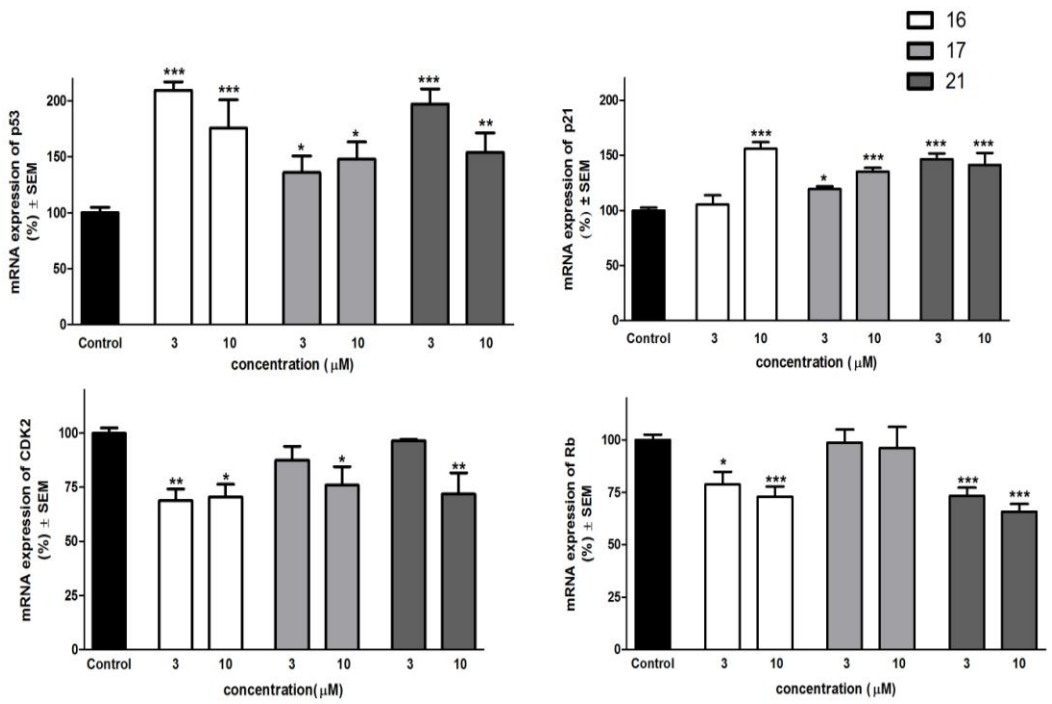


Figure 14. Expression of p53, p21, CDK2 and Rb mRNA after incubation for 24 hours with compounds **16**, **17** and **21**. *****, ****** and ******* indicate $p<0.05$, $p<0.01$ and $p<0.001$, respectively, as compared with the control conditions.

4.2.6 Western blot studies

According to the RT-PCR results the protein levels of phosphorylated Rb (p-Rb) have been determined by Western blot analysis (**Fig.15**). All of the tested compounds (**16**, **17** and **21**) reduced the level of p-Rb protein compared to the control value. Compound **21** exerted a pronounced decrease in protein level even at 3 μM. The action of **16** and **17** proved less marked.

