Environmental Toxicology and Pharmacology 30 (2010) 31–36

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

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

Guo-Fei Wang[a,1], Yue-Wei Guo[b], Bo Feng[a,1], Liang Li[b], Cai-Guo Huang[a,*], Bing-Hua Jiao[a]

[a] Department of Biochemistry and Molecular Biology, Second Military Medical University, 800 Xiangyin Road, Shanghai 200433, People's Republic of China
[b] State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, People's Republic of China

ARTICLE INFO

Article history:
Received 23 November 2009
Received in revised form 26 February 2010
Accepted 10 March 2010
Available online 17 March 2010

Keywords:
Tanghinigenin
Apoptosis
Fas
FasL
HL-60
Caspase

ABSTRACT

Tanghinigenin, a cardiac glycoside, is isolated from the seeds of *Cerbera manghas* L. In this study, we demonstrated that tanghinigenin 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 tanghinigenin 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 tanghinigenin-induced HL-60 cell apoptosis.

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 tanghinigenin against HL-60 human promyelocytic leukemia cells and its underlying molecular mechanisms. In addition, to the authors's knowledge, this is the first report describing the cytotoxicity and apoptosis-inducing effects of tanghinigenin 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-diphenylterazolium 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.

* Corresponding author. Tel.: +86 21 81870970x8020; fax: +86 21 65334344.
E-mail address: huangcaiguo@hotmail.com (C.-G. Huang).
[1] These authors contributed equally to this work.

1382-6689/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.etap.2010.03.012

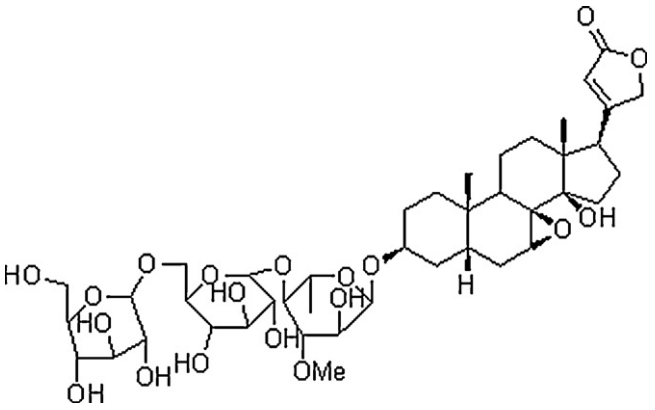

Fig. 1. Chemical structure of tanghinigenin.

2.2. Chemicals

Tanghinigenin (purity ≥ 99% by ^{1}H-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 (1.3 kg, dry weight) of *C. manghas* L. were cut into pieces and extracted exhaustively with MeOH (3 × 15 L). The MeOH extract was concentrated *in vacuo* to give a residue which was dissolved in H_2O (1000 ml) and the solution was partitioned consecutively between H_2O and petroleum ether, H_2O and EtOAc, H_2O 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 (9:1–0:100) to give 6 fractions (Fr.1–Fr.6) on the basis of TLC checking. Fraction 3 (17.0 g) was further purified by Sephadex LH-20 (chloroform/MeOH 1:1), followed by CC on silica gel and eluted with a gradient of chloroform–MeOH (9:1–3:2) to give GHSC-72 (9.0 mg), GHSC-74 (187 mg), GHSC-75 (870 mg) and GHSC-79 (tanghinigenin) (12 mg).

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% CO_2. Cells growing to 60–80% confluency were trypsinized with 0.25% trypsin–EDTA, counted and placed down at the desired density for treatment. Tanghinigenin was dissolved in dimethylsulfoxide (DMSO) and further diluted in PBS. The final DMSO concentration in medium was 0.1% and did not affect cell function and assay systems.

