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Influence of betulin and 28-*O*-propynoylbetulin on proliferation and apoptosis of human melanoma cells (G-361)*

Wpływ betuliny i 28-*O*-propynoilobetuliny na proliferację i apoptozę ludzkich komórek czerniaka linii G-361

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Introduction: Pentacyclic triterpenes are a group of compounds known to have anticancer activity. One of the best characterized triterpenes is betulin, which can be isolated from bark of birch trees and modified into new compounds with various interesting medical properties. Betulin is involved in activation of the caspase cascade and promotes cell death. The aim of the study was to investigate the effect of betulin and its acetylenic derivative, 28-*O*-propynoylbetulin, on proliferation and apoptosis in a human melanoma cell line.

Materials and Methods: The G-361 melanoma cell line was used. To evaluate growth arrest and caspase-3 activity, cells were treated with betulin and its derivative at a wide range of concentrations from 0.1 to 10 μg/mL.

Results: Betulin and 28-*O*-propynoylbetulin inhibited cell proliferation in a concentration-dependent manner. The cell cycle analysis revealed an increase of the sub-G1 cell fraction (representing dead cells) after incubation of cells with betulin and 28-*O*-propynoylbetulin. The observed cytotoxic effects were more pronounced for 28-*O*-propynoylbetulin. Activity of caspase-3 in 28-*O*-propynoylbetulin treated cells was nearly 2-fold greater compared to cells incubated with betulin.

Discussion: Our results show that betulin and 28-*O*-propynoylbetulin were effective in inhibition of cell growth and induction of apoptosis in a human melanoma cell line. The addition of the propynoyl group at the C-28 hydroxyl group of betulin led to a greater proapoptotic and antiproliferative effect in comparison to unmodified betulin. These observations suggest that the obtained derivative is a potent anti-melanoma agent.

Key words: **malignant melanoma • triterpenes • betulin • 28-*O*-propynoylbetulin • caspase-3 activity**

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Abbreviations: **α-MSH –** α-melanocyte stimulating hormone, **Ac-DEVD-pNA** – acetyl-Asp-Glu-Val-Asp-pNA, **Bak** – Bcl2-antagonist/killer, **Bax** – Bcl-2–associated X protein, **cAMP –** adenosine 3',5',-cyclic monophosphate, **MC-1 –** cell membrane melanocortin receptor 1, **NaB** – sodium butyrate, **PI** – propidium iodide, **pNA** – p-nitroaniline.

Introduction

In 2012, there were at least 100,300 new cases of malignant melanoma in Europe. Malignant melanoma incidence and mortality are constantly rising [12]. The most common risk factors include exposure to UV radiation, fair skin, dysplastic nevi syndrome, age and family history. In view of the significant treatment resistance, conventional therapy strategies are insufficient. Therefore, there is a great demand for more effective anti-melanoma agents [11,16,20]. It has been discovered that a group of natural compounds called triterpenes, widely distributed in nature, has potential anti-cancer activity [16,18].

Betulin [lup-20(29)-ene-3β,28-diol, **1**] is a pentacyclic lupane-type triterpene alcohol found in the bark of birch trees [29]. Betulin (**1**) can be easily isolated from the plant material but it has limited solubility in aqueous media. Fortunately, it can be easily converted into more soluble derivatives such as betulinic acid (**2**) and others. The chemical modifications of betulin (**1**) can be done within the secondary hydroxyl group at position C-3, primary hydroxyl group at C-28 and alkene moiety at position C-20 [8,23,24].

A number of studies have been reported describing antiviral, antibacterial, antimalarial, hepatoprotective and anti-inflammatory activities of betulin derivatives [15,30]. Recent data have described the anti-HIV activity after application of these compounds. Extensive studies indicated that they can act as e.g. membrane fusion or virus maturation inhibitors. Betulin (**1**) and its derivatives induced apoptosis in various cancer cell types, including glioblastoma, leukaemia and lung carcinoma [1,3]. Betulin (**1**) is involved in the mitochondria-mediated caspase-9 activation pathway (intrinsic pathway of apoptosis) by triggering Bak and Bax translocation which results in depolarization of the mitochondrial membrane potential. These processes result in the release of cytochrome c and proapoptotic proteins into the cytosol followed by activation of the caspase cascade (-9, -3, -7) and cell death [19,20]. Betulin derivatives are also involved in triggering cancer cell death.

