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The plant-derived triterpenoid tingenin B is a potent anticancer agent due to its cytotoxic activity on cancer stem cells of breast cancer *in vitro*

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ABSTRACT

Despite the rapid advances in chemotherapy regimens, the outcome of patients with breast cancer is not satisfactory. One of the reasons of this dissatisfaction is that subsets of cells in tumors which referred as cancer stem cells (CSCs) show and/or gain resistance to therapies. Thus, compounds that target CSCs are urgently needed. Since some are already used in the clinic, natural products have great potential for further development as anti cancer drugs. The aim of this study is to investigate the cytotoxic activity of tingenin b (or 22β-hydroxytingenone) which is a quinone-methide triterpenoid structurally related to tingenone, against breast CSCs (stem-cell enriched population from MCF-7 cell line, MCF-7s). It has been found that tingenin b was cytotoxic against MCF-7s (IC_{50} value for 48 h was found to be 2.38 μM) by inducing apoptosis. It was evident by Annexin V staining positivity, decreased mitochondrial membrane potential and Bcl-2 dephosphorylation with a concomitant increase in Bax protein expression. In addition, endoplasmic reticulum stress was also found to be involved in tingenin b-induced cell death. In conclusion, the results warrant further studies aimed at elucidating and corroborating its possible use in the treatment of breast cancer.

1. Introduction

During the past decade cancer stem cells (CSCs) have been discovered in a variety of solid tumors including breast, brain, prostate, colorectal, head &neck, lung, ovary, melanoma, and are considered as a notable subpopulation within the tumor bulk that is required to initiate and maintain tumors [1–10]. It has been reported that CSCs are responsible for chemoherapy resistance [11] and also hypothesized that they are associated with tumor metastasis. Chemotherapy resistance has been shown to result from a variety of events including inhibition of apoptosis [12]. In breast cancer, the expression of Bcl-2 mRNA was found to be significantly higher in 231-CSCs (breast cancer stem cells of MDA-MB-231) than the parental cells [13]. In another study, the anti-apoptotic protein survivin resulted to be overexpressed in MCF-7s (breast cancer stem cells of MCF7) as compared with MCF7 cells [14]. Therefore, induction of apoptosis in CSCs could be regarded as a therapy approach.

Given the fact that CSCs are resistant toward standard chemotherapies, natural compounds may bear potential to eradicate these cells. Natural products play a crucial role in the field of cancer, since some are already used in the clinic. They have been also found to inhibit CSCs [15]. Among them, quinone-methide triterpenes have been studied over the last several decades and isolated from *Celastraceae* [16] and *Hippocrateaceae* [17] families. Also they have

Abbreviations: Bcl2, B-cell lymphoma 2; CSC, Cancer stem cell; ccK18, M30, Caspase cleaved keratin 18; K18, Keratin 18; ATP, Adenosine-5'-triphosphate; ER, endoplasmic reticulum; PARP, Poly (ADP-ribose) polymerase; c-PARP, Cleaved-Poly (ADP-ribose) polymerase; PI, Propidium iodide; PBS, Phosphate buffered saline; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; HPLC, High Performance Liquid Chromatography; HRP, Horseradish peroxidase; RPMI, Roswell Park Memorial Institute medium; SFA, Sphere formation assay.

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found to exhibit anticancer effect against breast cancer [18–21]. Among them, tingenone has shown to be cytotoxic against several cancer types including breast [21–23]. The tingenin b (or 22β-hydroxytingenone) which is structurally related to tingenone, has antibacterial [24], antiparasitic [25] and anticancer activity [26,27]. However, its cytotoxic activity against any type of CSC and the mechanism of cell death remained to be identified. The present study was carried out to evaluate the *in vitro* cytotoxicity and the cell death mechanism of tingenin b in breast CSCs (stem-cell enriched population from MCF-7 cell line, MCF-7s).

As a first time in the literature, we have presented that tingenin b had a promising cytotoxic activity by inducing apoptosis which is possibly related to mitochondrial injury. In fact, ER stress seems to be involed in tingenin b induced apoptosis. Our results suggest that tingenin b may be a promising compound due to its cytotoxic activity and apoptosis and/or ER stress inducing effect against breast CSC although *in vivo* experiments are required for the proof-of concept.

