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Apoptotic activity of betulinic acid derivatives on murine melanoma B16 cell line

Wing-Keung Liu[a,*], Joyce C.K. Ho[a], Florence W.K. Cheung[a], Bonnie P.L. Liu[a], Wen-Cai Ye[b], Chun-Tao Che[c]

[a]*Department of Anatomy, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, People's Republic of China*
[b]*Department of Phytochemistry, China Pharmaceutical University, Nanjing, People's Republic of China*
[c]*School of Chinese Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, People's Republic of China*

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Abstract

The mitochondrion plays a crucial role in the process of apoptosis and has thus become one of the targets for the search for potential chemotherapeutic agents. Betulinic acid [3β-hydroxy-lup-20(19)lupaen-28-carbonic acid], a lupane-type triterpene which is abundant in many plant species, has been shown to exert a direct effect on the mitochondria and subsequent apoptosis in melanoma cells. Chemical synthesis and modification of betulinic acid are being explored to develop more potent derivatives. We present here the apoptotic activity of several natural derivatives of betulinic acid which were isolated from the roots of a Chinese medicinal herb, *Pulsatilla chinensis* (Bge) Regel [Ye, W., Ji, N.N., Zhao, S.X., Liu, J.H., Ye, T., McKervey, M.A., Stevenson, P., 1996. Triterpenoids from *Pulsatilla chinensis*. Phytochemistry 42, 799–802]. Of the five compounds tested, 3-oxo-23-hydroxybetulinic acid was the most cytotoxic on murine melanoma B16 cells (IC_{50}=22.5 μg/ml), followed by 23-hydroxybetulinic acid and betulinic acid (IC_{50}=32 and 76 μg/ml, respectively), with lupeol and betulin exhibiting the weakest cytotoxicity ($IC_{50}\geq$100 μg/ml). Exposure of B16 cells to betulinic acid, 23-hydroxybetulinic acid and 3-oxo-23-hydroxybetulinic acid caused a rapid increase in reactive oxidative species production and a concomitant dissipation of mitochondrial membrane potential in a dose- and time-dependent manner, which resulted in cell apoptosis, as demonstrated by fluorescence microscopy, gel electrophoresis and flow-cytometric analysis. Cell cycle analysis further demonstrated that both 3-oxo-23-hydroxybetulinic acid and 23-hydroxybetulinic acid dramatically increased DNA fragmentation at the expense of G1 cells at doses as low as 12.5 and 25 μg/ml, respectively, thereby showing their potent apoptotic properties. Our results showed that hydroxylation at the C3 position of betulinic acid is likely to enhance the apoptotic activity of betulinic acid derivatives (23-hydroxybetulinic acid and 3-oxo-23-hydroxybetulinic acid) on murine melanoma B16 cells.

Keywords: Melanoma; Betulinic acid derivative; Apoptosis; Reactive oxidative species; Mitochondrial membrane potential

1. Introduction

Betulinic acid [3β-hydroxy-lup-20(19)lupaen-28-carbonic acid] is a lupane-type triterpene which was first isolated from the stem bark of an East African evergreen tree, *Ziziphus mauritiana* Lam. (Rhamnaceae) and which is abundant in the white birch (Pisha et al., 1995). It possesses selective apoptotic activity toward melanoma cells (Pisha et al., 1995; Wick et al., 1999; Fulda and Debatin, 2000) and also toward tumor cells of neuroectodermal origin (Schmidt et al., 1997; Fulda et al., 1999). Betulinic acid-induced apoptosis is not exerted through a ligand/receptor system but is mediated by a decrease in mitochondrial permeability (Fulda et al., 1998), the release of mitochondrial cytochrome *c* into the cytosol (Fulda and Debatin, 2000), the formation of reactive oxidative species and the activation of crm-A-insensitive caspase activity (Wick et al., 1999). Recently, betulinic acid was also found to inhibit the replication of human immunodeficiency virus (HIV) (Hashimoto et al.,

* Corresponding author. Tel.: +852 2609 6896; fax: +852 2603 5031.
E-mail address: ken-liu@cuhk.edu.hk (W.-K. Liu).

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1997; Vlietinck et al., 1998; Holz-Smith et al., 2001). Because of its potential therapeutic action against melanoma and human immunodeficiency virus replication (De Clercq et al., 1996; De Clercq, 2000), a number of modified betulinic acid compounds were synthesized for clinical trial (Soler et al., 1996; Hashimoto et al., 1997; Jeong et al., 1999).

