

Uvangoletin induces mitochondria-mediated apoptosis in HL-60 cells *in vitro* and *in vivo* without adverse reactions of myelosuppression, leucopenia and gastrointestinal tract disturbances

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Abstract. This study investigated the cytotoxic effect of uvangoletin on HL-60 cells, and the effects of uvangoletin on myelosuppression, leucopenia, gastrointestinal tract disturbances and the possible cytotoxic mechanisms by using CCK-8, flow cytometry, western blot, xenograft, cyclophosphamide-induced leucopenia, copper sulfate-induced emesis and ethanol-induced gastric mucosal lesions assays. The results of CCK-8, flow cytometry and western blot assays indicated that uvangoletin showed the cytotoxic effect on HL-60 cells and induced the apoptosis of HL-60 cells by downregulating the expression levels of anti-apoptotic proteins (Survivin, Bcl-x1 and Bcl-2), upregulating the expression levels of pro-apoptotic proteins (Smac, Bax, Bad, c-caspase-3 and c-caspase-9), and promoting the release of cytochrome c from mitochondria to cytoplasm. Further, the results of xenograft assay suggested that uvangoletin inhibited the HL-60-induced tumor growth without adverse effect on body weight of nude mice *in vivo* by regulating the expression levels of above apoptotic proteins. The results indicated that the reductions of WBCs count and thighbone marrow granulocytes percentage in cyclophosphamide-induced leucopenia assay, the incubation period and number of emesis in copper sulfate-induced emesis assay and the gastric mucosal lesions in ethanol-induced gastric mucosal lesions assay were not

exacerbated or reversed by uvangoletin. In conclusion, the research preliminarily indicated that uvangoletin induced apoptosis of HL-60 cells *in vitro* and *in vivo* without adverse reactions of myelosuppression, leucopenia and gastrointestinal tract disturbances, and the pro-apoptotic mechanisms may be related to mitochondria-mediated apoptotic pathway.

Introduction

Leukemia (cancer of the blood) is a malignant disease of hemopoietic tissue. If the progenitor cells of white blood cells (WBCs) lose the ability to differentiate and mature, the malignant clonal hyperplasia and accumulation of the progenitor cells of WBCs will occur in bone marrow and other hematopoietic tissues, and then the liver, spleen, lymph nodes and other tissues or organs are invaded and destroyed. Finally, the normal hematopoietic function of body is inhibited resulting in leukemia (1-3). Leukemia is one of the leading causes correlated with death of people with malignant cancer (4). Although the diagnosis and therapy of leukemia are rapidly developed (5), there is still high mortality in leukemia patients (6,7).

Chemotherapy is an important therapy for treating leukemia (8,9). Although chemotherapy has been successfully used to treat leukemia, there are still many problems in chemotherapy of leukemia such as the adverse reactions of myelosuppression, leucopenia and gastrointestinal tract disturbances (emesis and gastric mucosal lesions) (10-12). Therefore, exploring new, effective and safe chemotherapy drugs to treat leukemia is important and urgent.

Sarcandrae Herba, also named as Zhongjiefeng, is derived from the whole grass of *Sarcandra glabra* (Thunb.) Nakai (Chloranthaceae). Many studies indicated that Zhongjiefeng can be used to treat cancer (13-16). It was reported that uvangoletin, a dihydrochalcones from Zhongjiefeng, shows cytotoxic effect on human promyelocytic leukemia (HL-60) cells in MTT assay (17). Based on the previous studies, our

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team further investigated the cytotoxic mechanisms of uvangoletin on HL-60 cells by CCK-8, flow cytometry, western blot and xenograft assays. Furthermore, the effects of uvangoletin on myelosuppression, leucopenia and gastrointestinal tract disturbances were assessed by cyclophosphamide-induced leucopenia, copper sulfate-induced emesis and ethanol-induced gastric mucosal lesions assays.

Materials and methods

Plant material. Zhongjiefeng was purchased from Chinese herbal medicine market in Bozhou, Anhui province, China in 2011 and identified by Rong Gong. A voucher specimen for *Sarcandra glabra* is stored in Shanxi Academy of Medical Sciences for future reference.

Chemicals and reagents. Analytical grade ethanol, cyclohexane, chloroform, ethyl acetate and silica-gel were purchased from Qingdao Haiyang Chemical Co., Ltd. (Qingdao, China). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and RPMI-1640 media were purchased from Invitrogen (Carlsbad, CA, USA). Cell Counting Kit-8 (CCK-8 kit), Annexin V-FITC/PI apoptosis assay kit and Enhanced BCA Protein Assay kit were obtained from Beyotime Biotechnology (Haimen, China). Primary antibodies for β -actin, Survivin, Bcl-x1, Bcl-2, Smac, Bax, Bad, cytochrome c, cleaved-caspase-3 (c-caspase-3) and c-caspase-9 were purchased from Abcam (Cambridge, UK). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody was obtained from Cell Signaling Technology (Beverly, MA, USA). Cyclophosphamide and copper sulfate were obtained from the 12th Shanghai pharmaceutical factory and Zhengzhou chemical reagent first factory.

