

## RESEARCH COMMUNICATION

# 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  $\mu\text{g/ml}$  for 4 h) showed significant increase in reactive oxygen species production and decreased mitochondrial transmembrane potential as detected by dichlorohydrofluorescein diacetate and 3,3'-dihexyloxycarbocyanine 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  $\text{Ca}^{2+}$  contents (using compartment-specific fluorescent  $\text{Ca}^{2+}$  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

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### 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'-dihexyloxycarbocyanine iodide (DiOC<sub>6</sub>), 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  $\lambda_{\text{max}}$  254 or  $\lambda_{\text{max}}$  366 nm).

*Plant materials and isolation of 4'-hydroxycinnamaldehyde*  
Air-dried powder (5 g) 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, <sup>1</sup>H NMR, <sup>13</sup>C NMR, DEPT, DQF-COSY, HMQC 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% CO<sub>2</sub>. Cells (1 x 10<sup>6</sup>) 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 10<sup>6</sup>) were treated with 4'-HCA at 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% CO<sub>2</sub> atmosphere. Cells were treated with DMSO for 30 min and absorbance (540 nm) of the cell lysate was measured using microtiter plate reader (Biotek, 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'-dichloro-2,7-dimethylfluorescein diacetate (DCFH-DA) (for ROS detection) were added for 15 min at 37 °C before cells were subjected to flow cytometry. Cells (1 x 10<sup>6</sup>) 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 FACScan 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 10<sup>6</sup> 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

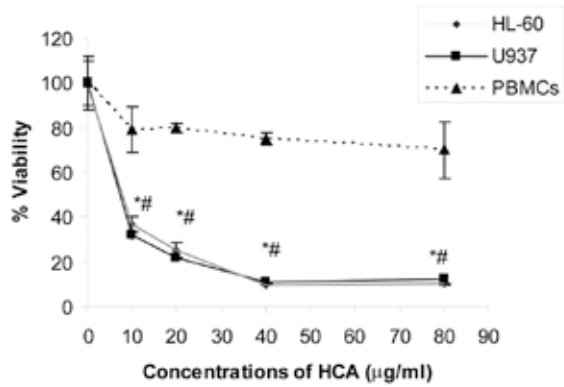
4'-HCA-treated cells were washed once in ice cold PBS and incubated at 4 °C for 10 min with ice-cold cell lysis buffer (250 mM sucrose, 70 mM KCl, 0.25% Triton X-100 in PBS containing complete mini protease inhibitor cocktail). Following centrifugation at 20,000 x g for 20 min, supernatant (50 µg, determined 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 (1:1000), and with rabbit polyclonal antibodies to Bcl-xL (1:1000), 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 Ca<sup>2+</sup> levels

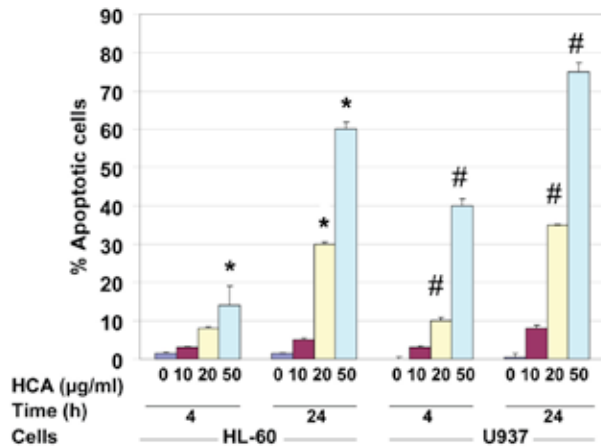
Cytosolic and mitochondrial Ca<sup>2+</sup> level was determined using 1 µM fluorescent dye Fluo3-AM in FITC setting and 250 nM fluorescent dye Rhod2-AM in PE setting respectively. After treatment with 4'-HCA for 4 h, cells were incubated with fluorescent dye for 15 min at 37 °C, washed with PBS containing 10 mM glucose and analyzed immediately by flow cytometry. In each analysis, 10,000 events were recorded and analyzed by FACScan (Becton Dickinson, USA).