2.4. Cell proliferation assay

The effect of tanghinigenin on the proliferation of HL-60 cells was determined using the MTT assay as previously described (Mosmann, 1983). In brief, cells (2×10^5 cells/well) were seeded in 96-well microtiter plates in the presence of the designated doses of tanghinigenin. After exposure to the drug for 12, 24 and 48 h, 50 μl MTT solution (2 mg/ml in PBS) was added to each well, and the plates were incubated for an additional 4 h at 37 °C. MTT solution in medium was aspirated. To achieve solubilization of the formazan crystal formed in viable cells, 200 μl DMSO was added to each well. The optical density of each well was measured at 550 nm using a microplate reader (Model 550, Bio-Rad, USA). The effect of tanghinigenin on growth inhibition was assessed as percent cell proliferation where cells treated with the vehicle were taken as 100% viable. IC_{50} value is the concentration of the compound required to reduce the absorbency to 50% versus control cells. Each plate contained multiple wells of a given experimental condition and multiple control wells. This procedure was replicated for 2–4 plates/condition.

2.5. DNA fragmentation analysis

HL-60 cells (2×10^6 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 sarkosinate and 1 mg/ml proteinase K] for 3 h at 56 °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 50 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.

2.6. Fluorescence morphological examination

Apoptotic morphology was studied by staining the cells with a combination of the fluorescent DNA-binding dyes acridine orange (AO) and ethidium bromide (EB). Cells were harvested and washed three times with PBS, after being incubated with different concentrations of tanghinigenin for 48 h, and were then stained with 100 μg/ml AO and EB (AO/EB) for 2 min. Then the color and structure (Pitrak et al., 1996) of the different cell types were observed under a fluorescence microscope (Olympus, IX70, Japan).

2.7. Annexin V-FITC/PI 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 $CaCl_2$] at a concentration of 1×10^6 cells/ml. Five microliters Annexin V-FITC and 10 μl PI (1 μg/ml) were then added to these cells, analyzed by FACScalibur 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.

2.8. Analysis of caspase-3, -8 and -9 activities

Caspases activities were measured using Caspase Activity Kit according to the manufacturer's instructions. Briefly, cells were washed with cold PBS, resuspended in lysis buffer and left on ice for 15 min. The lysate was centrifuged at 16,000 × *g* at 4 °C for 15 min. Activities of caspase-3, -8 and -9 were measured using substrate peptides Ac-DEVD-*p*NA, Ac-IETD-*p*NA and Ac-LEHD-*p*NA, respectively. The release of *p*-nitroanilide (*p*NA) was qualified by determining the absorbance with a microplate reader (Model 550, Bio-Rad, USA) at 405 nm. Each plate contained multiple wells of a given experimental condition and multiple control wells.

2.9. Western blot analysis

Cells were scraped from the culture vessels, washed twice with PBS, and then suspended in 30 μl Western blot lysis buffer containing 50 mM Tris–HCl (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 0.1% Trion X-100, and 1% SDS at 0–4 °C for 15 min. After centrifugation at 12,000 × *g* for 5 min at 4 °C, the supernatants were collected, and the protein concentrations were determined using bicinchoninic acid (BCA) Protein assay (Beyotime Biotechnology, Haimen, China). Equal amounts (50 μg of protein) of lysate were subjected to 12% SDS-PAGE. After electrophoresis, protein blots were transferred to a nitrocellulose membrane (Pall Life Sciences, Ann Arbor, MI) using an electro-blotting apparatus (Bio-Rad). The membrane was blocked with 5% non-fat milk in TBST (Tris Buffered Saline with Tween 20) solution, and incubated overnight with the corresponding primary antibodies in the blocking solution at 4 °C. After 3 washes with TBST solution, the membrane was incubated at room temperature for 1 h, with horseradish peroxidase-conjugated secondary antibody diluted with TBST solution (1:3000). The signals of detected proteins were visualized on an enhanced chemiluminescence reaction (ECL) system (Millipore).

2.10. Statistical analysis

Data are reported as mean ± S.D. All experiments were done at least three times, and three or more independent observations were made on each occasion. Statistically significant values were compared using Student's *t*-test for single comparison and *p*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of tanghinigenin on cell proliferation

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 IC_{50} 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.

