N-(β-Elemene-13-yl)tryptophan methyl ester induces apoptosis in human leukemia cells and synergizes with arsenic trioxide through a hydrogen peroxide dependent pathway

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Abstract

β-Elemene is an active component of herb medicine \textit{Curcuma Wenyujin} and \textit{N}-(β-elemene-13-yl)tryptophan methyl ester (ETME) was synthesized for increasing its antitumor activity. ETME induced apoptosis in human leukemia HL-60 and NB4 cells at concentrations less than 40 \textmu M. The apoptosis induction ability of ETME was associated with the production of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), the decrease of mitochondrial membrane potential, and the activation of caspase-3 that was blocked by catalase. ETME in combination with arsenic trioxide (As\textsubscript{2}O\textsubscript{3}), an agent used to treat acute promyelocytic leukemia, synergistically induced apoptosis in both cell lines by enhanced production of H\textsubscript{2}O\textsubscript{2}. These data suggest that ETME induces apoptosis and synergizes with As\textsubscript{2}O\textsubscript{3} in leukemia cells through a H\textsubscript{2}O\textsubscript{2}-dependent pathway.

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1. Introduction

β-Elemene is an active component of herb medicine \textit{Curcuma Wenyujin} Y.H with antitumor activities and its emulsion formulation has been developed as a complementary agent for cancer treatment in China [1–3]. However, the antitumor activity of β-elemene is modest that is probably due to its poor water-solubility. To improve its antitumor activity, we have performed structure modification of β-elemene and found that introduction of oxygen or nitrogen groups into β-elemene evidently increased its antiproliferative activities in leukemia cells [4]. Recently we have synthesized a group of β-elemene amino acid conjugates and found that β-elemene tryptophan methyl ester and β-elemene tryptophan were more active to inhibit human HL-60 leukemia cell growth than β-elemene conjugated with other amino acids (unpublished data).

The apoptotic effects and the mechanisms of action of \textit{N}-(β-elemene-13-yl)tryptophan methyl ester (ETME, Fig. 1) were investigated in human...
leukemia HL-60 and NB4 cells. ETME induced apoptosis in both HL-60 and NB4 cell lines that was in association with the increase of intracellular levels of hydrogen peroxide (H$_2$O$_2$) which was completely blocked by addition of catalase. Arsenic trioxide (As$_2$O$_3$) induced complete remission in acute promyelocytic leukemia (APL) patients, but not other types of myeloid leukemia patients [5–8]. APL-derived NB4 cells were more sensitive to As$_2$O$_3$-induced apoptosis than other leukemia cell lines [9,10]. Previously we have found that As$_2$O$_3$ induced apoptosis in NB4 cells through a H$_2$O$_2$ mediated pathway due to the inhibition of glutathione peroxidase [11]. In this study we have found that ETME plus As$_2$O$_3$ synergistically induced apoptosis in both HL-60 and NB4 cell lines which was correlated with the augmented production of H$_2$O$_2$. These data suggest that ETME in combination with As$_2$O$_3$ might be useful for the treatment of leukemia patients which are not responsive to As$_2$O$_3$ alone.

2. Materials and methods

2.1. Reagents

ETME was synthesized using β-elemene and l-tryptophan methyl ester. β-Elemene was extracted from the essential oils of *Curcuma Wenyujin* with a purity of 99.1%. Tryptophan was obtained from Amresco, Inc. (Solon, OH). N-acetylcysteine (NAC), ethidium bromide (EB), acridine orange (AO), propidium iodide (PI) and catalase were purchased from Sigma Chemical Co. (St. Louis, MO). Rhodamine-123 (Rh123) and 5,6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were obtained from Molecular Probes (Eugene, OR). Antibody to poly-(ADP-ribose)-polymerase (PARP) was obtained from Boehringer Mannheim (Indianapolis, IN) and antibodies to caspase-3, Bcl-2, actin and Bax were obtained from BD Biosciences (San Diego, CA).

