Triterpenoid pristimerin induced HepG2 cells apoptosis through ROS-mediated mitochondrial dysfunction

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Summary

Purpose: To investigate the anticancer properties implicated in a natural triterpenoid (pristimerin)-induced apoptosis and inhibited proliferation in human hepatocellular carcinoma (HCC) HepG2 cell line.

Methods: The cytotoxic activity of pristimerin in HepG2 cells was determined by MTT assay. Apoptotic morphology was observed by fluorescence microscope with Hoechst 33258 staining and percent apoptosis was measured by annexin V/PI double staining. DiOC6 for mitochondrial potential (ΔΨm) and DCFH-DA for reactive oxygen species (ROS) were determined by flow cytometry. Changes of apoptotic-related proteins were analysed by Western blot.

Results: Pristimerin exerted a potent cytotoxic effect on HepG2 cells. After HepG2 cells were treated with pristimerin, typical apoptotic bodies, increasing the proportion of apoptotic annexin V-positive cells and activation of caspase-3 were detected in a dose-dependent manner. It was intriguing that pristimerin increased the generation of ROS with a collapse of the mitochondrial membrane potential in the cells. In addition, there was significant change in other mitochondrial membrane proteins triggered by pristimerin, such as Bcl-2 and Bax. Pristimerin also effectively induced subsequent release of cytochrome C from mitochondria into the cytosol, downregulated EGFR protein expression and inhibited downstream signaling pathways in HepG2 cells. Pretreatment with N-acetylcysteine (NAC) blocked ROS generation and resulted in loss of mitochondrial membrane potential, release of cytochrome C and apoptosis induced by pristimerin.

Conclusion: These data indicate that ROS play an essential role in the induction of apoptosis by pristimerin in HepG2 cells.

Key words: bcl-2 apoptosis, HepG2, hepatocellular carcinoma, pristimerin, ROS

Introduction

HCC is a common and aggressive malignant tumor worldwide, with morbidity showing a rising trend [1]. Currently, surgery and chemotherapy are the main treatment options for HCC, but the curative effect of the existing chemotherapeutic drugs is not good enough and they have numerous side-effects [2,3]. Therefore, novel effective chemotherapeutic agents are warranted, particularly those derived from natural products.

Pristimerin is a quinonemethide triterpenoid compound which has been found in various species belonging to Celastraceae and Hippocrateaceae families and has long been used as anti-inflammatory, antioxidant, antimalarial, and insecticidal agent [4,5]. It has been reported that pristimerin has promising clinical potential both as a therapeutic and chemopreventive agent for cancer [6]. Indeed, pristimerin induces apoptotic cell death in certain human cancer cell lines, including breast and lung cancer [7] and acute myeloid leukemia [8]. Although evidence has been accumulated that the mode of cell death induced by pristimerin is
caspase-dependent apoptotic cell death, the involved mechanisms of action, especially cross-talk between signaling pathway and apoptotic cell death machinery, is largely unknown.

In this article we describe the results of our investigation on the anti-HCC activity of pristimerin, which might provide experimental evidence for clinical therapy in HCC.

**Methods**

**Chemicals and reagents**

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA), 3,3’-dihexyloxacarbocyanine iodide (DiOC6), Hoechst 33258 and the antioxidant NAC were purchased from Sigma (St. Louis, MO, USA). ApopNexin FITC Apoptosis Detection Kit was purchased from Chemicon (Temecula, CA, USA). Antibodies against caspase-3, caspase-9, cytochrome C and Bcl-2 were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against EGFR and Bax were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Akt, p-ERK1/2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-mouse IgG-horseradish peroxidase, and anti-rabbit IgG-horseradish peroxidase were purchased from KangChen Biotechnology (Shanghai, China). All tissue culture supplies were purchased from Life Technologies (Carlsbad, CA, USA). Other routine laboratory reagents of analytical or high-performance liquid chromatography grade were obtained from Whiga Biotechnology (Guangzhou, China). Pristimerin (Figure 1A) with a purity of >98% was purchased from the PI & PI Technology Inc. (Guangzhou, China).

**Cell lines and cell culture**

The human HCC cell line HepG2 was purchased from American Type Culture Collection. Cells were cultured in DMEM medium, which contains 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum. All cells were cultured in a humidified atmosphere incubator of 5% CO₂ and 95% air at 37 °C.

