Introduction

Numerous efforts have been made to search for effective agents against cancer, in particular from herbal medicines. Deoxypodophyllotoxin and podophyllotoxin (POD) are two well-known naturally occurring aryltetralin lignans. Both compounds are cytotoxic (1), and their derivatives such as etoposide (ETO) (2, 3), teniposide (4), and etopophos have potential clinical use as anti-tumor agents (5, 6). However, due to the drug resistance developed by cancer cells as well as side effects associated with the use of these agents in the clinic (including myelosuppression, neutropenia, and nausea), the search for new effective anticancer analogs remains an intense area of research (7, 8).

Justicia procumbens (J. procumbens) is a traditional herbal remedy used to treat fever, pain, and cancer (9). The bioactive justicins isolated from J. procumbens in the past few years included diphyllin, 6′-hydroxy justicidin A (HJA), and chinensinaphthol methyl ether (CME), which each have a chemical structure similar to that of POD (10–12). These justicins were proved to have a wide spectrum of biological activities, such as cytotoxicity (13–15), antimicrobial (16), anti-platelet (17), and antiviral activities (18). A new justicin 6′-hydroxy justicidin C (HJC) was for the first time isolated from J. procumbens. To date, there have been no reports about the therapeutic activity of HJC and how it mediates its anti-cancer effect.

Mitochondria play a central role in various pathophysiological processes of cancer cells, in particular apoptosis. Most anti-tumor drugs can induce apoptosis in different types of tumor cells. There are two major apoptotic pathways known to date — the intrinsic pathway and the extrinsic pathway, which both result in activation of effector caspases as the final step. The intrinsic apoptotic pathway involves an increase in mitochondrial membrane permeability and an increased release of cytochrome c into the cytoplasm, which in turn activates caspase-9 and caspase-3, resulting in apoptotic damage (19, 20). The extrinsic pathway is initiated by the activation of death receptors that involves the formation of a death-
inducing signaling complex (DISC). DISC formation results in the activation of caspase-8, which activates caspase-3 (21).

In the present study, we investigated whether HJC could modulate the survival and proliferation of cancer cells. Our on-going research indicated that HJC was cytotoxic against various tumor cells, and human leukemia K562 cell line was one of the most sensitive cell lines to the anti-tumor effect of HJC. In addition, the activity of HJC on several cell lines was better than that of HJA and CME. Therefore, in the present study, we further illustrate whether HJC treatment can induce apoptosis and clarify the related mechanism of HJC.

Materials and Methods

Materials

POD, ETO, and paclitaxel (TAX) were purchased from Sigma (St. Louis, MO, USA). HJC was isolated from *J. procumbens* and identified by UV, IR, and ¹H and ¹³C-NMR (12, 22). The cycle TEST™ PLUS DNA reagent kit, FITC active caspase-3 apoptosis kit, and FITC Annexin V apoptosis detection kit were purchased from BD Pharmingen (San Diego, CA, USA). The SOD activity assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

HJA and HJC were obtained by the following procedures: The dried plant (5 kg) of *J. procumbens* was extracted with hot 95% ethanol, 3 times successively. After removal of the solvent by evaporation, the residue was suspended in water and defatted with petroleum ether. The aqueous layer was further extracted with ethyl acetate. The resulting ethyl acetate extract (68 g) was subjected to silica gel CC using the chloroform–methanol system (100:2) to yield 70 fractions. Fractions 23–26 (3 g) were combined and subjected to CC on silica gel, Sephadex LH-20 gel, followed by preparative reverse-phase C18 HPLC using an acetonitrile–water system (30:70 to 40:60) to yield compounds HJA (pale yellow amorphous powder, 30 mg) and HJC (pale yellow amorphous powder, 8 mg) (12).

