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Characterization of antiproliferative and antimetastatic properties of novel terpene derivatives

Ph.D. Thesis

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SCIENTIFIC PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

1. Ozsvár, D.; Nagy, V.; Zupkó, I.; Szakonyi, Z. Stereoselective Synthesis and Antiproliferative Activity of Steviol-Based Diterpen Aminodiols. *Int J Mol Sci.* **2019**, *21* (1): e184. https://doi.org/10.3390/ijms21010184.
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2. Ozsvár, D.; Nagy, V.; Zupkó, I.; Szakonyi, Z. Synthesis and Biological Application of Isosteviol-Based 1,3-Aminoalcohols. *Int J Mol Sci.* **2021**, *22* (20): e11232. https://doi.org/10.3390/ijms222011232.
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3. Raji, M.; Le, T. M.; Huynh, T.; Szekeres, A.; Nagy, V.; Zupkó, I.; Szakonyi, Z. Divergent Synthesis, Antiproliferative and Antimicrobial Studies of 1,3-Aminoalcohol and 3-Amino-1,2-Diol Based Diaminopyrimidines. *Chem Biodivers.* **2022**, *19* (5): e202200077. https://doi.org/10.1002/cbdv.202200077.
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4. Raji, M.; Le, T. M.; Csámpai, A.; Nagy, V.; Zupkó, I.; Szakonyi, Z. Stereoselective Synthesis and Applications of Pinane-Based Chiral 1,4-Amino Alcohol Derivatives. *Synthesis.* **2022**, *54* (17): 3831–3844. https://doi.org/10.1055/s-0040-1719887.
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5. Nagy, V.; Mounir, R.; Szebeni, G. J.; Szakonyi, Z.; Gémes, N.; Minorics, R.; Germán, P.; Zupkó, I. Investigation of Anticancer Properties of Monoterpene-Aminopyrimidine Hybrids on A2780 Ovarian Cancer Cells. *Int J Mol Sci.* **2023**, *24* (13): e10581. https://doi.org/10.3390/ijms241310581.
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II. Kulmány, Á. E., Herman, B. E., Zupkó, I., Sinreih, M., Rižner, T. L., Savić, M., Oklješa, A., Nikolić, A., Nagy, V., Ocsovszki, I., Szécsi, M., & Jovanović-Šanta, S. Heterocyclic Androstane and Estrane D-ring Modified Steroids: Microwave-Assisted Synthesis, Steroid-Converting Enzyme Inhibition, Apoptosis induction, and Effects on Genes Encoding Estrogen Inactivating Enzymes. *J Steroid Biochem Mol Biol.* **2021,** 214: e105997. https://doi.org/10.1016/j.jsbmb.2021.105997

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1. Introduction

1.1. Epidemiology and overview of malignant disorders

According to the estimates of the World Health Organization (WHO), malignant disorders (or cancer) represents one of the major health burdens globally.[1] Besides cardiovascular disease, they are second leading cause of death worldwide, and still a critical and unsolved public health issue. Based on the most recent survey by the Global Cancer Observatory (GLOBOCAN), 19.3 million new cancer cases and almost 10.0 million cancer-related deaths occurred in 2020 alone[1]; while in 2000, 10.1 million new cases and 6.2 million tumor-related deaths were estimated.[2] Based on the comparison of the epidemiological data corresponding to the two time periods, it could be concluded that the last twenty years were characterized by increasing trends in the development of new cancer cases and cancer-related mortality.[3] Moreover, several studies suggested that the global cancer burden will continue to rise considerably in the future.[2] The number of new cases may reach 23.6 million by 2030.[4,5] In developed countries, the risk of cancer may raise to 78 % among the population over 55 years of age, respectively.[6] Several factors, including aging, and population growth , as well as the prevalence and distribution of the main risk factors related to cancer, may be associated with the rapidly growing cancer incidence and mortality globally.[1,7]

The complex pathophysiology of gynecological malignancies has not been completely understood. However, several risk factors have been identified, including genetic predisposition (e.g., due to mutations of the breast cancer genes BRCA1/BRCA2), hormone replacement therapy after menopause, human immunodeficiency virus (HIV) infection, human papillomavirus (HPV) infection, and other corresponding health conditions.[8–11] These factors may play a major role in the mutation of the DNA content in cells, subsequently leading to uncontrolled cell division and the development of a malignancy.[12,13]

Among females, breast cancer is the most frequently diagnosed type among cancers, as it accounts for 25% of all cases and 15% of all cancer-related deaths.[7,14] While cervical cancer is the fourth most commonly diagnosed tumor, cancer of the cervix is the second most commonly diagnosed malignancy after breast cancer in developing countries, where it is the third leading cause of cancer death following breast and lung cancers.[14] Regarding the causative factors, cervical tumors are most commonly caused by infections and persistence of a high-risk HPV virus, especially HPV genotypes 16 or 18. During the last twenty years,

vaccination and screening methods became widely available in developed countries, therefore the number of deaths have decreased considerably.[15]

Ovarian cancer is also a notable type of gynecological malignancies and the 7th most commonly detected cancer in women.[14] Based on epidemiological data, it accounts for only 3 percent of female cancers; despite this fact, ovarian cancer is the most lethal female reproductive cancer and accounts for the highest mortality rate, with 5-year survival rates below 45%.[16] Although the efficacy of ovarian cancer treatment has improved and there were some promising advances in cancer research in the last decade, 5-year survival after treatment is still remains below 30%.[17] Ovarian cancer is not a specific term, it encompasses several types of malignant disorders that all arise from the cells of the ovaries in the female reproductive system. There are three main classes of ovarian cancer: epithelial, germ cell and specialized stromal cell tumors. In the majority of cases, tumors are of epithelial origin. Based on WHO recommendations, epithelial ovarian carcinomas (EOC) may be divided into several morphological categories according to cell type: serous carcinomas (SC), mucinous carcinomas (MC), endometrioid carcinomas (EC), and clear-cell carcinomas (CCC), transitional-cell Brenner tumors, mixed, and undifferentiated type.[18,19] These subtypes may be further distinguished in their etiology, morphology, molecular biology and prognosis. However, they are treated as a single entity in clinical practice, as the standard treatment consists of cytoreductive surgery and chemotherapy with platina/taxane combination.[18] While the response rate to the first line therapy is usually good (around 80-90%), most of patients will have a recurrence of cancer and resistance of chemotherapy, thus, a cycle of repeated surgeries and additional rounds of chemotherapy have to be applied.[20] In addition, robust methods for early detection are not available, and the lack of specific symptoms – together with the reluctance of patients to seek medical attention – may be a contributor factor to delayed diagnosis. Thus, the majority of women are diagnosed when the cancer has already metastasized to other tissues.[20–22] The presence of metastases is often associated with poor survival rates. Several studies have reported that patients with a distant metastasis had decreased overall 5-year survival rates compared to the patients with localized or regional disease.[23] The major risk factors of ovarian cancer are a family history of the disease and genetic predisposition. If an ovarian carcinoma occurred in one first degree relative, a woman's lifetime risk will raise by 5%. Women with specific gene mutations (e.g., BRCA1 or BRCA2), are evidently at higher risk of ovarian cancer (23 - 54 %).[24] During the last decade, several additional gene mutations have been identified, namely, mutation of MLH1, MSH2, RAD51C, RAD51D, and BRIP1 genes, they also have elevated risk of ovarian cancer.[25] There are some nongenetic risk factors as well,

such as postmenopausal hormone therapy (particularly if used for more than five years), delayed childbearing, endometriosis, and lifestyle factors, for instance, obesity and smoking.[24,26]

Although rapid advancements in personalized medicine and in extensive preventive, diagnostic and therapeutic strategies could be observed in the last decades, the number of new cancer cases continues to grow worldwide. Therefore, the development of novel treatment options and anticancer agents are eagerly awaited.

1.2. Anticancer agents from natural sources

A considerable amount of literature has been published on natural products, which are a vital source of leads for anticancer drug development. The flora plays a major role is the discovery of antitumor agents and active substances. Since the early 1940s, the research and development for agents that may treat cancer have been a major challenge for all aspects of chemistry and pharmacology.[27] Plants have a long history for being using in cancer treatment. In general, most of the materials screened were pure compounds of synthetic origin (e.g., mustard nitrogen: 1943[28], methotrexate: 1948[29]), however, in the last decades, it has been recognized that natural products are an excellent source of complex chemical structures, possessing a wide variety of biological activities.[30] A large amount of natural products have been introduced into clinical trials, as well as into the clinical practice, and also has been used as lead or model molecules for structure optimization and for the development of safer and more tolerable drugs.[31] More than half a million test substances – natural and synthetic compounds – have been screened between 1962 and 1980 in the USA by the National Cancer Institute (NCI). As a result, several anticancer agents had been discovered, namely, the vinca alkaloids (vinblastine, vincristine, and the semi-synthetic vinorelbine), synthetic derivatives of epipodophyllotoxin (etoposide and teniposide), in addition to the derivatives of camptothecin (irinotecan and topotecan).[32] It should be mentioned that paclitaxel (Taxol®), which was also discovered in this period, was approved for clinical use against ovarian cancer in 1992.[33] In the last 20 years, some agents have been introduced in clinical practice, e.g., flavopirodol has been shown to be a potent cyclin-dependent kinase (CDK) inhibitor, omacetaxine is a protein synthesis inhibitor, and combretastatin A4 have affect tubulin polymerization.[34–36]

1.3. Terpenoids

Terpenoids, are the largest class of natural products, representing an important group of secondary plant metabolites. They are composed of five carbon isoprene units, and may be divided into subclasses based on their structure.[37] Terpenoids act as alkylating agents by addition to intracellular targets, typically sulfhydryl groups of proteins, thereby inhibiting the function of enzymes required for cell proliferation.[38] They are able to target several stages of cancer development including cell proliferation, angiogenesis, and metastasis, and they can induce programmed cell death.[39] The anticancer effect of several terpenoids is mediated through targeting the nuclear factor κB (NF-κB), Janus kinases-signal transducer and activated of transcription proteins (JAK-STAT), activator protein-1 (AP-1), metalloproteinases (MMPs), DNA topoisomerase I and II, blocking the endoplasmic reticulum (ER) calcium ATPase pump, proteasome inhibition, activating p53, and modulating DNA minor grooves.[40] Terpenoids are reported to induce cell death by a special form of apoptosis, which is called autophagy. It is a self-eating process, characterized by the formation of double-membrane vacuoles, autophagosomes, as well as merging with lysosomes, which can lead to degradation of vital biomolecules, thus, organelles of the cell become dysfunctional.[41]

1.3.1. Diterpenoids

Based on the number of carbons included by the isoprene units they contain, terpenoids may be divided into distinct groups. In recent decades, the diterpenoid stevioside with a complex *ent*-kaurane skeleton and three glucose moieties has been the focus of scientific attention.[42,43] Stevioside originates from the plant *Stevia rebaudiana*, which is commonly used in food chemistry as sweetener. A wide variety of biological activities of steviol have been described, including its antihypertensive, antitumor and immunomodulatory action.[44–46] These properties are associated with secondary plant metabolites, such as steviol glycosides (SGs), which are extracted from the leaf of the plant.[47] Several SGs has been identified, including stevioside, which also has anticancer properties.[48] A large and growing body of literature has investigated the anti-tumor effect of these derivates. Recently, *in vivo* studies have confirmed the lack of carcinogenicity of the glycosides. Interestingly, their potential as anticancer agents has also been revealed.[49] Based on literature data, it is hypothesized that stevioside induces a reactive oxygen-species (ROS)-mediated apoptosis.[50]

1.3.2. Monoterpenoids

Monoterpenes exhibit a wide variety of biological activities including anti-inflammatory, antibacterial and antioxidant. Numerous scientific works have explored bioactivity of monoterpenes, in particular anticancer potential, which have been proved in vitro in several cancer cell lines. [51,52] Monoterpenes and their derivates are key factors in the development of new biological active compounds. The structure of monoterpenes consists of two lined isoprene unit.[53] In medicinal chemistry, there are several studies which has been focused on coupling between aminoalcohols and pyrimidine. The presence of pyrimidine can lead to more diverse pharmacological properties, for instance several amino- or diaminopyrimidines are well-known bearing anti-inflammatory, anti-HIV, antiproliferative, or even antimalarial activity.[54–56]

In the development of novel compounds with natural sources, there are so many possibilities of structural modification. So, it can result in further promising terpenoid based compounds for anticancer investigations.

2. Aims and Objectives

During our experiments, our principal aim was to characterize the antiproliferative properties of recently synthesized diterpene, and monoterpene-based derivates on a panel of human gynecological cell lines *in vitro*. During our studies, potent derivatives have been identified, which were selected for further evaluation. Thus, the additional aim of our experiments was to examine the antiproliferative and antimetastatic characteristics, and to the determine the underlying mechanism of action of these promising compounds via *in vitro* experiments on an ovarian adenocarcinoma (A2780) cell line.

The purpose of the performed experiments were the following:

- Characterization of the antiproliferative potential of diterpene and monoterpene hybrids test substances against a panel of human gynecological cancer cell lines by a standard MTT-assay.
- Determination of the inhibitory concentration (IC_{50}) values in the case of the most potent compounds.
- Estimation of tumor selectivity by measuring the growth inhibitory percentage against a non-cancerous mouse fibroblast cell line (NIH/3T3).
- Detection of pro-apoptotic properties of the most effective compounds by Hoechst 33258/propidium iodide fluorescent double staining (HOPI).
- Characterization of the elicited alterations in cell cycle progression of the A2780 cell line by flow cytometry.
- Determination of the effects of the selected compounds on microtubules by a cell-free tubulin polymerization assay.
- Determination of the antimetastatic potential of the most potent compounds in wound healing and Boyden chamber assays, respectively.

3. Materials and Methods

3.1. Chemicals

Altogether, 86 derivates had been investigated in this study. The steviol-based diterpene aminodiols, isosteviol-based 1,3-aminoalcohols and monoterpene-aminopyrimidine hybrids were synthesized in the Institute of Pharmaceutical Chemistry (Faculty of Pharmacy, University of Szeged, Szeged), as described previously.[42,43,57,58] The chemical structure of the compounds involved of this study are presented in **Figure 1-7**. 10 mM stock solutions of the tested agents were prepared with dimethyl sulfoxide (DMSO) for all *in vitro* experiments. The highest applied DMSO concentration of the medium (0.3 %) did not exceed toxic range, so did not have influence on the cell proliferation.