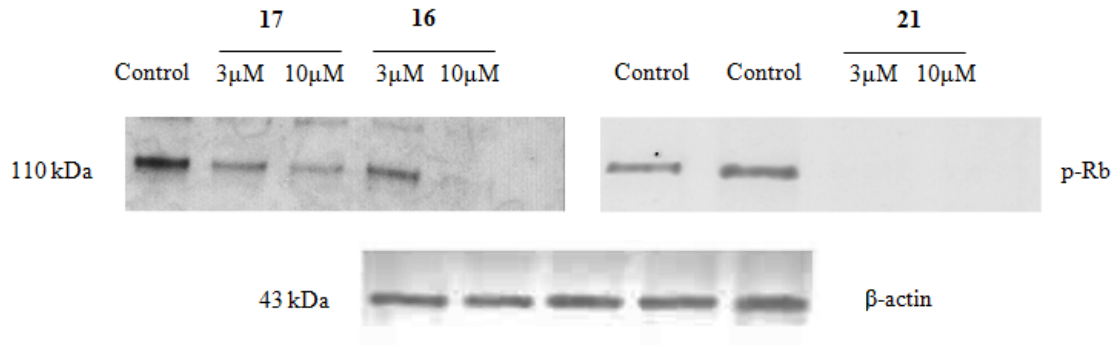


Figure 15. Upper panel in western blot studies can be seen the expression of phosphorylated Rb (p-Rb) protein level after 24 hours treatment with compounds **16**, **17** and **21**. Lower panel β-actin used as a loading control.

5 DISSCUSSION

Since cancer leads the mortality statistics worldwide research for novel reliable anticancer agent is a crucial task. A broad spectrum of natural products and their synthetic analogs are reported as potent experimental agent against some types of cancer but most of these molecules are aborted in a later phase of drug development.

Digitalis glycosides have long been used for the treatment of congestive heart failure, and convincing observational data bear witness to their anticancer action [47][48]. The early reports have been reinforced and the mechanism of this action has been postulated, leading to the generally accepted belief that the indications of cardiac glycosides may be extended to some cancers in the near future [49]. Diosgenin and many related steroidal alkaloids present in the Solanum and Dioscorea genera exhibit potent anticancer effects, and may be suggested as starting structures for novel synthetic antiproliferative drugs [18][50].

The oxime function has a widely accepted place in the design and synthesis of novel anticancer drugs, including agents with a steroidal skeleton. Isoflavones are a well-characterized group of natural compounds with a variety of biological activities, including anticancer effects. Introduction of an oxime function onto their natural skeleton may lead to an outstandingly effective derivative [23]. (*Z*)- and (*E*)-cholest-4-en-7-one oximes and their lactam derivatives were reported to exert antiproliferative action against HeLa and chronic myelogenous leukemia (K-562) cells, and the apoptosis-inducing capacity was additionally evidenced by means of morphological and biochemical approaches [51]. Huang *et al*. have reported the synthesis and screening of a set of ring A-modified cholestanes bearing an oxime function at position 6. Some of these compounds exhibited considerable activities against HeLa, human liver (SMMC 7404) and human gastric carcinoma (MGC 7901) cells [52]. These oximes are stated to exhibit anticancer properties similar to those of the currently investigated 16-oximes.

Estrogens may be responsible for carrying a pharmacophore moiety to estrogen receptor-expressing cells, and therefore determining the molecular targeting of the agent [53][54]. We earlier reported on some estrane-based antiproliferative compounds with presumably hormone-independent actions [55][56]. The most intensively investigated estrane is an endogenous estradiol metabolite, 2-methoxyestradiol, which does not exhibit hormonal

activity, but seems to be highly effective against a broad range of cancer cell lines *in vitro*, and some limited but promising *in vivo* data are also available [19].

The aim of the present study was the design, synthesis and pharmacological investigation of novel estrone-16-oximes. We have found no previous report describing anticancer effects of compounds from this class. The screening of the 63 newly synthetized compounds against three human cancer cell lines pointed to some structure-activity relationships. As a general rule, though with a few exceptions, the β orientation of the 13-methyl group is preferred. 3-Benzyl substitution typically favors the α position of the 13-methyl function. An unsubstituted oxime is generally preferred over an alkyl-substituted one, but an aromatic group (e.g. benzyl) may be considered. The hydroxy group on ring A may be unsubstituted, sulfamoyloxylated or substituted with an aromatic group (benzyl or *p*-methoxybenzyl). From the data relating to the anticancer efficacy, four compounds were selected for further investigations. Two of them (**3a** and **3e**) may be regarded as HeLa-selective, with limited action on A2780 cells; the molecules containing substituted oxime groups (**10h** and **11a**) exerted substantial action on MCF7 and A431 cells too. Tumor selectivity is one of the most critical challenges in the development of a novel anticancer agent. Although the MTT assay on intact human fibroblasts cannot be viewed as a thorough toxicological evaluation, it is undoubtedly advantageous that three of the four selected compounds did not exert substantial action on MRC-5 cells.

