RESEARCH ARTICLE

Terpinen-4-ol Induces Autophagic and Apoptotic Cell Death in Human Leukemic HL-60 Cells

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Abstract

**Background:** Terpinen-4-ol, a monoterpenoid, is found as the main component of essential oil extracts from many plants. In this study, apoptotic and autophagic types of cell death induced by terpinen-4-ol and associated mechanisms were investigated in human leukemic HL-60 cells. **Materials and Methods:** The cytotoxicity of human leukemic U937 and HL-60 cells was determined by MTT assay. Cytochrome c release, expression of Bax, Bcl-2, Bcl-xl and cleaved Bid were determined by Western blotting. Cell morphology was examined under a transmission electron microscope. LC3-I/II, ATG5 and Beclin-1 levels were detected by immunoblotting. **Results:** Terpinen-4-ol exhibited cytotoxicity to human leukemic HL-60 but not U937 cells. The apoptotic response to terpinen-4-ol in HL-60 cells was due to induction of cytochrome c release from mitochondria and cleavage of Bid protein after the stimulation of caspase-8. There was a slightly decrease of Bcl-xl protein level. The characteristic cell morphology of autophagic cell death was demonstrated with multiple autophagosomes in the cytoplasm. At the molecular level, the results from Western blot analysis showed that terpinen-4-ol significantly induced accumulation of LC3-I/II, ATG5 and Beclin-1, regulatory proteins required for autophagy in mammalian cells. **Conclusions:** Terpinen-4-ol induced human leukemic HL-60 cell death was via both autophagy and apoptosis.

**Keywords:** Terpinen-4-ol - HL-60 cells - apoptosis - autophagic cell death - leukemia

Introduction

Apoptosis is a form of cellular suicide that is essential for development and tissue homeostasis of all multicellular organisms. Caspases, a family of cysteine-dependent aspartate directed-proteases, play critical roles in initiation and execution of apoptosis. Two relatively well-characterized apoptotic pathways have been identified. The first pathway is mediated by death receptors, such as Fas or tumor necrosis factor (TNF) receptor (Guo et al., 2002). In the intrinsic pathway, the integrity of mitochondrial membranes is primarily controlled by a balance between the antagonistic actions of the pro-apoptotic and anti-apoptotic members of the Bcl-2 family (Lewis et al., 2005). Change in expression of the Bcl-2 family members resulting in a decrease of anti-apoptotic (e.g., Bcl-2, Bcl-xl) and an increase of pro-apoptotic (e.g., Bax, Bak) proteins may cause mitochondrial release of several pro-apoptotic molecules. After cytochrome c and Smac/Diablo release from mitochondria, cytosolic activation of intrinsic caspase cascade is promoted through formation of apoptosome and inhibition of the inhibitor-of-apoptosis proteins (IAPs), respectively. Caspases sequence-specifically cleave various endogenous cellular substrates and induce morphological and biochemical features of apoptosis including cell shrinkage, DNA fragmentation, chromatin condensation, and membrane blebbing (Karmakar et al., 2007).

Macroautophagy (autophagy) is an evolutionarily well-maintained catabolic process where a cell self-digests its cytoplasmic contents (literally “eats itself”). In eukaryotic cells, autophagy is a key mechanism for long-lived protein degradation and organelle turnover, and serves as a critical adaptive response that recycles energy and nutrients during starvation or cellular stress (Rubinsztein et al., 2007). The role of autophagy as a survival mechanism in response to these diverse stressors has been well established. Moreover, it has become increasingly clear that a basal level of autophagy serves as housekeeping functions vital for maintaining cellular homeostasis; especially, the failure to clear protein aggregates or damaged organelles by autophagy has been implicated in multiple pathological conditions, including cancer (Chen and Debnath, 2010). Terpinen-4-ol is a terpene that usually found as a main component in essential oil from plants, such as rhizomes of *Cassumunar* ginger; Plai in Thai; (*Zingiber montanum* (Koenig) Link ex Dietr.) (Paisooksantivatana and Bua-in, 2009), leaves and terminal branches of *Melaleuca alternifolia* (Myrtales: *Myrtaceae*) (Pazyar et al., 2013). Terpinen-4-ol has many pharmacological activities such as anti-inflammation (Hart et al., 2000; Jeenapongsa et al., 2003), and anti-hypersensitivity (Lahlou et al., 2003). Furthermore, bactericidal (Loughlin et al., 2008) and...
antifungal properties (Mondello et al., 2006) have also been found in terpinen-4-ol. Moreover, cytotoxicity of terpinen-4-ol on cancer cell lines, e.g., melanoma, has been reported (Calcabrini et al., 2004); which potently induces cell cycle arrest, apoptosis and necrotic cell death (Greay et al., 2010). There is no report of the clinically curative effect of terpinen-4-ol on cancer treatment, but terpinen-4-ol contained anti-inflammatory activity. A pivotal causative role of inflammation on cancer development is well established. Hence, the anti-cancer activity and mechanism of cell death induced by terpinen-4-ol were studied in human leukemic HL-60 cell model.