Betulin (**1**) can bind to the cell membrane melanocortin receptor (subtype MC1) in mouse melanoma cells. MC1 receptors are expressed mainly in skin, melanoma and immune cells. They belong to G-protein-coupled receptors and are involved in cAMP generation. Betulin (**1**) connection with MC1 receptor antagonizes α-melanocyte stimulating hormone and leads to inhibition of cAMP synthesis. On the one hand, α-MSH can act as a metastasis blocker (reducing cell migration and invasion) but on the other, it can reduce the ability of immune cells to detect tumour cells (downregulation of the expression of adhesion molecules). The role of α-MSH and cAMP needs to be more thoroughly investigated but it has been shown that the higher the density of MC1 receptors, the greater is cAMP production in human melanoma cells [22,28].

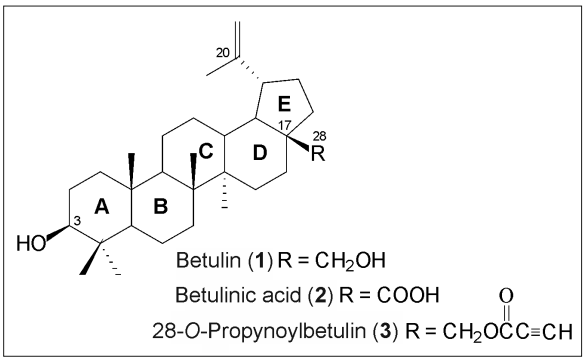

Fig. 1. Chemical structure of betulin (**1**), betulinic acid (**2**) and 28-*O*-propynoylbetulin (**3**)

The aim of the study was to investigate the effect of betulin (**1**) and its derivative called 28-*O*-propynoylbetulin (**3**) (Fig. 1) on proliferation and apoptosis in the G-361 human melanoma cell line.

Materials And Methods

Chemicals

Betulin (**1**) with a purity of ≥98% was purchased from Sigma-Aldrich. 28-*O*-Propynoylbetulin (**3**) was synthesized

via esterification of the C-28 hydroxyl group of betulin (**1**) with propynoic acid and characterized by standard spectroscopic methods. The ^{1}H-NMR, ^{13}C-NMR, IR and MS (EI) spectral data of compound **3** were reported in the literature by Boryczka [4].

Cells

The malignant melanoma G-361 cell line was purchased from LGC Promochem (Łomianki, Poland). It was obtained from the skin of 31-year-old male patient. The cells were grown in medium containing the following composition: 90% McCoy's medium (Sigma-Aldrich), 10% fetal bovine serum (PAA), 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma-Aldrich), and 10 mM HEPES (Sigma-Aldrich). The cells were cultivated at 37°C in a humidified atmosphere containing 5% CO_2.

Cell proliferation

To assess cell proliferation, the "In Vitro Toxicology Assay Kit, Sulforhodamine B Based" (Sigma-Aldrich) was used. Cells were seeded at an initial density of 10^3 cells per well in 200 μl of culture medium in 96-well plates. After 24 hours of incubation, test reagents – betulin (**1**) and 28-*O*-propynoylbetulin (**3**) – were added for the next 72 hours. The range of concentrations for both reagents was from 0.1 to 10 μg/mL. At the end of the incubation period, cells were washed with phosphate-buffered saline (PBS, Sigma-Aldrich), fixed in trichloroacetic acid (10% TCA) and stained with sulforhodamine B. After dissolution of the incorporated dye, absorbance was measured at 570 nm and 690 nm (reference wavelength) using the MRX Revelation plate reader (Dynex Technologies).

Cell cycle analysis

To study the cell cycle, G-361 cells were plated at an initial density of 10^6 cells per 100-mm diameter dish in 15 mL of culture medium. Cells were allowed to attach and grow for 24 hours prior to exposure to test reagents. Cells were incubated with compounds **1** and **3** (both at concentrations of 3 μg/mL and 10 μg/mL) for 48 hours. Control cultures were treated with vehicle (0.2% DMSO). Sodium butyrate (NaB) at a concentration of 1 mM was used as a positive control. NaB is known to block cell cycle progression in G1 and G2 phases.