**LC-MS/MS validation**

The UHPLC system consisted of an LC-20AD pump, a DGU-20 A3 degasser, an SIL-20AC autosampler and a CTO-20A column oven (Shimadzu, Kyoto). The UHPLC separation was performed on an Agilent Extent-C18 column (2.1 mm × 50 mm, 1.8 μm) with a gradient elution by a mobile phase consisting of 0.1% formic acid aqueous solution (A) and acetonitrile (Fisher, USA) with 0.1% formic acid (B) with the following gradient: 0.01–2.00 min, linear from 80% A / 20% B to 60% A / 40% B; 2.00–4.00 min, linear from 60% A / 40% B to 55% A / 45% B; 4.00–7.00 min, linear from 55% A / 45% B to 52% A / 48% B; 7.00–9.00 min, linear from 52% A / 48% B to 50% A / 50% B; 9.00–9.01 min, switch from 50% A / 50% B to 80% A / 20% B; and hold 80% A / 20% B until 10.00 min. The injection volume was 4 μl. The UHPLC system was coupled with an API 5500 Qtrap mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) via a Turbo IonSpray ionization interface. Following optimization of the setting parameters, the instrument was operated in the positive mode with an ion spray voltage of 5.5 kV, curtain gas pressure of 35 psi, nebulizer gas pressure of 50 psi, heater gas pressure of 50 psi, and the source temperature set at 550°C. The curtain, nebulizer, heater, and collision gases were all nitrogen. The multiple reaction monitoring (MRM) conditions were m/z 411.1 → 362.9, m/z 411.1 → 381.5. The declustering potential (DP), collision cell exit potential (EXP), and collision energy (CE) was 90 V, 13 V, and 30 V, respectively. The data were collected and analyzed by the Analyst Data Acquisition and Processing software (Version 1.6; Applied Biosystems, Foster City, CA, USA).

**Cell cultures**

Human leukemia K562, human promyelocytic leukemia HL-60, mouse lymphocytic leukemia L1210, and P388D1 mouse macrophage cell lines were obtained from Cancer Institute & Hospital, Chinese Academy of Medical Sciences. The cell lines were cultured in RPMI-1640 (GIBCO BRL, Grand Island, NY, USA) medium containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were maintained at 37°C in an atmosphere of 5% carbon dioxide–95% air.

**Cell viability assay and morphological evaluation**

Cytotoxicity was determined using a modified 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay (23). HJC was dissolved in dimethylsulfoxide (DMSO; Sigma, St. Louis, MO, USA) and the final concentration of DMSO was 0.1% (v/v). DMSO (0.1%) without HJC was used as the control. HJC was added to the culture medium at 356

---

**Materials and Methods**

**Materials**

POD, ETO, and paclitaxel (TAX) were purchased from Sigma (St. Louis, MO, USA). HJC was isolated from *J. procumbens* and identified by UV, IR, and ¹H and ¹³C-NMR (12, 22). The cycle TEST™ PLUS DNA reagent kit, FITC active caspase-3 apoptosis kit, and FITC Annexin V apoptosis detection kit were purchased from BD Pharmingen (San Diego, CA, USA). The SOD activity assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

HJA and HJC were obtained by the following procedures: The dried plant (5 kg) of *J. procumbens* was extracted with hot 95% ethanol, 3 times successively. After removal of the solvent by evaporation, the residue was suspended in water and defatted with petroleum ether. The aqueous layer was further extracted with ethyl acetate. The resulting ethyl acetate extract (68 g) was subjected to silica gel CC using the chloroform–methanol system (100:2) to yield 70 fractions. Fractions 23–26 (3 g) were combined and subjected to CC on silica gel, Sephadex LH-20 gel, followed by preparative reverse-phase C18 HPLC using an acetonitrile–water system (30:70 to 40:60) to yield compounds HJA (pale yellow amorphous powder, 30 mg) and HJC (pale yellow amorphous powder, 8 mg) (12).