2.2. Cell lines

HL-60 and NB4 cells were cultured in RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 μg/ml streptomycin, 1 mmol/L L-glutamine and 10% (v/v) heat-inactivated fetal bovine serum as we described previously [11].

2.3. Cell growth inhibition

Cells were seeded at 1.0 × 10$^5$ cells/mL and incubated with various concentrations of ETME for 72 h. Total cell number in each group was counted by hemocytometer and the cell viability was estimated by trypan blue exclusion [12].

2.4. Quantitation of apoptotic cells

Apoptotic cells were determined by morphologic observation and fluorescence-activated cell sorting (FACS) analysis after staining with PI [12]. For morphologic observation, cells were stained with AO and EB and assessed by a fluorescence microscope as described previously [12]. Briefly, 1 μL of stock solution containing 100 μg/mL AO and 100 μg/mL EB was added to 25 μL of cell suspension. Cells with nuclear shrinkage, blebbing and apoptotic bodies were counted as apoptotic cells. The percentage of apoptotic cells was calculated after counting total 300 cells. For FACS analysis with PI staining, cells were fixed with ice-cold 70% ethanol at a density of 1 × 10$^5$ cells/mL and treated with 200 μg/mL RNase for 30 min at 37 °C. PI was then added to the solution at a final concentration of 50 μg/mL and the DNA content was quantitated by flow cytometry (Becton Dickinson, San Jose, CA) with an excitation wavelength of 488 nm and an emission wavelength of 625 nm. Data were analyzed using CELLQuest (Becton Dickinson) software.

2.5. Determination of intracellular H$_2$O$_2$ amount

Intracellular H$_2$O$_2$ amount was monitored by flow cytometry after staining with DCFH-DA. In the present study, cells in logarithmic growth (1 × 10$^5$ cells/mL) were labeled with 5 μmol/L DCFH-DA for 1 h and then treated with or without ETME or As$_2$O$_3$ at 37 °C for different time points. After washing with PBS, cells were analyzed by flow cytometry (Becton Dickinson) with excitation and
emission wavelengths of 495 and 525 nm, respectively. Cells stimulated with 100 μM H₂O₂ for 1 h were used as a positive control [11].

2.6. Measurement of mitochondrial membrane potential (MMP)

MMP was assessed by the retention of Rh123, a membrane-permeable fluorescent cationic dye that is selectively taken up by mitochondria and is proportional to the MMP. Briefly, cells treated with ETME for different times were collected and incubated with 0.3 μg/mL Rh123 in dark for 20 min at room temperature. After washing with PBS, the cells were analyzed by flow cytometry (Becton Dickinson) with excitation and emission wavelengths of 495 and 535 nm, respectively.

2.7. Western blot analysis

Protein extracts (50 μg) prepared with RIPA lysis buffer (50 mmol/L Tris–HCl, 150 mmol/L NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% NP-40, 0.5% sodium deoxycholate, 1 mmol/L phenylmethyl sulfonyl fluoride [PMSF], 100 μmol/L leupeptin and 2 μg/mL aprotinin, PH 8.0) were separated on an 8% or 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were stained with 0.2% Ponceau S red to assure equal protein loading and transfer. After blocking with 5% nonfat milk, the membranes were incubated with the polyclonal antibody to PARP or the monoclonal antibodies to caspase-3, Bcl-2, Bax, or β-actin overnight at 4 °C. Immunocomplexes were visualized by ECL Western Blotting Detection reagents (Amersham Biosciences, England, UK). Protein contents in the lysate were determined by Bradford protein binding assay [13].

2.8. Statistics

The Student’s t-test (Microsoft Excel, Microsoft Corporation, Seattle, WA, USA) was performed to determine the significance between groups. A p-value of less than 0.05 (p < 0.05) was considered as statistically significant.

