Research Paper

Pristimerin induces apoptosis and inhibits proliferation, migration in H1299 Lung Cancer Cells

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Abstract

**Background:** The natural occurring pristimerin, a quinonemethide triterpenoid, is extracted from a variety of species of the *Celastraceae* and *Hippocrateaceae* family. This research investigated the *in vitro* anti-cancer potential of pristimerin on NSCLC cells NCI-H1299 and elucidated the molecular mechanism.

**Methods:** Cell growth inhibition by pristimerin was assessed using the MTT assay. Apoptosis was detected using the Annexin V/propidium iodide (PI) test. The colony forming assay was used to investigate the anti-proliferative effects of pristimerin. Wound healing assay and the transwell cell migration assay were utilized to determine the inhibitory effects of migration and invasion, respectively. Western blot was used to detect the protein expression, and real-time-quantitative (RT-q) PCR was used to analyze the mRNA expression.

**Results:** The results showed that pristimerin inhibited the proliferation of H1299 cells with an IC50 value of 2.2 ± 0.34 µM and induced apoptosis in a dose-dependent manner. The colony formation ability was reduced in a dose-dependent manner. A marked inhibition of migration and invasion against H1299 cells was observed in a dose- or time-dependent manner. Moreover, the decreased protein levels of vimentin, F-actin, integrin β1, matrix metalloproteinase (MMP2) and Snail revealed the potential inhibition of epithelial-to-mesenchymal transition (EMT). The regulated mRNA levels of integrin β1, MMP2 and Snail indicated the great potential in the treatment of NSCLC.

**Conclusion:** In conclusion, our study demonstrated that pristimerin suppressed NSCLC cells NCI-H1299 *in vitro*, exhibited potent activities of proliferation inhibition and apoptosis induction. Furthermore, the treatment of pristimerin decreased migration and invasion of H1299, which was correlated with EMT-related proteins and mRNA.

**Key words:** Anti-lung cancer, NSCLC, pristimerin, anti-apoptosis, migration, invasion

Introduction

Lung cancer is one of the most common malignant tumor throughout the world, accounting for the highest diagnostic rate (11.6%) and cancer death rate (18.4%), approximately 80% of which belongs to non-small cell lung cancer (NSCLC) [1, 2]. Although the targeted molecular therapy and immunotherapy has made great progresses [3], until recently, for early-stage lung cancer patients, chemotherapy remained the non-operative first-line approach (other than radiofrequency ablation and
standard radiotherapy) [4]. Traditional chemotherapeutic drugs are still faced with drug-resistance and related-toxicities and active development of new anti-cancer agents is warranted and promising.

Natural product contains abundant resources of bioactive agents, the anti-cancer activities of natural product which attracts increasing attention [5-8]. The natural occurring pristimerin, a quinonemethide triterpenoid, is isolated from varied Celastraceae and Hippocrateaceae family [9-11]. Recently, pristimerin has been reported to widely induce apoptosis and autophagy, inhibit angiogenesis and metastasis against cancer [12]. However, the potential efficacy of pristimerin against lung cancer is relatively deficient. In this article, we investigated the in vitro anti-cancer potential of pristimerin on NSCLC cells NCI-H1299 and elucidated the molecular mechanism.

Materials and Methods

Cell culture and reagents

The human lung adenocarcinoma epithelial cell line NCI-H1299 (lack expression of p53 protein) was obtained from iCell Bioscience Inc., Shanghai, China, and authenticated by short-tandem repeat (STR) profiling. The cells were maintained in RPMI-1640 medium supplemented with 100 μM streptomycin, 100 U/mL penicillin and 10% fetal bovine serum (FBS) (TIANHANG Biotech Co., Ltd., Zhejiang, China). Cells were cultured in a humidified atmosphere containing 5% CO2 at 37°C. Pristimerin (Fig. 1A) with a purity of >98% was purchased from the Spring & Autumn Biological Engineering Co., Ltd. (Nanjing, China).

