Gambogic acid induces cell apoptosis through endoplasmic reticulum stress triggered inhibition of Akt signaling pathways in extranodal NK/T-cell lymphoma cells

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[ABSTRACT] As the chemotherapeutic resistance of extranodal NK/T-cell lymphoma (ENKTL) rises year by year, searching for novel chemoprevention compounds has become imminent. Gambogic acid (GA) has recently been shown to have anti-tumor effects, but its role and underlying mechanism in ENKTL are rather elusive. In the present study, we showed that GA inhibited the cell growth and potently induced the apoptosis of ENKTL cells in vitro in a time- and concentration-dependent manner. Furthermore, GA induced cell death through endoplasmic reticulum stress (ERS) mediated suppression of Akt signaling pathways and finally the release of the caspase-3 proteases. Overall, our data provided evidences supporting GA as a potential therapeutic agent for ENKTL, which may facilitate further preclinical development of anti-tumor drugs.

[KEY WORDS] Gambogic acid; Apoptosis; Extranodal NK/T-cell lymphoma; Endoplasmic reticulum stress

Introduction
Extranodal NK/T-cell lymphoma (ENKTL) is identified as a distinct zoon in the World Health Organization (WHO) classification [1]. ENKTL is common, especially in Asia, where it accounts for about 10% of lymphomas [2-3]. Previous reports have shown poor effect of the existing chemotherapy in ENKTL cells [2-4]. Therefore, novel and more effective agents are urgently needed to improve the efficacy in the treatment of ENKTL.

Gambogic acid (GA) is a small molecule extracted from the traditional Chinese medicine gamboge [5]. Accumulating findings have demonstrated that GA possesses antitumor virtue [6], and several molecular targets of GA have been identified in tumors [7]. However, the effect of GA on ENKTL remains elusive until now.

In the present study, we explored the effect of GA in ENKTL cells and the underlying molecular mechanism. We demonstrated for the first time that GA exerted cytotoxicity against ENKTL cells. Our results showed that the apoptosis of ENKTL cells induced by GA was mediated by ERS and ERS-triggered Akt signaling pathway.

Materials and Methods
Reagents
Roswell Park Memorial Institute 1640 (RPMI 1640) medium, Glutamine, TRizol reagent, fetal bovine serum (FBS), OPTI-MEM medium and Lipofectamine 2000 transfection reagent were purchased from Life Technologies Corporation (Invitrogen, Carlsbad, CA, USA). First strand cDNA synthesis kit and all quantitative real-time PCR reagents were from Applied Biosystems (Foster City, CA, USA). Phosphate buffered saline (PBS), penicillin/streptomycin, agarose, Triton X-100, BSA, DEPC-water, isopropanol, chloroform and 0.25% trypsin-0.02% ethylene diamine tetraacetic acid (EDTA),
were purchased from Sangon Biotech (Sangon Biotech Corporation, Shanghai, China). Small interfering RNAs were obtained from GenePharma Co., Ltd. (GenePharma, Shanghai, China). 4×Protein SDS-PAGE Loading Buffer was obtained from Takara Biotechnology Co., Ltd. (Takara, Dalian, China). GA (S2448) was obtained from Selleck Chemicals (Selleck, Houston, TX, USA) and diluted in DMSO (dimethyl sulfoxide) for experimental use. 3(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT), crystal violet staining solution and annexin V/PI were purchased from Jiancheng Institute of Biotechnology (Jiancheng, Nanjing, China).

**Cell culture**

ENKTL cell lines SNK-6 and SNK-8 were provided from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and hatched in RPMI 1640 medium containing 10% FBS, 2 mmol·L⁻¹ of glutamine, 0.5 μg·mL⁻¹ of penicillin (equivalent to 100 U) and 0.1 mg·mL⁻¹ of streptomycin and incubated in a 37 °C incubator with 5% CO₂.