#### Statistical analysis

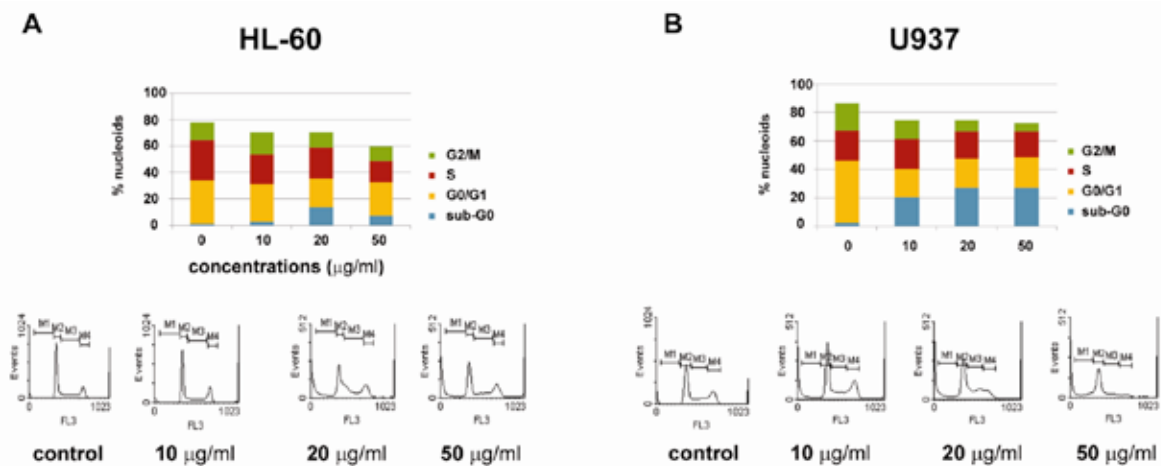
Results are expressed as mean ± SEM. Statistical



**Figure 1. Cell Viability of Human Leukemic Cells and PBMCs after Treatment with 4'-HCA.** Cytotoxicity of 4'-HCA on HL-60, U937 and PBMCs for 24 h was determined by MTT assay. Data are shown as mean ± S.E.M., obtained from 3 independent experiments conducted in triplicate. \*, #, p < 0.05, compared to untreated HL-60 and U937 cells respectively



**Figure 2. 4'-HCA-induced Apoptosis of HL-60 and U937 Cells.** Cells were treated with 4'-HCA at indicated concentrations for 4 or 24 h and apoptotic cells were examined by treatment with PI staining and visualization under a fluorescence microscope. Percentage of apoptotic cells is expressed as mean and S.E.M. of 200 cells from 3 independent experiments conducted in duplicate. \*, #, p < 0.05, compared to untreated HL-60 and U937 cells, respectively



**Figure 3. Histogram and Analysis of Percent Cells in Various Cell Cycle Stages of 4'-HCA-treated Human Leukemic Cells.** HL-60 (A) and U937 cells were treated with 4'-HCA for 24 h, stained with PI and processed by flow cytometry. Representative histograms and data are shown from three independent experiments

difference between control and treated group was determined by one-way ANOVA (Kruskal Wallis analysis) at limit of p < 0.05 from 3 independent experiments conducted in triplicate.