**LC-MS/MS validation**

The UHPLC system consisted of an LC-20AD pump, a DGU-20 A3 degasser, an SIL-20AC autosampler and a CTO-20A column oven (Shimadzu, Kyoto). The UHPLC separation was performed on an Agilent Extent-C18 column (2.1 mm × 50 mm, 1.8 μm) with a gradient elution by a mobile phase consisting of 0.1% formic acid aqueous solution (A) and acetonitrile (Fisher, USA) with 0.1% formic acid (B) with the following gradient: 0.01–2.00 min, linear from 80% A / 20% B to 60% A / 40% B; 2.00–4.00 min, linear from 60% A / 40% B to 55% A / 45% B; 4.00–7.00 min, linear from 55% A / 45% B to 52% A / 48% B; 7.00–9.00 min, linear from 52% A / 48% B to 50% A / 50% B; 9.00–9.01 min, switch from 50% A / 50% B to 80% A / 20% B; and hold 80% A / 20% B until 10.00 min. The injection volume was 4 μl. The UHPLC system was coupled with an API 5500 Qtrap mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) via a Turbo IonSpray ionization interface. Following optimization of the setting parameters, the instrument was operated in the positive mode with an ion spray voltage of 5.5 kV, curtain gas pressure of 35 psi, nebulizer gas pressure of 50 psi, heater gas pressure of 50 psi, and the source temperature set at 550°C. The curtain, nebulizer, heater, and collision gases were all nitrogen. The multiple reaction monitoring (MRM) conditions were m/z 411.1 → 362.9, m/z 411.1 → 381.5. The declustering potential (DP), collision cell exit potential (EXP), and collision energy (CE) was 90 V, 13 V, and 30 V, respectively. The data were collected and analyzed by the Analyst Data Acquisition and Processing software (Version 1.6; Applied Biosystems, Foster City, CA, USA).

**Cell cultures**

Human leukemia K562, human promyelocytic leukemia HL-60, mouse lymphocytic leukemia L1210, and P388D1 mouse macrophage cell lines were obtained from Cancer Institute & Hospital, Chinese Academy of Medical Sciences. The cell lines were cultured in RPMI-1640 (GIBCO BRL, Grand Island, NY, USA) medium containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were maintained at 37°C in an atmosphere of 5% carbon dioxide–95% air.

**Cell viability assay and morphological evaluation**

Cytotoxicity was determined using a modified 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay (23). HJC was dissolved in dimethylsulfoxide (DMSO; Sigma, St. Louis, MO, USA) and the final concentration of DMSO was 0.1% (v/v). DMSO (0.1%) without HJC was used as the control. HJC was added to the culture medium at 356
days of incubation with different concentrations of HJC, the cells were analyzed using a light microscope BX 41 (Olympus Corporation, Tokyo) with the magnification factor of 400.

**Measurement of SOD activity**

Superoxide anion (\( \text{O}_2^- \)) was generated as a mixture of the enzyme xanthine oxidase and its substrate xanthine. Enzymatic formation of superoxide anion was detected on the basis of cytochrome \( c \) reduction by xanthine oxidase plus xanthine. The activity of SOD was detected by its ability to reduce cytochrome \( c \), which causes an increase in absorbance at 550 nm. K562 cells (\( 1 \times 10^5 \) cells/ml) were plated in 96-well plates and allowed to attach for 24 h. HJC was added at different final concentrations that ranged from 8.41 to 135 \( \mu \text{M} \). After 48 h of incubation, K562 cells were collected and resuspended in PBS to adjust the cell number to \( 1 \times 10^5 \). The cell suspension was treated for three freeze-thaw cycles (thawed from the temperature of \(-80^\circ\text{C}\) by bringing them to room temperature), and the supernatant was collected to measure the intracellular SOD activity at 550 nm by using an automatic microplate reader.

**Intracellular ROS quantification**

The level of intracellular reactive oxygen species (ROS) was determined by the change of fluorescent probe dichlorofluorescein diacetate (DCFH–DA). Briefly, \( 1 \times 10^5 \) K562 cells were cultured into Petri dishes and then treated with the indicated concentrations of HJC (8.41, 33.7, and 135 \( \mu \text{M} \)) for 24 h. Cells were washed with PBS and were incubated with 10 mM DCFH–DA for 30 min at 37°C. Subsequently, cells were washed 3 times with PBS and ROS level was determined by flow cytometry.