Animals. Nude mice (5-6 weeks old, 20 ± 2 g), and rats (200 ± 20 g) were purchased from the SLRC Laboratory Animal Company (Shanghai, China). Pigeons (300-450 g) were obtained from Henan medical university laboratory animal center. All animal treatments were strictly consistent with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. All assays were carried out with the approval of the Second Hospital of Shanxi Medical University (protocol no. SXMU2013049).

Extraction and isolation of uvangoletin. *Sarcandrae Herba* was extracted thrice for 1 h with refluxing 95% ethanol, and then the 95% ethanol was combined and concentrated under reduced pressure to afford a crude extract. Then the crude extract was subjected to column chromatography over silica-gel column (100-200 mesh), eluted with systemic gradient of cyclohexane-ethyl acetate to give 6 fractions. Fraction 4 was subjected to column chromatography over silica-gel column (100-200 mesh), eluted with chloroform-ethyl acetate to give uvangoletin. The purity and structure of uvangoletin were verified and identified by HPLC and nuclear magnetic resonance (NMR) data. Each 100 kg *Sarcandrae Herba* can produce 3.06 g uvangoletin. The extraction and isolation of uvangoletin were completed in Push Bio-Technology (Chengdu, China).

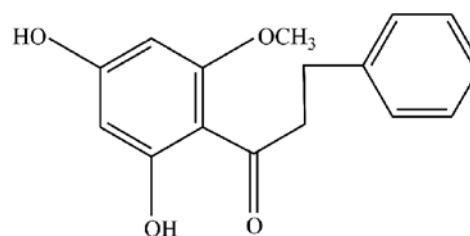


Figure 1. Chemical structure of uvangoletin.

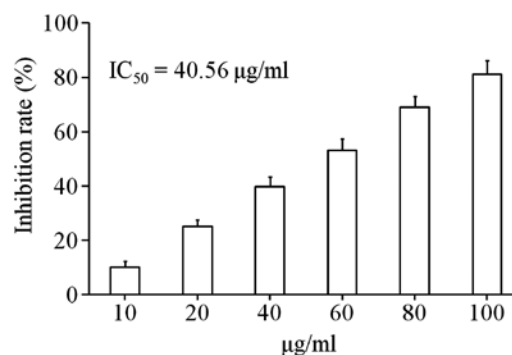


Figure 2. Cytotoxic effects of uvangoletin on HL-60 cells.

Uvangoletin was dissolved in 0.5% DMSO to give appropriate concentration for different assays.

Cell culture. HL-60 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with antibiotics (100 U/ml streptomycin and 100 U/ml penicillin) and 10% FBS at 37°C in 5% CO₂/95% air. HL-60 cells were sub-cultured until reaching logarithmic growth phase.

Cytotoxic assay. The cytotoxic effect of uvangoletin on HL-60 cells was assessed by CCK-8 assay. Briefly, HL-60 cells were seeded on 96-well culture plates (2×10^4 /well) with RPMI-1640 medium. After incubation for 4 h, HL-60 cells were treated with uvangoletin (0, 10, 20, 40, 60, 80 and 100 µg/ml) for 48 h and 0 µg/ml was considered as control group. CCK-8 was added to the 96-well culture plates, and cells were cultured for another 4 h. Then the optical densities (OD) of control and treatment groups were measured at 450 nm using Bio-Rad Model 680 microplate reader (Hercules, CA, USA). The cytotoxic effect of uvangoletin on HL-60 cells was evaluated by the inhibition rate, calculated as in Eq 1. Inhibition rate (%) = [(OD control - OD treatment)/OD control] x 100 (1).

Apoptosis assay. After treatment with uvangoletin (0, 20, 40 and 60 µg/ml) for 48 h, HL-60 cells were harvested and then washed with phosphate-buffered saline (PBS) solution. The washed HL-60 cells were re-suspended in cell staining buffer and then stained with Annexin V-FITC/PI. Subsequently, the stained HL-60 cells were analyzed by FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). The Annexin V-positive and PI-negative cells were considered as the early apoptotic cells, and the Annexin V-positive and