Materials and Methods

Chemicals

(+)-Terpinen-4-ol, MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide, propidium iodide (PI) and 3,3′-dihexyloxacarbocyanine iodide (DiOC₆) were obtained from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium, DEVD-AFC (Asp-Glu-Val-Asp-7-amino-4-trifluoroacetomethyl) and IETD-AFC (Ile-Glu-Thr-Asp-amino-4-trifluoroacetomethyl) were obtained from Invitrogen, USA. Mouse monoclonal anti-Bcl-2, anti-cytochrome c, anti-Beclin-1 and anti-Bax; rabbit polyclonal anti-Bid, anti-LC3-1/II and anti-Bcl-xl; rabbit monoclonal anti-ATG5 antibodies and horseradish peroxidase (HRP) conjugated secondary antibodies were purchased from Abcam, Cambridge, UK. Mouse monoclonal antibodies to cytochrome c and β-actin were obtained from Santa Cruz Biotechnology, CA, USA. Annexin-V-FLUOS staining kit was obtained from Roche, Basel, Switzerland. BCA protein assay kit was obtained from Biorad, Hercules, CA, USA. SuperSignal West Pico Chemiluminescent Substrate was purchased from Pierce, Rockford, IL, USA.

Cell culture

Human monocytic leukemia U937 cells and human promyelocytic leukemia HL-60 cells were gifts from Dr. Watchara Kasinroek (Faculty of Associated Medical Science, Chiang Mai University) and Assoc. Prof. Dr. Sukathida Ubol (Department of Microbiology, Faculty of Science, Mahidol University), respectively. 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% CO₂.

Cytotoxicity test

HL-60 and U937 cells were plated at 2×10⁴ cells per well in 96-well plates. The cells were grown in the presence of terpinen-4-ol at concentrations ranging from 10-160 µM for 24 h. Following terpinen-4-ol treatment, cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, MTT dye solution was added and incubated in CO₂ 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 (IC₅₀ and IC₃₀) were determined (Banjerdpongchai et al., 2008). In each experiment, determinations were carried out in triplicate.

Determination of reactive oxygen species (ROS) production

Human leukemic HL-60 cells (5×10⁵ cells/mL in 24-well culture plate) were treated with various concentrations of terpinen-4-ol, respectively. The cells were cultured at 37°C under 5% CO₂ atmosphere for 24 h. The hydrogen peroxide (H₂O₂, 3% (v/v)) was used as positive control. The cells were washed twice with PBS then the cells were loaded with 10 µM ofDCFH2-DA solution and incubated for 15 minutes before proceeded to flow cytometer (Banjerdpongchai et al., 2011).

Determination of caspases-8 activity

Human leukemic HL-60 cells (1×10⁶ cells/well in 24-well culture plate) were treated with various concentrations of terpinen-4-ol for 4 h. Then the treated cells were washed with ice-cold PBS and centrifuged at 200×g at room temperature for 5 min. Then the cell pellets were lysed with lysis buffer for 15 minutes on ice. The cell suspension was then centrifuged at 10,000×g for 10 minutes and 50 µL supernatant was transferred to 96-well plate. The reaction buffer was prepared by adding 10 µL of DTT to 1 mL of 2× reaction buffer. The reaction buffer, 50 µL, was added to each well. Caspase-8 fluorogenic substrate (IETD-AFC), 5 µL, was added and incubated at 37°C for 1 h. The fluorescence was measured at excitation wavelength of 400 nm and emission wavelength of 505 nm by fluorescence plate reader (Biotek, USA) (Banjerdpongchai et al., 2010).

