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Evaluation of the oncopharmacological potentials of the novel A-ring modified 13α-estrone derivatives

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PUBLISHED MANUSCRIPTS RELATED TO THE SUBJECT OF THE THESIS:

I. Jójárt R, **Ali H**, Horváth G, Kele Z, Zupkó I, Mernyák E. Pd-catalyzed Suzuki-Miyaura couplings and evaluation of 13α-estrone derivatives as potential anticancer agents. *Steroids*. 2020; 164: 108731. https://doi.org/10.1016/j.steroids.2020.108731

IF: 2.688 / **Q2**

II. Traj P, **Ali H**, Németh A, TD Sámuel, Tömösi F, Tea LR, Zupkó I, Mernyák E. Transition metal-catalysed A-ring C-H activations and C(sp2)-C(sp2) couplings in the 13α-oestrone series and in vitro evaluation of antiproliferative properties. *J Enzyme Inhib Med Chem*. 2021; 36(1): 895-902. https://doi.org/10.1080/14756366.2021.1900165

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III. Kovács É, **Ali H**, Minorics R, Traj P, Resch V, Paragi G, Bruszel B, Zupkó I, Mernyák E. Synthesis and antiproliferative activity of steroidal diaryl ethers. *Molecules*. 2023; 28(3):1196. https://doi.org/10.3390/molecules28031196

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IV. **Ali H**, Traj P, Szebeni GJ, Gémes N, Resch V, Paragi G, Mernyák E, Minorics R, Zupkó I. Investigation of the antineoplastic effects of 2-(4-Chlorophenyl)-13α-estrone sulfamate against the HPV16-positive human invasive cervical carcinoma cell line SiHa. *Int J Mol Sci*. 2023; 24(7):6625. https://doi.org/10.3390/ijms24076625

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II. Krstić G, Saidu Mb, Bombicz P, De S; **Ali H**, Zupkó I, Berkecz R, Rédei D, Hohmann J. Pauciflorins A–E, unexpected chromone-monoterpene-derived meroterpenoids from *Centrapalus pauciflorus*. *J Nat Prod*. Published online 2023. https://doi.org/10.1021/acs.jnatprod.2c01132

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1 INTRODUCTION

1.1. Cancer; epidemiology and therapeutic interventions

Cancer is a worldwide progressive health condition that occurs not only due to demographic changes, but also as a consequence of globalization, unhealthy life style and transition in risk factors.[1] Estimation of the global cancer burden in 204 countries from 2010 to 2019 indicated that, cancer was the second cause of death followed by cardiovascular disorders where the largest percentage in morbidity and mortality rates were documented specifically in low income countries.[2] Studies examined the link between human development and cancer incidence concluded that, future cancer burden is expected in less developed countries with a 100% increase until 2030.[3]

Association between socioeconomic status and cancer incidence and mortality rate of 27 cancer types in 175 countries revealed that, cancer incidence is positively associated with the socioeconomic levels of populations for all cancer types in both sexes.[4] Studies analyzed the major risk factors that contribute to cancer incidence worldwide indicated that, smoking, alcohol usage, low fruit-vegetables intake as well as obesity, are considered major risk factors in countries with low socioeconomic status. Among these, Europe and central Asia seem to have the highest proportion rate of cancer incidence due to risk factors exposure.[5]

Cancer researchers have been attempting for the last decades to thoroughly understand the biology of cancer including molecular mechanisms underlying its development, invasiveness and metastasis. This seems to facilitate developing effective approaches for designing and manufacturing effective therapeutics, however, drug resistance continues to be the main challenge. To overcome this obstacle, conventional chemotherapeutic agents with different mechanisms of action were combined. This approach worked remarkably for certain types of cancers mainly lymphoma, breast and testicular cancers.[6] However, almost 50 years after using the conventional chemotherapy, surgical recession of cancerous tissues and radiotherapy, it seems to be not sufficient to cure many types of cancer.[7] Therefore, different treatment strategies have been developed to manage this concern.

In January 2020, cancer researchers in the tenth edition of the annual congress on anticancer innovative therapy shared their latest knowledge concerning future challenges in the field of

cancer therapy. It was concluded that, in addition to the conventional chemotherapy, more attention should be paid to developing other novel therapeutic approaches for treating cancers and minimizing the possibility for development of multidrug resistance. Examples include epigenetic drugs, cell cycle inhibitors, CAR-T cells as well as miRNAs.[8]

Recent studies have demonstrated the validity of immunomodulatory agents to combat cancer in clinical trials. The initial success was represented by estrogen receptor (ER) antagonist as well as human epidermal growth factor receptor 2 (HER2) inhibitors.[9] The utilization of monoclonal antibodies that agonize or antagonize specific targets such as CD40 is considered another fruitful approach. Another kind of intervention belong to this group is represented by immune checkpoints such as cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) as well as programmed cell death-1 (PD-1) and its corresponding ligand (PD-L1).[10] The immunomodulating agents seem to improve the clinical outcomes in cancer patients particularly when administered with chemotherapeutic or antiangiogenic agents.[11]

Recently, other kinds of therapeutic interventions have come to attention including nanotechnology. It's based on utilizing nanoparticles that have the ability to carry biomolecules such as DNA, RNA, proteins and drugs, to the targeted sites and subsequently enhancing the efficacy of therapeutic agents.[12] In addition, photothermal therapy is also regarded as one of the promising interventions in cancer therapy. It's mechanism of action involves converting light to heat that is able to destroy various types of cancer growths. Although there are no clinical trials yet, promising in vitro and in vivo evidence suggest its efficiency.[13]

1.2. Uterine cervical carcinoma; an overview

Uterine cervical carcinoma (UCC) is the third most common cancer type among women globally, with a disproportionate high prevalence in less-developed countries.[14] The incidence rate in Europe is 10.6 per 100.000 where the western territories have lower incidence rates due to the implementation of well-developed screening and prevention programs.[15] A worldwide analysis used the global cancer observatory database indicated that, approximately 570.000 cases of cervical cancer and 311.000 deaths occurred in 2018 only. The global estimated age-standardized incidence is 13.1 per 100.000 women making it as one of the most abundant types after breast and colorectal cancers.[16] A cohort study estimating the invasive cervical carcinoma rate (ICC) in

European women taking antiretroviral therapy between 1996 and 2014 showed that, the incidence rate was 66 per 100.000.[17] In Hungary, the annual number of cervical cancer patients and prevalence of care utilization in 2018 was 26 per 100000 population hospitalized in acute inpatient care.[18]

In 1996, the World Health Organization along with the European Research Organization on Genital Neoplasia consensus conference on cervical cancer, identified human papillomavirus (HPV) as one of the primary causes of cervical cancer.[19] So far, scientists have identified 30 HPV types that may be transmitted through sexual intercourse. Of these, two are most commonly observed in the cervical malignant cells including HPV16 accounting for 50% of cases in USA and Europe, and HPV18 for 30% of cases. Worldwide, the HPV have been implicated in 99.7% of cervical squamous cell carcinoma cases.[20]

Although it is well known that HPV was detected in the majority of cervical cancer cases, however, additional genetic and epigenetic changes are required for disease progression.[21] Recent advances in cervical cancer biology revealed that, epigenetic alterations due to aberrant DNA methylation and histone modifications have been strongly linked to the cervical carcinogenesis and metastasis. The same study suggested that, interest should be given to study the utilization of these changes as biomarkers for disease progression, prognostic values and also as therapeutic targets.[22]

Concerning the prevention of the uterine cervical carcinoma, the new German S3 guidelines published in 2016 concluded that, HPV based screening may provide better protection than cytology alone through early detection of the premalignant disease prior to the progression. Therefore, women aged 30 years and older should be screened every 3-5 years.[23] Nowadays, primary prevention includes the 9-valent vaccine that provides protection against approximately 90% of cervical cancer, is available in Europe from May 2016 and administered in a 2-dose schedule.[24] Regular cervical cancer screening should be a key part in global efforts to reduce the morbidity and mortality rate in women with cervical carcinoma.

1.3. Steroids; from ordinary functions to anticancer therapy

Steroids are biologically active compounds with diverse physiological actions. The core chemical structure of steroid molecule is composed of four fused rings (A, B, C and D) where each of them displays a specific variation based on the existence of functional groups in that ring. Since their identification for the first time in 1935, steroids have been shown to exert a variety of actions in the clinical interventions owing to their potent anti-inflammatory and immune-modulating effects.[25]

Briefly, steroids are classified into three major categories; sex hormones, corticosteroids as well as neuroprotective. Sex hormones (androgens, progesterone and estrogens) play an important role in reproduction and development of secondary sex characteristics. With regards to corticosteroids, they are categorized into; glucocorticoids which maintain metabolism with stress response, and mineralocorticoids that regulate acid-base balance. Lastly, neuroprotective steroids that interact with androgen receptors to up-regulate bone and muscle synthesis.[26]

All steroids are synthesized by *de novo* steroidogenesis, a biological process by which steroids are generated from cholesterol as a preliminary precursor exist in the steroidogenic organs; adrenal cortex, placenta and gonads. It's important to mention that, cholesterol is obtained from three major sources; biosynthesis from acetate in the endoplasmic reticulum, hydrolysis of cholesteryl esters stored in the lipid droplets by the cholesteryl aster hydrolase and lastly, the free cholesterol found in the plasma membrane.[27]

Focusing on steroid hormones, they are lipophilic molecules involved in various cellular events via interacting with nuclear receptors (NRs). They exert their biological effects through two major mechanisms; genomic and non-genomic.[28] In genomic pathway, steroid hormones interact with their counterpart receptors expressed in the nucleus and the cytoplasm of the target cells. This results in the conformational changes in DNA at specific hormone responsive elements (HREs) and consequently regulation of the gene transcription.[29] Non-genomic pattern involves their interaction with NRs that are also located in the plasma membrane of the cells leading to the propagation of signal transduction through kinase pathways.[30]

Estrogens are considered the molecules of interest. They play a major role in the regulation of female reproductive physiology as well as the development of the secondary sex characteristics. Three endogenous estrogens exist; estradiol (E2), estriol (E3) and estrone (E1) where the latest

represents the weak form of endogenous estrogen biosynthesized from cholesterol.[31] Clinically, estrane based agents have been used to treat defects associated with gonadotropin hormone dysfunctions, atrophic vaginitis and prevention of osteoporosis related to estrogen deficiency.[32]

In addition to their ordinary hormonal functions, certain estrogens possess other biological activities including neuroprotective, antiangiogenic as well as anticancer effects. Among the estrogens that exhibit the previously mentioned biological effects, is 13α-estrone, which is an isomer of the estrone.[33, 34] The main risk in the design of estrane based anticancer agents is the possible hormonal side effects. The probable mechanism to overcome this obstacle and minimize the undesirable estrogenic activity, is the transformation of natural estrogens into their core-modified analogs.[35] It was shown that inversion in the configuration at C-13 results in a modified conformation, therefore, analogs of 13α-estrone don't possess estrogenic behavior.[36] It is important to mention that certain enzymes involved in estrogen biosynthesis can be inhibited by 2- and/or 4-substitued 13α-estrone derivatives.[37] Moreover, the organic anion transporter protein (OATP2B1), which is one of the key players in intestinal drug absorption and transport, might also be inhibited by certain 13α-estrone derivatives.[38]

Concerning the antiproliferative potential of 13α-estrones, our research group (Zupkó and colleagues) has been studying in vitro investigation for nearly a decade. It was concluded that, certain derivatives display outstanding growth inhibition against a panel of human adherent gynecological cancer cell lines.[39-41] Investigation of the mechanism of action of certain compounds demonstrated their capability to induce cell cycle blockade at G2/M phase.[42] Some derivatives induced apoptosis via the intrinsic pathway.[43] These data suggest that 13α-estrones are of great value owing to their multiple bioactive affects without estrogenic behavior.

2 AIMS AND OBJECTIVES

Throughout the duration of our experiments, preliminary assessment for numerous novel A-ring modified 13α-estrone derivatives had been performed. Several candidate compounds were identified and the most potent antiproliferative agent was chosen for further evaluation. The aim of the present study was to investigate in vitro antineoplastic properties of the most potent compound against the HPV16 positive human invasive cervical cancer cell line (SiHa) as well as to explore its mechanism of action.