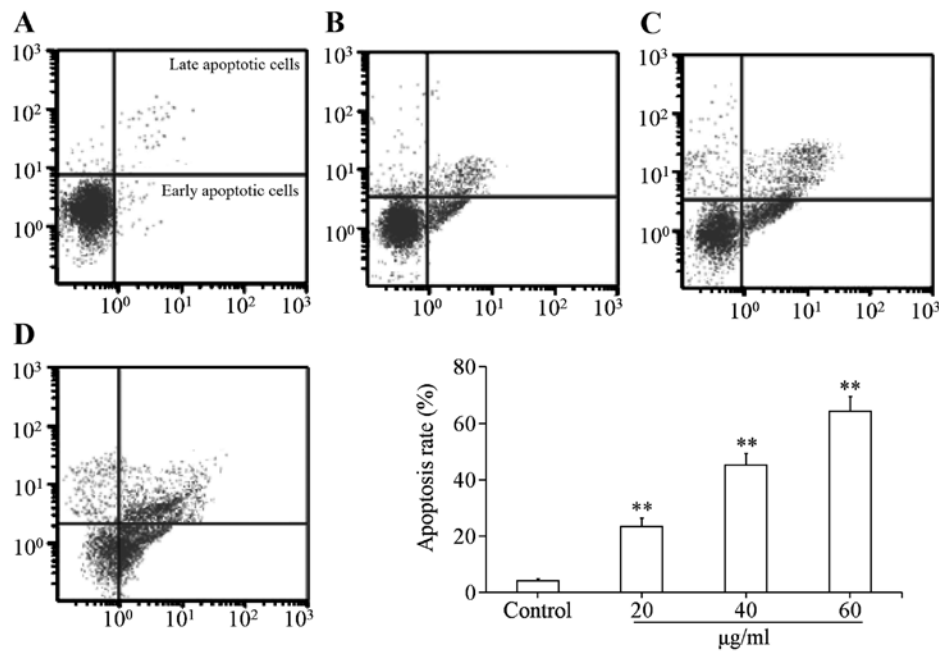


Figure 3. Effects of uvangoletin on the apoptosis of HL-60 cells. (A-D) Control, 20 $\mu\text{g/ml}$ uvangoletin, 40 $\mu\text{g/ml}$ uvangoletin and 60 $\mu\text{g/ml}$ uvangoletin. The apoptotic cells are the sum of early apoptotic cells plus late apoptotic cells. ** $p < 0.01$, compared with control group.

PI-positive cells were considered as the late apoptotic cells. The apoptotic cells were the sum of the early apoptotic cells plus the late apoptotic cells.

Western blot assay. After treatment with uvangoletin (0, 20, 40 and 60 $\mu\text{g/ml}$) for 48 h, total proteins of HL-60 cells were extracted, and its concentration was determined by Enhanced BCA Protein Assay kit. Total proteins ($\sim 40 \mu\text{g}$) were separated by 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS/PAGE) and then transferred to PVDF membrane. After being blocked with 5% fat-free milk, the PVDF membrane was incubated with corresponding primary antibodies and subsequently with HRP-conjugated goat anti-rabbit antibody. Lastly, the proteins were detected by chemiluminescence. β -actin was used as internal reference standard for western blot assay.

Xenograft assay. Nude mice were randomly divided into two groups ($n=8$): control group and uvangoletin group. HL-60 cells (2×10^6 cells/mouse) were subcutaneously injected in the right flank of nude mice. When the HL-60-induced tumors grew to appropriate diameter (2-3 mm), the nude mice were treated with 0.5% DMSO in control group and 40 mg/kg uvangoletin in uvangoletin group once a day for 20 days by intragastric administration (i.g.). Then the width and length of tumor and body weight of nude mice were separately measured on days 0, 5, 10, 15 and 20 by vernier caliper and electronic balance. Then, the nude mice were immediately sacrificed, and their tumor tissues were used for western blotting. The tumor volume was calculated as in Eq 2. Tumor volume = (width² x length)/2 (2).

Cyclophosphamide-induced leucopenia assay. Mice were randomly divided into five groups ($n=10$): normal group, control group and uvangoletin (20, 40 and 60 mg/kg) groups.

Control and uvangoletin groups were treated with 80 mg/kg cyclophosphamide once a day for 3 days by intraperitoneal injection (i.p.), and normal group was treated with normal saline once a day for 3 days by i.p. Whereas, normal and control groups were treated with 0.5% DMSO once a day for 7 days by i.g., and uvangoletin groups were treated with 20, 40 and 60 mg/kg uvangoletin once a day for 7 days by i.g. After 2 h of treatment on the 7th day, mice were immediately sacrificed to obtain thighbone marrow and orbital blood. Thighbone marrow was smeared and stained by Wright's stain, and then it was monitored by a microscope (18). The effects of uvangoletin on cyclophosphamide-induced myelosuppression were evaluated by thighbone marrow granulocytes percentage. Orbital blood was used to determine the WBC count, and evaluation of the effects of uvangoletin on cyclophosphamide-induced leucopenia (19).

Copper sulfate-induced emesis assay. Pigeons were randomly divided into four groups ($n=10$): control group and uvangoletin (20, 40 and 60 mg/kg) groups. Control group was treated with 0.5% DMSO once a day for 7 days by i.g., and uvangoletin groups were treated with 20, 40 and 60 mg/kg uvangoletin once a day for 7 days by i.g. Then copper sulfate (300 mg/kg) was administered orally to pigeons of all groups after 0.5 h of treatment on the 7th day. Finally, the effects of uvangoletin on copper sulfate-induced emesis were evaluated by incubation period and number of emesis within 1 h.