**Cell-cycle analysis**

K562 cells were seeded at a density of \( 1 \times 10^5 \) cells/ml in 12-well plates and incubated with different concentrations of HJC for 48 h. After exposure, \( 10^6 \) cells were harvested, washed with ice-cold PBS twice, centrifuged, resuspended, and fixed with 70% ethanol overnight at 4°C. Then the fixed cells were washed and resuspended with PBS again, treated with 100 \( \mu \text{l} \) of 100 \( \mu \text{g/ml} \) RNase at room temperature for 10 min, and stained with 200 \( \mu \text{g/ml} \) PI for 20 min in the dark. PI-stained cells were assayed using FACS Canto Becton Dickinson Flow Cytometry and cell cycle distributions were analyzed with the ModFit program. All of the samples were assayed in triplicate, and the fraction of every cell cycle phase was calculated.

**Annexin V/PI staining and flow cytometry analysis**

Apoptosis is a normal physiologic process that occurs during embryonic development as well as in maintenance of tissue homeostasis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35 – 36-kDa \( \text{Ca}^{2+} \)-dependent phospholipid-binding protein that has a high affinity for PS and binds to cells with exposed PS. Annexin V may be conjugated to fluorochromes including FITC. This format retains its high affinity for PS and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis.

K562 cells were plated in 12-well plates at a density of \( 5 \times 10^5 \) cells per well. The cells were treated with or without varying concentrations (0, 8.41, 33.7, and 135 \( \mu \text{M} \)) of HJC in complete medium for 48 h. At the end of each treatment, cells were collected and quantitative apoptotic death assay was performed by Annexin V and PI staining (Molecular Probes) following the manufacturer’s protocol, and apoptotic cells were then analyzed immediately by flow cytometry using the FACS (BD Pharmeden).

**Caspase-3 activity assay**

The caspase-3 enzymatic activity was measured using the caspase-3 activity assay kits (BioVision, San Francisco, CA, USA) according to the manufacturer’s instructions. In brief, cells were seeded at a density of \( 5 \times 10^5 \) cells/ml in 12-well slides. After treating with or without varying concentrations (0, 8.41, 33.7, and 135 \( \mu \text{M} \)) of HJC for 12 h, cells were collected and washed twice with ice-cold PBS and then resuspended in BD Cytofix/CytopermTM solution at a concentration of \( 1 \times 10^6 \) cells / 50 \( \mu \text{l} \). The cells were incubated for 20 min on ice and then washed twice with Perm/WashTM buffer at a volume of 0.5 ml buffer / \( 1 \times 10^6 \) cells at room temperature. The resuspended cells above were incubated at a concentration of 20 \( \mu \text{g/ml} \) antibody / \( 1 \times 10^6 \) cells for 30 min at room temperature. After that, each test was washed in 1.0 ml Perm/WashTM buffer and then resuspended in 0.5 ml Perm/WashTM buffer and analyzed by FACS.

**Statistical analysis**

All experimental data are shown as means ± S.D. and accompanied by the number of experiments. Analysis was performed using one-way ANOVA followed by Dunnett’s post-hoc test, and the significant difference was set at *\( p < 0.05 \), **\( p < 0.01 \).
**Results**

**MRM-IDA-EPI validation of HJC**

As shown in Fig. 1, A and B, no interferences from endogenous substances were observed at the retention times of 5.44 min. EPI scan showed that the main fragment ions were 112.8, 129.1, 173.0, 231.0, 247.1, 263.1, 305.0, 362.9, 379.0, and 395.0. This study successfully determined the antitumor component HJC and further authenticated its chemical structure.

**HJC inhibits the growth of leukemia K562 cells**

Initially, the cytotoxicity of HJC was investigated with different doses of HJC (0.13, 0.26, 0.53, 1.05, 2.11, 4.22, 8.41 16.9, 33.7, 67.5, and 135 μM) in human leukemia K562 cells by MTT assay. As shown in Fig. 2B, HJC inhibited the viability of K562 cells in a dose-dependent manner. The results show that after HJC treatment for 48 h, the 50% inhibition concentration of HJC by MTT assay was 15.2 μM. Accordingly, the concentrations of HJC used in the following study were 8.41, 33.7, and 135 μM. The loss of mitochondrial transmembrane potential is a critical step in the process of apoptosis. Typical morphological features of apoptosis, such as cell surface blebbing, were observed under light microscopic examination of K562 cells treated for 2 days with 8.41 μM of HJC. Necrotic cells were detected under light microscopic examination of K562 cells treated for 2 days with 33.7 and 135 μM of HJC (Fig. 2A).