Apoptosis is a process of cell death which is characterized by chromatin condensation, DNA fragmentation and formation of apoptotic bodies (Kerr et al., 1972). It is generally believed that betulinic acid induces loss of the mitochondrial transmembrane potential and interferes with the permeability transition pore complex, by which mitochondrial apoptotic factors are released into the cytosol for cleavage of caspases and activation of the apoptotic machinery (Fulda et al., 1998). In the present study, we compared the cytotoxicity of five natural betulinic acid derivatives and revealed that 3-oxo-23-hydroxybetulinic acid and 23-hydroxybetulinic acid exhibited more potent apoptotic activity by increasing the formation of intracellular reactive oxidative species and reducing the mitochondrial membrane potential of murine melanoma B16 cells.

2. Materials and methods

2.1. Cell cultures

The murine melanoma B16 cell line (NBL6323) and murine connective tissue fibroblast L929 cell line (CCL1) were obtained from the American Type Culture Collection and maintained in F10 medium and RPMI medium, respectively, supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 IU/ml penicillin. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 48 h for comparison of cytotoxicity between betulinic acid and its derivatives. In order to compare the cell specificity of betulinic acid, B16 cells and murine L929 fibroblasts were incubated with betulinic acid for 72 h, and the MTT-based cytotoxicity assay was performed as described (Liu et al., 2004).

2.2. Test compounds

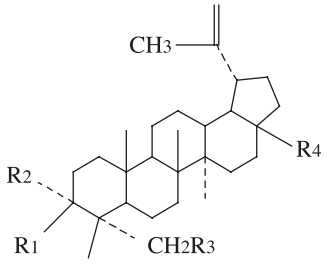
Betulinic acid and its derivatives, including lupeol, 23-hydroxybetulinic acid, betulin and 3-oxo-23-hydroxybetulinic acid, are lupane-type triterpenes which were isolated from the roots of a Chinese medicinal herb, *Pulsatilla chinensis* (Bge) Regel (Ye et al., 1996). The molecular mass of these five compounds ranges from 442 to 472, as determined by mass spectrometry and elemental analysis (Fig. 1). The compounds were dissolved in dimethyl sulfoxide (DMSO) to make a stock solution, from which appropriate concentrations were prepared with culture medium before each experiment. The final concentration of DMSO did not exceed 0.5% in any experiment.

2.3. Cell proliferation assay

B16 melanoma cells (2×10^4 cells/0.1 ml/well) were treated with a serial dilution of betulinic acid in 96-well culture plates (Costar, USA) or 8-chamber culture slides (Nunc 177402, USA) for 48 h. During the last 4 h, cells were reacted with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) at 37 °C for colorimetric MTT-based cytotoxicity assay. The reaction product, formazan, was extracted with DMSO, and the absorbance was read at 540 nm (Liu et al., 2002). Data represent the mean values and standard deviations of triplicate assays in at least one experiment.

2.4. Fluorescence staining for morphological observation

B16 cells were treated with a serial dilution of betulinic acid, 23-hydroxybetulinic acid and 3-oxo-23-hydroxybetulinic acid in eight-chamber slides for 48 h, washed briefly with phosphate-buffered saline before they were fixed with



Compounds	R_1	R_2	R_3	R_4	Formula	MW	IC_{50} (μg/ml)[μM]
Lupeol (LP)	OH	H	H	CH_3	$C_{30}H_{50}O$	426	>100 [>234.7]
Betulin (BN)	OH	H	H	CH_2OH	$C_{30}H_{50}O_2$	442	100 [226.2]
Betulinic acid (BA)	OH	H	H	COOH	$C_{30}H_{48}O_3$	456	76 [166.6]
23-hydroxybetulinic acid (OHBA)	OH	H	OH	COOH	$C_{30}H_{48}O_4$	472	32 [67.8]
3-oxo-23-hydroxybetulinic acid (PH-1)	O		OH	COOH	$C_{30}H_{46}O_4$	470	22.5 [47.9]

Fig. 1. Chemical structures and cytotoxicity (IC_{50} for 48 h) of betulinic acid and its derivatives.

buffered formalin, stained with 0.01% acridine orange in 0.06 M phosphate buffer, pH 6.0, and differentiated with 0.1 M calcium chloride. Fluorescence micrographs were taken on a fluorescence microscope (Axioskop, Zeiss, Germany) with a 450–490-nm excitation block filter and a 520-nm barrier filter (Liu et al., 2002).