Assay of ethanol-induced gastric mucosal lesions. Rats were randomly divided into four groups ($n=10$): control group and uvangoletin (20, 40 and 60 mg/kg) groups. All groups were treated according to the treatments in copper sulfate-induced emesis assay. Rats were managed with ad libitum, but had free access to water for 12 h on the 7th day. After 2 h of treatment on the 7th day, rats were administered orally with 0.8 ml 75%

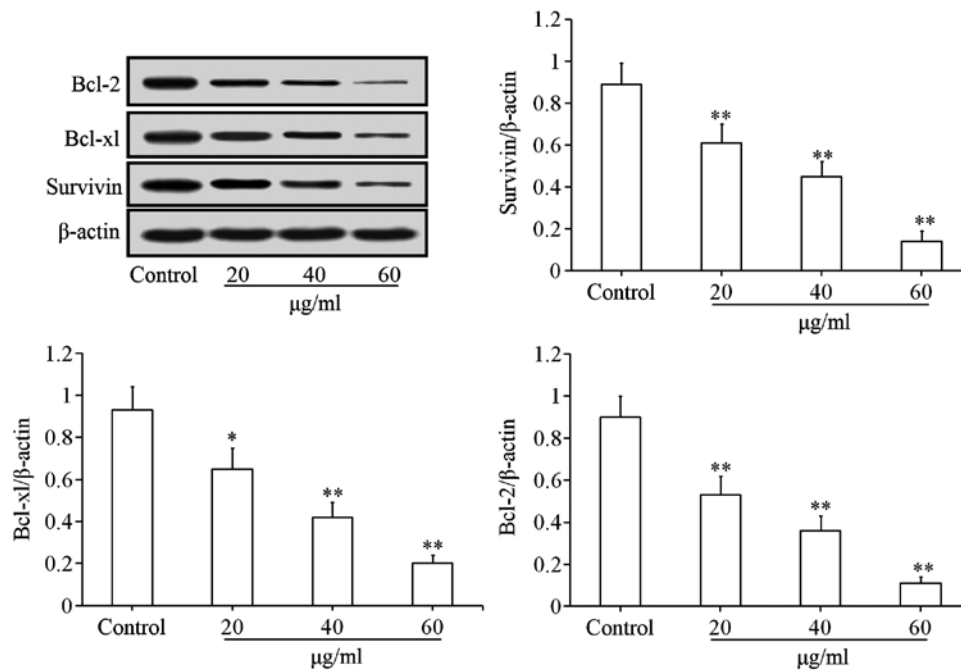


Figure 4. Effects of uvangoletin on the expression levels of anti-apoptotic proteins (Survivin, Bcl-xl and Bcl-2) in HL-60 cells. * $p < 0.05$, ** $p < 0.01$, compared with control group.

Table I. Effects of uvangoletin on the WBC count and thighbone marrow granulocytes percentage in cyclophosphamide-induced leucopenia model.

Groups	WBC count ($10^9/l$)	Thighbone marrow granulocytes percentage (%)
Normal	117.3±16.1	67.5±9.1
Control	68.7±14.8 ^a	57.4±4.9 ^a
20 mg/kg uvangoletin	70.3±15.2	54.4±3.8
40 mg/kg uvangoletin	66.7±13.9	59.3±5.2
60 mg/kg uvangoletin	73.7±16.1	58.5±4.6

^a $p < 0.01$, compared with normal group.

ethanol. After another 1 h, rats were immediately sacrificed and the stomach was fixed by 1% formaldehyde for 0.5 h. The gastric mucosal lesions were directly observed. Ulcer index, used to evaluate the effects of uvangoletin on 75% ethanol-induced gastric mucosal lesions, was calculated according to the existing method and gastric mucosal lesions area (mm^2) (20).

Statistical analysis. All data are presented as mean \pm standard deviation. The difference between two groups was analyzed by one-way ANOVA on SPSS 21.0. The difference was considered as statistically significant when p -value was < 0.05 .

Results

Purity and identification of uvangoletin. The purity of target analyte was verified by area normalization method of HPLC,

and the results indicated that its purity was $>99.3\%$. Then the target analyte was identified as uvangoletin (Fig. 1) by comparing its NMR data with the existing literature (21,22).

Uvangoletin shows cytotoxic effect on HL-60 cells. The cytotoxic effect of uvangoletin on HL-60 cells was assessed by CCK-8 assay. As shown in Fig. 2, after treatment with uvangoletin (0, 10, 20, 40, 60, 80 and 100 $\mu g/ml$) for 48 h, the cytotoxic effect of uvangoletin on HL-60 cells was significantly observed, and the IC_{50} value was 40.56 $\mu g/ml$. Moreover, the cytotoxic effect of uvangoletin on HL-60 cells was positively related to its concentration.

