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Induction of Apoptosis in Human Leukaemic Cell Lines K562, HL60 and U937 by Diethylhexylphthalate Isolated from *Aloe vera* Linne

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Abstract

We investigated the effect of diethylhexylphthalate (DEHP) from *Aloe vera* Linne on the apoptosis of human leukaemic cell lines K562, HL60 and U937 to examine its pharmacological activity.

At a level of 10 μg mL^{-1} DEHP a significant anti-leukaemic effect was observed for all three cell lines, as measured by clonogenic assay. After treatment with 10 μg mL^{-1} DEHP for 4 h, agarose gel electrophoresis and flow cytometric analysis confirmed the occurrence of apoptosis.

These results indicate that DEHP isolated from *Aloe vera* Linne has a potent anti-leukaemic effect, and thus represents a new type of pharmacological activity with respect to human leukaemic cells.

Aloe is widely used in the manufacture of food products, beverages, pharmaceuticals and cosmetics. Among some of the characteristics of aloe are its aromatic properties, bitter taste, the cathartic activity of the anthraquinones it contains and its ability to reduce inflammation and accelerate wound healing, as well as other pharmacological activity, such as emolliency (Saito et al 1989; Schmidt & Greenspoon 1991; Vázquez et al 1996; Kim & Lee 1997; Zhang & Tizard 1996). The means by which its components are responsible for these effects are, however, unclear (Grendlay & Reynolds 1986). In addition to the general components, the identified trace components of *Aloe vera* Linne include 17 alkylbenzenes, 19 fatty acids and 35 dehydroabietic acid derivatives. It has been reported that alkylbenzenes from n-hexane extracts of *Aloe vera* Linne have membranolytic activity, and that fatty acids and dehydroabietic acid derivatives have anti-tumour and anti-ulcer activity (Yamaguchi et al 1993).

In a previous paper we reported the purification of diethylhexylphthalate (DEHP) isolated from *Aloe vera* Linne, and its inhibition of the growth of various tumour cell lines and prevention of AF-2-induced mutation on *Salmonella typhimurium* TA98 and TA100 (Lee et al 2000). In this paper we report the colony-forming inhibition and induction of apoptosis of DEHP isolated from *Aloe vera* Linne for the trial of a new pharmacological activity on human leukaemic cells.

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Materials and Methods

Cell lines and cell culture

Three human leukaemic cell lines (K562, HL60 and U937) were used for the examination of the biological effect of DEHP. The cell lines were maintained in a standard culture medium: RPMI 1640 (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (JRH Bioscience) for K562 and U937, and RPMI 1640 supplemented with 20% heat-inactivated foetal bovine serum for HL60.

Clonogenic assay

A modification of the method described by Kern et al (1993) was used. $CaCl_2$ and 2-mercaptoethanol were excluded, and vitamin C (0·3 mM) was added to the medium.

The cells to be tested were suspended in 0·3% agar in enriched CMRL 1066 (Gibco BRL) supplemented with 15% horse serum, 2% foetal bovine serum (FBS), penicillin-streptomycin (100 units mL^{-1}), glutamine (4 mM), insulin (2 units mL^{-1}) and vitamin C (0·3 mM). Prior to plating, asparagine (0·6 mg mL^{-1}) and DEAE–dextran (0·5 mg mL^{-1}) were added to the cells with or without various concentrations of DEHP. One millilitre of this suspension was pipetted into each underlayer of 0·5% agar and enriched McCoy's 5A medium containing 10% FBS, 5% horse serum, 2·2% sodium pyruvate, L-serine (42 ng mL^{-1}), 2% glutamine and penicillin-streptomycin (20 units mL^{-1}). The number of cells plated was 3×10^3, 1×10^4 and 3×10^3 in 35 mm petri dishes for K562, HL60 and U937, respectively. After 10 days of culture, the numbers of colonies (colony size of diameter $\geq 50\ \mu m$) were counted.

