Escin Sodium Induces Apoptosis of Human Acute Leukemia Jurkat T Cells

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Escin sodium has been used in the clinic as an antioedematous, antiexudative and vasoprotective agent for many years and has shown excellent tolerability. However, little is known about its anticancer activity. This is a report for the first time that escin sodium exerts a cytotoxic effect on human acute leukemia Jurkat T cells via the induction of apoptosis rather than cell cycle arrest. Escin sodium activated the initiator caspase-8, -9, and the effector caspase-3, degraded poly (ADP-ribose) polymerase (PARP) and attenuated the expression of Bcl-2. In addition, escin sodium inhibited the growth of cancer cells in a selective manner with Jurkat cells most sensitive to it. Taken together, the data show that escin sodium possesses potent apoptogenic activity toward human acute leukemia Jurkat T cells. Copyright © 2011 John Wiley & Sons, Ltd.

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INTRODUCTION

Defective apoptosis represents a major causative factor in the development and progression of cancer (Ferreira et al., 2002; Kasibhatla and Tseng, 2003). The ability of tumor cells to evade engagement of apoptosis can play a significant role in their resistance to conventional therapeutic regimens (Kasibhatla and Tseng, 2003). Therefore, induction of apoptosis in cancer cells is one of the strategies of anticancer therapy. Over the past decades, much effort has been invested into the search for agents that can differentially induce apoptotic death in cancer cells (Ferreira et al., 2002; Kasibhatla and Tseng, 2003; Sun et al., 2004; Khan et al., 2007).

Escin, a natural mixture of triterpene saponins extracted from dry ripe fruit of the traditional Chinese medicine Fructus Aesculi collected from a China dispensatory, and from dry ripe fruits of horse chestnut crude drug collected from Phamacopeia Germanica abroad, consists of A, B, C and D escin (Sirtori, 2001; Fu et al., 2005; Zhou et al., 2009). Escin sodium was launched in 1994 in China and the indication for its use was edema that had resulted from trauma or an operation (Fu et al., 2005). In addition, the major mechanisms of escin sodium in the treatments of chronic venous insufficiency (CVI) and edema have been well clarified (Frick, 2000; Sirtori, 2001). However, few reports focus on the anticancer effects of escin sodium (Zhou et al., 2009; Tan et al., 2010). Mechanisms of escin sodium inhibited growth have never been investigated in human acute leukemia Jurkat T cells.

As a natural product used in the clinic for many years, escin sodium is more readily available, affordable and safe than some new drugs, which is highly encouraging for the further development of escin sodium as an alternative to antileukemic agents.

Accordingly, the present study investigated the antiproliferative and induction of apoptotic effects of escin sodium on human acute leukemia Jurkat T cells and suggests a possible mechanism of escin sodium-induced apoptosis. The results of the MTT cell viability assay showed that escin sodium exhibited potent concentration- and time-dependent antiproliferative effects in Jurkat cells. The apoptotic effects were analysed by Hoechst 33258 staining, DNA fragmentation assay and Annexin V-FITC/PI (propidium iodide) staining assay. Morphological evidence of apoptosis, a significant increase of Annexin V+ and PI− cells (early apoptotic) and apoptotic DNA fragmentation, were observed in cells treated with escin sodium. Escin sodium activated caspase-8, -9, and the effector caspase-3, degraded PARP. In addition, escin sodium decreased the expression of anti-apoptotic protein (Bcl-2). These results indicate that escin sodium is a potent natural inhibitor of proliferation and inducer of apoptosis in Jurkat cells.