The detailed objectives of the conducted experiments are clarified as follows;

- Assessment of the antiproliferative potential of various 13α-estrone derivatives against a panel of human adherent gynecological cancer cell lines as well as determination of their half maximum inhibitory concentration (IC_{50}) values via MTT assay.
- Estimation of tumor selectivity by measuring the growth inhibitory percentage against non-cancerous mouse fibroblast cell line (NIH/3T3).
- Investigation of the mechanism of action of the selected compound by assessing its influence on cell cycle distribution.
- Determining the ability of the chosen compound to induce morphological changes represented by necrosis and apoptosis using Hoechst 33258/propidium iodide fluorescent double staining (HOPI).
- Demonstrating the proapoptotic activity through the colorimetric determination of caspase-3 enzyme activity.
- Determining the effects of the candidate compounds on microtubules thorough performing cell-free tubulin polymerization assay.
- Exploring the antimetastatic capacity particularly on cell migration and invasion by conducting both wound healing and Boyden chamber assays respectively.

3 MATERIALS AND METHODS

3.1. Chemical structures of the novel A-ring substituted 13α-estrone derivatives

A total of 47 newly synthesized 13α-estrone derivatives have been investigated in this study.[44-46] Modifications were mainly substituted in the A-ring of the hormonally inactive 13α-estrone core. The novel compounds were categorized into three sets based on the nature of the substituents introduced. Syntheses of all novel 13α-estrone derivatives were carried out by colleagues of the Institute of Organic Chemistry, University of Szeged, Hungary.

3.1.1 A-ring halogenated 13α-estrones

The first set of compounds (**1**-**17**) represents 3-hydroxy or 3-benzyloxy 2- or 4-(substituted) phenyl derivatives (**Figure 1**). Modifications were applied at positions C-2 and C-4 of the A-ring of the hormonally inactive 13α-estrone core.[44]

3.1.2 Steroidal diaryl ethers

The newly synthesized compounds in this set (**18**-**29**) includes the novel 13α-estrone derivatives arylated directly at the *C*-3-*O* function.[45] The chemical structures are illustrated in **Figure 2**.

3.1.3 Carbamate, sulfamate and pivalate derivatives of 13α-estrone and their 17-deoxy counterparts

The third set of compounds (**30**-**47**) includes newly synthesized 3-*O*-carbamoyl, -sulfamoyl, or –pivaloyl derivatives of 13α-estrone and their 17-deoxy counterparts.[46] The chemical structures of this group of compounds are illustrated below **Figure 3**.
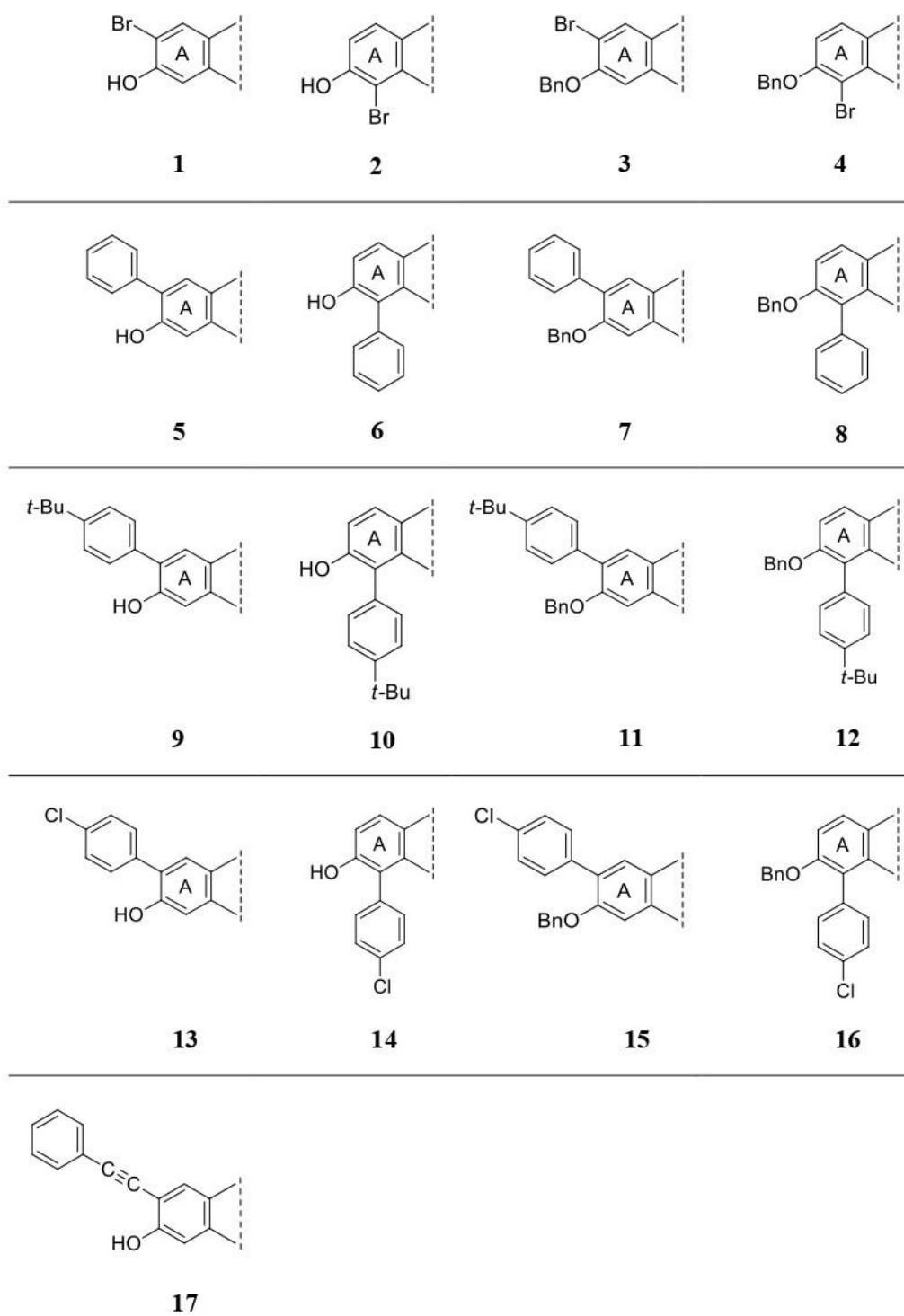
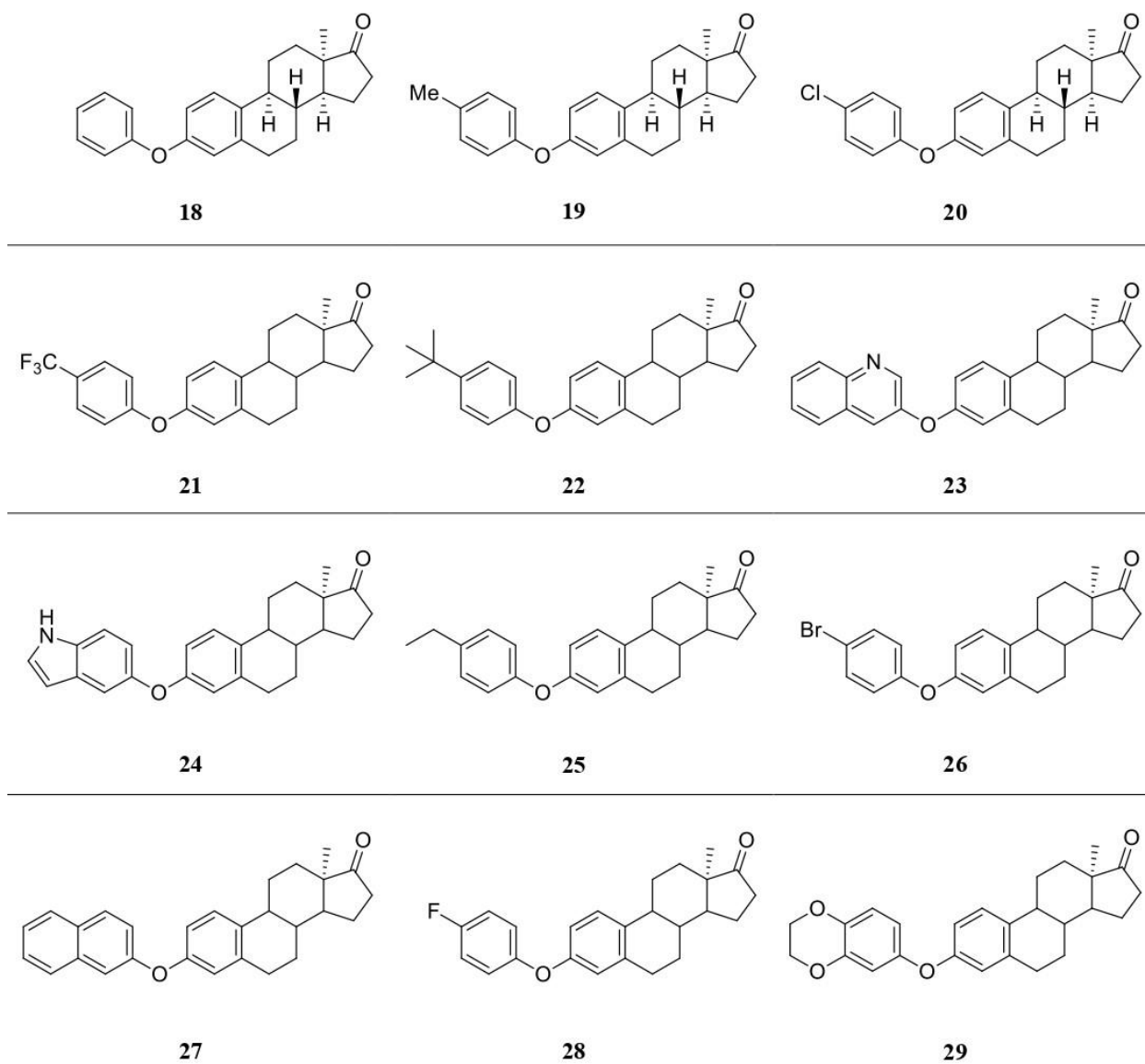**Figure 1**: Chemical structures of the A-ring halogenated 13α-estrone derivatives

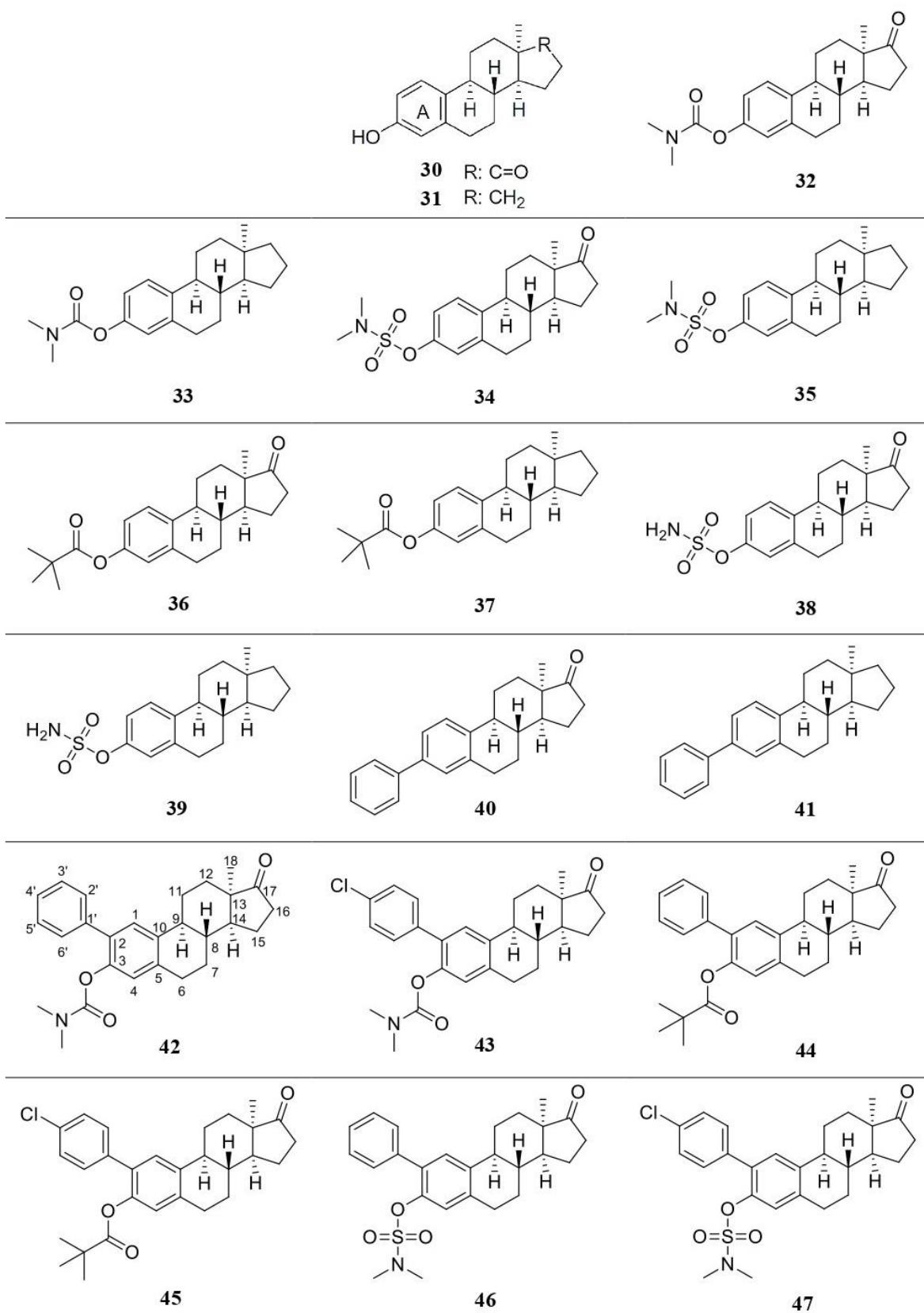**Figure 2**: Chemical structures of steroidal diaryl ethers