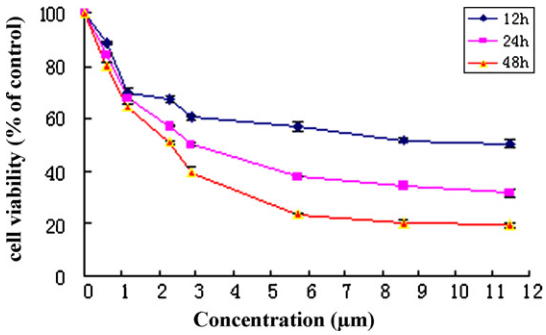

Fig. 2. The cells (2×10^5/well) treated with different concentrations of tanghinigenin were cultured for 12, 24, and 48 h, respectively. The percentage of cell viability was determined by MTT assay. Data are presented as means ± S.D., $N = 3$.

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).

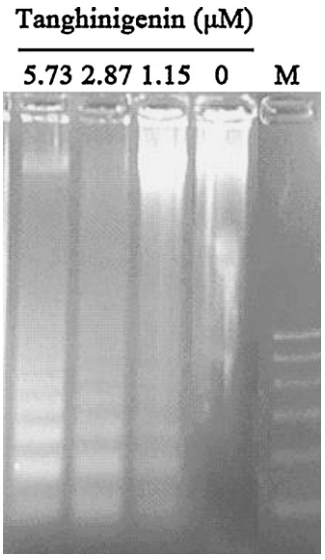

Fig. 4. DNA fragmentation of HL-60 cells exposed to tanghinigenin. HL-60 cells were incubated with 0, 1.15, 2.87, and 5.73 μM of tanghinigenin for 48 h. M, 100 bp ladder. DNA ladders reflecting the presence of DNA fragments were viewed on EB-stained gel. A typical result from three independent experiments is presented.

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

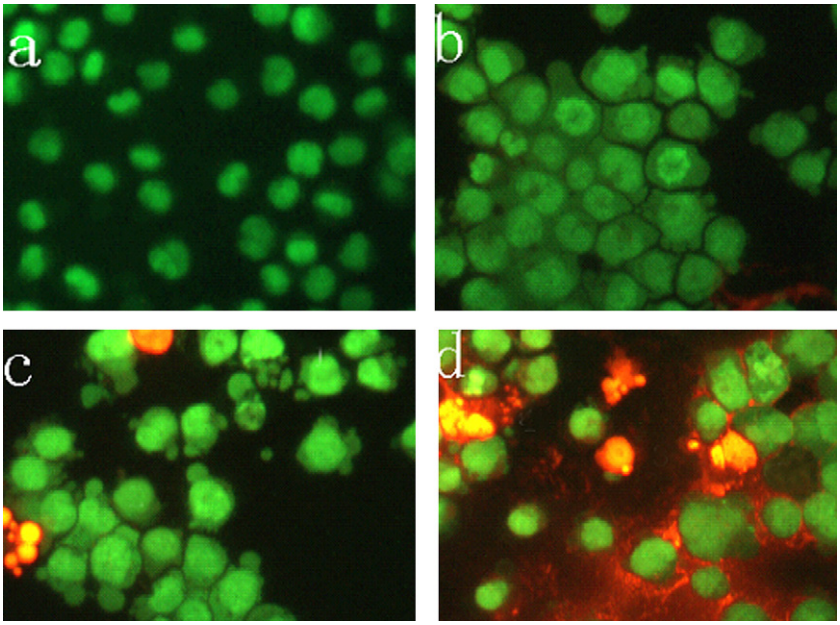

Fig. 3. Tanghinigenin induces apoptotic morphological changes on HL-60 cells. After being treated with indicated concentrations of tanghinigenin for 48 h, HL-60 cells were harvested, washed with PBS, and stained with AO/EB (100 μg/ml). Cell morphology was observed under fluorescence microscopy. Green 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×).