MATERIALS AND METHODS

Abs and reagents. Escin sodium (Lot: ZL-09010A), consisting of escin sodium A, B, C and D, was obtained from Luye Pharmaceutical Company (Yantai, China) (Fig. 1). Anti-caspase-8, anti-caspase-9, anti-caspase-3,
anti-PARP, anti-Bcl-2, anti-Bax, anti-Bcl-XL and anti-GAPDH were purchased from Cell Signalling Technology (Beverly, MA). Horseradish peroxidase-conjugated secondary antibodies (goat-anti-rabbit and goat-anti-mouse) were obtained from Santa Cruz Biotechnology (CA, USA). (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ribonuclease (RNase), proteinase K and propidium iodide (PI) were purchased from Sigma–Aldrich (St Louis, MO, USA). Hoechst 33258 was purchased from KeyGEN Biotechnology, (Nanjing, China). All other reagents and chemicals used were of analytical grade.

**Cell culture.** The human acute leukemia Jurkat T cells (clone E6–1), human lung adenocarcinoma A549 cells, human ovarian carcinoma SK-OV-3 cells, obtained from the American Type Culture Collection, and human hepatocellular carcinoma HepG2 cells, obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). The human pancreatic carcinoma PANC-1 cells, human neuroglioma SHG-44 cells and human breast cancer MDA-MB-231 cells, obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, were maintained in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). All cells were cultured in a humidified atmosphere with a 5% CO₂ incubator at 37 °C.

**MTT assay.** Cell viability was assessed using the MTT assay. The cells (5 × 10⁴ for Jurkat cells, 1 × 10⁵ for HepG2 cells, 7 × 10⁴ for PANC-1 cells, 6 × 10⁴ for SHG-44 cells, 4 × 10⁵ for A549 cells, 1 × 10⁴ for MDA-MB-231 cells and 5 × 10³ for SK-OV-3 cells) were placed in 100 μL of media with or without indicated concentrations of escin sodium in each well of a 96-well flat-bottomed microtiter plate in triplicate cultures and incubated for the indicated time at 37 °C in an incubator with a 5% CO₂ atmosphere. MTT was prepared at 5 mg/mL in phosphate-buffered saline (PBS) and added to each well. The cell cultures were continued for another 4 h at 37 °C. DMSO was added to each well, and the absorbance was measured at 570 nm and 630 nm wavelengths using a microculture plate reader. The cell viability was expressed as a percentage of absorbance in cells with indicated treatments to that in cells with medium control treatment.

**Detection of apoptotic cells by Annexin V-FITC and PI staining.** Cells were stained simultaneously with FITC-conjugated Annexin V and PI to enable the discrimination of intact cells (A⁻/PI⁻), early apoptotic cells (A⁺/PI⁻) and late apoptotic or necrotic cells (A⁺/PI⁺). Briefly, 1 × 10⁶ Jurkat cells were stained with annexin V-FITC and PI for 15 min in the dark and analysed by a FACSScan laser flow cytometer (FACSCalibur, Becton Dickinson, USA).

**Detection of morphological apoptosis.** Jurkat cells were seeded in 24-well culture dishes overnight prior to treatment with escin sodium for 24 h. Apoptotic nuclear morphology was assessed using Hoechst 33258. After harvesting, the cells were washed twice with PBS, and fixed with 4% formaldehyde for 30 min at 4 °C, and then washed and stained with Hoechst 33258 at room temperature for 10 min. The cells were again washed and resuspended in PBS for morphological observation with a fluorescence microscope (Zeiss Axio Observer A1) at 340 nm to determine nuclei fragmentation and chromatin condensation.

**DNA fragmentation assay.** Jurkat cells (1 × 10⁶) were seeded in 6-well culture dishes overnight and treated with the indicated concentrations of escin sodium for 17 h, and then collected by centrifugation at 367 × g for 5 min at 4 °C. The pellets were lysed by DNA lysis buffer (20 mM EDTA, 100 mM Tris, pH 8.0, 0.8% (w/v) SDS) and then incubated with RNase (125 U/mL) for 2 h at 37 °C, followed by incubated with proteinase K (5 mg/mL) at 50 °C overnight. Finally, the mixture was centrifuged and