Fig. 2. ETME, but not β-elemene nor tryptophan, inhibited cell growth and induced cytotoxicity at low concentrations in both HL-60 and NB4 cells. (A) Cell growth inhibitory effects of ETME. (B) Cytotoxicity of ETME. (C) Cell inhibitory effects of β-elemene (Ele), tryptophan (Try) and ETME in HL-60 cells. (D) Cytotoxicity of Ele, Try and ETME in HL-60 cells. Cells were treated with the indicated agents at the indicated concentrations for 3 days. Cell growth inhibition was determined by directly counting cell number using a hemocytometer and the cell viability was determined by trypan blue exclusion. Values shown are means ± SE of three independent experiments. *p < 0.05 and **p < 0.01 compared to the combination group of Ele plus Try.
3. Results

3.1. ETME inhibited cell growth and induced cytotoxicity in human leukemia cells

The cell growth inhibition was determined by directly counting cell number and the viability was determined by trypan blue exclusion in both HL-60 and NB4 cells after treatment with different concentrations of ETME. ETME inhibited cell growth in a dose-dependent pattern at a concentration range of 10–40 μmol/L (Fig. 2A). The viability was not affected by ETME at a concentration of less than 20 μmol/L. ETME at a concentration of 30 μmol/L significantly decreased the viability in both HL-60 and NB4 cells (Fig. 2B). In contrast, β-elemene and tryptophan did not evidently inhibit cell growth nor induce cytotoxicity in HL-60 cells at a concentration of 60 μM alone or their combination (Fig. 2C and D). Tryptophan methyl ester had a similar activity as that of tryptophan, which did not inhibit cell growth nor had an enhanced effect when in combination with β-elemene (data not shown). These data suggest that the cell growth inhibition and cytotoxicity of ETME is not due to the simple addition of β-elemene and tryptophan methyl ester.

3.2. ETME induced apoptosis in both HL-60 and NB4 cells

To determine whether ETME-induced cytotoxicity is due to apoptosis induction, the apoptotic effects of ETME treatment were investigated based on the morphologic observation after staining with AO and EB in both cell lines treated with ETME at concentrations of 0, 20, 30 and 40 μmol/L for 12 h. Both HL-60 and NB4 cells were sensitive to ETME-induced apoptosis and that 55% and 74% of apoptotic cells were detected after treatment with 40 μmol/L of ETME for 12 h, respectively (Fig. 3A). The number of apoptotic cells was increased in both cell lines after treatment with ETME for a prolonged time (Fig. 3B). To confirm the apoptotic effects of ETME treatment, the hypodiploid DNA (SubG1) was determined using flow cytometry after staining with PI. The numbers of cells in SubG1 were increased in both HL-60 and NB4 cells after treatment with ETME (Fig. 3C).

3.3. ETME-induced apoptosis was correlated with the H$_2$O$_2$ production that was inhibited by catalase

The H$_2$O$_2$ amount was determined in both HL-60 and NB4 cells after treatment with ETME at a concentration of 40 μmol/L for 8 h. The amount of H$_2$O$_2$ was evidently...
increased in both NB4 and HL-60 cells (Figs. 4A and 5A). A
time-dependent effect of ETME on \( \text{H}_2\text{O}_2 \) production was
observed in NB4 cells. The intracellular levels of \( \text{H}_2\text{O}_2 \) in
NB4 cells after treatment with ETME for 2, 4 and 8 h were
increased to 77.8%, 81.7% and 83.7%, respectively. Pre-
treatment with catalase in NB4 cells blocked ETME-
induced \( \text{H}_2\text{O}_2 \) accumulation (Fig. 4A) and apoptosis (Fig.
4B). Pretreatment with the antioxidant NAC only had
slightly blockade effects on ETME-induced \( \text{H}_2\text{O}_2 \) produc-
tion and apoptosis (Fig. 4A and B). Similar results were
obtained in HL-60 cells and that catalase but not NAC pre-
treatment blocked ETME-induced \( \text{H}_2\text{O}_2 \) accumulation
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obtained in HL-60 cells and that catalase but not NAC pre-
treatment blocked ETME-induced \( \text{H}_2\text{O}_2 \) accumulation
(Fig. 5A) and apoptosis (Fig. 5B). ETME decreased the
MMP and induced the cleavage of PARP that were blocked
by catalase but not by NAC (Fig. 5C and D). The levels of
Bcl-2 and Bax were not influenced by these treatments (Fig.
5D). These data suggest that \( \text{H}_2\text{O}_2 \) production is a mediator of ETME-induced apoptosis which results in the decrease
of MMP and the activation of caspase-3.