**Comparison of the effects of HJA and HJC on K562, HL-60, L1210, and P388D1 cells**

To compare the activity between HJC and HJA, the cytotoxicity of the 2 compounds, derived from *J. procumbens*, was investigated in K562, HL-60, L1210, and P388D1 cell lines. After incubation for 2 days, the inhibition ratios of the cells induced by different concentrations of HJC and HJA were delineated (Fig. 3). The results demonstrated that HJC exhibited more cytotoxicity than that of HJA on K562 and HL-60 cell lines, and the IC\textsubscript{50} of HJC on the HL-60 cell line was 20.1 ± 2.3 μM. In contrast, HJA exhibited more cytotoxicity than that of HJC on L1210 and P388D1 cell lines, and the IC\textsubscript{50} of HJA on L1210 and P388D1 cell lines were 4.8 ± 2.4 and 4.8 ± 2.4 μM, respectively.

**HJC inhibits the SOD activity of leukemia K562 cells**

Whether HJC can change the redox system in leukemia K562 cells was also examined. Compared with the control, the activity of SOD in human leukemia K562

---

**Fig. 1.** Chemical structure of 6′-hydroxy justicidin C (HJC), etoposide (ETO), paclitaxel (TAX), and podophyllotoxin (POD). A) typical chromatograms of HJC; B) full-scan product ion spectra of [M+H]+ ions and fragmentation schemes for HJC.
Fig. 2. Growth-inhibitory effect of HJC on the viability of K562 cells evaluated by MTT assay. A) Photomicrographs of K562 cells exposure to different concentrations of HJC for 48 h. a) control group, b) 8.41 μM HJC, c) 33.7 μM HJC, d) 135 μM HJC. B) Effect of HJC on proliferation of K562 cells. The cell viability was determined by the MTT assay. Each data represents the mean ± S.D. from 3 independent experiments, each at least in triplicate.

Fig. 3. Comparison of the effects of HJA and HJC on K562, HL-60, L1210, and P388D1 cell lines (the closed circles represent HJC, the closed rectangles represent HJA). The percent of cell inhibition was determined by the MTT assay. The IC50 of HJC on HL-60, K562, L1210, and P388D1 was 20.1 ± 2.3, 15.2 ± 1.2, 19.2 ± 2.1, and 15.8 ± 1.9 μM, respectively; the IC50 of HJC on HL-60, K562, L1210, and P388D1 was 35.6 ± 2.8, 43.9 ± 2.9, 4.8 ± 2.4, and 4.8 ± 1.4 μM, respectively.
J Luo et al

cells decreased remarkably under the increase of HJC concentration (Fig. 4A). Specifically, the activity of SOD decreased 35.6%, 65.8%, and 73.5% at the concentration of 8.41, 33.7, and 135 μM, respectively.

HJC enhances ROS levels in leukemia K562 cells

As shown in Fig. 4B, after HJC treatment, the intracellular ROS levels increased remarkably compared with the level in the control group. Values were expressed as the mean ± S.D. **P < 0.01, compared with the control group.

Effect of HJC on the SOD activity and ROS levels in K562 cells. A) Effect of HJC on SOD activity in K562 cells. K562 cells were treated with different concentrations of HJC and POD for 48 h. B) Intracellular ROS quantification in K562 cells. Values were expressed as the mean ± S.D. **P < 0.01, compared with the control group.

94.08% of total cells compared to 6.99% in the control group. However, K562 cells treated with 8.41 – 135 μM HJC and 22.7 μM ETO for 48 h increased the subG0 phase in a dose-dependent manner as higher concentrations of HJC led to significantly higher percentage of cells in the subG0 phase. Cells in G2/M phase were hardly found in this group. The apoptosis percentage of K562 cells exposed to 8.41, 33.7, and 135 μM HJC was 6.35%, 14.72%, and 30.52%, respectively (Fig. 5D).

