Tanghinigenin from seeds of *Cerbera manghas* L. induces apoptosis in human promyelocytic leukemia HL-60 cells

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**Abstract**

Tanghinigenin, a cardiac glycoside, is isolated from the seeds of *Cerbera manghas* L. In this study, we demonstrated that tanghinigen reduced the viability of human promyelocytic leukemia HL-60 cells in a time- and dose-dependent manner, and efficiently induced apoptosis in HL-60 cells as evidenced by the Annexin V/PI binding assay, DNA fragmentation and AO/EB staining studies. In addition, stimulation of HL-60 cells with tanghinigen induced a series of intracellular events including the activation of caspase-3, -8, and -9, as well as up-regulation of Fas and FasL protein level. Taken together, caspase activation and Fas/FasL interaction was found to be involved in tanghinigen-induced HL-60 cell apoptosis.

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1. Introduction

Tanghinigenin ([Yamauchi et al., 1987; Fig. 1]) is an active component of the seeds of *Cerbera manghas* L., and belongs to the class of steroid-like compounds designated as cardiac glycosides. Their continued efficacy in the treatment of congestive heart failure and dysrhythmia is well documented ([Hamad et al., 2007; Nelson et al., 2008]). However, there is little knowledge about the role of this category of compounds in the prevention and/or treatment of proliferative diseases such as cancer. New findings in the last five years have demonstrated that these compounds are involved in complex cell-signal transduction mechanisms, inducing selective control of human tumors without interfering with normal cellular proliferation ([Stenkvist, 2001; Trevisi et al., 2004; Li et al., 2006]), and as such, represent a promising candidate for targeted cancer chemotherapy.

Fas (also known as Apo-1/CD95) is a transmembrane protein of the tumour necrosis factor/nerve growth factor receptor superfamily that transmits an apoptotic signal in susceptible normal and neoplastic cells. On cross-linking and oligomerization by its ligand (Fasl), Fas recruits, via a cytoplasmic domain of ~70 amino acid residues (the death domain), an adaptor molecule known as Fas-associated death domain (FADD; Schulze-Osthoff et al., 1998).

FADD, in turn, allows the recruitment of the proenzyme form of caspase-8, also known as FADD-like interleukin-1β-converting enzyme, resulting in proteolytic autoactivation of caspase-8 ([Muzio et al., 1998; Salvesen and Dixit, 1999]). The complex formed by the cross-linked receptor Fas, FADD, and procaspase-8 has been named as the death-inducing signaling complex (DISC). Caspase-8 acts directly on downstream caspases such as caspase-3, which is responsible for the cleavage of death substrates and execution of apoptosis ([Scaffidi et al., 1998]).

In this study, we sought to investigate the antiproliferative and apoptotic effects of tanghinigen against HL-60 human promyelocytic leukemia cells and its underlying molecular mechanisms. In addition, to the author’s knowledge, this is the first report describing the cytotoxicity and apoptosis-inducing effects of tanghinigen in vitro.

2. Materials and methods

2.1. Reagents

Reagents used in the present study included RPMI 1640 medium, newborn calf serum (NCS), penicillin, streptomycin, trypsin–EDTA (GIBCO Laboratories, Grand Island, USA); Dimethylsulfoxide (DMSO), propidium iodide (PI), Annexin V-FITC apoptosis detection kit, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Ethidium bromide (EB), acridine orange (AO), RNase A (Sigma Chemical Co., St. Louis, MO, USA); Quick Apoptotic DNA Ladder Detection Kit (Biovision, PA, USA); rabbit polyclonal anti-Fas, anti-FasL antibodies, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Santa Cruz, CA, USA); caspase-3, -8 and -9 activity kits ( Beyotime Institute of Biotechnology, Jiangsu Province, China); 100 bp ladder (TaKaRa Shuzo, Tokyo, Japan); all other chemicals were of analytical grade.