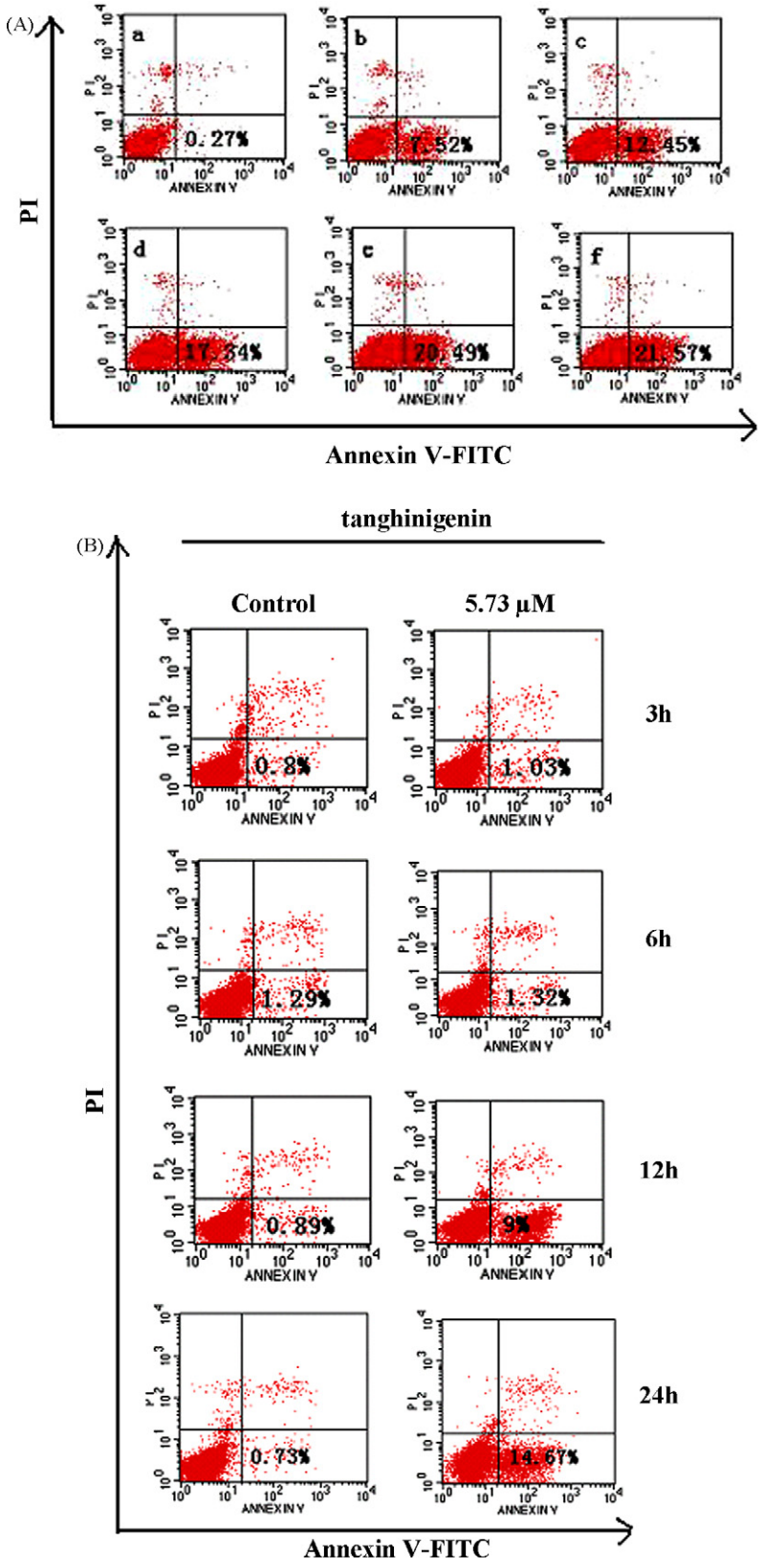

Fig. 5. Tanghinigenin-induced apoptosis in HL-60 cells. (A) Tanghinigenin-induced apoptosis in HL-60 cells in dose-dependent manner. Exponentially growing cells were treated with different concentration of tanghinigenin for 24 h. (B) Tanghinigenin-induced apoptosis in HL-60 cells in time-dependent manner. 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.