3.4. ETME in combination with As\(_2\)O\(_3\) have enhanced
cytotoxic effects

As\(_2\)O\(_3\) has been shown to have variant cytotoxic effects in both leukemia cell lines at therapeutic concentrations
(1–2 \( \mu \text{M} \)) [11]. The combined effects of ETME with low
concentrations of As\(_2\)O\(_3\) on cell growth and cytotoxicity were determined. As\(_2\)O\(_3\) at 1 or 2 \( \mu \text{M} \) in combination with ETME had enhanced cell growth inhibitory and cytotoxic
effects (Fig. 6).

3.5. ETME plus As\(_2\)O\(_3\) synergistically increased the levels
\( \text{H}_2\text{O}_2 \) and induced apoptosis in both HL-60 and NB4 cells.

The apoptotic effects of ETME in combination with
As\(_2\)O\(_3\) were determined in both HL-60 and NB4 cells. ETME at 25 \( \mu \text{M} \) in combination with 1 or 2 \( \mu \text{M} \)
As\(_2\)O\(_3\) synergistically induced apoptosis after treatment of
24 h (Fig. 7A). ETME (25 \( \mu \text{M} \)) plus As\(_2\)O\(_3\) (1 \( \mu \text{M} \))
induced apoptosis in 37.3% of NB4 cells after treatment of
24 h and ETME (25 \( \mu \text{M} \)) plus As\(_2\)O\(_3\) (2 \( \mu \text{M} \))
induced apoptosis in 59.3% of HL-60 cells after treatment of
24 h (Fig. 7A). The \( \text{H}_2\text{O}_2 \) amount was determined in both
HL-60 and NB4 cells after treatment with ETME, As\(_2\)O\(_3\) or their combination. As\(_2\)O\(_3\) treatment at 1 or
2 \( \mu \text{M} \) for 15 h only minimally increased the amount of
\( \text{H}_2\text{O}_2 \) in both NB4 and HL-60 cells. Intracellular amount
of \( \text{H}_2\text{O}_2 \) was increased 20.8% in NB4 cells and 5.3% in
HL-60 cells after treatment with ETME alone at a concentra-
tion of 25 \( \mu \text{M} \). The intracellular amount of \( \text{H}_2\text{O}_2 \) was increased 62.1% in NB4 cells after treatment with
1 μM As₂O₃ plus 25 μM ETME and 72% in HL-60 cells after treatment with 2 μM As₂O₃ plus 25 μM ETME (Fig. 7B).

In order to further study the mechanism of the combined treatments, the protein levels of PARP, caspase-3, Bcl-2 and Bax were determined by Western blot analysis. Combination treatment of ETME with As₂O₃ induced the cleavage of PARP and decreased the levels of pro-caspase-3 (Fig. 7C). The levels of Bcl-2 and Bax protein were not changed after treatment with each agent alone or their combinations (Fig. 7C). These data suggest that the combined apoptotic effect of ETME with As₂O₃ is due to an enhanced H₂O₂ production and the activation of capase-3.