Figure 3: Carbamate, sulfamate and pivalate derivatives of 13α-estrone

3.2. Tumor cell lines and culture

In this study, a panel of human adherent gynecological cancer cell lines were used to conduct in vitro experiments. As a preliminary step, stock solutions (10 µM) of the test compounds were prepared and dissolved in dimethyl sulfoxide (DMSO). Breast cancer cell lines (MCF-7, MDA-MB-231), ovarian (A2780), cervical (HeLa) and non-cancerous mouse fibroblast cells were purchased from the European Collection of Cell Cultures, Salisbury, UK. The cervical cancer cell line (SiHa) was obtained from the American Tissue Culture Collection, Virginia, USA. All cells were maintained in minimal essential medium supplemented with 10% of fetal bovine serum (FBS), 1% of non-essential amino acids and 1% of antibiotic-antimycotic mixture, in humidified air with 5% of CO_2 at 37 ºC. All other mediums, chemicals and supplements were purchased from Lonza group Ltd. T, Basel, Switzerland, unless otherwise specified.

The detailed characterization of the cancer cell lines that are used in this study is summarized in **Table 1**:

Cell line	Characteristics
MCF-7	Human breast adenocarcinoma derived from the pleural effusion, express both progesterone and estrogen receptors.
MDA-MB-231	Triple negative human breast cancer cell line, don't express progesterone and estrogen receptors as well as human epithelial growth factor receptor 2 (HER-2).
SiHa	HPV16 positive human cervical carcinoma cell line.
HeLa	HPV18 positive human cervical carcinoma cell line.
A2780	Human ovarian carcinoma cell line, derived from an ovarian endometroid adenocarcinoma of untreated patient.
NIH/3T3	Non-cancerous mouse embryonic fibroblast cell line

Table 1: Characteristics of the utilized cancer cell lines.

3.3. Antiproliferative assay

The growth inhibitory effects of the test compounds against a panel of previously described cancer cell lines was investigated using the standard MTT assay.[47] Initially, cells were seeded into 96-well plates at a density of 5000/ well and incubated overnight in a previously described lab conditions. As a preliminary assessment, cells were treated with the fresh medium containing 10 or 30 μM concentrations of the test compounds. After 72 hours of incubation, 20 μl of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was added to each well and incubated for 4 hours. Formazan crystals in living cells were produced by the intact mitochondrial reductase and precipitated as purple crystals. The supernatant (medium and MTT solution) in all wells were then removed and 100 μl of DMSO was added to each well to dissolve the precipitated formazan crystals. The plates were then put in the shaker for approximately 60 minutes at 37 °C. Lastly, the samples were assayed at 545 nm using a microplate reader (BMG, LAbTech, Ortenberg, Germany) where the untreated cells served as controls. As a next step, the active compounds that elicited more than 50% of inhibition undertook a series of dilutions where the sigmoidal dose response curves were fitted to determine their half maximum inhibitory concentration (IC_{50}) values. All in vitro experiments were conducted twice with at least five parallel wells to ensure consistency and accuracy of results. Cisplatin (Ebewe Pharma GmbH, Unterach, Austria) was used as a positive control.

3.4. Cell cycle analysis by flowcytometry

To determine the mechanism of action of the selected test compound, cell cycle analysis was conducted to measure the cellular DNA contents of cells using flowcytometry. Cells were seeded into a 24-well plate at a density of 80.000 cells/well and incubated overnight. The cells were then treated with 50 μl of fresh medium containing the desired concentrations of the test compound and incubated for 24 and 48 hours respectively. Next, the cells were washed with phosphate buffer saline (PBS) and harvested with trypsin. The supernatants were then removed, and the cells were centrifuged at 1400 rpm for 5 minutes. The DNA contents of the cells were stained by 300 μl of propidium iodide (PI) solution (10 $\mu g/ml$ PI, 0.1% triton-X, 0.1% sodium citrate and 10 $\mu g/ml$ RNase-A dissolved in PBS), and stored in a dark place for 30 minutes. Eventually, the samples were analyzed by a FACS caliber flow cytometer where at least 20.000 events per sample was

evaluated for each analysis. The obtained data were analyzed using ModFit LT 3.3.11 software (Bedford, Massachusetts, USA). Untreated cells were considered as control whereas the hypodiploid sub-G1 cell population was regarded as apoptotic cells.

3.5. Hoechst 33258/propidium iodide fluorescent double staining (HOPI)

Fluorescent staining with DNA-specific dyes was performed to detect the necrotic and apoptotic morphological changes in cells induced by the test compound using HOPI method. SiHa cells were initially seeded into 24-well plate at a density of 50.000 cells/well and incubated overnight. The cells were then treated with the desired concentrations of the test compound and incubated for 24 hours in cell culturing conditions described previously. Following treatment, the cells were stained with medium containing lipophilic Hoechst 33258 [HO] (5 µg/ml) and hydrophilic Propidium iodide [PI] (1 µg/ml) and incubated in a dark place for 60 minutes. Then, the medium was refreshed in samples and eventually appropriate images (5 per each condition) were taken by QCapture Pro software (QImaging, BC, Canada) and Nikon Eclipse TS100 fluorescent microscope equipped with appropriate filters for Hoechst 33258 (excitation: 360/40 nm bandpass filter, emission: 460/50 nm bandpass and 400 nm dichromatic mirror) and PI (excitation: 500/20 nm bandpass filter, emission: 520 nm longpass filter and 515 nm dichromatic mirror). In each sample, cell nuclei emitting fluorescence were counted and the intact, necrotic and apoptotic cell proportions were expressed as percentages.

3.6. Caspase-3 activity

To evaluate the proapoptotic property of the test compound and its ability to induce the programmed cell death, colorimetric caspase-3 activity was measured using commercially available kit (Abnova, Taipei, Taiwan). Cells were seeded into the culture flasks at a density of 10^7 as a control and $1.5*10^7$ as treated cells and incubated overnight. Then, the cells were treated with the desired concentrations of the test compound and incubated for 24 hours. Using a cell scrubber, the cells were carefully scrubbed, harvested, counted, washed with PBS and subsequently centrifuged at 3000 rpm for 15 minutes at the room temperature. The pellets were then re-suspended in lysis buffer (100 µl/10^7 cells) and incubated on ice for 20 minutes. Later, the

cell lysates were again centrifuged at 600 g at 4 °C for 15 minutes. Finally, the cell lysates for each sample (5 µl supernatant + 10 µl substrate + 90 µl assay buffer) were pipetted in to a 96-well plate and incubated overnight according to the manufacturer's instructions. The absorbance of the cleaved substrate that is directly proportional to the amount of the active caspase-3 was measured by a microplate reader at 405 nm. The detected amount of caspase-3 in treated cells at different concentrations was compared to untreated cells which served as a control.

3.7. Tubulin polymerization assay

To assess the influence of the test compound on the microtubule system, cell-free tubulin polymerization assay was performed using commercially available kit (Cytoskeleton Inc., Denver, Colorado, USA). The assay was conducted on a pre-warmed 96-well microplate where each testing condition was represented by two parallel wells. Then, 10 µl of general tubulin buffer (GTB) was added to the first two wells representing the negative control whereas the same amount of paclitaxel was added to the next two wells as a positive control. After adding the desired compound concentrations, the polymerization reaction was initiated by adding 100 µl of tubulin to each well. The absorbance values of samples were immediately measured per each minute at 340 nm using a 60 minutes kinetic measurement protocol. To demonstrate changes in polymerization of tubulin induced by the test compound, a polymerization curve was fitted to the measured data. The highest difference between three absorbance values at two consecutive time intervals was considered as maximum rate of tubulin polymerization (Vmax).

3.8. Cell migration assay

To assess the influence of the test compound on cell migration, a wound healing assay was conducted using specific chambers (ibidi GmbH, Martinsried, Germany). Cells were seeded into the inserts at a density of 50.000 cells /well and incubated overnight. After that, the inserts were gently removed by a forceps and the wells were washed twice by 5 ml of PBS to remove non adherent cells. The cells were then treated with a fresh medium (2% FBS) containing the desired compound concentrations and incubated for 24 and 48 hours respectively. Migration of cells towards the wound closure site was visualized using a CCD camera (QImaging MicroPublisher Color RTV5.0, Teledyne Photometrics, Tucson, Arizona, USA). The percentage of cell migration

(wound closure %) was calculated based on the photos taken at different intervals (0, 24 and 48 hours) using ImageJ software version 1.53a (National Institutes of Health, Bethesda, MD, USA).

3.9. Invasion assay

The anti-invasive capacity of the test compound was assessed using Boyden chamber assay. The test is based on using specific Boyden chambers (BD Biosciences, Bedford, MA, USA) that act as in vitro model for the tumor microenvironment consisting of 2 thin layers of matrigel for cancer cell suspension and chemo-attractants separated by a PET membrane with a pore size of 8 μM. The serum free medium containing both cell suspension and the desired compound concentrations was pipetted on the upper layer of the chamber whereas the medium (10% FBS) in the lower layer of the chamber served as a chemo-attractant. After 24 hours of incubation, the supernatants were carefully removed from the upper site of the membrane and then was cleaned from non-invading cells using a cotton swab soaked in PBS. The membrane was then washed twice with PBS and fixed with the ice-cold ethanol (96%). The invading cells were stained by crystal violet stain (1%) and kept in a dark place for approximately 30 minutes. Eventually, several appropriate images were taken (at least 5 per each sample) and the percentage of invading cells was calculated in treated cells compared to untreated controls.

3.10. Statistical analysis

Statistical data analysis in all cell-based in vitro experiments was performed by one way analysis of variance followed by Dunnet test. Data were expressed as mean values ± standard error of mean (SEM) using GraphPad Prism version 5.01 (GraphPad, San Diego, CA, USA). *P* values were calculated to display the statistical differences between study groups. The *P* values < 0.05 and < 0.01 were considered statistically significant and highly significant respectively.

4 RESULTS

4.1 Antiproliferative activity and tumor selectivity of the A-ring substituted 13α-estrone derivatives

4.1.1 A-ring halogenated 13α-estrones

All the novel compounds in this study were initially tested against previously described cancer cell lines to determine their cytotoxic potential using the standard MTT assay. To determine the tumor selectivity, the compounds were also tested against the non-cancerous mouse fibroblast cell line (NIH/3T3).