**MTT assay**

Cell viability was evaluated by MTT assay following the previous reports [8-9]. The absorbance at 490 nm (A₄₉₀) of each well was detected on a microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA). Each experiment was repeated in triplicate.

**Apoptosis analysis by flow cytometry**

The cells were seeded into 6-well plates at a determined density that would yield 50% confluence in 48 h. Then either DMSO or GA (final concentration at 1.5 μmol·L⁻¹) was added. After treatment for 48 h, the cells were collected, washed twice with PBS, and hatched for 15 min with a admixture containing FITC labeled annexin V and propidium iodide in the binding buffer. The fluorescence was tested using Flow cytometer (BD Biosciences, San Jose, CA, USA). Apoptosis rate was compared on the basis of the following formula: apoptosis rate = number of apoptotic cells/total number of cells.

**RNA extraction and real-time quantitative PCR**

Two million of cells were harvested and the RNA was extracted by adding 1 mL of TRIzol Reagent for 1 min, following the directions provided by the manufacturer. The quality of RNA was checked by electrophoresis on a denatured agarose gel and the concentration of total RNA was read on a spectrophotometer at A₂₆₀. Total RNA (1 μg) was reverse transcribed into First-Strand cDNA using the complementary DNA Synthesis Kit. The PCR primers were showed in Table 1. The reaction condition of PCR was same as previous reports [10]. GAPDH was selected for internal reference.

**Protein sample preparation and Western blotting analysis**

The cells were harvested and washed with ice-cold PBS thrice. Cell pallets were homogenized in extraction buffer and hatched for 30 min at 4 °C, followed by centrifugation for 10 min at 14 000 r·min⁻¹. Concentration of total protein was ratified by Bradford assay. The total protein (100 μg/lane) was resolved in 12% SDS-polyacrylamide gels, and was then transferred onto PVDF membranes (0.22 μm, Bio-rad, Hercules, CA, USA) in 25 mmol·L⁻¹ of Tris-base, 190 mmol·L⁻¹ of glycine and 20% methanol. After blocking with 10% skim milk and 0.1% Tween 20 in TBS for 1 h, the membranes were reacted with anti-cleaved-caspase-3 (1 : 2000), anti-pro-caspase-3 (1 : 2000), anti-GRP78 (1 : 1000), anti-ATF4 (1 : 1000), anti-CHOP (1 : 1000), anti-phospho-Akt-(pSer473) (1 : 2000), anti-total-Akt (1 : 2000), anti-GAPDH (1 : 2000) or anti-beta-actin (1 : 1000) antibodies, respectively, at 4 °C overnight [12-13]. After binding with horse radish peroxidase (HRP)-coupled goat anti-rabbit or goat anti-mouse IgG (1 : 1000) at 37 °C for 1 h, the blots were visualized by enhanced chemiluminescence (Inmmobilon Western HRP, Millipore Bio-technology, Billerica, MA, USA). All the antibodies were purchased from Cell Signaling Technology (Cell Signaling, Beverly, CA, USA).

**Transient transfection and RNA interference of CHOP**

To induce RNA interference-mediated knockdown of CHOP, siRNA sequences were constructed. For transient transfection, 2 × 10⁶ SNK-6 or SNK-8 cells were plated into 6-well plates 24 h before transfection. The transfection was executed using Lipofectamine 2000 reagent and OPTI-MEM medium according to the manufacturer’s suggestions. The RNA interference sequences were 5’-AAGAACCAGCAGAGGUCACAA-3’ for CHOP [11].

**Statistical analysis**

All the experimental values were represented as means ± SD unless otherwise indicated. Phenotypes of cells and expression difference for independent samples were analyzed by ANOVA using SPSS package for Windows (Version 19.0; Chicago, IL, USA). Differences were determined to be statistically significant at P < 0.05.