HJC increased the subG0 phase of K562 cells

As shown in Fig. 5B, K562 cells treated with 28.5 μM POD and 12.0 μM TAX for 48 h were arrested at metaphase phase. At the dose of 28.5 μM of POD, the percentage of cells in metaphase phase reached 94.08% of total cells compared to 6.99% in the control group. However, K562 cells treated with 8.41 – 135 μM HJC and 22.7 μM ETO for 48 h increased the subG0 phase in a dose-dependent manner as higher concentrations of HJC led to significantly higher percentage of cells in the subG0 phase. Cells in G2/M phase were hardly found in this group. The apoptosis percentage of K562 cells exposed to 8.41, 33.7, and 135 μM HJC was 6.35%, 14.72%, and 30.52%, respectively (Fig. 5D).

HJC-induced apoptosis is related with a caspase-dependent pathway

Active caspase-3, a marker for cells undergoing apoptosis, consists of a heterodimer of 17- and 12-kDa subunits, which is derived from the 32-kDa pro-enzyme. FITC Anti-Active Caspase-3 Antibody has been reported to specifically recognize the active form of caspase-3 in human and mouse cells.

Human leukemia K562 cells were left untreated or treated for 12 h with HJC to induce apoptosis. Cells were permeabilized, fixed, and stained for active caspase-3 as described in the accompanying Staining Protocol. Cells were then analyzed by flow cytometry. As shown in

FITC Annexin V staining precedes the loss of membrane integrity, which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with FITC Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) to allow the investigator to identify early apoptotic cells (PI negative, FITC Annexin V–positive).

The apoptotic death assay employing Annexin V/PI staining followed by FACS analysis clearly showed a dose-dependent apoptotic effect of HJC in K562 cells (Fig. 5A). As shown in the representative FACS analysis scatter-grams, Annexin V/PI staining of control cells showed a large viable cell population with the same staining also for early apoptotic, late apoptotic, and dead cells. However, treatment of cells with HJC at the dose of 8.41, 33.7, or 135 μM for 48 h resulted in a strong shift from live cells to an early and late apoptotic cell population (Fig. 5C). Specifically, the early apoptotic percentage of K562 cells exposed to 8.41, 33.7, and 135 μM HJC was 8.05%, 7.99%, and 23.34%, respectively; the viable cell population exposed to 8.41, 33.7, and 135 μM HJC was 85.12%, 85.12%, and 64.70%, respectively. The positive reference drug ETO induced apoptosis at the concentration of 22.7 μM (the viable cell population was 72.88%), but the apoptosis was not increased when the dose of ETO increased to 90.6 μM (the viable cell population was 70.64%).

Effect of HJC on apoptosis in K562 cells

HJC increases the subG0 phase of K562 cells

As shown in Fig. 5B, K562 cells treated with 28.5 μM POD and 12.0 μM TAX for 48 h were arrested at metaphase phase. At the dose of 28.5 μM of POD, the percentage of cells in metaphase phase reached 94.08% of total cells compared to 6.99% in the control group. However, K562 cells treated with 8.41 – 135 μM HJC and 22.7 μM ETO for 48 h increased the subG0 phase in a dose-dependent manner as higher concentrations of HJC led to significantly higher percentage of cells in the subG0 phase. Cells in G2/M phase were hardly found in this group. The apoptosis percentage of K562 cells exposed to 8.41, 33.7, and 135 μM HJC was 6.35%, 14.72%, and 30.52%, respectively (Fig. 5D).
HJC Causes Apoptosis in K562 Cell Line

Fig. 5. Effect of HJC on apoptosis in K562 cells by flow cytometry. All the results shown in this figure are representative of 3 experiments with similar results. A) PI analysis of K562 cells treated with different concentrations of HJC for 24 h. B) HJC caused strong apoptotic death of K562 cells. Representative FACS analysis scattergrams of Annexin V/PI–stained control and cells treated with 0, 8.41, 33.7, or 135 μM HJC for 48 h showed 4 different cell populations. C) Statistical analysis of the percentage of early and late apoptosis caused by HJC. D) The apoptosis rate and change of cell cycle of K562 cells treated with HJC for 24 h. Cells were plated in 12-well plates at a density of 1 × 10^4 cells per well.

