

## RESEARCH ARTICLE

# Induction of Intrinsic and Extrinsic Apoptosis Pathways in the Human Leukemic MOLT-4 Cell Line by Terpinen-4-ol

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## Abstract

Terpinen-4-ol is a terpene found in the rhizome of Plai (*Zingiber montanum* (Koenig) Link ex Dietr.). In this study apoptogenic activity and mechanisms of cell death induced by terpinen-4-ol were investigated in the human leukemic MOLT-4 cell line. Terpinen-4-ol exhibited cytotoxicity in MOLT-4 cells, with characteristic morphological features of apoptosis by Wright's staining. The mode of cell death was confirmed to be apoptosis by flow cytometric analysis after staining with annexin V-FITC and propidium iodide. A sub-G1 peak in DNA histograms of cell cycle assays was observed. Terpinen-4-ol induced-MOLT-4 cell apoptosis mediated through an intrinsic pathway involving the loss of mitochondrial transmembrane potential (MTP) and release of cytochrome c into the cytosol. In addition, terpinen-4-ol also induced apoptosis via an extrinsic pathway by caspase-8 activation resulting in the cleavage of cytosolic Bid. Truncated-Bid (tBid) translocated to mitochondria and activated the mitochondrial pathway in conjunction with down-regulation of Bcl-2 protein expression. Caspase-3 activity also increased. In conclusion, terpinen-4-ol can induce human leukemic MOLT-4 cell apoptosis via both intrinsic and extrinsic pathways.

**Keywords:** Terpinen-4-ol - human leukemic cells - apoptosis - mitochondrial pathway - extrinsic pathway

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## Introduction

Apoptosis is a process by which cells undergo organized self-destruction without an inflammatory response. Apoptotic cell morphologies include membrane blebbing, nuclear and cytoplasmic condensation and apoptotic bodies (Schimmer et al., 2001). In the extrinsic pathway, ligation of the death receptors stimulates the activation of the initiator caspase-8, which then triggers downstream events either by directly activating caspase-3 or Bid cleavage. Truncated-Bid in turn initiates the mitochondrial pathway. Pro-apoptotic Bid targets the mitochondria and regulates other apoptotic-related protein such as Bcl-2, Bcl-xL and Bax (Reyes-Zurita et al., 2011). The intrinsic pathway plays a key role in regulating cell death through mitochondria. Mitochondrial outer membrane permeabilization (MOMP) decides the "point of no return" of apoptotic cell death and triggers the release into the cytoplasm of proteins that mediate cell death, such as cytochrome c. This leads to the activation of caspase-9, which then triggers effector caspase-3 activation, resulting in apoptotic cell death (Kang and Reynolds, 2009).

Terpinen-4-ol is the main component from rhizomes of "Plai" in Thai (*Zingiber montanum* (Koenig) Link ex Dietr.) (Bua-in and Paisooksantivatana, 2009). It is used as herbal medicine with several activities, such as anti-inflammation (Hart et al., 2000), anti-hypersensitivity

(Lahlou et al., 2003), anti-bacterial (Loughlin et al., 2008) and anti-fungal infection (Mondello et al., 2006). Furthermore, it can impair the growth of human M14 melanoma cells (Calcabrini et al., 2004) as well as induces necrotic cell death coupled with apoptotic cell death and arrests cell cycle at G1 stage in mouse malignant mesothelioma AE17 and murine melanoma B16-F10 cells (Greay et al., 2010). Further studies of potential anti-cancer drugs with a variety of cell lines are necessary, because there are high variability responses in different cancer cells following treatment with cytotoxic agents. The aims of this study were to investigate mode of cell death and the mechanisms of terpinen-4-ol-induced cytotoxicity in human leukemic MOLT-4 cell line.

## Materials and Methods

(+)-Terpinen-4-ol, histopaque, MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide, propidium iodide (PI) and 3,3'-dihexyloxycarbocyanine iodide (DiOC<sub>6</sub>) were obtained from Bio-rad, Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium, DEVD-AFC (Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin) and IETD-AFC (Ile-Glu-Thr-Asp-amino-4-trifluoromethylcoumarin) were obtained from Invitrogen, USA. Mouse monoclonal anti-Bcl-2, rabbit polyclonal anti-Bid antibodies and horseradish peroxidase (HRP) conjugated secondary antibodies were purchased from Abcam, Cambridge, UK.