2.5. DNA extraction and electrophoresis

Cells treated with betulinic acid and its derivatives (3×10^6) were collected and incubated in 0.2 ml lysis buffer (pH 7.5) containing 10 mM Tris–HCl, 1 mM EDTA, 1% sodium dodecyl sulfate and 100 μg/ml proteinase K at 37 °C for 24 h. The lysates were extracted with phenol and chloroform before they were treated with 1 μg/ml RNase A at 37 °C for 30 min. DNA was then purified, precipitated, separated on a 1.5% agarose gel containing 0.1 μg/ml ethidium bromide and visualized on a UV transilluminator.

2.6. Flow-cytometric cell cycle analysis

Cells were treated with betulinic acid and its derivatives for 48 h and a cell suspension of 100,000 cells was fixed with 70% alcohol for 15 min at 4 °C, treated with RNase A and stained with 1.0 μg/ml propidium iodide (PI, Boehringer Mannheim, Germany). The red fluorescence of DNA-bound PI in individual cells was measured at 488 nm with a Beckman Coulter Altra flow cytometer, and the results were analyzed using Expo32 software (Beckman Coulter, USA)(Liu et al., 2002).

2.7. Measurement of the production of reactive oxidative species

The formation of intracellular reactive oxidative species by B16 cells was measured using an oxidation-sensitive fluorescent probe (H_2DCF-DA, D399 Molecular Probe, Oregon, USA) (Brubacher and Bols, 2001). B16 cells were plated at 3×10^5/750 μl supplemented RPMI medium in each well of a six-well culture plate overnight, and the cultures were then incubated in culture medium containing 5 μg/ml H_2DCF-DA for 10 min at 37 °C before being co-cultured with serial concentrations of betulinic acid or its derivatives for 30 min. The H_2DCF-DA in the cytoplasm of the cell was first deacetylated by cellular esterase to H_2DCF, which was converted to a green fluorescent product DCF by intracellular reactive oxidative species produced by treated B16 cells. The intensity of fluorescence was measured by flow cytometry with excitation at 488 nm and emission at 535 nm, and the results were analyzed using Expo32 software.

2.8. Measurement of NADPH oxidase activity

NADPH-oxidase activity was determined using a luminol-enhanced chemiluminescence system that allows determination of intracellularly generated reactive oxidative species. Since significant reactive oxidative species production was measured after treatment with betulinic acid and its derivatives from 1 to 4 h, B16 cells (5×10^4 cells/well) were exposed to betulinic acid and its derivatives for 1 and 4 h, washed twice with PBS, and 5 μM lucigenin and NADPH (100 μM) were added, and luminescence was measured every 30 s for 20 min in a luminometer (Berthold, Germany). Changes in luminescence are expressed as relative luminescent units per 10^6 cells per minute. No changes in luminescence were observed in the absence of NADPH, and addition of betulinic acid to lucigenin in PBS in the absence of cells did not increase chemiluminescence.

2.9. Determination of mitochondrial transmembrane potential ($\Delta\Psi_m$)

The $\Delta\Psi_m$ of B16 cells after treatment with either betulinic acid or its derivatives was measured using a mitochondria-specific probe, chloromethyl-X-Rosamine (CMXRos, Mitotracker Red) (M7512, Molecular Probe), according to the method described by Gilmore and Wilson (1999). Briefly, B16 cells were treated with serial concentrations of betulinic acid and its derivatives for 1, 2 and 4 h. During the last 15 min, cells were incubated with 100 nM CMXRos in the culture medium at 37 °C in the dark before they were rinsed with phosphate-buffered saline and subjected to flow-cytometric analysis immediately with excitation at 488 nm and emission at 610 nm. The results were analyzed using Expo32 software.

3. Results

3.1. Cytotoxicity of betulinic acid and its derivatives

Fig. 2A shows the cytotoxicity of the five compounds, with 3-oxo-23-hydroxybetulinic acid being the most toxic compound (IC_{50}=22.5 μg/ml), followed by 23-hydroxybetulinic acid, betulinic acid, betulin and lupeol (IC_{50}=32, 76, 100 and >100 μg/ml, respectively). Hydroxylation and oxidation at positions R1, R2 and R3 interfered with the cytotoxicity of this lupane-type triterpene. The hydroxylation of the methyl group at R4 did not affect cytotoxicity, as indicated by lupeol and betulin (both $IC_{50}\geq100$ μg/ml); however, carboxylation at R4 (i.e., betulinic acid) markedly enhanced cytotoxicity (IC_{50}=76 μg/ml of betulinic acid vs. ≥100 μg/ml of lupeol and betulin). The alteration of ring 1 was important for the induction of apoptosis in B16 cells, as demonstrated by fluorescence microscopy and gel electrophoresis. Normal B16 cells with an oval nucleus adhered to the culture plate, while after 3-oxo-23-hydroxybetulinic acid and 23-hydroxybetulinic acid treatment, cells showed nuclear morphology changes associated with apoptosis. Prominent apoptotic bodies were observed in B16 cells treated with 25 μg/ml 3-oxo-23-hydroxybetulinic acid for 48