Uvangoletin induces apoptosis of HL-60 cells. The flow cytometry analysis was used to investigate whether the cytotoxic effect of uvangoletin on HL-60 cells was related to apoptosis. As shown in Fig. 3, after treatment with uvangoletin (0, 20, 40 and 60 $\mu g/ml$) for 48 h, apoptosis of HL-60 cells was

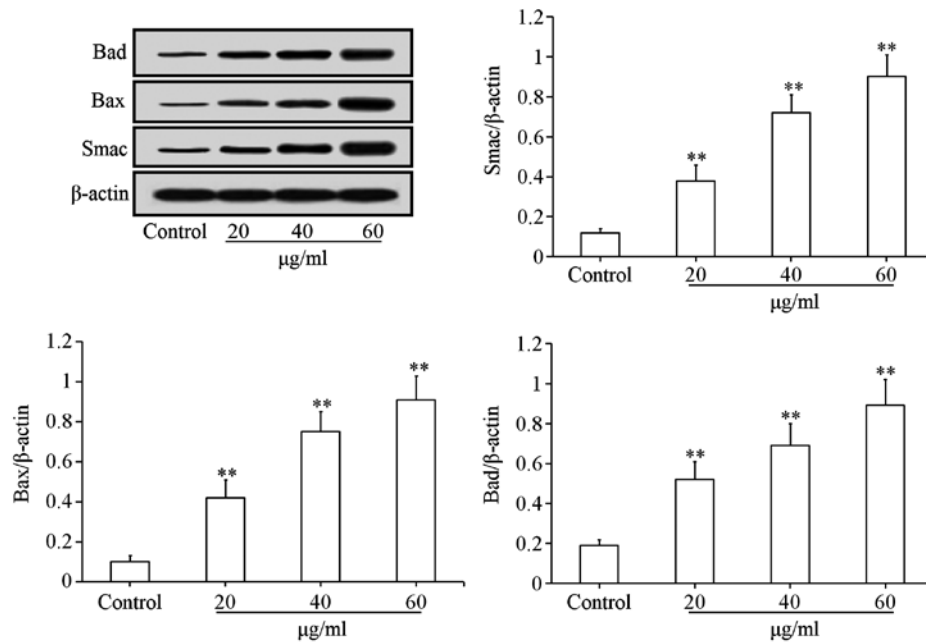


Figure 5. Effects of uvangoletin on the expression levels of pro-apoptotic proteins (Smac, Bax and Bad) in HL-60 cells. ** $p < 0.01$, compared with control group.

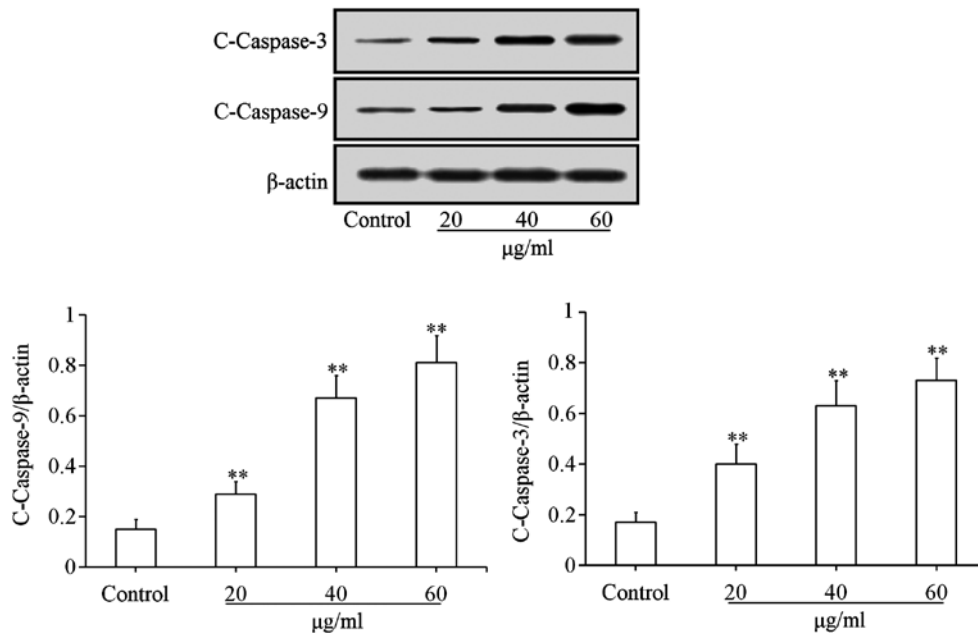


Figure 6. Effects of uvangoletin on the expression levels of pro-apoptotic proteins (c-caspase-3 and c-caspase-9) in HL-60 cells. ** $p < 0.01$, compared with control group.

significantly ($p < 0.01$) induced, compared with control group. The results indicated that the cytotoxic effect of uvangoletin on HL-60 cells was related to apoptosis.

