4’-Hydroxycinnamaldehyde from *Alpinia galanga* (Linn.) Induces Human Leukemic Cell Apoptosis via Mitochondrial and Endoplasmic Reticulum Stress Pathways

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Abstract

Rhizomes of *Alpinia galanga* (Linn.) or ‘Kha’ in Thai are used in food and as folk medicine in South and Southeast Asia. The aims of this study were to identify the mechanism of cell death of human leukemic HL-60 and U937 cells induced by 4’-hydroxycinnamaldehyde (4’-HCA) isolated from *A. galanga*. 4’-HCA was cytotoxic to both cell lines in a dose-dependent manner (p<0.05) as demonstrated by MTT assay. Apoptosis induced by 4’-HCA was demonstrated by a variety of methods: visualization of propidium iodide (PI)-stained cells under fluorescence microscope, detection of subdiploid cells by PI-staining and flow cytometry, and assay of active caspase-3 using a specific fluorogenic substrate. 4’-HCA-treated cells (10 and 50 μg/ml for 4 h) showed significant increase in reactive oxygen species production and decreased mitochondrial transmembrane potential as detected by dichlorohydrofluorescein diacetate and 3,3’-dihexyloxacarbocyanine iodide respectively, together with flow cytometry. The apoptotic death involved cytochrome c release, increase in Bax level and concomitant decreases in levels of Bcl-2 and Bcl-xL (using Western blotting), and elevation in cytosolic and mitochondrial Ca²⁺ contents (using compartment-specific fluorescent Ca²⁺ dyes). These results indicate that 4’-HCA induces apoptosis of human leukemic cell through a combination of mitochondrial and ER stress pathways.

Keywords: 4’-HCA - *Alpinia galanga* (Linn.) - apoptosis - human leukemic cells - mitochondria - ER stress

Introduction

Rhizomes of *Alpinia galanga* (Linn.) are cooked as spice in many food preparations in Southeast Asia (Pancharoen et al., 2000). They are used also as folk medicine with several activities, such as anti-microbial, carminative, stomachic, anti-rheumatic, anti-flatulent and anti-itching (Matsuda et al., 2003). Our previous study showed that hexane extract of *Alpinia galanga* (Linn.) induces human leukemic HL-60 cells to undergo apoptosis via caspase-3, PI3-K and MEK pathway (Banjerdpongchai et al., 2008). 4’-Hydroxycinnamaldehyde (4’-HCA) was purified from an acetone extract of *A. galanga* (Linn.) and investigated for its mechanism of apoptosis in HL-60 and U937 cells, demonstrating its action was via mitochondrial and for HL-60 also via ER stress pathways.

Materials and Methods

Chemicals and reagents

RPMI 1640 was obtained from Gibco (Invitrogen), Carlsbad, California, USA, fetal bovine serum, caspase-3 detection kit from Invitrogen, USA, MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide), propidium iodide, 2’,7’–dichlorohydrofluorescein diacetate (DCFH-DA), 3,3’-dihexyloxacarbocyanine iodide (DiOCA), proteinase K, ribonuclease A and histopaque were obtained from Sigma-Aldrich, St. Louis, MO, USA. Complete mini protease inhibitor cocktail was from Roche, Basel, Switzerland. Mouse monoclonal antibodies to cytochrome c, Bax and Bcl-2 and rabbit polyclonal antibody to Bcl-xL, horseradish peroxidase (HRP) conjugated secondary antibodies were obtained from Abcam, Cambridge, UK. SuperSignal West Pico Chemiluminescent Substrate was obtained from Pierce, Rockford, IL, USA. Rhod2-AM and Fluo3-AM were obtained from Molecular Probes, Eugene, OR, USA. Silica gel (Merck No. 7734, Mesh 70-230 ASTM) was purchased from Merck, Darmstadt, Germany. Thin layer chromatography (TLC, silica gel 60 PF254, 20 x 20 cm, 0.2 mm) was used to examine separated fractions. Compounds were visualized by ultraviolet light (either at λmax 254 or λmax 366 nm).