2. Materials and methods

2.1. Source and characterization of tingenin b

Tingenin b ((6αS,6βS,8αR,9S,11R,12αS,14αR)-3,9-dihydroxy-4,6α,6β,8α,11,14α-hexamethyl-7,8,9,11,12,12α,13,14-octahydropicene-2,10-dione) was obtained from *Maytenus* sp. by following the procedure already described [16]. *Mytenus* sp. root bark was kindly provided by Universitade Federal do Pernambuco, Recife, Brazil. The dried ethanol extract, prepared from the root bark of *Maytenus* sp., was chromatographed on silica gel column, using chloroform as eluent to give pure tingenone (4.5 g) and impure tingenin b (350 mg). Two further purifications on a silica gel column with a mixture of hexane/ethyl acetate = 75:25 (v/v), were necessary to obtain pure tingenin b (200 mg). The compound crystallized from ethyl acetate as red prisms; m.p. 196–197 °C; $[\alpha]_D^{22}$ = −484.6; IR, ν_{max} ($CHCl_3$) 3700-3300, 1705, 1650, and 1590 cm^{-1}; UV, ($CHCl_3$) λ_{max} (logε): 250 (4), and 422 (4.08) nm; ESI-MS $(M-H)^+$ 436.3 *m/z*; ^{1}H NMR, (400 MHz, $CDCl_3$): δ (ppm) 7.03 (1H, brd, J = 6.7 Hz, C6-H), 6.53 (1H, s, C1-H), 6.38 (1H, d, J = 6.7 Hz, C7-H), 4.53 (1H, s, C22α-H), 2.65 (1H, m, C20-H), 2.23 (2H, m, C11β-H, and C16β-H), 2.21 (3H, s, C23-H), 2.20 (1H, m, C19α-H), 2.00 (1H, m, C11α-H), 1.86 (1H, m, C15α-H), 1.85 (1H, m, C12α-H), 1.83 (1H, m, C12β-H), 1.80 (1H, m, C18-H), 1.77 (1H, m, C19β-H), 1.65 (1H, m, C15β-H), 1.61 (1H, m, C16α-H), 1.50 (3H, s, C24-H), 1.35 (3H, s, C25-H), 1.05 (3H, d, J = 6.30 Hz, C28-H), 0.96 (3H, s, C26-H), 0.85 (3H, s, C27-H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 213.5 (C21), 178.4 (C2), 168.5 (C8), 164.7 (C10), 146.1 (C3), 133.6 (C6), 127.7 (C5), 119.8 (C1), 118.1 (C7), 117.3 (C4), 76.4 (C22), 45.0 (C18), 44.8 (C17), 44.3 (C14), 42.6 (C9), 40.9 (C20), 40.6 (C13), 39.1 (C24), 34.0 (C11), 32.0 (C19), 29.9 (C12), 29.5 (C16), 28.3 (C15), 24.9 (C27), 21.6 (C25), 20.5 (C26), 14.7 (C28), 10.3 (C23). HPLC purity: ≥96%.

2.1.1. Chemicals and instruments

The solvents, having an analytical grade or HPLC grade, were purchased from Sigma Aldrich. All reagents were used without further purification. Chromatography was carried on silica gel (70–230 mesh). All chromatography fractions were monitored by thin-layer chromatography (TLC), and silica gel plates with fluorescence F254 were used.

Melting point was taken in open capillaries on a Büchi Melting Point B-545 apparatus and was uncorrected. Infrared spectrum was recorded in $CHCl_3$ on a FT-IR spectrometer. UV spectrum was recorded on a JASCO V-550 spectrophotometer. ^{1}H NMR and ^{13}CNMR spectra were recorded using a Bruker 400 Ultra ShieldTM spectrometer (operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C) using $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal standard. Chemical shifts are reported in parts per million. Multiplicities are reported as follows: singlet (s), doublet (d), multiplet (m), and broad doblet (brd). Mass spectrometry was performed using a Thermo Finnigan LXQ linear ion trap mass spectrometer, equipped with an electrospray ionization (ESI) ion source bearing a steel needle.

HPLC analytical separation was performed on a Waters 2690 Separation Module, equipped with a Rheodyne Model 8125 20-μl injector and a Model M486 programmable multi-wavelength detector (PDA). Chromatographic data were collected and processed using the Empower Chromatography Manager software. Column: Phenomenex Luna C18, 5 μm (250 × 4.6 mm); eluent: aqueous trifluoroacetic acid 1% (v/v)/methanol = 80:20 (v/v); isocratic mode; flow rate: 1.0 mL/min; UV detection at 430 nm; retention time (R_t) = 23.50 min.

2.2. Culture of breast cancer stem cells (MCF-7s) cells

To obtain floating mammospheres (MCF-7s cells) that contain cancer stem cells, we applied the spheroid colony formation method as previously published [28] and cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Cytotoxicity and apoptosis assays

2.3.1. ATP viability assay

The ATP assay is a luminometric assay that reliably measures the viability of cells [29]. MCF-7s cells were seeded at a density of 5×10^3 cells per well of 96-well ultralow surface cell culture plate. After 72 h, tingenin b was added at different concentrations (at the range of 0.19–6.25 μM) and cells were then incubated with tingenin b for 48 h. At the end of treatment, to extract intracellular ATP from the cells, 5× ATP-releasing reagent (a detergent-based reagent) was added and after mixing thoroughly cells were incubated at room temperature for 30 min. At the end of the incubation, 50 μL of suspension was transferred into white opaque 96-well plate and 50 μl luciferin-luciferase mixture (FLAAM, ATP Bioluminescent Somatic Cell Assay Kit, Sigma, Germany) was added to cell suspension. Luminescent signal was measured using a count integration time of 1 s at luminometer (Bio-Tek, Vermont, USA). Cell viability of treated cells was calculated in reference to the untreated control cells using the formula as viability (%) = [100 × (Sample RLU)/(Control RLU)]. Assay was performed at least two times and the results were given as mean ± SD of independent experiments.

2.3.2. Sphere formation assay (SFA)

SFA shows the effect of the compounds on the formation process of mammospheres, not the effect on the already-formed mammospheres. Briefly, MCF-7s cells were plated in 96-well ultralow surface cell culture plates at a density of 5×10^3 cells per well and then treated with tingenin b at different concentrations (at the range of 0.04–6.25 μM). Cells were incubated with these doses for 7 days. At the end of treatment, to easily visualize the plates, cells were then incubated with MTT at 37 °C for an additional 4 h. The number of mammospheres were counted and calculated via normalization of treatment groups to negative control (untreated) cells and expressed as percentage.

2.3.3. Fluorescence imaging (Double staining with Hoechst 33342 and propidium iodide, PI)

In order to examine the nuclear morphology and membrane integrity of cells, two fluorescent dyes, Hoechst 33342 and PI, were used, respectively. Cells were seeded at a density of 5×10^3 cells per well of 96-well ultralow surface cell culture plates and treated with