



23-Hydroxybetulinic acid-mediated apoptosis is accompanied by decreases in *bcl-2* expression and telomerase activity in HL-60 Cells

Zhao-Ning Ji^a, Wen-Cai Ye^b, Guo-Ging Liu^a, W.L. Wendy Hsiao^{c,*}

^aDepartment of Pharmacology, China Pharmaceutical University, Nanjing 210009, China

^bDepartment of Phytochemistry, China Pharmaceutical University, Nanjing 210009, China

^cDepartment of Biology, The Hong Kong University of Science and Technology, Hong Kong China

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Abstract

23-Hydroxybetulinic acid, a derivative of betulinic acid, was investigated for its apoptotic effect and the associated telomerase activity in human leukemia HL-60 cells. Apoptosis and *bcl-2* were determined by flow cytometry analysis. A PCR-based telomeric repeat amplification protocol assay was used to detect telomerase activity. Results showed that 23-hydroxybetulinic acid induced growth arrest and apoptotic cell death in HL-60 cells. The apoptotic events were associated with concurrent down-regulation of *bcl-2* and the telomerase activity. Our data suggest that 23-hydroxybetulinic acid may be a potential cytotoxic agent for treatment of cancer.

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Introduction

Pulsatilla chinensis (Bunge) Regel is a Chinese medicinal herb for “blood-cooling” and detoxification in traditional Chinese medicine, and as such has been used for the treatment of amoebic dysentery and malaria [1]. We have isolated one of the compounds, 23-hydroxybetulinic acid (23-HBA) from the root of the plant [2]. Structurally, it closely resembles betulinic acid (BetA), which has been identified as

* Corresponding author. Tel.: +86-2358-7341; fax: +86-2358-1559.

E-mail address: bowhsiao@ust.hk (W.L.W. Hsiao).

an anti-tumor and apoptosis-inducing natural product [3,4]. Our study showed that the cytotoxic activities of 23-HBA against K-562 human leukemia and human HeLa cells are similar to that of BetA [5]. Apoptosis is a physiological mode of cell death in regulating development and homeostasis. Impaired apoptosis is associated with many different disease states including cancer, in which apoptosis is suppressed. Telomerase is a ribonucleoprotein that adds repeated sequence of TTAGGG to the end of telomeres and thereby prevents their shortening. Telomerase activation has been reported in 98% of established immortal cell lines and in about 90% of tumor tissues tested, but little or none is detectable in normal somatic cells [6,7]. This suggests that activation of telomerase may play an important role in carcinogenesis and immortalization. Both increased telomerase activity and enhanced resistance to apoptosis have been widely reported in human tumor cells [8,9], but little is known about any specific linkage between telomerase activity and apoptosis. Some studies suggested that telomerase activity was mechanistically involved in the regulation of apoptosis in both physiological and pathological settings [8–10].

In this study, we report the cytotoxicity and apoptotic response induced by 23-HBA in human leukemia HL-60 cells. The telomerase activity during apoptosis was examined by telomeric repeat amplification protocol (TRAP) assay, and B cell leukemia/lymphoma 2 gene (*bcl-2*) was analyzed to determine whether their expressions correlated with telomerase activity. We found that 23-HBA inhibited telomerase activity and suppressed the expression of *bcl-2* in HL-60 cells. The inhibition of telomerase activity of HL-60 cells was closely associated with the apoptotic events induced by 23-HBA.

Materials and methods

Chemicals

The chloroform-soluble fraction of methanol extracts of *Pulsatilla chinensis* (Bunge) Regel roots was fractionated by silica gel and Sephadex LH-20 to obtain an analytically pure sample of 23-HBA (Fig. 1) [2].

Cell culture and viability assay

Human leukemia HL-60 cell line was obtained from the Shanghai Institute Cell Biology. The cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (Gibco),

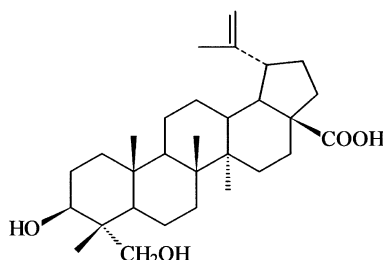


Fig. 1. Structure of 23-hydroxybetulinic acid.

100,000 unit/l penicillin and 100 mg/l streptomycin (Gibco), incubated in a humidified CO₂ (5%) incubator at 37 °C. The cytotoxicity of 23-HBA was measured by MTT assay [11]. Briefly, 90 µl of cell suspension containing 5×10^4 exponentially growing HL-60 cells were seeded in 96-well plates. 23-HBA (10 µl per well) was added immediately to achieve the desired concentration of 1–1000 µM. The plates were cultured at 37 °C for 3, 6, 12 and 24 h. Twenty µl of stock solution (5 mg/ml) of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Fluka) was added to each well and incubated for 4 h. A lysis solution (10% sodium dodecyl sulfate, 5% isobutanol and 12 mM HCl) was added to the plates and incubated for 12 h. The optical density was then measured at 570 nm using an ELISA plate reader (Hua Dong Electronic CO, Nanjing, China). All MTT assays were performed in four replicated wells.