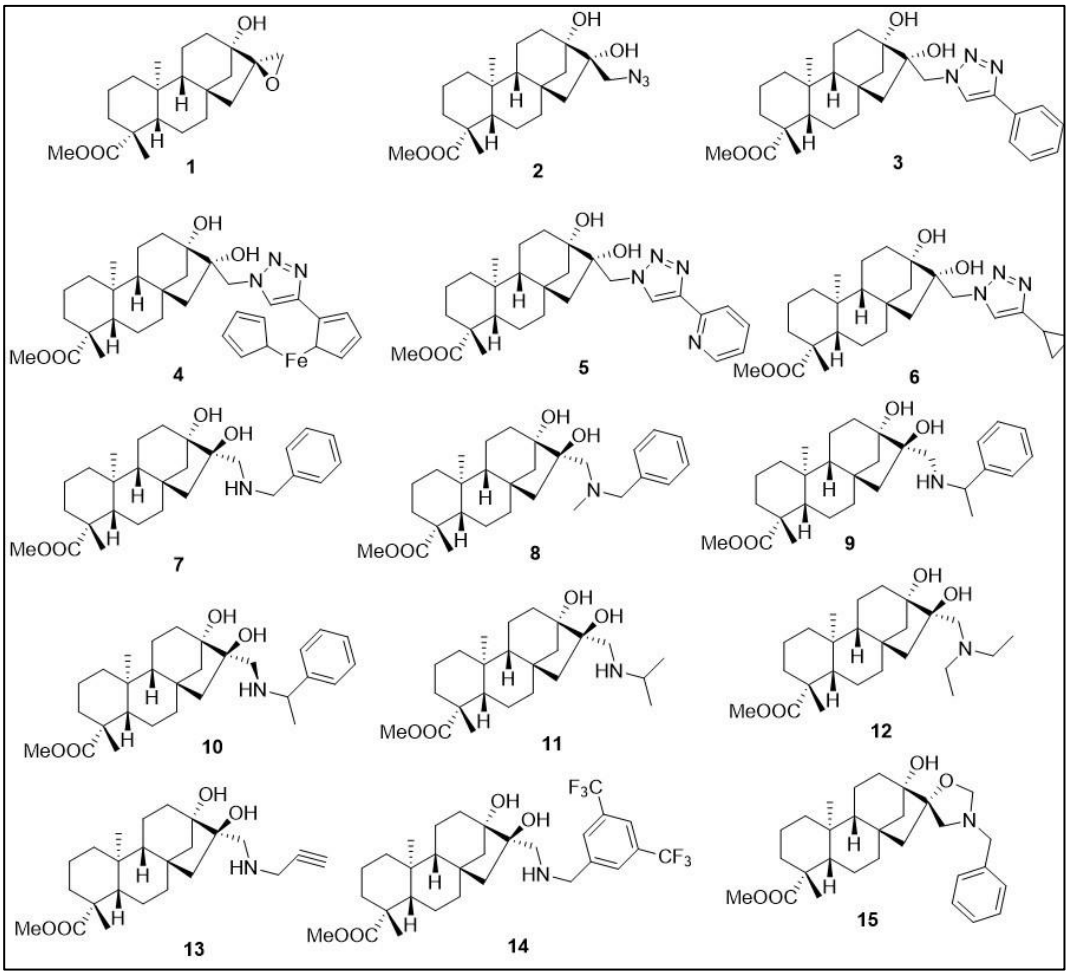


Figure 1A. Chemical structures of steviol type-aminodiols (**1-15**)

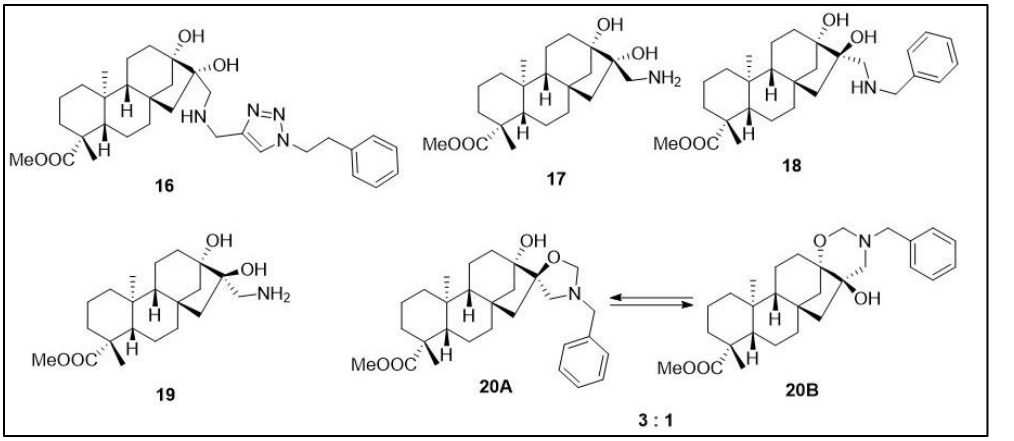


Figure 1B. Chemical structures of steviol type-aminodiols (**16-20B**)

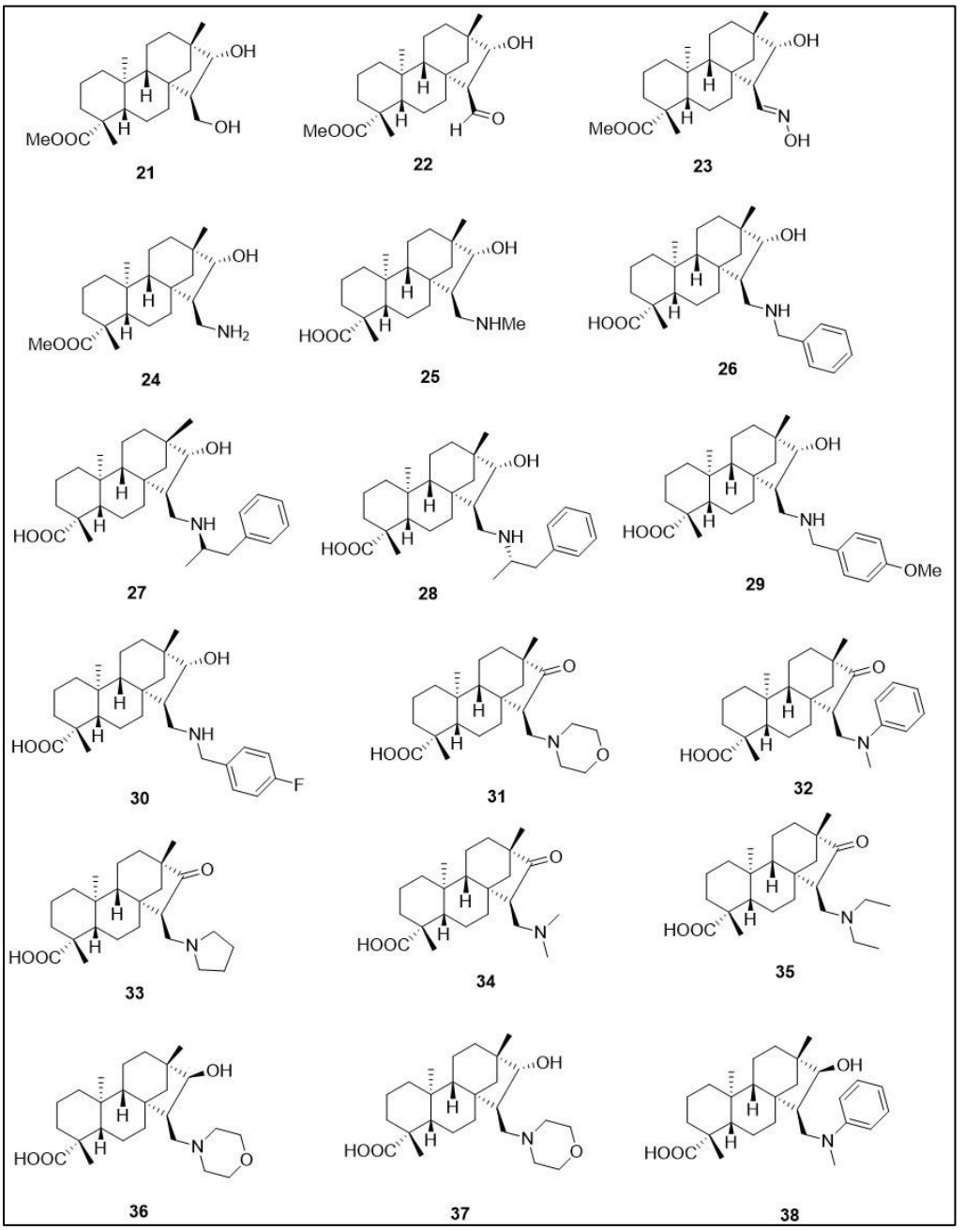


Figure 1C. Chemical structures of steviol type-aminodiols (**21-38**)

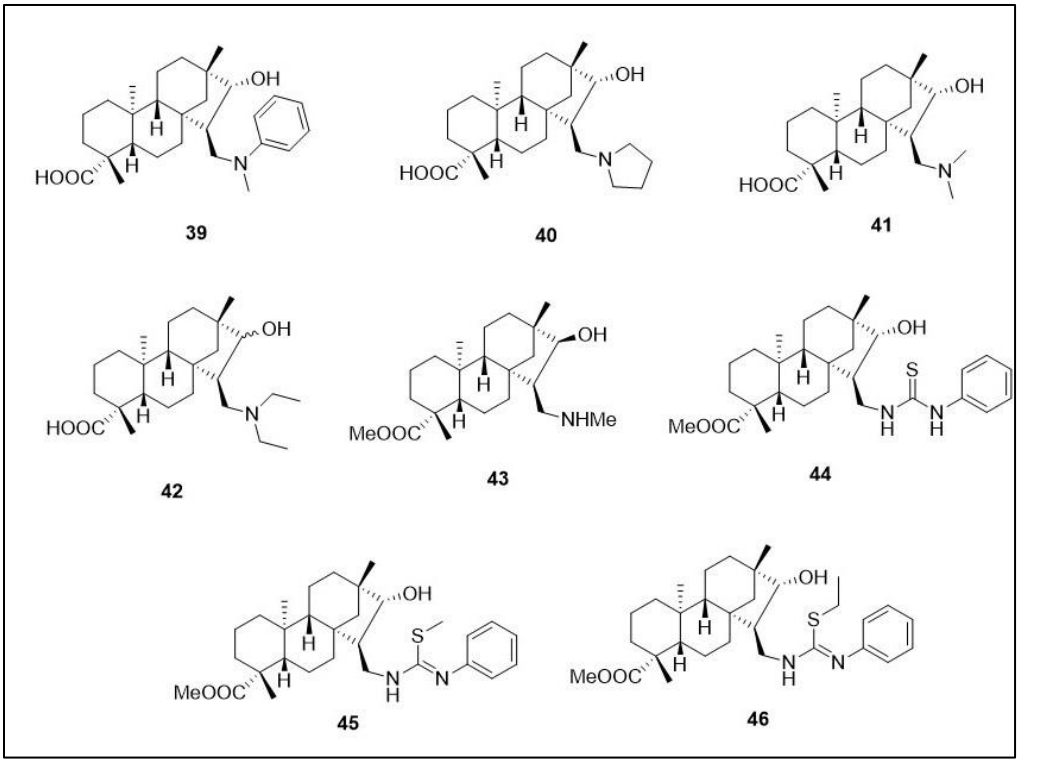


Figure 2. Chemical structures of isosteviol-based 1,3-aminoalcohols (**39-46**)

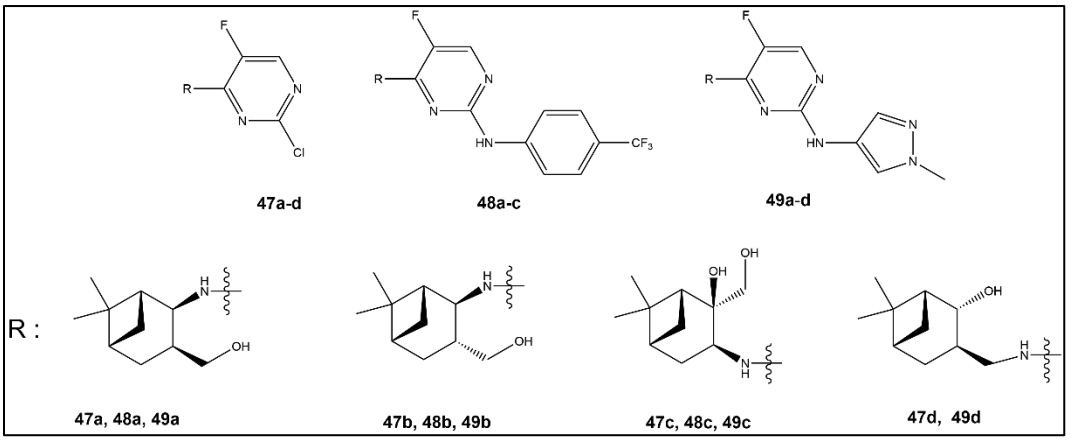


Figure 3. Chemical structures of 5-fluoro-2,4-diamino pyrimidine derivatives (**47a-49d**)

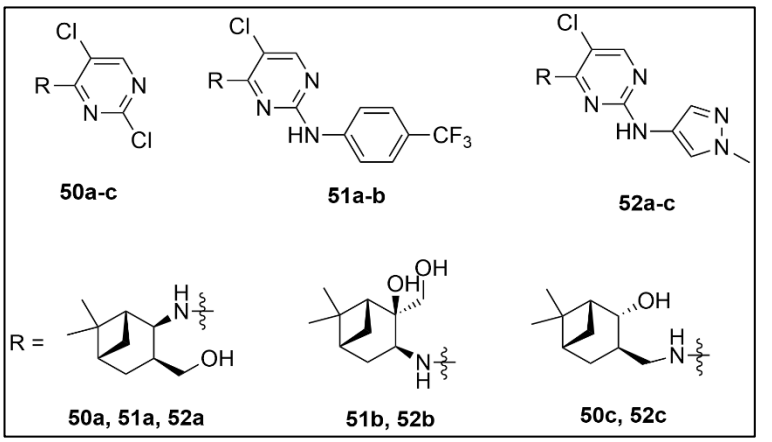


Figure 4. Chemical structures of 5-chloro-2,4-diamino pyrimidine derivatives (**50a-52c**)