**Results**

**Inhibitory effects of GA on the growth of ENKTL cells**

To investigate whether GA possesses any potential for treatment of ENKTL, we examined its effect on proliferation of ENKTL cells. By the treatment with different concentrations of GA (0, 0.375, 0.75, 1.5, 2.25, and 3 μmol·L⁻¹), it was observed that the cell proliferation was concentration- and time-dependently inhibited by GA (Fig. 1B). It was further demonstrated that 1.29 and 1.06 μmol·L⁻¹ of GA was closely approaching an IC₅₀ concentration against SNK-6 and SNK-8 cells. Statistical results showed that treatment with 1.5 μmol·L⁻¹ GA for 48 h resulted in a significant inhibitory effect, compared with the control (P < 0.05).

**Apoptotic effects of GA on ENKTL cells**

To confirm the induction of apoptosis in GA-treated cells, apoptotic cells counting by Annexin V/PI staining using flow cytometry and Western blotting for analysis of apoptosis-related proteins such as cleaved caspase-3 were carried out. As shown in Fig. 2, 1.5 μmol·L⁻¹ of GA contributed to the apoptosis of ENKTL cells. As shown in Figs. 2A and 2B, treatment with increasing concentrations of GA resulted in a concentration-dependent increase in the cleaved-caspase-3,
Fig. 1  Inhibitory effects of gambogic acid (GA) on the growth of ENKTL SNK-6 and SNK-8 cells. (A) Structure of GA. (B) SNK-6 and SNK-8 cells on 96-well plate were stimulated by 1.5 μmol·L⁻¹ GA for 24, 48, 72, and 96 h. The values shown are means ± SD, and all assays were repeated in triplicate.

Fig. 2  Apoptotic effects of GA on SNK-6 and SNK-8 cells. (A) After the treatment with GA at 1.5 μmol·L⁻¹ for 24 h, all the cells were harvested and the total protein was extracted and prepared for SDS-PAGE. The protein levels of cleaved-Caspase-3, pro-Caspase-3, and beta-actin were analyzed by Western blotting. The values shown are means ± SD, and all assays were repeated in triplicate, **P < 0.01 vs 0 μmol·L⁻¹. (B) The cells were stained with Annexin V/PI and the apoptosis was determined by flow cytometry. The values shown are means ± SD, and all assays were repeated in triplicate, **P < 0.01 vs control.
which was the evidence of apoptotic induction. Additionally, after flow cytometry, treatment with GA (1.5 μmol·L⁻¹, 48 h) resulted in more apoptotic deaths in SNK-6 and SNK-8 cells than the control (Fig. 2B). Taken together, these results indicate that GA played a crucial role in inducing apoptosis in ENKTL cells.

**Apoptotic effects of GA on ENKTL cells through endoplasmic reticulum stress (ERS)**

To clarify whether the ERS is involved in GA-induced apoptosis in ENKTL cells, we examined the markers of ERS in SNK-6 and SNK-8 cells. As shown in Fig. 3, the addition of GA to SNK-6 and SNK-8 cells increased the levels of GRP78, ATF4, and CHOP. Taken together, the apoptotic effect of GA on ENKTL cells was through ERS.

**Inhibitory effects of GA on the activation of Akt by induction of CHOP**

To further elucidate the molecular mechanism underlying GA-induced apoptosis, we examined the phosphorylation status of Akt in SNK-6 and SNK-8 cells treated with various concentrations of GA. Based on Western blotting analyses using antibodies specific to phospho-Akt at the serine-473 sites (p-AKT), total-Akt (t-Akt), and GAPDH, the phosphorylation of Akt in GA-treated cells appeared to be inhibited in a concentration-dependent manner, compared to that in untreated control cells. Furthermore, knockdown of CHOP expression by siRNA (siCHOP) markedly reversed the dephosphorylation of Akt induced by GA, indicating that ERS inhibited Akt phosphorylation through the up-regulation of CHOP. Collectively, these results displayed that the inhibition of Akt phosphorylation resulted in the anti-proliferative effect of GA in gastric cancer cells (Fig. 4).