In-vitro anti-leukaemic activity was defined in terms of the ratio of the number of colonies surviving on the DEHP-treated plates to those on the control plates. The agreement index for anti-leukaemic activity was defined to be less than 30% colony forming (Clark & Van Hoff 1984).

DNA extraction and electrophoresis

Cells (2×10^6) were centrifuged in an eppendrof tube at 13 000 *g* for 5 min, washed with cold PBS (phosphate buffered saline) and lysed in 1 mL lysis buffer (50 mM Tris, 20 mM EDTA, 2% SDS, 50 $\mu g\ mL^{-1}$ proteinase K and 50 $\mu g\ mL^{-1}$ RNase) at 50°C for 3 h. DNA was then extracted with 0·5 mL phenol, followed by 0·5 mL phenol/-chloroform (1 : 1, v/v) and 0·5 mL chloroform/isoamylalcohol (24 : 1) extraction for removal of protein and residual phenol. The addition of two volumes of 100% ethanol then precipitated the DNA. The samples were frozen at −70°C for 30 min. The DNA precipitates, which were recovered by centrifugation at 13 000 *g* at 4°C for 10 min, were dried by vacuum suction and resuspended in TE buffer. Before electrophoresis, loading buffer (10 mM EDTA, 0·25% (w/v) bromophenol blue and 50% (v/v) glycerol) was added to each sample in a 1 : 5 ratio. The DNA was then loaded into the wells of a 2% agarose gel and electrophoresed for 1 h at 50 V in TBE buffer. Finally, the DNA was stained with ethidium bromide and visualized under ultraviolet light (Chang & Yung 1996).

Cell viability

Modified MTT assay was used as an indication of cell viability (Monks et al 1991). Cells (1×10^4 cells per 100 μL) were inoculated in 96 well plates. After 24 h, 100 μL DEHP was applied to the culture wells and the cultures were incubated at 37°C in a time-dependent manner (1, 2, 3, 4 and 8 h). After these times, 50 μL of MTT solution was added to the wells. After incubation for 4 h at 37°C, 250 μL was removed from each well and 100 μL of dimethyl sulphoxide was added to solubilize the MTT-formazan product. The optical densities were read on an automated spectrophotometric plate reader at the single wavelength of 570 nm.

Detection of CD95 receptor expression

After treatment, approximately 2×10^5 cells were collected, washed twice with cold PBS and then incubated for 45 min on ice with 80 μL of anti CD95/FITC (Ancell Co.). The stained cells were washed three times, fixed with 1% paraformaldehyde and analysed by flow cytometry on an FACScalbur using CellQuest software (Backton-Dickinson). The stained cells were excited at 488 nm with a laser beam from an argon ion laser (Friesen et al 1996).

Results

Colony-forming inhibition

The data in Table 1 show that DEHP inhibits colony formation of human leukaemic cell lines K562, HL60 and U937. The level of suppression increases with increasing concentrations of DEHP. When the concentration of DEHP reached 500 $\mu g\ mL^{-1}$, all colony formation of the cell lines used was clearly inhibited, resulting in 0% colony formation. At a concentration of 100 $\mu g\ mL^{-1}$ DEHP, the colony-forming rate of U937, but not of K562 and HL60, reached a level of 0%. At a concentration of 10 $\mu g\ mL^{-1}$ DEHP, K562, HL60 and U937 had colony-forming rates of 24, 25 and 18%, respectively. In this assay system, colony formation for

Table 1. Colony-forming effects of DEHP isolated from *Aloe vera* Linne in human leukaemic cell lines by clonogenic assay.

Cell line	Cell type	Concentration of DEHP ($\mu g\ mL^{-1}$)					
		500	100	50	10	5	1
K562	Human leukaemia	0[a]	4[a]	10[a]	24[a]	34	57
HL60	Human leukaemia	0[a]	12[a]	22[a]	25[a]	30[a]	60
U937	Human leukaemia	0[a]	0[a]	8[a]	18[a]	24[a]	39

[a]Sensitive, i.e. colony-forming ≤30%. Data are means from five separate experiments.

