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Original Article

Molecular Pathway of Psoralidin-Induced Apoptosis in HepG2 Cell Line*

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ABSTRACT **Objective:** To test the role of psoralidin in human liver cancer HepG2 cells *in vitro*. **Methods:** Cell viability was assessed by methylthiazolyldiphenyl-tetrazolum bromide assay and apoptotic cells were labeled by annexin V then sorted by flow cytometry. Protein expressions of caspase-3, caspase-8, caspase-9, Bax, Bid, Bcl-2, Bcl-xL and p53 were examined by western blot while activity of caspase-3, -8 and -9 were also determined. **Results:** Psoralidin reduces cell viability greatly in a time dependent manner (64%, 40%, 21%, 12% at 2, 6, 24 and 48 h treatment with 64 μmol/L psoralidin respectively) and up-regulates activities of caspase-3, -8 and -9 in a concentration dependent manner (between 4 to 64 μmol/L). Psoralidin also increases the expression of pro-apoptosis genes Bax, Bid and p53 while decreases the expression of pro-survival genes Bcl-2 and Bcl-xL, both in a concentration dependent manner between 4 and 64 μmol/L (P<0.05 at 16 and 64 μmol/L). Caspase-3 inhibitor (Ac-DEVD-CHO at concentrations between 10 to 20 μmol/L), p53 inhibitor (pifithrin-α at 5 μmol/L) and cyclosporin A can attenuate the apoptotic effect of psoralidin. **Conclusion:** The cytotoxic role of psoralidin might work through both intrinsic and extrinsic apoptotic pathway.

KEYWORDS psoralidin, apoptosis, HepG2, mitochondria, p53, cyclosporin A

Psoralidin is one of the major coumarins isolated from the seeds of *Psoralea corylifolia* (PCL) which has been used as alternative medicine for the management of various diseases such as cardiovascular diseases, inflammatory diseases and nephritis.[1-4] Apart from psoralidin, PCL extracts include psoralen, isopsoralen, corylin, bavachin, isobavachin and bakuchiol, etc.[4]

Psoralidin possess cytotoxic activity against SNU-1 and SNU-16 stomach carcinoma cell lines.[5] In another study, psoralidin was found to be a cytotoxic against the HT-29 and MCF-7 human cancer cell lines.[6] Psoralidin can enhance tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in prostate cancer cells and in HeLa cells to exert its anti-tumor effect.[7,8] Psoralidin inhibited epithelial-mesenchymal transition markers (β-catenin and vimentin) and up-regulated E-cadherin expression, resulting in reduced migration and invasion. Psoralidin administration results in the induction of apoptosis in prostate cancer cells.[9,10] Additionally, psoralidin was reported to be able to inhibit expression of tumor necrosis factor (TNF)-alpha and its downstream prosurvival signaling molecules such as nuclear factor (NF)-kappaB and Bcl-2 and simultaneously induces the death receptor-mediated apoptotic signaling to activate caspase cascade and lead to apoptosis.[11]

Apoptosis has specific morphological change and its defects can result in many human pathological conditions, including cancer, autoimmune diseases and neurodegenerative disorders.[12,13] Apoptosis can be triggered by various intra or extra cellular stimuli. Pro-apoptotic members such as Bax and Bak as well as anti-apoptotic members include Bcl-2 and Bcl-xL all involved in intra cellular stimuli which finally activate caspase-9, -3 and -7 to initiate apoptosis (Intrinsic pathway).[14,15] Extra stimuli work use death receptor on plasma membrane to form a large death-inducing signaling complex to activate caspase-8.[16-18] In liver, caspase-8 employs either extrinsic pathway and

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intrinsic pathway.[19] Caspases belong to a family of cysteine proteases that play essential roles in apoptosis, necrosis, and inflammation.[20] Bcl-2 family include the pro-apoptotic mediators, Bax and Bid, and the anti-apoptotic effectors, Bcl-2, Bcl-xL.[21] Bid is activated through proteolytic processing, then translocates to mitochondria and leads to disruption of the organelles and the release apoptogenic molecules such as cytochrome C to start up apoptosis.[22-24]

The aim of this study was to explore the pro-apoptotic role of psoralidin through both intrinsic and extrinsic apoptotic pathway in HepG2 cell.

METHODS

Reagents

Psoralidin was isolated and purified from PCL (purity > 98% by high performance liquid chromatography) by Dr. WANG Yue-fei at Tianjin University of Traditional Chinese Medicine.