Concerning the first set of compounds (**1-17**) [**Table 2**], a structural activity relationship was observed between bromo derivatives (**1, 3**) and (**2, 4**). The 3-OH compounds (**1 and 2**) didn't exert a remarkable growth inhibition against cancer cell lines. The 4-bromo regioisomer (**4**) of benzyl ethers exerted outstanding antiproliferative effects against the estrogen receptor positive MCF-7 as well as a lower inhibition against the rest of the cell lines. The other 3-OBn regioisomer (**3**) exhibited a weak antiproliferative action. Concerning tumor selectivity, compound (**4**) displayed a limited growth inhibition ($< 50\%$) although the other cell lines were highly sensitive to the reference agent cisplatin. A comparison between phenylated 3-OH (**5, 6**) and brominated 3-OH (**1, 2**) indicated that phenyl substitution instead of introduction of a bromine improved the antiproliferative potential (only for **5**) where the latter seems to be more effective than (**6**). With respect to (**7**), it exerted moderate effects that could be due to the existence of the 3-OBn functional group. Compounds bearing 3-OH and 4-*tert*-butylphenyl groups (**9** and **10**) displayed higher potency compared to (**5 and 6**), while their 3-OBn derivatives (**11** and **12**) displayed a very weak growth inhibition. The 2-(4″-chlorophenyl) 3-OH compound (**13**) exerted substantial growth inhibitory effects on both MCF-7 and HeLa and was more potent compared to (**14**). In case of 2-(4″-chlorophenyl)-3-OBn compounds (**15** and **16**), the second derivative exerted more potent effects against all the tested cell lines. Lastly, the 2-phenylethynyl derivative (**17**) exerted somehow mild effects which could be due to the presence of the short linear linker between the two phenyl functions.

| Comp. | Conc. (μM) | inhibition (%) ± SEM   [calculated IC50 value (μM)] | | | | | |
		MCF-7	MDA-MB-231	HeLa	SiHa	A2780	NIH/3T3
1	10	-	-	-	-	-	n.t
	30	24.26 ± 0.76	22.85 ± 1.97	32.26 ± 0.52	20.72 ± 3.56	-	
2	10	-	-	-	-	-	n.t
	30	51.92 ± 1.61	42.09 ± 1.31	54.81 ± 1.14	-	44.21 ± 1.09	
3	10	-	-	-	-	-	n.t
	30	-	-	48.57 ± 1.40	-	-	
4	10	76.67 ± 0.30	54.41 ± 0.88	60.23 ± 0.42	59.04 ± 1.51	47.78 ± 2.10	30.59 ± 0.47
	30	79.45 ± 0.85 (0.52)	62.36 ± 0.67 (3.91)	68.16 ± 0.62 (2.85)	63.04 ± 0.41 (2.74)	54.75 ± 1.06 (6.85)	29.55 ± 0.36
5	10	44.72 ± 0.73	31.50 ± 1.77	36.82 ± 0.98	33.49 ± 0.90	35.73 ± 1.21	35.48 ± 1.33
	30	71.54 ± 0.40 (13.12)	48.97 ± 0.42	63.67 ± 0.30 (17.06)	54.36 ± 0.37	45.89 ± 0.96	40.04 ± 1.48
6	10	-	-	-	-	-	n.t
	30	-	-	27.29 ± 1.02	-	-	
7	10	32.11 ± 1.90	-	51.26 ± 0.20	25.45 ± 2.09	25.44 ± 1.06	-
	30	74.50 ± 1.16 (16.87)	76.16 ± 1.0 (18.92)	75.53 ± 1.18 (8.98)	70.06 ± 1.48 (18.56)	73.42 ± 0.41 (18.62)	-
8	10	-	-	-	-	-	n.t
	30	27.35 ± 1.36	-	-	-	27.14 ± 0.94	
9	10	47.97 ± 0.98	42.24 ± 1.02	66.29 ± 0.87	44.03 ± 1.15	52.46 ± 0.20	45.30 ± 2.71
	30	66.68 ± 0.71 (12.82)	53.23 ± 0.35	77.67 ± 0.42 (6.69)	51.95 ± 0.78	68.13 ± 0.55 (9.41)	51.68 ± 1.96
10	10	-	-	38.70 ± 1.67	-	35.83 ± 1.92	-
	30	96.93 ± 0.33 (15.73)	91.02 ± 0.59 (17.19)	97.96 ± 0.15 (11.19)	92.43 ± 0.88 (16.9)	97.93 ± 0.17 (10.38)	-
11	10	-	-	24.51 ± 1.62	-	-	n.t
	30	28.21 ± 0.85	34.85 ± 0.52	52.36 ± 1.25	20.00 ± 0.79	64.77 ± 1.82	
12	10	-	-	-	-	-	n.t
	30	32.81 ± 1.33	-	-	-	24.65 ± 2.81	
13	10	53.41 ± 0.23	25.95 ± 0.43	73.65 ± 0.22	50.85 ± 0.30	24.97 ± 0.52	26.47 ± 1.33
	30	59.51 ± 0.22 (5.33)	61.92 ± 0.67 (18.32)	72.46 ± 0.81 (3.33)	53.45 ± 0.95 (13.24)	33.61 ± 0.74	28.17 ± 0.25
14	10	-	-	-	-	20.20 ± 1.13	-
	30	88.41 ± 1.35 (19.76)	52.83 ± 1.31	80.24 ± 0.21 (17.91)	72.66 ± 1.09 (21.19)	82.92 ± 0.45 (14.97)	20.9 ± 0.42
15	10	-	-	-	-	-	n.t
	30	36.29 ± 1.21	22.91 ± 0.55	56.17 ± 1.12 (29.97)	47.26 ± 0.3	34.63 ± 0.48	
16	10	48.56 ± 0.32	-	47.46 ± 1.65	-	-	-
	30	77.13 ± 0.40 (9.95)	33.67 ± 1.39	64.12 ± 2.00 (10.57)	62.24 ± 0.94 (21.96)	55.04 ± 0.61 (26.24)	-
17	10	23.51 ± 1.95	-	-	-	-	n.t
	30	69.00 ± 0.32 (21.51)	29.66 ± 0.59	59.89 ± 1.16 (27.68)	46.18 ± 0.14	78.34 ± 0.12 (20.82)	
Cisplatin	10	53.03 ± 2.29	-	42.61 ± 2.33	88.64 ± 0.50	83.57 ± 1.21	91.80 ± 0.39
	30	86.90 ± 1.22 (5.78)	71.47 ± 1.20 (19.13)	99.93 ± 0.26 (12.43)	90.18 ± 1.78 (7.84)	95.02 ± 0.28 (1.30)	93.68 ± 0.20 (2.70)

Table 2: Antiproliferative properties of the A-ring halogenated 13α-estrone derivatives

4.1.2 Steroidal diaryl ethers

Based on MTT results, compound (**18**) displayed a potent growth inhibitory actions against both MCF-7 and HeLa, however, the later cell line seemed to be more sensitive. All compounds in this set demonstrated potent antiproliferative activities against HeLa with calculated IC_{50} values ranging 5-10 µM [**Table 3**]. On the other hand, the growth inhibition was not improved in compounds bearing fluorine functional group (**28**, **21**). The inhibitory potential of naphthyl derivative (**27**) was similar to its phenyl counterpart (**18**) except SiHa which was more sensitive to **27**. Concerning nitrogen-containing heterocyclic compounds, **23** exerted substantial antiproliferative activity against all tested cell lines except SiHa. The other two compounds (**29** and **24**) were less potent than **23**.

All compounds were tested against non-cancerous NIH-3T3 cell line as well. Interestingly, none of them displayed a growth inhibition of more than 50% even at a higher concentration (30 µM) indicating tumor selectivity.

4.1.3 3-*O*-carbamoyl, -sulfamoyl, or –pivaloyl 13α-estrone derivatives and their 17-deoxy counterparts

Concerning the antiproliferative action of the 3rd set of compounds, 13α-estrone (**30**) displayed weaker inhibitory effects than its 17-deoxy counterpart (**31**) [**Table 4**].

The carbamate derivatives (**32, 33**) didn't exert strong inhibitory effects. However, the 17-deoxy pivaloyl derivative (**37**) exerted substantial growth inhibitory potential against MCF-7 and HeLa. Among the current set of compounds, the 3-*O*-sulfamoyl group was considered the most potent. Sulfamates bearing H_2N-function (**38, 39**) generally exerted less inhibitory effects compared to *N,N*-dimethyl derivatives (**34, 35**). The 3-deoxy-3-phenyl derivatives (**40, 41**) possessed weak antiproliferative potential. Next, the determination of antiproliferative effects was continued testing 2-(4-subst.phenyl)-13α-estrone derivatives (**42-47**). To summarize, the carbamate compound group (**32, 42** and **43**) didn't result in outstandingly active compounds concerning antiproliferative action. Moreover, the 2-phenyl pivalate (**44**) displayed higher effects against MCF-7 compared to its 17-deoxy counterpart (**36**). Lastly, the 2-(4-chlorophenyl)-13α-estrone

| Comp. | Conc. (μM) | inhibition (%) ± SEM   [calculated IC50 value (μM)] | | | | | |
		MCF-7	MDA-MB-231	HeLa	SiHa	A2780	NIH/3T3
18	10	24.82 ± 3.78	-	60.24 ± 0.58	-	23.00 ± 1.32	-
	30	81.42 ± 1.50 (8.54)	25.30 ± 1.82	92.02 ± 1.38 (5.53)	-	58.48 ± 1.03 (23.81)	38.75 ± 1.21
19	10	-	-	56.69 ± 1.29	-	39.64 ± 1.84	-
	30	29.28 ± 1.26	33.34 ± 1.75	83.29 ± 1.34 (7.99)	-	62.15 ± 1.15 (16.67)	43.34 ± 2.17
20	10	-	-	60.72 ± 1.24	-	34.93 ± 2.85	-
	30	37.51 ± 1.44	30.28 ± 2.24	75.40 ± 1.42 (5.78)	38.91 ± 1.69	51.48 ± 1.93 (26.17)	-
21	10	35.08 ± 0.69	27.06 ± 2.93	67.46 ± 0.46	31.80 ± 2.77	36.13 ± 1.39	-
	30	45.42 ± 3.30	39.67 ± 2.39	80.74 ± 0.58 (6.90)	45.55 ± 2.63	52.60 ± 1.00 (23.47)	45.47 ± 0.28
22	10	24.75 ± 0.74	-	65.28 ± 1.02	-	30.84 ± 2.24	-
	30	30.77 ± 1.67	32.93 ± 1.88	77.06 ± 0.93 (7.11)	41.14 ± 2.38	56.98 ± 1.07 (23.65)	24.07 ± 1.67
23	10	49.68 ± 2.46	24.07 ± 2.47	76.18 ± 1.49	22.46 ± 2.36	48.66 ± 1.90	35.48 ± 1.33
	30	74.67 ± 0.51 (8.78)	58.28 ± 1.39 (22.95)	90.11 ± 0.80 (3.98)	48.33 ± 1.27 (30.80)	71.21 ± 1.35 (11.54)	49.87 ± 0.89
24	10	-	-	49.40 ± 1.97	-	-	n.t
	30	42.25 ± 1.33	-	71.63 ± 1.45 (9.59)	-	28.02 ± 3.57	
25	10	43.99 ± 1.77	-	62.38 ± 1.19	27.24 ± 2.60	-	-
	30	58.64 ± 1.08 (16.44)	33.16 ± 0.95	88.14 ± 0.79 (5.13)	43.55 ± 0.86	45.08 ± 1.75	21.99 ± 2.03
26	10	48.82 ± 2.18	-	65.97 ± 0.61	20.31 ± 3.10	-	-
	30	59.91 ± 1.73 (11.98)	28.28 ± 1.39	80.70 ± 0.76 (5.21)	38.42 ± 1.30	39.58 ± 2.60	30.92 ± 2.15
27	10	-	29.61 ± 3.01	73.86 ± 1.48	28.80 ± 2.10	42.23 ± 0.71	-
	30	43.11 ± 2.035	41.87 ± 0.85	77.08 ± 1.34 (5.52)	54.33 ± 0.83 (23.90)	51.25 ± 1.43 (25.25)	24.15 ± 0.60
28	10	49.02 ± 0.85	-	30.14 ± 3.05	-	-	n.t
	30	60.22 ± 1.68 (13.28)	-	37.78 ± 3.86	-	46.50 ± 2.43	
29	10	29.08 ± 1.88	20.72 ± 1.33	61.30 ± 0.98	20.79 ± 3.34	48.44 ± 0.49	-
	30	63.02 ± 1.09 (18.91)	24.10 ± 3.15	74.01 ± 0.80 (9.16)	38.58 ± 2.61	69.17 ± 0.50 (11.47)	46.16 ± 2.11

Table 3: Antiproliferative properties of steroidal diaryl ethers

sulfamate (**47**) displayed an outstanding growth inhibition against all cell lines in which both HPV18 and HPV18 positive invasive uterine cervical cancer cell lines (SiHa and HeLa) were substantially inhibited in very low micromolar ranges (IC$_{50}$= 2.71 and 2.28 μM) respectively.

Overall, based on the antiproliferative data, **47** was the most potent antiproliferative agent among all tested groups, specifically against HVP16 positive human cervical cancer cell line (SiHa). Therefore, it was selected for further evaluation and to explore its mechanism of action.