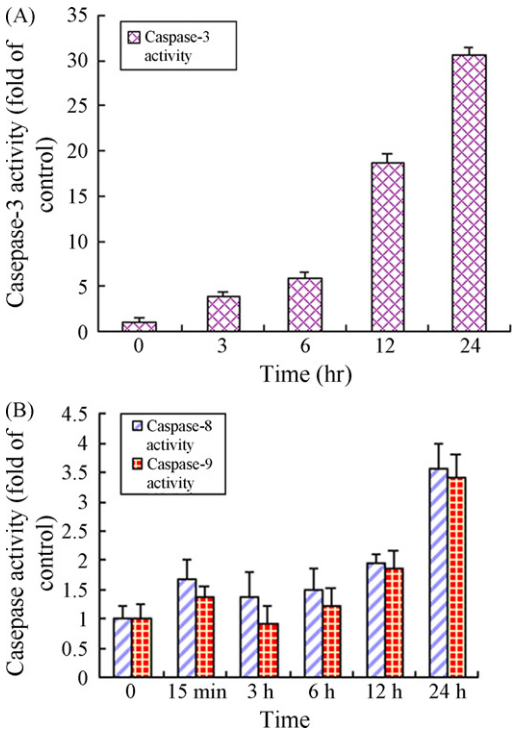

Fig. 6. Activation of caspase-3, -8, -9. Cells were incubated with 5.73 μM tanghinigenin for various time periods. Activation of caspase-3, -8, and -9 was analyzed by caspase-3, caspse-8 and caspase-9 Activity Kits as described in the materials and methods. Each date represents the mean ± S.D. of three 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).

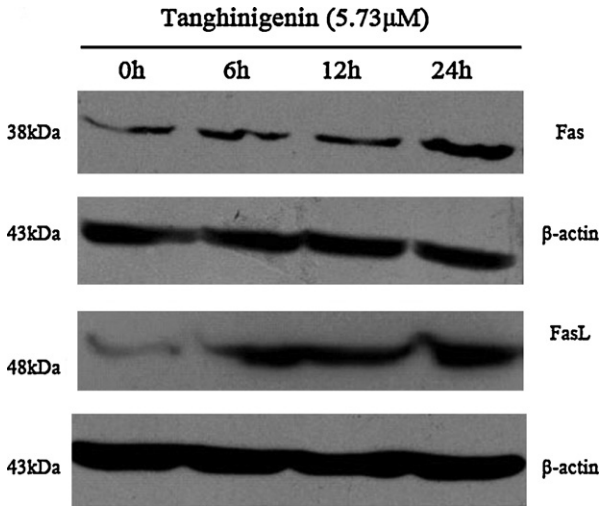

Fig. 7. Expressions of Fas and FasL in HL-60 cells after tanghinigenin exposure. Western blot analysis of Fas and FasL in HL-60 cells exposed to 5.73 μM tanghinigenin for various time periods. The molecular weight (kDa) of each protein is indicated to the left of the image. β-Actin was used as an internal loading control. Each experiment was performed in triplicate.

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 in 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.

Acknowledgement

This work was supported by the National Basic Research Program of China (973 Program) (No. 2010CB833804).

References

Friesen, C., Herr, I., Krammer, P.H., Debatin, K.M., 1996. Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nat. Med. 2, 574–577.

Fulda, S., Sieverts, H., Friesen, C., Herr, I., Debatin, K.M., 1997. The CD95 (APO-1/Fas) system mediates drug-induced apoptosis in neuroblastoma cells. Cancer Res. 57, 3823–3829.

Gratas, C., Tohma, Y., Barnas, C., Taniere, P., Hainaut, P., Ohgaki, H., 1998. Up-regulation of Fas (APO-1/CD95) ligand and downregulation of Fas expression in human esophageal cancer. Cancer Res. 58, 2057–2062.