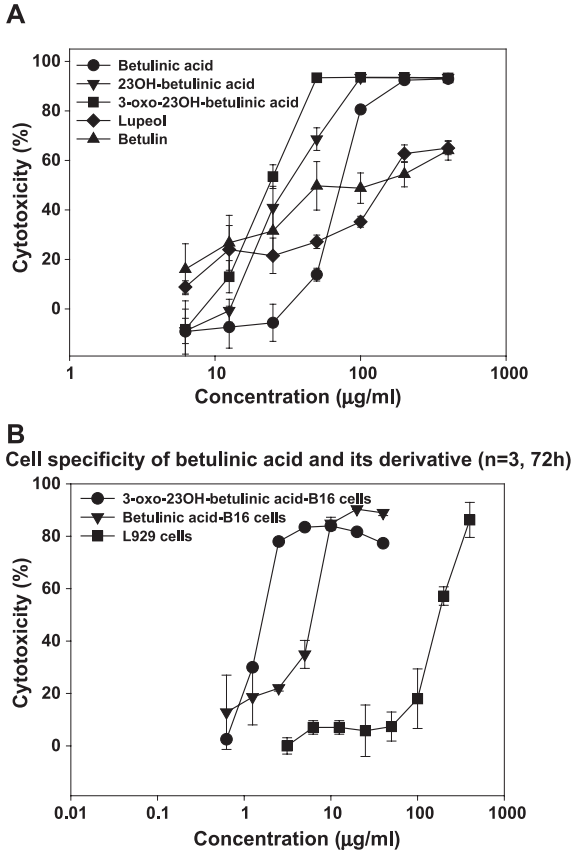


Fig. 2. Cytotoxicity of betulinic acid and its derivatives on murine melanoma B16 cells for 48 h. The data are means±standard deviation (n=3) (A). Comparison of the cytotoxicity of betulinic acid and 3-oxo-3OH-betulinic acid on murine B16 melanoma cells and murine L929 fibroblasts for 72 h shows that B16 cells are more susceptible to the cytotoxicity of betulinic acid and 3-oxo-3OH-betulinic acid (IC_{50}=8 and 1 μg/ml, respectively) by at least two orders of magnitude (IC_{50}>100 μg/ml for both compounds) for L929 cells (B).

h (Fig. 3A and B). DNA fragmentation was also demonstrated in these cells by DNA gel electrophoresis (Fig. 4). All these features suggest that betulinic acid and its

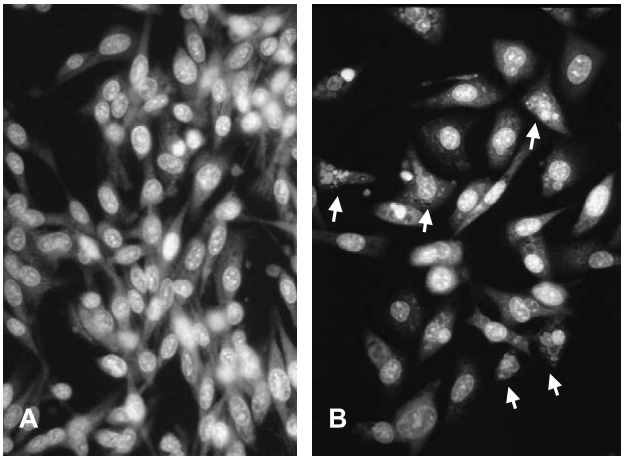


Fig. 3. Light microscopy of normal murine melanoma B16 cells (A) and typical apoptotic B16 cells (arrows) after treatment with betulinic acid and its derivatives for 48 h (350×).

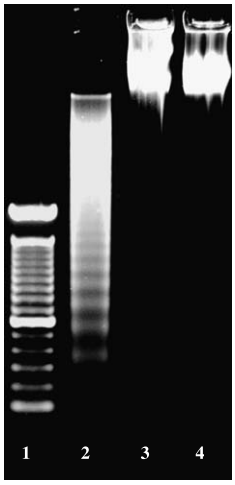


Fig. 4. Typical DNA ladder is prominent in B16 cells treated with 50 μg/ml betulinic acid or its derivatives (lane 2), but not in the cells treated with the same amount of lupeol and betulin (lanes 3 and 4). The 100-bp DNA ladder marker is shown in lane 1.

derivatives induce cell death in B16 cells through the apoptotic pathway.