Effects of uvangoletin on mitochondria-mediated apoptotic proteins. To investigate the pro-apoptotic mechanisms of uvangoletin on HL-60 cells, the effects of uvangoletin on the expression levels of anti-apoptotic proteins (Survivin, Bcl-x1 and Bcl-2) and pro-apoptotic proteins (Smac, Bax, Bad, c-caspase-3 and c-caspase-9), and the release of cytochrome c from mitochondria to cytoplasm in HL-60 cells

were studied by western blot assay. As shown in Fig. 4, after treatment with uvangoletin (0, 20, 40 and 60 $\mu\text{g/ml}$) for 48 h, the expression levels of Survivin, Bcl-x1 and Bcl-2 in HL-60 cells were significantly ($p < 0.01$ or $p < 0.05$) downregulated, compared with control group. As shown in Figs. 5 and 6, after treatment with uvangoletin (0, 20, 40 and 60 $\mu\text{g/ml}$) for 48 h, the expression levels of Smac, Bax, Bad, c-caspase-3 and c-caspase-9 in HL-60 cells were significantly ($p < 0.01$) upregulated, compared with control group. As shown in Fig. 7, after treatment with uvangoletin (0, 20, 40 and 60 $\mu\text{g/ml}$) for 48 h, the release of cytochrome c from mitochon-

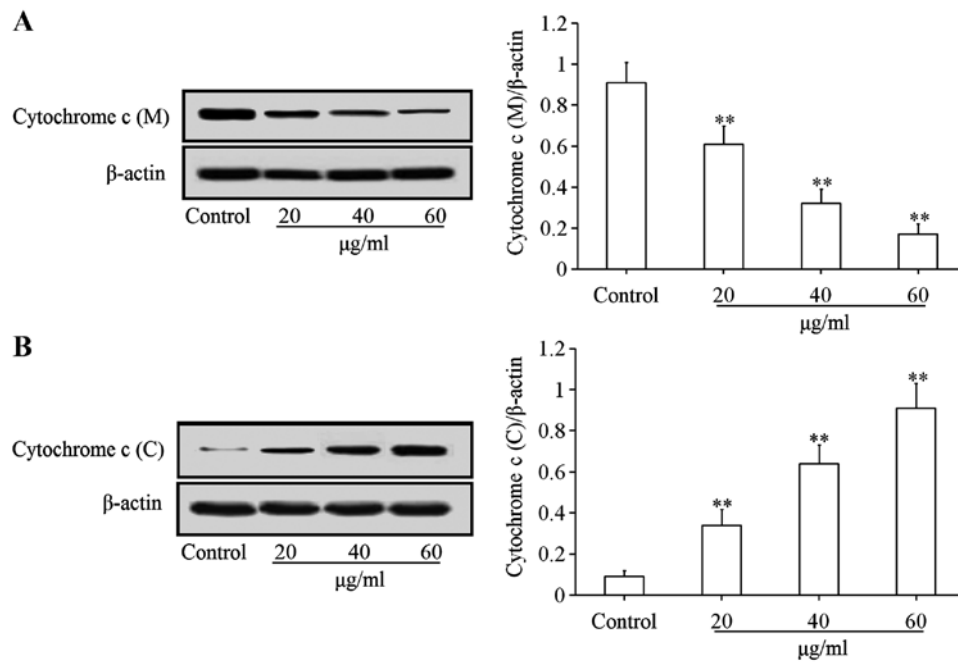


Figure 7. Effects of uvangoletin on the release of cytochrome c from mitochondria to cytoplasm in HL-60 cells. (A and B) Cytochrome c in mitochondria [Cytochrome c (M)] and in cytoplasm [Cytochrome c (C)]. ** $p < 0.01$, compared with control group.

Table II. Effects of uvangoletin on the incubation period and number of emesis in copper sulfate-induced emesis model and the ulcer index in 75% ethanol-induced gastric mucosal lesion model.

Groups	Incubation period of emesis (min)	Number of emesis (with 1 h)	Ulcer index
Control	16.2 \pm 5.3	54.7 \pm 13.1	63.7 \pm 12.5
20 mg/kg uvangoletin	15.9 \pm 4.8	53.1 \pm 12.8	64.8 \pm 13.6
40 mg/kg uvangoletin	17.1 \pm 5.7	49.5 \pm 12.4	59.7 \pm 11.2
60 mg/kg uvangoletin	16.4 \pm 4.9	50.3 \pm 14.6	65.4 \pm 15.4

dria to cytoplasm in HL-60 cells was significantly ($p < 0.01$) increased, compared with control group.

Effects of uvangoletin on HL-60-induced tumors in nude mice. Xenograft assay was used to study the effects of uvangoletin on HL-60-induced tumors *in vivo*. After treatment with 40 mg/kg uvangoletin once a day for 20 days, the HL-60-induced tumor volumes in nude mice were significantly ($p < 0.01$ or $p < 0.05$) inhibited (Fig. 8B), compared with control group, and the growth of body weight of nude mice was not affected by the treatment (Fig. 8A). Additionally, the expression levels of anti-apoptotic proteins (Survivin, Bcl-x1 and Bcl-2) in tumor tissues were significantly ($p < 0.01$) downregulated (Fig. 9A), and the expression levels of pro-apoptotic proteins (Smac, Bax, Bad, c-caspase-3 and c-caspase-9) in tumor tissues were significantly ($p < 0.01$) upregulated (Fig. 9B and C), compared with control group.