Fig. 6. Effects of HJC on caspase-3 activity in K562 cells. The changes of active caspase-3 were detected by flow cytometry in K562 cells after treatment with 8.41, 33.7, and 135 μM HJC for 48 h.
In addition, when the concentration of ETO was 90.6 μM, the percentages of active caspase-3-positive cells were increased 8.37%, 23.21%, and 35.20% when treated with 8.41, 33.7, 135 μM HJC.

Discussion

HJC is a new compound extracted from the *J. procumbens* plants and has been identified to have a basic molecular structure similar to that of the classic anti-tumor drug POD. Previous study showed that the content of HJC in *J. procumbens* was lower than those in JB, CME, HJB, and HJA (24). However, our research showed that the IC50 of HJC on K562 and HL-60 cell lines was lower than that of HJA (Fig. 3), which indicated that the change in the binding position of the oxygen would influence the cytotoxicity to specific tumor cells. In addition, our ongoing study indicated that the oral absolute bioavailability of HJC was higher than that of HJA (25) (36.0% ± 13.4%) and CME (26) (3.2% ± 0.2%), making HJC a promising candidate for leukemia therapy. Furthermore, HJC was shown to induce apoptosis in human leukemia K562 cells for the first time, and this effect involves caspase activation.

Mitochondria are responsible for generation of substantial amounts of superoxide caused by electron leakage from the oxidative phosphorylation pathway. SOD is the first line of defense against ROS, protecting cells from the peroxidation injury induced by transferring one radical (O2·−) to the next (H2O2) (27). In this study, the activity of SOD was decreased significantly in K562 cells after the treatment of HJC. Analysis of ROS content and mitochondrial transmembrane potential (Δψm), using specific fluorescent probes in flow cytometry, showed that HJC can mediate ROS production and the loss of Δψm.

ETO, a topoisomerase II inhibitor, is extensively used in the treatment of leukemia. However, K562 cells are known to be less sensitive to ETO than other cell lines (28). Jiang et al. used flow cytometry to measure the apoptosis rate induced by continuous exposure to ETO (10 μM) in K562 cells and found that apoptosis was barely detected 24 h after exposure to ETO (29). In this study, the cytotoxicity of POD and ETO were not significant when the concentration was less than 20 μM. In addition, when the concentration of ETO was 90.6 μM, both the early and late apoptosis percentages detected were lower than that of 135 μM HJC.

The inhibition of cancer cell proliferation and induction of apoptosis are important mechanisms for a chemotherapeutic agent (30). A number of proteins are known to be involved in the regulation of apoptosis through the mitochondrial pathway and in turn promote activation of the downstream molecules in the caspase cascade, such as the Bel-2 family proteins (31).

Caspases are a family of cysteine proteases that cleave target proteins at specific aspartate residues, constituting key components of the apoptotic pathway. Two distinct pathways of apoptosis have been identified: mitochondria-initiated apoptosis that occurs through caspase-9 and the death receptor–mediated pathway that requires caspase-8 (32). Caspase-3 is a key effector molecule in the caspase-dependent cell apoptosis pathway that cleaves a number of cellular proteins, leading to apoptotic changes. Thus activation of the enzymatic activity of caspases-3 might provide a mechanism to initiate the apoptotic program (33). In this study, HJC treatment induced apoptosis in K562 cells in a dose-dependent manner. The activity of 33.7 μM HJC was equal to that of 48.0 μM TAX and stronger than that of 90.6 μM ETO.

Our results indicated that HJC raised the enzymatic activity of caspase-3 significantly. We concluded that HJC-induced apoptosis in K562 cells via the activation of caspase-dependent pathway through the caspase-3 mitochondrial pathway. This study presents a potential novel alternative to human leukemia K562 cell therapy.

Acknowledgments

Financial support for this work from the Beijing Natural Science Foundation of China (711106110034) is gratefully acknowledged.

References

5. Ayres DC, Loike JD. Lignans: chemical, biological, and clinical properties. Cambridge, New York: Cambridge University Press;
HJC Causes Apoptosis in K562 Cell Line

1990.