Transmission electron microscopy

HL-60 cells (3×10⁶ cells/well in 6-well culture plate) were treated with terpinen-4-ol at IC₅₀ concentration and treated with rapamycin (30 nM) as a positive control for autophagic cell death. The cells were cultured at 37°C under 5% CO₂ atmosphere for 24 h. Then the treated cells were washed with ice-cold PBS and centrifuged at 200×g at room temperature for 5 min. The cell pellet was fixed in 1% glutaraldehyde in PBS for overnight. The cells were washed triple times with PBS and were embedded in 2% agarose. Then, fine pieces of embedded cells were fixed in 2% osmium tetroxide in PBS for 1 h and were washed twice with PBS. After that, the samples were dehydrated in 50% ethyl alcohol twice each for 5 minutes, in 70% ethyl alcohol twice each for 5 minutes, in 85% ethyl alcohol twice each for 5 minutes, in 95% ethyl alcohol twice each for 10 minutes and 100% ethyl alcohol thrice each for 5 minutes, in propylene oxide twice each for 10 minutes, in propylene oxide:resin (2:1) for 30 minutes and in propylene oxide:resin (1:1) for 90 minutes, respectively. The pellets were transferred to fresh pure resin in embedding molds and incubated overnight at room temperature. The blocks were polymerized at 60°C for 2 days. Then, the blocks were selected for an appropriate area and were cut into approximately 80 nm sections with a diamond knife and stained with uranyl acetate and lead citrate. The samples were observed with a...
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× g for 15 minutes. The precipitates were removed and the solutions were 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 as previously described (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-Bid, anti-Bax, anti-Bcl-xl, anti-Bcl-2, or to PVDF membranes and probed with anti-ATG5, anti-Beclin-1, and anti-LC3-I/II antibodies; followed by a specific 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 a densitometer.

Statistical analysis

Data were expressed as mean±SD (standard deviation). Statistical analysis was determined by one-way ANOVA, Bonferroni correction and Student’s t-test. Statistical significance was considered at limit of p<0.05 from 3 independent experiments conducted in triplicate.

Results

Cytotoxic effect of terpinen-4-ol on human leukemic HL-60 and U937 cells

Percent cell viability of HL-60 and MOLT-4 cells were reduced by terpinen-4-ol with a dose-dependence manner, whereas terpinen-4-ol had no cytotoxic effect on U937 as shown in Figure 1 and our previous report (Khaw-on and Banjerdpongchai, 2012). The IC_{50} and IC_{50} values (inhibitory concentration at 20% and 50%) of the terpinen-4-ol on HL-60 cells were 12 and 30 μM, respectively, which were used for further experiments. When compared to MOLT-4 cells from our previous report, terpinen-4-ol was toxic to HL-60>MOLT-4>U937 cells. Terpinen-4-ol-induced HL-60 cell cytotoxicity was investigated further for the type of cell death and mechanisms involved.

Effect of terpinen-4-ol on ROS production and cytochrome c release

Since the cytotoxicity of cancer cells can be caused by ROS, HL-60 cells treated with terpinen-4-ol were determined for ROS generation by using DCFH2-DA and flow cytometry. It was found that the fluorescence intensity of DCF significantly increased at 10 and 40 μM in HL-60 cells (Figure 2A). When incubated with terpinen-4-ol, ROS was produced in HL-60 cells markedly and there was an increase of cytochrome c release at IC_{50} and IC_{50} concentrations of terpinen-4-ol (Figure 2B).

Terpinen-4-ol-induced apoptosis via truncated bid and caspase-8-activated extrinsic pathway

Caspase-8 is the initiator caspase of the extrinsic apoptosis pathway, which is triggered by several death receptors. In order to obtain further evidence for supporting the involvement of caspase-8 in terpinen-4-ol-triggered apoptosis in human leukemic cells, caspase-8 activity was determined. The caspase-8 activity significantly increased for 1.27 folds in cells treated with terpinen-4-ol compared to control (Figure 3A). It has been shown that Bid is the substrate of caspase-8 and truncated Bid (tBid) can trigger Bax activation (Guo et al., 2002). Therefore, proapoptotic t-Bid was evaluated at 6, 12 and 24 hours after terpinen-4-ol exposure by Western blot. Truncated Bid (tBid) was shown to increase time-dependently in terpinen-4-ol-treated HL-60 cells (Figure 3B).

Figure 1. Effect of Terpinen-4-ol on Cell Viability in Human Leukemic Cells. HL-60 and U937 cell lines HL-60 and U937 cells (3×10^3 cells/ml) were treated with terpinen-4-ol at various concentrations for 24 hours. Cell viability was evaluated by MTT assay. The Y-axis shows the percent of cell survival, and the X-axis shows various concentrations of terpinen-4-ol. The mean±SD of three independent experiments performed in triplicate are shown. *p<0.05, vs control treated without terpinen-4-ol