Effects of uvangoletin on cyclophosphamide-induced leucopenia in mice. As shown in Table I, after treatment with cyclophosphamide, the WBC count and thighbone marrow granulocytes percentage of control group were significantly

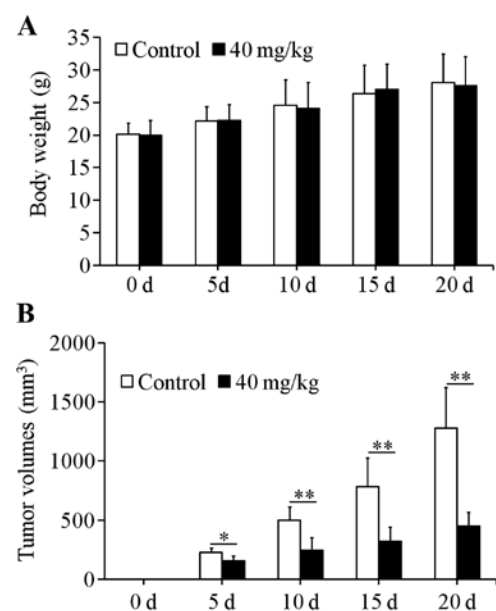


Figure 8. Effects of uvangoletin on the body weight (A) of nude mice and the volumes of HL-60-induced tumor (B). * $p < 0.05$, ** $p < 0.01$, compared with control group.

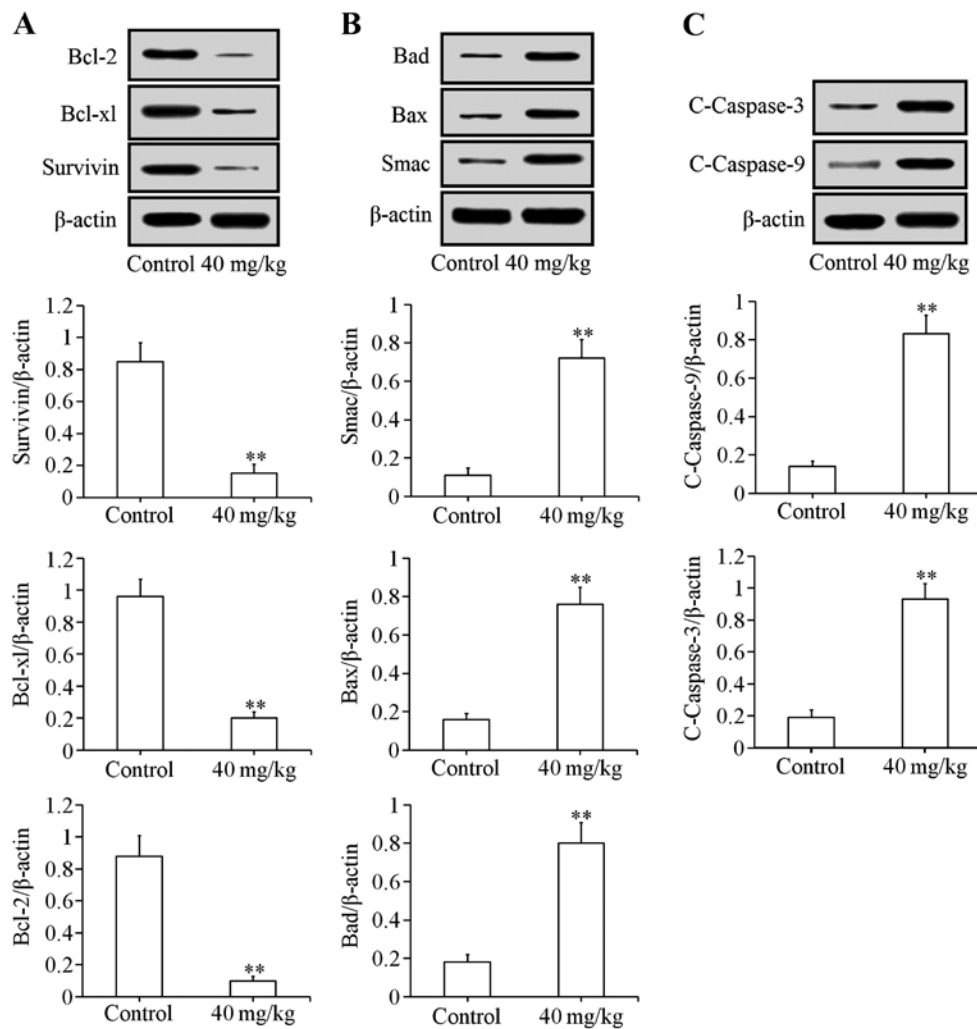


Figure 9. Effects of uvangoletin on the expression levels of apoptotic proteins in HL-60-induced tumor tissues. (A-C) The expression levels of anti-apoptotic proteins (Survivin, Bcl-xl and Bcl-2), pro-apoptotic proteins (Smac, Bax and Bad) and pro-apoptotic proteins (c-caspase-3 and c-caspase-9). ** $p < 0.01$, compared with control group.

($p < 0.01$) decreased, compared with normal group, and it suggested that the modeling was successfully established. After treatment with uvangoletin, the reduction of WBC count and thighbone marrow granulocytes percentage were not significantly exacerbated or reversed, compared with the control group.