Modified Eagle's medium (MEM), fetal bovine serum (FBS), trypsin and penicillin-streptomycin solution were obtained from Hyclone (Logan, USA). Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was bought from Sigma (St Louis, USA). Dead cell apoptosis kit (V13241) with annexin V/propidium iodide (PI) was bought from Invitrogen (Grand Island, USA). Primary antibodies of Bax (2772), Bid (2002), Bcl-xL (2764), Bcl-2 (2876) were obtained from Cell Signaling Co. Ltd. (Boston, USA). Primary antibodies of caspase-3 (PAA626Hu01) and caspase-8 (PAA853Hu01) were bought from Cloud-clone Co. Ltd. (Wuhan, China). Primary antibody of caspase-9 (sc-8355) was bought from Santa Cruz (Texas, USA). Primary anti-p53 antibody (ab32389) was bought from Abcam (Cambridge, Cambridgeshire, UK). Dimethyl sulphoxide (DMSO), phosphate balanced solution (PBS), pifithrin-α, Ac-DEVD-CHO, Z-VAD-FMK, cyclosporin A, and caspase-3, -8, -9 assay kits were purchased from Beyotime Institute of Biotechnology (Haimen, China). RIPA lysis buffer, protease inhibitor and bicinchoninic acid protein assay kit were purchased from Dalian Meilun Biotech Co., Ltd (Dalian, China). Polyvinylidene fluoride (PVDF) transfer membrane was purchased from Millipore (Billerica, USA).

Cell Culture and MTT Analysis

HepG2 cell was purchased from Cell Culture Center, Institute of Biochemistry and Cell Biology, Chinese Academy of Life Sciences (Shanghai, China). HepG2 cells were maintained in MEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 ℃ in a water-saturated atmosphere of 5% CO_2 humidified incubator (HERA cell 150, Thermo, USA).

HepG2 cells were seeded in a 96-well microtiter plate at density of 0.3×10^5 cells/mL (100 μL, 0.3×10^4 per well). After 24 h of cell attachment, cells were treated with various concentrations of psoralidin for 24 h. Cell viability was evaluated by MTT assay. Briefly, 20 μL of MTT solution was added to each well (final, 0.5 mg/mL) for 4 h. Medium was removed, then 200 μL of DMSO was added to each well and absorbance was determined at 570 nm using microplate reader (InfiniteM200, Teacan, Swissland). Cell viability was expressed as a percentage of the control. Each concentration and time point treatment had been repeated using 3 independent plates.

Western Blot Analysis

Cells lysate protein was separated by electrophoresis on a 10% polyacrylamide gel at 200 V for various minutes and transferred to PVDF membranes at 250 mA. The membranes were incubated with primary antibody at 4 ℃ overnight, and with secondary antibody (IgG-HRP) for 2 h.

Flow Cytometry Analysis Using Annexin V/PI Double-Staining

HepG2 cells were seeded at 5×10^5 cells per well in 6-well microplates. After 24 h of cell attachment, with the indicated concentrations of psoralidin (4, 16 and 64 μmol/L) for 24 h, cell apoptosis was detected by annexin V/PI apoptosis detection kit following the manufacturer's instructions. Briefly, the medium was removed and the cells were gently trypsinized, washed with PBS, resuspended in binding buffer and incubation with annexin V and PI for 10 min at room temperature in the dark. Cells were analyzed by flow cytometer (Epics Altria, Beckman Coulter, Germany) within 1 h.

Measurement of Caspase-3, -8 and -9

Caspase-3, -8 and -9 activities were assayed using the kit. Through cleavage of the specific substrate for caspase-3 (Ac-DEVD-pNA), caspase-8 (Ac-IETD-pNA) or caspase-9 (Ac-LEHD-pNA) releasing the chromophore, p-nitroaniline (pNA). The absorbance at 405 nm was used to quantify the caspases activities, and the values of caspases

activities were normalized of control.

Statistical Analysis

Data were shown as mean ± standard deviation ($\bar{x} \pm s$). Statistical analysis was performed according to the Student's *t*-test and one-way ANOVA analysis followed by Dunnett's multiple comparison tests using SPSS 17.0. The probability values of $P<0.05$ were considered as significance.

RESULTS

Psoralidin-Induced Apoptosis in HepG2 Cells

When it was used in a final concentration between 0–128 μmol/L, psoralidin was shown to reduce cell viability in a concentration dependent manner in various incubation periods (Figure 1A). Cell viability was 64%, 40%, 21%, 12% at 2, 6, 24 and 48 h respectively, treated with 64 μmol/L psoralidin. Comparing different incubation time, effect of psoralidin on cell fate was also timely dependent although it did not show significant difference between 24 and 48 h of incubation time in low final concentration. The flow cytometry results confirmed the concentration dependent manner (between 4–64 μmol/L) of psoralidin on cell viability, which has been shown by MTT assay, when psoralidin was incubated with cells for 24 h (Figure 1B).