A set of additional *in vitro* experiments was devoted to an experimental approach to the possible mechanism of action of these agents. Most of the currently available drugs used in anticancer treatment have the capacity to induce programmed cell death either by fortifying apoptotic signaling or by inhibiting antiapoptotic signaling [57]. Treatment-dependent morphological changes were recorded and qualitatively evaluated by means of Hoechst 33258 - PI fluorescent staining after incubation for 24 hours. Typical apoptotic markers, such as cellular shrinkage, nuclear condensation and increased membrane permeability, were detected, especially at the highest concentrations of **3a** and **3e**. Correspondingly, flow cytometric cell cycle analysis was performed for a quantitative determination of the DNA content of HeLa cells as a function of the treatment. After a short exposure, **3a** and **3e** resulted in a marked suppression of the synthetic phase, and the subdiploid population increased only at high concentration (30 μM). The same compounds elicited more pronounced cell cycle

perturbation, including the accumulation of subG1 cells, after a longer incubation. It is therefore concluded that a period of 24 hours is sufficient for the development of the morphological hallmarks of apoptosis, but not for the complete activation of the self-decomposing enzymatic procedure, which may explain why the appearance of cells with subdiploid DNA requires 48 hours. Besides apoptosis induction, a substantial contribution of necrosis can not be excluded, especially at higher concentration.

Compounds **3a** and **3e** significantly increased the caspase-3 activity, confirming the induction of programmed cell death. Although caspase-independent cell death can manifest apoptotic morphology, and crucial caspases, including caspase-3, may be involved in non-lethal intracellular signaling, the assessment of executioner caspase activity remains an important part of apoptosis detection [58].

Since **3a** and **3e** exerted a substantial inhibition of DNA synthesis, comparable to that of the reference agent cisplatin, a further set of experiments was devoted to the determination of cycle-regulating factors at the mRNA level by means of a RT-PCR technique. The transition from the G1 to the S phase is tightly regulated by the expression of Rb, CDK4 and CDK6, and p16 factors, the latter regarded as crucially important [59]. The importance of this cyclin D – CDK4 – CDK6 – p16 – Rb – E2F pathway is indicated by the fact that it has been found to be altered in virtually all human tumors [60]. An innovative agent intervening in this pathway may therefore be considered especially advantageous. In view of our results, it seems conceivable that treatments with the two best compounds lead to the up-regulation of p16 (also referred to as CDK4 inhibitor). Since Rb is typically regulated postsynthetically by phosphorylation, its overall activity can not be fully characterized by determining its expression at the mRNA level [33]. Our RT-PCR data were therefore supplemented with Western blot analyses, which indicated that the two most effective compounds have the capacity to decrease the expressions of Rb and pRb at a protein levels, and also the proportion of pRb. Since the expression of significant members of a pathway parallel to that detailed above, including CDK2, p21, p27 and p53, did not exhibit any appreciable treatment-dependent differences, the signal mechanism via proteins p16 and CDK4 governing the phosphorylation of Rb may be suggested as a predominant mechanism of action of the tested estrone analogs.