Table 4: Antiproliferative properties of carbamate, sulfamate and pivalate derivatives							
Comp.	Conc. (μM)	inhibition (%) ± SEM [calculated IC50 value (μM)]					
		MCF-7	MDA-MB-231	HeLa	SiHa	A2780	NIH/3T3
30	10	23.04 ± 1.25	-	-	-	-	n.t
	30	29.05 ± 2.75	-	23.38 ± 1.56	-	24.39 ± 2.24	
31	10	25.69 ± 2.26	18.34 ± 2.01	33.82 ± 0.92	-	-	-
	30	95.96 ± 0.54 (13.65)	96.18 ± 0.43 (14.17)	99.01 ± 0.97 (8.56)	96.04 ± 0.32 (15.80)	97.80 ± 0.85 (13.69)	97.05 ± 0.15 (15.29)
32	10	27.18 ± 1.67	-	23.78 ± 1.28	32.27 ± 1.03	21.99 ± 1.12	n.t
	30	38.53 ± 1.26	-	66.10 ± 1.87 (17.26)	35.01 ± 0.94	45.81 ± 0.92	
33	10	-	-	-	-	-	n.t
	30	26.60 ± 1.97	-	54.70 ± 1.35	22.26 ± 1.49	36.57 ± 1.99	
34	10	60.47 ± 2.62	-	67.84 ± 0.86	58.35 ± 0.66	23.38 ± 1.50	23.98 ± 2.16
	30	82.67 ± 1.15 (5.28)	42.14 ± 1.23	69.78 ± 1.08 (6.67)	60.32 ± 1.09 (13.21)	40.16 ± 2.09	44.12 ± 2.35
35	10	62.39 ± 1.61	24.89 ± 1.46	56.34 ± 0.69	55.25 ± 1.27	24.89 ± 2.05	32.77 ± 0.61
	30	88.70 ± 1.44 (5.54)	57.66 ± 1.67 (24.15)	60.84 ± 1.50 (9.49)	58.80 ± 0.83 (13.07)	48.72 ± 2.53	47.71 ± 0.92
36	10	48.02 ± 0.67	-	49.48 ± 1.41	-	-	n.t
	30	60.59 ± 0.77 (14.60)	36.93 ± 2.25	66.08 ± 0.92 (13.11)	52.15 ± 0.77	30.81 ± 0.67	
37	10	53.74 ± 0.44	32.70 ± 0.51	66.02 ± 1.43	49.83 ± 1.12	-	29.96 ± 0.99
	30	60.39 ± 0.87 (9.14)	35.72 ± 0.47	68.20 ± 1.16 (6.34)	50.91 ± 1.84	35.21 ± 1.76	33.69 ± 1.23
38	10	21.30 ± 0.31	-	46.89 ± 1.72	-	-	n.t
	30	29.57 ± 1.32	44.55 ± 1.07	66.55 ± 1.20 (11.80)	-	46.78 ± 1.89	
39	10	33.48 ± 1.58	-	73.19 ± 2.04	-	-	-
	30	81.72 ± 1.03 (10.69)	35.91 ± 1.02	85.41 ± 0.96 (6.90)	84.63 ± 1.23 (21.49)	83.48 ± 0.60 (16.86)	25.02 ± 2.20
40	10	22.97 ± 2.41	-	-	-	27.25 ± 1.20	n.t
	30	42.59 ± 2.77	-	63.61 ± 2.00 (19.62)	20.84 ± 1.54	42.05 ± 1.04	
41	10	-	-	-	-	-	n.t
	30	23.15 ± 0.77	-	21.20 ± 2.10	25.27 ± 0.57	-	
42	10	23.48 ± 1.63	-	-	21.84 ± 1.76	33.53 ± 2.15	n.t
	30	87.72 ± 0.70 (13.95)	67.95 ± 0.92 (20.83)	52.74 ± 2.13	48.65 ± 1.95	89.68 ± 0.54 (12.99)	
43	10	20.53 ± 3.05	-	48.03 ± 1.44	20.00 ± 1.57	-	24.62 ± 1.15
	30	58.51 ± 2.17 (24.01)	21.22 ± 2.19	65.31 ± 1.49 (13.48)	47.84 ± 0.80	55.00 ± 0.96	42.70 ± 1.32
44	10	25.85 ± 1.93	-	48.76 ± 1.50	-	20.22 ± 2.27	-
	30	77.95 ± 2.01 (11.80)	31.40 ± 2.86	58.91 ± 0.76 (15.20)	41.32 ± 2.08	55.19 ± 0.36 (25.54)	32.11 ± 2.70
45	10	66.09 ± 1.84	-	55.99 ± 1.62	-	-	-
	30	79.06 ± 3.18 (7.16)	-	62.70± 1.49 (8.23)	37.25 ± 2.01	48.58 ± 1.14	-
46	10	61.11 ± 2.36	29.40 ± 0.71	62.78 ± 0.47	48.91 ± 1.60	31.83 ± 1.45	21.04 ± 1.09
	30	75.83 ± 2.58 (6.72)	37.59 ± 0.70	69.39 ± 0.80 (7.53)	55.37 ± 0.77 (15.95)	41.57 ± 2.10	25.72 ± 2.72
47	10	57.84 ± 1.56	35.66 ± 0.64	81.11 ± 0.67	78.53 ± 2.53	50.01 ± 1.04	34.27 ± 1.93
	30	81.64 ± 2.61 (6.36)	65.31 ± 1.94 (16.34)	95.45 ± 0.80 (2.28)	91.44 ± 0.94 (2.71)	76.55 ± 1.01 (10.60)	50.57 ± 1.14

In addition to ability of the test substance to remarkably inhibit cell proliferation, determining tumor selectivity was also considered a crucial step in our study. For that purpose, the tumor selectivity index (TSI) for compound **47** was calculated according to the following equation; $IC_{50[cancerous]}/IC_{50[non-cancerous]}$. The TSIs (except for HeLa) were less than 1 but more than 0.1 indicating that, this compound has moderate tumor selectivity. **Table 5** demonstrates the calculated tumor selectivity indices for compound **47** versus cisplatin against the utilized cell lines.

Cell lines	IC_{50} malignant/IC_{50} NIH/3T3	IC_{50} cisplatin/IC_{50} NIH/3T3
MCF-7	0.264	2.140
MDA-MB-231	0.680	7.085
HeLa	0.094	4.603
SiHa	0.112	2.903
A2780	0.441	0.481

Table 5: Tumor selectivity index of compound **47** on the utilized cell lines

4.2. Cell cycle analysis

To investigate the mechanism of action of compound **47**, cell cycle analysis was performed using flow cytometry. SiHa cells were treated with 2 and 4 µM of the test compound and were incubated for 24 and 48 hours respectively. Cell cycle disturbance was induced at 2 and 4 µM concentrations after 24 hours post treatment. It was characterized by a significant elevation in G2/M and S cell populations in the expense of G0/G1 in a dose dependent manner. Moreover, a significant elevation in hypo-diploid subG1 cell population was observed particularly at a higher concentration (4 µM). The same trend was demonstrated after 48 hours of exposure with the test compound [**Figure 4**].
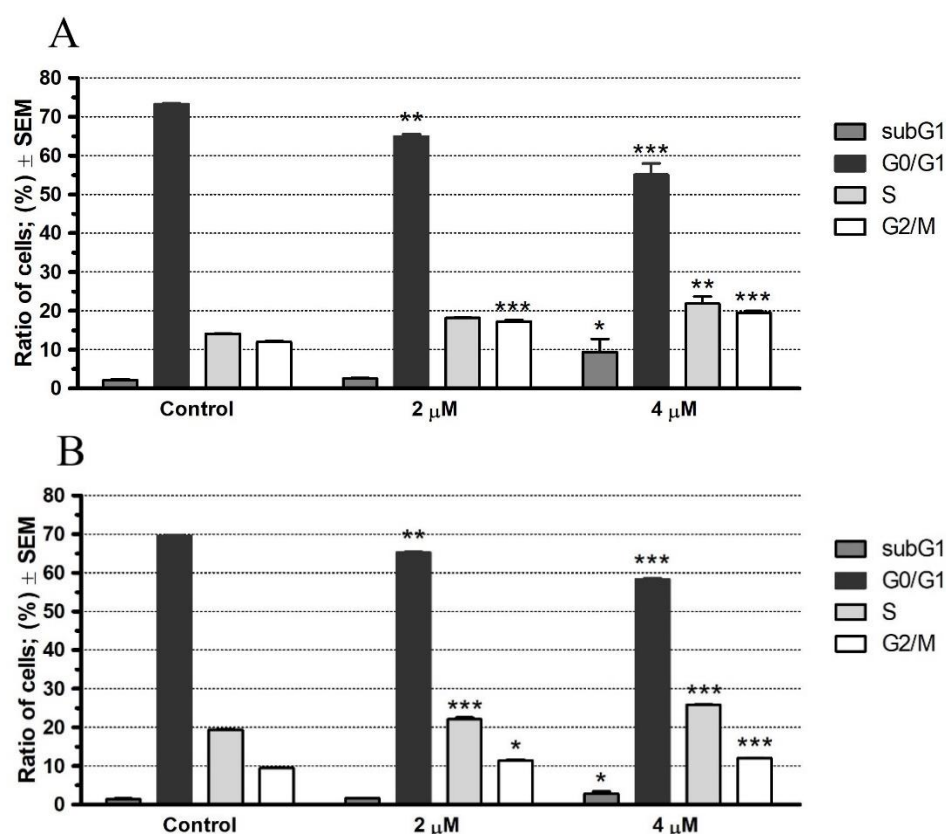
Figure 4. Cell cycle analysis by flowcytometry.

Compound **47** displayed a cell cycle disturbances, characterized by an increased rate of the S and G2/M cell populations at the expense of G0/G1. The upper and lower panels show the effects of **47** on cell cycle phases at 24 (**A**) and 48 (**B**) hours post treatment respectively.

** and *** indicate significance at $p < 0.01$ and $p < 0.001$, respectively

4.3. HOPI fluorescent double staining

To determine the ability of the test compound to induce morphological changes represented by necrosis and apoptosis, HOPI staining was performed as previously described. Changes in cell morphology and membrane integrity were observed in SiHa cells after 24 hours post treatment. Based on our results, fluorescent images demonstrated a remarkable reduction in viable intact cell population and a significant elevation in cells emitting light blue fluorescence (apoptotic) due to DNA condensation as well as necrotic cells emitting red fluorescent due to damaged cell membrane [**Figure 5**]. The obtained data suggest that, compound **47** was able to induce programmed cell death in a concentration dependent manner.
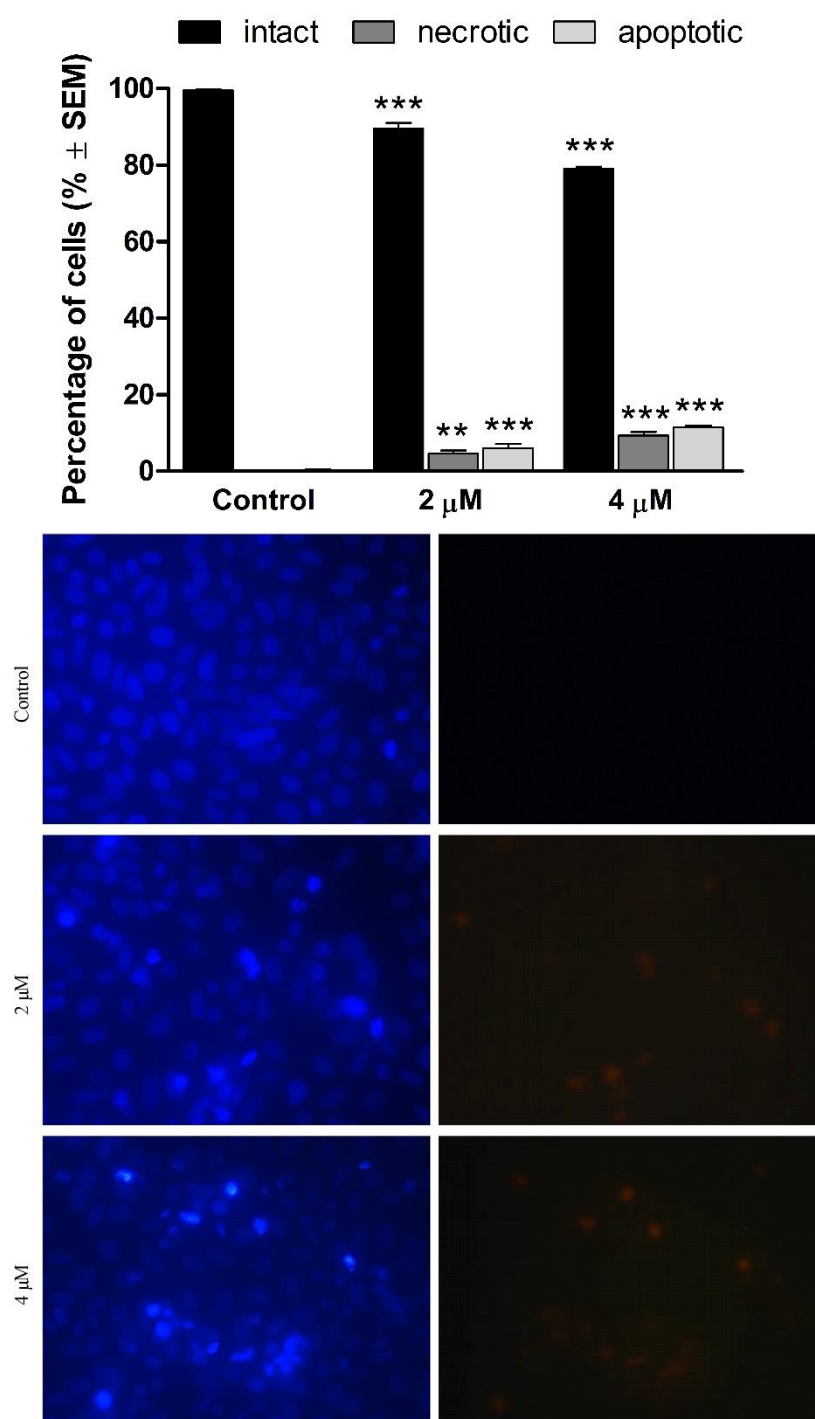
Figure 5. Morphological changes of SiHa after 24 hours of exposure to compound **47** visualized by HOPI staining. A significant decrease in viable cells as well as a remarkable increase in both necrotic and apoptotic cell populations were induced by **47**.
Upper panel: percentages of viable, necrotic and apoptotic cell populations
Lower panel: representative fluorescent images (10x magnification)
** and *** indicate significance at p < 0.01 and p < 0.001, respectively

