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Characterization of antiproliferative and antimetastatic properties of novel terpene derivates

Summary of Ph.D. Thesis

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Characterization of antiproliferative and antimetastatic properties of novel terpene derivates

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1. Introduction

According to the estimates of the World Health Organization (WHO), malignant disorders (or cancer) represent one of the major health burdens globally. Besides cardiovascular disease, they are second leading cause of death worldwide, and still a critical and unsolved public health issue. Based on the latest survey by Global Cancer Observatory (GLOBOCAN), 19.3 million new cancer cases and almost 10.0 million cancer-related deaths occurred in 2020. Based on statistical data of las twenty years, it could be concluded that epidemiological data indicate increasing trends in the development of cancer Moreover, several studies suggested that the global cancer burden will continue to rise considerably in the future.The number of new cases may reach 23.6 million by 2030. 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. Whereas cervical cancer is the fourth most commonly diagnosed cancer, regarding the causative factors, cervical tumors are mostly caused by HPV virus, especially HPV 16 and HPV 18 strains. During last two decades, vaccination and screening methods became widely available in developed countries, therefore the number of death cases have decreased. Ovarian cancer is also a notable type of gynecological malignancies and the 7th most commonly detected cancer in women. Despite this fact, ovarian cancer is the most lethal female reproductive cancer and accounts for the highest mortality rate. 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%. The standard treatment of ovarian cancer consists of cytoreductive surgery and chemotherapy with platina/taxane combination. While the response rate to the first-line therapy is usually good (around 80-90%), most patients will have a recurrence of cancer and resistance of chemotherapy. In addition, robust methods for early detection are not available, and the lack of specific symptoms can contribute to delayed diagnosis.

A considerable amount of literature has been published on natural products, which are a vital source of leads for anticancer drug development. Many natural products have been introduced into the 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. Terpenoids, are the largest class of natural products, represent an important class of secondary plant metabolites. They act as alkylating agents by addition to intracellular targets, typically sulfhydryl groups of proteins, thereby inhibiting the function of enzymes required for proliferation. In addition, they can target several stages of cancer development including cell proliferation, angiogenesis, and metastasis and they can induce programmed cell death.

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. Several potent derivates had been identified, as a result, two potent monoterpenes-based compounds were selected for further evaluation.

The purpose of the performed experiments were the followings:

- Characterization the antiproliferative potential of diterpene and monoterpene derivates against human gynecological cancer cell lines by 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 non-cancerous mouse fibroblast cell line (NIH/3T3).
- Detection of proapoptotic properties of the most effective compounds by Hoechst 33258/propidium iodide (HOPI) fluorescent double staining.
- Characterization of the elicited alterations in cell cycle progression of A2780 cell line by flow cytometry.
- Determination of the effects of the selected compounds on microtubules by cell-free tubulin polymerization assay.
- Determination of the antimetastatic potential of the most potent compounds by wound healing and Boyden chamber assays respectively.

3. Material 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. The chemical structure of the most potent and widely investigated compounds of this study are presented in **Figure 1.**

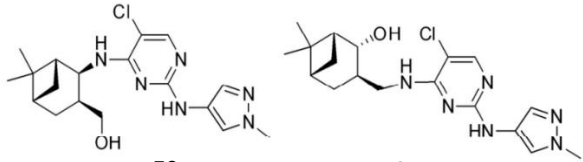

Figure 1. Structures of the investigated monoterpene-based diaminopyrimidine analogs.

3.2. Cell cultures and culturing conditions

To assess the antiproliferative potential, a panel of human adherent gynecological cell lines were utilized, including cervical cancer cell lines (Hela, SiHa), breast cancer cell lines (MDA-MB-231, MCF-7), ovarian (A2780) and non-cancerous mouse fibroblast cells (NIH/3T3). All cell line were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA) and 1% antibiotic-antimycotic solution, at 37^0C, under humidified air circumstances with 5% CO_2.