In case of nonsteroidal 17β-HSD1 inhibitors confirmed the importance of inhibition of 17β-HSD1 thereby reduced the level of 17β-estradiol. Cancers of the female reproductive organs, including the breast, the cervix and the ovaries, are frequently hormone-dependent malignancies. Estrogens play a crucial role in the development and progression of these disorders. In postmenopausal women, adrenal steroids are converted to estrogens by a set of enzymes such as aromatase and the reductive isoforms of 17β-HSD [61]. 17β-HSD1 is the best-characterized isoform of reductive 17β-HSDs responsible for the local generation of the most potent natural estrogen 17β-estradiol, while the oxidative isoforms (17β-HSD2, 4 and 14) govern the opposite reaction, leading to the substantially less potent estrone. It is generally considered that the tissue-specific expressions of the reductive and oxidative isoforms play pivotal roles in the *in situ* estrogen exposure and consequently determine the progression of hormone-dependent disorders. It has been reported that tumor growth in a murine xenograft model was stimulated by estrone when 17β-HSD1-expressing MCF7 cells were used, whereas proliferation of parental MCF7 cells can be stimulated by estradiol only. Consequently, this growth stimulation could be prevented by a specific 17β-HSD1 inhibitor without exerting substantial effect on the uterine weight [62]. These data clearly demonstrate that 17β-HSD1 blockade is an attractive and selective point for intervention in estrogen-dependent cancers.

Since the targeted disorders are estrogen-dependent proliferative states (e.g. gynecological cancers and endometriosis), a direct antiproliferative property of these pharmacons can be considered advantageous. It has not yet been investigated whether the combination of 17β-HSD1 inhibition and antiproliferative property in one molecule could lead to superior effects compared to pure 17β-HSD1 inhibitors. It might have a positive impact delaying drug resistance as observed nowadays in case of SERMs or aromatase inhibitor treatment [63].

Since no direct antiproliferative effect was considered during the development of the tested 17β-HSD1 inhibitors, identification of compounds combining these two properties was the goal of this study.

The currently investigated ten compounds are highly active 17β-HSD1 inhibitors (IC_{50} 8–143 nM, **Table 3.**)[37][39]. Although representing different chemical classes, the chosen 17β-HSD1 inhibitors, designed as steroidomimetics, all share a hydrophobic central core,

mimicking the B and C steroidal ring, and two phenolic substituents, mimicking the two polar oxygens of estrone. In the optimization process to increase their potency, it appeared that these moieties are necessary for high 17β-HSD1 inhibition [64]-[66]. These 10 compounds differ in the nature of the hydrophobic central core, which influence not only the electronic density on the whole compound but also the overall geometry of the compounds: Thiophene **16**–**19**, thiazole **20** and phenyl rings **21** lead to a linear shape while the derivatives **22**–**25** with a naphthalene group are more globular.

Many of the chemically related natural product classes (e.g. flavonoids, lignans and chalcones) exhibit pronounced antiproliferative capacities which are reflected in diet-related morbidity of some cancers [67]. In addition, a reduced risk of breast, ovarian and colorectal cancer was found for high intake of flavonoids [68].

The antiproliferative effects of the 17β-HSD1 inhibitors (compounds **16**–**25**) were tested in 4 different cell lines: HeLa, A2780, MCF7 and MRC-5 under standard condition. Based upon their antiproliferative activity in HeLa cells where higher responses are observed, the compounds can be classified in three groups: highly active (**21** and **18**), middle active (**16**, **17**, **20**, **22**) and low or inactive (**19**, **23**, **24**, **25**). Interestingly, the structure activity relationship (SAR) study on the central core shows that both phenyl **21** and 2,5-substituted thiophene **18** as central core are good, while the naphthalene (**23**, **24**, **25**) is not. In addition, the 2,4-thiophene **19** is 3 times less active than the thiazole **20**. As the different central cores will induce different electronic repartitions in the molecule, these results highlight the influence of the electronic density on the activity. Comparison of **18** (highly active) and **17** (middle active) also indicates the importance of the substitution pattern on the hydroxyphenyl rings: Only a fluorine atom leads to good activity and only one, and this molecular feature seems to be valid for **21** as well.