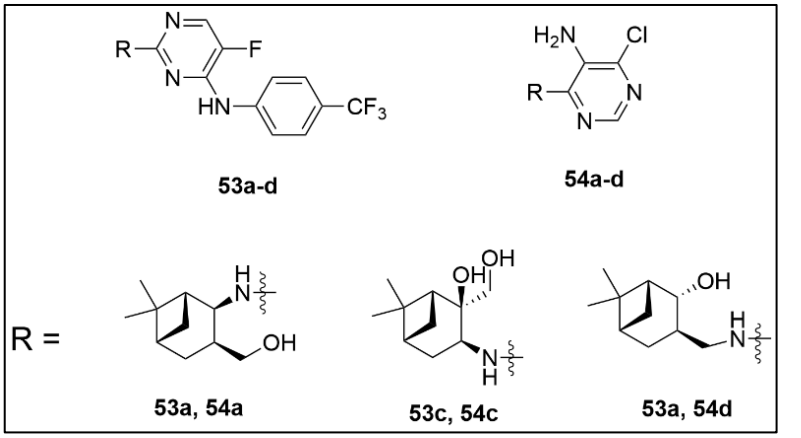


Figure 5. Chemical structures of 5-fluoro-2,4-diaminopyrimidines and 6-chloro-4,5-diamino pyrimidines (**53a-54d**)

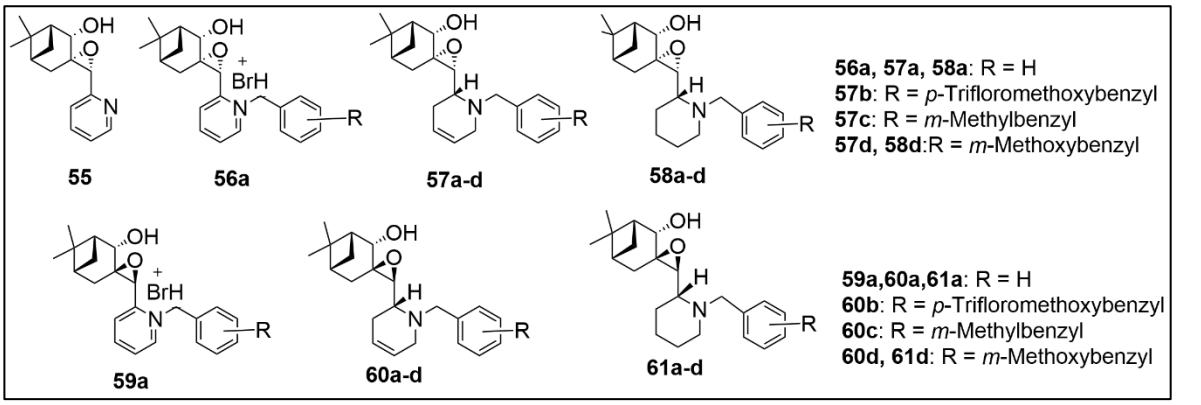


Figure 6. Chemical structures of 4-amino-2,3-epoxy-1-ol derivatives (**55-61d**).

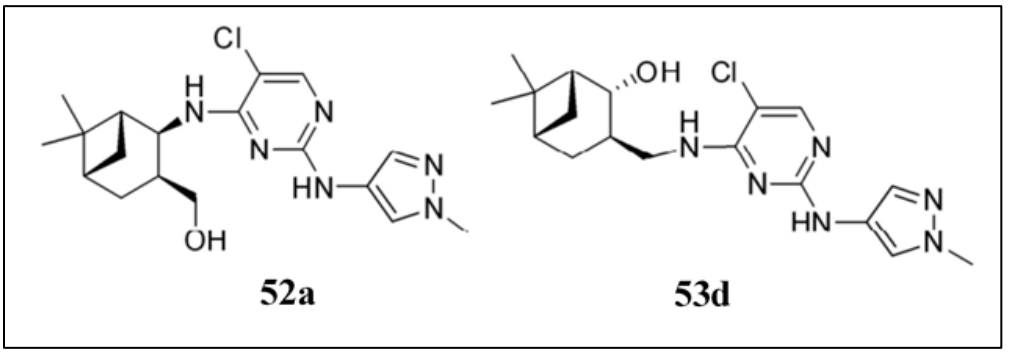


Figure 7. Chemical structures of the selected compounds.

3.2. Cell cultures and culturing conditions

To assess the antiproliferative potential of the tested compounds, a panel of human adherent gynecological cell lines was used. Cervical cancer cell lines (Hela), breast cancer cell lines (MDA-MB-231, MCF-7), ovarian (A2780) and non-cancerous mouse fibroblast cells were purchased from the European Collection of Cell Cultures (Salisbury, UK). A cervical cancer cell line (SiHa) was obtained from the American Tissue Culture Collection (ATCC; Virginia, USA). All cell lines were maintained in minimal essential medium (MEM), supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acid (NEAA) mixture and 1% antibiotic-antimycotic solution, at 37°C, under humidified air circumstances with 5% CO_2. In addition, all media, chemicals, and supplements, if otherwise not specified, were purchased from Lonza group Ltd. (Basel, Switzerland). The characteristics of cell lines utilized during the experiments are summarized in **Table 1**.

Table 1. Overview of utilized cell lines.

Cell Line	Origin and Characteristics
A2780	Established from an ovarian endometroid adenocarcinoma tumor in an untreated patient, epithelial morphology, cells grow as a monolayer
MDA-MB-231	Established from a pleural effusion, a metastatic mammary adenocarcinoma, triple negative breast cancer
MCF-7	Derived from pleural effusion from an adenocarcinoma, epithelial-like morphology, cells grow as monolayers
HeLa	Isolated from a cervical carcinoma, HPV-18 positive, the oldest immortalized cell line
SiHa	HPV-16 positive human cervical carcinoma
NIH/3T3	Isolated from mouse embryo, fibroblast cell line

3.3. MTT assay (Antiproliferative assay)

To determine the proliferation and viability of cells upon treatment with the tested compounds, a standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed.[59] Cells were seeded onto 96-well microtiter plates at a density of 5000 cells/well. After an overnight incubation, cells were treated with elevating concentration of test compounds (0.1-30 μM), and incubated for 72 hours under cell culture conditions, as described above. Subsequently, the MTT solution (5 mg/mL in phosphate buffer) was added to the wells, and incubated for 4 hours. The supernatant was removed, and to dissolve the precipitated formazan crystals, 100 μL of dimethyl sulfoxide (DMSO) were added. Lastly, absorbance values were recorded by a microplate-reader (BMG, LAbTech, Ortenberg, Germany) at 545 nm. Untreated cells were used as a control, and cisplatin was included as a clinically applied reference compound. Six-point concentration-

response curves were fitted to the measured points, and the IC_{50} values were calculated by the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Two independent measurements were performed with five parallel wells.

3.4. Hoechst 33258–propidium iodide fluorescent double staining

To characterize the changes in cell morphology and cell membrane integrity upon treatment with the tested compounds, fluorescent double staining was performed. Briefly, a suspension of A2780 cells were seeded into 6-well plates at a density of 300,000 cells/well and were incubated under cell culture conditions overnight, then treated with the specified concentrations of test compounds for 24 h and 48h. After the designated incubation time, the cells were stained by lipophilic Hoechst 33258 (1 μg/mL, HO) and hydrophilic propidium iodide (3 μg/mL, PI) for 1 h in the dark, under the same cell culture circumstances. Then, the medium was refreshed in the wells, and images were taken by Nikon Eclipse Ti-u microscope (Nikon Instruments Europe, Amstelveen, The Netherlands) equipped with appropriate optical blocks. Images were analyzed by NIS-Elements BR software (Nikon Instruments Europe, Amstelveen, The Netherlands). Nuclei emitting fluorescence were counted, and the proportion of intact, apoptotic, and necrotic cell populations were expressed as percentages (%).

3.5. Cell cycle analysis by flow cytometry

To obtain more detailed results concerning the mechanism of action of the tested compounds, and their influence on the cell cycle of ovarian adenocarcinoma cells, cell cycle analysis was performed with 24 and 48 h of designated incubation time. The A2780 cell line was seeded onto 24-well plates (80.000 cells/well). After an overnight incubation, the samples were treated with increasing concentration of test compounds for 24 h or 48 h. Following this, a washing step with phosphate buffer saline (PBS) and trypsinization process were performed. Cells were pooled with the collected supernatants and were centrifuged at 1400 rpm for 5 min at room temperature. Pellets were resuspended and stained by DNA staining solution, which consisted of 10 μg/mL RNase A, and 0.1 % sodium citrate dissolved in PBS, for 30 min in dark, at room temperature. Detection of DNA content from at least 20.000 cells/sample were carried out by a FACSCalibur flow cytometer, and the recorded data were analyzed by the Kaluza Analysis 357 Software (Beckman Coulter,

Brea, CA, USA). Untreated cells were considered as controls. Hypodiploid subG1 phases were regarded as a sign of a late apoptotic event.

3.6. Tubulin polymerization

To determine the direct effect of potent compounds on the microtubular system, an *in vitro* tubulin polymerization assay was performed (Cytoskeleton Inc., Denver, CO, USA). According to the manufacturer's recommendations, the assay reactions were performed on pre-warmed (37°C), UV-transparent 96-well microplate. Then, 10 µL of solutions of test compound, was placed on the wells supplemented with 2 mM $MgCl_2$, 0.5 mM ethylene glycol tetra-acetic acid (EGTA), 1 mM guanosine triphosphate (GTP) and 10.2% glycerol. Ten µL of general tubulin buffer were used as an untreated control. The polymerization reaction was initiated by adding 100 µL of 3.0 mg/mL tubulin in 80 mM PIPES, pH 6.9, to each sample. Absorbance of the samples was recorded per minute by a microplate reader (BMG Labtech, Ortenberg, Germany) at 340 nm, and a 60-min kinetic measurement protocol was applied. The maximum reaction rate (V_{max}; Δabsorbance/min) of tested compounds was calculated by the highest difference between the measured absorbances at two consecutive time points. According to the manufacturer's recommendation, a clinically applied reference agent, paclitaxel (PAC), was used at a high concentration (10 µM). Each sample was prepared in two parallels.

3.7. Wound healing assay

To determine the antimetastatic properties, namely, the influence of the tested compounds on cell migration, wound-healing assays was carried out by using the Ibidi Culture-Insert with two wells (Ibidi GmbH, Gräfelfing, Germany). Suspension of the A2780 cells was prepared in minimal essential medium supplemented with 2% FBS, 1% NEAA, and 1% penicillin/streptomycin mixture. Firstly, cells were seeded at a density of 500.000 cells into each chamber of special silicone inserts on 12-well microplates. Cells were incubated for 48 hours until forming a confluent monolayer, and the inserts were gently removed. A washing step with PBS was implemented to remove non-adherent cells. In the following steps, solutions of test compounds were prepared in a medium supplemented with 2% FBS, then added onto the cells, and incubated for 24 h and 48 h respectively. Lastly, images of the wound area at different times (0, 24, and 48 h post-treatment) were taken by Nikon Eclipse TS100 fluorescence microscope. The size of the cell-free area was

determined manually along the wound edge by the ImageJ version 1.53a software (National Institutes of Health, Bethesda, MD, USA). The migration rate was calculated based on comparison the wound closure of untreated controls and treated wells.

3.8. Boyden chamber assay

The Boyden chamber assay was performed to assess to the invasion ability of ovarian adenocarcinoma cells upon treatment with the selected test compounds. According to the protocol, BD BioCoat Matrigel Invasion Chambers (BD Biosciences, Bedford, MA, USA) are required to this assay, which containing a 8 µm pore size polyethylene terephthalate (PET) membrane and a thin layer of Matrigel basement matrix, which act as an *in vitro* model of the extracellular environment. The suspension of the A2780 cells, prepared in serum-free medium supplemented with desired concentrations of test compounds, was injected into the upper chamber onto the pre-hydrated membrane. The medium supplemented with 10% FBS was used as a chemoattractant in the lower compartment. After 24 h and 48 h of incubation at 37°C and 5% CO_2, supernatants and non-invading cells were eliminated by scrubbing with a cotton swab. Additionally, inserts were washed with PBS twice and fixed in ice-cold ethanol (96%) for 15 minutes, and stained with 1% crystal violet dye for 30 minutes in the dark at room temperature. A Nikon Eclipse TS100 microscope was utilized to take images (at least 3 photos per insert). To determine the invasion rate, invading cells were counted and compared to untreated control samples.

3.9. Statistical analysis

For statistical analyses, GraphPad Prism version 5.01 software (GraphPad, San Diego, CA, USA) was used. The statistical significance was estimated by one-way analysis of variance (ANOVA), followed by the Dunnett post hoc-test. In the Results section, all data are presented as means ± standard error of the mean (SEM) of at least three replicates. *, ** and *** indicate $p< 0.05$, $p< 0.01$ and $p< 0.001$ compared to control samples, respectively.

4. Results

4.1. Antiproliferative activity of diterpene- and monoterpene-based derivates

As previously described, 4 sets of compounds were involved in this study. To determine their antiproliferative properties against the selected cancer cell lines, a standard MTT assay was performed. Firstly, compounds were applied in 10 μM and 30 μM to assess their percentages of grow inhibition (%). Then, IC_{50} values were determined only in the case of potent derivates.

4.1.1. Steviol-type aminodiols

In the first set of compounds, twenty derivatives were tested on a panel of human adherent cell lines (HeLa, SiHa, MDA-MB-231 and A2780) by MTT-assay (**Table 2)**. Based on these data, some conclusions could be drawn concerning the structure–activity relationships of the tested compounds. The presence of the aromatic ring is a requirement of the cell-growth inhibiting effect; in addition, the N-benzyl substituent is also an essential part of the compounds, which exerted a remarkable antiproliferative effect on cancer cell lines.[42] From this set of compounds, 9 derivatives (**4**, **7–10**, **14–16** and **18**) exhibited remarkable antiproliferative properties. Based on their IC_{50} values, **15** was the most effective against all of cancer cell lines, as its IC_{50} values were under 2.0 μM. However, it was not found to be a stable compound in the follow-up investigations.

