



Pulmonary, gastrointestinal and urogenital pharmacology

12-Deoxyphorbol 13-palmitate mediated cell growth inhibition, G2-M cell cycle arrest and apoptosis in BGC823 cells

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ABSTRACT

The highly toxic monomer 12-deoxyphorbol 13-palmitate (G) was extracted from the roots of *Euphorbia fischeriana*. Our experimental data confirmed studies showing that 12-deoxyphorbol 13-palmitate had certain antitumor activities. The MTT method, soft agar experiments, and nude mouse tumor experiments proved that 12-deoxyphorbol 13-palmitate inhibited the growth of BGC823 cells. We found that the drug could induce cell cycle arrest at the G2-M checkpoint in BGC823 cells. The compound also induced apoptosis as assayed by Annexin-V-FITC/PI dual labeling, AO/EB dyeing, and caspase-3 and caspase-9 activity. The reduction in expression of cyclin B1 protein and the increased activity of reactive oxygen species were observed in BGC823 cells treated with 12-deoxyphorbol 13-palmitate for 24 h. In addition, we found down-regulation of cdc2/cyclin B, cyclin A and p-chk1 in tumor cells. There was also up-regulation of Bax, p53, p21, and IκB-α and down-regulation of Bcl-2 and NF-κB by WB. Our studies may define a novel mechanism by which 12-deoxyphorbol 13-palmitate inhibits tumor cell growth and induces apoptosis. The results of our current studies provided strong experimental evidence for the use of 12-deoxyphorbol 13-palmitate as a potential preventive and/or therapeutic agent in cancer.

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1. Introduction

Euphorbia fischeriana Steud is a type of *Lang-du* from Traditional Chinese Medicine. In China, *Lang-du* has been used to treat patients with cancer, edema and ascites for many years (Ogura et al., 1978; Wang et al., 2010). Jolkinolide B and 17-hydroxy-jolkinolide B found in *E. fischeriana* Steud have demonstrated a wide range of biological activities *in vitro*. These two novel natural compounds had low toxicity and potent anti-cancer effects in a variety of cancer cells (Luo and Wang (2006); Lin et al., 2012; Yan et al., 2008; Wang et al., 2009). 12-Deoxyphorbol 13-palmitate is one of these new anti-tumor traditional Chinese medicine monomers from *Euphorbia fischeriana* Steud. In a recent report, 12-deoxyphorbol 13-palmitate was described without molecular mechanism studies and potential biological effects.

We therefore sought to explore its biological influence on gastric cancer cells *in vitro* and *in vivo*.

2. Materials and methods

2.1. Plant extracts and purification

The drug (purity > 99%) (Fig. 1) was kindly provided by Professor Shu-Jun Zhang (Chemical Engineering Institute, Qiqihar University). 12-Deoxyphorbol 13-palmitate was extracted from the roots of *Euphorbia fischeriana* with ethyl acetate. It was isolated using column chromatography and found to have a molecular weight of 586.84 g/mol by gas chromatography (Shimadzu, Kyoto, Japan). The chemical formula of 12-deoxyphorbol 13-palmitate (C₃₆H₅₈O₆) was elucidated from its nuclear magnetic resonance spectrum. The drug was dissolved in dimethyl-sulfoxide (DMSO) to make a 1 mg/ml stock solution, which was further diluted to the appropriate concentration with culture medium before each experiment. Control experiments contained only DMSO.

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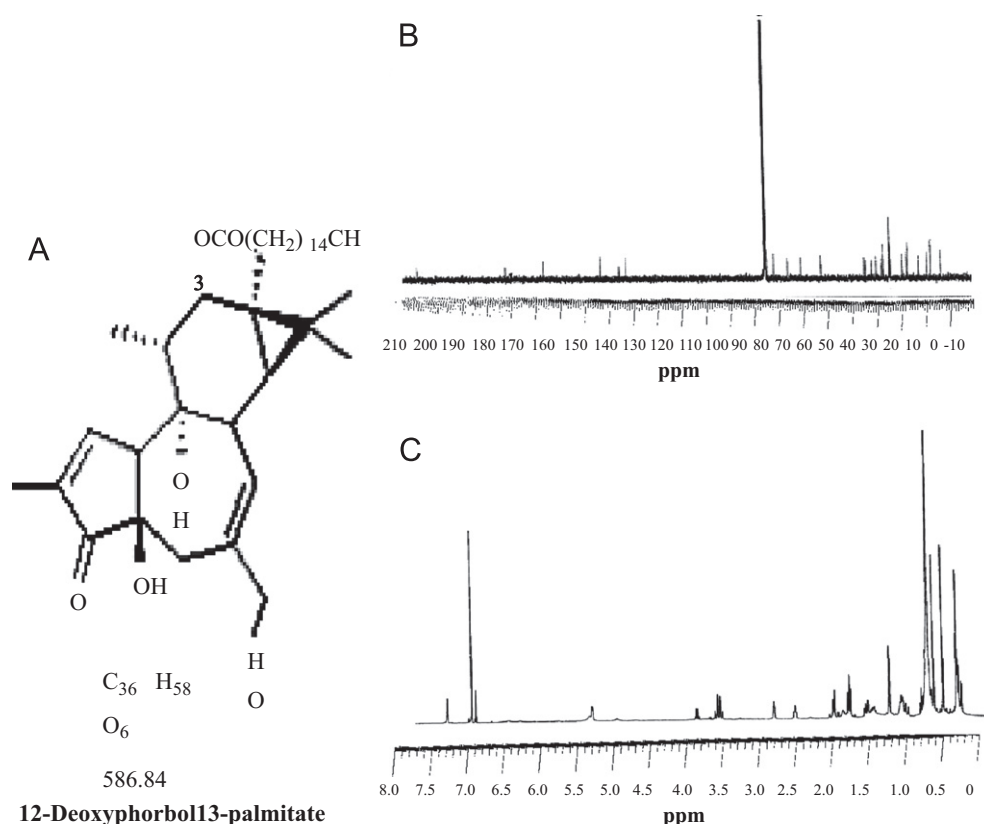