Cell viability assay

The MTT assay was utilized to evaluate the cytotoxic activity of pristimerin. 4 × 10^3 cells were transferred into each well of 96-well plates in a final volume of 190 μL. After cells attached for 24 h, 10 μL pristimerin were added to the medium at a series of different concentrations for 72 h incubation. 20 μL MTT (5 mg/mL) was added to each well for 4 h reaction. Next, the supernatant was discarded, and the formed formazan product was dissolved in 100 μL DMSO/well. The optical density (OD) was detected at an absorbance wavelength of 540 nm using a Model 550 Microplate reader (Bio-Rad Laboratories, Berkeley, CA, USA), with reference filter of 655 nm. The IC50 value was used to express the inhibitory action of pristimerin, which was obtained according to the concentration-response curve using Bliss software. Percent survival rate was obtained using the formula as described previously [5].

Annexin V/ propidium iodide (PI) test

Annexin V-FITC/PI double staining assay was applied to determine apoptosis, using an Annexin V-FITC/PI apoptosis detection kit (KeyGen Biotech Co., Ltd., Nanjing, China). H1299 cells were cultured in the presence of 0.9, 1.8 and 3.6 μM pristimerin for 48 h, then collected and resuspended in 0.5 mL binding buffer/sample for 5 min. Subsequently, 5 μL FITC-labeled Annexin-V and 5 μL PI were added for 15 min staining at 37°C in dark. The stained samples were measured using a flow cytometer (Beckman Coulter, Brea, CA, USA) and analyzed by CytExpert software version 2.2 (Beckman Coulter, Brea, CA, USA). The rate of apoptosis (%) was determined by dividing the number of apoptotic cells by the number of total cells observed, and multiplying with 100 [7]. All experiments were repeated three times.

Colony forming assay

Cells were split and transferred into 12-well plates with 400 cells/well. Until the colonies of 30-40 cells formed, cells were incubated with pristimerin of 1.8 μM for 24, 48 and 72 h, respectively. The wells were washed three times with PBS, then fixed with methanol and stained with 0.5% (w/v) crystal violet. Images were captured using ChemiDoc™ XRS+ (Bio-Rad Laboratories, Berkeley, CA, USA). The colony forming assay was repeated three times in duplicate.

Wound healing assay

Cells were digested using trypsin and resuspended in serum-free medium. The cells were transferred into 6-well plates and grown to reach confluence overnight. Subsequently, linear wounds were scraped with pipette tips in the cell monolayer. Next, the debris was removed and the edge of the wound was smoothed by washing the cells once. The cells were incubated with pristimerin (0.9, 1.8 and 3.6 μM), respectively. After 0, 24 and 48 h of incubation, wounds were observed and photographed using a light microscope (Olympus Life Science, Tokyo, Japan) at ×40 magnification. Cell motility was quantified by the percentage of the repaired area. The percent wound closure (%) was obtained through the following formula: percent mobility rate = migrated cell surface area/total surface area × 100 [13].

Invasion assay

The bottom of transwell apparatus (Corning Costar, Corning, NY, USA) was pre-coated with matrigel (Corning Costar, Corning, NY, USA), which was diluted by 5 times with basal medium. Cells were transferred into the upper chamber of the transwell apparatus at a density of 8 × 10^5 cells/well in 200 μL

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of serum-free RPMI-1640 medium, which contained the pristimerin (0.9, 1.8 and 3.6 μM). The lower chamber contained 600 μL RPMI-1640 medium adding 10% FBS, which was a chemoattractant. After incubation of 48 h, cells that migrated into the lower chamber were fixed with methanol and stained with 0.5% (w/v) crystal violet [14]. Cells on the top layer were removed using a cotton swab, and the migrated cells were photographed using an inverted microscope (Leica Microsystems, Wetzlar, Germany) at ×200 magnification. Nine random fields were acquired to quantify attached and migrated cells. The cell number was quantified by IMAGE-J software (version 1.51j8, Bethesda, MD, USA) [http://imagej.nih.gov/ij/]. Cell counts were expressed as the mean number of cells per field of view.