4.4. Determination of caspase-3 activity

The significant elevation in hypo-diploid population in cell cycle analysis provided a preliminary clue about the ability of compound **47** to induce apoptosis. Therefore, after confirming the proapoptotic effects of the test compound evidenced by HOPI staining, it was a crucial step to identify the apoptotic pathway. For that purpose, a colorimetric determination of caspase-3 enzyme activity was performed. SiHa cells were treated with 2 and 4 µM concentrations and incubated for 24 hours. A 3-fold increase in caspase-3 enzyme activity was detected after 24 hours of incubation particularly at higher concentration (4 µM) indicating that the apoptosis was induced via the intrinsic pathway [**Figure 6**].
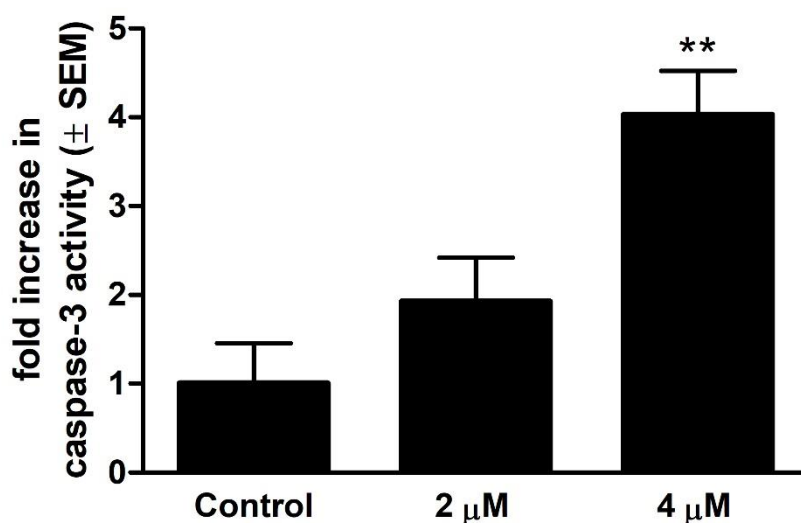
Figure 6. Caspase-3 activity

The proapoptotic property of the test compound was confirmed by assessing caspase-3 activity on SiHa cells after 24 hours post treatment. At a concentration of 4 µM, compound **47** induced a 3-fold increase in caspase-3 enzyme activity compared to untreated control cells.
** indicates significance at $p < 0.01$.

4.5. Tubulin polymerization

To illustrate the effects of the test compounds on microtubules, a photometric cell-free tubulin polymerization assay was conducted. The previously conducted computational simulations investigated the existence of conformational spaces of the ligands and their ability to bind to the taxoid binding sites.[45] Based on these calculations, compounds (**23**, **34**, **46** and **47**) were chosen for tubulin polymerization assay. Two concentrations were chosen (125 and 250 μM) based on the previously calculated IC_{50} values as well as the manufacturer instructions. A significant increase in tubulin polymerization was induced by all test compounds compared to the negative control. Next, it was essential to calculate the maximum rate of tubulin polymerization (Vmax) for each compound to determine their efficacy of polymerizing tubulin. The calculated Vmax values were significantly higher in all compounds even at lower concentrations, however, they were lower than the reference agent paclitaxel [**Figure 7**]. The obtained data suggest that, the antiproliferative potential of the tested compounds are elicited through the disturbance of tubulin polymerization.

4.6. Wound healing

To illustrate the effects of compound **47** on cell migration, wound healing assay was conducted in a serum reduced medium (2% FBS) using special silicon inserts as in vitro model of wound induction. SiHa cells were initially treated with 0.5 and 1 μM and incubated for 24 and 48 hours respectively. Data obtained from images were used to calculate the cell migration percentage towards the wound closure site (wound closure %). Compound **47** exerted a statistically significant decrease in cell migration compared to untreated controls after 24 and 48 hours of incubation respectively [**Figure 8**]. The obtained results suggest that, the test compound has potent anti-migratory effects at both 0.5 and 1 μM concentrations.
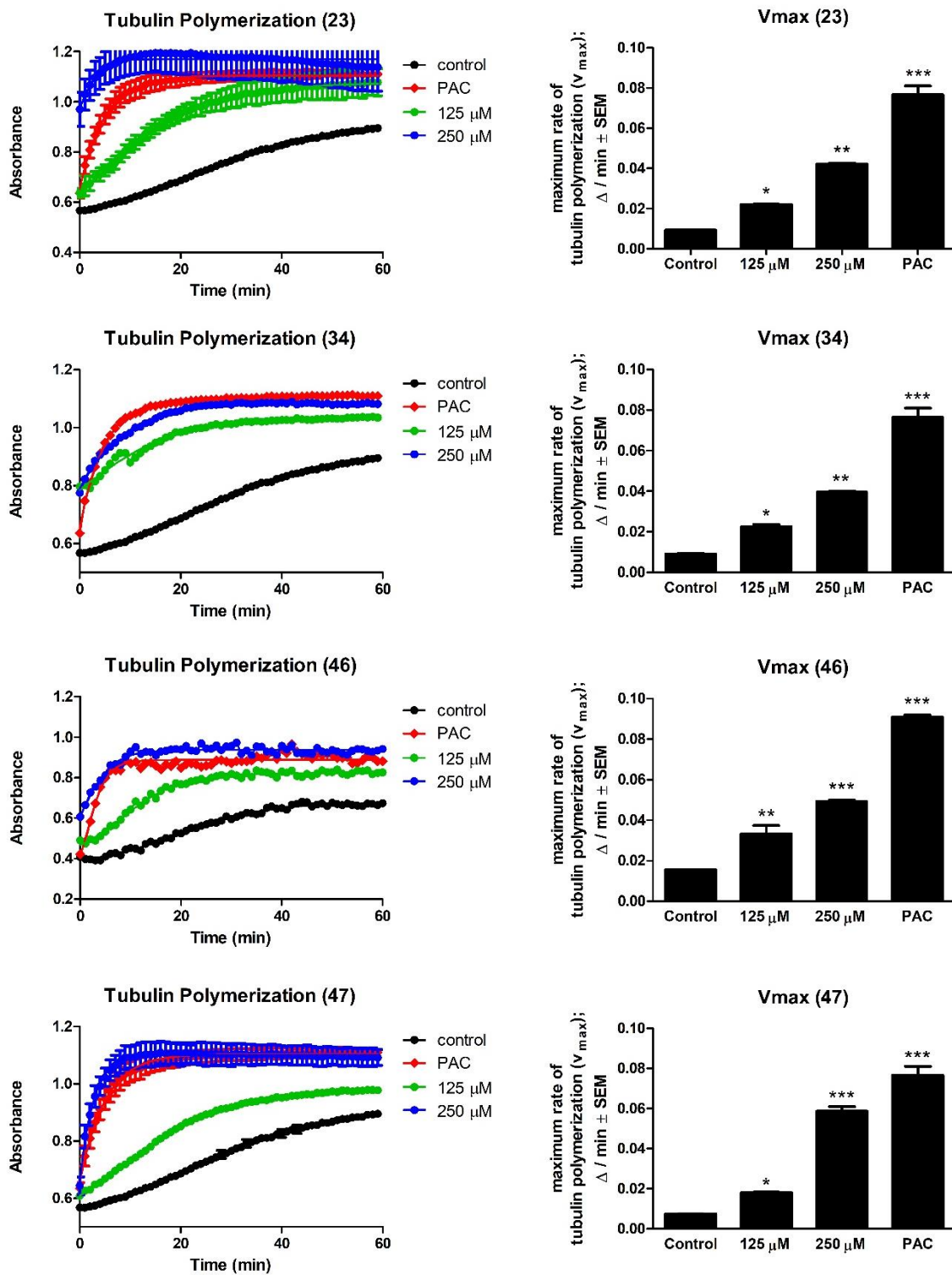
Figure 7: Tubulin polymerization assay. **Left panels**: trends of tubulin polymerization compared to the positive control (paclitaxel 10 μM) and to vehicle. **Right panels**: calculated maximum rates of tubulin polymerization (Vmax). *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

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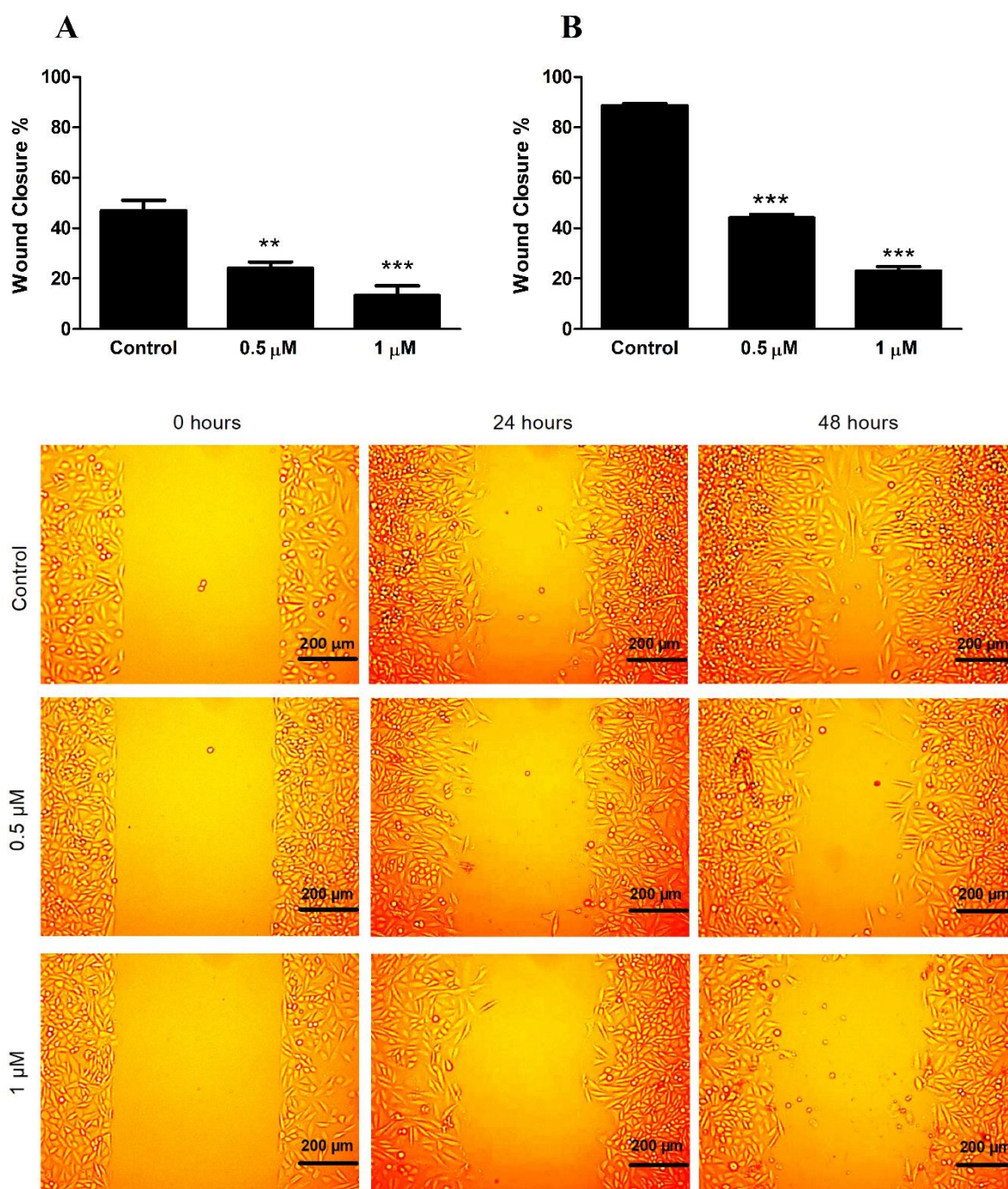
Figure 8: Cell migration (wound healing) assay. **Upper panels**: effects of compound **47** on SiHa cell migration at 24 (**A**) and 48 (**B**) hours post-treatment. **Lower panel**: representative images of the antimigratory effects of **47** at 0, 24 and 48 h post-treatment. ** and *** indicate significance at p < 0.01 and p < 0.001, respectively.