3.2. Cell cycle analysis

A decrease in the G1 population and a concomitant increase in the subG1 population were observed in B16 cells after treatment with 3-oxo-23-hydroxybetulinic acid, 23-hydroxybetulinic acid and betulinic acid as compared with betulin and lupeol (Fig. 5A–G). Both 3-oxo-23-hydroxybetulinic acid and 23-hydroxybetulinic acid started to induce DNA fragmentation in B16 cells at 12.5 and 25 μg/ml, respectively. Such changes were only observed with >50 μg/ml betulinic acid, and no changes were detected with lupeol and betulin at 100 μg/ml.

3.3. Production of reactive oxidative species

The production of intracellular reactive oxidative species by B16 cells after treatment with betulinic acid and its derivatives for 2 h was measured fluorometrically using the oxidation-sensitive fluorochrome, H_2DCF-DA. Fig. 6A shows the dose-dependent increase in reactive oxidative species in B16 cells, with 3-oxo-23-hydroxybetulinic acid and 23-hydroxybetulinic acid being the most potent compounds, with reactive oxidative species production reaching a peak at 25 and 50 μg/ml, respectively, and declining to an undetectable level thereafter, possibly reflecting the death of the cells. The reactive oxidative species production induced by betulinic acid increased gradually, reaching a peak level similar to that for 3-oxo-23-hydroxybetulinic acid or 23-hydroxybetulinic acid at 100 μg/ml. B16 cells produced relatively low levels of reactive oxidative species in response to the other two compounds, betulin and lupeol. A comparison of the production by the three more potent compounds further demonstrated that the level of reactive