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Mouse monoclonal antibodies to cytochrome c and beta-actin were obtained from Santa Cruz Biotechnology, USA. Annexin-V-FLUOS staining kit was obtained from Roche, Basel, Switzerland. BCA protein assay kit was obtained from Bio-rad, Hercules, CA, USA. SuperSignal West Pico Chemiluminescent Substrate was purchased from Pierce, Rockford, IL, USA.

#### *Cell culture and Cytotoxicity test*

Human acute lymphoblastic leukemia MOLT-4 cells were gifts from Dr. Watchara Kasinroek (Faculty of Associated Medical Science, Chiang Mai University, Thailand). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density gradient centrifugation using the standard protocols. The cells were cultured in 10% fetal bovine serum in RPMI-1640 medium supplemented with penicillin G (100 units/ml) and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. MOLT-4 cells were grown in the presence of terpinen-4-ol at concentrations ranging from 10 to 160 µM. Following terpinen-4-ol treatment, cell viability was assessed by MTT (3-(4, 5-dimethyl)-2, 5-diphenyl tetrazolium bromide) assay. Briefly, MTT dye solution was added and incubated in CO<sub>2</sub> incubator for 4 h. Then, 100 µl of DMSO was added to dissolve the dye crystals. Absorbance was measured by using a microplate reader (Biotek, USA) at 570 nm. The percentage of cell viability was calculated and 20% and 50% inhibitory concentrations (IC<sub>20</sub> and IC<sub>50</sub>) were determined.

#### *Determination of cell cycle distribution and Apoptosis assay*

The effect of terpinen-4-ol on MOLT-4 cell cycle phase distribution was assessed using flow cytometry. Briefly, after cell treatment with terpinen-4-ol for various times, cells were harvested and thereafter washed twice with cold PBS, and centrifuged. The pellet was resuspended in 500 µl cold 70% methanol in PBS for overnight at 4°C. The cells were centrifuged at 750 x g for 5 min, pellets were washed twice with cold PBS, suspended in 500 µl PBS, incubated with 10 µl RNAase (20 µg/ml final concentration) and stained with PI (50 µg/ml final concentration) overnight at 4°C before analysis by flow cytometry. DNA histograms were further analyzed using FACS DIVA software (Becton Dickinson, USA) for cell cycle analysis. Percentage of cells in each phase was evaluated.

Apoptosis was determined with the annexin-V-FLUOS staining kit according to the manufacturer's instructions. To examine apoptotic cell morphology by using the Wright-Giemsa staining method, cells were stained with Wright-Giemsa dye and observed under light microscope (Jia et al., 2003).

#### *Reduction of mitochondrial transmembrane potential ( $\Delta\Psi_m$ )*

Reduction of mitochondrial transmembrane potential ( $\Delta\Psi_m$ ) during the induction of apoptosis was examined by using 3,3-dihexyloxycarbocyanine iodide (DiOC<sub>6</sub>) (Zamzami et al., 2007). Cells were harvested after terpinen-4-ol treatment, and DiOC<sub>6</sub> was added to final

concentration of 40 nM. After 15 minutes of incubation at 37°C, the cells were washed and analyzed by flow cytometry technique.

#### *Assay of caspase-3 and caspase-8 activity*

Caspase-3 and -8 activities were detected by using fluorogenic DEVD-AFC (Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin) and IETD-AFC (Ile-Glu-Thr-Asp-7-amino-4-trifluoromethylcoumarin), respectively, in 96-well plate. Enzyme-catalyzed release of AFC was measured by a fluorescence plate reader (Bio-tek, USA) using 355 nm excitation and 460 nm emission wavelengths.

#### *Western blot analysis*

After treatment, cells were washed twice with PBS and lysed in 100 µl of RIPA buffer at 4°C for 15 minutes, then centrifuged at 12,000 x g for 15 minutes. The precipitates were removed and the solutions stored at -80°C. Concentration of protein was determined by the BCA protein assay kit using bovine serum albumin as a standard. Western blot analysis was conducted by a method described previously (Banjerdpongchai et al., 2010). Briefly, cellular protein 50 µg was loaded onto 15% SDS-polyacrylamide gels. The protein bands were transferred to nitrocellulose membranes and probed with anti-cytochrome c, anti-Bcl-2 and anti-truncated Bid, followed by a horseradish peroxidase-conjugated secondary antibody. Detection of the antibody reactions was performed by enhanced chemiluminescent reagents and chemiluminescence was exposed to the X-ray films. The bands were analyzed by densitometer.

#### *Statistical analysis*

Data were expressed as mean±S.D. (standard deviation). Statistical difference between control and treated group was determined by the one-way ANOVA (Kruskal Wallis analysis) in triplicate of three independent experiments. Statistical significance is expressed as \*p <0.05, \*\*p <0.01.