Figure 2. The Effect of Terpinen-4-ol on ROS Production (A) and Mitochondrial Release of Cytochrome c to Cytosol (B) in HL-60 Cells. HL-60 cells were treated with terpinen-4-ol for 4 hours and incubated with DCFH2-DA (A). The fluorescence intensity was analyzed by flow cytometer. *p<0.05 and *p<0.01, vs control treated without terpinen-4-ol. Mitochondrial release of cytochrome c in terpinen-4-ol-induced HL-60 cells was determined by Western blot analysis using total cell lysates (B). Representative data from three independent experiments with the same pattern are shown.
Effect of terpinen-4-ol on cellular morphology in HL-60 cells

The electron micrographs after fixation of terpinen-4-ol-treated HL-60 cells demonstrated the vacuolous changes of cytoplasm (autophagosomes and secondary lysosomes) without chromatin condensation (Figure 4B-D) as confirmed by positive control of treatment with an autophagy inducer, rapamycin (Figure 4E and 4F). This indicated that autophagic cell death was executed in terpinen-4-ol-treated HL-60 cells by morphological criterion. Most of the autophagosomes contained lamellar structures (Figure 4C) or residual digested materials (Figure 4D) as same as those shown in rapamycin-treated HL-60 cells (Figure 4E and 4F).

Figure 4. Transmission Electron Micrographs of Terpinen-4-ol-treated HL-60 Cells. Untreated (A) or treated with terpinen-4-ol (at IC_{50} concentration) HL-60 cells (B-D) or 100 nM rapamycin-treated cells (E) and (F) were harvested, fixed, and examined under the transmission electron microscope. Arrow heads and arrows indicate autophagosomes or secondary lysosomes, respectively, whereas N indicates nucleus

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Effect of terpinen-4-ol on expressions of Bcl-2 family proteins in HL-60 cells

Bcl-2 family proteins play important roles in both apoptotic and autophagic cell death (Rikiishi, 2012). To investigate whether terpinen-4-ol causes alteration in the levels of Bcl-2 family members, the expression of Bcl-2 family proteins in terpinen-4-ol-treated HL-60 cells was examined. The expression levels of Bcl-2 and Bax did not significantly change, whereas the protein level of Bcl-xl slightly decreased (Figure 5A).

Figure 5. Bcl-2 Family and Autophagy-related Protein Expressions in Terpinen-4-ol-treated HL-60 Cells. HL-60 cells were treated with terpinen-4-ol at IC_{50} concentration for the indicated times. Bax, Bcl-2, Bcl-xl (A), LC3-I/II, Beclin-1, and ATG5 (B) expressions were analyzed by Western blot using the cell lysates. Representative results of the same pattern from three independent experiments are shown

Induction of autophagic cell death by terpinen-4-ol in HL-60 cells

LC3-I/II, Beclin-1 and ATG5 are recognized as positive regulators of autophagy induction in eukaryotic cells (He and Klionsky, 2009). As the morphology of terpinen-4-ol-treated HL-60 cells was found to be characteristic of autophagic cell death, it is possible that terpinen-4-ol exerts its antileukemic effect by triggering autophagic cell death in HL-60 cells.
ol-treated HL-60 cells was typical of autophagic cell death (from transmission micrographs as shown in Figure 4), the biochemical criteria to confirm the autophagic cell death are determined, viz., LC3-I/II, Beclin-1 and ATG5 protein expressions (Bursch, 2001). HL-60 cells were treated with terpinen-4-ol at IC50 concentration for 6, 12 and 24 h and protein expression levels of LC3-I/II, Beclin-1 and ATG5 were assessed by Western blot. As shown in Figure 5B, the expression levels of LC3-I/II, Beclin-1 and ATG5 proteins increased time-dependently after inducing HL-60 cells with terpinen-4-ol, which confirmed autophagic cell death execution.

Discussion

The contribution of terpinen-4-ol as an anti-cancer agent and the underlying signaling pathways of different types of cell death remain unknown. In this study, a more comprehensive illustration of terpinen-4-ol-induced cell death including apoptosis and autophagy in HL-60 cells was produced. The results of MTT assay demonstrated that terpinen-4-ol treatment significantly induced cell death in human leukemic HL-60 cells but not in U937 cells. The MTT assay indicated that HL-60 cells exhibited the greatest sensitivity to terpinen-4-ol-induced cytotoxicity with the lower level of IC50 (30 µM) compared to MOLT-4 cells (155 µM, in our previous study) (Khaw-on and Banjerdpongchai, 2012).