After 48 hours cells were trypsinized and washed twice with ice-cold PBS, filtered (20 μm Syringe Filcons Non-Sterile, BD Biosciences) and fixed in 70% ice-cold ethanol while vortexing at low speed. The samples were stored at 4°C overnight. Directly before the assay, cells were treated with RNase A (10 mg/ml, Sigma-Aldrich, for 1 hour at 37°C), stained with propidium iodide (PI, Calbiochem, 1 mg/ml) and then analysed in a flow cytometer (FACS AriaII, Becton Dickinson). DNA histograms were examined with BD FACSDiva software (Becton Dickinson) and showed the distribution of cells in cell cycle phases. Additionally, the amount of polyploids was estimated.

Caspase-3 activity

To determine the proapoptotic caspase-3 activity, the "Caspase-3 Assay Kit, Colorimetric" (Sigma-Aldrich) was used. Cells were plated at the density of 10^6 per 100-mm diameter dish and incubated in standard medium for 24 hours. The investigated reagents (**1,** 10 μg/mL and **3,** 10 μg/mL) were added for the next 24 hours. After this period, cells were scraped from the dishes and lysed. Cell lysates were centrifuged (16000 g; 10 min) and stored at -80°C. The assay is based on hydrolysis of the substrate, Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Asp-pNA), by active caspase-3. The absorbance of released p-nitroaniline (pNA) was measured at 405 nm using the MRX Revelation plate reader (Dynex Technologies). Results are expressed relative to cellular protein content, which was determined spectrophotometrically by Bradford's method [6].

Results

Cell proliferation

In order to elucidate the influence of tested compounds on cell proliferation, cells were incubated in the presence of various concentrations (0.1, 0.3, 1, 3, 10 μg/mL) of betulin (**1**) and compound **3**. The effect of 72 hours of incubation is shown in Fig. 2. The lowest concentrations (0.1 and 0.3

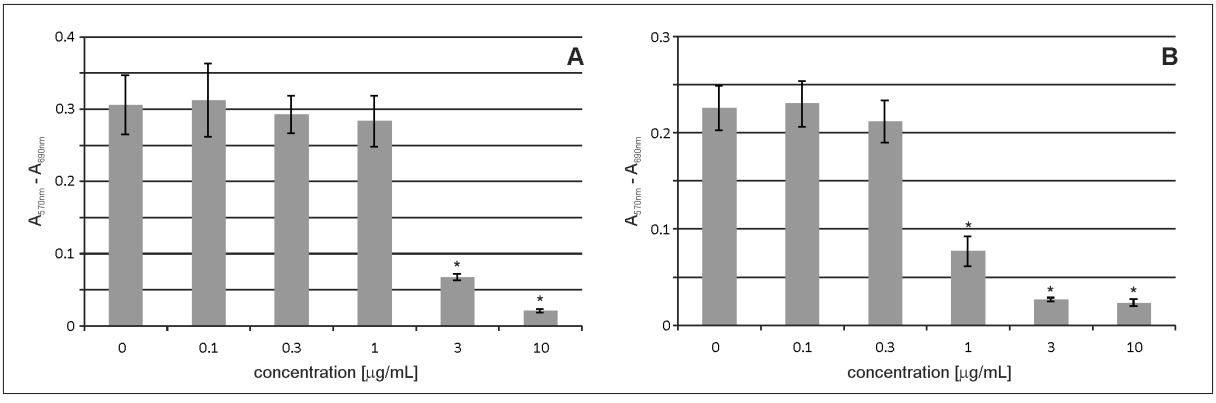

Fig. 2. Growth of G-361 cell line cultured in the presence of various concentrations of betulin (**1**) (A) and 28-*O*-propynoylbetulin (**3**) (B). Each bar represents the mean ± SD; *p < 0.05 compared with control