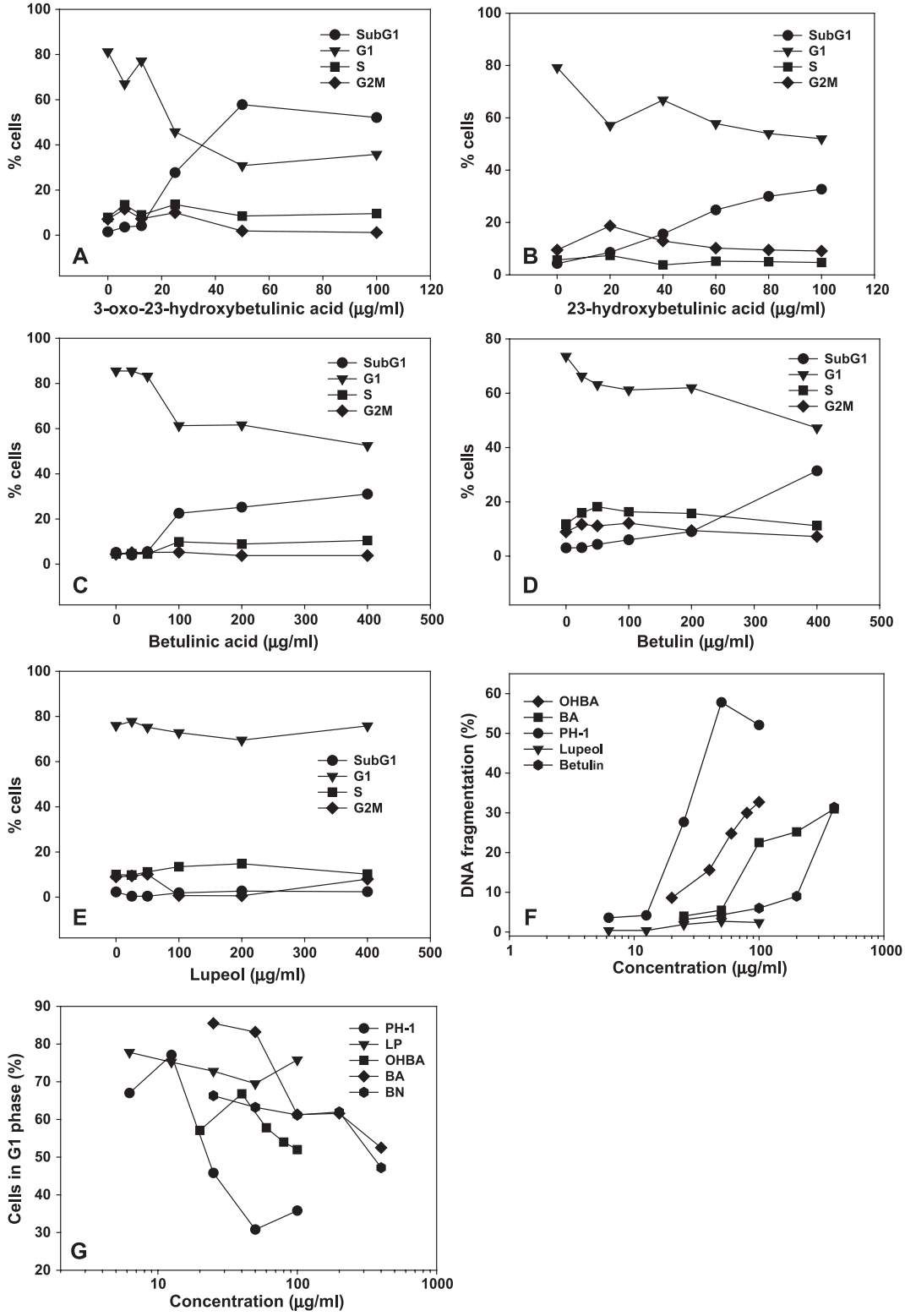


Fig. 5. Cell cycle analysis of B16 melanoma cells treated with betulinic acid and its derivatives for 48h (A–E). A comparison of DNA fragmentation and cells in G1 phase induced by these five compounds after 48 h is shown in (F) and (G).

oxidative species increased in a time-dependent manner in the first 4 h of exposure and the rank order of potency was 3-oxo-23-hydroxybetulinic acid>23-hydroxybetulinic acid->betulinic acid (Fig. 7A).

3.4. Mitochondrial membrane potential ($\Delta\Psi_m$) of B16 cells

Evidence from previous studies has demonstrated that treatment of betulinic acid resulted in the dissipation of the

mitochondrial membrane potential (Fulda and Debatin, 2000). Our laboratory therefore investigated whether the stronger apoptotic activity of betulinic acid derivatives could be attributed to the dissipation of the mitochondrial membrane potential of B16 cells. Fig. 6B shows that $\Delta\Psi_m$ initially increased in all treated B16 cells and then declined to an undetectable level in a dose-dependent manner, except for in lupeol- or betulin-treated cells, where the mitochondrial membrane potential remained elevated. No changes in $\Delta\Psi_m$ were measured in B16 cells treated with ≥100 μg/ml lupeol and betulin. The dissipation of mitochondrial membrane potential in the 3-oxo-23-hydroxybetulinic acid, 23-hydroxybetulinic acid and betulinic acid groups occurred in a time-dependent manner (Fig. 6B) and was inversely correlated with the increased production of reactive oxidative species (Fig. 7A).

3.5. Measurement of NADPH oxidase activity

Betulinic acid and its derivatives had a weak effect on lucigenin-dependent chemiluminescence in B16 cells, which responded with a minimal release of reactive oxidative species. A relatively low level of NADPH oxidase activity (<10%) was measured in B16 cells in both the presence and the absence of betulinic acid and its derivatives (Fig. 7B),

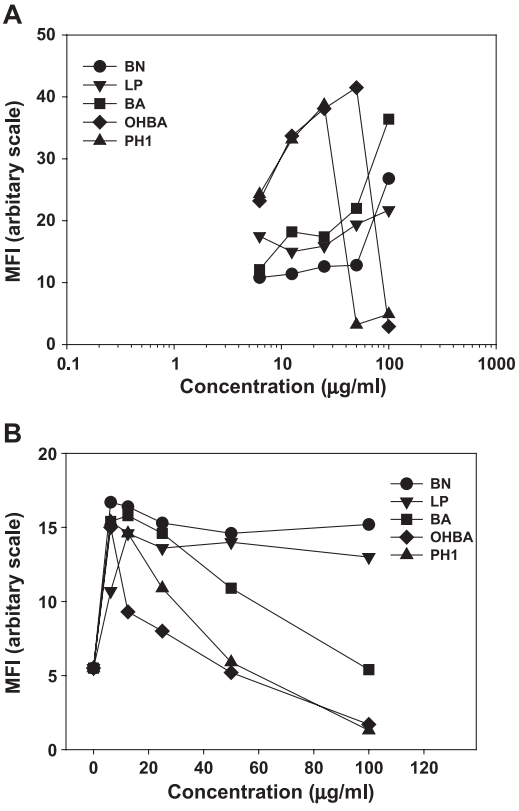


Fig. 6. Effect of betulin (BN), lupeol (LP), betulinic acid (BA), 23-hydroxybetulinic acid (OHBA) and 3-oxo-23-hydroxybetulinic acid (PH-1) on reactive oxidative species (reactive oxidative species) production (A) and mitochondrial membrane potential (B) in B16 cells for 2 h.