3.3. MTT assay (Antiproliferative assay)

To determine the proliferation and viability of cells upon treatment with test compounds, standard MTT assay was performed. Cells were seeded onto 96-well microplates at a density of 5000 cells/well. After an overnight incubation, cells were treated with elevated concentration of test compounds (0.1-30 μM), and incubated for 72 hours under cell culture conditions, as noted previously. Subsequently, 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to the wells and incubated for 4 hours. To dissolve the precipitated formazan crystals, dimethyl sulfoxide (DMSO) was added into wells. Lastly, absorbance values were recorded by a microplate-reader at 545 nm. Untreated cells were used as a control, and cisplatin was used as a clinically applied reference compound. Six-point dose-response curves were fitted to the measured points, and the IC_{50} values were calculated by the GraphPad Prism 5.0 software. Two independent measurements were performed with five parallel wells.

3.4. Hoechst 33258–propidium iodide (HOPI) fluorescent double staining

To characterize the changes in cell morphology and cell membrane integrity upon treatment with test compounds, fluorescent double staining was performed. Briefly, suspension of A2780 cells were seeded into 6-well plates, and incubated under cell culture conditions for an overnight, then treated with the desired concentrations of test compounds for 24 h and 48 h. After the appropriate 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 equipped with appropriate optical blocks. 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 a more detailed mechanism of action of test compounds, and their influence on cell cycle of ovarian adenocarcinoma cells, cell cycle analysis by flow cytometry was performed. Cell suspension was seeded onto 24-well plates. After an overnight incubation, the samples were treated with increasing concentration of test compounds for 24 h and 48 h. Then, 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. Untreated cells were considered as control, as well as 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. According to the manufacturer's recommendations, the assay reactions were performed on pre-warmed (37^0C), UV-transparent 96-well microplate. Then, test compound solutions were placed on the wells, general tubulin buffer were used as untreated control, and paclitaxel (PAC) served as the reference compound. Absorbance of the samples was recorded by a microplate reader at 340 nm, and a 60-min kinetic measurement protocol was applied. The maximum reaction rate (Vmax; Δabsorbance/min) of tested compounds was calculated by the highest difference between the measured absorbances at two consecutive time points.

3.7. Wound-healing assay

To determine the antimetastatic properties, namely, the influence of tested compounds on cell migration, Wound-healing assays was carried out by using the ibidi Culture-Insert. Suspension of A2780 cells was prepared in minimal essential medium supplemented with 2% FBS. Subsequently, cells were seeded into each chamber of special silicone inserts on 12-well microplates. After the incubation time, inserts were gently removed, and 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 software. The migration rate was calculated based on comparison the wound closure of untreated controls and treated wells.

3.8. Boyden chamber assay

The most widely accepted cell migration technique, namely the Boyden-chamber assay, was performed to assess to the invasion ability of ovarian adenocarcinoma cells upon treatment with potent test compounds. According to protocol, BD BioCoat Matrigel Invasion Chamber are required to this assay, which are containing an 8 µm pore size polyethylene terephthalate (PET) membrane and a thin layer of Matrigel basement matrix, and act as an *in vitro* model of the extracellular environment. Suspension of 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 cell culture condition, 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, 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 was used. The statistical significance was estimated by one-way analysis of variance (ANOVA), followed by the Dunnett post-test. In the Results section, all data are presented as means ± 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 assay

As described above, 3 set of compounds were screened by standard MTT-assay. Altogether, 24 compounds exhibited remarkable antiproliferative activities. They had compared each other according to structure–activity relationships and growth inhibition percentage. Our goal was to find candidates against ovarian cancer cell line,

so only two potential compounds (**52a** and **53d**) were selected. The investigation of tumor selectivity in intact human fibroblast cell lines revealed that the selectivity of the test compounds for the cancerous cells are comparable with reference agent (cisplatin)**. (Table 1-2.)**

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 1. Calculated IC_{50} values of both test compounds and cisplatin, measured by MTT assay after incubation for 72 h on gynecological cancer and intact fibroblast 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

Table 2. Tumor selectivity index of selected compounds on the utilized cell lines

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.