In addition, it was determined on HeLa and MCF7 cells in steroid-free medium. Although the calculated IC_{50} tended to be higher in that latter case, the differences were not sufficiently large to indicate a pivotal role of the estrogen exposure of the utilized cells. The estrogen receptor expressions and steroid metabolic capacities of these cell lines are in good agreement with our findings. HeLa and A2780 cells express no estrogen receptors while MCF7 cell line is reported as estrogen receptor positive [69][70]. This crucial difference is not reflected in the IC_{50} values. Furthermore, most of our experiments were performed on HeLa

cells, because this cell line proved to be the most sensitive toward the tested agents. HeLa cells have recently been characterized with very low activities of aromatase and reductive 17β-HSD (types 1, 5, 7 and 12) and a moderate steroid sulfatase capacity [71].

In MCF7 breast cancer cells, reductive 17β-HSD12 and steroid sulfatase are highly expressed, but mRNA levels of 17β-HSD1 and 17β-HSD2 were low or not detected, respectively [72]. It was concluded that in these cells estradiol can be formed from estrone and estrone sulfate. Human ovarian cell line A2780 expresses functional reductive 17β-HSD, which can be substantially stimulated by interleukin-6 and basic fibroblastic growth factor, but the increased rate of the estrone-estradiol conversion has no impact on the viability of the treated cells [73]. All these published data indicated that no relationship can be revealed between 17β-HSD1 activity and the proliferative character of the cancer cell lines used. Moreover, all of these compounds inhibited 17β-HSD1 and 17β-HSD2 in the nM and nM–μM ranges, respectively, *in vitro* without exhibiting substantial binding affinity to the α and β types of human estrogen receptors [37][39]. As antiproliferative action of the compounds was found for cancer cell lines not or hardly expressing 17β-HSD1, this effect seems to be an additional, beneficial feature independent of the interference with the metabolism of endogenous estrogens.

The cancer selectivity of the tested agents deserves special consideration. Since none of the molecules inhibited substantially the growth of fetal fibroblast MRC-5 cells a relatively advantageous safety profile may be expected.

Since many of active agents inhibited cell growth in relevant concentration range (i.e. the μM range), the mechanism of their action was also approximated. The apoptosis-inducing property of the tested drugs was therefore regarded as a crucial point of our experiments. Fluorescent staining with Hoechst 33258 and with PI is suitable for the qualitative detection of nuclear condensation and perturbation of the membrane functions, respectively. An apparent contradiction between the results of these morphological studies and the flow cytometry analyses after the 24 hours treatment was revealed: in spite of the clear nuclear condensation and increase in membrane permeability, no substantial subG1 cell populations were evidenced. It is concluded that 24 hours is not enough for complete activation of the enzymatic machinery of the apoptotic self-decomposition. Instead, a significant depression of the synthetic phase of the cell cycle was displayed. After a longer incubation period (48

hours), a concentration-dependent increase in the hypodiploid fraction was detected, which is a generally accepted hallmark of apoptosis [41].

Induction of programmed cell death was confirmed by demonstration of significantly increased caspase-3 activities in the cases of **20** and **21**.

The inhibition of nucleic acid synthesis was confirmed by means of BrdU incorporation assay. Compounds **16**–**18** and **20**–**22** decreased the DNA turnover in a comparable manner to the reference agent cisplatin. This behavior indicates a point of intervention in the upstream events of the G1-S transition. In the next set of experiments, therefore, the expressions of four relevant cell cycle-regulating factors were followed through a RT-PCR technique with three selected compounds (**16**, **17** and **21**) at the mRNA level. Although it is proved that compounds **16**, **17** and **21** exert their action via p53 – CDK2 pathway, the exact site of action cannot be deduced from our results.

Genistein, one of the most extensively investigated isoflavones, has been reported to induce apoptosis in breast cancer cell lines with the up-regulation of p21 and p53 tumor suppressors. Inhibition of protooncogene HER-2 protein tyrosine phosphorylation has been reported recently and the tyrosine-kinase blockade has been suggested as the basis of the anticancer effect of genistein [74]. Many of the polyphenol-type phytochemicals isolated from green tea, including catechins and resveratrol, exert their cytostatic action by a direct inhibition of specific receptor tyrosine kinases [75]. On the basis of these data, kinase inhibition can be speculated as a basic antiproliferative mechanism of the currently investigated agents.