**Western blot**

Cultured cells from different samples were collected and washed twice with ice-cold PBS. The pellet was vortexed and lysed in lysis buffer (Cell Signaling Technology, Danvers, MA, USA), supplemented with Proteinase Inhibitor Cocktail (Cell Signaling Technology, Danvers, MA, USA) and phenylmethanesulfonyl fluoride (PMSF) (Cell Signaling Technology, Danvers, MA, USA). 25 μg/lane proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto PVDF membranes (Millipore, Boston, MA, USA). The membranes were then blocked with TBST buffer containing 5% non-fat milk, subsequently incubated with primary antibodies overnight at 4°C. After 3 times washed with TBST buffer, the membranes were incubated to bind horseradish peroxidase (HRP) conjugated secondary antibodies for 2 h at room temperature. Blots were visualized by Western Lightening® Plus-ELC kit (PerkinElmer, Waltham, MA, USA) and exposed by ChemiDoc™ XRS+ (BIO-RAD, Berkeley, CA, USA). Antibodies used in this study included: rabbit anit-Integrin beta 1 (1:1000 dilution) (#9699, Cell Signaling Technology), rabbit anit-MMP2 (1:1000 dilution) (ab92536, Abcam), rabbit anit-MMP7 (1:1000 dilution) (ab180714, Abcam), mouse anti-F-actin (0.2 μg/mL) (ab4792, Abcam), rabbit anti-Snail+Slug (1:1000 dilution) (ab180714, Abcam), mouse anti-GAPDH (1:10000 dilution) (MB001, Bioworld Technology), GAPDH (1 A6) monoclonal antibody (1:10000 dilution) (MB001, Bioworld Technology), Anti-rabbit IgG, HRP-linked Antibody (1:2000 dilution) (#7074, Cell Signaling Technology) and HRP-conjugated goat anti-mouse (1:5000 dilution) (BS12478, Bioworld Technology).

**mRNA extraction, reverse transcription and real-time-quantitative (RT-q) PCR**

Total RNA of H1299 cells stimulated with pristimerin (0.9, 1.8, 2.7 and 3.6 μM) after 24 h was extracted using TRizol (Thermo, Waltham, MA, USA). The PrimeScript™ RT Master Mix (Takara Biomedical Technology, Kusatsu, Japan) was used to transcribe mRNAs into cDNA. The forward and reverse primers (Takara Biomedical Technology, Kusatsu, Japan) were designed to detect the expression of integrin β1, matrix metallopeptidase 2 (MMP2) and Snail. SYBR® Premix Ex Taq™ (TliRNaseH Plus) (Takara Biomedical Technology, Kusatsu, Japan) was utilized for RT-qPCR and GAPDH was used to normalize mRNA expression. RT-qPCR was carried out using a QuantStudio 5 (Thermo Fisher scientific, Waltham, MA, USA). The program setting for qPCR is as below: firstly, initial denaturation at 95°C for 30 sec; then 40 reaction cycles were followed by denaturation at 95°C for 5 sec, annealing at 60°C for 34 sec, and then elongation at 72°C for 30 sec [15]. The data were calculated using the 2^ΔΔCq method and GAPDH was used as control gene for normalization [16]. The primer sequences designed are listed in Table 1.

**Table 1. The primer sequences employed in RT-qPCR**

| Primer name  | Primer sequence (5’–3’) | Company            |
|--------------|-------------------------|--------------------|
| GAPDH Forward | GGAAGTCAGTCAGGGACATCA  | Invitrogen, Inc.   |
| GAPDH Reverse | GTCAATGGATGGCAAATATCCACT | Invitrogen, Inc.   |
| Integrin β1 Forward | GACGCCGCGCGGAAAAGATG | Takara, Inc.       |
| Integrin β1 Reverse | GCCACCCACCAATTGCGCCC  | Takara, Inc.       |
| Snail Forward | CCAGACCCACTCATGTCAGAAG | Takara, Inc.       |
| Snail Reverse | GCCAGGTTAGGAGGAGGAAGA  | Takara, Inc.       |
| MMP2 Forward | CTATGCGCAATGCCGAAAGA   | Takara, Inc.       |
| MMP2 Reverse | TTCAAGTAATTGGGAGCCCTTGA | Takara, Inc.       |

