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Elemene induces apoptosis and regulates expression of *bcl*-2 protein in human leukemia K562 cells[1]

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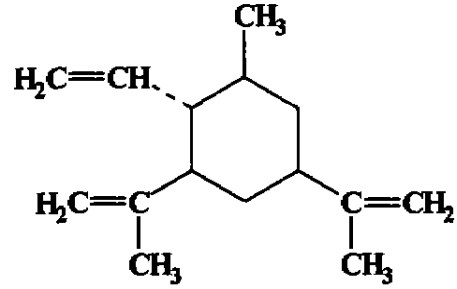
KEY WORDS elemene; leukemia K562; apoptosis; *bcl*-2 genes; DNA damage; cultured tumor cells; agar gel electrophoresis; flow cytometry

ABSTRACT

AIM: To study the antitumor action of elemene (Ele) and its mechanism. **METHODS**: Inhibition of proliferation was measured with a colorimetric 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Morphological assessment of apoptosis was performed with fluorescence microscope. DNA fragmentation was assessed by agarose gel electrophoresis and flow cytometry. The levels of *bcl*-2 protein was measured with flow cytometry. **RESULTS**: Exposure of exponentially growing K562 cells to Ele 65 – 520 μmol·L^{-1} for 48 h resulted in growth arrest. The values of IC_{50} and 95 % confidence limits were 220 (152 – 319) μmol·L^{-1}. After treatment of K562 cells with Ele 130 μmol·L^{-1}, marked morphological changes including "Apo bodies" reduction in volume were observed with fluorescence microscope. Agarose gel electrophoresis of DNA from cells treated with Ele for 48 h revealed "ladder" pattern. The levels of *bcl*-2 protein in K562 cells treated with Ele for 48 h were obviously decreased. **CONCLUSION**: Ele induces apoptosis of K562 cells, which is related with the down-regulation of *bcl*-2 protein in K562 cells.

INTRODUCTION

Elemene [Ele, cyclohexane, 1-ethenyl-1-methyl-2,4-bis (1-methylethenyl)] was isolated from the root of *Curcuma zedoaria*. In animal models, Ele exerted antitumor effects on ascitic tumors. Clinical studies showed that Ele was one of the active agents in the management of human lung cancer and malignant pleural and peritoneal effusions. It had no toxicity on bone marrow, liver, heart, and kidney[1,2]. To gain more information on the therapeutic potential of Ele, this paper was to study whether Ele could induce apoptosis in human leukemia K562 cells and its mechanism of antitumor activity.



Elemene
$C_{15}H_{24}$, M_r 204, mp 114 – 118 ℃

MATERIALS AND METHODS

Reagents Ele emulsion injection was

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purchased from Dalian Jingang Pharmaceutical Factory (lot № 9505238). 3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) and RPMI-1640 were the products of Sigma. Anti-*bcl*-2 antibody was purchased from Dako Co.

Cell culture and drug treatment K562 cells were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences, and were maitained in RPMI-1640 supplemented with 10 % heat-inactived calf serum, benzylpenicillin 100 kU·L^{-1}, and streptomycin 100 mg·L^{-1} in a humidified atmosphere containing 5 % CO_2 at 37 ℃. Exponentially growing cells (3×10^8 cells·L^{-1}) were exposed to Ele 65 – 520 μmol·L^{-1} for 48 h. The K562 cells incubated with daunorubicin (Dau) 10 μmol·L^{-1} for 48 h were positive control[3]. After treatment, cells were harvested by centrifugation at 2000 × *g* and washed with PBS.

Cell growth and morphological assessment Cell growth was determined by a colorimetric MTT-assay[4]. For observation of chromatin condensation, the cells were collected by centrifugation at 2000 × *g* and stained by 4′,6-diamido-2-phenylindole hydrochloride[5]. The condensed chromatin parts containing some cytoplasm that appeared as "dots" under a fluorescence microscope were called "Apo bodies."

DNA content The DNA degradation which often precedes visibly detectable apoptosis was determined directly using flow cytometry[6]. The apoptotic cells were calculated by determing the percentage of cells with a DNA content less than that of G_1 phase. The detected cells were just the same as those measured in MTT assay.

DNA gel electrophoresis[7] After 48-h incubation with Ele, fragmented DNA was analyzed by electrophoresis. The cellular DNA was extracted, dialyzed, and electrophoresed in 1.8 % agarose gel. DNA was visualized with ethidium bromide.