Flow cytometry analysis

Cells were collected and fixed in 70% ethanol at 4 °C overnight. Subsequently, cells were treated with Tris buffer (pH 7.4) containing 1% RNase A and stained with propidium iodide (PI) (5 µg/ml). Distribution of cells with different DNA contents was determined by a flow cytometry (FacsCalibur, Becton Dickinson) and the data were analyzed by multicycle DNA content and cell cycle analysis software (Modfit LT 2.0)[12].

Indirect immunofluorescence assay of bcl-2 expression

The cellular level of *bcl-2* protein was measured by flow cytometry [13]. Briefly, 10^6 cells were washed twice in cold PBS before the addition of 20 µl FITC (fluorescein isothiocyanate)-conjugated mouse anti-human *bcl-2* antibody. After 30 min incubation at room temperature in the dark, cells were rinsed twice and resuspended in 500 µl PBS and used for flow cytometry analysis. A FACScan flow cytometry (Becton Dickinson) equipped with an argon laser (488 nm) was used to measure fluorescence. Data were analyzed using Cellquest software (Becton Dickinson).

Telomeric repeat amplification protocol (TRAP) assay

Telomerase activity was assayed by the TRAP method as described previously [6,14] with minor modification. Generally, HL-60 cells treated with 23-HBA (10 µM) for 3, 6, 12 and 24 h were washed twice in PBS (pH 7.4), and suspended in cold 3-[(3-cholamidopropyl) dimethyl ammonio]-1-propane-sulphonate(CHAPS) lysis buffer. After 30 min of incubation in ice, the lysate was centrifuged at 12,000 g for 30 min at 4 °C, and the supernatant was rapidly frozen and stored at –80 °C. The concentration of protein was measured using the Bradford protein assay. One µg of protein extract was used for each TRAP assay. The TRAP assay was carried out in 50 µl of reaction mixture containing 1 µg extract, 20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 63 mM KCl, 0.005% Tween-20, 1 mM EGTA, 50 µM dNTP mixture, 0.1 µg TS primer (5'-AATCCGTCGAGCAGAGTT-3') and 2 units/µl of Taq polymerase. After a 30 min incubation at 30 °C for telomerase-mediated extension of the TS primer, 0.1 µg of CX oligonucleotides (5'-CCCTTACCCTTACCCTTACCCTAA-3') was added. The reaction mixture was then subjected to 30 cycles of PCR amplification (94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s). The reaction products were examined by electrophoresis on a 12% nondenaturing polyacrylamide gel in 1 × Tris-borate-EDTA (TBE) buffer at 200 V for 2 h. The gel was then stained with 0.2% silver nitrate for 10

min, scanned and analyzed by Smart View Bioimage Analysis System (Furi Company, Shanghai, China).

Results

Effect of 23-HBA on the growth of HL-60 cells

A concentration-dependent inhibition of cell growth was seen in cultures treated with 23-HBA for 12 h at dosages ranging from 1–1000 μM (Table 1A). The time course study indicated that at 10 μM , 23-HBA induced detectable effects within rather short exposure periods (3 to 6 h) (Table 1B). After the addition of 23-HBA to the cultures for 3, 6, 12 and 24 h, cell viability progressively decreased as shown in Table 1B.

Detection of apoptosis by flow cytometry

To determine whether apoptosis is involved in the anti-proliferation effect of 23-HBA, HL-60 cells were treated with 10 μM 23-HBA for 3, 6, 12 and 24 h, and subjected to flow cytometry analysis for apoptotic activity. Results showed that a typical sub- G_1 peak, representing the apoptotic cell population, began to appear after 3h of treatment with the drug. The percentage of the apoptotic cells increased as treatment duration increased. The percentage of apoptotic cells of the cultures treated with 23-HBA for 3, 6, 12, and 24 h was 1.1, 3.6, 11.0 and 49.2%, respectively (Fig. 2). Based

Table 1
Cytotoxicity of 23-hydroxybetulinic acid in HL-60 cells assessed by MTT assay