Table 2. Antiproliferative properties of aminodiol derivates

Compound	Conc. (μM)	Growth Inhibition (%) ± SEM			
		Calculated IC_{50} (μM)			
		A2780	HeLa	SiHa	MDA-MB-231
1	10	- *	-	-	-
	30	-	-	26.81 ± 2.02	-
2	10	-	-	-	-
	30	-	-	-	-
3	10	-	27.07 ± 1.12	-	-
	30	38.38 ± 2.53	34.72 ± 0.32	-	-
4	10	49.78 ± 1.28	43.19 ± 2.20	96.41 ± 0.41	-
	30	85.21 ± 0.53	51.98 ± 2.65	96.55 ± 0.30	52.04 ± 0.85
	IC_{50}	10.18	21.89	4.64	29.90
5	10	22.24 ± 1.36	-	-	-
	30	49.35 ± 0.58	26.80 ± 0.62	28.85 ± 0.69	34.62 ± 3.14
6	10	-	25.61 ± 3.14	-	-
	30	48.15 ± 0.68	28.46 ± 1.98	-	36.30 ± 3.34
7	10	54.36 ± 3.34	37.41 ± 0.57	-	-
	30	99.24 ± 0.16	98.58 ± 0.17	96.42 ± 0.44	98.45 ± 0.06
	IC_{50}	6.68	9.37	24.68	26.16

8	10	35.57 ± 2.31	39.90 ± 2.76	-	-
	30	65.79 ± 3.17	52.72 ± 2.04	-	23.77 ± 1.59
	IC_{50}	17.34	23.49		
9	10	84.73 ± 0.84	60.46 ± 1.65	92.54 ± 0.77	94.09 ± 0.59
	30	98.75 ± 0.17	98.81 ± 0.10	96.89 ± 0.93	98.30 ± 0.24
	IC_{50}	4.19	4.79	6.07	4.32
10	10	89.99 ± 1.16	92.25 ± 0.99	91.23 ± 0.89	97.00 ± 0.16
	30	98.87 ± 0.19	98.59 ± 0.07	94.34 ± 0.62	98.27 ± 0.20
	IC_{50}	4.91	3.96	6.54	4.39
11	10	-	-	-	-
	30	-	39.18 ± 1.84	20.41 ± 2.30	-
12	10	-	-	-	-
	30	29.07 ± 1.42	27.44 ± 1.06	20.83 ± 2.29	-
13	10	20.75 ± 0.77	-	-	-
	30	43.94 ± 2.99	30.21 ± 0.96	-	26.32 ± 1.04
14	10	72.12 ± 1.13	69.15 ± 2.86	59.69 ± 1.52	97.68 ± 0.13
	30	99.14 ± 0.12	98.25 ± 0.22	91.88 ± 1.34	98.26 ± 0.23
	IC_{50}	6.25	5.73	7.84	4.76
15	10	98.53 ± 0.16	98.72 ± 0.11	96.50 ± 0.32	97.56 ± 0.42
	30	98.97 ± 0.09	98.87 ± 0.03	97.06 ± 0.31	98.48 ± 0.40
	IC_{50}	1.07	1.05	1.62	1.25
16	10	47.33 ± 0.91	42.91 ± 1.19	-	-
	30	98.83 ± 0.23	98.95 ± 0.22	94.82 ± 0.05	95.81 ± 0.24
	IC_{50}	9.78	10.39	14.95	9.15
17	10	-	-	-	-
	30	-	-	-	-
18	10	52.78 ± 2.29	56.41 ± 0.96	86.27 ± 1.83	83.98 ± 0.41
	30	99.08 ± 0.06	99.01 ± 0.72	90.88 ± 1.03	98.09 ± 0.13
	IC_{50}	8.60	4.13	8.58	6.58
19	10	-	23.98 ± 2.06	20.95 ± 1.64	-
	30	-	44.56 ± 1.21	28.13 ± 0.75	89.42 ± 1.00
20	10	-	20.53 ± 0.36	-	-
	30	22.70 ± 0.56	28.92 ± 0.53	-	-
Cisplatin	10	83.57 ± 1.21	42.61 ± 2.33	88.64 ± 0.50	67.51 ± 1.01
	30	95.02 ± 0.28	99.93 ± 0.26	90.18 ± 1.78	87.75 ± 1.10
	IC_{50}	1.30	12.43	7.84	3.74

Note: cell growth inhibition values less than 20% were considered negligible and are not given numerically

4.1.2. Isosteviol-based 1,3-aminoalcohols

Concerning the second set of compounds (**21**–**46**), MCF-7 was also added to the cell line panel, on which the prepared diterpene analogues were tested by the means of MTT assay as given in **Table 3**. Based on the MTT results, it was shown that an amino functional group was essential to antiproliferative properties. The IC_{50} values of the primary amine (**24**) and secondary amine derivates (**25**–**30)** were comparable to that of the reference agent (cisplatin). In contrast, the oxime and aldehyde analogues did not elicit relevant effect on cancer cell proliferation. As previously mentioned, the N-benzyl substitution was essential

for remarkable antiproliferative properties, as previously shown, cervical cell lines were especially sensitive to these agents. Aminoketone analogues (**31**–**35)** were much more effective, while reduced analogues with tertiary amino function (**36**–**43**) did not have any relevant anticancer effect. IC_{50} values of the phenylthioureido analogue (**44**) were between 10 and 23μM, while the thioether compounds (**45**–**46)** exerted only negligible activity.

Table 3. Antiproliferative properties of isosteviol-based 1,3-aminoalcohols derivates

Comp.	**Conc.**	**Growth Inhibition (%) ± SEM**				
	(μM)	**[Calculated IC_{50} (μM)]**				
		HeLa	**SiHa**	**MDA-MB-231**	**MCF-7**	**A2780**
21	10	-*	-	-	-	-
	30	-	-	-	-	33.22 ± 2.23
22	10	-	-	-	-	-
	30	21.47 ± 2.18	26.54 ± 0.84	-	-	36.90 ± 2.48
23	10	-	-	-	-	-
	30	-	-	-	-	-
24	10	94.71 ± 0.13	80.91 ± 0.70	93.18 ± 0.84	92.41 ± 1.34	93.66 ± 0.39
	30	95.06 ± 0.34	91.52 ± 0.49	97.15 ± 0.23	91.89 ± 0.43	93.77 ± 0.10
	IC_{50}	4.11	4.73	5.25	4.13	6.52
25	10	38.90 ± 2.34	24.71 ± 2.36	44.70 ± 1.61	78.69 ± 0.56	64.75 ± 3.04
	30	100.18 ± 0.66	100.08 ±2.32	96.15 ± 1.23	100.87 ±0.75	101.13 ±0.55
	IC_{50}	11.6	13.48	10.7	6.11	1.36
26	10	96.04 ± 0.17	99.54 ± 0.50	90.29 ± 0.55	94.27 ± 0.39	97.16 ± 0.38
	30	96.21 ± 0.46	100.06 ±0.42	96.20 ± 0.38	95.32 ± 0.93	97.38 ± 0.47
	IC_{50}	5.47	6.43	5.37	7.44	7.96
27	10	100.09 ± 0.29	100.23 ±0.85	96.39 ± 0.67	100.88 ±0.42	101.01 ±0.30
	30	100.24 ± 0.45	100.23 ±0.68	99.76 ± 1.44	101.25 ±0.65	101.07 ±0.35
	IC_{50}	3.09	4.75	7.34	4.36	4.21
28	10	99.49 ± 0.45	101.90 ±0.44	97.45 ± 0.72	99.48 ± 0.77	100.05 ±0.75
	30	99.69 ± 0.46	102.41 ±0.52	96.13 ± 1.15	100.41 ±0.30	101.23 ±0.67
	IC_{50}	2.92	4.95	8.28	4.34	4.29
29	10	97.80 ± 1.27	101.65 ±0.66	101.67 ± 0.90	99.85 ± 0.53	100.77 ±0.22
	30	98.16 ± 1.61	102.49 ±0.67	99.45 ± 0.92	100.44 ±0.46	100.78 ±0.35
	IC_{50}	2.55	4.37	5.58	2.51	4.04
30	10	98.04 ± 1.42	102.03 ±0.47	99.68 ± 0.67	100.08 ±0.21	100.16 ±0.61
	30	99.81 ± 0.54	102.91 ±0.41	99.15 ± 0.80	100.12 ±0.44	100.55 ±0.50
	IC_{50}	2.75	4.19	4.40	2.14	3.81
31	10	-	-	-	-	-
	30	-	40.01 ± 2.19	-	-	86.09 ± 1.83
	IC_{50}	-	-	-	-	27.31
32	10	-	30.12 ± 2.84	-	-	-
	30	34.61 ± 1.67	53.90 ± 2.24	-	-	-
33	10	-	-	-	-	-
	30	-	-	-	-	-
34	10	27.72 ± 0.98	-	-	-	-

	30	35.73 ± 2.29	20.30 ± 0.47	-	-	34.07 ± 0.95
35	10	-	-	-	-	-
	30	26.53 ± 1.76	-	-	-	-
36	10	49.69 ± 2.54	33.90 ± 2.39	-	-	49.13 ± 2.44
	30	47.40 ± 2.02	39.9 ± 2.05	-	29.04 ± 2.24	81.24 ± 0.62
37	10	25.07 ± 2.30	-	22.32 ± 1.15	-	-
	30	43.36 ± 3.25	76.41 ± 1.03	27.31 ± 0.82	64.26 ± 2.09	68.03 ± 0.38
38	10	50.54 ± 2.19	44.71 ± 1.76	52.87 ± 0.50	37.27 ± 2.22	36.64 ± 0.53
	30	76.81 ± 1.25	76.26 ± 0.47	86.13 ± 0.57	88.14 ± 2.32	89.64 ± 0.82
	IC_{50}	9.17	12.2	9.20	17.29	8.16
39	10	49.71 ± 1.38	48.68 ± 2.18	46.26 ± 1.63	31.24 ± 1.57	29.09 ± 1.16
	30	82.06 ± 0.66	99.45 ± 0.39	89.73 ± 0.62	89.44 ± 1.12	96.18 ± 0.48
	IC_{50}	13.65	14.34	9.26	17.24	14.93
40	10	45.56 ± 0.46	-	41.84 ± 0.52	31.24 ± 0.92	-
	30	67.21 ± 0.52	25.66 ± 2.03	51.39 ± 1.78	84.31 ± 1.23	79.72 ± 1.03
41	10	37.91 ± 0.44	25.33 ± 2.07	35.57 ± 0.40	27.02 ± 1.04	-
	30	57.71 ± 0.84	75.04 ± 0.44	40.20 ± 0.82	74.21 ± 0.62	68.28 ± 1.15
42	10	42.49 ± 0.68	21.14 ± 1.35	36.28 ± 0.59	-	-
	30	65.17 ± 0.92	79.86 ± 1.06	48.41 ± 1.42	72.25 ± 0.65	68.28 ± 1.13
43	10	24.65 ± 1.66	-	-	-	-
	30	49.66 ± 2.16	45.36 ± 1.87	21.00 ± 0.35	75.64 ± 3.20	74.59 ± 1.16
44	10	51.56 ± 1.59	-	-	28.57 ± 2.62	45.83 ± 1.85
	30	98.88 ± 0.33	92.46 ± 1.51	95.47 ± 0.35	94.16 ± 0.01	95.59 ± 0.24
	IC_{50}	9.97	6.91	22.96	14.31	10.16
45	10	-	30.40 ± 3.38	41.15 ± 2.07	-	21.39 ± 3.18
	30	92.08 ± 0.77	90.27 ± 0.86	95.37 ± 0.51	44.31 ± 0.48	94.66 ± 0.44
	IC_{50}	17.86	12.69	12.31		13.83
46	10	-	-	-	21.97 ± 2.57	-
	30	62.35 ± 1.07	51.53 ± 2.31	52.63 ± 2.43	81.02 ± 3.02	68.62 ± 1.85

Note: cell growth inhibition values less than 20% were considered negligible and are not given numerically

4.1.3. Pinane-based 1,4-amino alcohol derivates

The third set of compounds of our *in vitro* studies, were the newly synthesized 1,4-amino alcohol derivatives (altogether 25 derivatives), were tested on multiple cancer cell lines well. Compounds with a primary amino functional group on the pyrimidine ring or monoamino-substituted pyrimidines elicited negligible effects (**50b**, **54c**, and **54d**), i.e. their growth inhibition percentage did not exceed 60% at 30 μM concentration (**47a**–**c**, **50a**–**b**, and **54c**–**d**). On other hand, compound **54a** was selective for HeLa cells. In comparison with the effect of *p*-CF_3-phenyl and 4-pyrazolyl substituents at the 2-amino function, the *p*-CF_3-phenyl substituents appeared to be more potent than the 4-pyrazolyl substituent in the case of the 6-F analogues (**48a**/**49a**, **48c**/**49c** and **51b**/**52b**). Even though, an opposite relationship was observed in the case of 6-chloro-substituted diaminopyrimidines (**51a**/**52a**). The 1,3-aminoalcohol function at the pinane system did not show any remarkable influence on

antiproliferative activity, therefore if could be confirmed that the *cis* configuration of the 1,3-aminoalcohol moiety is preferred (**47a**/**47b**, **48a**/**48b**). Interestingly, the chloro substitution at the diaminopyrimidine skeleton exhibited more pronounced effects on the growth of cancer cells than the corresponding fluoro-derivatives (**49a**/**52a**). Taken together, analogue **52a** exerted higher cell growth inhibition than cisplatin against all cell lines. In the case of compound **53d**, more than 90% cell growth inhibition was shown. Both molecules may be considered as starting points for promising anticancer agents.[57] The growth inhibition percentages of pinane-based 1,4-amino alcohol derivates are summarized in **Table 4**.