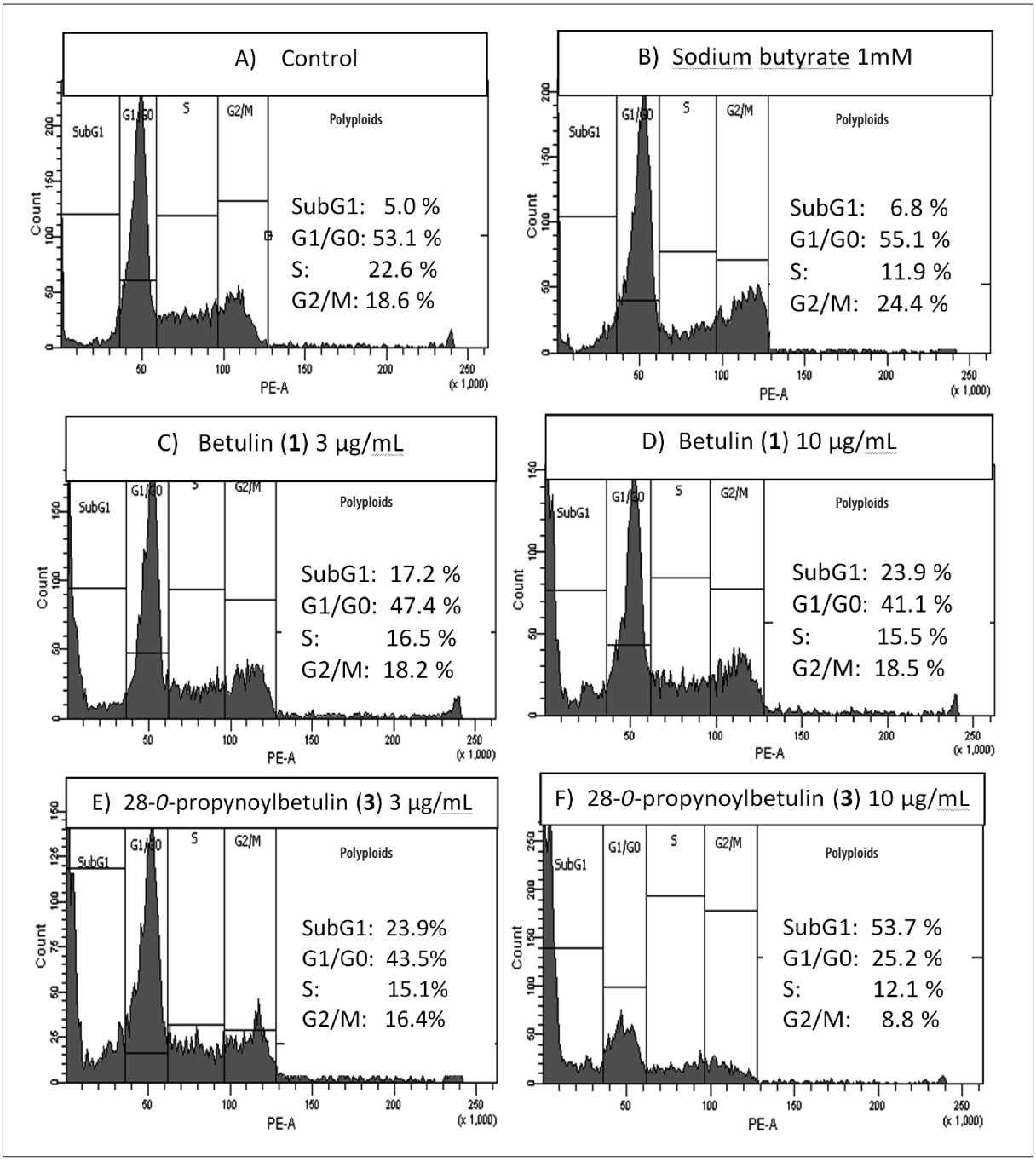

Fig. 3. Cell cycle analysis of G-361 cells incubated with B) sodium butyrate (1 mM), C) betulin (**1**) (3 µg/mL), D) betulin (**1**) (10 µg/mL), E) 28-*O*-propynoylbetulin (**3**) (3 µg/mL), F) 28-*O*-propynoylbetulin (**3**) (10 µg/mL); A) untreated control cells

µg/mL) of both reagents did not cause a significant change in the cell growth rate, compared to the control group. In comparison to 1 µg/mL betulin (**1**), treatment with derivative **3** at 1 µg/mL resulted in a significant reduction of the cell proliferation. At concentrations above 1 µg/mL, both tested substances caused concentration-dependent inhibition of cell growth. 28-*O*-Propynoylbetulin (**3**) and betulin (**1**) maximally inhibited cell proliferation when added to the cells at a concentration of 10 µg/mL.

Cell cycle analysis

As shown in Fig. 3, exposure of G-361 cells to the tested compounds for 48 hours resulted in alterations in the cell cycle phase distribution. Under control conditions 22.6% of the cells were in the S phase (Fig. 3.A). Cell incubation with 1 mM NaB, a well-known inhibitor of cell division, resulted in a decrease of the percentage of cells in S phase to 11.9% (Fig. 3.B). Simultaneously, accumulation of cells

in both G1 and G2/M phases took place. Additionally, NaB caused an increase in the percentage of cells in the sub--G1 fraction from 5.0% to 6.8%, indicating some augmentation of cell death. The histograms of G-361 cells treated with betulin (**1**) and derivative **3** revealed large increases in the sub-G1 fraction, indicating significant induction of cell death (Fig. 3.C-F). The amount of dead cells was concentration-dependent and was higher in cultures treated with 28-*O*-propynoylbetulin (**3**) than in cultures incubated with betulin (**1**). Massive cell death in the cultures treated with the highest concentration (10 μg/mL) of compounds **3** and **1** was also manifested by a significant increase in the amount of cells that rounded up, detached from the dish and floated in the medium (data not shown).