There are several studies systematically examines the roles of terpinen-4-ol in apoptotic and autophagic cell death induction. Apoptosis induced by terpinen-4-ol and its underlying mechanism have been previously studied, for example, terpinen-4-ol induces apoptosis in human non-small cell lung cancer (NSCLC) cells (Wu et al., 2012). Terpinen-4-ol is able to induce apoptosis in human leukemia MOLT-4 cells through both the mitochondria-mediated intrinsic pathway indicating by the cytochrome c release and the loss of mitochondrial transmembrane potential (MTP) (Khaw-on and Banjerdpongchai, 2012). Moreover, it has revealed that terpinen-4-ol induces apoptosis in human leukemic MOLT-4 cells in vitro via caspase-8-activated extrinsic pathways and also cross talked to intrinsic pathway by the presence of truncated Bid protein. Thus, terpinen-4-ol-treated HL-60 cells underwent apoptotic cell death as evidenced in MOLT-4 cells.

Apoptotic cells show alteration in morphological features from viable cells. This can be differentiated from a normal cell under the light and fluorescence microscope (Singh, 2000). In autophagic cell death, the light micrographs also show the specific morphologies (Saeki et al., 2000). Therefore, the simple way to distinguish the modes of cell death of terpinen-4-ol-treated cells is the examination of cell morphology at the fine and ultrastructural level. When treated with terpinen-4-ol, autophagic characteristics were found in HL-60 cells with vacuolar changes (Figure 4B-D).

It is clear that mitochondrial permeability transition (MPT) pore formation is characterized by the loss of mitochondrial transmembrane potential (MTP) and plays a key role in determining whether the cell lives or dies (Zorov et al., 2009). However, in terpinen-4-ol-treated HL-60 cells, cytochrome c release was demonstrated. The decrease of Bcl-xl and the release of cytochrome c indicated the mitochondrial pathway of apoptosis in terpinen-4-ol-treated HL-60 cells. But Bcl-2 and Bax protein expression levels did not alter. The exact function and mechanism of the abrupt change in the mitochondria induced by terpinen-4-ol require further investigation at the molecular levels.

Reactive oxygen species (ROS), including oxygen radicals and their reactive products, are known to react with biological molecules such as proteins, lipids and DNA, leading to cellular and tissue damage (Girard-Lalancette et al., 2009). It is well known that ROS can induce both apoptosis and autophagy. The redox environment of the cell is crucial for controlling both apoptosis and autophagy. ROS might be the cause or the effect of mitochondrial changes in the death pathways. ROS usually are generated in the mitochondria in the electron transport chain. ROS cause the damage of mitochondria and link to the mitochondrial pathway of apoptosis. The high level of ROS is often intimately associated with apoptotic cell death (Laurent et al., 2005). The way to prove the causative role of ROS can be performed by the pretreatment with radicals scavenging molecules such as glutathione or N-acetylcysteine. A recent report has established the role of ROS generated in mitochondria in the regulation of autophagy. This pathway, induced by the anti-cancer agent sodium selenite, leads to mitochondrial damage, ROS production, and selective degradation of mitochondria via autophagosomes and cell death in malignant glioma cells (Kim et al., 2007).

In the present study, it was found that in HL-60 cells terpinen-4-ol could significantly induce ROS generation and the autophagic cell death, which led to the remove of damaged organelles. ROS and oxidative damage might be generated in the autolysosomes and microsomes, as well as mitochondria. Terpinen-4-ol-exposed HL-60 cells could be induced to undergo apoptosis through oxidative stress.

The oxidative stressor induces two independent apoptotic pathways within pancreatic acinar cells, viz., the classical mitochondrial calcium-dependent pathway that is initiated rapidly in the majority of cells, and a slower, caspase-8-mediated pathway that depends on the lysosomal activities of cathepsins and is used when the caspase-9 pathway is disabled (Baumgartner et al., 2007). The present study demonstrated that HL-60 cells induced high caspase-8 activity and led to the cleavage of Bid protein without the MTP reduction. This could be induced by ROS generation and autophagosomal enzymes, such as cathepsin but further study is needed to elucidate this hypothesis. It has also been reported that Bcl-2 family proteins might interact with the autophagic proteins (ATGs) or Beclin-1 to turn on/off or switch the mode of cell deaths between apoptosis and autophagic cell death (Nishida et al., 2008; Kang et al., 2011; Fiorini et al., 2013; Naves et al., 2013). ATG5 also plays as a switch on autophagy to apoptosis (Yousufi et al., 2006) and JNK regulates dissociation of Bcl-2 and Beclin-1 (Wei et al., 2008). Whether these findings exist in terpinen-4-ol-induced HL-60 cell death, it requires further investigation.

In conclusion, terpinen-4-ol induced autophagic cell death by activating LC3-I/II, Beclin-1 and ATG5 proteins and apoptosis as evidenced by the increase of tBid expression, cytochrome c release and caspase-8 activity in human leukemia HL-60 cells (Figure 6).
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