**Statistical analysis**

All experiments were performed in triplicate unless otherwise stated. The differences between groups were analyzed using ANOVA followed by Tukey’s post-hoc test. Statistical differences in each assay were analyzed by SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). Differences between groups were considered statistically significant if P < 0.05.

**Results**

**Pristimerin exhibits a notable cytotoxicity against H1299 cells**

Cell growth inhibition by pristimerin was assessed using MTT assay. Pristimerin (Fig 1A) inhibited the proliferation of H1299 cells with an IC50 value of 2.2 ± 0.34 μM. The growth inhibition occurred in a dose-dependent manner (Fig. 1B). The results
indicated the cytotoxicity of pristimerin against H1299 cells.

**Pristimerin shows a potent apoptotic-inducing effect against H1299 cells**

To investigate whether pristimerin is able to induce apoptosis of H1299 cells. The cells were incubated with 0.9, 1.8 and 3.6 μM of pristimerin for 48 h, then apoptosis was detected by flow cytometry based on annexin V and PI staining to discriminate healthy cells from early/late apoptotic cells and necrotic cells. The results showed that the rates of apoptosis were 0.61 ± 0.11, 5.08 ± 0.90, 29.79 ± 0.88 and 80.64 ± 3.92% for control, 0.9, 1.8 and 3.6 μM of pristimerin, respectively (Fig. 2).

**Pristimerin decreases H1299 cells colony formation**

The colony forming assay was performed to further investigate the anti-proliferative effects of pristimerin. The results showed that incubation of H1299 cells with pristimerin for 24, 48 and 72 h reduced the colony formation in a time-dependent manner which was characterized by specific colony numbers and sizes of H1299 cells (Fig. 3).

**Pristimerin reduces H1299 cells migration and invasion**

Wound healing assay was applied to evaluate the inhibitory effect of pristimerin on NSCLC cells migration. After H1299 cells were exposed to pristimerin for 24 h at a concentration of 0.9, 1.8 and 3.6 μM, the mobility ratios with respect to control were 38.03 ± 3.71, 35.67 ± 3.23, 25.54 ± 5.76 and 4.91 ± 1.47%, respectively. Moreover, after exposure to pristimerin for 48 h at the same series of concentration, the mobility ratios were 65.66 ± 6.42, 58.61 ± 3.72, 47.36 ± 6.18, 11.92 ± 3.63%, respectively (Fig. 4). The result suggested that pristimerin markedly inhibited H1299 cells migration in a dose-dependent manner.

**Invasion is another process of cancer proliferation and metastasis.** Transwell assay was utilized to analyze the role of pristimerin in regulating the invasion of NSCLC cells. Consistent with the results of migration assay, pristimerin markedly inhibited NSCLC cells invasion. After treatment of H1299 cells with 0.9, 1.8 and 3.6 μM pristimerin for 48 h, the number of invaded cells decreased (Fig. 5). The above results indicated that pristimerin reduced cell invasion in a dose-dependent manner.

**Pristimerin regulates protein expression**

Cancer metastasis is a complicated process, which involved motility, adhesion and disruption of polarity of cell. To investigate whether pristimerin inhibited EMT of H1299 cells, western blot was used to detect the protein express of vimentin, F-actin, integrin β1, MMP2 and Snail. After H1299 cells were incubated with 0.9, 1.8 and 3.6 μM pristimerin for 48 h, the protein expression of vimentin, F-actin, integrin...
β1, MMP2 and Snail were decreased in a dose-dependent manner (Fig. 6).