4.7. Boyden chamber assay

To determine the anti-invasive capacity of the test compound, Boyden chamber assay was performed using special chambers as in vitro model for tumor microenvironment. SiHa cells were treated with a series of concentrations (0.25, 0.5 and 1 µM) and incubated for 24 hours. Image based data were analyzed per each sample to calculate the invasion percentage of cancer cells. Based on our results, compound **47** displayed a substantial dose dependent decrease in invasion % compared to untreated samples after 24 hours of exposure [**Figure 9**]. Data suggest that, compound **47** exerted a significant anti-invasive capacity at 0.5 and 1 µM concentrations respectively. Moreover, the anti-invasive effects are obviously starts at 0.5 µM and are substantially lower than the IC_{50} value.
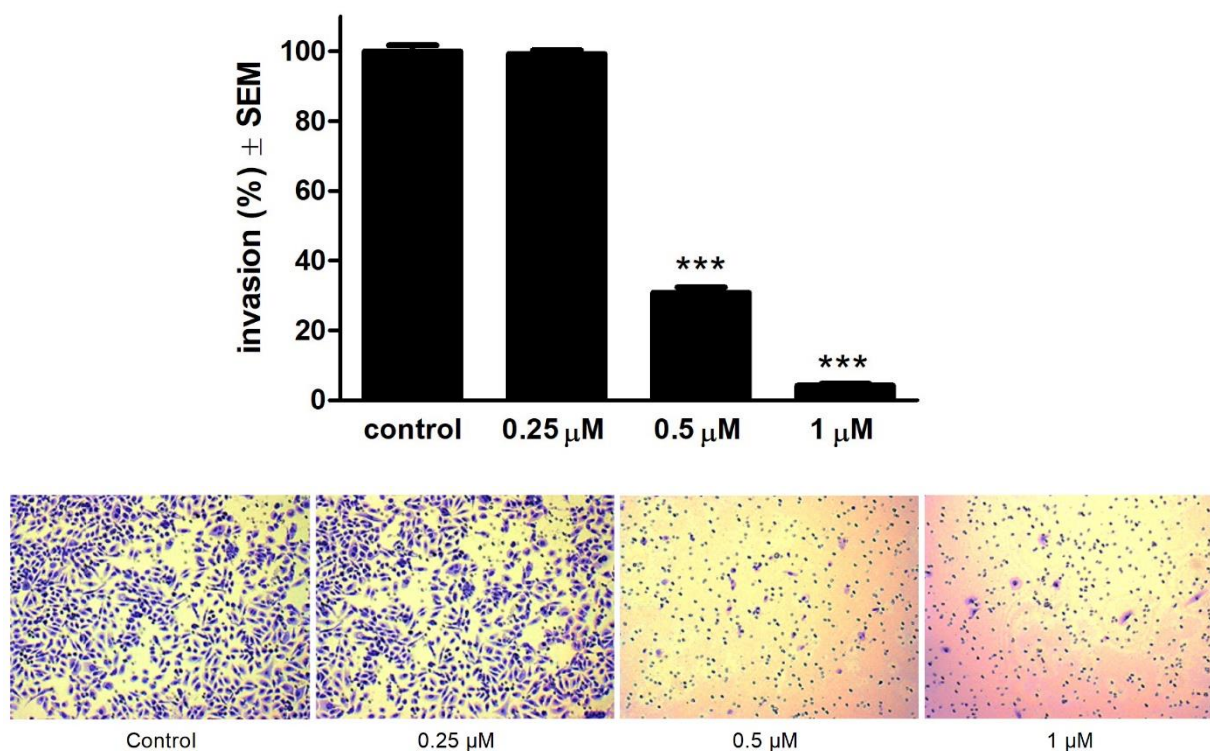
Figure 9: Boyden chamber assay.

Compound **47** elicited a significant anti-invasive effect on SiHa cells, at subantiproliferative concentrations. **Upper panel**: anti-invasive effect of **47** at 24 h post-treatment. *** indicates significance at $p < 0.001$. **Lower panel**: microscopic images showing the density of invasive cancer cells after crystal violet staining (10× magnification).

5 DISCUSSION

It is well documented that steroids demonstrate a pivotal role in several biological processes via genomic and non-genomic signaling pathways.[48] However, their major contribution in progressing multiple gynecological carcinomas such as breast and ovarian cancers has also been well evidenced.[49, 50] The hallmark mechanism that links estrogen to cancer progression is highlighted by its ability to enhance cell proliferation through cyclin G1 overexpression.[51] Therefore, antiestrogen therapy that based on targeting estrogen receptors and inhibiting cell proliferation, has been widely used for treatment of various hormonal dependent gynecological carcinomas mainly breast and ovarian cancers.[52-54] Pharmaceutical research have shaded a particular focus on steroid molecules because of their unique structural framework. In addition to their ability to penetrate cell membrane through the previously mentioned signaling pathways, substituting functional groups at specific positions resulted in a remarkable improvement in biological activities.[55, 56]

Nevertheless, there is also another concept that contradicts the common notion concerning the orchestrating role of estrogen in cervical carcinoma. It was concluded that, estrogen has no or little influence on SiHa, because these cells lack and/or possessing very low expression rate of estrogen receptors. Studies examined the expression of ER in normal uterine cervical tissues versus the HPV16 positive cervical carcinoma indicated that, the ER expression in the tested samples was decreased to undetectable levels.[57, 58] Moreover, immunohistochemical analysis revealed no marked difference in ER expression in normal tissues compared to HPV16 positive UCC.[59]

The aim of the present study was to investigate the antineoplastic effects of the newly synthesized A-ring modified 13α-estrone derivatives, with a particular focus on determining in vitro antiproliferative, antimetastatic and proapoptotic properties of the most potent agent; 2-(4-chlorophenyl)-13α-estrone sulfamate (compound **47**) against a panel of human gynecological cancer cell lines specifically SiHa.

Steroid-based anticancer agents constitute an essential group of cancer therapeutics. They are either natural, synthetic or even semisynthetic. They exert mechanism of action through different pathways ranging from receptor antagonism to the direct antiproliferative potential. Cyproterone acetate was reported as a first steroid with anticancer effects that came into research attention in

1968. Since then, many steroid-based agents have been synthesized and tested, however, not all of them were practically used because of the unexpected adverse side effects.[60] Main categories of anticancer agents of steroid origin are summarized below.

Estradiol-related compounds with direct antiproliferative activity are described as agents that exert cytotoxic activity without interfering with steroids hormonal receptor pathways. The light had been shade on this line of research since the 90s when 2-methoxyestradiol (2-ME2) was discovered as a first antiprolferative agent of estrane origin with direct inhibitory effects against MCF-7.[61] Further investigations involving its mechanism of action revealed that, the antiproliferative capacity of 2-ME2 is mediated by cell cycle arrest, activation of both intrinsic and extrinsic apoptotic cascades.[62,63] 2-ME2 proved to exhibit antiangiogenic activity mediated through down-regulation of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF).[64] Moreover, in vitro studies indicated that 2-ME2 possess antimetastatic potential through inhibiting cancer cell migration, invasion and cell adhesion which could be associated with its antitubulin effects.[65]

Since 2-ME2 has limited biological activity and rapid metabolism in vivo, intensive research have been conducted towards synthesizing analogs that could overcome this obstacle. So far, more than 100 analogs have been tested and proved to have better antiproliferative activity than the original compound.[66] It was indicated that substitution of a small functional group at C-2 position of the A-ring seems to improve antiproliferative potential. Moreover, C-3 modification of the A-ring via substituting free hydroxyl group results in metabolic degradation. Concerning the D-ring, it was shown that numerous substitutions can be performed at positions C-16 and C-17 without influencing antiproliferative activity of the target analog.[67]

Conjugates of estradiol-related compound and anticancer agents came to attention for the first time in 1972 when the first therapeutic conjugate of estrogen with the anticancer agent estramustine was introduced. It is believed that these kinds of conjugates improve the efficiency of the clinical activity and site-specific delivery of the target agents.[68] Until so far, sixteen conjugates have been tested against various cancer cell lines. However, leukemia together with the ER-positive and ER-negative breast cancer cells seems to be most sensitive.[69]

It is well known that steroid sulfatase (STS) plays a crucial role in the biosynthesis of estrone from estrone sulfate and also dehydroepiandrosterone (DHEA) from dehydroepiandrosterone sulfate

(DHEAS).[70] These molecules may promote cell proliferation particularly in hormonal dependent breast cancers. In addition, elevated STS levels were documented in other cancer type's mainly ovarian and endometrial carcinomas. Therefore, targeting STS was a key element to block this synthetic cascade. The first STS inhibitor; estrone-3-*O*-sulfamate (EMATE) came into clinical practice in 1994 which then became so called the first generation of STS inhibitors along with its analogue (E2MATE).[71] Later, 2nd generation inhibitors were introduced having dual mechanism of action represented by enzyme inhibition as well as direct antiproliferative activity through inhibition of tubulin polymerization. Recently, the 3rd generation agents which are conjugated with aromatase inhibitors have also been tested and under clinical development.[72]

Besides 2-ME2 and its analogs, another category of estrane based compounds with A-ring modified have also been shown to exert in vitro antiproliferative activity against various cancer cell lines of gynecological origin. However, their mechanism of action seems to be different than 2-ME2 and its analogs. Most of these compounds were tested by Zupkó and colleagues for over than a decade.

The antiproliferative potentials of diverse groups of the core-modified 13α-estrone derivatives have been previously reported by our research group. These modifications were mainly substituted at positions C-2, C-4 and/or 3-OH of the hormonally inactive 13α-estrone core molecule. Certain compounds displayed remarkable growth inhibitory effects with submicromolar IC_{50} values.[44] It was observed that, nature, size and polarity of the introduced functional groups, prominently influences the growth inhibitory action of the synthesized compounds. In addition, introduction of the benzyl or benzyltriazolyl onto 3-OH significantly improved the antiproliferative action.[73-76] Therefore, a further attention was paid on testing 13α-estrone core derivatives bearing the above mentioned functions. As a consequence, various novel compounds have been synthesized having both enzyme inhibitory and antiproliferative actions.[77]

In addition to the antiproliferative activity, it was previously reported that, certain A-ring halogenated 13α-estrone derivatives are considered very promising compounds due to their diverse biological activities. The 17β-hydroxysteroid dehydrogenase 1 (17β-HSD1) and steroid sulfatase (STS) involved in estradiol biosynthesis could be inhibited by certain halogenated derivatives.[78] Moreover, the 3-bromo-3-hydroxy derivative also displayed dual inhibitory effects against 17β-HSD1 and the human organic-transporting polypeptide (OATP2B1) transporter.[79]

In this study, 3 sets of the newly synthesized compounds were tested against a panel of gynecological cancer cell lines. Beginning with the A-ring halogenated derivatives (**1-17**), certain compounds (**4, 13** and **9**) have been identified as potent antiproliferative agents. Among the tested cell lines, the estrogen receptor positive MCF-7 and the HPV18 positive HeLa were considered the most sensitive. None of the promising agents displayed a substantial growth inhibition against NIH/3T3 indicating their tumor selectivity. The most potent compound (**4**) displayed a remarkable antiproliferative activity against all tested cell lines but MCF-7 was considered the most sensitive. However, the most potent phenylated compound (**13**) exerted outstanding growth inhibition against MCF-7 and HeLa. It is worth mentioning that, compounds (**4**) and (**13**) behaved similarly concerning both breast cancer cell lines MCF-7 and MDA-MB-231. However, (**13**) differentiated between two uterine cervical carcinoma cell lines HeLa and SiHa. Based on the above explanation, it can be concluded that, substitution in the A-ring of the 13α-estrone core seems to improve the antiproliferative action.