Fig. 1. Structure of 12-deoxyphorbol 13-palmitate (a) with carbon (b) and proton (c) nuclear magnetic resonance spectra.

2.2. Cells and cell culture

The human gastric cancer cell line BGC823 was obtained from the Laboratory of Molecular Oncology, Beijing Cancer Hospital/Institute. Cells were incubated in complete DMEM (Gibco BRL, Grand Island, NY, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA) in a humidified atmosphere at 37 °C and 5% CO₂.

2.3. Cell viability analysis

Cells (1×10^4 /ml) were cultured in 96-well chamber slides for 24 h before use. Culture medium was replaced with fresh medium containing the drug concentrations ranging from 0 to 80 µg/ml, and then fresh medium with drug was added every 24 h up to 72 h. MTT (Sigma-Aldrich, St. Louis, MO, USA) was added into each well and cultured for another 4 h, and the supernatant was discarded and replaced with 100 µl DMSO. When the crystals were dissolved, the absorbance values were read on an enzyme-labeled mini reader II (Bio-Rad, Hercules, CA, USA) at 570 nm. This procedure was repeated three times.

2.4. Soft agar cloning experiment

The single-cell suspensions of cell lines were cultured in 60 mm dishes at a density of 2×10^3 cells per well in triplicate and then incubated with 12-deoxyphorbol 13-palmitate ranging from 0 to 40 µg/ml for 4 weeks. Cells were dead when the liquid concentration of the drug was 80 µg/ml. The cells were then stained with the vital tetrazolium dye INT for 12 h (Kajiwar et al., 2008). Soft agar was fixed with 100 µl methanol-acetic acid (3:1, v/v). The number of colonies was counted under a light microscope, but only colonies containing more than 50 cells were counted.

2.5. Cell Cycle Analysis

Cells were seeded in 60-mm culture dishes and grown to 50% confluence. Subsequently, the cells were cultured in serum-free medium for 24 h and then incubated with 0, 10, 20, or 40 µg/ml of 12-deoxyphorbol 13-palmitate for 24 h in complete medium. The cells were harvested by trypsinization, centrifuged at 2000 g for 5 min, washed in PBS, and resuspended in cold 70% ethanol. Finally, 1 ml propidium iodide stain solution (20 µg/ml PI, 100 µg/ml DNase free RNase A) was added to the samples and analyzed on a FACScan instrument (Becton Dickinson, San Francisco, CA) within 30 min (Tsai et al., 2010). Data on 10,000 cells were acquired and processed using Lysis II software (Becton Dickinson).

2.6. Flow cytometric analysis of apoptosis

Cells were seeded in 6-well plates at a density of 1.2×10^6 cells/well. After 24 h of 12-deoxyphorbol 13-palmitate treatment at 0, 10, 20, and 40 µg/ml, cells were collected. The cells were then washed twice with cold PBS, and 1×10^6 cells were resuspended in 500 µl of $1 \times$ binding buffer. One hundred microliters of cell suspension was transferred to a 5 ml culture tube and incubated with 10 µl of Annexin V antibodies and 10 µl of propidium iodide containing 300 µg/ml RNase (sigma, MO, USA) (Tsai et al., 2010). The cells were gently vortexed and incubated for 15 min at room temperature in the dark. Four hundred microliters of $1 \times$ binding buffer was added to each tube and the cells were analyzed by flow cytometry within 1 h.

2.7. Fluorescence Microscopy

Cells were plated on 13×13 mm coverslips in cell culture dishes. Twenty-four hours after plating, cells were fixed with 4% paraformaldehyde for 10 min, blocked with 1% BSA for 30 min,

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