Table 4. Growth inhibition percentage of pinane-based 1,4-amino alcohol derivates

Comp.	Conc.	Growth Inhibition (%) ± SEM				
	(μM)	Hela	SiHa	MBA-MB-231	MCF7	A2780
47a	10	36.67 ± 1.41	–*	–	–	–
	30	57.49 ± 0.73	22.37 ± 1.32	–	43.58 ± 1.35	30.38 ± 1.51
47b	10	–	–	–	–	–
	30	42.22 ± 0.76	–	–	–	–
47c	10	–	–	–	–	–
	30	29.62 ± 1.91	22.04 ± 3.41	–	31.47 ± 1.93	–
47d	10	–	–	–	–	–
	30	44.46 ± 2.42	–	32.41 ± 1.52	–	–
48a	10	43.44 ± 0.58	27.96 ± 1.12	–	–	23.62 ± 3.32
	30	98.69 ± 0.22	95.07 ± 0.99	98.51 ± 0.21	98.30 ± 0.25	99.05 ± 0.33
48b	10	–	–	–	–	–
	30	76.92 ± 0.30	50.99 ± 1.73	27.12 ± 1.31	65.79 ± 0.26	62.41 ± 2.13
48c	10	39.66 ± 1.40	–	41.87 ± 0.66	–	–
	30	76.21 ± 0.81	72.46 ± 1.91	68.83 ± 1.38	56.85 ± 2.56	66.22 ± 1.20
49a	10	29.79 ± 3.82	–	37.75 ± 1.99	25.47 ± 1.91	62.17 ± 0.23
	30	49.39 ± 1.92	53.95 ± 1.78	61.97 ± 1.11	75.99 ± 2.31	91.11 ± 0.12
49b	10	42.07 ± 2.44	24.67 ± 1.73	–	35.79 ± 2.41	–
	30	74.39 ± 1.00	47.04 ± 1.46	48.28 ± 1.71	69.19 ± 1.63	74.74 ± 1.09
49c	10	29.09 ± 0.88	33.77 ± 2.88	–	32.37 ± 3.38	–
	30	51.39 ± 1.15	38.49 ± 1.65	–	45.18 ± 1.12	–
49d	10	24.12 ± 1.89	30.58 ± 1.68	–	25.63 ± 0.71	64.95 ± 2.94
	30	52.81 ± 0.72	57.85 ± 1.08	25.81 ± 0.72	54.94 ± 0.69	80.19 ± 0.49
50a	10	–	–	–	–	–
	30	45.96 ± 1.42	–	–	–	45.16 ± 2.21
50b	10	–	–	–	–	–
	30	–	–	–	–	–
50c	10	20.14 ± 2.00	–	–	–	–
	30	37.38 ± 3.02	–	–	–	–
51a	10	39.89 ± 2.83	–	27.65 ± 2.22	34.83 ± 2.85	79.14 ± 1.91
	30	98.99 ± 0.25	96.59 ± 0.65	96.65 ± 0.30	94.70 ± 0.33	98.28 ± 0.24

51b	10	60.29 ± 1.51	40.49 ± 2.10	-	53.41 ± 0.44	42.58 ± 1.04
	30	97.89 ± 0.14	95.45 ± 0.49	94.97 ± 0.19	90.84 ± 0.46	96.97 ± 0.16
52a	10	99.13 ± 0.19	93.06 ± 0.65	72.79 ± 1.09	84.74 ± 0.32	95.17 ± 0.99
	30	100.20 ± 0.11	94.31 ± 0.68	86.75 ± 0.92	90.75 ± 0.54	97.06 ± 0.20
52b	10	–	24.07 ± 1.38	-	33.75 ± 2.41	34.81 ± 2.94
	30	63.50 ± 1.64	57.89 ± 1.07	20.18 ± 2.70	66.07 ± 1.30	80.61 ± 0.92
52c	10	45.07 ± 2.92	33.07 ± 1.54	23.28 ± 1.75	46.35 ± 2.67	84.72 ± 0.62
	30	67.74 ± 1.33	68.87 ± 1.23	50.51 ± 1.90	75.95 ± 0.92	93.28 ± 0.40
53a	10	–	–	–	–	29.81 ± 1.66
	30	29.62 ± 2.13	–	39.11 ± 3.06	30.24 ± 0.64	60.64 ± 0.46
53c	10	–	–	–	27.80 ± 1.85	30.59 ± 1.59
	30	97.72 ± 0.42	100.45 ± 1.39	99.17 ± 0.54	96.12 ± 0.84	100.00 ± 0.44
53d	10	29.02 ± 2.26	–	27.15 ± 1.34	–	26.19 ± 1.88
	30	95.06 ± 0.47	90.39 ± 0.57	93.88 ± 0.53	90.52 ± 1.03	91.75 ± 3.12
54a	10	95.92 ± 0.39	-	-	37.88 ± 2.62	48.96 ± 2.03
	30	97.96 ± 0.25	26.42 ± 2.67	-	39.68 ± 2.22	55.91 ± 1.16
54c	10	–	–	–	–	–
	30	–	–	–	–	–
54d	10	38.61 ± 2.29	–	–	–	–
	30	56.06 ± 0.89	–	–	–	23.18 ± 1.37

Note: cell growth inhibition values less than 20% were considered negligible and are not given numerically

4.1.4. 4-amino-2,3-epoxy-1-ol derivatives

The other half of monoterpene based derivates, namely, 4-amino-2,3-epoxy-1-ol, were screened on the same cell line panel, as described above. According to the pharmacological activities of the tested analogues, compounds containing a pyrimidine ring (**55**, **56a**, and **59a**) had no considerable antiproliferative properties. Full saturation of the pyrimidine ring (**58a**–**d**, and **61a**–**d**) resulted in almost negligible activities, i.e. approximately 30-50% of cell growth inhibition. The substituent on the phenyl ring did not seem to affect the activity of the obtained analogues. The trans orientation of the epoxy function seems to be preferred, especially in the case of **60c**. Substituents of the benzyl ring on the tetrahydropyridine function did not seem to influence the activity of the obtained analogue. Non-substituted compounds (**60a**) elicited a remarkable inhibition against ovarian cancer cell line. In comparison with cisplatin, many compounds (**57d**, and **60a**–**d**) exhibited considerable antiproliferative activities against the cancer cell line; however, cancer type selectivity was not sown.[58] Results of MTT assay are summarized in **Table 5**.

Table 5. Growth inhibition percentage of 4-amino-2,3-epoxy-1-ol derivatives

Comp.	Conc. (μM)	Growth Inhibition (%) ± SEM				
		HeLa	SiHa	MDA-MB-231	MCF-7	A2780
55	10	-*	-	-	-	-
	30	-	-	-	-	-
56	10	-	-	-	-	-
	30	-	-	-	-	-
57a	10	-	-	28.16 ± 0.43	-	-
	30	33.85 ± 2.00	77.01 ± 1.65	41.10 ± 3.07	-	47.73 ± 1.52
57b	10	-	40.56 ± 1.64	55.68 ± 1.28	44.74 ± 1.50	39.31 ± 3.16
	30	95.59 ± 0.74	75.21 ± 0.64	90.09 ± 0.81	84.97 ± 3.05	95.45 ± 1.16
57c	10	-	-	-	-	-
	30	24.25 ± 1.32	-	37.22 ± 1.22	33.41 ± 1.74	64.09 ± 1.77
57d	10	-	54.15 ± 2.35	71.67 ± 2.17	48.99 ± 2.71	90.38 ± 0.89
	30	85.79 ± 0.26	92.76 ± 0.58	89.93 ± 0.86	97.97 ± 1.46	95.99 ± 1.56
58a	10	-	-	-	-	-
	30	38.88 ± 2.71	-	24.39 ± 1.22	-	-
58b	10	-	-	-	-	-
	30	22.53 ± 1.47	-	-	-	-
59a	10	-	-	-	-	-
	30	29.42 ± 2.35	-	26.89 ± 1.58	-	24.47 ± 2.50
60a	10	-	28.96 ± 3.12	-	21.50 ± 2.28	91.37 ± 1.51
	30	76.19 ± 2.61	88.38 ± 0.71	43.86 ± 0.65	86.14 ± 2.84	98.18 ± 0.47
60b	10	25.63 ± 1.32	38.97 ± 2.73	58.49 ± 2.78	29.26 ± 3.00	33.46 ± 1.93
	30	83.98 ± 1.63	85.38 ± 1.54	93.95 ± 0.75	98.26 ± 2.28	99.07 ± 0.46
60c	10	-	47.99 ± 3.03	69.33 ± 1.95	56.93 ± 2.35	88.65 ± 0.58
	30	87.67 ± 2.24	93.03 ± 1.72	94.80 ± 0.14	96.07 ± 1.47	97.45 ± 0.41
60d	10	-	23.09 ± 2.86	44.44 ± 1.21	62.03 ± 2.17	77.22 ± 2.85
	30	66.61 ± 1.54	92.58 ± 1.91	89.26 ± 0.88	97.45 ± 1.35	99.37 ± 1.19
61a	10	-	-	-	-	-
	30	51.48 ± 0.32	35.17 ± 0.78	36.42 ± 0.59	21.15 ± 2.55	-
61b	10	-	-	27.30 ± 1.74	-	-
	30	24.25 ± 2.76	-	40.40 ± 2.85	20.90 ± 1.69	26.22 ± 2.42

Note: cell growth inhibition values less than 20% were considered negligible and are not given numerically

4.1.5. Overview of the results for the antiproliferative assays

As described above, 4 sets of compounds were screened by the standard MTT-assay. Several potent compounds with remarkable antiproliferative activity have been identified. Our goal was to find promising candidates against ovarian cancer cell lines, thus, concerning structure–activity relationships and growth inhibition percentages, two analogues (**52a** and **53d**) were selected for further evaluation in subsequent experiments. Additionally, the other potent candidates may also be utilized to design and synthesize for novel anticancer agents.

4.1.6. Antiproliferative properties of selected compounds

In view of the chemical similarities and antiproliferative activity of the two monoterpene derivatives, they were chosen for the further experiments to determine their mechanism of the action. After all, IC_{50} values of **52a** and **53d** were determined on the cancer cell lines, their values were comparable with the clinically applied control cisplatin. To obtain information about tumor selectivity of the selected compounds, they were tested additionally against the non-cancerous fibroblast cell line (NIH/3T3). Tumor selectivity index was calculated (TS index) according to the following formula: IC_{50}[cancerous]/IC_{50}[non-cancerous]. It has been revealed that the selectivity of the novel analogues is comparable with the referent agents', i.e., they have moderate tumor selectivity. Results are presented in **Tables 6** and **7**.

Table 6. Antiproliferative properties of compounds **52a** and **53d**

Cell Line	**Calculated IC_{50} values (μM)**		
	compound **52a**	compound **53d**	cisplatin
HeLa	1.39	7.69	12.14
SiHa	2.14	16.70	5.31
MDA-MB-231	1.78	27.35	10.17
MCF-7	1.49	16.27	8.05
A2780	0.77	2.86	1.30
NIH/3T3	4.36	8.05	3.96

Table 7. Tumor selectivity index of selected compounds on the utilized cell lines

Cell Line	**Tumor selectivity index**		
	compound **52a** [IC_{50} malignant/IC_{50} NIH/3T3]	compound **53d** [IC_{50} malignant/IC_{50} NIH/3T3]	IC_{50} **cisplatin**/IC_{50} NIH/3T3]
HeLa	0.32	0.95	3.06
SiHa	0.49	2.07	1.34
MDA-MB-231	0.41	3.39	2.56
MCF-7	0,34	2.02	2.03
A2780	0.17	0.35	0.32

4.2. Hoechst-Propidium double staining

Fluorescent nuclei staining was performed, using Hoechst 33258 and propidium iodide dyes, after 24 h or 48 h exposure to 0.5, 1, 2 or 4 μM of compound **52a**, and 1, 2, 4 or 5 μM of compound **53d**, in order to characterize the changes in cell morphology and cell membrane integrity of ovarian cancer cells induced by tested compounds. Fluorescent images were taken from the same field by two different optical blocks, which is required to Hoechst or propidium-iodide. For quantitative analysis, cells with intact, apoptotic, and

necrotic features were counted, then the ratio of cells with different morphological markers was calculated. In general, the statistical evaluation revealed an elevated number of apoptotic and necrotic cells after 48 h, as well as a significant decline in viable cells, with a concentration-dependent manner in the case of both compounds. (**Figures 8.** and **9.**)

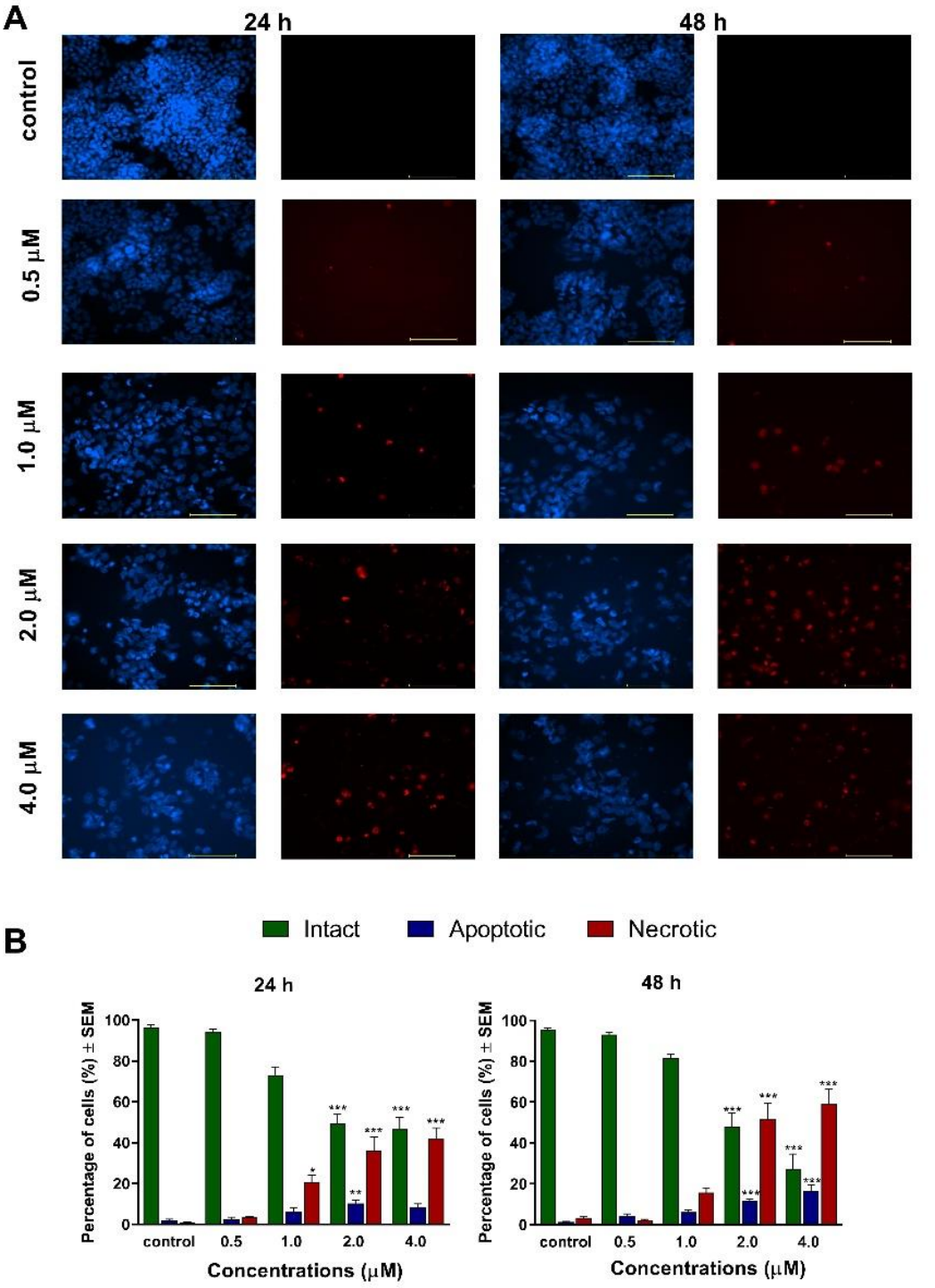


Figure 8. Morphological changes in A2780 cells after 24 h or 48h of exposure to compound **52a**, visualized by HOPI double staining. **Panel A:** representative pairs of images of the same field; blue fluorescence (left panels) indicates Hoechst 33258, and red fluorescence (right panels) shows propidium accumulation. The bar in the figure indicates 100 μm. **Panel B:** percentages of intact,

apoptotic, and necrotic cell populations. *, ** and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