4.2 Hoechst-Propidium double staining

Fluorescent nuclei staining was performed, using Hoechst 33258 and propidium iodide dyes, after 24 h and 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. In general, the statistical evaluation revealed a concentration-dependently elevated ratio of apoptotic and necrotic cells after 48 h, accompanied by a significant decline in the ratio of viable intact cells, in the case of both compounds. It could be observed that **52a** induced a more pronounced apoptotic or necrotic damage compared to **53d.** (**Figure 2.**)

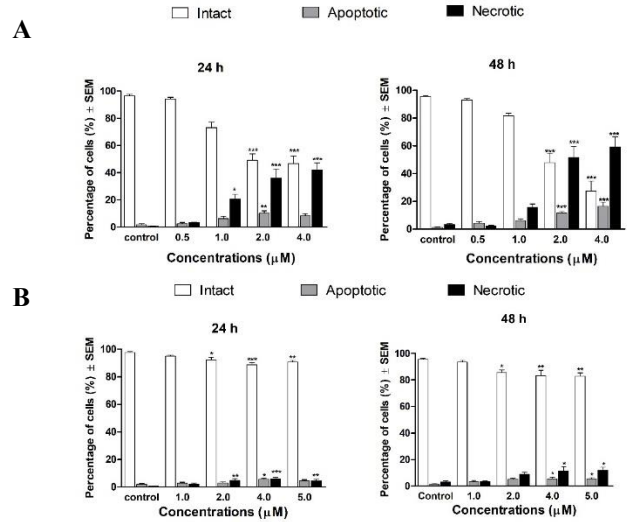

Figure2. Percentages of intact, apoptotic, and necrotic cell populations exposure to (**A**): compound **52a** (**B**): compound **53d**, after 24 h and 48 h incubation time. *, ** and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared to control. Data are from three independent experiments performed in triplicate; non-significant changes are not shown.

4.3. Cell cycle analysis

The effects of test compounds on the cell cycle were investigated by flow cytometry after 24 h and 48 h of incubation. The ratio of 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 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 and dose-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 could suggest that **52a** and **53d** induce a marked cell cycle arrest in the G2/M in a dose-dependent manner. (Figure **3.**)

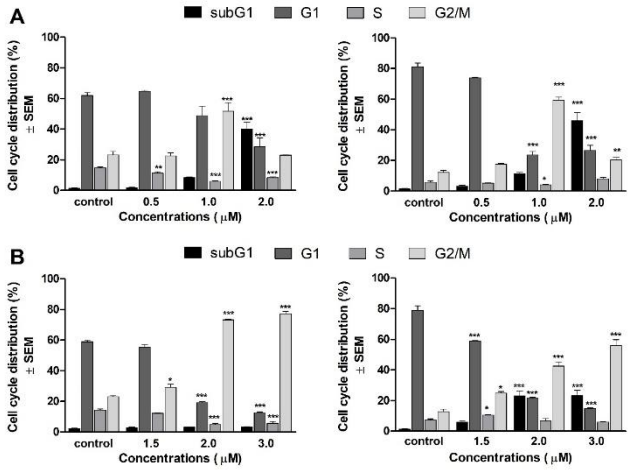

Figure 3. Effect of tested compounds on cell cycle distribution of A2780 cells (A): treated with 0.5, 1, and 2 μM of **52a** for 24 h and 48 h (B): treated with 1.5, 2.0, and 3.0 μM of **53d** for 24 h and 48 h. *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared to control. Data are from three independent experiments performed in triplicate, non-significant changes are not shown.

4.4. Tubulin polymerization

A cell-free photometric assay was used to determine the direct effects of the test compounds on microtubule formation. The relatively high applied concentrations were calculated according to manufacturer's recommendations. In comparison with control, the highest concentration of both test compounds induced a significant acceleration of tubulin polymerization. In addition, a considerable rise could be recorded in the Vmax value at 350 μM of **52a** and 300 μM of **53d.** (Figure **4.**)