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2.2. Chemicals

Tanghinigenin (purity ≥ 99% by 1H-Nuclear Magnetic Resonance (NMR) spectroscopy and Liquid Chromatography–Mass Spectrum (LC–MS)) was purified by Shanghai Institute of Materia Medica, the Chinese Academy of Sciences according to the previously described method (Yamauchi et al., 1987). Briefly, fresh seeds of C. manghas L. were cut into pieces and extracted exhaustively with MeOH (3 × 15 L). The MeOH extract was concentrated to give a residue which was dissolved in H2O (1000 mL) and the solution was partitioned consecutively between H2O and petroleum ether, H2O and EtOAc, H2O and n-BuOH. The n-BuOH extract (62 g) was separated by column chromatography (CC) on silica gel (100–200 mesh). The column was eluted with a gradient of chloroform–MeOH (9:1–3:2) to give tanghinigenin (12 mg).

2.2.3. Cell culture and drug preparation

Human promyelocytic leukemia cell line HL-60 was purchased from the cell bank of Shanghai Institute of Cell Biology (Shanghai, China). HL-60 cells were maintained in RPMI 1640 medium containing 10% heat-inactivated NCS and 100 U/ml penicillin + 100 μg/ml streptomycin. Cells were grown in a 37°C incubator supplied with 95% room air and 5% CO2. Cells growing to 60–80% confluency were trypsinized with 0.25% trypsin–EDTA, counted and placed down at the desired density for treatment.

2.3. Cell proliferation assay

To assess the effect of tanghinigenin on cell proliferation, MTT assay was used. A typical dose- and time-dependent inhibition of cell growth was observed in HL-60 cells with an IC50 of approximately 2.15 μM following treatment with tanghinigenin for 48 h (Fig. 2). The result demonstrated that tanghinigenin had a significant inhibitory effect on proliferation of HL-60 cells.

2.4. Cell proliferation assay of apoptotic cells

Apoptosis was determined by staining cells with Annexin V-FITC and PI labeling, because Annexin V can identify externalization of phosphatidylserine during the progression of apoptosis, and therefore can detect cells in early stages of apoptosis. To quantitate apoptosis, prepared cells were washed twice with cold PBS and then re-suspended in 500 μl binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl2 at a concentration of 1 × 106 cells/ml. Five microliters Annexin V-FITC and 10 μl PI (1 μg/ml) were then added to these cells, analyzed by FACS Calibur flow cytometry (Becton Dickinson), and calculated by CellQuest software. Viable cells were negative for both PI and Annexin V and apoptotic cells were positive for Annexin V and negative for PI, while late apoptotic dead cells displayed both high Annexin V and PI labeling. Non-viable cells, which underwent necrosis, were positive for PI and negative for Annexin V.

2.5. DNA fragmentation analysis

HL-60 cells (2 × 106 cells/ml) under different treatments were collected, washed with PBS twice and then lysed in 100 μl lysis buffer [50 mM Tris (pH 8.1); 10 mM EDTA; 0.5% sodium sarcosinate and 1 mg/ml proteinase K] for 3 h at 37°C and treated with RNase A (0.5 μg/ml) for another 1 h at 37°C. DNA was extracted by phenol:chloroform:isoamyl alcohol (v/v/v, 25:24:1) before loading and analyzed by 1.8% agarose gel electrophoresis in the presence of 0.1 μg/ml ethidium bromide (EB). The agarose gel was run at 30 V for 90 min in Tris–borate/EDTA electrophoresis buffer (TBE). Approximately 30 μg DNA was loaded in each well, visualized under UV light and photographed.

Fig. 1. Chemical structure of tanghinigenin.
Fig. 2. The cells (2 × 10^5/well) treated with different concentrations of tanghinigenin were cultured for 12, 24, and 48 h, respectively. The percentage of cell viability was determined by MTT assay. Data are presented as means ± S.D., N = 3.

3.2. Effect of tanghinigenin on apoptosis

To determine whether the growth inhibitory activity of tanghinigenin was related to the induction of apoptosis, morphological assay of cell death was investigated using the AO/EB staining for fluorescence microscopy. After HL-60 cells were exposed to various concentration of tanghinigenin for 48 h, different morphological features were analyzed. Uniformly green live cells with normal morphology were seen in the control group (Fig. 3a), whereas green early apoptotic cells with nuclear margination and chromatin condensation occurred in the experimental group with 1.15 and 2.87 μM tanghinigenin (Fig. 3b and c), orange later apoptotic cells with fragmented chromatin and apoptotic bodies were seen when tanghinigenin was applied at the concentration of 2.87 and 5.73 μM (Fig. 3c and d). The result suggested that tanghinigenin was able to induce marked apoptotic morphology in HL-60 cells.