**Cell viability assay**

Cells were harvested during the logarithmic growth phase and seeded in 96-well plates at a density of 1.5×10⁴/mL in a final volume of 190μL/well. After 24 h incubation, 10 μL pristimerin full-range concentration was added to 96-well plates. After 68 h treatment, 20 μL MTT (5 mg/mL stock solution of saline) were added to each well for 4 h. Subsequently, the supernatant was removed and MTT crystals were solubilized with 100μL anhydrous DMSO in each well. Thereafter, cell viability was measured by model 550 microplate reader (Bio-Rad) at 540 nm, with 655 nm as reference filter. The 50% inhibitory concentration (IC50) was determined as the anticancer drug concentration causing 50% reduction in cell viability and calculated from the cytotoxicity curves (Bliss’ software). Percent cell survival was calculated using the following formula: survival (%) = [(mean experimental absorbance) / (mean control absorbance)]×100.

**Assessment of apoptosis morphology by Hoechst 33258 staining**

HepG2 cells were treated with the indicated concentrations of pristimerin in the absence or presence of 5 mmol/L NAC for 24 h. Both floating and trypsinized adherent cells were collected, washed once with ice-cold PBS, fixed with 1 mL of 4% paraformaldehyde for 20 min, and washed once with ice-cold PBS. Then, the cells were incubated in 1 mL PBS containing 10 μmol/L

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**Figure 1.** A: Chemical structure of pristimerin; B: Pristimerin showed potent cytotoxicity to human HCC HepG2 cells. Mean±SD of at least triplicate determinations. Each experiment was done in 6 replicate wells.
Hoechst 33258 at 37 °C for 30 min, washed twice, and observed using fluorescence microscopy with standard excitation filters (Leica Dmrb) in random microscopic fields at × 400 magnification.

Annexin V/propidium iodide double-staining assay

HepG2 cells were treated with the indicated concentrations of pristimerin in the absence or presence of 5 mmol/L NAC for 24 h. Both floating and attached cells were collected, washed with ice-cold PBS twice, and resuspended in 200 μL of 1× binding buffer containing annexin V (1:50 according to the manufacturer’s instruction) and 40 ng/sample propidium iodide (PI) for 15 min at 37 °C in the dark. Then, the number of viable, apoptotic, and necrotic cells was quantified by flow cytometer (Becton Dickinson) and analyzed by the Cell-Quest software. Cells were excited at 488 nm and emission of annexin V at 525 nm and PI were collected through 610 nm band-pass filters. At least 10,000 cells were analyzed for each sample. Percent apoptosis was calculated as follows: [(number of apoptotic cells) / (number of total cells observed)]×100.

Measurement of ROS generation

DCFH-DA was used as ROS capture in the cells. It is cleaved intracellularly by nonspecific esterases to form 2,7-dichlorodihydrofluorescein (DCFH), which is further oxidized by ROS and becomes a highly fluorescent compound 2,7-dichlorofluorescein (DCF). Thus, the fluorescence intensity of DCF is proportional to the amount of ROS produced by the cells. In the present study, HepG2 cells were exposed to 2 μmol/L pristimerin in the absence or presence of 5 mmol/L NAC for 24 h, respectively. 6 × 10^6 cells were harvested, washed with PBS, and incubated with 50 μmol/L DCFH-DA at 37 °C for 20 min in the dark. After washing once with ice-cold PBS, cells were harvested and kept on ice for immediate detection by flow cytometry at the excitation wavelength of 488 nm and emission wavelength of 530 nm. The average intensity of DCF stands for intracellular ROS levels.

Determination of membrane potential of mitochondria (Ψm)

Changes of Ψm (ΔΨm) were monitored by flow cytometry with the mitochondrial tracking fluorescent dye DiOC6 after HepG2 cells were exposed to 2 μmol/L pristimerin in the absence or presence of 5 mmol/L NAC for 24 h, respectively. 6 × 10^6 cells were harvested, washed with PBS, and incubated with 40 μmol/L DiOC6 at 37 °C for 20 min in the dark. Then, the cells were washed twice, resuspended in 1 mL PBS, and analyzed by FACS Calibur flow cytometer with excitation wavelength of 484 nm and emission wavelength of 501 nm [9]. The data obtained from flow cytometry were analyzed by CellQuest software and expressed as mean fluorescence intensity. The expressed data were the results of at least three independent determinations.