![Figure 1. Chemical structure of escin sodium.](image_url)
Cell cycle analysis. Jurkat cells were seeded in 6-well culture dishes overnight and treated with the indicated concentrations of escin sodium for 24 h. The cells were harvested, washed twice in PBS, and lysed for 5 min at 4 °C with ice cold RIPA buffer (1% NP-40 in 150 mM NaCl, 50 mM Tris (pH 7.5), 2 mM EDTA). The equalized amounts of proteins from each sample were subjected to SDS-polyacrylamide gel electrophoresis. Protein bands were then transferred to polyvinylidene difluoride membranes. The membranes were blocked in TBBS (PBS with 0.05% Tween 20) containing 5% (w/v) nonfat dry milk, washed in TBPS and then incubated with primary antibody. After washing, the membranes were incubated with secondary antibody conjugated with IgG horseradish peroxidase (HRP). Immune complexes were detected by the enhanced chemiluminescence system. GAPDH was used as the loading control.

Statistical analysis of data. Three or more separate experiments were performed. All statistical analysis was performed using the software SPSS 11.5 for Windows (Chicago, IL, USA). Statistical comparisons were made by Student’s t-test. Data were expressed as mean ± SD. Values of p < 0.05 indicated statistical significance.
proenzyme and the appearance of the 37 kDa form of cleaved caspase-9. Moreover, exposure of Jurkat cells to escin sodium led to a reduction of the protein level of the 32 kDa caspase-3 precursor. As a downstream target of active caspase-3 during induction of apoptosis, PARP has been reported to be cleaved into two fragments (Nicholson et al., 1995). The detection of the degradation of PARP coincided with the activation of caspase-3. The amount of 116 kDa PARP decreased and the 85 kDa degraded product increased after treatment with escin sodium for 14 h. All these results strongly indicated that the activation of caspase cascade was involved in escin sodium mediated Jurkat cells apoptosis.

**Intrinsic pathway is involved in escin sodium-induced apoptosis**

The Bcl-2 protein family has been demonstrated to play a critical role in the regulation of apoptosis. This large family comprises apoptosis regulating proteins that modulate the intrinsic (mitochondrial) pathway, including antiapoptotic proteins, such as Bcl-2, Bcl-XL; and other proapoptotic proteins, such as Bax (Brunelle and Letai, 2009). Bcl-2 is an upstream effector molecule in the apoptotic pathway and is identified as a potent suppressor of apoptosis. Bcl-2 has been shown to form a heterodimer complex with the proapoptotic member Bax, thereby neutralizing its proapoptotic effects. Therefore, the ratio of Bax: Bcl-2 is a decisive factor and plays an important role in determining whether cells will undergo death or survival (Elmore, 2007; Gustafsson and Gottlieb, 2007). So, the expression of Bcl-2 in control and escin sodium-treated whole cell extracts was determined. Western blot analysis clearly showed a concentration-dependent suppression of Bcl-2 expression (Fig. 4b). However, Bax gene is mutated in Jurkat cells and consequently these cells do not have Bax protein (Meijerink et al., 1998; Shawgo et al., 2008) (Fig. 4b). As a result, the decrease of Bcl-2 expression in
Jurkat cells reflected an increase of the ratio of Bax: Bcl-2 to some extent. In contrast, escin sodium did not affect the expression of Bcl-XL, another proapoptotic protein (Fig. 4b). The reduction of Bcl-2 expression, together with the activation of caspase-9 described above, suggested that escin sodium-induced apoptotic cell death was mediated through the intrinsic pathway.

**Cell cycle phase distribution analysis of escin sodium-treated cells**

To assess the correlation between escin sodium-induced proliferation inhibition and cell cycle blockage, the effects of the escin sodium on cell cycle distribution were determined by flow cytometry. It was found that 20, 30 μg/mL escin sodium, which exerted cytotoxic effect on Jurkat cells, did not produce cell cycle arrest (Fig. 5a). However, lower concentration of escin sodium caused a concentration dependent increase in the G0/G1 phase cell population slightly, accompanied by a concomitant decrease in the S and G2/M phases’ cell population. As shown in Fig. 5b, the results indicated that, compared with the control, 2.5, 5, 10 μg/mL escin sodium increased the population of G0/G1 phase from 46.44% ± 2.16% to 48.37% ± 1.84%, 52.93% ± 0.74%, 57.30% ± 1.51% after 24 h treatment, respectively.