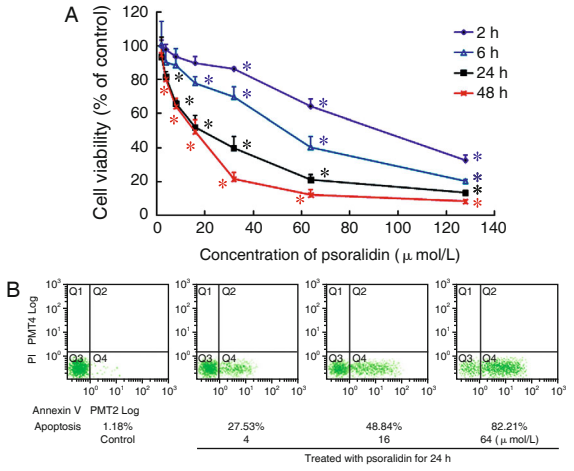

Figure 1. Cytotoxicity and Apoptosis Induced by Psoralidin

Notes: A: MTT assay shows that psoralidin reduces cell viability in a timely and concentration dependent manner, $^{*}P<0.05$, compared with the value without psoralidin; B: Flow cytometry assay confirms that psoralidin increases cell apoptosis rate in a concentration dependent manner.

Activation of Caspases by Psoralidin

Activities of both caspase-8 and -9 rise with psoralidin administration (4, 16, 64 μmol/L) in a concentration dependent manner (Figures 2A and 2B). These results indicated that psoralidin was involved in both intrinsic and extrinsic apoptotic pathway. Unsurprisingly, activity of one of their downstream targets from both pathways, caspase-3, is also marked increase in a similar pattern as caspase-8 and -9 (Figure 2A). As a result, caspase-3 inhibitor (Ac-DEVD-CHO) and general caspase inhibitor (Z-VAD-FMK, Figure 2B) can blunt the cytotoxic effect of psoralidin. Immunoblot confirmed the up-regulation of caspase-3 and -8 as well as the process of caspase-9 by psoralidin in a concentration dependent manner.

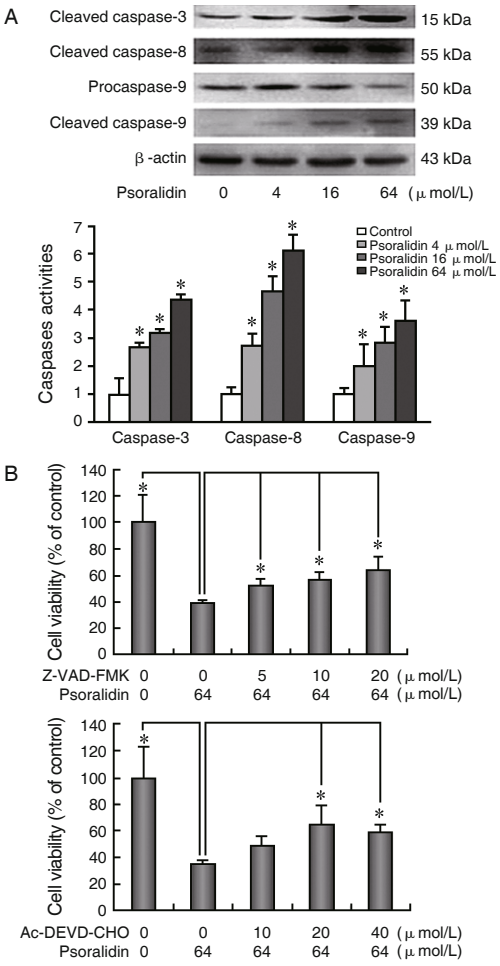

Figure 2. Expressions and Activities of Caspase-3, -8, and -9

Notes: A: Psoralidin up-regulates activities of caspase-3, -8 and -9 in a concentration dependent manner. The immunoblot results shows up-regulation of caspase-3 -8 and -9. B: The cytotoxicity of psoralidin can be down-regulated by the caspase-3 inhibitor, Ac-DEVD-CHO and the general caspase inhibitor, Z-VAD-FMK. $^{*}P<0.05$, compared with the control

Up-Regulation of Bax, Bid and Down-Regulation of Bcl-2, Bcl-xL

Here, it was shown that pro-apoptotic factors Bax and Bid increase upon psoralidin administration in a dose dependent manner. Psoralidin of 16 and 64 μmol/L demonstrated statistical difference from

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control (P<0.05, Figure 3A). On the other hand, level of pro-survival factors Bcl-2 and Bcl-xL drop with psoralidin administration, also in a dose dependent manner (Figure 3B). When final concentration of psoralidin reach 64 μmol/L, both Bcl-2 and Bcl-xL abated significantly compare to control (P<0.05).