Whole-cell lysates and Western blot analysis

After HepG2 cells were exposed to indicated concentrations of pristimerin in the absence or presence of 5 mmol/L NAC for 24 h, whole cells were harvested and washed twice with ice-cold PBS, and the pellet was vortexed and 1× lysis buffer [50 mmol/L Tris- HCl (pH 6.8), 10% glycerol, 2% SDS, 0.25% bromphenol blue, and 0.1 mol/L DTT] was added for 100 μL/5 × 10^6 cells. After heated at 95 °C for 20 min, the lysates were centrifuged at 12,000 rpm for 10 min and the supernatant was collected. The protein concentration was determined by nucleic acid-protein analyzer (Beckman). Equal amount of lysate protein was separated on 8-12% SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Pall). The nonspecific binding sites were blocked with 5% nonfat dry milk for 2 h. The membranes were incubated overnight at 4 °C with specific primary antibodies. Then, the membranes were washed three times with TBST buffer and incubated at room temperature for 1 h with horseradish peroxidase-conjugated secondary antibody. After three washes with TBST buffer, the immunoblots were visualized by the enhanced Phototope-Horseradish Peroxidase Detection Kit purchased from Cell Signaling Technology (Danvers,MA,USA) and exposed to Kodak medical X-ray processor [10].

Subcellular fractionation for Western blot analysis of cytosolic cytochrome C

After HepG2 cells were exposed to indicated concentrations of pristimerin in the absence or presence of 5 mmol/L NAC for 24 h, whole cells were harvested by centrifugation at 1,000 rpm for 5 min. The pellets were washed twice with ice-cold PBS, suspended with 5-fold volume of ice-cold cell extract buffer [20 mmol/L L4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES-KOH; pH 7.5), 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 250 mmol/L sucrose, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 0.02 mmol/L aprotinin], and incubated for 40 min at 4 °C. Then, the cells were centrifuged at 1,200 rpm for 10 min at 4 °C; the supernatant was subsequently centrifuged at 12,000 rpm for 15 min at 4 °C and the final supernatant was used as cytosolic fraction of cytochrome C. Then, 5×loading buffer [250 mmol/L Tris-HCl (pH 6.8), 50% (v/v) glycerol, 10% (w/v) SDS, 0.5% (w/v) bromphenol blue, and 5% (w/v) DTT] was added to the above obtained supernatant and the mixture was boiled at 100 °C for 15 min. Thus, the protein solution was used for identification of cytosolic cytochrome C by Western blot with 15% SDS-PAGE and blotting onto polyvinylidene difluoride membrane. The cytochrome C protein was detected by using anti-cytochrome C antibody in the ratio of 1:1,000 [11].
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Statistics

For each protocol, three independent experiments were performed. Results were expressed as the mean ± standard error of the mean (SEM). Statistical calculations were performed by using SPSS16.0 software. Differences in measured variables between experimental and control groups were assessed by the Student’s t-test. p < 0.05 was indicative of significant difference and p < 0.01 was indicative of very significant difference.

Results

Pristimerin exerted potent cytotoxicity against HepG2 cells

The cytotoxicity of pristimerin to HepG2 cells was measured by MTT assay. Pristimerin inhibited cell proliferation in a concentration-dependent manner in HepG2 cells after 72 h treatment (Figure 1B). The IC50 of pristimerin was 1.44 ± 0.24 μmol/L for HepG2 cells. The data suggested that pristimerin exhibited potent cytotoxicity against HepG2 cells.

Pristimerin induced apoptosis in HepG2 cells

To observe the morphologic characteristics of apoptosis, cells were stained with Hoechst 33258 after HepG2 cells were exposed to 1.0-4.0 μmol/L pristimerin for 24 h and detected by fluorescence microscopy. Control cells showed even distribution of the stain and round homogeneous nuclei features. Apoptotic cells increased gradually in a dose-dependent manner and displayed typical changes including reduction of cellular volume, bright staining and condensed or fragmented nuclei (Figure 2A-D). For further assessment of apoptosis induced by pristimerin, we examined the exposure of phosphatidylserine on the cell surface by using annexin V/PI double staining. Flow cytometric analysis revealed that the percentage of apoptotic cells with annexin V-positive but PI-negative cells increased gradually with concentration in pristimerin-treated cells. The early percent apoptosis was 0.73 ± 0.47%, 17.17 ± 1.63%, 26.23 ± 1.89%, 38.53 ± 2.15% (Figure 3A). Western blotting also revealed pristimerin-induced growth inhibition and apoptosis in HepG2 cells, showing activation of caspase-3 and downregulation of EGFR (Figure 5A).

NAC blocked pristimerin-induced ROS generation and the loss of Ψm

After HepG2 cells were pretreated with 5 mmol/L NAC for 6 h prior to 2 μmol/L pristimerin exposure, the ROS generation and changes of Ψm (ΔΨm) were monitored by flow cytometry. Our results demonstrated that the increase of ROS and the collapse of Ψm were observed after HepG2 cells exposure to 2 μmol/L pristimerin. Importantly, pretreatment with NAC effectively blocked pristimerin-induced ROS generation and the loss

Figure 2. HepG2 cells apoptosis morphological changes were examined by Hoechst 33258 staining. A-D: Pristimerin-mediated HepG2 cell apoptosis and morphologic changes in a dose-dependent manner. E-H: NAC suppresses pristimerin induced formation of apoptotic bodies. HepG2 cells were treated with 1.0-4.0μmol/L pristimerin in the absence or presence of 5 mmol/L NAC for 24 h; apoptotic bodies were observed under fluorescence microscope at ×400 magnification.
of membrane potential (Figure 4).