We also analyzed whether DNA fragmentation, another hallmark of apoptosis, was induced by tanghinigenin in HL-60 cells. Exposure of cells to tanghinigenin (1.15, 2.87, and 5.73 μM) for 48 h led to evident DNA fragmentation as indicated by the formation of DNA ladder in the agarose gels, whereas control showed no evident DNA ladder (Fig. 4).

To further confirm that it was tanghinigenin that induced cell apoptosis, HL-60 cells were stained with Annexin V-FITC and PI and analyzed by flow cytometry. As shown in Fig. 5A, tanghinigenin increased the number of early apoptotic cells (Annexin V-positive and PI-negative proportion cells) in a dose-dependent manner at 24 h in HL-60 cells. The number of early apoptotic cells in HL-60 cells was elevated to approximately 80% at 24 h following the treatment of 10 μM tanghinigenin, as compared with the tanghinigenin-untreated group. Furthermore, as shown in Fig. 5B, tanghinigenin increased the number of early apoptotic cells in a time-dependent manner at the concentration of 5.73 μM in HL-60 cells. The number of early apoptotic cells in HL-60 cells was elevated to approximately 18% at 24 h following the treatment of 5.73 μM tanghinigenin.

Fig. 3. Tanghinigenin induces apoptotic morphological changes on HL-60 cells. After being treated with indicated concentrations of tanghinigenin for 48 h, HL-60 cells were harvested, washed with PBS, and stained with AO/EB (100 μg/ml). Cell morphology was observed under fluorescence microscopy. Green live cells showed normal morphology in control (a); green early apoptotic cell show nuclear margination and chromatin condensation with 1.15 μM (b) or 2.87 μM (c) tanghinigenin treatment. Orange later apoptotic cells showed fragmented chromatin and apoptotic bodies after 2.87 μM (c) or 5.73 μM (d) tanghinigenin treatment (magnification 100×).

Fig. 4. DNA fragmentation of HL-60 cells exposed to tanghinigenin. HL-60 cells were incubated with 0, 1.15, 2.87, and 5.73 μM of tanghinigenin for 48 h. M, 100 bp ladder. DNA ladders reflecting the presence of DNA fragments were viewed on EB-stained gel. A typical result from three independent experiments is presented.
Fig. 5. Tanghinigenin-induced apoptosis in HL-60 cells. (A) Tanghinigenin-induced apoptosis in HL-60 cells in dose-dependent manner. Exponentially growing cells were treated with different concentration of tanghinigenin for 24 h. (B) Tanghinigenin-induced apoptosis in HL-60 cells in time-dependent manner. Exponentially growing cells were treated with 5.73 μM tanghinigenin for 0, 3, 6, 12 and 24 h. Cells with Annexin-V and PI staining were measured with a FACScalibur flow cytometer. Bottom right quadrant, Annexin V+/PI−, early apoptotic cells; top right quadrant, Annexin V+/PI+, late apoptotic cells. Each experiment was performed in triplicate.
Fig. 6. Activation of caspase-3, -8, -9. Cells were incubated with 5.73 μM tanghinigenin for various time periods. Activation of caspase-3, -8, and -9 was analyzed by caspase-3, caspse-8 and caspase-9 Activity Kits as described in the materials and methods. Each date represents the mean ± S.D. of three independent experiments. (A) Activation of caspase-3 in time-dependent manner. (B) Activation of caspase-8 and caspase-9 in time-dependent manner.

tanghinigenin, as compared with the tanghinigenin-treated group at 0 h.

Above all, these results indicate that cytotoxic effects observed in response to tanghinigenin were associated with induction of apoptotic cell death.

3.3. Effect of tanghinigenin on caspase-3, -8 -9 like activities in HL-60 cells

In order to examine the role of caspases in the apoptosis induced by tanghinigenin, we measured the proteolytic activity of the executioner caspase-3 and the initiator caspase-8 and -9. Fig. 6 shows that tanghinigenin treatment caused a time-dependent increase in caspase-3, -8 and -9 proteolytic activities. A significant increase in the activity of caspase-3 was observed after 3 h of exposure to tanghinigenin, remaining this activity elevated for at least 24 h (Fig. 6A), and activation of both caspase-8 and caspase-9 was evident after 15 min of incubation and persisted up to 24 h of treatment (Fig. 6B).