![Figure 3](image-url)
Cytotoxic effects of escin sodium on several cancer cells

Since escin sodium has shown its potential as an antileukemic agent, its cytotoxic effects on a range of cancer cells were further investigated. The cells were treated with 20–80 μg/mL escin sodium for 72 h, except Jurkat cells and HepG2 cells for 48 h, and then MTT assays were carried out (Fig. 6). At the highest concentration of 80 μg/mL, significant cytotoxic effects were observed in five out of the six solid tumor cell lines incubated with escin sodium (p < 0.05), while human ovarian carcinoma SK-OV-3 cells were absolutely resistant to it. Reductions in cell viability occurred in all the tumor cells sensitive to escin sodium except SHG-44 incubated with 60 μg/mL escin sodium. In addition, reductions of cell viability were observed at 40 μg/mL for A549, PANC-1 and HepG2 cells. Compared with the solid tumor cells, Jurkat cells were most sensitive to escin sodium. At the lowest concentration of 20 μg/mL, a mean reduction of 64.36% cell viability was observed in Jurkat cells, and at the higher concentrations, the viabilities of Jurkat cells were almost negligible.

DISCUSSION

Apoptosis is one of the most vital pathways through which chemo preventive agents inhibit the overall growth of cancer cells (Ferreira et al., 2002). Our understanding of the complexities of apoptosis and the mechanisms evolved by tumor cells to resist engagement of cell death has focused research effort into the development of strategies designed selectively to induce apoptosis in cancer cells (Kasibhatla and Tseng, 2003). Therefore, this study evaluated the chemo preventive potential of escin sodium in human acute leukemia Jurkat T cells, as well as their effects on apoptosis, and suggest a possible mechanism of Jurkat induced apoptosis.

The growth-inhibitory activity of escin sodium for Jurkat cells was exerted in both time- and concentration-dependent manners. Treatment of Jurkat cells with escin sodium resulted in a markedly increased accumulation of sub-G1 phase cells in a concentration-dependent manner. The induction of apoptotic effects of escin sodium on Jurkat cells was confirmed by Hoechst 33258 staining, DNA fragmentation assay and annexin V-FITC/PI staining assay. Nuclear condensation and granular apoptotic bodies were observed by Hoechst 33258 staining. After escin sodium treatment, Jurkat cells exhibited a loss of DNA integrity, showing a typical ladder pattern of multiples of 180–200 base pairs characteristic of apoptosis. Cells treated with escin sodium showed a decrease of vital cells (A−/PI−) and a significant increase of annexin V staining (A+/PI+) (Lazebnik et al., 1994). Furthermore, the mechanism underlying escin sodium-mediated apoptosis in Jurkat cells was investigated. Western blot experiments indicated that expression of Jurkat cells to escin sodium strongly stimulated the activation of upstream caspase-8 and caspase-9 in a concentration-dependent manner. In addition, after treatment with escin sodium, the cleavage of PARP was detected along with the activation of caspase-3. This cleavage leads to inactivation of PARP, thus preventing a futile DNA repair cycle (Lazebnik et al., 1994). Escin sodium markedly decreased Bcl-2 levels, which led to an increase in the Bax/Bcl-2 ratio.