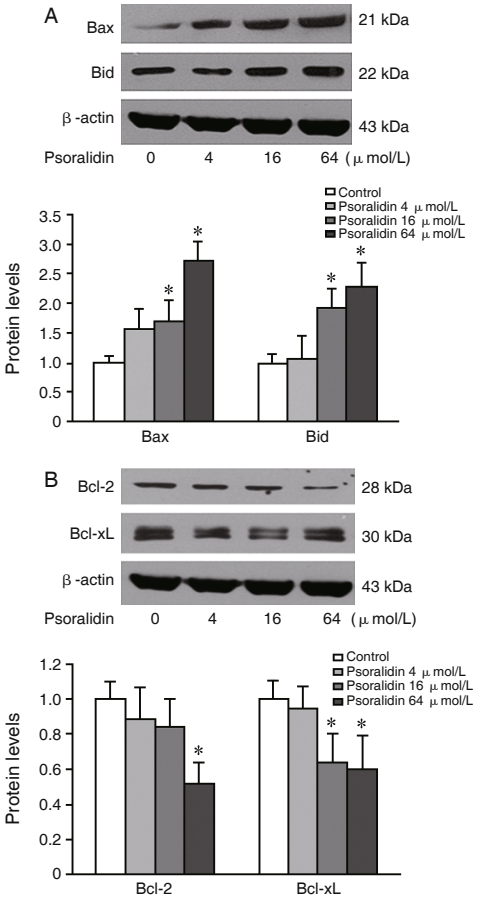

Figure 3. Effect of Psoralidin on Bax, Bid, Bcl-2 and Bcl-xL

Note: *P<0.05, compared with the control

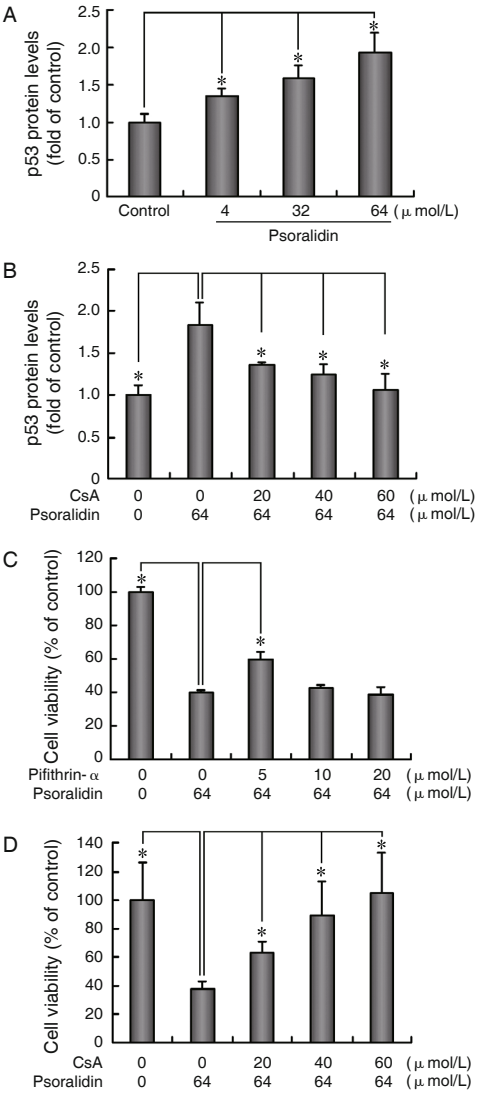

Figure 4. The Role of p53 and CsA in Apoptosis Induced by Psoralidin

Notes: Psoralidin boost p53 expression which can be inhibited by CsA. Pifithrin-α, a p53 inhibitor, can antagonize the cytotoxic effect of psoralidin. CsA also rescues the cytotoxic effect of psoralidin but in a concentration dependent manner.

p53 Dependent Apoptosis

As shown in Figure 4A, p53 expression can be up-regulated with increased psoralidin administration. By contrast, CsA can antagonize the effect of psoralidin to boost p53 expression (Figure 4B). What's more, low concentration (5 μmol/L) but not high concentration (10 and 20 μmol/L) of small molecule inhibitor of p53, pifithrin-α, can rescue the cytotoxic effect of psoralidin to HepG2 cells (Figure 4C). Moreover, CsA also reverse the cytotoxic effect of psoralidin as pifithrin-α did but in a dose dependent manner (Figure 4D).