**Discussion**

Up to now, studies on GA have focused on various types
Fig. 4 The activity of Akt in response to GA. (A) SNK-6 and SNK-8 cells were incubated with different concentrations of GA for 24 h. Protein levels were detected by western blotting. Blots were representatives of three replicates. (B) Expression of CHOP in SNK-6 and SNK-8 cells, after transfection with si-NC (control) or siRNA CHOP (siCHOP), was detected by qRT-PCR. Data showed that siCHOP exhibited significant inhibition effect. **P < 0.01 vs control. (C) SNK-6 and SNK-8 cells were transfected with siCHOP and then incubated with 1.5 μmol·L⁻¹ GA for 24 h. The protein level of phosphorylated Akt was detected by Western blotting. The blots are typical examples of three replicates. **P < 0.01 vs control.
of cancers [14-16]. However, there are no documentations reporting the roles of GA in ENKTL and the mechanism. In this work, we evaluated the anti-proliferative effects of GA in ENKTL cells, aiming for its therapeutic use. We initiatedly demonstrated that GA exerted potent inhibitory effects on ENKTL cells. Furthermore, we revealed that GA promoted apoptosis of ENKTL cells, shown by Western blotting and flow cytometry detections.

At molecular level, we identified that the protein levels of cleaved-Caspases, which are associated with apoptosis, were increased following GA treatment in a concentration- and time-dependent manner. As to the signaling pathways, since the activity of Akt plays crucial roles in promoting the incidence and development of ENKTL carcinogenesis, several proteins involved in these pathways were examined to uncover the explicit signal transduction involved in GA mediated cellular effects.

It was observed that the levels of ERS-related proteins were increased and the phosphorylation of Akt were suppressed after the treatment of GA, indicating that the anti-proliferation effects of GA on ENKTL carcinogenesis were through ERS mediated down-regulation of Akt signaling pathway.

It has been well documented that ERS induces apoptosis in various cancer cells, and CHOP plays a potential role in ERS-induced apoptosis [17]. However, the apoptosis pathway downstream of CHOP is not well established, especially in ENKTL. Recently, it has been revealed that CHOP is involved in ERS-induced apoptosis in a variety of cells [18-19]. CHOP is identified as an important regulator of cell viability [20]. Therefore, interference with the action of CHOP may provide potential therapeutic solutions. In the present study, the expression of CHOP was elevated after the treatment of GA in a concentration- dependent manner. These findings indicated that CHOP is essential for ERS-induced apoptosis in ENKTL cells.

Until now, the molecular mechanism underlying CHOP-mediated apoptosis remains unclear [21]. It has been reported that CHOP could directly interact with Akt and suppress its phosphorylation in cancer cells [22]. On the other hand, another study has demonstrated that ERS significantly inhibits the phosphorylation of Akt [23]. In the present study, we found that siRNA mediated knockdown of CHOP markedly reversed the de-phosphorylation of Akt which had been inhibited after the intervention by GA. Therefore, it was confirmed that CHOP was the intermediate molecule mediating the inhibition of phosphorylation of Akt by ERS in ENKTL cells.

Recently, Akt signaling pathway has been reported as a key factor that is closely related to cell proliferation [24]. It is also well known that CHOP induces the transcriptional activity of Akt by mediating its phosphorylation [25]. In the present study, it was shown that Akt phosphorylation was down-regulated by ERS and CHOP induced by GA. Therefore, it was confirmed that the cell apoptosis by ERS in SNK-6 and SNK-8 cells was mediated through the CHOP triggered Akt signaling pathways. These findings may facilitate the understanding of the pathway of the apoptosis induced by GA.

In conclusion, based on the presented data, we propose the following scheme by which GA induces the apoptosis of ENKTL cells. GA induced ERS and therefore induced the expression of CHOP. Afterwards, CHOP suppressed Akt signaling pathway. Taken together, this work shed light on the apoptotic role of GA against ENKTL cells. Further exploration using animal models or clinical evaluations is required to confirm the scheme proposed in this report.

References


