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Influence of 28-*O*-propynoylbetulin on proliferation and apoptosis of melanotic and amelanotic human melanoma cells*

Wpływ 28-*O*-propynoilobetuliny na proliferację i apoptozę ludzkich komórek czerniaka melanotycznego i amelanotycznego

Anna Kaps^{1, B, E, F, G}, Ewa Chodurek^{1, A, B, C, F}, Arkadiusz Orchel^{1, D, E},
Marzena Jaworska-Kik^{1, B, F}, Ewa Bębenek^{2, B, E}, Stanisław Boryczka^{2, A, G},
Janusz Kasperczyk^{1, A, G}

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

¹Department of Biopharmacy, Medical University of Silesia in Katowice, School of Pharmacy with the Division of Laboratory Medicine, Sosnowiec, Poland

²Department of Organic Chemistry, Medical University of Silesia in Katowice, School of Pharmacy with the Division of Laboratory Medicine, Sosnowiec, Poland

Summary

Introduction:

A relatively new approach in treatment of malignant melanoma is the use of betulin and its synthetic derivatives that have anticancer properties. The aim of the study was to determine the effect of an acetylenic derivative of betulin, 28-*O*-propynoylbetulin, on cell growth and apoptosis induction in human melanotic and amelanotic melanoma cells.

Materials and methods:

The A2058 and C32 cell lines were incubated with 28-*O*-propynoylbetulin (working solutions from 0.1 to 10 µg/ml). To evaluate cell proliferation, a sulforhodamine B based assay was conducted. In order to elucidate the early stages of apoptosis in both melanoma cell lines, caspase-3 activity was evaluated.

Results:

The administration of 28-*O*-propynoylbetulin at a concentration equal to or less than 1 µg/ml did not cause a statistically significant change in the cell proliferation in either melanoma cell line (compared to control, $p > 0.05$). Higher concentrations of the compound (3 and 10 µg/ml) inhibited the cell growth (in comparison to control, $p < 0.05$). These results corresponded with caspase-3 activity results that revealed an increase of enzyme activity after 24-hour incubation with 3 and 10 µg/ml of the compound (compared to control, $p < 0.05$).

Discussion:

The study revealed that 28-*O*-propynoylbetulin may have diverse effects on melanoma cells and could be a strong inhibitor of cell growth (C32 cells) or exert a more potent proapoptotic effect (A2058 cells). These findings support the possibility of the use of EB5 in different anti-melanoma approaches.

Key words:

malignant melanoma, triterpenes, 28-*O*-propynoylbetulin, caspase-3-activity

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Author's address: Anna Kaps, PhD, Department of Biopharmacy, School of Pharmacy with the Division of Laboratory Medicine, Medical University of Silesia in Katowice, 8 Jedności St., 41-200 Sosnowiec, Poland, phone: +32 364 10 62, e-mail: annakulczycka.sum@gmail.com

Abbreviations: **Ac-DEVD-CHO** – caspase-3 inhibitor, acetyl-Asp-Glu-Val-Asp-al; **Ac-DEVD-pNA** – caspase-3 substrate, acetyl-Asp-Glu-Val-Asp p-nitroanilide; **AIF** – apoptosis-inducing factor; **Bak** – bcl-2 homologous antagonist/killer; **Bax** – bcl-2-like protein 4; **DMSO** – dimethyl sulfoxide; **EB5** – 28-*O*-propynoylbetulin; **Smac/DIABLO** – second-mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein-binding protein with low PI; **SRB** – sulforhodamine B; **TCA** – trichloroacetic acid.

INTRODUCTION

Neoplastic diseases represent one of the greatest medical challenges of the 21st century. Worldwide, malignant melanoma (*melanoma malignum*) causes about 65 000 deaths annually. There are almost 200 000 new cases of malignant melanoma per year [8]. Melanoma is derived from melanocytes, pigment cells of neuroectodermal origin, which undergo malignant transformation [3]. About 2-8% of melanoma cases are amelanotic melanoma (*melanoma amelanoticum*) [16]. Amelanotic phenotype is characterized by a small amount or none of the pigment melanin in cells [13]. The exact mechanism by which the pigment is lost remains to be elucidated. It is believed to occur by changes in tyrosinase, an enzyme involved in melanogenesis. Transformation of L-tyrosine into melanin by tyrosinase not only generates cytotoxic intermediates but also chelates chemotherapeutic agents and decreases the effectiveness of antimelanoma therapy. The use of inhibitors, such as *N*-phenylthiourea or *D*-penicillamine, could suppress the melanogenesis and sensitize melanoma cells to a chemotherapeutic agent [21].