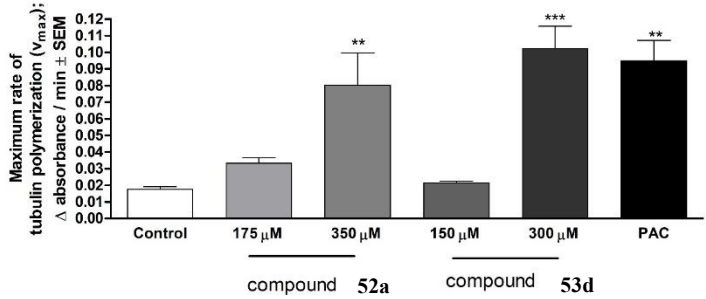

Figure 4. The calculated maximum rate of tubulin polymerization of the tested compounds in a cell independent experimental system, in vitro. Paclitaxel (PAC) was included as a reference agent Results are means ± SEM of the data on two separate measurements with duplicates. ** and *** indicate significance at $p < 0.01$ and $p < 0.001$, respectively, compared to control. Findings are based on the results of two independent experiments, both performed in duplicate. Non-significant changes are not shown.

4.5. Wound healing assay

To determine the anti-migratory effect of test compounds, changes in the size of 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. Treatment with

52a, no significant differences were observed after 24h. However, a significant change was obtained after 48 h at both concentrations. On the other hand, treatment with compound **53d** induced a considerable inhibition at 1.5 µM, as well as 2.0 µM after 24 and 48 hours of treatment compared to the control. In conclusion, our results revealed that both analogs have antimigratory properties. (Figure **5.**)

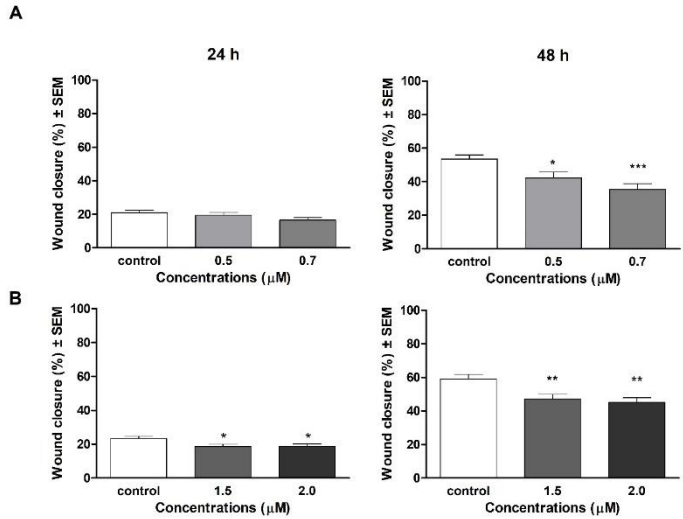

Figure 5. Effect of compounds on the migratory capacity of A2780 cells after 24 and 48 hours analyzed by wound healing assay. (**A**): treatment with compound **52a**, (**B**): treatment with compound **53d**. Rate of migration was calculated as the ratio of wound closure in treated samples compared to control samples. Results are mean values ± SEM of the data on three separate measurements with triplicates. *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Findings are based on the results of 4 independent experiments, all performed in triplicate. Non-significant changes are not shown.

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 of cellular migration with a special Boyden chamber assay as the extracellular environment of primary tumors, to study cellular invasion. Both compounds elicited a modest action on the invasion of A2780 cells after 24 h of incubation. In contrast, a more prolonged incubation period (48 h) revealed impressive results for both compounds. (Figure **6.**)