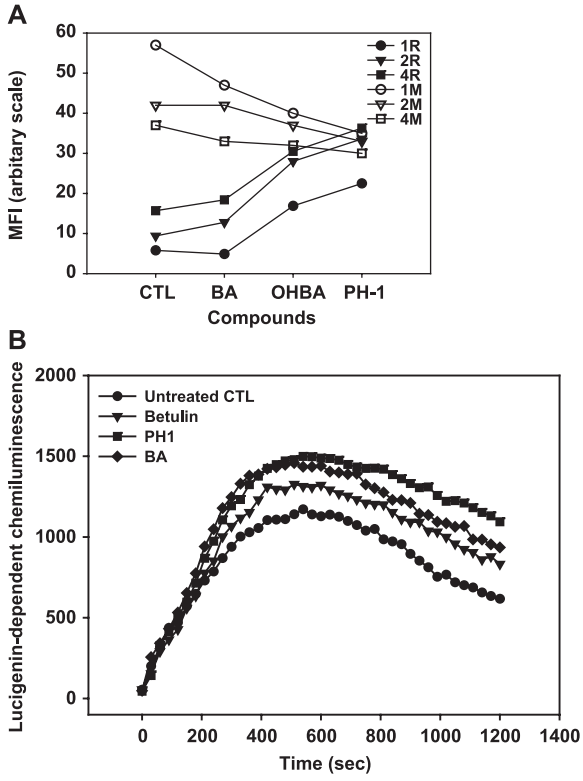


Fig. 7. Changes in reactive oxidative species production (solid symbols) and mitochondrial membrane potential (opened symbols) in B16 cells treated with betulinic acid (BA), 23-hydroxybetulinic acid (OHBA) and 3-oxo-23-hydroxybetulinic acid (PH-1) for 1, 2 and 4 h, respectively (A). No remarkable activation of lucigenin-dependent NADPH oxidase activity was measured in B16 cells treated with betulinic acid and its derivatives for 2 h. Cells without drug treatment were used as untreated control, and no changes in luminescence were observed in the absence of NADPH, indicating the activity is NADPH dependent (B).

which may be a result of a very low level of activatable NADPH oxidase in murine cells (Bylund et al., 2003).

4. Discussion

The mitochondrion plays an essential role in the process of apoptosis (Fesik, 2000; Gottlieb, 2000). When cells receive intracellular or extracellular signals leading to apoptosis, prominent mitochondrial alterations, including changes in membrane permeability, decrease in membrane potential, swelling, disruption and the release of caspases and endonucleases, are observed and result eventually in DNA fragmentation and cell death. The mitochondrion is thus a novel target for the development of anticancer drugs: for example, arsenite and betulinic acid are excellent examples currently under preclinical evaluation (Costantini et al., 2000). Betulinic acid is a naturally occurring lupane-type triterpene which was first isolated from the stem bark of an East African evergreen tree, *Z. mauritiana*, and which is also very abundant in white birch (Pisha et al., 1995). Betulinic acid induces disruption of mitochondrial

membrane potential in melanoma cells (Fulda et al., 1998) and neuroblastoma cells (Fulda et al., 1999), both of neuroectodermal origin, and ultimately triggers apoptosis of these cancer cells. Apoptosis is normally associated with cytoplasmic shrinkage, nuclear alteration and DNA fragmentation, which were clearly demonstrated in melanoma cells treated with betulinic acid and its derivatives in the present study (Figs. 3 and 4) and in other studies (Fulda et al., 1998, 1999). This apoptotic activity, however, is not associated with cell growth arrest (Fig. 5) but required the translation of preexisting RNA, production of reactive oxidative species, mitochondrial membrane potential disruption and activation of caspases (Wick et al., 1999), indicating a crucial role for reactive oxidative species and mitochondria in betulinic acid-induced apoptosis. Flow-cytometric analysis using oxidation-sensitive fluorochrome, H_2DCF-DA, demonstrated a dramatic increase in intracellular reactive oxidative species in B16 cells after treatment with 3-oxo-23-hydroxybetulinic acid and 23-hydroxybetulinic acid for 2 h, peaking at 50 and 100 μg/ml, respectively. This increase was two- to four-fold higher than that elicited by betulinic acid, betulin and lupeol (Fig. 6A). However, betulinic acid derivatives only activated less than 10% lucigenin-dependent reactive oxidative species in B16 cells (Fig. 7B), strongly indicating that mitochondria but not NADPH oxidase was the source of reactive oxidative species in murine melanoma B16 cells. The possibility that there is a relatively low level of activatable NADPH oxidase in murine cells cannot be excluded (Bylund et al., 2003).