Indirect immunofluorescence The levels of *bcl*-2 protein was measured by flow cytometry[8]. Antigen density was measured as mean cell fluorescence intensity (FI) on a linear scale, and percentage of positive cells was determined by flow cytometry.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and analyzed by *t*-test.

RESULTS

Cell growth and morphological assessment Ele produced a concentration-dependent inhibition of K562 cell growth after 48-h treament (Tab 1). IC_{50} (95 % confidence limits) were 220 (152 – 319) μmol·L^{-1}.

Tab 1. Effect of elemene (Ele) and daunorubicin (Dau) on proliferation and apoptosis in K562 cells at 48 h. $n = 5$ **experiments** (3×10^8 **cells·L^{-1}, 100 μL/well).** $\bar{x} \pm s$.
$^aP > 0.05$, $^cP < 0.01$ *vs* control.

Drugs/ μmol·L^{-1}		MTT /A540	Inhibition /%	Apoptotic cells/%
Control		2.03 ± 0.09	0	0.9 ± 0.2
Ele	65	1.59 ± 0.08^c	21.9 ± 0.1^c	0.8 ± 0.3^a
	130	1.26 ± 0.04^c	37.8 ± 1.8^c	20.2 ± 2.5^c
	260	0.60 ± 0.17^c	70.5 ± 7.1^c	42.4 ± 8.5^c
	520	0.36 ± 0.06^c	82.2 ± 2.6^c	100.0 ± 0.0^c
Dau	10	0.65 ± 0.11^c	67.7 ± 5.2^c	32.6 ± 5.8^c

When K562 cells were treated with Ele 130 μmol·L^{-1} for 48 h, the "dotted" chromatin, which was condensed chromatin and divided into "Apo bodies" appeared in a large cell subpopulation under the fluorescence microscope (Fig 1).

DNA fragmentation Agarose gel electrophoreses of DNA extracted from cells treated with Ele 130 – 520 μmol·L^{-1} revealed a "ladder" pattern. In comparison, there was no obvious DNA fragmentation from K562 cells treated with Ele 65 μmol·L^{-1} (Fig 2).

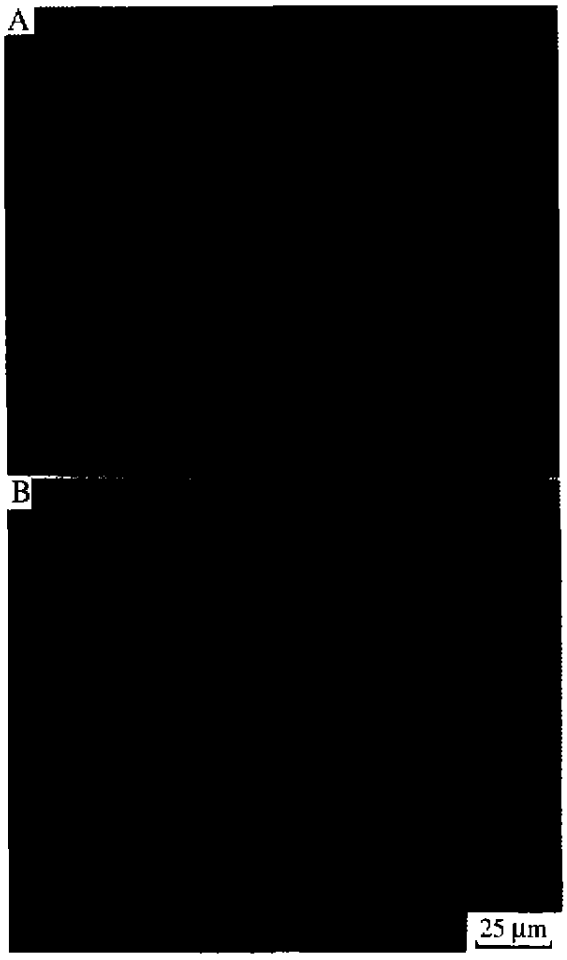

Fig 1. Fluorescence of K562 cells stained with DAPI, ×400. A) Control; B) cells treated with elemene 130 μmol·L^{-1} for 48 h.

