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EFLDO induces apoptosis in hepatic cancer cells by caspase activation *in vitro* and suppresses tumor growth *in vivo*

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ABSTRACT

To study the apoptosis induced by EFLDO (ent-3α-formylabieta-8(14), 13(15)-dien-16,12β-olide), extracted from the *Euphorbia lunulata* Bge, in the HepG2 cell line and to study the antitumor activity of this compound *in vivo*, Cell viability and migration were evaluated with CCK-8 (2-(2-methoxy-4-nitrophenyl)-3- (4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) and wound healing assays, respectively. In addition, the cell cycle was examined using flow cytometry after propidium iodide (PI) staining. Apoptosis was analyzed by using the Annexin V/PI staining assay. Pro-caspase activation and apoptosis protein expression were evaluated by western blotting. A HepG2 xenograft model in nude mice was also established to study the antitumor activity of EFLDO *in vivo*. Immunohistochemical analysis was used to detect the expression of Ki67 in the tumors *in situ*. EFLDO could induce dose- and time-dependent apoptosis in HepG2 human hepatic cancer cells. Activation of caspases 3, 8, and 9 played an important role in EFLDO-induced apoptosis *in vitro*. Decreased levels of Bcl-2 and Survivin and increased level of BAX were also involved in this process. Furthermore, EFLDO could inhibit HepG2 tumor growth in nude mice, and the proliferation characteristics, reflected by the Ki67 index, were suppressed significantly. The results indicated that EFLDO could induce apoptosis in hepatic cancer cells by caspase activation *in vitro* and suppress tumor growth *in vivo*.

1. Introduction

Cancer is considered to be an uncontrolled proliferation of cells, and this imbalance between the proliferative and apoptotic activity of cells occurs as a consequence of a series of genetic changes [1]. As a malignant tumor, hepatocellular carcinoma (HCC) is reported to be associated with a high mortality rate, based on the statistical report of malignant tumors worldwide in 2015 [2]. In areas with cancer registries, liver cancer was found to be the fourth most common cancer in China, with an incidence of 28.71/100000. Furthermore, mortality caused by liver cancer ranked second and third in a study of all cancer-related deaths in urban and rural areas, respectively [3]. Now, methods for the treatment of liver cancer include surgical resection, liver transplantation, radiotherapy and chemotherapy [4–5], however, the mortality rate remains high after these treatments. Given these circumstances, new therapeutic approaches with better efficacy and safety are urgently needed. With relatively few side effects and broad applications in a variety of diseases, including cancer, natural products have been receiving a great deal of attention lately [6–10]. *Euphorbia lunulata* Bge, a species of *Euphorbia L.,* is traditionally used in folk medicine for treating asthma, bronchitis, gastric carcinoma and breast carcinoma [11]. Diterpenoids, the characteristic constituents of *Euphorbia L.* plants [12], were found to have pharmacological activities including anticancer [13–15], antimicrobial [16], and antiviral activities [17]. We recently isolated EFLDO from the ethanolic extract of *Euphorbia lunulata* Bge; EFLDO is a kind of ent-abietane-type diterpene lactone [18]. In this study, we studied the activity of EFLDO on the proliferation and migration of cancer cells *in vitro* and the anticancer effect of EFLDO on a xenograft model of human hepatic cancer in nude mice. Furthermore, potential mechanisms responsible for the antitumor effect of EFLDO were studied.

2. Materials and methods

2.1. Cell culture

Human hepatocellular carcinoma cell line HepG2, human large-cell lung cancer NCI-H460, human breast cancer line MCF-7, human colonic

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carcinoma cell line HCT-116, human erythroleukemia cell line K562 and human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC, Manassas USA). In addition, these cell lines were cultured in Dulbecco's modified Eagle's medium (Hyclone, Thermo Fisher Scientific Inc., Massachusetts, USA) containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were cultivated in a fully humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Reagents and antibodies

EFLDO (ent-3α-formylabieta-8(14),13(15)-dien-16,12β-olide, Fig. 1, $C_{21}H_{28}O_4$, MW 367.2) was purified and identified from the ethanolic extract of *Euphorbia lunulata* Bge in our lab. EFLDO was dissolved in dimethyl sulfoxide (DMSO) to prepare a 10 μg/mL stock solution, which was stored at −20 °C. The Annexin V-FITC Apoptosis Detection Kit was obtained from EnoGene (New York, USA). Primary antibodies against human cleaved caspase-3, human cleaved caspase-8, human cleaved caspase-9, Bcl-2, Survivin, Bax, and the internal control protein GAPDH and horseradish-peroxidase-conjugated secondary antibodies were obtained from EnoGene (New York, USA).

2.3. Cell viability assay

For the cell viability assay, the cells were seeded in a 96-well plate at a density of 1×10^4/well and then treated with EFLDO for 72 h. Then, 10 μL of CCK-8 solution (EnoGene, New York, USA) was added to each well. The plates were then incubated at 37 °C for 4 h. The absorbance of each well was measured by an enzyme-linked immunosorbent assay reader at 450 nm. The growth inhibition curve and IC_{50} value were analyzed by GraphPad Prism 5 software (GraphPad Software, San Diego, USA).

