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Erianin induces apoptosis in human leukemia HL-60 cells

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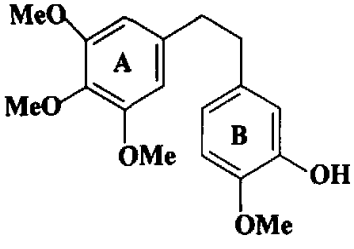
KEY WORDS erianin; leukemia; HL-60 cells; apoptosis; DNA fragmentation; cell cycle; *bcl*-2 genes

ABSTRACT

AIM: To investigate the effect of erianin on human HL-60 cell line and explore its mechanism of apoptosis *in vitro*. **METHODS**: Inhibition of proliferation was measured with colorimetric MTT assay. The morphologic changes were observed by fluorescence and electron microscopes. DNA fragmentation was visualized by agarose gel electrophoresis, and the DNA degradation was determined by flow cytometry. Immunohistochemical analysis was used to identify the expression of *bcl*-2 and *bax* genes. **RESULTS**: The growth of human HL-60 cells was significantly inhibited by erianin 20 - 81.9 nmol/L during 72 h treatment ($P < 0.01$). The IC_{50} value was 38 nmol/L after a 24-h exposure to erianin, while that of vincristine, the positive control, was 101 nmol/L. The typical morphologic changes were observed and the nuclear DNA fragmentation exhibited "ladder" pattern. The cell cycle of HL-60 cells was arrested in G_2/M phase, and expression of *bcl*-2 gene was decreased while that of *bax* was increased. **CONCLUSION**: Erianin showed potent inhibitory activity on the proliferation of HL-60 cells. The inhibition might be relative to the apoptosis induced by erianin and the altered expression of *bcl*-2 and *bax* genes in HL-60 cells.

INTRODUCTION

Erianin [Eri, dihydrocombretastatin A-4 (1c), 3-hydro-4, 3′, 4′, 5′-quamythloxy-stilbene] is a natural product extracted from *Dendrobium chrysotoxum* Lindl in Yunnan province[1], which belongs to the stilbene ahd dihydrostilbene derivative series — named combretastatins. Eri was also extracted from the *Combrum caffrum* in South Africa. The combretastatins exert antiproliferative effect on many carcinoma cell lines with low toxicity[2-5], and the target is the microtubules in cells. They can inhibit tubulin polymerization and stimulate tubulin-dependent GTP hydrolysis. As the structure of the combretastatins is similar to that of colchicine, they can competitively inhibit the binding of colchicines to the protein, which means that they can bind to the same site on the tubulin[6]. There is good correlation between the inhibitory effect on tubulin polymerization and the effect of antitubulin, but the correlation between cytotoxicity and inhibition in tubulin assays *in vitro* is not notable[7,8], which may suggest that there are other mechanisms involved in the cytotoxicity of the drugs. In this study, we were prompted to investigate the mechanism of Eri from a new aspect, apoptosis.



Chemical structure of erianin
$C_{18}H_{22}O_5$, M_r 318

MATERIALS AND METHODS

Reagents Eri was kindly provided by Prof WANG Zheng-Tao (Department of Natural Pharmaceutical Chemistry, China Pharmaceutical University). Eri was dissolved in Me_2SO and the final concentration of Me_2SO was less than 0.01 %. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was the product of Fluka and RPMI-1640 was

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from Gibco. Vincristine was obtained from Wanle Pharmaceutical Factory, Shenzhen, China.

Cell culture HL-60 cell line purchased from Shanghai Institute of Cell Biology was maintained in RPMI-1640 supplemented with 10 % calf serum, benzylpenicillin 100 kU/L, and streptomycin 100 mg/L, at 37 ℃ in 5 % CO_2 and 95 % air.

MTT assay[9] After incubation with different concentrations of Eri, inhibition of HL-60 cell proliferation was measured with colorimetric MTT assay. Single-cell suspensions were prepared, and then dispersed in replicate 40-well microtiter plates with a cell density of 1×10^8/L and a total volume of 200 μL per well. Eri 12.5 – 81.9 nmol/L and vincristine 72 – 344 nmol/L were added immediately. The absorbance was measured on DJ-3022 ELISA Micro-Plate Reader at 570 nm.

Morphological examination Cells were treated with Eri 64 nmol/L for 24 h, and identified under an Olympus BHF-342 fluorescence microscope after stained with AO/EB[10]. Cells treated and harvested as above were fixed and viewed with a Hitachi H-300 electron microscope[11].