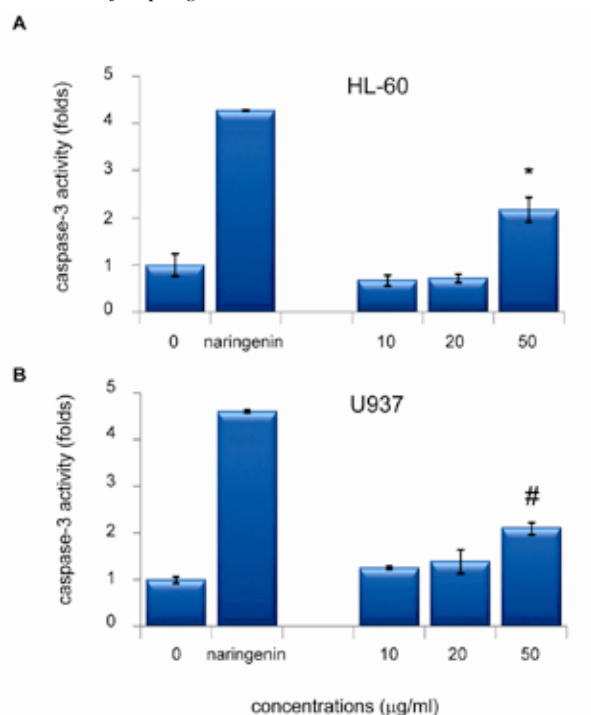
## Results

### Cell cytotoxicity with apoptotic induction

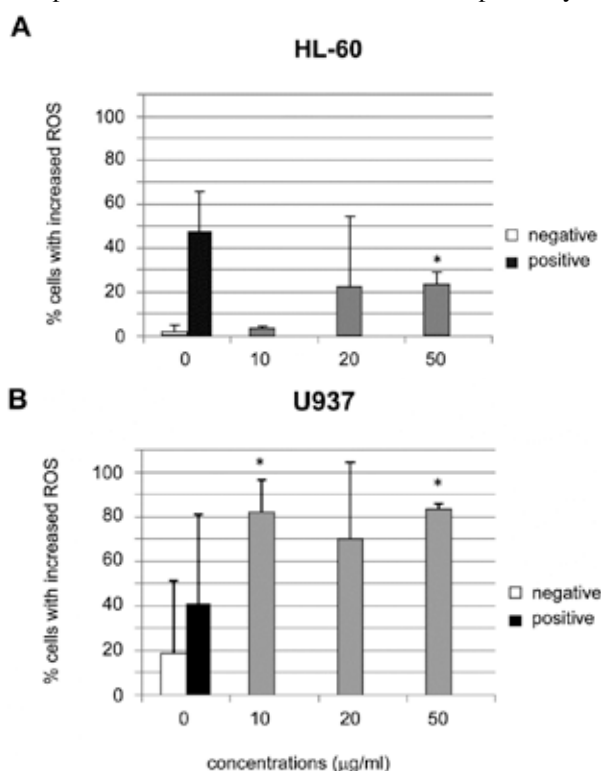
Cell viability was evaluated in HL-60 and U937 cells after incubation with 4'-HCA for 24 h using MTT assay. 4'-HCA was cytotoxic to both HL-60 and U937 cells (Figure 1), but not to PBMCs up to 80 µg/ml, which was the maximum concentration that could be dissolved in DMSO (vehicle control). Apoptotic cells (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

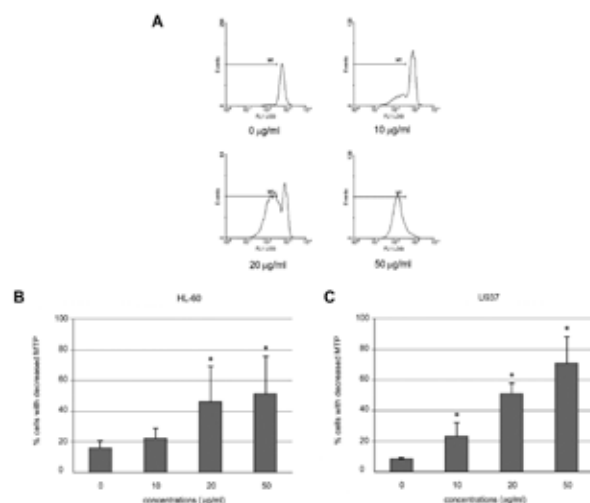
It has been reported that curcumin sensitizes non-small cell lung cancer cell anoikis through ROS-mediated Bcl-2 down-regulation (Pongrakhananon et al., 2010), but curcumin and capsaicin repress ROS generation in primary human keratinocytes (Becatti et al., 2010). In the present study 4'-HCA induced 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



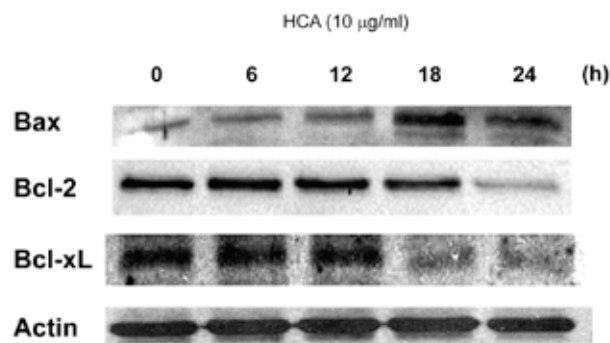
**Figure 4. Caspase-3 Activity of 4'-HCA-treated HL-60 and U937 Cells.** Caspase-3 activity in HL-60 (A) and U937 (B) cell lysates was determined using caspase-3-specific fluorogenic substrate as described in Materials and Methods with naringenin (250 µg/ml) as positive control. Data are mean ± S.E.M. \*, #, p < 0.05, compared to untreated HL-60 and U937, respectively



**Figure 5. ROS Production in 4'-HCA-treated HL-60 and U937 Cells.** HL-60 (A) and U937 cells (B) were treated with 4'-HCA for 4 h and ROS production was determined by using 2',7'-dichloro-dihydrofluorescein diacetate and flow cytometry. Data are mean ± S.E.M. Positive control is hydrogen peroxide (15 mM) treatment for 30 min. \* p < 0.05, compared to untreated HL-60 and U937