Plant materials and isolation of 4’-hydroxycinnamaldehyde

Air-dried powder (5 kg) of *A. galanga* rhizomes,  

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specimen BKF No. 102287, obtained from the National Park, Wildlife and Plant Conservation Department, Ministry of Natural Resources and Environment, Bangkok, Thailand was percolated with hexane (17 l) at room temperature with five repetitions taking for up to 7 days. Then, the residue was subsequently percolated with acetone (17 l) under the same conditions. Then, filtration and evaporation were applied to produce acetone (52.92 g) extract, which was subjected to silica gel column chromatography (1.8 kg), using a gradient of n-hexane and ethylacetate. Fractions were analyzed using thin-layer chromatography (TLC) and selected fractions were combined to yield a pale yellow crystal of 4'-HCA (MS, IR, UV, 1H NMR, 13C NMR, DEPT, DQF-COSY, HMBC and HMBC spectra, as well as elemental analysis, were identical with literature data (Phitak et al., 2009).

Cell culture

Human promyelocytic leukemic HL-60 and promonocytic U937 cells were gifts from Dr. Sukhathida Ubol and Dr. Watchara Kasinroek respectively. Cells were cultured in 10% fetal bovine serum in RPMI-1640 medium supplemented with penicillin G (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere containing 5% CO₂. Cells (1 x 10⁶) were treated with 10-80 µg/ml of 4'-HCA for indicated durations.

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood obtained from adult volunteers by density gradient centrifugation using histopaque according to standard protocols. Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. PBMCs (3 x 10⁶) were treated with 10-80 𝜇g/ml of 4’-HCA for indicated concentrations and durations.

Cytotoxicity assay

Following 4'-HCA treatment, cell viability was assessed by MTT assay (Su et al., 2002). In brief, cell suspension was incubated with MTT solution (100 µg/ml) for 4 h at 37 °C in a humidified 5% CO₂ atmosphere. Cells were treated with DMSO for 30 min and absorbance (540 nm) of the cell lysate was measured using microtiter plate reader (Bio-tek, USA). Number of viable cells was calculated from untreated cells and the data were expressed as percent cell viability.

Fluorescence microscopy

Human leukemic cells were treated with 4’-HCA (10, 20, 50 µg/ml) for 4 and 24 h, and then cytospun onto glass slides. After air drying, cells were fixed with absolute methanol for 10 min at −20 °C, washed twice with phosphate-buffered saline (PBS) and air-dried. Propidium iodide (200 µg/ml) was applied to the fixed cells for 10 min at room temperature. After washing with PBS and drying, slides were mounted with 90% glycerol and examined under fluorescence microscope (Olympus, Japan). Apoptotic cells (condensed nuclei and fragmented cells) were recorded from a total of 200 cells per slide. Three independent experiments were performed in duplicate.

Determination of mitochondrial transmembrane potential, reactive oxygen species (ROS) production and DNA fragmentation

Either 40 nM 3,3'-dihexyloxacarbocyanine iodide (DiOC6) (for mitochondrial transmembrane potential determination) or 5 µM 2',7’-dichlorodihydrofluorescein diacetate (DCFH-DA) (for ROS detection) were added for 15 min at 37 °C before cells were subjected to flow cytometry. Cells (1 x 10⁶) were re-suspended in PBS containing PI (50 µg/ml), 0.1% Triton X-100 and 0.1% sodium citrate. Cells then were analyzed in a FACSScan equipped with a 488 nm argon laser using CellQuest software (Becton-Dickinson, USA). Data were depicted as histograms and the percentage of cells displaying hypodiploid DNA content was determined. Percentage of cells in each phase was also evaluated to determine the existence of cell cycle arrest.