Due to aggressiveness of melanoma and high resistance to conventional treatment [22], there is an urgent need to discover new drug-like compounds that exhibit potent antitumoral activity and will be well tolerated *in vivo*. In recent years, there has been a growing interest in natural compounds belonging to the group of triterpenes, which may be potential therapeutic agents in melanoma treatment [6]. Betulin and its natural carboxyl derivative called betulinic acid are naturally occurring compounds that are strong inducers of the mitochondria-dependent pathway of apoptosis and have a wide range of biological activities (e.g. anti-inflammatory, antibacterial, antimalarial, anticancer) [14]. Their anticancer properties have been confirmed in leukemia, melanoma, neuroblastoma, glioblastoma and medulloblastoma, among others [10]. Betulin [lup-20(29)-ene-3 β ,28-diol], a lupane-type pentacyclic triterpene, is composed of four six-membered rings and one five-membered

ring in *trans* configuration. It has three reactive moieties including a primary hydroxyl group at C-28, a secondary hydroxyl group at C-3, and an isopropenyl group at C-19 [2]. Due to the high amount of betulin in the bark of birch, its isolation is relatively easy and inexpensive. Despite a wide range of biological activities, the drug efficacy is limited by poor aqueous solubility. Enhancement of the anticancer potential of a betulin-based treatment strategy will require the design and synthesis of betulin derivatives with better bioavailability [4,15]. The influence of betulin and its synthetic derivatives on biological functions of melanoma cells (e.g. cell proliferation, programmed cell death) should be examined [18].

The aim of the study was to evaluate the effect of 28-*O*-propynoylbetulin, an acetylenic betulin derivative, on cell proliferation and caspase-3 activity in melanotic (A2058) and amelanotic (C32) melanoma cells.

MATERIAL AND METHODS

Chemicals

28-*O*-Propynoylbetulin (EB5) was synthesized via esterification of the C-28 hydroxyl group of betulin (Sigma-Aldrich) with propynoic acid. The compound was obtained according to our previously reported method [4]. Stock solutions were prepared by dissolving the test compound in DMSO (Sigma-Aldrich). The working solutions from 0.1 to 10 μ g/ml were prepared immediately before use.

Cells

Human melanoma cell lines were purchased from ATCC (LGC Standards). The A2058 cell line was obtained from the skin and lymph nodes of a 43-year-old Caucasian man with melanotic melanoma. The C32 cell line was derived from the skin of a 53-year-old Caucasian adult with amelanotic melanoma. Both cell lines were grown in medium containing 90% MEM (Sigma-Aldrich), 10%