**Figure 6. Reduction of Mitochondrial Transmembrane Potential in 4'-HCA-treated HL-60 and U937 Cells.** Cells were treated with 4'-HCA for 4 h and mitochondrial transmembrane potential was measured using DiOC<sub>6</sub> and flow cytometry. Histograms of U937 cells with decreased MTP are shown in (A). Percent cells with decreased MTP of HL-60 (B) and U937 cells (C) are shown as mean + S.E.M.. \*, #, p < 0.05, compared to untreated HL-60 and U937 cells respectively

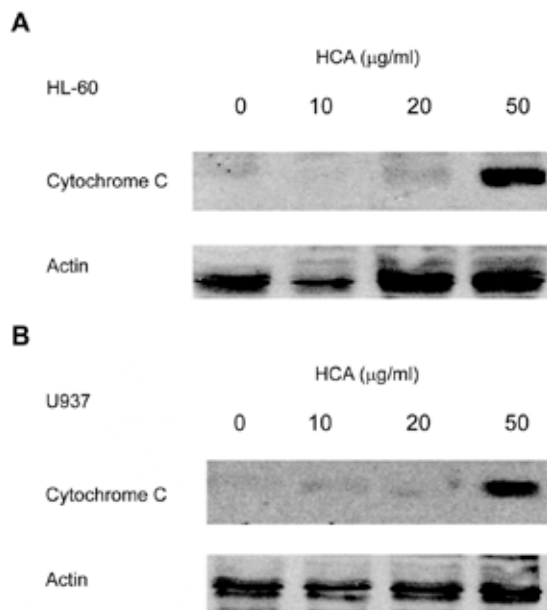


**Figure 7. Bcl-2 Family Protein Expression in 4'-HCA-treated HL-60 Cells.** HL-60 cells were exposed to 10 µg/ml of 4'-HCA for 6, 12, 18 and 24 h. Levels of Bcl-2, Bcl-xL and Bax were examined by Western blot as described in Materials and Methods. Representative results are shown of 3 independent experiments

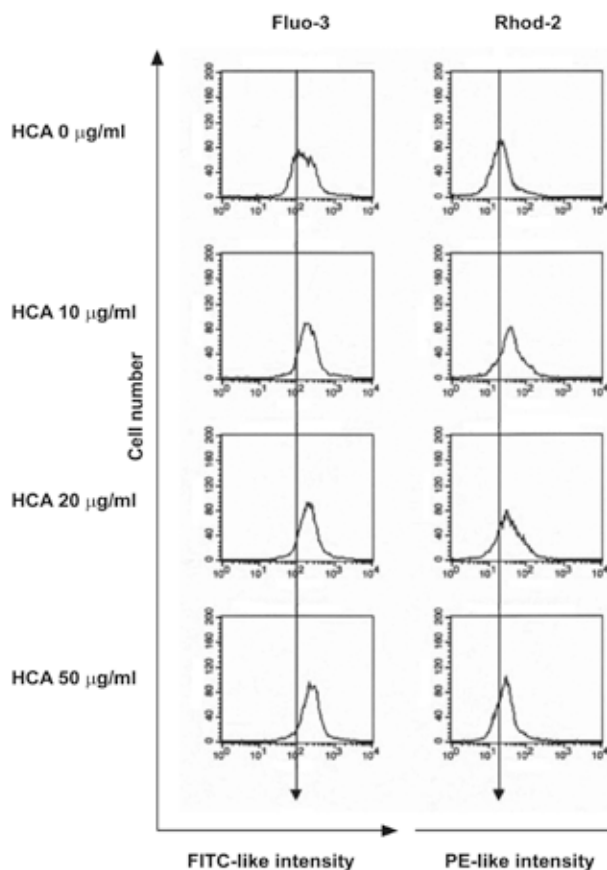
increase in ROS contributes to the perturbation to the mitochondrial transmembrane potential, but the results indicate mitochondrion as a target intracellular organelle of 4'-HCA-mediated apoptosis (and possibly 2'-HCA as well).