Hamad, E., Mather, P.J., Srinivasan, S., Rubin, S., Whellan, D.J., Feldman, A.M., 2007. Pharmacologic therapy of chronic heart failure. Am. J. Cardiovasc. Drugs 7, 235–248.

Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y., Nagata, S., 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 66, 233–243.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., Wang, X., 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91, 479–489.

Li, J., Zelenin, S., Aperia, A., Aizman, O., 2006. Low doses of ouabain protect from serum deprivation-triggered apoptosis and stimulate kidney cell proliferation via activation of NF-κB. JASN 17, 1848–1857.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. J. Immunol. Methods 65, 55–63.

Muller, M., Strand, S., Hug, H., Heinemann, E.M., Walczak, H., Hofmann, W.J., Stremmel, W., Krammer, P.H., Galle, P.R., 1997. Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wildtype p53. J. Clin. Invest. 99, 403–413.

Muzio, M., Stockwell, B.R., Stennicke, H.R., Salvesen, G.S., Dixit, V.M., 1998. An induced proximity model for caspase-8 activation. J. Biol. Chem. 273, 2926–2930.

Nelson, A., Juan, C.D., Olga, S., Harvey, B.P., 2008. Heart failure drug digitoxin induces calcium uptake into cells by forming transmembrane calcium channels. PNAS 105, 2610–2615.

Pitrak, D.L., Tsai, H.C., Mullane, K.M., Sutton, S.H., Stevens, P., 1996. Accelerated neutrophil apoptosis in the acquired immunodeficiency syndrome. J. Clin. Invest. 98, 2714–2719.

Robert, A., Newman, Yang, P.Y., Alison, D., Pawlus, Keith, I., BlocK, 2008. Cardiac glycosides as novel cancer therapeutic agents. Mol. Interv. 8, 36–49.

Salvesen, G.S., Dixit, V.M., 1997. Caspases: intracellular signaling by proteolysis. Cell, 91443–91446.

Salvesen, G.S., Dixit, V.M., 1999. Caspase activation: the induced-proximity model. Proc. Natl. Acad. Sci. U.S.A. 96, 10964–10967.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H., Peter, M.E., 1998. Two CD95 (Apo-1/Fas) signaling pathways. EMBO J. 17 (6), 1675–1687.

Schempp, C.M., Simon-Haarhaus, B., Termeer, C.C., Simon, J.C., 2001. Hypericin photo-induced apoptosis involves the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and activation of caspase-8. FEBS Lett. 493, 26–30.

Schulze-Osthoff, K., Ferrari, D., Los, M., Wesselborg, S., Peter, M.E., 1998. Apoptosis signaling by death receptors. Eur. J. Biochem. 254, 439–459.

Stenkvist, B., 2001. Cardenolides and cancer. Anticancer Drugs 12, 635–636.

Tillman, D.M., Petak, I., Houghton, J.A., 1999. A fas-dependent component in 5-fluorouracil/leucovorin-induced cytotoxicity in colon carcinoma cells. Clin. Cancer Res. 5, 425–430.

Trauth, B.C., Klas, C., Peters, A.M., Matzku, S., Moller, P., Falk, W., Debatin, K.M., Krammer, P.H., 1989. Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science 245, 301–305.

Trevisi, L., Visentin, B., Cusinato, F., Pighin, I., Luciani, S., 2004. Antiapoptotic effect of ouabain in human umbilical vein endothelial cells. Biochem. Biophys. Res. Commun. 321, 716–721.

Wilhelm, S., Georgios, S.B., 2007. Endogenous and exogenous cardiac glycosides: their roles in hypertension, salt metabolism, and cell growth. AJP-Cell Physiol. 293, C509–C536.

Yamauchi, T., Abe, F., Wan, A.S.C., 1987. Studies on cerbera V minor glycosides of 17-alpha digitoxigenin from the stems of genus *Cerbera*. Chem. Pharm. Bull. 35, 4993–4995.