4. Discussion

β-Elemene and tryptophan only had minimal cell growth inhibitory effects without inducing cytotoxicity in HL-60 cells at a concentration less than 60 μM (Fig. 2C and D). The combination treatment of β-elemene and tryptophan did not have additive or enhanced cell growth inhibition and cytotoxicity (Fig. 2C and D). Similarly β-elemene and tryptophan methyl ester did not have an additive and an enhanced cell growth inhibitory effect at a concentration of 60 μM (data not shown). These data suggest that the cell growth inhibitory and cytotoxic effects of ETME are not due to the simple combination of β-elemene with tryptophan methyl ester. It has been found that structurally modified derivatives of tryptophan at the carboxy group had an increased antiproliferative effects in tumor cells [14,15]. N-[(Trimethylamine-boryl-carbonyl]-L-tryptophan and naphthalene)sulfonyl-DL-tryptophan (A-91) have been reported to inhibit cell growth of mouse leukemia cells in vitro and in vivo [16,17]. From 16 synthesized β-elemene amino acid derivatives we found that only N-(β-elemene-13-yl)trypto-
phan and ETME have evidently increased antiproliferative effects in HL-60 cells (unpublished data). These observations suggest that the antiproliferative and cytotoxic effects of ETME might be due to an augmented effect of β-elemene on the activity of tryptophan after conjugation.

ETME treatment evidently induced apoptosis in both HL-60 and NB4 cells (Fig. 3). The amount of H$_2$O$_2$ in both HL-60 and NB4 cells were evidently increased which was correlated with the ETME-induced apoptosis (Figs. 4 and 5). To confirm whether H$_2$O$_2$ is the mediator of ETME-induced apoptosis, both HL-60 and NB4 cells were pretreated with catalase which diminishes H$_2$O$_2$. Catalase pretreatment blocked ETME-induced apoptosis and H$_2$O$_2$ accumulation in both HL-60 and NB4 cells (Figs. 4 and 5). The antioxidant NAC which works through increasing the intracellular levels of GSH did not block ETME-induced apoptosis and H$_2$O$_2$ production (Figs. 4 and 5). ETME decreased the levels of MMP and activated caspase-3 in both HL-60 and NB4 cells that were inhibited by catalase pretreatment (Fig. 5C and D). These data suggest that ETME induces apoptosis through generating H$_2$O$_2$ followed by mitochondria-mediated caspase-3 activation.

It has been shown that tryptophan generates superoxide anion under light exposure [18]. Near-ultraviolet irradiation of saturated, oxygenated solutions of tryptophan generates H$_2$O$_2$ [19] and tryptophan has been found to increase ROS production in the cell culture media [20]. 3-Hydroxyanthranilic acid (3HAA), one of tryptophan metabolites, has been found to induce apoptosis in T cells through generation of intracellular ROS, downregulation of MMP and activation of caspases [21]. 3HAA has been found to generate reduced redox-active metal ions and hydrogen peroxide by Fenton type chemistry [22]. Therefore ETME may generate H$_2$O$_2$ in cell culture medium or in cells through metabolism that need to be further determined.

As$_2$O$_3$ induces disease remission in APL patients [5–7] and induces apoptosis in APL leukemia cells at low concentrations [9,11,23]. Although APL NB4 cells are highly sensitive to As$_2$O$_3$-induced apoptosis, other leukemia cell lines such as HL-60 cells are not sensitive to As$_2$O$_3$-induced apoptosis at the therapeutic concentrations (1–2 μM) [9,11]. Moreover it has been found that other types of acute myeloid leukemia patients do not respond to As$_2$O$_3$ treatment [8]. Previously we found that As$_2$O$_3$ induced apoptosis through the generation of H$_2$O$_2$ by inhibition of glutathione peroxidase [11]. To increase the apoptotic effect of As$_2$O$_3$ without increasing its concentrations, combination treatments have been approached. It has been found that
ascorbic acid, catechin and trolox, which can produce H$_2$O$_2$, have an augmented effect on As$_2$O$_3$ apoptosis induction [24–26]. Similar to these combination treatments of As$_2$O$_3$ plus ETME have a synergistic apoptotic effect in both NB4 and HL-60 cells that was correlated with the enhanced H$_2$O$_2$ production, the PARP cleavage and the caspase-3 activation without influencing the levels of Bcl-2 and Bax protein (Fig. 7). Our data indicate that ETME alone or in combination with As$_2$O$_3$ induces apoptosis in leukemia cells through generating H$_2$O$_2$ followed by mitochondria-mediated activation of caspase-3.

References