Next, we investigated steroidal diaryl ethers (**18-29**) that were synthesized via direct arylation at the *C*-3-*O* function as reported previously. Among this group, compound **23** was highlighted as the most potent antiproliferative agent against all the tested cell lines. The substantial improvement in its biological activity could be due to the substitution of a nitrogen heteroatom into the introduced moiety. It should be emphasized that certain newly synthesized agents belong to this group exerted more growth inhibitory potential compared to the reference agent Cisplatin. Moreover, the HPV18 positive HeLa was considered the most sensitive cell line to almost the majority of compounds.

With regards to the last set (**30-47**), the carmabate, pivalate and sulfamate derivatives were synthesized by substituting *N*- and/or *O*-containing DGs to the phenolic 3-OH functions of the 13α-estrone and its 17-deoxy counterparts. Within this group, certain potent antiproliferative agents were identified (**34, 46 and 47**) and interesting structural activity relationships were detected. Compound **34** (bearing N, N-dimethyl pharmacophore combined with 17-keto group) displayed a potent antiproliferative action against both MCF-7 and HeLa. Moreover, the cell growth inhibitory potential was substantially improved by introducing 4-cholophenyl moiety onto C-2 position of the A-ring. Both HPV18 and HPV16 positive cervical cells were substantially inhibited by **46** and **47** in a very low micromolar range.

It is important to declare that, an interesting structural relationship between compounds (**34, 46 and 47**) was noticed. No modification at C-2 was done in compound **34**, whereas the 2-phenyl functional group was substituted in compounds **46 and 47**. Based on the above explanation, it can be concluded that the antiproliferative potential of *C*-3-*O* modified 13α-estrone derivatives greatly depends on the nature of the introduced moieties. Moreover, 4-chlorophenylation together with N, N-dimethyl pharmacophore substantially improved the antiproliferative potential against HPV16 positive cervical cells (SiHa). Compared to others, compound **47** proved to be the most potent agent particularly against HPV16 positive SiHa, therefore, it was used for further analysis.

Following identification of the most potent antiproliferative agent, it was also essential to determine its tumor selectivity. For that purpose, **47** was tested against the non-cancerous mouse fibroblast cell line (NIH/3T3). The growth inhibition % was not more than 50 even at a higher concentration (30 µM). Furthermore, the tumor selectivity index (TSI) was measured to determine the selectivity status of the candidate compound. The TSI was recorded less than 10 but more than 1 indicating that, our candidate compound (**47**) has moderate tumor selectivity.

Identifying the mechanism of action of the newly synthesized candidate drugs, is considered a crucial step in pharmaceutical research. Based on this concept, we attempted to demonstrate the effects of compound **47** on cell cycle distribution. The test compound was able to induce cell cycle disturbance characterized mainly by a statistically significant elevation in both G2/M and S phases in the expense of G0/G1 cell population. It is worth mentioning that, elevation of subG1 cell population particularly at a higher concentration was also noticed providing a preliminary clue regarding the capability of compound **47** to induce apoptosis.

Programmed cell death (apoptosis) is an orchestrated cellular process in many physiological and pathological conditions. In cancer biology, it is well known that apoptosis doesn't occur frequently due to down regulation of p53, a tumor suppressor gene, which subsequently enhances cell proliferation, invasion and metastasis.[80, 81] In addition to its role in cancer progression, defects in apoptosis may result in resistance to cytotoxic cancer therapies.[82, 83] Therefore, understanding the molecular mechanisms that regulate apoptosis and developing agents that trigger apoptosis, may provide novel opportunities in the field of cancer therapeutics.

To investigate the ability of compound **47** to induce apoptosis and to study the morphological alterations upon necrosis and apoptosis, the treated SiHa cells were stained with fluorescent

Hoechst and PI double staining. A significant reduction in viable cells as well as a significant increase in both necrotic and apoptotic cell populations was noticed. These data suggest that our test compound exhibits proapoptotic effects.

Understanding the mechanism of apoptosis is a crucial step in determining the precise approach by which a drug exerts its proapoptotic effects. Since apoptosis is orchestrated by caspases, it is achieved via two main pathways; extrinsic and intrinsic.[84] Concerning the first mechanism, apoptosis is initiated when the death ligands (TNF and FasL) bind to their counterpart receptors (Fas and TNFR1) initiating intracellular death domains represented by Fas-associated death domain (FADD), TNF-associated death domain as well as cysteine proteases mainly caspase-8.[85] Secondly, apoptosis by the intrinsic pathway is usually initiated within the target cell when the internal stimuli such as oxidative stress molecules, DNA damage and hypoxia increase the mitochondrial permeability and release the proapoptotic molecules mainly the cytochrome c into the cytoplasm, eventually activating caspase-3.[85, 87] Based on the above explanation, the significant fold increase in caspase-3 enzyme activity in our treated SiHa cells 24 hours post treatment suggests that, compound **47** could induce apoptosis via the intrinsic pathway.

Numerous epidemiological studies highlighted the association of metastasis with the high proportion of cancer-related deaths worldwide. It is estimated that, metastasis is the primary cause of death in more than 90% of cancer patients.[88] Furthermore, it is also responsible for the development of chemotherapeutic drug resistance.[89] Concerning survival rates in metastatic uterine cervical carcinoma, studies indicated that, the 5-year survival rate in metastatic status was 16.5% compared to 91.5% in non-metastatic cancer patients.[90] The initiation of metastasis involves a series of events including migration of cancer cells from primary tumor sites via blood circulation and eventually invading distant organs after escaping immune surveillance.[91] Studies on experimental models of metastasis as well as patient-derived tumor gene expression have identified genes expressed on cancer cells that promote metastasis.[88] It's believed that these metastasis-promoting genes enhance cell migration, invasion, extravasation and resistance to stromal and metabolic stresses.[89]

Metastasis in cervical cancer is categorized into; hematogenous in which the disseminated cells invade distant organs via blood stream mainly lungs and bones. Secondly, the lymphatic metastasis that involves invading regional lymph nodes.[92, 92] Regarding the risk status of

metastasis, studies emphasized that, cancer patients with the hematogenous metastasis have a higher risk of death compared to the lymphatic type.[94]

Since the cell migration and invasion are considered key players in metastasis, the in vitro antimetastatic properties of our test compound was assessed using wound healing and Boyden chamber assays. As previously described, compound **47** substantially inhibited cell migration in a dose dependent manner after 24 and 48 hours of exposure. Moreover, it also displayed dose and time dependent anti-invasive effects in micro molar ranges compared to untreated samples. Based on the obtained data, it can be concluded that, compound **47** exhibit potent antimetastatic effects even at very low concentrations.

Mircotubules (MTs) are major components of the cytoskeleton. Because of their crucial role in cell division, it has been considered as a highly attractive target for anticancer drug design.[95] Tubulin-binding agents (TBAs) such as vinca alkaloid and taxanes are widely used chemotherapeutic drugs for treatment of a diverse range of cancers including breast, lung, prostate and ovary.[96] Generally, TBAs interfere with MT functions by inhibiting cell proliferation and preventing formation of mitotic spindle, leading to mitotic arrest in the metaphase transition and subsequently apoptosis.[97] TBAs are generally divided into two distinct categories; MT-stabilizing and MT-destabilizing agents. The first group such as taxanes (e.g. paclitaxel) stabilize microtubules through binding to the β-tubulin subunit of α/β-tubulin of the MT wall, whereas destabilizing agents (e.g. vinca alkaloids) target the same site but in the tubulin heterodimer.[98, 99] Despite the considerable success of TBAs in cancer therapy, drug resistance still remains the main challenge. Studies emphasized that the development of resistance towards TBAs is associated with alterations in tubulin system including changes in tubulin isotype expression, posttranslational modifications of tubulin, development in mutations and changes in the expression levels of MT-related proteins.[100, 101]

To explore the effects of our test compounds on MTs, computational simulations were conducted [45, 102] to investigate the ligand binding character to the taxoid binding site. Based on these protein-ligand interaction results, compounds **23**, **34**, **46** and **47** were chosen as candidate agents for tubulin polymerization assay (TPA). As expected, all the four compounds displayed a disturbance in tubulin polymerization characterized by increase in the maximum rate of tubulin polymerization (Vmax). This trend was concentration-dependent even at the lower concentration. Accordingly, it

can be concluded that, compounds **23**, **34**, **46** and **47** exert potent effects on microtubular system through stabilizing microtubules. Moreover, the antiproliferative properties of these compounds may be elicited through disturbance in tubulin polymerization.

6 SUMMARY

As concluding remarks, the main findings in this study can be summarized below;

- Among all the tested groups of the A-ring modified 13α-estrone derivatives, compound **47** was highlighted as the most potent antiproliferative agent. It exerted an outstanding growth inhibitory effects against the HPV16 positive human invasive uterine cervical cancer cell line (SiHa) in a low micromolar range ($IC_{50} = 2.71$ μM).

- The tumor selectivity index was between 0.1-10 indicating moderate tumor selectivity.

- The mechanism of action of the candidate agent (**47**) was investigated by cell cycle analysis using flowcytometry. Cell cycle disturbance was induced characterized by increase in the G2/M and S phases in the expense of the G0/G1 cell population.

- Based on the significant elevation in subG1 population in cell cycle analysis, the proapoptotic effects were further evidenced by Hoechst/PI fluorescent double staining as well as caspase-3 measurement. It was shown that, compound **47** was able to induce apoptosis via the intrinsic pathway.

- The antimetastatic capacity was determined by cell migration and Boyden chamber assays. Compound **47** demonstrated potent antimetastatic properties even at submicromolar ranges.

- Tubulin polymerization assay indicated that, compounds **23**, **34**, **46** and **47** possess direct effects on the microtubules through disturbance of tubulin polymerization. Moreover, the antiproliferative effects may be elicited through increase in maximum rate of tubulin polymerization.

  To the best of our knowledge, compound **47** is considered the first known 13α-estrone derivative with such a high potency against SiHa described in the literature. Therefore, it should be considered as a model for designing new anticancer agents targeting cervical carcinomas.

7 GLOSSARY OF ABBREVIATIONS AND ACRONYMS

- **17β-HSD1 :** 17β-hydroxysteroid dehydrogenase 1
- **2-ME2:** 2-methoxyestradiol
- **ANOVA:** Analysis of variance
- **bFGF:** Basic fibroblast growth factor
- **CAR-T:** Chimeric antigen receptor T cells
- **CASPASE:** Cysteine-aspartic protease
- **CTLA-4:** Cytotoxic leukocyte antigen 4
- **DHEA:** Dehydroepiandrosterone
- **DHEAS:** Dehydroepiandrosterone sulfate
- **DMSO:** Dimethyl sulfoxide
- **DNA:** Deoxyribonucleic acid
- **E1:** Estrone
- **E2:** Estradiol
- **E3:** Estraiol
- **EMATE:** Estrone 3-*O*-sulfate
- **ER:** Estrogen receptor
- **ERE:** Estrogen responsive element
- **FADD :** Fas-associated death domain
- **Fas:** Apoptosis-mediating surface antigen
- **FasL:** Fas ligand (CD178)
- **FBS:** Fetal bovine serum
- **G1:** First growth phase in the cell cycle
- **G2/M:** Second growth phase in the cell cycle / mitotic phase
- **HER2:** Human epidermal growth factor 2
- **HO:** Hoechst stain
- **HPV:** Human papillomavirus
- **HRE:** Hormone responsive element

- **IC$_{50}$:** Half maximal inhibitory concentration

- **ICC:** Invasive cervical carcinoma

- **miRNAs:** microRNAs

- **MTs:** Microtubules

- **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

- **NR:** Nuclear receptor

- **OATP2B1:** Organic anion-transporting polypeptide 2

- **P:** Probability value (level of statistical significance)

- **p53:** Tumor suppressor gene

- **PBS:** Phosphate buffer saline

- **PD-1:** Programmed death 1

- **PET:** Polyethyline terephthalate

- **PI:** Propidium iodide

- **PR:** Progesterone receptor

- **RNA:** Ribonucleic acid

- **S:** Synthesis phase in the cell cycle

- **SEM:** Standard error of mean

- **STS:** Steroid sulfatase

- **SubG1:** Hypodiploid cell population

- **TBA:** Tubulin polymerization assay

- **TBAs:** Tubulin binding agents

- **TNF:** Tumor necrosis factor

- **TNFR1:** Tumor necrosis factor receptor 1

- **TSI:** Tumor selectivity index

- **UCC:** Uterine cervical carcinoma

- **VEGF:** Vascular endothelial growth factor

- **V*max* :** Maximum rate of tubulin polymerization

- **WHO :** World Health Organization

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8 AKNOWLEDGMENTS