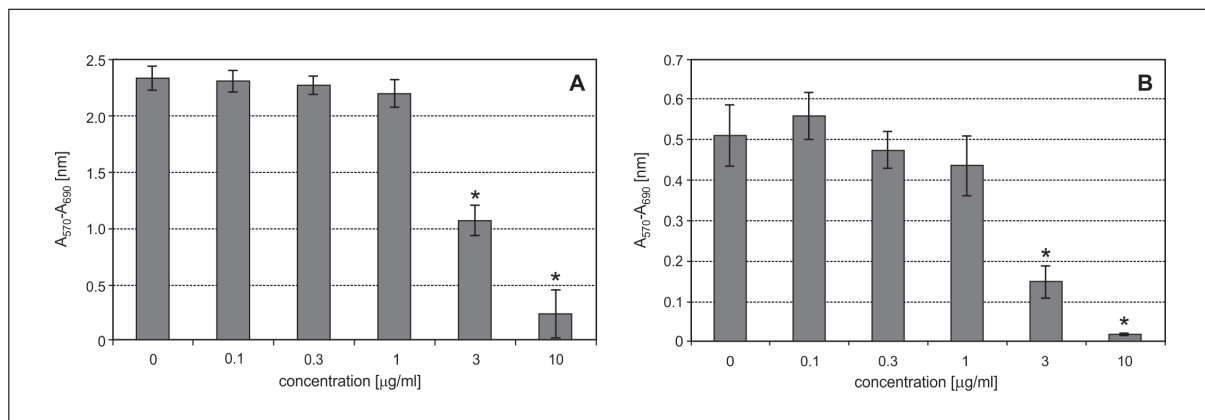


Fig. 1. The effect of various concentrations of 28-*O*-propynoylbetulin on growth of A2058 (A) and C32 (B) cells. Each bar represents the mean \pm SD; * $p < 0.05$ compared with control

fetal bovine serum (PAA), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich). The cells were cultivated at 37°C in a humidified 5% CO₂ atmosphere.

Cell proliferation

To assess the effect of 28-*O*-propynoylbetulin on cell proliferation, the “In Vitro Toxicology Assay Kit, Sulforhodamine B based” (Sigma-Aldrich) was used according to the manufacturer’s protocol. Cells were seeded onto 96-well plates at 10³ cells/well in 200 µl of growth medium and incubated at 37°C in a humidified 5% CO₂ atmosphere. After a 24-hour period, the culture medium was changed and 28-*O*-propynoylbetulin (at concentrations from 0.1 to 10 µg/ml) was added to the medium for the next 72 hours. Control cultures were treated with vehicle (0.2% DMSO). After 3 days, cells were washed with phosphate-buffered saline (PBS, Sigma-Aldrich), fixed with trichloroacetic acid (10% TCA) and stained with sulforhodamine B (0.4% SRB). After solubilization of the dye in 10 mM Tris solution, the plates were read on an MRX Revelation plate reader (Dynex Technologies) at 570 nm and 690 nm (reference wavelength).

Caspase-3 activity

To estimate the proapoptotic caspase-3 activity, the “Caspase-3 Assay Kit, Colorimetric” (Sigma-Aldrich) was used in accordance with the manufacturer’s instructions. Cells were plated onto a 100-mm diameter dish at density of 3x10⁶ cells/dish in 15 ml of growth medium and incubated at 37°C in 5% CO₂ atmosphere. The next day, the tested compound (at concentrations of 3 and 10 µg/ml) was added for the next 24 hours. Control cultures were treated with 0.2% DMSO. Sodium butyrate (Merck) at 10 mM final concentration was used as a positive control. After this period, cells were scraped from the dishes, lysed, centrifuged (16000g; 10 min) and supernatants were stored at -80°C. The assay, based on the measurement of *p*-nitroaniline release resulting hydrolysis from the peptide substrate (Ac-DEVD-pNA) by active caspase-3, was performed on 96-well plates. The plates were read on

the MRX Revelation plate reader, and the absorbance of released *p*-nitroaniline was measured at 405 nm. Specificity of the assay for caspase-3 was verified by adding to the part of wells a specific enzyme inhibitor (Ac-DEVD-CHO). The caspase-3 activities in cell lysates were calculated relative to cellular protein content (determined by Bradford’s method [5], Sigma-Aldrich).

Statistical analysis

The data obtained from 3 independent series of experiments were expressed as mean values \pm standard deviations. Statistical significance analysis was based on analysis of variance (ANOVA) followed by a Tukey post hoc test. The *p*-value of < 0.05 was considered significant. Statistical analysis was performed using Statistica 10 PL software for Windows (StatSoft, Poland).

RESULTS

Cell proliferation

In order to elucidate the effect of the betulin derivative 28-*O*-propynoylbetulin on proliferation of melanoma cells, cell lines were incubated in the presence of various concentrations (0.1, 0.3, 1, 3, 10 µg/ml) of the tested compound (Fig. 1). In both cell lines the concentrations less than or equal to 1 µg/ml did not cause a significant change in the cell growth, compared to the control group ($p > 0.05$). However, in cultures exposed to higher EB5 concentrations (3 and 10 µg/ml), cell proliferation was significantly reduced ($p < 0.05$), and that effect was concentration dependent. However, a careful look at the graphs reveals that the extent of inhibition of cellular growth varied clearly in both cell lines. Treatment with 3 µg/ml EB5 resulted in the reduction of C32 cell quantity to 28.8% of the control (Fig. 1B). In the same conditions, growth of the A2058 cell line was reduced merely to 45.9% of the control (Fig. 1A). A similar pattern of cell response was seen when EB5 was used at the concentration of 10 µg/ml. C32 cell number was reduced to 3.6% of the control, whereas the amount of A2058 cells was reduced to 10.3% of the control.