K562, HL60 and U937 was inhibited at levels of DEHP of 500 to 10 $\mu g\,mL^{-1}$ to more than 70% of the controls. In contrast, none of the cell lines showed significant inhibition of colony formation at 1 $\mu g\,mL^{-1}$ DEHP.

Induction of apoptosis

Before the detection of apoptosis, we detected the cell viability of 10 $\mu g\,mL^{-1}$ DEHP in a time-dependent manner (Table 2). In this experiment, we found that cell viability decreased over time. After 4 h, the cell viabilities of DEHP were 28, 22 and 27% for K562, HL60 and U937, respectively. However, cell viability of DEHP in cell lines tested did not decrease significantly within 3 h after the start of treatment with 10 $\mu g\,mL^{-1}$ DEHP. Following the induction of apoptosis, the apoptotic DNA fragments produced by DEHP were analysed by agarose gel electrophoresis (Figure 1). 7-Aminoactinomycin D (7-AAD, 1 $\mu g\,mL^{-1}$) was used as a positive control in the detection of apoptosis. The DEHP treatments produced a typical DNA ladder pattern of apoptosis. DNA fragmentation in K562, HL60 and U937 cells was observed at a concentration of 10 $\mu g\,mL^{-1}$ DEHP 4 h after the start of treatment with DEHP. In this DNA fragmentation, DEHP exerted a pattern that was similar to that of 7-AAD, the positive control.

To clarify the type of DEHP-induced growth inhibition, we investigated CD95 cell surface expression during treatment with 10 $\mu g\,mL^{-1}$ DEHP under the same conditions as resulted in DNA fragmentation. The percentage of apoptotic cells in each cell line was 82% for K562, 85% for HL60 and 88% for U937 (Figure 2).

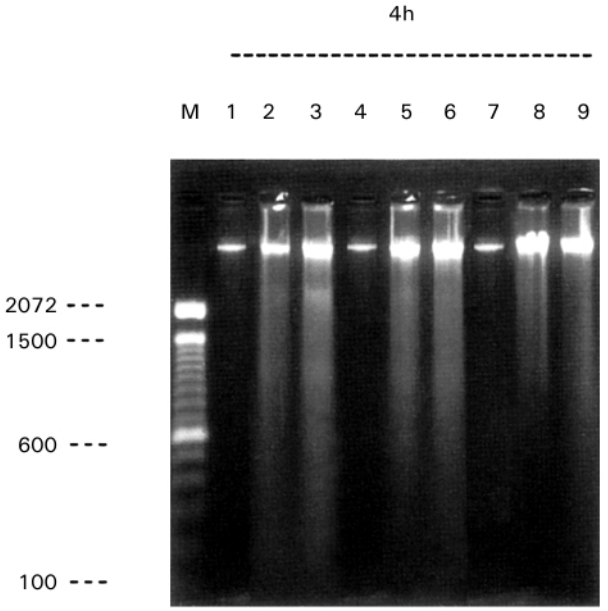

Figure 1. Detection of DNA fragmentation in K562, HL60 and U937 cells treated for 4 h with 10 $\mu g\,mL^{-1}$ DEHP by agarose gel electrophoresis. M = 100-bp ladder marker. Lanes 1, 4 and 7 = controls (1 = K562; 4 = HL60; 7 = U937). Lanes 2, 5 and 8 = positive control (7-AAD, 1 $\mu g\,mL^{-1}$). Lanes 3, 6 and 9 = 10 $\mu g\,mL^{-1}$ DEHP treatment. After treatment with DEHP, DNA fragments were analysed by 2% agarose gel electrophoresis.