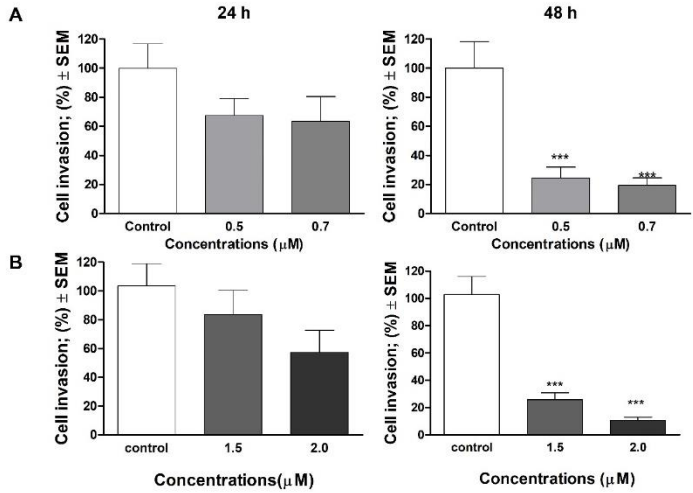

Figure 6. Cell invasion capacity of A2780 cells through matrigel coated Boyden chambers. Statistical analysis of the percentage of invasive cells after 24 h and 48 h incubation time compared to control samples. (**A**): treatment with compound **52a**; (**B**): treatment with compound **53d**. *** indicates significance at $p < 0.001$. Findings are based on the results of at least 4 independent experiments, all performed in duplicate. Non-significant changes are not shown.

5. Discussion

Since malignant disorders are the second leading cause of death worldwide and cancer represent a huge burden on healthcare system, pharmacological investigation of anticancer derivates is one of the greatest challenges. To find an appropriate agent for therapy is even much more complex task in cancer research nowadays, because of the presence of metastasis. As previously described, many natural products have been introduced and used in clinical therapy.

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. Based on literature data, terpenoids can exhibit anticancer effect by arrest of cell cycle, as well as initiation of apoptosis.

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

The exploration of the mechanism of action of newly synthesized compounds is generally required in pharmaceutical research. In this regard, the apoptosis inducing effects of the selected compounds were proven by two independent methods, namely, fluorescent double staining and cell cycle analysis. Morphological signs of programmed cell death were assumed by Hoechst33258/PI staining. Chromatin condensation in early apoptotic cells and loss of membrane integrity in necrotic and secondary necrotic cells were visualized under a fluorescent microscope after 24 h and 48 h incubation. Statistical analysis revealed the concentration-dependent proapoptotic effect of both analogs on A2780 cell line. Consequently, elevation of the concentration lead to the formation of late apoptotic or secondary necrotic cells with loss of membrane function.

To assess the mechanism of action of test compounds, cell cycle was performed on A2780 cell line. Both test compounds induced a concentration-dependent disturbance of cell cycle. In general, the most noticeable consequence of treatment was the elevation of G2/M populations and the accumulation of hypodiploid (subG1) cells

at the expense of the G1 population. After 48 h incubation, the increase in the G2/M population was much more substantial, which can be indicated 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 both analogs (**52a** and **53d**) on the A2780 cell line. This finding aligns with recent studies showing that terpenoids typically cause G2/M arrest of the cell cycle and apoptotic cell death in different human cancer cell lines.

The direct effect of the tested compounds on tubulin polymerization is another crucial step forward to explore their mechanism of their action. During mitosis, tubulin polymerization is an important progress, the imbalance between polymerization and depolymerization of microtubule can result in the termination of cell division. This shift can be regarded as a target for anticancer drugs. We can distinguish two group, one of them exert their antitumor effect by binding microtubular proteins, which can disturb tubulin polymerization, disrupting the microtubule assembly during mitosis, and the metaphase arrest of the cell. The other group is the tubulin-binding agents such as paclitaxel, by stabilizing the microtubule polymer and preventing microtubules from disassembly, which can result in the cell cycle arrest, especially in the G0/G1 and G2/M phases. Several studies reported that terpenoids induced pronounced cell cycle arrest via direct influence on tubulin polymerization. Based on our findings, both compounds caused a disturbance on tubulin polymerization which is characterized by an increase in the maximum rate of tubulin polymerization (Vmax). 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.

Antimetastatic properties of tested compounds is crucial part of the development of anti-cancer drugs. Metastasis formation have been identified as major contributing factors for poor patient prognosis and cancer-related death globally. As noted previously, ovarian cancer accounts for the highest mortality rate because of five-year survival rates below 45%. Metastasis formation is a complex process, it consists of including changes in cell adhesion, migration, and invasion. The prevention or inhibition of this multistep process is crucial in the future of tumor therapy. In this study, the *in*

vitro 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 h 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. In conclusion our findings revealed that, besides their antiproliferative, proapoptotic, and tubulin-disrupting activities, compounds **52a** and **53d** also can be potent antimigratory agents because of their significant inhibitory effects on cell migration and invasion.

In summary, the tested compounds can 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.