DNA gel electrophoresis[11] After incubation with Eri 64 nmol/L for 24 h, DNA fragmentation was analyzed by electrophoresis. The cellular DNA was extracted, dialyzed, and separated by electrophoresis on 2 % agarose gel, then visualized under UV light after staining with ethidium bromide 5 mg/L.

Flow cytometry[11] Quantitative detection of apoptotic cells and analysis of cell cycle distribution in cultures were performed directly by flow cytometry. The sub-G_1 peak formed by cells reduced DNA content and represented the presence of apoptotic cells. The cells were allowed quantitative comparison of the degree of apoptosis induced by different treatments. In this study, the cells were stained with propidium iodide (PI) 50 mg dissolved in PBS containing RNase A 100 mg/L, and 0.1 % Triton X-100. The fluorescence of individual cell was measured with FACS Calibur flow cytometry.

Immunohistochemical analysis[12] The changes on expression of *bcl*-2 and *bax* genes were performed by immunohistochemical assay. After fixed by acetone at 4 ℃, the cells were treated with anti-Bcl-2 or anti-Bax mouse monoclonal antibody. Biotinylated Ig, avidin-biotin complex, and 3-amino-9-ethylcarbazole substrate solutions were used according to the manufacture's instruction. The positive rate of the reactions was assessed: 200 consecutive cells in three fields were counted, 1 % – 9 % (±), 10 % – 29 % (+), 30 % – 49 % (++), >50 % (+++).

Statistics The results were expressed as $x \pm s$, and assessed by t-test.

RESULTS

Growth inhibition HL-60 cells exposed to Eri 12.5 – 81.9 nmol/L revealed evident antiproliferative action after 24 h, 48 h, and 72 h in a concentration-dependent manner. With the negative logarithm of drug concentrations against cell viability in linear regression assay, we got the IC_{50} value of Eri 24 h as 38 nmol/L while that of vincristine was 101 nmol/L (Tab 1).

Tab 1. Effects of Eri on the growth of HL-60 cells by colormetric MTT assay. $n = 3$ **experiments.** $x \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* **control group.**

Concentration/ nmol·L^{-1}		$A_{570\ nm}$ 24 h	48 h	72 h
Eri	81.9	0.41 ± 0.09^c	0.36 ± 0.05^c	0.18 ± 0.03^c
	51.2	0.47 ± 0.03^c	0.39 ± 0.04^c	0.28 ± 0.01^c
	32	0.54 ± 0.04^c	0.49 ± 0.04^c	0.37 ± 0.03^c
	20	0.61 ± 0.21^c	0.69 ± 0.03^c	0.79 ± 0.10^c
	12.5	0.84 ± 0.04^b	1.44 ± 0.13^b	1.84 ± 0.07^a
Vincristine	344	0.43 ± 0.04^c	0.43 ± 0.05^c	0.26 ± 0.06^c
	153	0.47 ± 0.01^c	0.50 ± 0.08^c	0.3 ± 0.6^c
	102	0.52 ± 0.01^c	0.53 ± 0.03^c	0.33 ± 0.09^c
	86	0.55 ± 0.06^c	0.64 ± 0.03^c	0.38 ± 0.04^c
	72	0.61 ± 0.04^c	0.75 ± 0.05^c	0.58 ± 0.21^c
Control		1.07 ± 0.12	1.82 ± 0.14	1.85 ± 0.17

Occurrence of apoptosis After staining with AO/EB, four types of cell morphology were visualized by fluorescence microscope, ie, live and dead cells with normal chromatin, the early and later apoptotic cells with condensed and fragmented chromatin (Fig 1). Furthermore, the ultrastructural features of a typical apoptotic HL-60 cell characterized by cell volume reduction, cytoplasm shrinkage, condensation, fragmentation of chromatin, and presence of apoptotic bodies under electron microscope (Fig 2).

DNA isolated from HL-60 cells which had been cultured with Eri 64 nmol/L for 24 h showed the characteristic "ladder" pattern of apoptosis. A comparison with molecular weight markers indicated that the fragments were multiples of approximately 180 – 200 bp (Fig 3).

HL-60 cells treated with Eri 64 nmol/L for 0 h, 24 h, 30 h, and 36 h were analyzed with flow cytometry. The occurrence of sub-G_1 peak represented the presence of

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apoptotic cells, and Eri led to a remarkable increase in the apoptotic rate in a time-dependent manner from 3.16 % up to 36.52 % during a certain time range (0–30 h) (Tab 2).

Tab 2. Effects of Eri 64 nmol/L on the cell cycle by flow cytometry.