Discussion

The phthalates DEHP and dibutylphthalate (DBP) are found in food packaging, blood packs and other environmental packaging. Over a period of time, the biological effects of phthalate esters such as DEHP and DBP have been well documented. DBP has been reported to have pharmacological activity as a purging agent on leukaemia (Wang et al 1996). DEHP is a hepatic carcinogen in rats, but does not induce mutations in bacteria or mouse lymphoma cells in-vitro or chromosomal aberrations in Chinese hamster cells, human leukocytes or human foetal lung cells (Morita et al 1997). DEHP was shown to be anti-mutagenic by the spot test in mice C57BL (Fahrig & Steinkamp-Zucht 1996).

In this study, the DEHP isolated from *Aloe vera* Linne was found to exert significant anti-leukaemic activity. The K562, HL60 and U937 cell lines had colony-forming rates of 24, 25 and 18% respectively, by clonogenic assay, at concentrations of 10 $\mu g\,mL^{-1}$ DEHP. Agarose gel electrophoresis and flow cytometry were used to identify the type of cell death caused by DEHP treatment. We selected a dose of 10 $\mu g\,mL^{-1}$ DEHP as a reference, and this showed a colony-forming rate of 30% or lower on all tested cell lines.

We investigated the induction of apoptosis in K562, HL60 and U937 cell lines at times selected in a time-dependent manner using an MTT assay by flow cytometry. Evidence of apoptotic DNA frag-

Table 2. Cell viability (%) of K562, HL60 and U937 cells treated with 10 $\mu g\,mL^{-1}$ DEHP in a time-dependent manner using an MTT assay.

Cell line	Cell type	Exposure time (h)				
		1	2	3	4	8
K562	Human leukaemia	100	100	86	28	29[a]
HL60	Human leukaemia	96	90	79	22	22
U937	Human leukaemia	97	86	70	27	28

[a]Optical density value of cell viability of tested cell line without DEHP treatment was expressed as 100% survival. Data are means of five separate experiments.