Regardless of cell death, betulin (**1**) seems to arrest the cell cycle at G2/M phase. After subtracting the sub-G1 fraction under control conditions, the percentages of G1, S and G2/M phase cells were 56%: 24%: 20%, respectively. In cultures treated with compound **1** at concentrations of 3 μg/mL and 10 μg/mL, the cell cycle phase distributions were 58%: 20%: 22% and 55%: 21%: 25%, respectively. Similar accumulation of cells in G2/M phase was not observed in the cultures incubated with compound **3**.

Caspase-3 activity

In order to determine whether the observed cell death was apoptotic, the impact of the studied compounds on caspase-3 activity in G-361 cells was assayed. Betulin (**1**) and 28-*O*-propynoylbetulin (**3**) were used at a concentration of 10 μg/mL and the cells were exposed to the compounds for 24 hours. Both substances significantly stimulated the enzyme activity compared to the control (Fig. 4), however, **3** was a much more powerful inducer than betulin (**1**) (activity of caspase-3 in derivative **3** treated cells was nearly 2-fold greater compared to cells incubated with betulin (**1**)). The magnitude of increases of enzyme activity was proportional to the number of dead (sub-G1) cells determined by flow cytometry. Therefore, we concluded that treatment with substances **1** and **3** resulted in apoptosis induction in G-361 cell cultures.

Our results clearly show that betulin (**1**) and its derivative 28-*O*-propynoylbetulin (**3**) were effective inhibitors of cell proliferation and activators of apoptosis in G-361 melanoma cell culture. These effects were concentration-dependent and more pronounced for the betulin derivative. The low concentrations (0.1-1 μg/mL) of betulin (**1**) and 28-*O*--propynoylbetulin (**3**) were insufficient to significantly reduce cell growth. The best antiproliferative effect was seen at concentrations above 1 μg/mL (3 and 10 μg/mL) of both compounds.

Discussion

Malignant melanoma is the most dangerous cutaneous cancer. A lot of anti-melanoma therapies including surgery, chemotherapy, immunotherapy, and targeted therapy have been developed. The anti-cancer strategies are based

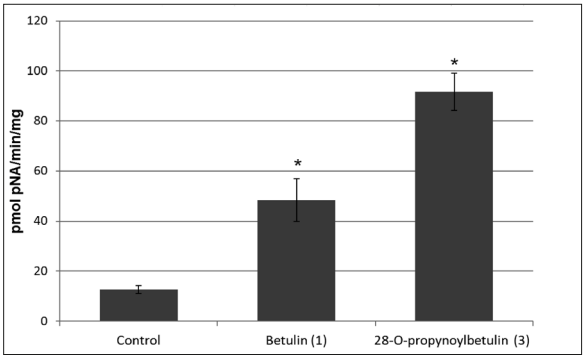

Fig. 4. The effect of 24 hours of exposure to 10 μg/mL betulin (**1**) and 10 μg/mL 28-*O*-propynoylbetulin (**3**) on caspase-3 activity in G-361 cells. Each bar represents the mean ± SD; ∗p < 0.05 compared with control

on elimination of tumour cells by e.g. suppression of cell proliferation and induction of apoptosis [2,10]. Apoptosis is a result of extrinsic or intrinsic activation pathways. In both cases, activation of proapoptotic enzymes called caspases ultimately leads to cell death. The most important effector caspase is caspase-3, which is responsible for chromatin condensation, DNA degradation, protein (vimentin, gelsolin, fodrin, poly(ADP-ribose)polymerase) cleavage and cell blebbing [26]. In cancer treatment, these processes should be promoted only in tumour cells without a negative influence on normal cells [32].