Effects of uvangoletin on copper sulfate-induced emesis in pigeons and 75% ethanol-induced gastric mucosal lesions in rats. As shown in Table II, after pigeons were treated with copper sulfate and rats were treated with 75% ethanol, the emesis in pigeons and the gastric mucosal lesions in rats were observed in the control group. After treatment with uvangoletin, the incubation period and number of emesis and the gastric mucosal lesions were not significantly exacerbated or reversed, compared with the control group.

Discussion

In the present study, we investigated the cytotoxic effect of uvangoletin on HL-60 cells, the effects of uvangoletin on myelosuppression, leucopenia and gastrointestinal tract

disturbances and the possible cytotoxic mechanisms for the first time.

The CCK-8 assay is a commonly used method to assess the cytotoxic effects of the target drug on target cells, and the flow cytometry analysis is a commonly used method to assess whether the cytotoxic effects of the target drug on target cells is related to apoptosis (23,24). The results of CCK-8 and flow cytometry assays (Figs. 2 and 3) indicated that the cytotoxic effect of uvangoletin on HL-60 cells was related to apoptosis. Then the effect of uvangoletin on mitochondria-mediated apoptotic pathway in HL-60 cells was assessed to explore the pro-apoptotic mechanisms of uvangoletin.

The mitochondria-mediated apoptotic pathway is a research hotspot in apoptosis of cancer cells (23-26). The anti-apoptotic proteins (Survivin, Bcl-xl and Bcl-2), pro-apoptotic proteins (Smac, Bax, Bad, Bid, c-caspase-3 and c-caspase-9) and release of cytochrome c from mitochondria to cytoplasm play important roles in the mitochondria-mediated apoptotic pathway. When mitochondria are stimulated by apoptotic stimuli, the release of cytochrome c and Smac from mitochondria to cytoplasm are promoted, and the release is inhibited by Bcl-2 or Bcl-xl, whose functions are inhibited by Bax, Bad or

Bid (25). The cytochrome c, dATP, Apaf-1 and procaspase-9 in the cytoplasm form apoptosome, and then procaspase-9 is activated to generate c-caspase-9 (27). Finally, caspase-3 is activated by c-caspase-9 to generate c-caspase-3, which induces apoptosis of cells (25). However, Survivin inhibits the activation and function of c-caspase-3 (28). The function of Survivin is inhibited by Smac.

In summary, apoptosis of cells result from the interaction of the mitochondria-mediated apoptotic proteins. The results of western blotting (Figs. 4-7) indicated that pro-apoptotic mechanisms of uvangoletin on HL-60 cells were related to the mitochondria-mediated apoptotic pathway by downregulating the expression levels of Survivin, Bcl-x1 and Bcl-2, upregulating the expression levels of Smac, Bax, Bad, c-caspase-3 and c-caspase-9, and promoting the release of cytochrome c from mitochondria to cytoplasm in HL-60 cells. Further, the results of the xenograft assay (Figs. 8 and 9) suggested that uvangoletin inhibited the HL-60-induced tumor growth without adverse effect on body weight of nude mice *in vivo* by regulating the mitochondria-mediated apoptotic pathway.

The myelosuppression, leucopenia and gastrointestinal tract disturbances (emesis and gastric mucosal lesions) are main adverse reactions in chemotherapy of leukemia (10-12). In the present study, the effects of uvangoletin on myelosuppression, leucopenia and gastrointestinal tract disturbances were investigated. Cyclophosphamide is a commonly used chemotherapy drug in the clinic (29), and cyclophosphamide-induced leucopenia model in mice is a commonly used method (30) to study the effect of the target drug on the WBC count and the thighbone marrow granulocyte percentage. The copper sulfate-induced emesis model in pigeons and ethanol-induced gastric mucosal lesion model in rats are two commonly used methods to evaluate the effects of the target drug on emesis and gastric mucosal lesions (31,32). The results (Tables I and II) indicated that reductions of the WBC count and thighbone marrow granulocytes percentage in cyclophosphamide-induced leucopenia assay, the incubation period and number of emesis in copper sulfate-induced emesis assay and the gastric mucosal lesions in ethanol-induced gastric mucosal lesions assay were not exacerbated or reversed after treatment with uvangoletin for 7 days, and it preliminarily indicated that uvangoletin had not adverse reactions such as myelosuppression, leucopenia and gastrointestinal tract disturbances.

In conclusion, the research preliminarily indicated that uvangoletin induced apoptosis of HL-60 cells *in vitro* and *in vivo* without adverse reactions of myelosuppression, leucopenia and gastrointestinal tract disturbances, and the pro-apoptotic mechanisms of uvangoletin on HL-60 cells were related to mitochondria-mediated apoptotic pathway. Uvangoletin may be considered as a potent chemotherapy drug to treat leukemia without adverse reactions of myelosuppression, leucopenia and gastrointestinal tract disturbances, and there is a need to further investigate uvangoletin in this regard.

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