In addition, as the mode of action of the compounds could not be completely elucidated, it is not clear whether the antiproliferative effects observed are either off-target effects or specific effects. From the experiments described in this study, it is believed that the compounds studied can interfere with the cell cycle regulating machinery presumably at multiple levels in cancer cells without affecting the viability of intact cells.

17β-HSD1 inhibition is a very promising approach for the treatment of estrogen-dependent diseases as it will decrease the level of the active estrogen estradiol in the target cell. However, estradiol is so potent that only traces are able to activate cell proliferation. As it might difficult to abolish completely the estradiol presence by 17β-HSD1 inhibition, it will be beneficial to use an agent which shows also antiproliferative properties and suppresses the

cell growth via another mechanism. The antiproliferative potency of these compounds is limited but it might be sufficient to complete the 17β-HSD1 inhibitory effect. Moreover, moderately potent cytostatic 16,17-secoandrostane derivatives with remarkable aromatase inhibitory action have been suggested as anticancer drug candidates indicating the favorable combination of direct antiproliferative and endocrine disruptor properties [76].

6 SUMMARY

In conclusion, our current results provide the first evidence that substituted estrone oximes may selectively suppress cancer cell proliferation by promoting apoptotic cell death and modulate the cell cycle progression. Although relatively high concentrations are needed to exert substantial activity, their cancer selectivity seems to be more beneficial than that of the reference agent cisplatin. Accordingly, the estrone oxime skeleton is suggested as an appropriate scaffold for the design and development of novel antiproliferative agents.

In summary, 17β-HSD1 blockade is an attractive and selective point for intervention in estrogen-dependent cancers by inhibiting the excessive formation of potent 17β-estradiol. These investigated nonsteroidal 17β-HSD1 inhibitor compounds exhibited pronounced antiproliferative activity. The proapoptotic effects of the tested 17β-HSD1 inhibitors have been evidenced. These results indicate that it is possible to combine direct antiproliferative activity with 17β-HSD1 inhibition resulting in novel agents. Having in hand such compounds, it might be possible to further investigate whether these molecules with a dual mode of action are superior to pure 17β-HSD1 inhibitors and might lead to the discovery of superior drugs for the treatment of estrogen-dependent proliferative disorders.

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8 ACKNOWLEDGEMENTS

I would like to express my special thanks to Prof. Dr. George Falkay and Dr. István Zupkó, the heads of the Department of Pharmacodynamics and Biopharmacy, for providing me with the possibility to work in this department.

I am also very grateful to my supervisor István Zupkó Ph.D for his help, advice, support and encouragement.

As our work required close co-operation, I owe my thanks to our collaborating partners. I am greatly indebted to Prof. Dr. Rolf W. Hartmann and his coworkers Dr. Sandrine Oberwinkler-Marchais and Dr. Martin Frotscher, Pharmaceutical and Medicinal Chemistry, Saarland University, Germany for the 17β-HSD1 inhibitors and the fruitful discussions. I express my gratitude for Prof. Dr. János Wölfling, Prof. Dr. Gyula Schneider and their colleagues, Department of Organic Chemistry, University of Szeged for the design and synthesis of estrone-16-oxime ethers. I am grateful for Prof. Dr. Reko Leino and his coworkers, Abo Akademi University, Finland for the synthesis of dihomoestradiol analogs.

I express my gratitude for all my colleagues and co-authors in the Department of Pharmacodynamics and Biopharmacy for creating a supportive and pleasant athmosphere.

I would like to express my thanks to Prof. Dr. László Dux and Dr. Róbert Gáspár for their previous support and help.

I thank to Judit Czintkota for her excellent technical assistance in my experimental work.

I would like to thank for the suppport of the Centenarium Foundation of Gedeon Richter Ltd.

I am deeply grateful for my family, my love and my friends for their patience and support.