A. Dosage dependence study			
23-HBA (μM)*	O.D. _{570 nm} , Mean \pm S.D. ⁿ		% Survival
0	1.30 \pm 0.05		–
1	1.07 \pm 0.04 ^c		82.31
10	0.97 \pm 0.05 ^c		74.61
100	0.692 \pm 0.023 ^c		53.23
1000	0.571 \pm 0.019 ^c		43.92
B. Duration of treatment			
Time (h)	O.D. _{570 nm} , Mean \pm S.D. ⁿ		% Survival
	– 23-HBA	+ 23-HBA	
0	0.96 \pm 0.01	0.97 \pm 0.02	–
3	0.98 \pm 0.03	0.93 \pm 0.04 ^a	94.81
6	1.01 \pm 0.05	0.87 \pm 0.01 ^c	86.44
12	1.05 \pm 0.04	0.72 \pm 0.03 ^c	68.57
24	1.33 \pm 0.05	0.62 \pm 0.02 ^c	46.61

n = 4 wells for each experiment; 10 μM of 23-HBA was applied to each experiment.

^a*P* > 0.05, ^b*P* < 0.05, ^c*P* < 0.01 vs control.

*The duration of treatment was 12 h; *n* = 4 wells for each experiment.

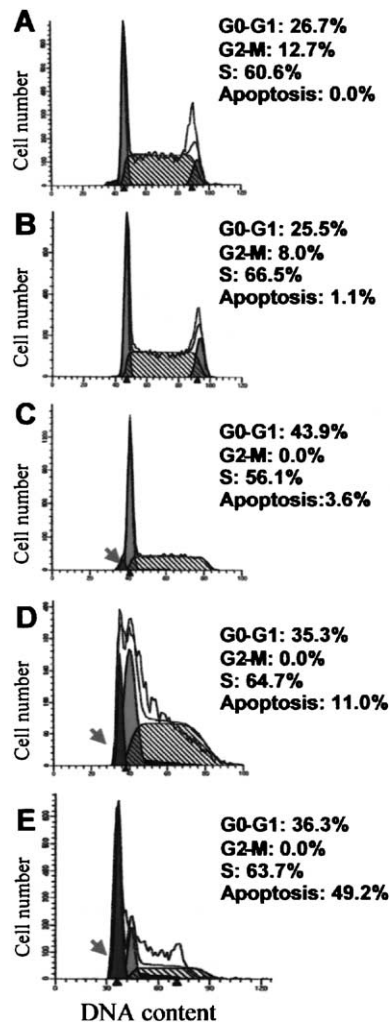


Fig. 2. Flow cytometry analysis of apoptotic cells treated of 23-HBA. 10^6 cells were treated with 23-HBA, stained with propidium iodide, and analyzed on a flow cytometer for identifying sub-G₁ peak (arrow bar) representing apoptotic cells. **A**, normal HK-60 cells; **B**, **C**, **D**, and **E** show cells treated with 10 μ M of 23-HBA for 3, 6, 12, and 24 h respectively. The number in the top right quadrant represents the percentage of apoptotic cells.

on the histograms shown in Fig. 2, 23-HBA also tended to arrest cells at the G₁ phase of the cell cycle.

Detection of bcl-2 expression using immunofluorescent staining

To understand the possible involvement of the apoptosis-modulating gene bcl-2, protein expression of the gene was analyzed in cells treated with 23-HBA by flow cytometry after immunofluorescent double staining of the cells with anti-bcl-2 antibody. As shown in the dot plots (Fig. 3), the percentage of the

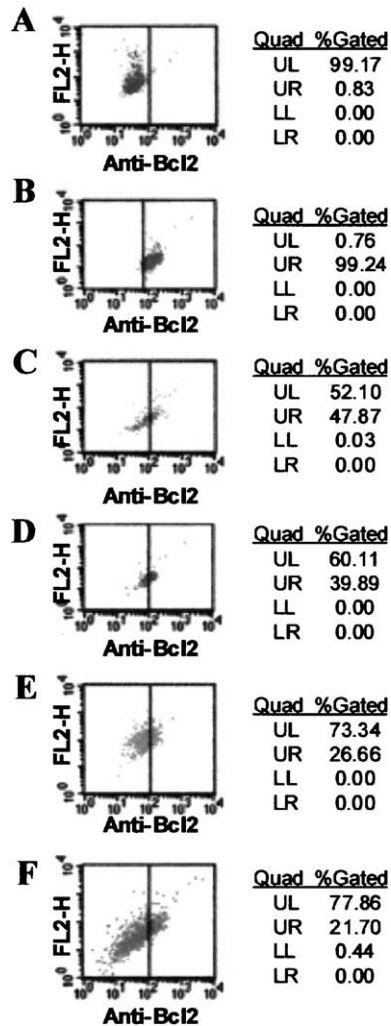


Fig. 3. Detection of *bcl-2* expression by flow cytometry. **A**. control (2nd antibody alone); **B**. normal HL-60 cells; **C**, **D**, **E**, and **F** show treatments of 10 μ M 23-HBA for 3, 6, 12, and 24 h respectively.

bcl-2 positive cells were declined from 99.24% in the no treatment control population to 47.87, 39.89, 26.66 and 21.70% in cultures treated with 23-HBA for 3, 6, 12, and 24 h respectively. The levels of *bcl-2* were in inverse relationship with the percentage of apoptotic cells present in the treated cultures (Fig. 3 and Fig. 2).