Time/h	G_0/G_1	S	G_2/M	Apoptosis/%
0	46.16	27.37	26.47	3.16
24	19.88	45.11	42.00	19.58
30	7.91	34.02	58.07	36.52
36	7.87	31.67	60.46	20.92

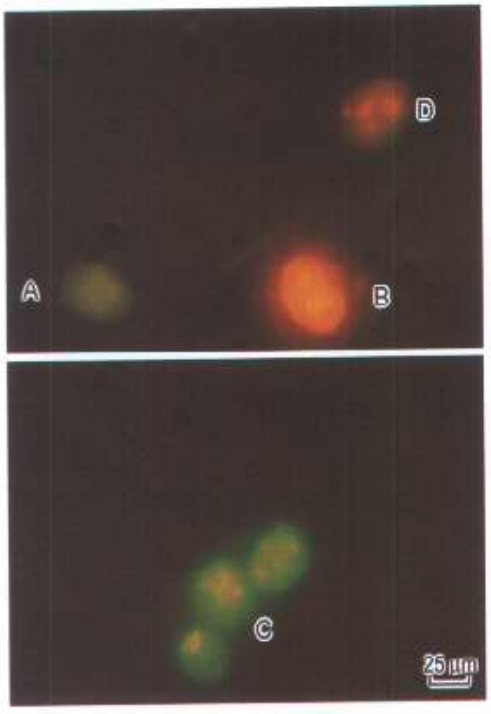

Fig 1. Fluorescence microscopy of HL-60 cells. Morphological changes were observed in cells treated with Eri 64 nmol/L for 24 h. A: live cell; B: dead cell; C: early apoptotic cell; D: later apoptotic cell. × 400.

Cell cycle distribution The obvious change in cell cycle distribution of nonapoptosis cells characterized by loss of G_0/G_1 phase and increase of G_2/M phase cells suggested that Eri 64 nmol/L led to the accumulation of HL-60 cells in G_2/M phase (Tab 2).

Expression of *bcl-2* and *bax* genes Bcl-2 and Bax were localized to cytoplasm and/or membranes. Expression of *bcl-2* genes, as analyzed by immunohistochemistry, was reduced in Eri-treated cells;

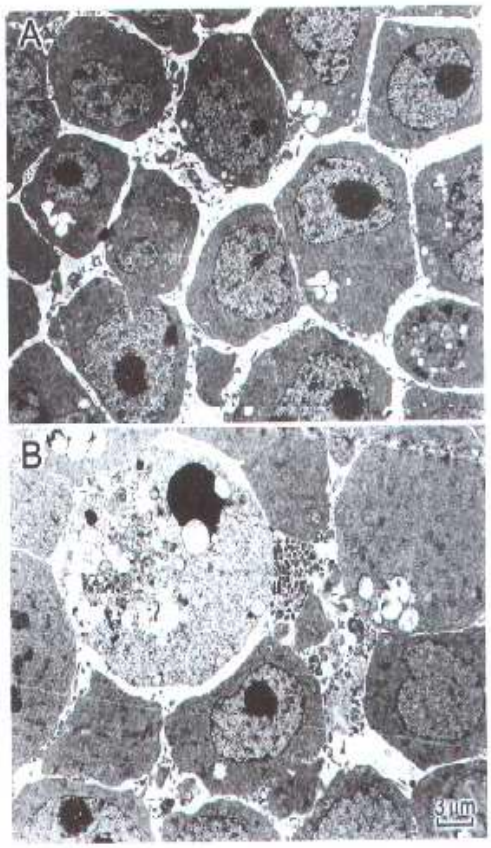

Fig 2. Electron microscopy of HL-60 cells. HL-60 cells treated with Eri 64 nmol/L for 24 h showed typical apoptotic character, such as cell shrinkage, condensation, and fragmentation of chromosomes. A: control; B: Eri-treated. × 3000.

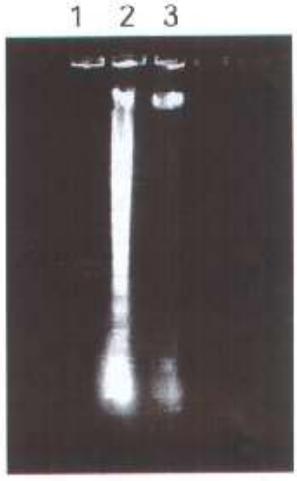

Fig 3. Agarose gel electrophoresis of DNA fragmentation. Lane 1: DNA marker (234 bp); Lane 2: HL-60 cells exposed to Eri 64 nmol/L for 24 h; Lane 3: HL-60 cells in control group.