DISCUSSION

In this study, it was shown that cytotoxic property of psoralidin is consistent with other reports.[5-8] However, psoralidin was shown to be able to lead cells derived from liver cancer to demise. As been demonstrated previously, psoralidin enhance TRAIL-mediated apoptosis while TRAIL is an important endogenous anticancer factor that induces apoptosis selectively in cancer cells.[7] TRAIL can trigger extrinsic apoptotic pathway through binding to death receptors. Indeed, it was found that the up-regulation of caspase-8 activity, an important player in extrinsic apoptotic pathway, in HepG2 cells. On the other hand, the activity of intrinsic apoptotic factor caspase-9 was also changed upon psoralidin administration which imply that psoralidin is involved in both intrinsic and extrinsic apoptotic pathway. Bid, a pro-apoptotic member of the Bcl-2

family that mediate cross talk between the extrinsic and intrinsic pathways of apoptosis, was also increased with psoralidin administration. This is another evidence that psoralidin play roles in both apoptotic pathway.

Simon, et al[25] revealed that p53 over-expression up-regulates Notch mRNA transcription and protein expression. This disparity can be explained by the finding that damaged cells entering apoptosis signal the surrounding unaffected cells to divide and compensate for the tissue loss and p53 play an important role in this apoptosis induced proliferation process.[26,27] It is likely that psoralidin inhibit Notch signaling which result in apoptosis, then the elevated p53 level increase Notch level to initiate apoptosis induce proliferation. This is very important in clinical application because psoralidin was indicated to be able to improve liver regeneration besides killing cancer cells selectively. What's more, cells committed to die via p53-dependent apoptosis can follow both mitochondrial pathway and death receptor pathway.[28,29] So our result, which showed psoralidin altered p53 expression, adds more weight to the hypothesis that psoralidin take part in both intrinsic and extrinsic pathway.

Psoralidin was demonstrated to inhibit lipopolysaccharide-induced inducible nitric oxide synthase expression[30] and to induce reactive oxygen species generation that lead to loss of mitochondria membrane and release of cytochrome C. Furthermore, psoralidin can induce quinone reductase activity who is an antioxidant but increase the sensitivity of cancer cells to TNF mediated apoptosis involved oxidative stress.[31,32] Psoralidin, therefore, might protect normal cells as antioxidant but lead abnormal cells to apoptosis through sensitizing them to TNF mediated apoptosis. Additionally, psoralidin inhibited the ionizing radiation-induced reactive oxygen species (ROS) activated cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) expression.[33] Hepatitis B virus X protein was known to be able to enhance and maintain liver cell proliferation via a positive feedback loop involving COX-2 and 5-LOX.[34] So psoralidin might act to disrupt the positive loop of hepatitis B development then play a role in preventing liver cancer besides its effect on treatment.

Cyclosporin A, which was widely used for organ transplantation and autoimmune disorders,[35,36] and it was reported that CsA was stated to inhibit apoptosis by preserving mitochondria structure therefore prohibit mitochondrial permeability transition pore opening.[37,38]

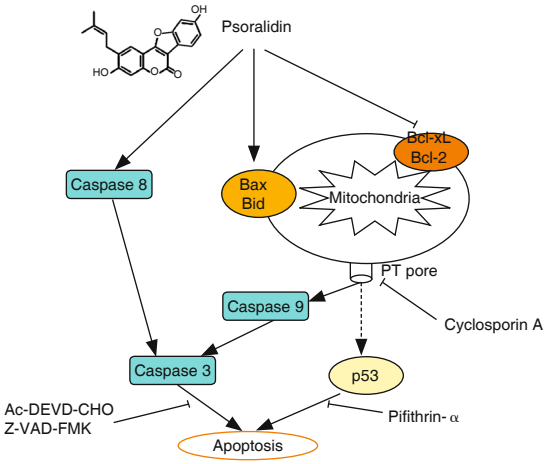

Figure 5. Systematic Graph Illustrates the Pathway Psoralidin Involved in Apoptosis

In summary, it was revealed in the study that the role of psoralidin in cell viability in HepG2 cells. It might involve in both intrinsic and extrinsic apoptotic pathway. Our study point the pharmacological effect of PCL in hepatocarcinoma to its monomer psoralidin and shed light on exploring detailed apoptotic pathway of psoralidin.

Conflict of Interest

There is no conflict of interest.

Author Contributions

Yu B and Zhou K designed the experiments; Wang AH, Zhou K, Chai LJ and Liu L performed the experiments; Yu B, Wang AH and Zhou K analyzed data; Yu B and Zhou K drafted manuscript.

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