Apoptotic cell death can be induced through the extrinsic or the intrinsic signaling pathways that are ultimately coupled to the activation of effector caspases (Gupta, 2003). In the extrinsic death receptor pathway, ADD recruits pro-caspase-8 by protein–protein interaction via homologous death effector domain (DED) to form a death-inducing signal complex (DISC). During DISC formation, pro-caspase-8 is autolytically cleaved to yield active caspase-8 (Nunez et al., 1998). The activation of caspase-8 in escin sodium treated-Jurkat
Cells indicated the involvement of an extrinsic apoptotic pathway. Apoptosis mediated through the mitochondrial pathway is regulated by the Bcl-2 family of proteins (Elmore, 2007). The increased ratio of the pro-apoptotic to anti-apoptotic Bcl-2 family members would elicit cytochrome c release, which is associated with Apaf-1 and procaspase-9 to form the apoptosome. Dimerization of caspase-9 induced the loss of the mitochondrial membrane potential (Chen et al., 2007). Knock-out of caspase-9 has been shown to delay the loss of mitochondrial membrane potential (Hakem et al., 1998). In the present study, caspase-9 activation, in accordance with the down-regulated expression of Bcl-2, suggested that mitochondrial signal pathways might be involved in this escin sodium-induced Jurkat cells apoptosis. Taken together, these data demonstrated that the apoptotic effect of escin sodium on Jurkat T cells was exerted by the activation of caspase-8, and the subsequent induction of mitochondria-dependent activation of caspase cascade.

The dose (20, 30 μg/mL) of escin sodium which exhibited a cytotoxic effect on Jurkat cells did not arrest cell cycle, while the dose (2.5, 5, 10 μg/mL) of escin sodium which arrested Jurkat cells in G0/G1 phase did not affect cell viability. These results suggested that the cell cycle arrest of Jurkat cells in G0/G1 phase by escin sodium did not account for cell killing and the growth suppression of Jurkat cells by escin sodium was not mediated via alterations in the cell cycle. Escin sodium exhibited various cytotoxic effects on different cancer cells, with Jurkat cells most sensitive to it and SK-OV-3 cells completely resistant to it. The

![Figure 5. Effect of escin sodium on cell cycle distribution.](image-url)

antitumor activity of escin sodium against HepG2 cells was consistent with the previous study (Zhou et al., 2009). Most of the cancer cell lines tested are sensitive to escin sodium. However, the mechanisms by which escin sodium elicits its chemo preventive properties remain largely unknown. We report here that escin sodium induced apoptosis of human acute leukemia Jurkat T cells with both extrinsic and intrinsic pathways involved. A recent paper reported that \( \beta \)-escin induces apoptosis in human hepatocellular carcinoma cells via inhibition of the STAT3 signaling pathway (Tan et al., 2010). Bcl-2 can block cell death induced by a variety of chemotherapeutic agents, in parallel with an increase in chemo resistance (Seitz et al., 2009). The down-regulation of the expression of Bcl-2 is likely linked with the ability of escin sodium to induce apoptosis in Jurkat cells and hepatocellular carcinoma cells (Zhou et al., 2009; Tan et al., 2010). The various cytotoxic effects of escin sodium on different cancer cells suggested that escin sodium exerted anticancer activity not extensively but selectively. However, the mechanism underlying escin sodium-mediated anticancer specificity remains unclear. Therefore, the focus of our next work is to find the target of escin sodium exactly.

In conclusion, this is the first report to demonstrate that escin sodium exerts a cytotoxic effect on human acute leukemia Jurkat T cells via the induction of apoptosis rather than arrest of the cell cycle. Escin sodium inhibits the growth of cancer cells in a selective manner. The apoptosis inducing ability of escin sodium, in conjunction with its excellent tolerability and safety profiles proven in clinical studies, could make it a potentially effective preventive and/or therapeutic agent against leukemia; however, additional experiments are necessary to better define the involvement of escin sodium and Jurkat cells in the specific activation of the apoptotic pathways. Further \textit{in vivo} evaluation of the antileukemic activity of escin sodium in a T-cell acute lymphoblastic leukemia xenograft model is now ongoing in our laboratories.

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**Conflict of Interest**

The authors have declared that there are no conflict of interest.

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