**Effect of NAC on pristimerin-induced release of cytochrome C, growth inhibition and apoptosis**

To examine the relationship between pristimerin-induced intracellular ROS production and cell growth inhibition and apoptosis, cells were pretreated with NAC to investigate the effects of intracellular ROS depletion on pristimerin-induced cell growth inhibition. HepG2 cells were exposed to 1.0-4.0 μmol/L pristimerin for 24 h following pretreatment with 5 mmol/L NAC for 6 h. The formation of apoptotic bodies increased following pristimerin exposure in HepG2 cells and this effect was attenuated with NAC pretreatment (Figure 2E-H). Annexin V/PI double staining revealed that NAC significantly inhibited the ability of pristimerin to induce apoptosis in HepG2 cells (Figure 3). After HepG2 cells were pretreated with 5 mmol/L NAC for 6 h prior to 2μmol/L pristimerin exposure, apoptosis-related proteins were detected by Western blotting analysis. Our results showed that NAC significantly prevented pristimerin-induced release of cytochrome C, caspase 9, caspase-3 activation and downregulation of EGFR, Akt, and p-ERK1/2 (Figure 5B). These results revealed a link between ROS formation and pristimerin-induced growth inhibition and apoptosis in HepG2 cells.

**Discussion**

In recent years, screening for active anticancer drugs from the Chinese medicine has become a hot spot in anticancer research. We hope that new and more effective anticancer drugs obtained from Chinese herbs can be developed to improve the prognosis of cancer patients. Some of the plants, such as *Maytenus chuchuhuasca* and *Maytenus laevis*, have traditionally been used in the treatment of arthritis and skin cancer in South America [12]. Pristimerin is a quinonemethide triterpenoid compound which has been found in various species belonging to *Celastraceae* and *Hippocrateaceae* families. It is known that pristimerin exhibits antimicrobial, antiinflammatory, and antitumor effects [13]. Many studies [6-8] have shown that pristimerin is a potent and broad-spectrum antitumor agent, with activity against a wide range of different human cancers. However, the mechanism of the anti-HCC activity of pristimerin was never explored. Our experimental results showed that pristimerin displayed potent cytotoxicity to human HCC HepG2 cells and the IC50 was $1.44 \pm 0.24$ μmol/L (Figure 1B).
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Recently, pristimerin has been shown to induce apoptosis in certain cancer cell lines [7]. Currently, it is not completely clear how pristimerin induces proteasome inhibition and apoptosis. Pristimerin-triggered caspase activation was confirmed in human breast cancer cells [7]. In our study, apoptosis assay showed that apoptotic cells induced by pristimerin displayed condensed and fragmented nuclei by Hoechst 33258 staining (Figure 2A-D). Annexin V/PI double staining assay further confirmed the results of Hoechst 33258 staining by showing that the important membrane alterations relating to apoptosis in HepG2 cells and the percent apoptosis increased in a dose-dependent manner (Figure 3A). Additionally, we showed that pristimerin induces caspase-dependent apoptosis in HepG2 cells, implying activation of caspase-3 (Figure 5A). Taken together, these results suggest that pristimerin is able to decrease the viability of HepG2 cells through induction of caspase-dependent apoptosis.

A variety of signaling pathways may be involved in apoptosis and the mitochondrial pathway is one of the major apoptosis pathways [14]. Mitochondria have been shown to play a central role in the apoptotic process, because both the intrinsic and the extrinsic pathway can converge at the mitochondrial level and trigger mitochondrial membrane permeabilization [15,16]. Release of cytochrome C and other proapoptotic proteins, such as SMAC/Diablo and EndoG from the mitochondria to cytosol is the limiting factor in the mitochondrial pathway and the mitochondrial dysfunction. Recent studies showed that treatment of breast cancer cells with pristimerin resulted in a rapid release of cytochrome C from mitochondria, which preceded caspase activation and the decrease of mitochondrial membrane potential. This process did not depend on Bcl-2 family (Bcl-2, Bcl-xl and Bax) protein levels and did