Assay of caspase-3 activity

Cleavage of the fluorogenic peptide substrate DEVD-AMC (Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) is indicative of caspase-3-like activity. Cell lysate (from 1 x 10⁶ cells) and substrate (50 µM) were combined in a standard reaction buffer and added to a 96-well plate. Enzyme-catalyzed release of AMC was measured by a fluorescence plate reader (Bio-tek, USA) using 355 nm excitation and 460 nm emission wavelengths.

Western blot analysis

4’-HCA-treated cells were washed once in ice cold PBS and incubated at 4 °C for 10 min with ice-cold cell lysis buffer (250 mM sucrose, 70 mM KCl, 0.25% Triton X-100 in PBS containing complete mini protease inhibitor cocktail). Following centrifugation at 20,000 x g for 20 min, supernatant (50 μg, determined by Bradford method) was separated by 17% SDS-PAGE and transferred onto nitrocellulose membrane. After treating with 5% non-fat milk in PBS containing 0.2% Tween-20, membrane was incubated with mouse monoclonal antibodies to cytochrome c (1:1,500), to Bax (1:200) and to Bcl-2 (1:1,000), and with rabbit polyclonal antibodies to Bcl-xL (1:1,000), followed by appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:20,000). Protein bands were visualized on X-ray film with SuperSignal West Pico Chemiluminescent Substrate.

FACS analysis for Ca²⁺ levels

Cytosolic and mitochondrial Ca²⁺ level was determined using 1 µM fluorescent dye Fluo3-AM in FITC setting and 250 nM fluorescent dye Rhod2-AM in PE setting respectively. After treatment with 4’-HCA for 4 h, cells were incubated with fluorescent dye for 15 min at 37 °C, washed with PBS containing 10 mM glucose and analyzed immediately by flow cytometry. In each analysis, 10,000 events were recorded and analyzed by FACSscan (Becton Dickinson, USA).

Statistical analysis

Results are expressed as mean ± SEM. Statistical
4’-Hydroxycinnamaldehyde from *Alpinia galanga* (Linn.) Induces Human Leukemic Cell Apoptosis

**Results**

**Cell cytotoxicity with apoptotic induction**

Cell viability was evaluated in HL-60 and U937 cells after incubation with 4’-HCA for 24 h using MTT assay. 4’-HCA was cytotoxic to both HL-60 and U937 cells (Figure 1), but not to PBMCs up to 80 µg/ml, which was the maximum concentration that could be dissolved in DMSO (vehicle control). Apoptotic cells (condensed nuclei and fragmented bodies) were observed in both cell lines incubated with 10 - 50 µg/ml of 4’-HCA for 4 h and 24 h in a dose and time dependent manner (Figure 2). Apoptosis induced by 4’-HCA was confirmed by FACS analysis, which showed subdiploid peak prior to G1 phase in both HL-60 and U937 cells together with the presence of G2/M arrest in 4’-HCA-treated U937 cells (Figure 3). Very recently Kim et al. (2010) have reported that 2’-HCA shows anti-proliferative activity via cell cycle arrest at the G2/M-phase and increases the number of cells in the sub-G1 phase in SCC-15 and HEp-2 oral cancer cells. Apoptosis in HL-60 and U937 cells due to 4’-HCA was confirmed by presence of active caspase-3 (Figure 4). Thus, cinnamaldehyde containing 2’- or 4’-hydroxy moiety has the ability to induce apoptosis of cancer cells.

**ROS production and reduction of mitochondrial transmembrane potential**

It has been reported that curcumin sensitizes non-small cell lung cancer cell anoikis through ROS-mediated Bcl-2 down-regulation (Pongrakhananon et al., 2010), but curcumin and capsaicin repress ROS generation in primary human keratinocytes (Becatti et al., 2010). In the present study 4’-HCA induced ROS production in HL-60 and U937 cells (Figure 5A and B). This was accompanied by a loss of mitochondrial transmembrane potential in a dose-dependent fashion, as measured by MTP assay (Figure 6). It is not possible to conclude whether the
increase in ROS contributes to the perturbation to the mitochondrial transmembrane potential, but the results indicate mitochondrion as a target intracellular organelle of 4'-HCA-mediated apoptosis (and possibly 2'-HCA as well).