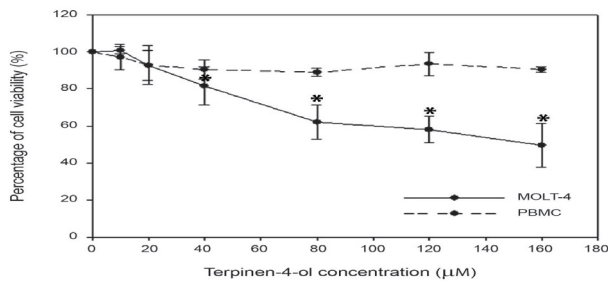
## **Results and Discussion**

#### *Cell cytotoxicity*

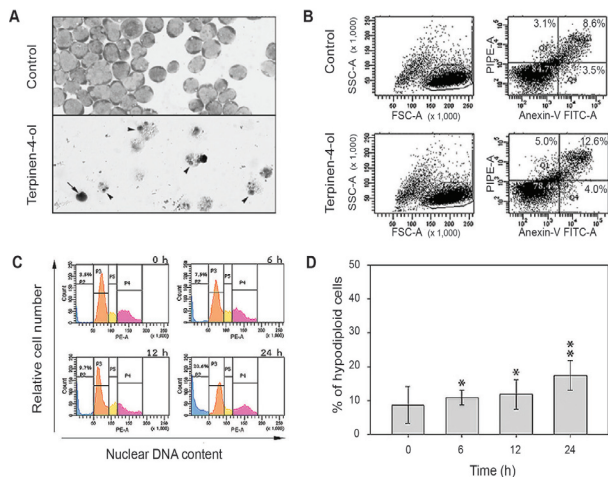
Terpinen-4-ol was cytotoxic to MOLT-4 cells in a dose dependent manner but not affect PBMCs as shown in Figure 1. The IC<sub>50</sub> and IC<sub>20</sub> (inhibitory concentration at 50% and 20%) values of terpinen-4-ol on MOLT-4 cells are 155 and 45 µM, respectively. Then the inhibitory concentration of terpinen-4-ol at 50% (IC<sub>50</sub>) or 20% (IC<sub>20</sub>) was used for further experiments.

#### *Effect of terpinen-4-ol on the apoptosis induction*

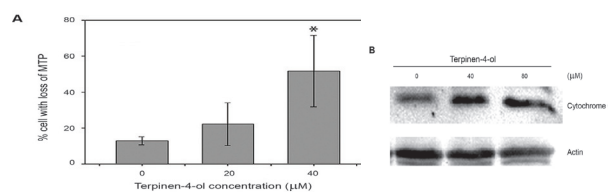
To examine whether terpinen-4-ol could induce apoptosis in MOLT-4 cells, the induction of apoptosis by terpinen-4-ol was examined for the morphology. The appearance of nuclear condensation and apoptotic bodies was demonstrated, which are characteristic of apoptotic cells by using Wright-Giemsa staining (Figure 2A). The increase in annexin-V-positive cell populations was found (Figure 2B). In addition, Sub-G1 DNA contents were



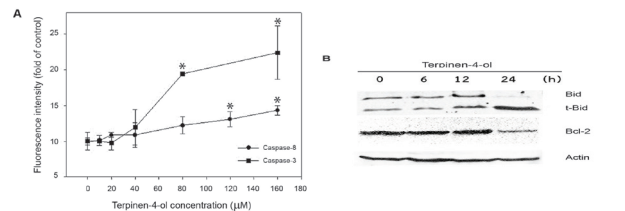
**Figure 1. The Cytotoxic Effect of Terpinen-4-ol on Human Leukemic MOLT-4 Cells and PBMCs.** MOLT-4 and PBMCs cells were cultured with terpinen-4-ol at various concentrations for 24 hours and cell viability was evaluated by MTT assay. The results are mean  $\pm$  S.D. of triplicates from three independent experiments, \* $p$  < 0.05 versus control.



**Figure 2. The Apoptotic Induction of Terpinen-4-ol-treated MOLT-4 Cells.** (A) MOLT-4 cells untreated and treated with terpinen-4-ol at 45  $\mu$ M for 24 h were stained with Wright-Giemsa dye. The apoptotic bodies (arrowhead) and nuclear condensation cells (arrow) were indicated. (B) Dot plot analysis of treated-cells after exposure to terpinen-4-ol at 155  $\mu$ M for 4 h was shown. The percentages of early apoptotic (right lower quadrant) and late apoptotic cells (right upper quadrants) were determined by Annexin V-FITC/PI staining and flow cytometry. (C) Representative cell cycle histograms of terpinen-4-ol-treated MOLT-4 cells were analyzed by flow cytometer. Percentages of sub-G1 populations increased accordingly to the concentrations of terpinen-4-ol. (D) Means of sub-G1 populations increased dose-dependently. Data were from three independent experiments with the same pattern, \* $p$  < 0.05, \*\* $p$  < 0.01 versus control.