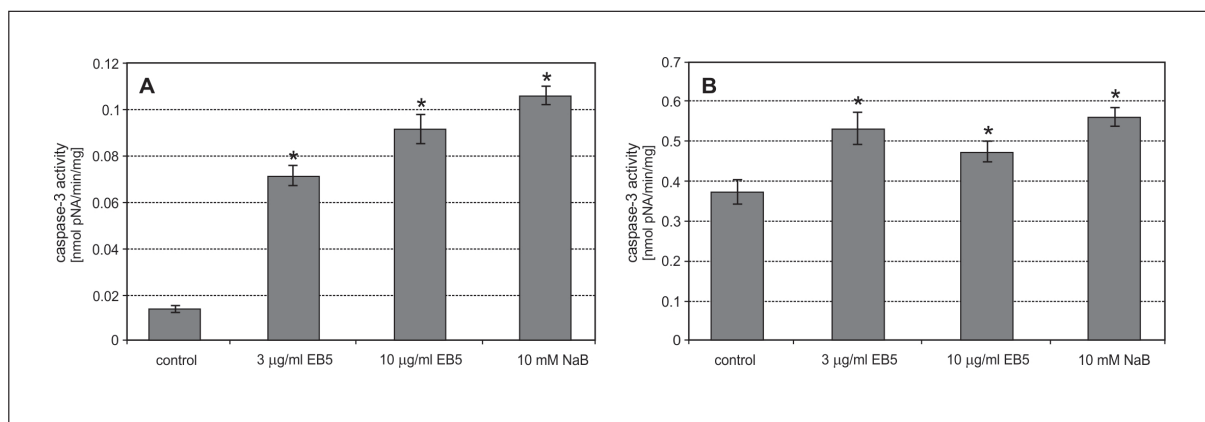


Fig. 2. The effect of 24 hours of exposure to 3 and 10 µg/ml 28-O-propynoylbetulin and 10 mM sodium butyrate on caspase-3 activity in A2058 (A) and C32 (B) cells. Each bar represents the mean \pm SD; * $p < 0.05$ compared with control

Caspase-3 activity

In order to determine the early stages of apoptosis, the impact of the studied compound on caspase-3 activity in human melanoma cells (A2058 and C32 cell lines) was estimated. The compound concentrations were selected based on the above described results, and the EB5 was used at apparently cytotoxic concentrations: 3 and 10 µg/ml. As a positive control 10 mM sodium butyrate was used; it is a well-known inhibitor of histone deacetylase and inducer of cell apoptosis via the caspase-8, -10 and -3 activation pathway [17].

The caspase-3 activity in melanotic (A2058) cells is presented in Fig. 2A. A statistically significant increase in the enzyme activity ($p < 0.05$) was observed in cells treated with both concentrations of EB5 and 10 mM sodium butyrate. Treatment with 28-O-propynoylbetulin at concentrations of 3 and 10 µg/ml led to 5.4 and 6.9-fold increase in caspase-3 activity, respectively. The observed cell responses were comparable to cells treated with sodium butyrate and indicate extensive cell apoptosis. As shown in Fig. 2B, 24-hour exposure of C32 cells to 28-O-propynoylbetulin also resulted in significant increases in caspase-3 activity in all the treated cell cultures. However, the cell responses were clearly weaker compared to melanotic cells. The enzyme activity was increased 1.3 and 1.5-fold above the control, after treatment with 3 and 10 µg/ml EB5 respectively.

