Malignant melanoma is highly resistant to chemotherapy and radiotherapy, particularly in the metastatic or relapse phase. Median survival time of metastatic patients is 6-9 months and the 5-year survival rate does not exceed 1-2%. In Poland, according to data from 2011, melanoma occupies 12th place in incidence of cancer among men and 13th place among women. Over the period 1990-2011, annual mortality among men suffering from melanoma increased from 301 to 679, and among women from 350 to 580. Predictions of cancer prevalence and mortality up to the year 2025 indicate that the number of melanoma cases in Poland will grow, especially in people over 65 years of age (1). Therefore, new possible treatment strategies are still searched for. Inhibition, delay or reversal of carcinogenesis using natural or semisynthetic compounds seems to be a promising approach (2, 3). Betulin is a natural compound with cytotoxic activity against cancer cells. It can be easily converted to various semisynthetic derivatives (4, 5).

Boryczka et al. (5) have synthesized a series of new acetylenic derivatives of betulin (containing one or two acetylenic groups at carbons C-3 and/or C-28. Their cytostatic activity was tested on human cell lines: SW707 (colorectal adenocarcinoma), CCRF/CEM (leukemia) and T47D (breast cancer). Additionally, some murine cell lines were used: P388 (leukemia), Balb3T3 (normal fibroblasts). All the tested acetylenic derivatives of betulin exhibited stronger antiproliferative action than the parent compound and 28-O-propynoylbetulin was the most effective.

Betulin \([\text{lup-20(29)-ene-3\beta,28-diol}}]\) belongs to the pentacyclic triterpenes of the lupane group. Betulin molecule is composed of four six-membered rings and one five-membered ring, and has three reactive moieties, which include isopropenyl group at C-19, the primary hydroxyl group at C-28, and the secondary hydroxyl group at C-3 (6, 7). Pentacyclic triterpenes constitute a group of substances extensively tested for their antitumor properties (8, 9). An important advantage of betulin and its derivatives is low toxicity against normal cells. The usefulness of betulin is limited by its poor solubility in water, which is 0.08 mg/mL (10, 11).

The use of G-361 melanoma cell line is a consequence our previous study showing that betulin and 28-O-propynoylbetulin inhibit proliferation and induce apoptosis and differentiation in melanoma cells (9). Accumulation of melanin as well as its structure are the main differentiation markers of the skin pigment cells. G-361 cells belong to melanotic...
cell lines, therefore, they are useful in studies of mechanisms controlling the cell differentiation. Moreover, that cell line enables studies of the role of melanin in melanoma etiology and therapy.

The aim of the study was to investigate the effect of acetylenic derivatives of betulin 2-7: 28-O-propynoylbeutin 2; 28-O-proparglyloxycarbylbeutin 3; 28-O-(3-butyloxycarbonyl)betulin 4; 3,28-O,O'-dipropynoylbetulin 5; 3,28-O,O'-di(proparglyloxycarbyl)betulin 6; 3,28-O,O'-di(3-butyloxycarbonyl)betulin 7 (Fig. 1) on cell proliferation in G-361 human melanoma cell line.

EXPERIMENTAL

Synthesis of acetylenic derivatives of betulin 2-7

Compounds 2-7 were obtained at the Department of Organic Chemistry, Medical University of Silesia in Katowice. All reactions were monitored by thin layer chromatography (TLC) using silica gel 60 254F plates (Merck) and a mixture of chloroform and ethanol (20 : 1 or 40 : 1, v/v) as an eluent. Spots were visualized on the plates by spraying with 5% sulfuric acid (VI) solution in ethanol and heating at 110°C. Derivatives of betulin 2-7 were purified by column chromatography on silica gel 60, < 60 µm (Merck) using a mixture of chloroform and ethanol (20 : 1 or 40 : 1, v/v) as an eluent. The chemical structure of compounds 2-7 was confirmed by the 1H-NMR, 13C-NMR, IR and MS (EI) spectral data and described in the paper by Boryczka et al. (5).

The esterification of betulin 1 with propynoic acid in dichloromethane in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) afforded mixtures of compounds 2 and 5. Derivatives 2 and 5 were separated by column chromatography to give pure 2 and 5 in 60% and 12% yields, respectively. The reactions of betulin 1 with the propargyl chloroformate and 3-butyln-1-yl chloroformate in benzene in the presence of pyridine gave a mixture of monoesters 3, 4 and diesters 6, 7. The resulting mixtures were separated by column chromatography to afford pure products 3, 4 and 6, 7 in 64–69% and 23–27% yields, respectively.

Cell culture

The G-361 human malignant melanoma cell line (LGC Promochem, Lomianki, Poland) was used in this study. G-361 melanotic cells were derived from the skin of 31-year-old Caucasian man. The G-361 cell line was grown in 90% McCoy's medium (Sigma-Aldrich), supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Cell proliferation

Stock solutions were prepared by dissolving the tested compounds 2-7 in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at a concentration of 5 mg/mL. They were used to prepare working solutions in culture medium immediately before use. To determine the cytotoxicity of acetylenic betulin derivatives 2-7, In Vitro Toxicology Assay Kit, sulforhodamine B based (Sigma-Aldrich) was used. Sulforhodamine B is an anionic dye which binds to amino acid residues of cellular proteins. The amount of bound dye in particular wells is proportional to the total cell biomass.