**Figure 3. Loss of Mitochondrial Transmembrane Potential ( $\Delta\Psi_m$ ) and Cytosolic Release of Cytochrome c in Terpinen-4-ol-induced MOLT-4 Cell Apoptosis.** (A) Mitochondrial transmembrane potential (MTP) was monitored by determining percentage of cells with  $\Delta\Psi_m$ , reflected by diminished uptake of DiOC<sub>6</sub> relative to untreated controls. (B) Western blot analysis of cytochrome c was performed in total cell lysates of MOLT-4 cells at indicated concentrations in the treatment with terpinen-4-ol. Representative data from three independent experiments are shown.



**Figure 4. Effect of Terpinen-4-ol Treatment on Caspase-8, -3 Activities and Cleavage of Bid and Expression of Bcl-2 in MOLT-4 Cells.** (A) Caspase-8 and -3 activities of terpinen-4-ol-treated MOLT-4 cells were shown. (B) Antiapoptotic Bcl-2 and proapoptotic truncated Bid (t-Bid) expression levels and cleavage were determined by Western blotting. Representative results of the same pattern from three independent experiments are shown.

measured by cell cycle analysis (Figure 2C). MOLT-4 cells treated with terpinen-4-ol exhibited time-dependent increase in hypodiploid (<2n DNA) population. Terpinen-4-ol significantly increased the percent accumulation of sub-G1 populations from control (8.70%) to 11.00, 11.83 and 17.47% at 0, 6, 12, and 24 h of treatment, respectively (Figure 2D).

#### *$\Delta\Psi_m$ loss and release of cytochrome c from mitochondria of MOLT-4 cells treated with terpinen-4-ol*

To study the mechanisms of apoptosis involved in terpinen-4-ol-induced MOLT-4 cell apoptosis, mitochondrial transmembrane potential and cytochrome c release were investigated by flow cytometry analysis and immunoblotting. In the untreated control cells, almost all cells were functionally active with high DiOC<sub>6</sub> fluorescence; percentage of cells with  $\Delta\Psi_m$  was low. Terpinen-4-ol at 40  $\mu$ M caused mitochondrial damage and percent cells with the decrease of mitochondrial transmembrane potential was about 50% (Figure 3A). The loss of MTP is largely due to the opening of mitochondrial permeability transition pores (PTP), which conducted the leakage of cytochrome c from mitochondria to the cytosol at 40  $\mu$ M (Figure 3B). These results suggest a role of the mitochondria in terpinen-4-ol-induced apoptosis.

#### *Terpinen-4-ol-induced apoptotic cell death activated caspase-3 and -8, Bid cleavage and Bcl-2 downregulation*

To address the role of caspases in terpinen-4-ol-induced MOLT-4 apoptosis, DEVD-AFC and IETD-AFC were used to determine caspase-3 and -8 activities, respectively. It revealed a significant increase in caspase-3 but not caspase-8 activity at 80  $\mu$ M of terpinen-4-ol. At 160  $\mu$ M, activation of caspase-8 significantly increased, but the extent was less than that observed in caspase-3 activity (Figure 4A).

Bcl-2 family proteins including proteins that suppress apoptosis, such as Bcl-2 and proteins that promote apoptosis, such as Bad, Bax, and Bid, have been reported to play a central role in regulating cytochrome c release from mitochondria (Brunelle and Letai, 2009). Terpinen-4-ol decreased Bcl-2 expression at 24 h treatment by immunoblotting (Figure 4B). Moreover, proapoptotic t-Bid was elevated at 24 hours after terpinen-4-ol exposure. Taken together, altered expression of Bcl-2 was involved with the presence of truncated-Bid as

a connection between the extrinsic and the intrinsic apoptotic pathways.

Terpinen-4-ol contains antiproliferative and antitumor effects in human nonsmall lung cancer cells (Wu et al., 2012). The present study revealed that terpinen-4-ol induced apoptosis in human leukemic cells *in vitro* via mitochondria-mediated intrinsic pathway and caspase-8-activated extrinsic pathways.

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