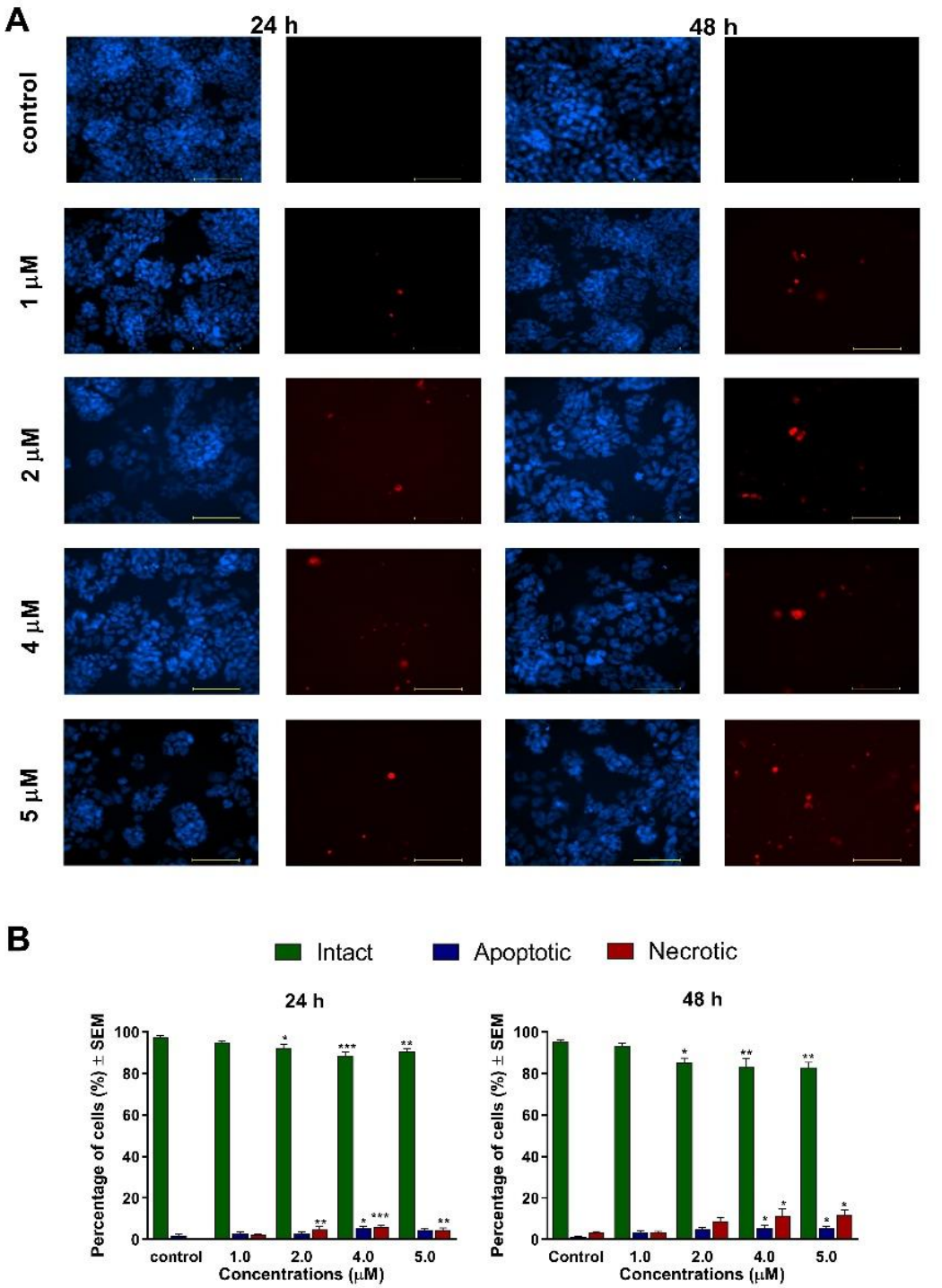


Figure 9. Morphological changes in A2780 cells after 24 h or 48h of exposure to compound **53d**, visualized by HOPI double staining. **Panel A:** representative pairs of images of the same field; blue fluorescence (left panels) indicates Hoechst 33258, and red fluorescence (right panels) shows propidium accumulation. The bar in the figures indicates 100 μm. **Panel B:** percentages of intact, apoptotic, and necrotic cell populations. *, ** and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

4.3. Cell cycle analysis

The effects of test compounds on the cell cycle distribution were investigated by flow cytometry after 24 h or 48 h of incubation. The applied concentrations were based on the calculated IC_{50} values (shown previously), and the cell populations in the various cell cycle phases (subG1, G1, S, and G2/M) were determined. A concentration-dependent rise of hypodiploid subG1 cell populations was regarded as a marker of induced apoptosis. Treatment with 1 μM of **52a** elicited a significant accumulation of cells in the G2/M phase ($p<0.001$), and a reduction in the S phase. Higher concentration resulted in serious cell cycle disturbance dominated by subG1 accumulation. After 48 h of incubation, the changes become more pronounced. Treatment with **53d** for 24 h significantly ($p<0.001$), and concentration-dependently decreased the G1 phase, whereas substantial elevation was recorded in the G2/M population. More prolonged incubation resulted in a gradual accumulation of hypodiploid cells. These results may suggest that **52a** and **53d** induce a marked cell cycle arrest in the G2/M in a dose-dependent manner (**Figure 10**).

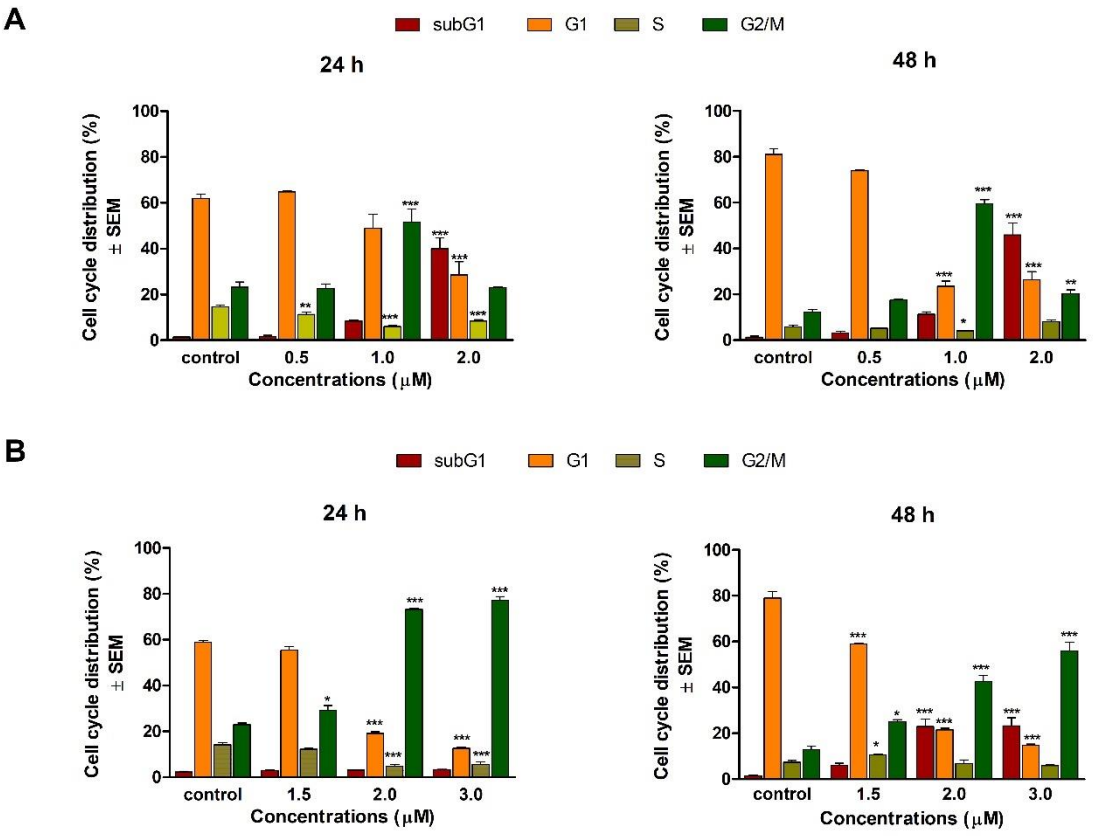


Figure 10. Effect of two selected compounds on cell cycle distribution of A2780 cells. **Panel A**: treated with 0.5, 1, and 2 μM of **52a** for 24 and 48 h. **Panel B**: treated with 1.5, 2, and 3 μM of **53d** for 24 and 48 h. *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

4.4. Tubulin polymerization

The direct effect of the test compounds on microtubule formation was determined by a specific photometric assay in a cell-free system. Based on calculated IC_{50} values, applied concentrations of compounds were determined according to the recommendations of the manufacturer; and paclitaxel was included as a reference agent. In comparison with untreated control samples, the highest concentrations of both compounds induced a significant acceleration of tubulin polymerization. Moreover, a considerable rise in the V_{max} value was recorded at 350 μM of **52a** and 300 μM of **53d** compared to the reference agent paclitaxel (**Figure 11**.).

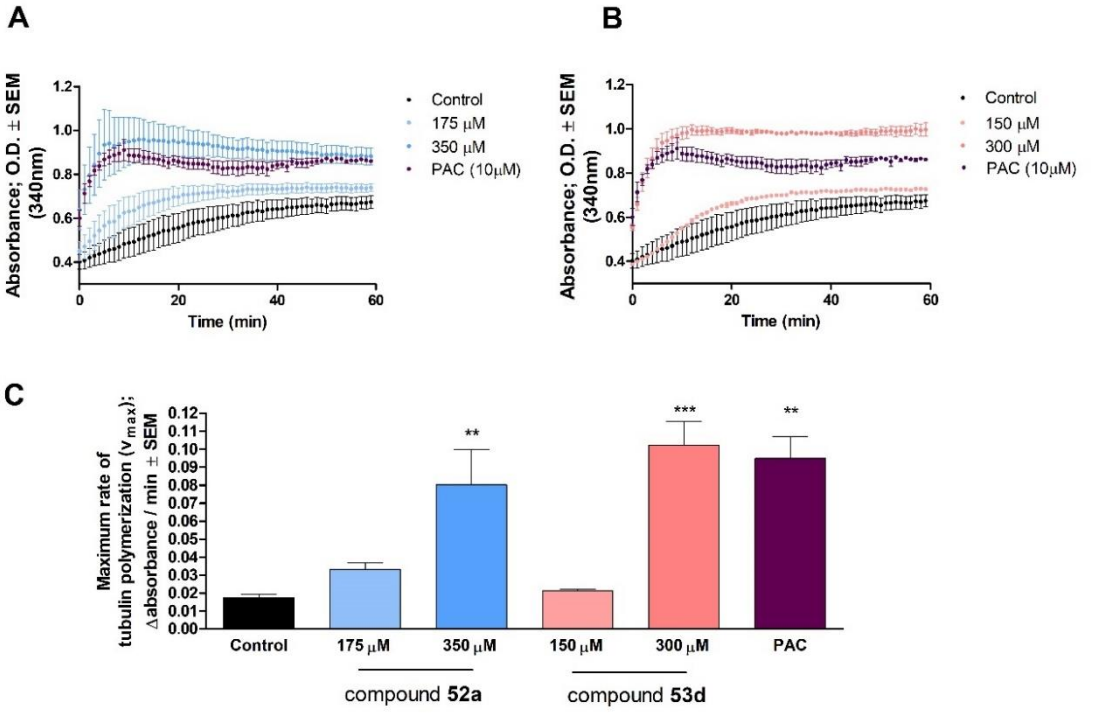
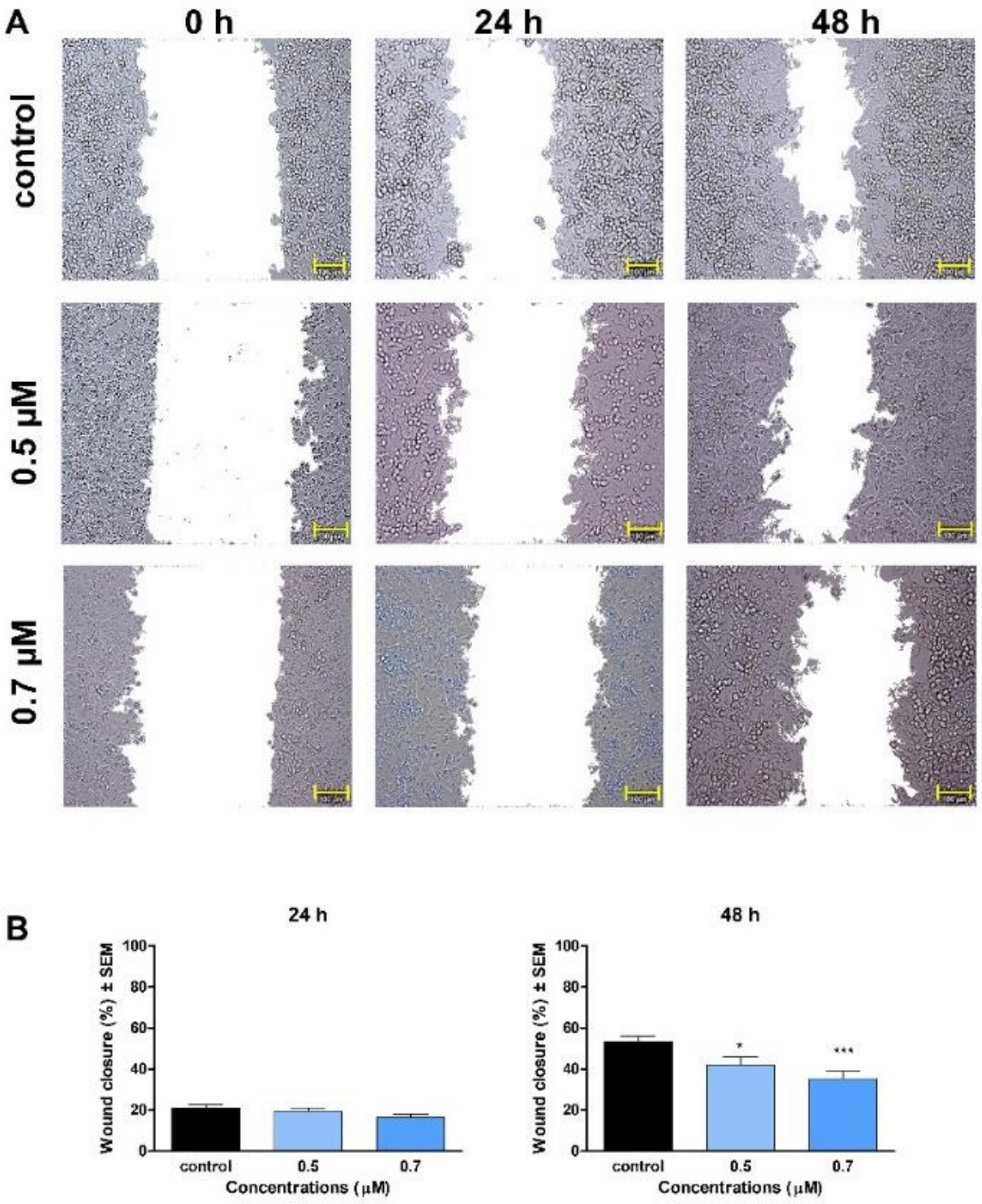


Figure 11. Compounds **52a** and **53d** affect tubulin polymerization (Panels **A** and **B**, respectively). Paclitaxel (PAC) was included as a reference agent. **Panel C:** calculated maximum rate values of tubulin polymerization. ** and *** indicate significance at $p < 0.01$ and $p < 0.001$, respectively. Results from two independent experiments performed in duplicate.