Effect of 23-HBA on telomerase activity of HL-60 cells

We also explored the possible linkage between *bcl-2* expression and telomerase activity in 23-HBA induced apoptosis using the TRAP assay. Addition of 23-HBA in HL-60 cells substantially reduced the telomerase activity (Fig. 4A). The percentage of relative activity of telomerase in the treated cells was

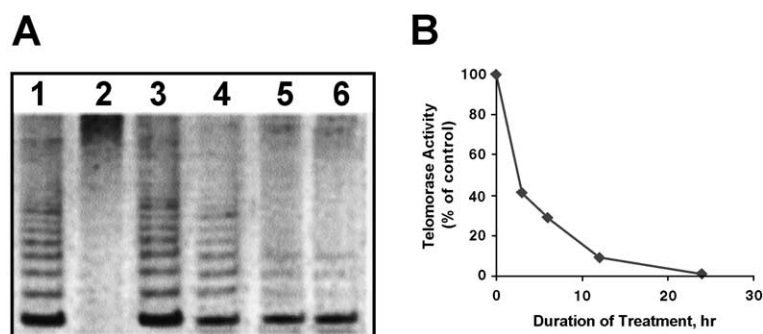


Fig. 4. Effect of 23-HBA on telomerase activity in HL-60 cells. **A.** TRAP assay of HL-60 cells treated with various duration of 23-HBA. Lane 1: normal HL-60 cells; Lane 2: a negative lysis buffer alone control; Lanes 3, 4, 5, 6: cell extracts treated with 10 μ M 23-HBA for 3, 6, 12 and 24 h respectively. **B.** Signal intensity in each lane (shown in Panel A) was measured by area integration of the first six ladders from the bottom of the gel using the Furi Electrophoresis Image Analysis System. Relative telomerase activities were quantified by comparing the signal intensity in each lane, and using normal HL-60 cells as 100%.

decreased from 100% in the control group to 41.43, 28.87, 9.28 and 1.03% in groups treated for 3, 6, 12, and 24 h respectively (Fig. 4B).

Discussion

The compound 23-HBA derived from the roots of *Pulsatilla chinensis* (Bunge) Regel, is a newly isolated derivative of BetA. BetA has been considered as a promising new anti-tumor agent [3,4]. Studies show that BetA along with other synthetic derivatives acts as a new family of cytotoxic agents against melanoma, neuroectodermal tumor cells, including neuroblastoma, medulloblastoma, glioblastoma, and Ewing's sarcoma cells which represent the most common solid tumors of childhood [3,15–17]. The mechanism of the drug action of BetA is not clear, however, studies suggest that the cytotoxic effect is mediated by apoptotic process [3,4]. The BetA induced apoptosis can be reversed by overexpression of the anti-apoptotic proteins *bcl-2* or *bcl-X(L)*. In this study, we demonstrated that 23-HBA acts as an effective cytotoxic agent against HL-60 cells by inducing growth arrest and apoptosis in concentration- and duration-dependent manners. Apoptosis is accompanied by a decrease in telomerase activity and down-regulation of the pro-apoptotic gene *bcl-2*.

The view that apoptosis is a tightly regulated self-destruction mechanism is widely accepted. However, the concept that telomerase may be an important factor in suppressing apoptotic signaling cascades was not known until recently. For instance, the stable expression of *bcl-2* in human cancer cells results in an increase of telomerase activity and decrease in apoptosis [9]. A few studies also show that agents inhibiting telomerase activities lead to the progressive telomere shortening and cause cells to undergo apoptosis [8,10,18,19]. All these data imply that telomerase may play an important role in modulating apoptotic cell death and may serve as a target for the discovery of anti-cancer drugs. In the present studies, concurrent inhibition of *bcl-2* expression and telomerase activity was observed 3 h after 23-HBA treatment, while the apoptotic cells only became detectable 6 h after drug treatment, suggesting that the down-regulation of *bcl-2* and telomerase may play a causative role in

23-HBA-induced apoptosis. While the precise nature of 23-HBA-mediated apoptosis remains to be determined, our data reveal that 23-HBA, a newly isolated derivative of BetA, may induce apoptosis through the telomerase–Bcl-2 cascade and may have a potential to be developed as an effective cytotoxic agent in cancer treatment.

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