Triterpenes are a group of compounds that can suppress cell proliferation and stimulate apoptosis. A widely naturally occurring representative of triterpenes is betulin (**1**). Poor aqueous solubility is the major problem associated with betulin (**1**) administration. It affects penetration of the compound into cells. Solving that problem involves the use of betulin (**1**) as a precursor and converting it into betulinic acid (**2**) and other derivatives [25,31]. For years, the majority of studies have described compound **1** as an inactive or weakly active anti-cancer compound. Hata et al. [14] showed that the lupane triterpenes with a carbonyl group at C-17 had greater cytotoxicity to melanoma (SK-MEL-28, G361), leukaemia (K562, U937, HL60) and neuroblastoma (NB-1, GOTO) cells compared to betulin (**1**). Further studies by Pyo *et al.* [27] and Gauthier et al. [13] evidenced induction of apoptosis in breast (MCF7), lung (A549), prostate (PC-3) and colorectal (DLD-1) cancer cells attributed to betulin (**1**) administration. Li et al. [19] revealed that betulin (**1**) is a stronger cell death inducer in human cervix carcinoma HeLa, hepatoma HepG2, breast cancer MCF7 and lung adenocarcinoma A549 cells than in prostate PC-3, carcinoma NCI-460 and hepatoma SK-HEP-1 cells. Drag et al. [9] compared the cytotoxic effect of betulin (**1**), betulinic acid (**2**) and extract from bark of birch tree on human gastric carcinoma (EPG85-257) and human pancreatic carcinoma (EPP85-181) drug-sensitive and drug-resistant cell lines. Although betulin (**1**) was the least active of all compounds, the results indicated that betulin (**1**) had a good inhibitory concentration value (IC_{50}) and had a significant cytotoxic impact on pancreatic and gastric carcinoma drug-resistant cell lines.

In the present study, the G-361 melanoma cell line was used to assess the antiproliferative and proapoptotic properties of betulin (**1**) and its acetylenic derivative 28-*O*--propynoylbetulin (**3**). This betulin derivative was chosen on the basis of a preliminary study that revealed its activity against melanoma cells (data not shown). The introduction of an alkyne motif into the betulin structure (the carbon-carbon triple bond) may have a significant influence on biological properties of the compound [5]. The 28-*O*-Propynoylbetulin (**3**) was obtained by addition of a propynoyl group to betulin (**1**) at the C-28 position. Boryczka et al. [4] have previously observed that compound **3** was the strongest cytotoxic agent against human leukaemia (CCRF/CEM) and murine leukaemia (P388) when compared to its precursor, other acetylenic betulins and cisplatin. 28-*O*-Propynoylbetulin (**3**) was less active against breast cancer (T47D) and colorectal cancer (SW707). In our study, we confirmed the ability of both compounds **1** and **3** to induce cell growth arrest and apoptosis. Li *et al.* [19] investigated the anti-cancer activity of betulin (**1**) in HeLa cells. Betulin (**1**) at 10 μg/mL induced apoptosis in HeLa cells e.g. via caspase-3 activation.

In our study, treatment of G-361 cells with betulin (**1**) resulted in significant caspase-3 activation. However, incubation of cells with 28-*O*-propynoylbetulin (**3**) led to apoptosis induction at a significantly higher level than with betulin (**1**) (nearly 2-fold greater). The flow cytometry analysis detected a considerable increase of the sub-G1 cell fraction after incubation with tested compounds and the percentage of sub-G1 cells corresponded well with activity of caspase-3 (higher for compound **3** and lower for betulin (**1**)).

Betulin and betulinic acid are believed to exert their cytotoxic effects primarily through the interaction with mitochondrial membrane, resulting in its increased permeability, the release of mitochondrial cytochrome c or apoptosis-inducing factor (AIF) into the cytosol and the activation of caspases, leading to apoptotic cell death [7,19]. Our findings showed that addition of the propynoyl group at the C-28 hydroxyl group of betulin (**1**) leads to a stronger proapoptotic effect and cell growth arrest in comparison to betulin (**1**). In that derivative, the terminal triple bond is located close to the carbonyl group, forming a relatively reactive configuration [4]. We suppose that the interaction of the propynoyl group with nucleophilic amine and thiol groups augments a disturbance of the mitochondrial membrane integrity leading to decreased cell viability. The obtained derivative seems to be a potent anti-cancer agent with concentration-dependent activity. The cytotoxic effect of betulin (**1**) and its acetylenic derivative **3** on melanoma cells has to be more carefully examined. Exploiting the mechanism of action of the analysed compounds may aid in development of new anti-melanoma strategies.

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The authors have no potential conflicts of interest to declare.