4.5. Wound healing assay

To determine the anti-migratory effect of tested compounds, changes in the size of the cell-free area were measured at 0, 24h, and 48h post-treatment using samples with reduced serum-containing medium. A reduction of cell-free gaps - produced by special silicon inserts - was demonstrated by image analysis of wound closure. In treatment with compound **52a,** no significant differences were observed after 24 h. However, a significant ($p < 0.05$ and $p < 0.01$) change was obtained after 48 h at both concentrations. On the other hand, inhibition in a concentration-dependent manner was recorded at 1.5 μM, as well as 2.0 μM with compound **53d** after 24 and 48 hours of treatment, as compared to the control. (Figure **12**.)



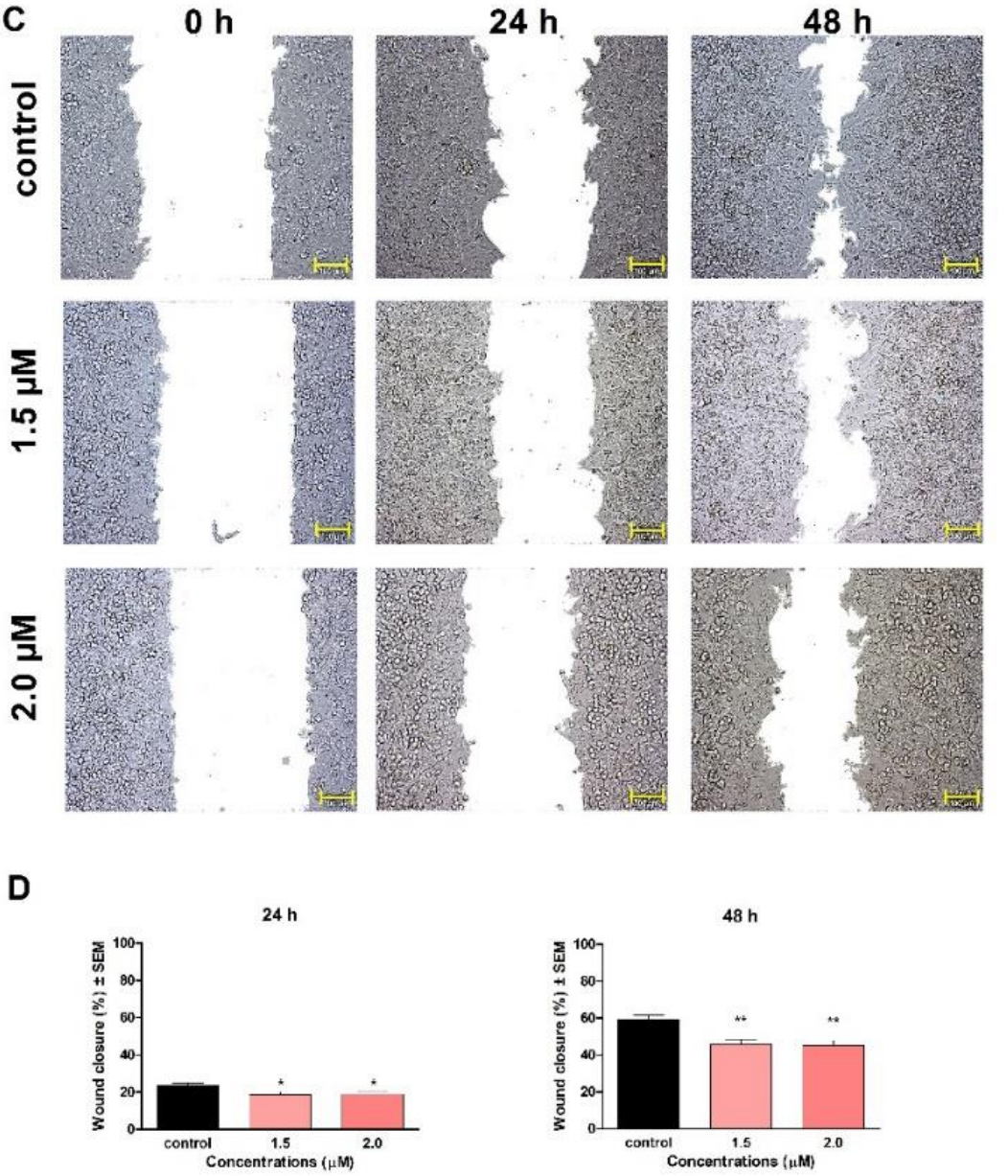


Figure 12. Effect of **52a** and **53d** on migration in A2780 cells. **Panel A:** representative images after 24 and 48 h treatments with **52a**. **Panel B:** calculated wound closure values determined after treatments with **52a**. **Panel C:** representative images after 24 and 48 h treatments with **53d**. **Panel D:** calculated wound closure values determined after treatments with **53d**.

The bar in the figures indicates 100 µm.

*, **, and *** indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; results from 4 independent experiments performed in triplicate

4.6. Boyden chamber assay

Besides cell migration, cellular invasion and infiltration of the surrounding tissues are crucial to the development of tumor metastases. Therefore, we supplemented our wound healing assay with a cellular migration test, i.e. a special Boyden chamber assay as the extracellular environment of primary tumors, to study cellular invasion. As a result, a noticeable decline in the invasive potential could be observed after 48 h post-treatment (**Figure 13A–B**).

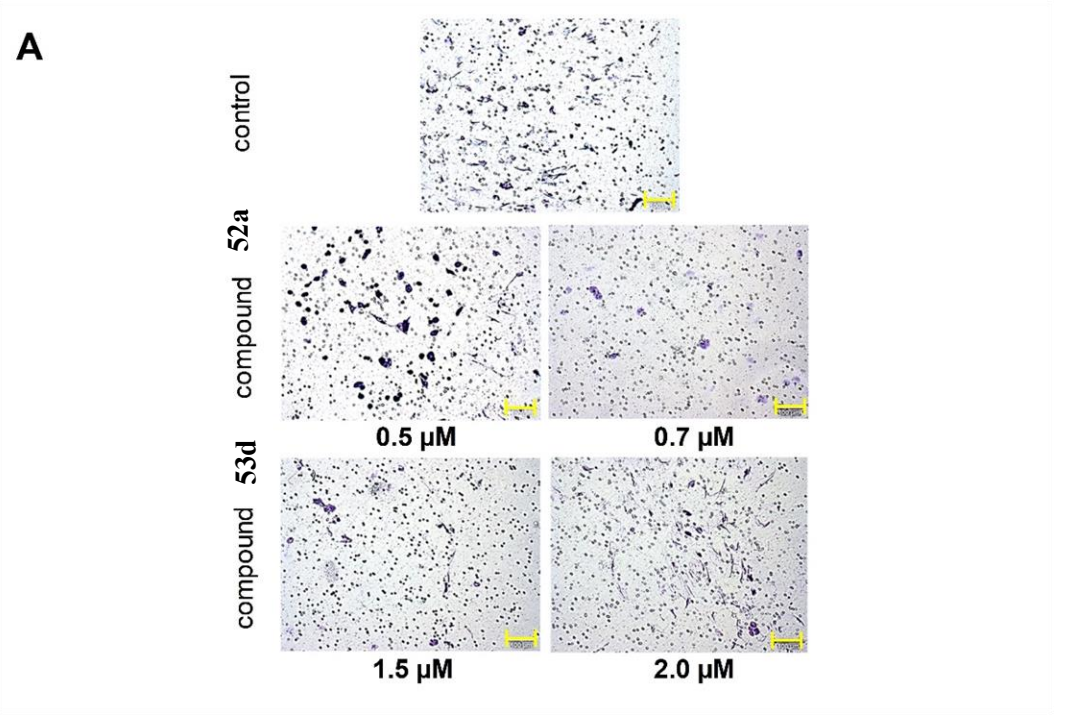


Figure 13A. Effects of compounds on the invasion of A2780 cells.
Panel A: representative pictures after 48 h treatments with **52a** and **53d**.
The bar in the figures indicates 100 µm.

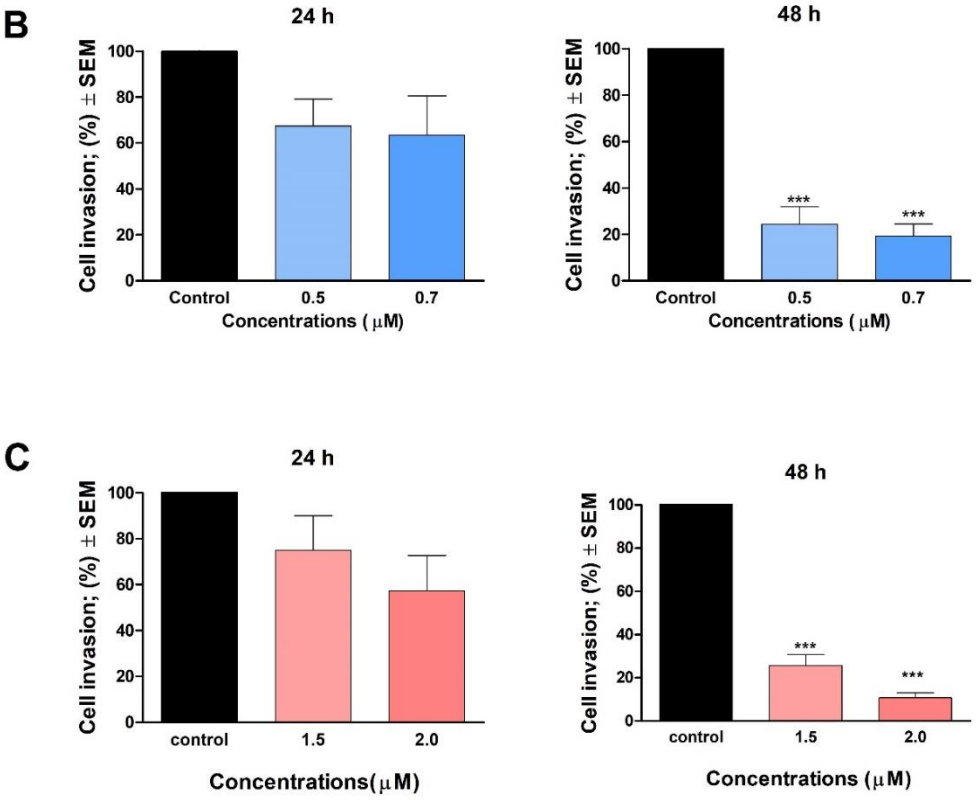


Figure 13B. Statistical analysis of Boyden chamber assay. **Panel B:** calculated number of invading cells treated with **52a**. **Panel C:** calculated number of invading cells treated with **53d**. *** displays significance at $p < 0.001$. Results from at least 4 independent experiments performed in duplicate.

5. Discussion

As malignant disorders are the second leading cause of death worldwide and represent a major and ever-growing burden on healthcare systems, pharmacological investigation of anticancer derivates is one of the greatest challenge of medicinal chemistry.[14] To find an appropriate agent for therapy is even much more complex task in cancer research nowadays, because of the presence and awareness of metastasis formation. As previously described, many natural products have been introduced and used in clinical therapy.[30,32] Terpenoids are the large group of secondary metabolites, which have been applied as medical agents for a long time, and they have wide variety of pharmacological activities. In recent years, a large volume of published studies reported their role as potential anticancer agents.[60–62] Based on literature data, terpenoids can exhibit anticancer effect by inducing cell cycle arrest , as well as initiation of apoptosis. Moreover, they may influence angiogenesis and metastasis by modulating intracellular signaling pathways.[63,64] The structural diversity of compounds with natural sources can be an advantage, which can give greater plasticity in interactions with cells. Also, they can exhibit remarkable antiproliferative action with hormone-independent mechanisms of activity. In clinical trials, the therapeutic effects of some terpenoids were previously tested. In the case of breast cancer, D-limonene reduced the expression of cyclin D1, which can inhibit the proliferation of tumor cells.[65] An other terpenoid, β-elemene was the most promising derivate in a clinical study, where applying it in several advanced cancers may result in high survival rate with significantly fewer side effects.[66] Although, there are some terpenoids which are unsuitable for clinical use, due to their limited bioavailability. In the case of semisynthetic diterpenoid taxanes, there are some derivatives, which are used as chemotherapeutic agents, such as paclitaxel or docetaxel. For instance, cabazitaxel has lower toxicity, and it can induce mitotic arrest by inhibiting microtubules.[67] It should be mentioned, that structural modifications of terpenoids may lead to new strategies in the development of analogues, that might enhance the bioavailability and efficacy of future anticancer terpenoids. Moreover, they can be lead compounds for the development of androgen or estrogen receptor modulators for use in hormone-responsive cancer chemoprevention and chemotherapy.[68]

The aim of the present study was the pharmacological characterization of newly synthesized terpenoid-based antiproliferative compounds on gynecological cancer cells. In the case of most potent compounds (**52a, 53d**), our goal was to further assess and evaluate their antiproliferative and antimetastatic properties on a ovarian cancer cell line (A2780).