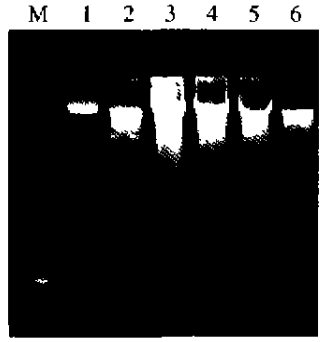

Fig 2. DNA fragmentation of K562 cells treated with Ele for 48 h. (M) Marker; (1) daunorubicin 10 μmol·L^{-1}; (2) control; (3) Ele 520 μmol·L^{-1}; (4) 260 μmol·L^{-1}; (5) 130 μmol·L^{-1}; (6) 65 μmol·L^{-1}.

DNA degradation by flow cytometry After exposure of K562 cells to Ele 130 – 260 μmol·L^{-1} for 48 h, there were apoptotic cells from 20.2 % to 42.4 % (Tab 1).

Expression of *bcl*-2 protein K562 cells incubated with medium alone were 89.1 % ± 1.2 % positive for the *bcl*-2 protein, and FI was 2.32 ± 0.07. Along with increase in the concentration of Ele, % of positive cells and FI decreased to 39.8 % ± 2.5 % and 1.39 ± 0.02, respectively (Tab 2).

Tab 2. Expression of *bcl*-2 protein in K562 cells treated with elemene for 48 h. $n = 4$ samples (5000 cells were measured in each sample). $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control.

Ele/μmol·L^{-1}	Positive cells/%	FI (lg)
0	89.1 ± 1.2	2.32 ± 0.07
65	90.2 ± 0.2^a	1.81 ± 0.01^c
130	66.5 ± 1.8^c	1.71 ± 0.02^c
260	39.8 ± 2.5^c	1.39 ± 0.02^c

DISCUSSION

Ele is a kind of anticancer agents. *In vitro*, Ele exerts growth-inhibitory effects on many kinds of human tumor lines, such as HL-60 cells, Hela, SPC-A_1 cells[1]. This work revealed the possible mechanisms that Ele exerted growth-inhibitory effects on K562 cells. Ultrastructural studies of the K562 cells treated with Ele showed that the cytoplasm and nucleus were extensively condensed. The morphological evidence presented here indicated that Ele induced apoptosis of K562 cells. The internucleosomal fragmentation of DNA, which resulted in a ladder type pattern comprising 180 bp intervals in gel electrophoresis, was a key molecular event in apoptosis[7]. In our study, a ladder of fragmented DNA from Ele-treated K562 cells was seen. In addition, DNA degradation characteristic was also determined rapidly in single cells using flow cytometry. So our

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experiment results revealed that Ele elicited apoptosis of K562 cells.

The oncogenes such as *bcl*-2 led to the development of tumor cells, and it may be related to the drug-resistance of tumor cells[9]. Our results showed that Ele not only induced the apoptosis of K562 cells, but also drastically decreased the expression of *bcl*-2 protein in K562 cells at the same time.

In conclusion, these results indicate that inducing apoptosis in tumor cells is one of the mechanisms for antitumor activity of Ele, and it is related with the decrement of *bcl*-2 protein expression. (03-106

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榄香烯诱导人白血病 K562 细胞凋亡及调控 *bcl*-2 蛋白的表达[1] R979.1 R733-705

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关键词 榄香烯; 白血病 K562; 细胞凋亡; *bcl*-2 基因; DNA 损伤; 培养的肿瘤细胞; 琼脂凝胶电泳; 流动血细胞计数

目的: 研究榄香烯的抗肿瘤作用机制. **方法**: 抑制细胞增殖采用 MTT 法; 用荧光显微镜观察细胞的形态学变化; DNA 电泳、流式细胞仪技术检测 DNA 断裂; 用流式细胞仪检测 *bcl*-2 蛋白的表达. **结果**: 榄香烯 65 - 520 μmol·L^{-1}明显抑制 K562 细胞增殖, IC_{50}(95 % 可信区间)为 220 (152 - 319) μmol·L^{-1}, 电泳可见 DNA 断裂形成的阶梯状条带, 形态学上表现为染色体聚集, 核固缩、断裂. 而 *bcl*-2 蛋白的水平明显下降. **结论**: 榄香烯诱导人白血病 K562 细胞凋亡, 这与 *bcl*-2 蛋白表达的下降有关.

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