*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



**Figure 8. Cytochrome c Release of 4'-HCA-treated HL-60 and U937 Cells.** HL-60 (A) and U937 cells (B) were treated with 4'-HCA at indicated concentrations for 4 h and cytochrome c release was determined using Western blot as described in Materials and Methods



**Figure 9. Effect of 4'-HCA on Cytosolic (Fluo3) and Mitochondrial (Rhod2) Ca<sup>2+</sup> Levels in HL-60 Cells.** HL-60 cells were incubated with Fluo3 (left panel) to measure cytosolic or Rhod2 (right panel) to measure mitochondrial Ca<sup>2+</sup> for 15 min after treatment with and without 4'-HCA for 1 h, and then subjected to flow cytometry as described in Materials and Methods. Representative data of three independent experiments are shown

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 Ca<sup>2+</sup> 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 Ca<sup>2+</sup> levels have been found in ER stressed cells (Zhang and Armstrong, 2007). Ca<sup>2+</sup> 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 Ca<sup>2+</sup> levels in both cytosolic and mitochondrial compartments respectively (Figure 9), but the Ca<sup>2+</sup> 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 Ca<sup>2+</sup> 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 Ca<sup>2+</sup> 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

- Banjerdpongchai R, Kongtawelert P, Pompimon W (2008). The effect of MEK and PI3-K inhibitors on HL-60 cell apoptosis induced by the extract of *Alpinia galanga*. *Acta Horticulturae*, **786**, 47-56.
- Becatti M, Prignano F, Fiorillo C, et al (2010). The involvement of Smac/DIABLO, p53, NF- $\kappa$ B and MAPK pathways in apoptosis of keratinocytes from perilesional vitiligo skin: protective effects of curcumin and capsaicin. *Antioxid Redox Signal*, **13**, 1309-21
- Hong SH, Kim J, Kim JM, et al (2007). Apoptosis induction of 2'-hydroxycinnamaldehyde as a proteasome inhibitor is associated with ER stress and mitochondrial perturbation in cancer cells. *Biochem Pharmacol*, **74**, 557-65.
- Kim SA, Sung YK, Kwon BM, et al (2010). 2'-Hydroxycinnamaldehyde shows antitumor activity against oral cancer in vitro and in vivo in a rat tumor model. *Anticancer Res*, **30**, 489-94.
- Korsmeyer SJ, Wei MC, Saito M, et al (2000). Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. *Cell Death Differ*, **7**, 1166-73.
- Lee CW, Lee SH, Lee JW, et al (2007). 2-hydroxycinnamaldehyde inhibits SW620 colon cancer cell growth through AP-1 inactivation. *J Pharmacol Sci*, **104**, 19-28.
- Lee SH, Lee CW, Lee JW, et al (2005). Induction of apoptotic cell death by 2'-hydroxycinnamaldehyde is involved with ERK-dependent inactivation of NF- $\kappa$ B in TNF- $\alpha$ -treated SW620 colon cancer cells. *Biochem Pharmacol*, **70**, 1147-57.
- Li J, Xia X, Ke Y, et al (2007). Trichosanthin induced apoptosis in HL-60 cells via mitochondrial and endoplasmic reticulum stress signaling pathways. *Biochim Biophys Acta*, **1770**, 1169-80.
- Matsuda H, Pongpiriyadacha, Y, Morikawa T, Och, M, Yoshikawa M (2003). Gastroprotective effects of phenylpropanoids from the rhizomes of *Alpinia galanga* in rats: structural requirements and mode of action. *Eur J Pharmacol*, **471**, 59-67.
- Pancharoen O, Prawat U, Tuntiwachwuttikul P (2000). Phytochemistry of the zingiberaceae. *Stud Nat Prod Chem*, **23**, 797-865.
- Phitak T, Choocheep K, Pothacharoen P, et al (2009). The effects of p-hydroxycinnamaldehyde from *Alpinia galanga* extracts on human chondrocytes. *Phytochemistry*, **70**, 237-43.
- Pongrakhananon V, Nimmannit U, Luanpitpong S, Rojanasakul Y, Chanvorachote P (2010). Curcumin sensitizes non-small cell lung cancer cell anoikis through reactive oxygen species-mediated Bcl-2 downregulation. *Apoptosis*, **15**, 574-85
- Roucou X, Montessuit S, Antonsson B, Martinou JC (2002). Bax oligomerization in mitochondrial membranes requires tBid (caspase-8-cleaved Bid) and a mitochondrial protein. *Biochem J*, **368**, 915-21.
- Scorrano L, Korsmeyer SJ (2003). Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. *Biochem Biophys Res Commun*, **304**, 437-44.
- Su WC, Chang SL, Chen TY, Chen JS, Tsao CJ (2002). Comparison in in vitro growth-inhibitory activity of carboplatin and cisplatin on leukemic cells and hematopoietic progenitors: the myelosuppressive activity of carboplatin may be greater than its antileukemic effect. *Jpn J Clin Oncol*, **30**, 562-67.