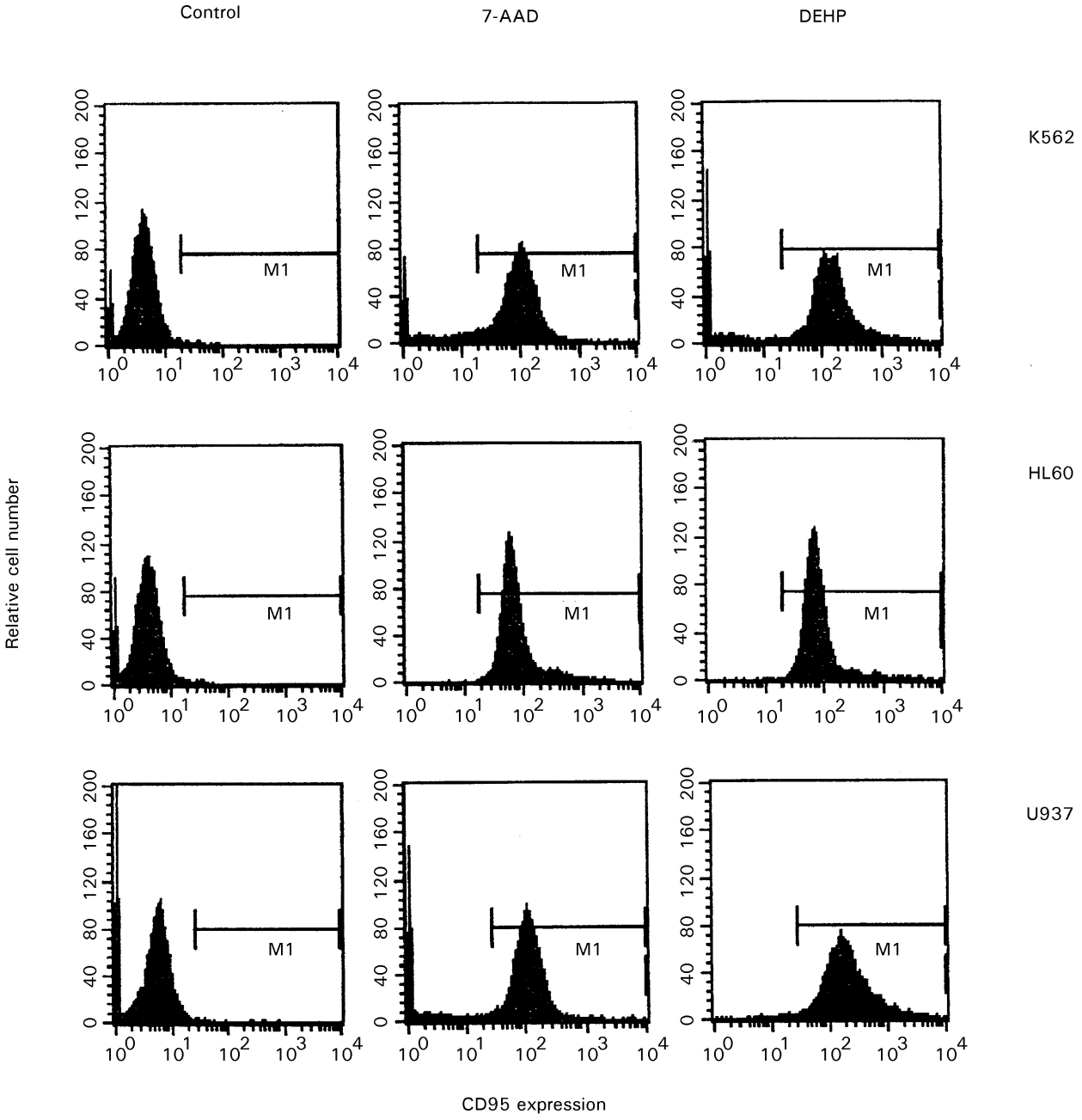

Figure 2. CD-95 receptor expression by flow cytometry in K562, HL60 and U937. Cells were either untreated (left-hand column), treated with 1 μg mL^{-1} 7-AAD (middle column) or treated with 10 μg mL^{-1} DEHP (right-hand column). M1 = Window for aptotic cells.

mentation was found in K562, HL60 and U937 4 h after the start of treatment with 10 μg mL^{-1} DEHP.

The CD95 system appears to be a major effect pathway by which cytotoxic compounds, as well as cytotoxic T-cells, mediate apoptosis (Rouvier et al 1993; Friesen et al 1996). Cytotoxic compounds in different tumour cell lines induce expression of CD95, and interaction of both molecules leads to apoptosis (Friesen et al 1996; Micheau et al 1997; Nagata 1997; Fulda et al 1998; Posovszky et al 1999). In this study we investigated the role of the CD95 system during DEHP treatment of human leukaemic cell lines in-vitro. Apoptosis was detected by flow cytometry with anti-CD95 (APO-1/FAS)/FITC under the same conditions of DNA fragmentation detection. CD-95-mediated apoptosis rose by 82% for K562, 85% for HL60 and 88% for U937 4 h after the start of treatment with 10 μg mL^{-1} DEHP. We found that apoptotic bodies exerted at 4 and 8 h, and examined until 48 h by phase microscopy (data not shown). At this concentration colony formation inhibition rates of 76,

75 and 82% by clonogenic assay were found, and exert 72, 78 and 73% cytotoxicity by MTT assay against K562, HL60 and U937, respectively.

These results prove that this apoptosis was induced by the cytotoxicity of DEHP and the anti-leukaemic effect of DEHP was mediated by the induction of apoptosis, at least in the clonogenic assay system. This study indicates that DEHP from *Aloe vera* Linne has a new pharmacological anti-leukaemic activity, and we believe that DEHP and *Aloe vera* are worthy of further study.

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