Our *in vitro* studies demonstrated that terpenoid-based compounds can exhibit a remarkable antiproliferative effect against different cancer cells.[42,43,57,69–71]

According to our first library of compounds (**1–20**), namely steviol type aminodiols, the standard MTT assay was performed on four human adherent cell lines (MDA-MB231, HeLa, SiHa, and A2780). Based on growth inhibition percentage, nine out of twenty compounds had been identified as potent antiproliferative agents (**4**, **7–10**, **14–16** and **18)**. Some of them proved to be more potent than cisplatin, which is used as clinically anticancer agent. A few molecules elicited no relevant effect (**1, 2** and **21**), therefore it could be concluded that the presence of aromatic ring is a requirement of the cell-growth inhibiting effect. Among 4-substituted triazoles (**3–6**), one of them (**4**) exerted substantial action, especially against cervical SiHa cells (IC_{50}: 4.64 μM). A large number of studies revealed that derivatives with triazole rings have antiproliferative and proapoptotic properties, thus, the triazole ring can be regarded as a pharmacophore when built onto a skeleton.[72] It was found also that *N*-benzyl substituent is an essential part within the molecule, in the case of aminodiol analogs with secondary or tertiary amino functions (**7–14**). The most potent compound (**15**), showed potency in case of all tested cell lines. Based on above data, it could be concluded that the design, synthesis, and investigation of further analogs with oxazolidine, may be relevant.[42]

In the next phase, a new library of isosteviol-based chiral 1,3-aminoalcohols and thioureido derivatives were synthesized (**21–46**), as reported previously.[43] Among these derivates, the diol (**21**), aldehyde (**22),** and oxime analogues (**23**) did not have relevant antiproliferative properties on cancer cells, consequently, an amino function seems to be essential for their biological activities in this regard. It should be noted that certain newly synthesized agents (**25–30)** exerted comparable or much more potent growth inhibitory potential compared to the reference agent cisplatin. In addition, the estrogen receptor positive MCF-7 and the HPV18 positive HeLa were considered the most sensitive cell lines. It has been proved that the *N*-benzyl substitution is essential for remarkable antiproliferative effect. As noted, cervical cell lines are especially sensitive to these agents. Derivates with aminoketone functions (**31–35**) did not inhibit the proliferation of cancer cells. Based on structure-activity relationship, **27-30** may be regarded as potent agents, against HPV18 positive HeLa and HPV16 positive SiHa cells.

In case of the third compound library, the antiproliferative properties of the prepared monoterpene-based diaminopyrimidine analogues (**47a–54d**) were determined. Unfortunately, it was shown that compounds with primary amino function on the pyrimidine ring or monoamino substituted pyrimidines were either ineffective (**50b, 54c,** and **54d**) or

exerted modest activities even at the highest concentrations tested (**47a**–**d**, **50a**, and **50b**). One compound (**54a**) was selective for the HPV18 positive HeLa, the growth-inhibitory percentage was above 90%. When comparing the effect of *p*-CF_3-phenyl and 4-pyrazolyl substituents at the 2-amino function, the *p*-CF_3-phenyl substituent have better activities than the 4-pyrazolyl substituent in the case of the 6-F analogs (**48a/49a, 48c/49c,** and **51b/52b**). Further observation also confirmed that the position of the monoterpene component in the diaminopyrimidine ring does not seem relevant to biological activity in this regard. Moreover, chloro-substitution on the diaminopyrimidine skeleton elicited the most pronounced effect on the proliferation of cancer cells (**52a** and **53d**). According to 4-tetrahydropyridine-2,3-epoxy-1-ol derivates (**55–61d**), the *in vitro* pharmacological studies confirmed that the 1,4-aminoalcohol function, together with the oxirane and tetrahydropyridine ring systems, were required for potent antiproliferative activity. However, the stereochemistry of the oxirane ring and the N-substituents on the tetrahydropyridine function had no influence on the antiproliferative effect.[57,58]

Based on our findings, concerning structure relationships and antiproliferative activities, two pinane-based 1,4-amino alcohol derivatives (**52a** and **53d**) were selected for further evaluation. Interestingly, analogue **52a** exerted higher growth inhibition than the reference agent, cisplatin. Both exhibited antiproliferative action on A2780 cell line, so both molecules may be considered as promising novel anticancer agent against ovarian adenocarcinoma. In this view, both were selected for further investigations to explore their mechanism of action and antimetastatic properties.

In the following step, to calculate IC_{50} values of **52a** and **53d**, the previously included cancer cell lines were applied. To determine their tumor selectivity was also essential for the following investigations; for that purpose, both compounds were assessed against non-cancerous mouse fibroblast cell line (NIH/3T3). The calculated IC_{50} values were four-fold higher in the case of both compounds, than against ovarian cancer. The determination of tumor selectivity index revealed that data comparable with values of cisplatin. Based on this result, our compounds have a moderate tumor selectivity.

The exploration of the mechanism of action of newly synthesized compounds is generally required and desirable in pharmaceutical research. In this regard, the apoptosis inducing effects of the selected compounds were proven by fluorescent double staining and cell cycle analysis. To identify morphological signs of programmed cell death, the HOPI method was utilized. Chromatin condensation in early apoptotic cells and loss of membrane integrity in necrotic and secondary necrotic cells were visualized under a fluorescent microscope by Hoechst 33258/PI staining after 24 h and 48 h incubation with different

concentrations of **52a** and **53d**. Morphological changes confirmed the concentration-dependent proapoptotic effect of both compounds on A2780 cell line. In the case of **52a**, elevation of the concentration has led to the formation of late apoptotic or secondary necrotic cells with loss of membrane function. Beside the detection of apoptosis, cell cycle analyses also provided important details about the mechanism of action of the tested terpenoid derivates. An increased subG1 population was related with apoptotic processes[73], and to confirm proapoptotic properties of analogues, flow cytometric analysis were performed on ovarian cancer cells. Both compounds exhibited significant elevation of cell number in the G2/M phase and indicated a cell cycle arrest at that phase. Furthermore, the increase in the G2/M population was typically more substantial after 48 h than after 24 h of incubation, indicating cell cycle arrest at the G2/M phase. In addition, the proportion of the hypodiploid subG1 fraction, regarded as the apoptotic cell population, increased in a concentration-dependent manner, supporting the proapoptotic property of **52a** and **53d** in the A2780 cell line. These finding are consistent with recently reported studies, highlighting that terpenoids typically cause G2/M arrest and apoptotic cell death in different human cancer cells.[64,74]

The direct effect of the tested compounds on tubulin polymerization was another crucial step forward to explore their mechanism of action. During mitosis, tubulin polymerization is an important process, the imbalance between polymerization and depolymerization of microtubule can result in the termination of cell division.[75] This phenomena can be regarded as a target for anticancer drugs, and is readily being utilized during therapy. We can distinguish two groups of such compounds: one of them are depolymerization agents such as nocodazole, they exert their antitumor effects by binding microtubular proteins, which can disturb tubulin polymerization, disrupting the microtubule assembly during mitosis, and the metaphase arrest of the cell.[76] The other group of compounds are the tubulin-binding agents such as paclitaxel, by stabilizing the microtubule polymer and preventing microtubules from disassembly. Paclitaxel arrests the cell cycle in the G0/G1 and G2/M phases and induces cell death in cancer.[37] Because **52a** and **53d** was detected to arrest cell cycle in the G2/M phase, it was rational to investigate the direct effect of these analogues on tubulin polymerization in an *in vitro* cell-free system. Moreover, many studies reported that terpenoids induced pronounced cell cycle arrest *via* direct influence on tubulin polymerization.[74,77] Both compounds caused a disturbance on tubulin polymerization, especially **53d** at 300 μM**,** which is referred by and increase in the maximum rate of tubulin polymerization (V_{max}). Consequently, an imbalance occurred between the polymerization and depolymerization of microtubules, leading to cell cycle

arrest and inducing apoptosis. Our results suggest that test compounds may have a paclitaxel-like microtubule-stabilizing effect.

As previously described, ovarian carcinoma has the highest case-to-fatality ratio among all gynaecologic malignancies.[26] It has been characterized by rapid growth, early metastasis development, and a generally aggressive disease course. There are not any specific, early symptoms that may warm patients or clinicians for the diagnosis. Unfortunately, a majority of patients present at an advanced stage, with extensive metastatic disease. There has been an increasing number of studies indicate that, the 5-year survival rates is under 20% in metastatic disease, in comparison with non-metastatic patient where it is above 90%.[24,78] Metastasis formation has been identified as major contributing factor for poor patient prognosis and cancer-related death globally.[23,79,80] During tumor progression, including invasive growth and metastasis development, cell invasion is crucial for cancer spread and for the formation of distant metastases. [81,82] To achieve reduction in the mortality of ovarian cancer, research for novel drug candidates capable of interfering with metastasis formation is eagerly required. The metastatic cascade refers to a multi-step process, involving cancer cell migration and invasion through the basal membrane to reach the extracellular matrix and neighboring tissue.[81] In the *in vitro* experimental system, antimetastatic properties of **52a** and **53d** were assessed by wound healing and Boyden chamber assays. Both compounds inhibited the invasion and migration of A2780 cells after 48 exposures. Our results also demonstrated that the inhibition of cell motility becomes obvious at 0.5 μM of **52a** and 1.5 μM of **53d**, which are below the concentration of substantial antiproliferative action. Our findings indicate that besides their antiproliferative, proapoptotic, and tubulin-disrupting activities, compounds **52a** and **53d** may also can be potent antimigratory agents, due totheir significant inhibitory effects on cell migration and invasion.[71]

In conclusion, our results demonstrate that, selected analogues are promising antiproliferative agents *in vitro*. During the cell cycle analysis, both compounds caused G2/M phase arrest, presumably, by stabilizing tubulin polymers, leading to programmed cell death. Besides their antiproliferative and proapoptotic properties, these analogues are suggested to show antimetastatic potential against the utilized ovarian cancer cell lines. Based on our findings, compounds **52a** and **53d** can also be regarded as potential new drug candidates with a promising new mechanism of action among terpenoids, possessing antiproliferative properties, and they may be utilized for the design of novel anticancer agents.

6. Summary

In this study, our finding can be summarized as follows:

- From the tested terpenoid compounds in our studies, a substantial portion of the compounds included in our experiments exerted pronounced antiproliferative properties with remarkable growth-inhibition percentages on breast and gynecological malignant cell lines (HeLa, SiHa, MDA-MB-231, MCF7, A2780).
- Two out of the 86 compounds were selected for further evaluation to explore their mechanism of action on an ovarian cancer cell line. Calculated IC_{50} values were 0.8-3.0 μM on A2780 cells.
- The calculated IC_{50} values of the tested compounds on NIH/3T3 non-cancerous cell line were four-fold higher than on A2780 cells, which may be interpreted as moderate tumor selectivity.
- The most potent compounds showed pronounced proapoptotic effects on the A2780 cell line, as evidenced by fluorescent double staining. Morphological changes confirmed the concentration-dependent proapoptotic effects of both compounds on ovarian carcinoma cells.
- According to cell cycle results, both terpenoids increased the cell number of the subG1 population, and caused cell cycle arrest in the G2/M phase.
- The tubulin polymerization assay indicated that both compounds have direct tubulin stabilization effects. Additionally, the antiproliferative effects may be elicited through increase in maximum rate of tubulin polymerization.
- The antimetastatic characteristic of our test substances was demonstrated by inhibiting cell migration, invasion below the concentration of substantial antiproliferative action.

In summary, the results have demonstrated that compounds are potent antiproliferative agents via our *in vitro* experiments. Both compounds had influence on the cell cycle, induced G2/M phase blockade by stabilizing tubulin polymers, which resulted in programmed cell death. Besides their proapoptotic effect, their antimetastatic potential was also confirmed on the utilized ovarian cancer cell lines. Based on the presented findings, compounds **52a** and **53d** may be regarded as new drug candidates with promising new mechanisms of action among the antiproliferative terpenoids and may potentially allow for the design of novel anticancer agents.

7. Glossary of Acronyms and Abbreviations

- **ANOVA:** analysis of variance
- **AP-1:** activator protein-1
- **ATCC:** American Type Culture Collection
- **ATP:** Adenosine-triphosphate
- **BRC1/BRC2:** breast cancer type 1 susceptibility protein gene / breast cancer type 2 susceptibility protein gene
- **BRIP1:** BRCA1 interacting protein 1
- **CCC:** clear-cell carcinomas
- **CDK:** cyclin-dependent kinase
- **CO_2:** carbon dioxide
- **Comp.:** compound
- **Conc.:** concentration
- **Cyclin D1:** cell cycle protein, tumor marker
- **DISC:** death-inducing signaling complex
- **DMSO:** Dimethyl sulfoxide
- **DNA:** Deoxyribonucleic acid
- **EC:** endometrioid carcinoma
- **ECACC:** European Collection of Authenticated Cell Cultures
- **EOC:** epithelial ovarian carcinomas
- **ER:** endoplasmic reticulum
- **FBS:** fetal bovine serum
- **G1:** first growth phase in cell cycle
- **G2/M:** second growth phase in the cell cycle / mitotic phase
- **GLOBOCAN:** Global Cancer Observatory
- **GLUT1:** glucose transporter 1
- **HEPA:** high-efficiency particulate absorbing filter
- **HER2**: human epidermal growth factor receptor 2
- **HIV:** human immunodeficiency virus
- **HOPI:** Hoechst 33258 – propidium iodide
- **HPV:** human papillomavirus
- **IARC:** International Agency for Research on Cancer
- **IC_{50}:** half maximal inhibitory concentration
- **JAK-STAT:** Janus kinases-signal transducer and activated of transcription proteins

- **MC:** mucinous carcinoma
- **MEM:** minimal essential medium
- **MLH1:** DNA mismatch repair protein
- **MMPs**: metalloproteinases
- **MSH2:** tumor suppressor gene
- **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide
- **NCI:** National Cancer Institute
- **NEAA:** non-essential amino acids
- **NF-κB:** targeting nuclear factor κB
- **p:** p-value, level of statistical significance
- **p53:** cellular tumor antigen p53
- **PAC:** paclitaxel
- **PBS:** phosphate buffer solution
- **PET:** polyethylene terephthalat
- **RAD51C:** gene for hereditary breast and ovarian cancer syndrome
- **RAD51D:** gene for hereditary breast and ovarian cancer syndrome
- **Ras-Raf-MAPK:** mitogen-activated protein kinase signal transduction cascade
- **ROS:** reactive oxygen-species
- **S:** phase of DNA replication
- **SC:** serous carcinoma
- **SEM:** standard error of the mean
- **SGs:** steviol glycoside
- **Sub-G1:** hypodiploid cell fraction
- **USA:** United States of America
- **VEGFR**: vascular endothelial growth factor receptor
- **V_{max} :** maximum initial rate of reaction
- **WHO:** World Health Organization

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