Betulinic acid is not only a highly selective inhibitor of human melanoma cells, but also of HIV, and thus, many betulinic acid analogs have been synthesized to study their specific inhibitory activities (Kim et al., 1998, 2000). They were either conjugated with amino acids (Jeong et al., 1999) or modified at the C3, C17 or C20 positions of betulinic acid to yield more soluble and cytotoxic compounds (Kim et al., 2000; Chowdhury et al., 2002). We have isolated several natural betulinic acid derivatives from the roots of a Chinese medicinal herb, *P. chinensis* (Bge) Regel (Ye et al., 1996): lupeol and betulin have a methyl group and methanol at C17, whereas 23-hydroxybetulinic acid and 3-oxo-23-hydroxybetulinic acid have a hydroxyl group at the C23 position. The hydroxylation of the methyl group at R4 did not affect cytotoxicity, as indicated by lupeol and betulin (both $IC_{50} \geq 100$ μg/ml); however, carboxylation at R4 (i.e., betulinic acid) markedly enhanced the cytotoxicity of the compounds (IC_{50}=76 μg/ml of betulinic acid vs. ≥100 μg/ml of lupeol and betulin). The alteration of ring 1 in both 23-hydroxybetulinic acid and 3-oxo-23-hydroxybetulinic acid is important to enhance apoptotic activity in B16 cells, as demonstrated by flow-cytometric analysis.

The oxidized moiety at R1 and R2 in 3-oxo-23-hydroxybetulinic acid further augmented the cytotoxicity of this C23 hydroxyl lupane-type triterpene (IC_{50}=22.5 μg/ml of 3-oxo-23-hydroxybetulinic acid vs. ≥32 μg/ml of 23-hydroxybetulinic acid). The order of cytotoxicity was correlated with reactive oxidative species production and with the dissipation of mitochondrial membrane potential in treated B16 cells (Fig. 6). It is noteworthy mentioning that the reactive oxidative species production in B16 cells treated with betulin, lupeol and even betulinic acid at doses up to 50 μg/ml was relatively low, whereas that elicited by treatment with 3-oxo-23-hydroxybetulinic acid and 23-hydroxybetulinic acid reached a peak level at 25 μg/ml and 50 μg/ml, respectively, and declined thereafter (Fig. 6A). Exposure to betulinic acid, lupeol and betulin remarkably increased the mitochondrial membrane potential within 2 h, and it remained at a high level with concentrations up to 100 μg/ml. However, the mitochondrial membrane potential of 23-hydroxybetulinic acid- and 3-oxo-23-hydroxybetulinic acid-treated B16 cells declined to a basal level at concentrations of 25–50 μg/ml, indicating that the cells were already committed to apoptotic pathways.

Betulinic acid is a selective inhibitor of human melanoma cells, e.g., MEL-1, MEL-2 and MEL-4 (IC_{50}=1, 2 and 4.8 μg/ml for 72-h cultures, respectively, Pisha et al., 1995), but not of certain murine cells (Fulda et al., 1999). The IC_{50} of betulinic acid for B16 cells in culture for 48 and 72 h was 76 (Fig. 2A) and 8 μg/ml (Fig. 2B), which is similar to 7.9 μg/ml for 72 h reported by Hata et al. (2002). A comparison of the cytotoxicity of betulinic acid and 3-oxo-23-hydroxybetulinic acid between murine melanoma B16 cells and murine L929 fibroblasts for 72 h further demonstrated that B16 cells are more sensitive to betulinic acid (IC_{50}=8 μg/ml) and 3-oxo-23-hydroxybetulinic acid ($IC_{50} \approx 1$ μg/ml) by two orders of magnitude ($IC_{50} \geq 100$ μg/ml for L929 cells for both compounds) (Fig. 7B), which strongly suggest the specificity of betulinic acid and its derivative for melanoma cells. Recent unpublished data for the more cytotoxic 3-oxo-23-hydroxybetulinic acid and 23-hydroxybetulinic acid in human neuroblastoma SY5Y cells prompts comparison of the mitochondrial disruptive effects of these compounds in human melanoma cells and in murine B16 cells.

Acknowledgments

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