Cells were seeded in 96-well plates (Nunc™ Micro Well Plates) at a density of 10^4 cells per well. After 24 h of incubation, the culture medium was removed and replaced with the fresh one containing the tested compounds 2-7 in the concentration range of 0.1-20 µg/mL. Then, the cells were cultured for the next 72 h. At the end of incubation period, cells were washed with phosphate-buffered saline (PBS, Sigma-Aldrich), fixed in trichloroacetic acid (10% TCA) and stained with sulforhodamine B. After the dissolution of the incorporated dye, absorbance was measured at 570 nm and 690 nm (reference wavelength) using the MRX Revelation plate reader (Dynex Technologies).
Figure 2. Growth of G-361 cell line cultured in the presence of various concentrations of acetylenic derivatives of betulin 2-7: A) 28-O-propynoylbetulin 2 and 3,28-O,7'-dipropynoylbetulin 5. B) 28-O-propargyloxycarbonylbetulin 3 and 3,28-O,7'-dipropargyloxycarbonylbetulin 6. C) 28-O-(3-butynyloxycarbonyl)betulin 4 and 3,28-O,7'-di(3-butynyloxycarbonyl)betulin 7. (Each bar represents the mean ± SD; *p < 0.05 compared with control)
Statistical analysis

Differences in cell proliferation were analyzed for statistical significance using analysis of variance (ANOVA) and the Kruskal-Wallis test. The p-value of < 0.05 was considered significant. Analysis was performed using Statistica 10.0 software for Windows (StatSoft, Poland).

RESULTS

In this study, the proliferation of G-361 melanotic melanoma cell line, was evaluated using the assay with sulforhodamine B. The range of concentrations of the tested compounds was from 0.1 to 20 µg/mL and the cells were treated for 72 h. It has been shown that 28-O-propynoylbetulin, 28-O-propargyloxy carbonylbetulin, and 28-O-(3-butynylcarbonyl)betulin have an impact on G-361 cells proliferation, and this effect depends on the concentration of the tested compounds (Fig. 2). 28-O-Propynoylbetulin at low concentrations (0.1 and 0.3 µg/mL) did not exert any influence on the growth rate of the cells as compared to control culture. Statistically significant inhibition of proliferation of the tested cell line was found in cultures exposed to higher concentrations of this compound (≥ 1 µg/mL).

The strongest growth inhibition was observed in cultures of G-361 cells exposed to 3, 10 and 20 µg/mL of derivative 2 (Fig. 2A). 28-O-Propargyloxy carbonylbetulin 3 at concentrations of 0.3, 1 and 3 µg/mL did not cause significant inhibition of cellular growth. Compound 3 significantly inhibited the cell proliferation (compared to control) at concentrations of 10 and 20 µg/mL (Fig. 2B). A similar effect was observed when cells were incubated with 28-O-(3-butynylcarbonyl)betulin 4 (Fig. 2C).

Treatment of G361 cell line with 3,28-O',O'-dipropynoylbetulin 5, 3,28-O',O'-di(propargyloxy carbonyl)betulin 6 and 3,28-O',O'-di(3-butynylcarbonyl)betulin 7 did not cause significant changes in cell proliferation in the whole range of tested concentrations (0.1-20 µg/mL) (Fig. 2A-C).

DISCUSSION

Melanoma is a malignant tumor derived from the melanocytic neuroectodermal cells, which undergo malignant transformation (12). The aggressiveness of melanoma is determined by their ability to metastasize rapidly, a high degree of proliferation of tumor cells, the broad genetic and epigenetic changes, and high resistance to conventional treatments (13).

Triterpenes are a group of compounds which can potentially inhibit the proliferation of tumor cells and induce their apoptosis. Betulin 1, as a representative of pentacyclic triterpenes, is widely distributed in nature (14-17). Li et al. (18) showed that betulin 1 is a potent inducer of cell death in human cervical carcinoma (HeLa), hepatoblastoma (HepG2), breast cancer (MCF7) and lung adenoma cells (A549).

Our studies have shown that fundamental to the cytotoxic effects of acetylenic derivatives of betulin is a hydroxyl group at C-3 position. It has been shown that monoesters 2-4, obtained by a replacement of the hydroxyl group at C-28 position of betulin 1 by alkynyl groups, exhibited the most potent cytotoxicity. The strongest cytotoxic activity against G-361 cell line showed 28-O-propynoylbetulin 2. These results are consistent with our previous observations, that 28-O-propynoylbetulin 2 has stronger apoptotic and cytotoxic effect on G-361 cells than betulin 1 (9). Boryczka et al. (5) demonstrated that 28-O-propynoylbetulin 2 was the most strongly acting compound on human leukemia cells (CCRF/CEM) compared with betulin 1 and other acetylenic derivatives of betulin and cis-platinum.

In our work, weaker cytotoxic effect with respect to the derivative 2 showed monoesters: 28-O-propargyloxy carbonylbetulin 3 and 28-O-(3-butynylcarbonyl)betulin 4, which only at a concentration of 10 and 20 µg/mL significantly inhibit growth of G-361 cells. The present study demonstrated that cytotoxicity of derivatives 3 and 4 at the highest concentration (20 µg/mL) was similar to 2. Instead, simultaneous esterification of the C-3 hydroxyl group (diesters 5-7) completely abolished the cytotoxic action of the compound (in concentration range 0.1-20 µg/mL).

The scientists are currently looking for new derivatives of betulin, in order to obtain the compounds with high anticancer activity in human melanoma cells. The present study demonstrated that simple changes in the structure of betulin 1 may provide new interesting compounds with potentiated antitumor activity.

The addition of the propynoyl group at the C-28 position of betulin 1 led to an increase in proapoptotic and cytotoxic effect against G-361 cells, in comparison to betulin 1 (9). Cytotoxicity of acetylenic derivatives of betulins 2-7 with respect to melanoma malignum cells, requires further studies. Understanding the mechanisms of action of the analyzed compounds can contribute to the development of new treatment strategies for melanoma. Another aspect requiring additional study is the influence of...
these substances on the composition and structure of pigment produced by melanocytes and its implications for the effectiveness of antineoplastic therapy.

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REFERENCES


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