DISCUSSION

Apoptosis is a key killing mechanism for most antitumor therapies. Since suppression of apoptosis during carcinogenesis is thought to play a central role in the development and progression of some cancers, improvement in the survival of cancer patients largely depends on finding strategies that are able to target tumor cell resistance and to induce apoptosis in these cells [9]. Results from recently conducted surveys show that betulin and its derivatives may possess multiple anticancer properties that cause inhi-

bition of proliferation and induction of apoptosis in cancer cells [4,15]. Betulin is a strong inducer of the intrinsic pathway of apoptosis by triggering Bak and Bax proteins' translocation to the mitochondria, which leads to depolarization of its membrane potential. This process results in release of cytochrome c, apoptosis-inducing factor (AIF) or second-mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein-binding protein with low PI (Smac/DIABLO). The cytochrome c triggers the caspase cascade by caspase-3 activation, and this is a key event in the triggering of apoptosis [10,18].

Our previous works demonstrated that simple changes in betulin structure may provide new compounds with better anticancer activity and bioavailability [2,4,18]. The anticancer properties of betulin and its synthetic derivatives were confirmed on human (e.g. T47D, A549, Lu1, A431, CCF/CEM, SW707) and murine (e.g. P388, Balb3T3) cell lines. The effect strongly depended on the type of cell line and structure of betulin [4,16]. Boryczka *et al.* [4] demonstrated that 28-O-propynoylbetulin (EB5) was the compound most strongly acting on human leukemia cells compared with betulin, other derivatives and *cis*-platinum. Orchel *et al.* [18] investigated the impact of EB5 on human malignant G-361 melanoma cells. *In vitro* studies, including cell growth, cell cycle and caspase-3 activity assessments, showed that EB5 was a powerful inducer of apoptosis in these cells. The activity of caspase-3 was nearly 2-fold greater in cells treated with 10 µg/ml of EB5 than pure betulin, and the sub-G1 fraction was increased to nearly 54% at the same time. Bębenek *et al.* [2] proved that the hydroxyl group at the C-3 position is responsible for the strong cytotoxic effect of acetylenic derivatives, and esterification of this group completely abolished the action of the compound.

In this study, we evaluated the effect of EB5 on cell growth and apoptosis in melanotic A2058 and amelanotic C32 melanoma cells. Both our observations and literature data indicate that, besides melanin synthesis, the two cell lines differ somewhat in their proper-

ties. A2058 cells proliferate and migrate rapidly and are highly invasive [12]. The C32 cell line has substantially smaller proliferation and migration rates [20]. Both cell lines are highly tumorigenic in nude mice. Incubation of A2058 cells with EB5 at concentrations of 3 and 10 µg/ml resulted in several-fold increases in caspase-3 activity, evidencing the potent proapoptotic activity of the compound. Levels of apoptosis in EB5-treated cell cultures were comparable to cells incubated with 10 mM sodium butyrate, a well-known apoptosis inducer in neoplastic cell lines [1,11]. Thus, A2058 cells, although possessing the active pathways of melanin synthesis [21], proved quite sensitive to the apoptosis-inducing action of EB5. This sensitivity could be potentially attributed to their high proliferation rate, as Pfarr *et al.* [19] have suggested that betulin is more cytotoxic in rapidly dividing cells. The apoptotic response of C32 cells to the tested substances was much more confined, and caspase-3 activity increased no more than 153% above the control level. On the other hand, as shown in Fig. 1, growth inhibitory action of 3 and 10 µg/ml EB5 seems to be more pronounced in C32 cells, compared with the A2058 cell

line. Paradoxically, it could be related to the extensive apoptosis of the A2058 cells. Donato *et al.* [7] have shown that apoptotic melanoma cells secrete factors that promote the growth of surviving cells and caspase-3 plays a significant role in the generation of these factors. This phenomenon can influence the treatment efficiency of melanoma cells, both *in vitro* and *in vivo*. Consequently, drugs with potent antiproliferative activity but moderate cell death inducing activity could be desirable in the melanoma treatment.

Many factors involved in malignant transformation are still unknown, and there is an urgent need to expand the knowledge about the molecular mechanisms of melanoma development and changes in proliferation, differentiation and apoptosis of melanoma cells. Identification and detailed knowledge of action of new potential chemotherapeutic agents that could be used in either the prevention or treatment of melanoma are still required. Elucidating the mechanism of apoptosis after 28-O-propynoylbetulin treatment will support a new therapeutic strategy to cure human melanomas, especially the amelanotic type.

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