Figure 4. NAC blocked pristimerin-induced ROS generation and loss of Ψm in HepG2 cells. A: NAC suppresses pristimerin-induced ROS generation. B: NAC suppresses pristimerin-induced collapse of the Ψm. Red area, black and green lines were representative of ROS or Ψm levels for control, pristimerin alone, and NAC+ pristimerin. C and D: ROS or Ψm levels of HepG2 cells, expressed as units of mean fluorescence intensity, were calculated as percentage of control. Mean ± SD of at least triplicate determinations. ** p< 0.01 vs control.
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In recent years it has become apparent that ROS play an important role during apoptosis induction [17]. Many studies have indicated that some anticancer agents (e.g., doxorubicin, epirubicin, and daunomycin) induce apoptosis in part with generation of ROS and the disruption of redox homeostasis [18]. Intracellular production of ROS can directly lead to activation of the mitochondrial permeability transition and to loss of mitochondrial membrane potential [19]. Pristimerin-induced mitochondrial cell death in cervical cancer cells was reported through ROS generation [20]. However, recent studies using pristimerin in breast cancer cells convey a different viewpoint. The generation of ROS in MDA-MB-231 cells was not affected by pristimerin [7]. Our research showed that pristimerin induced generation of ROS and subsequent loss of mitochondrial membrane potential in HepG2 cells (Figure 4). We tried to explain how chemically and biologically pristimerin influenced ROS generation. It is a known fact that most prooxidants/antioxidants behave differently depending on the experimental conditions and cell lines of different tissues of origin [11,21,22]. Therefore, the exact mechanisms by which pristimerin increases the intracellular ROS level and subsequent mitochondrial cell death remain to be elucidated. In our study, the increase of ROS, the collapse of the $\Psi_m$, the release of cytochrome C, caspase-9 and caspase-3 activation and downregulation of EGFR, Akt, and p-ERK1/2 were observed after HepG2 cells exposure to 2 μmol/L pristimerin. Also pretreatment with NAC significantly prevented pristimerin-induced ROS generation, loss of $\Psi_m$, activation of apoptosis-related proteins and downregulation of proliferation-related proteins (Figures 2-5). These results indicate that ROS-dependent activation by pristimerin is critically required for the mitochondrial dysfunction in HepG2 cells.

The ubiquitin-proteasome pathway is essential for many fundamental cellular processes, including cell cycle, apoptosis, angiogenesis, and differentiation [23]. In addition, the proteasome contributes to the pathologic state of several human diseases including cancer and AIDS, in which some regulatory proteins are either stabilized due to decreased degradation or lost owing to accelerated degradation [24]. Therefore, targeting disease-associated proteins for ubiquitination and degradation represents a promising alternative therapeutic strategy in cancer. Pristimerin is a natural analog of celastrol. Not surprisingly, it also targets the proteasome [25]. Nucleophilic susceptibility and in silico docking studies show that C6 of pristimerin is highly susceptible towards a nucleophilic attack by the hydroxyl group.

**Figure 5.** A: Pristimerin augmented the instability of EGFR and induced activation of caspase-3 in HepG2 cells in a dose-dependent manner. B: NAC reversed pristimerin-induced release of cytochrome C, growth inhibition and induced changes of apoptotic proteins related to mitochondrial pathway in HepG2 cells.

not require translocation of Bax to the mitochondria. In the present study, treatment of HCC HepG2 cells with pristimerin resulted in a rapid release of cytochrome C from mitochondria, which preceded caspase activation and cell apoptosis. This process also depended on Bcl-2 family (Bcl-2 and Bax) protein levels, showing that the ratio of Bcl-2 and Bax was downregulated (Figure 5B). Taken together, our results revealed that intrinsic pathways took part in the pristimerin-induced apoptosis in HepG2 cell lines.
of Thr1 of the proteasomal chymotrypsin subunit. EGFR-overexpressing cancer cells have been shown to activate downstream mitogen-activated protein kinase (MAPK) and PI3K (phosphatidylinositol 3-kinase)/Akt signaling pathway. As shown in Figure 5A, we discovered that pristimerin also inhibited the growth in HepG2 cells. Pristimerin blocked the expression of EGFR and Akt and this inhibition by pristimerin may be through the proteasome pathway. Such studies will be performed in the near future.

In summary, our findings demonstrated that pristimerin induced ROS-dependent mitochondrial cell apoptosis in human HepG2 cancer cells. The mechanisms might involve inhibition of expression of EGFR and Akt proteins. We concluded that pristimerin could be a lead compound for further drug development to overcome resistance in liver cancer patients. We believe that elucidating the molecular mechanisms used by naturally occurring products to regulate cell death is critical for both our understanding of cell death events and the development of cancer therapeutic agents.

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References