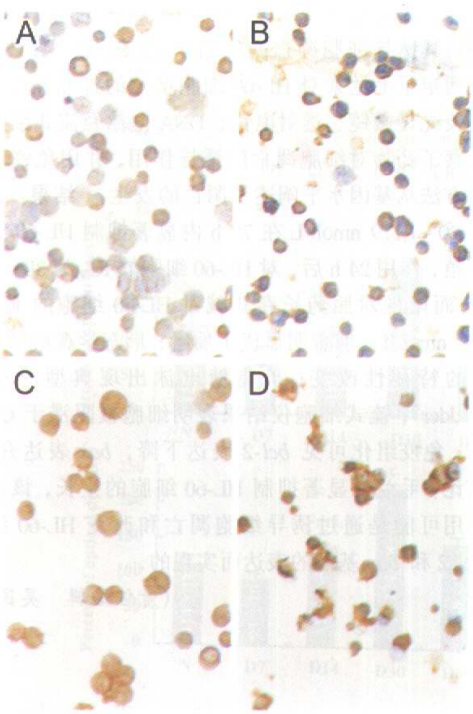

Fig 4. Immunohistochemical analysis for the expression of *bcl*-2 and *bax* genes in HL-60 cells. A: control group (*bcl*-2); B: erianin 64 nmol/L for 24 h (*bcl*-2); C: control group (*bax*); D: erianin 64 nmol/L for 24 h (*bax*). ×400.

while that of *bax* genes was increased in 24 h, suggesting that Eri could down-regulate the *bcl*-2 gene level while up-regulate the *bax* gene level (Fig 4).

DISCUSSION

In this study, we first observed the antiproliferative effect of Eri with the colorimetric MTT assay. The findings showed that growth of HL-60 cells was significantly inhibited by Eri *in vitro*. The antineoplastic effect of combretastatins has been proved to be concerned with their antimicrotubule activity. They could inhibit microtubule assembly, stimulate tubulin-dependent GTP hydrosis, hence result in an accumulation of cells in the metaphase[3,6]. In our work, research was carried out to investigate the mechanism of Eri from apoptotic aspect for the first time and explore its biochemical basis to the occurrence of apoptosis. Fluorescence and electron microscopy revealed the morphologic changes of apoptotic cells. Nuclear DNA of apoptotic cells displayed "ladder" appearance in the test of gel electrophoresis. Flow cytometric analysis of nuclear suggested the rate of apoptosis increased with the incubating time ranged from 0 h to 30 h. While after 30 h, rate of apoptosis was decreased, which may be due to the rise of secondary necrosis of apoptotic cells with time extension. HL-60 cells underwent the arrests of cell mitosis at G_2/M phase, which was consistent with the previous research[3,6]. According to the results of immunohistochemistry, the expression of *bcl*-2 genes, inhibitor of apoptosis, was decreased, while that of *bax* genes, inducer of apoptosis, was increased. In spite of some limitation of the present study, it may still offer insight into the mechanism of Eri, numerous hurdles, such as whether the agent elicit similar responses in HL-60 cells when treated *in vivo* and how Eri acts on the other carcinoma cells *in vitro* and *in vivo*.

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毛兰素诱导人白血病 HL-60 细胞的凋亡

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关键词 毛兰素; 白血病; HL-60 细胞; 细胞凋亡; DNA 断片; 细胞周期; *bcl*-2 基因

目的: 研究毛兰素对 HL-60 细胞增殖的抑制作用, 探讨其诱导细胞凋亡的机制. **方法**: 用 MTT 比色法测定了毛兰素对 HL-60 细胞增殖的抑制作用; 应用荧光显微镜、透射电镜、DNA 电泳及流式细胞仪观察了药物对细胞凋亡的诱导作用, 并用免疫组化的方法从基因水平阐述了凋亡的发生. **结果**: 毛兰素 20 – 81.9 nmol/L 在 72 h 内显著抑制 HL-60 细胞增殖, 作用 24 h 后, 对 HL-60 细胞的 IC_{50} 为 38 nmol/L, 而阳性对照药长春新碱对 HL-60 细胞的 IC_{50} 为 101 nmol/L, 前者明显优于后者; 形态学观察可见凋亡的特征性改变; 琼脂糖电泳出现典型的 DNA "ladder"; 流式细胞仪结果表明细胞被阻滞于 G_2/M 期; 免疫组化可见 *bcl*-2 表达下降, *bax* 表达升高. **结论**: 毛兰素显著抑制 HL-60 细胞的生长, 该抑制作用可能是通过诱导细胞凋亡和改变 HL-60 细胞 *bcl*-2 和 *bax* 基因的表达而实现的.

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