2.4. Wound healing assay

Cells were cultured in 60-mm tissue culture plates under standard conditions. Prior to being scratched with P200 pipette tips to create an artificial wound, cells were washed 3 times with 1 × phosphate-buffered saline (PBS). To remove any cellular debris, cells were washed 3 times. Then, the cells were incubated with EFLDO in fresh media. The wounds were photographed at 0, 24, 48, and 72 h after the cells had been scratched. The extent of wound closure was quantitated by measuring the wound areas obtained from five independent fields using ImageJ (v1.43u).

2.5. Cell cycle analysis

HepG2 cells were plated in 6-well plates at a density of 1×10^6 cells/well and cultured with EFLDO (0, 6.25, 12.5, 25, 50 and 62.5 μM for 24 h or 50 μM for 0,12,24 and 48 h). After two washes with PBS, the cells were resuspended in PI solution at 50 μg/mL and incubated for 30 min in the dark at room temperature. The DNA content

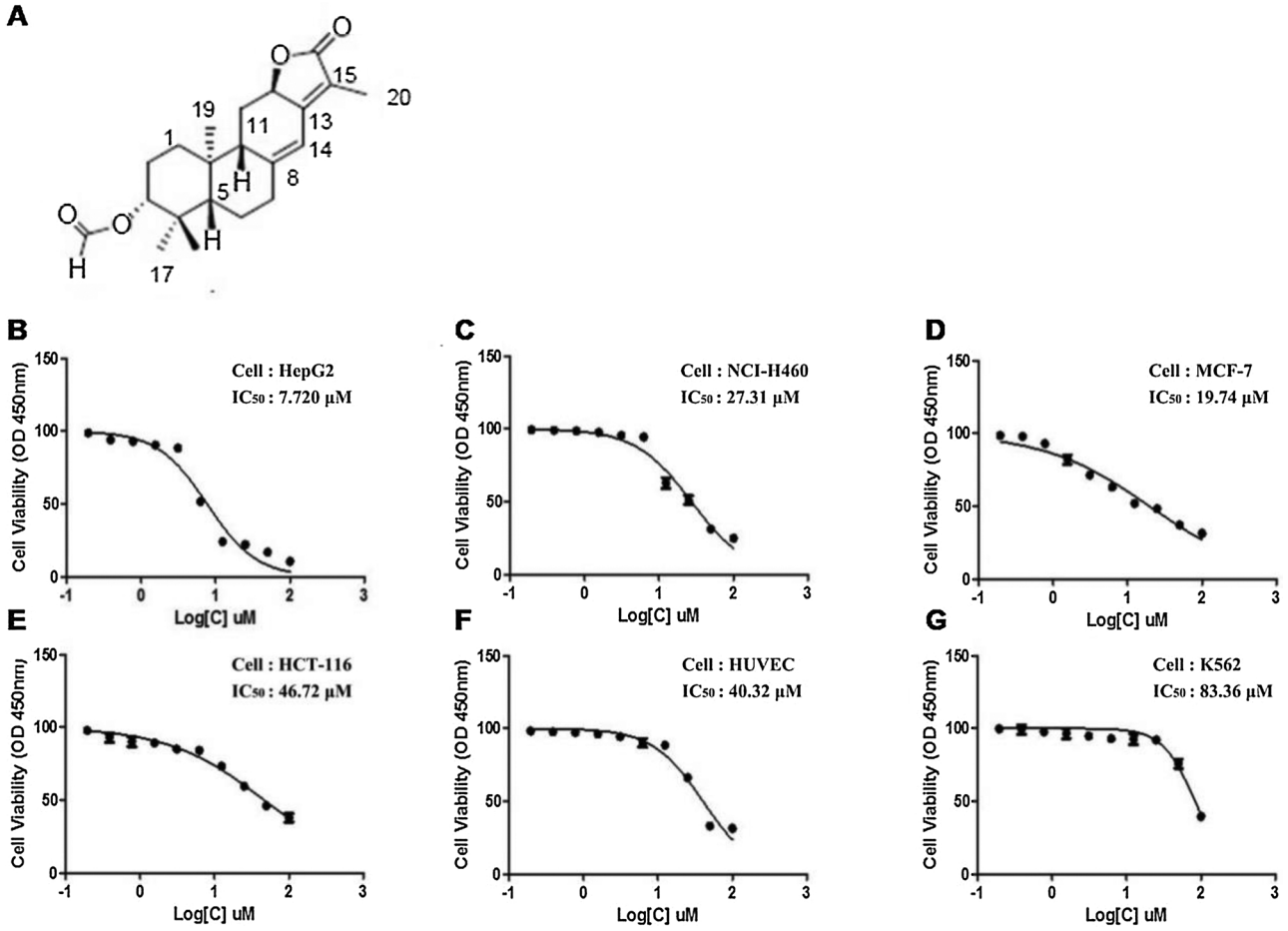

Fig. 1. EFLDO induced inhibition of proliferation in different tumor cells and HUVECs. (A) Chemical structure of EFLDO (EN, $C_{21}H_{28}O_4$, MW 367.2). (B–G) EFLDO induced inhibition of proliferation in different tumor cells and HUVECs. Cells were treated with various doses of EFLDO, and cell viability was determined by the CCK-8 assay. The IC_{50} growth-inhibitory concentration of EFLDO was 7.720 μM for HepG2 cells. (B) 27.31 μM for NCI-H460 cells (C) 19.74 μM for MCF-7 cells. (D) 46.72 μM for HCT-116 cells. (E) 40.32 μM for HUVECs (F) and 83.36 μM for K562 cells (G).