Release of cytochrome c and levels of Bax, Bcl-2 and Bcl-xL

The mitochondrial apoptotic signaling pathway involves Bax, a pro-apoptotic Bcl-2 family member, which induces permeabilization of the mitochondrial outer membrane allowing release of cytochrome c, which through the formation of apoptosome activates caspase-9 and ultimately caspase-3 (Korsmeyer et al., 2000; Roucou et al., 2002; Scorrano and Korsmeyer, 2003). Bax level in HL-60 cells was raised by treatment with 10 µg/ml of 4'-HCA in a time dependent manner (Figure 7), whereas
4'-Hydroxycinnamaldehyde from *Alpinia galanga* (Linn.) Induces Human Leukemic Cell Apoptosis

The levels of anti-apoptotic Bcl-2 and Bcl-xL were reduced (Figure 7). Cytochrome c release was observed in both HL-60 and U937 cells following treatment with 50 μg/ml of 4'-HCA for 4 h (Figure 8). The mechanism of apoptotic death by 2'-HCA in colon SW620 cancer cells is through ERK pathway-dependent NF-kappaB inactivation (Lee et al., 2005), and 2'-HCA can inhibit cancer cell growth by down regulating of c-Jun and c-Fos gene expression (Lee et al., 2007). However, whether this means that 4'-HCA and 2'-HCA have different mechanisms in apoptotic induction in cancer cells still needs further studies.

Cytosolic and mitochondrial Ca2+ status in 4'-HCA-treated HL-60 and U937 cells

Trichosanthin induces apoptosis in HL-60 cells via mitochondrial and endoplasmic reticulum stress signaling pathways (Li et al., 2007). Increases in cytosolic and mitochondrial Ca2+ levels have been found in ER stressed cells (Zhang and Armstrong, 2007). Ca2+ levels in both mitochondria and cytosol were measured in 4'-HCA-treated HL-60 and U937 cells using fluorescent dyes Rhod2-AM and Fluo3-AM. FACS histograms of Fluo3-AM- and Rhod2-stained HL-60 cells treated with 10, 20 and 50 μg/ml of 4'-HCA revealed the increase of Ca2+ levels in both cytosolic and mitochondrial compartments respectively (Figure 9), but the Ca2+ levels in both mitochondria and cytosol of treated U937 treated did not change (data not shown). From DNA microarray studies, 2'-HCA induces in human colon SW620 cell line expression of heat shock family and ER stress-responsive genes, as well as the gene of glucose-regulated protein, 78 kDa (GRP78) (Hong et al., 2007), supporting the apoptotic induction of SW620 cells by 2'-HCA via the ER stress genes whereas the increase of Ca2+ levels in both mitochondria and cytosol in HL-60 cells indicated the involvement of ER stress by 4'-HCA as well. However, the mechanism of different calcium response in U937 cells compared to HL-60 cells needs to be further elucidated.

4'-HCA extracted from *A. galanga* (Linn.) induced apoptosis in 2 human leukemic cell lines, HL-60 and U937, by up-regulating Bax gene expression, with concomitant down-regulation of Bcl-2 and Bcl-xL genes, and by causing a rise in ROS level, loss of mitochondrial transmembrane potential, release of cytochrome c into cytosol, elevation of mitochondrial and cytosolic Ca2+ levels, and activation of caspase-3. These data indicate that 4'HCA induces apoptosis through both mitochondria and ER stress pathways. The hexane extract of *A. galanga* (Linn) induces HL–60 apoptosis in a different mechanism, viz. via caspase-3, PI3-K and MEK pathway, because 4'-HCA is obtained from the acetone extract and the active compounds might not be the same. Further experiments in an in vivo model are required before further application of 4'-HCA in the clinical use can be considered.

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References