3.4. Changes on the Fas and FasL expressions after tanghinigenin exposure

To further investigate whether tanghinigenin-induced apoptosis occurred via Fas and FasL interaction, which is known to mediate the caspase-8-dependent apoptotic pathway, we examined the changes on the Fas and FasL expressions in HL-60 cells after tanghinigenin exposure. Western blot analysis demonstrated that the overall expression level of FasL was higher than that of Fas in HL-60 cells, but the protein levels of Fas and FasL were increased in tanghinigenin-treated cells compared with the untreated controls in a time-dependent manner (Fig. 7).

4. Discussion

Many efforts have been made to both search for compounds that can influence apoptosis and understand mechanisms of their actions. In recent years, more knowledge has been obtained about how cardiac glycosides induce cell death in human cancers. It was found in the present study that tanghinigenin, a cardiac glycoside, isolated from the seeds of *C. manghas* L., inhibited the proliferation of HL-60 cells markedly in a dose- and time-dependent manner (Fig. 2). HL-60 cells treated with tanghinigenin exhibited typical morphological features of apoptosis (Fig. 3). In addition, the apoptosis induced by tanghinigenin was also confirmed by observations of agarose gel electrophoresis (Fig. 4) and phosphatidylserine translocation (Fig. 5). Next, our focus was on cellular mechanisms whereby tanghinigenin induced apoptosis in HL-60 cells.

Caspase activation is thought to be important for cardiac glycoside-induced cell death in vitro and in vivo (Wilhelm and Georgios, 2007; Robert et al., 2008). Caspases are broadly grouped into initiator or effector caspases, according to the roles they play in the apoptosis inducing system (Salvesen and Dixit, 1997). The initiator caspases including typically caspase-8 and caspase-9, are activated by two alternative pathways. The first involves death receptor-mediated apoptosis through caspase-8. It is characterized by binding cell death ligands and cell death receptors, and subsequently activates caspase-8 and caspase-3 (Schempp et al., 2001). The second involves mitochondria-mediated apoptosis through caspase-9. The key element in the pathway is the liberation of the cytochrome C from mitochondria to the cytosol. Once cytochrome C is in the cytosol, cytochrome C together with Apaf-1 activates caspase-9, and the latter then activates caspase-3 (Li et al., 1997). In both pathways activation of caspase-3 plays the central role in the initiation of apoptosis (Salvesen and Dixit, 1999). Our study revealed that tanghinigenin treatment caused a time-dependent increase in caspase-3, -8 and -9 proteolytic activities as shown in Fig. 6, indicating that death receptor-mediated apoptosis and mitochondria-mediated apoptosis may be involved in tanghinigenin-induced apoptosis.

The death receptor pathway is triggered by members of the death receptor family, such as Fas receptor and tumor necrosis factor receptor (Trauth et al., 1989; Itoh et al., 1991). The expression of Fas and FasL has been demonstrated to cause a number of solid
tumors and hematological malignancies (Friesen et al., 1996; Gratas et al., 1998). The physiological role of the Fas/FasL pathway outside the immune system remains unclear, although the Fas system may play a role in drug-induced apoptosis in some cell types (Tillman et al., 1999; Fulda et al., 1997; Muller et al., 1997). Our data showed that the protein levels of Fas and FasL were increased in tanghinigenin-treated cells compared with the untreated controls in a time-dependent manner (Fig. 7). Release of FasL initiated apoptosis through Fas death receptor which subsequently activated caspase-8, indicating the Fas/FasL pathway play a role in tanghinigenin-induced apoptosis in HL-60 cells. In conclusion, the results of the present study firstly indicate that tanghinigenin inhibited proliferation and induced apoptosis in human promyelocytic leukemia HL-60 cells. Caspase activation and Fas/FasL interaction played an important role in tanghinigenin induced apoptosis of HL-60 cells. These in vitro findings suggest that this compound is potentially useful as an apoptosis inducer for treatment of human promyelocytic leukemia.

Conflict of interest
We declare that there are no conflicts of interest.

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References