Figure 3. Pristimerin of 1.8 µM leads to a significant inhibition of colony formation of H1299 cells in a time-dependent manner. A. H1299 cells were treated with different concentrations of pristimerin and colony efficiency was observed via a colony forming cell assay. B. Quantitative results of (A). Data were quantified as mean ± standard deviation. *P < 0.05, **P < 0.01, n ≥ 3.

Figure 4. Treatment of pristimerin decreases the migrative ability of H1299 cells in vitro in a dose-dependent manner. A. H1299 cells were treated with different concentrations of pristimerin for 24 h and 48 h, the mobility ratio was observed via wound healing assays. B. Quantitative results of (A). Data are presented as the mean ± standard deviation. *P < 0.05, **P < 0.01, n ≥ 3.

Figure 5. H1299 cells invasion is inhibited in vitro by pristimerin in a dose-dependent manner. A. The invasive ability of H1299 cells was detected via transwell assay. Following treatment of H1299 cells with different concentrations of pristimerin for 48 h, the number of invaded cells decreased in a dose-dependent manner. B. Quantitative results of (A). Data are presented as the mean ± standard deviation. *P < 0.05, **P < 0.01, n ≥ 3.

Figure 6. Pristimerin regulates migration and invasion-related proteins expression in H1299 cells. After H1299 cells were treated with pristimerin of 0.9, 1.8 and 3.6 µM for 48 h respectively, the whole-cell lysates were assayed by Western blot and corresponding antibodies. A. Down-regulations of vimentin, F-actin, integrin β1, MMP2 and Snail expression by pristimerin in a dose-dependent manner were observed. B. Data analysis of (A). Data are presented as the mean ± standard deviation. *P < 0.05, **P < 0.01, n ≥ 3.
Pristimerin affects mRNA expression

To determine whether the anti-proliferative, anti-migration and anti-invasive activities of pristimerin were related to the specific mRNA levels, RT-qPCR was used to determine the mRNA expression. After H1299 cells were incubated with pristimerin (0.9, 1.8, 2.7 and 3.6 μM) for 24 h, the mRNA levels of integrin β1, MMP2 and Snail were down-regulated in a dose-dependent manner (Fig. 7).

![Relative mRNA expression with pristimerin](image)

**Figure 7.** Pristimerin regulates migration and invasion-related mRNA in H1299 cells. A. After H1299 cells were treated with pristimerin of 0.9, 1.8, 2.7 and 3.6 µM for 24 h respectively, the mRNA expression was detected by RT-qPCR. Down-regulation of Snail, integrin β1 and MMP2 mRNA expression were observed. B. Data analysis of (A). Data are presented as the mean ± standard deviation. *P < 0.05, **P < 0.01, n ≥ 3.

Discussion

Pristimerin (Fig. 1A), a quinonemethide triterpenoid, kills cancer cell lines in a wide spectrum [12], as well as potent *in vivo* anti-cancer activities [17, 18]. However, its activities against lung cancer are insufficient of evidence to support. It was reported that pristimerin has shown activities of proliferation-inhibiting and apoptosis-inducing in NSCLC cells [11, 19]. At the present research, pristimerin exhibited a powerful cytotoxicity to H1299 cells, with IC₅₀ value of 2.2 ± 0.34 μM (Fig. 1B). Apoptosis and autophagy induced by pristimerin were widely revealed [20-22]. In our study, the annexin V-FITC/PI test was used to detect apoptosis. The results showed that apoptosis was strongly induced in a dose-dependent manner in H1299 cells (Fig. 2). Subsequently, colony forming cell assay was utilized to detect the anti-proliferative ability of pristimerin. The decreased cell numbers and sizes of H1299 cells showed that pristimerin time-dependently inhibited cell proliferation (Fig. 3).

Cancer metastasis is the most common reason that caused human cancer deaths, while only a minority of lung cancers are localised when diagnosis [23-26]. In order to investigate whether pristimerin inhibited motility of NSCLC cells, wound healing assay and transwell assay were applied. The experimental data indicated that pristimerin significantly decreased NSCLC cells migration in a dose-dependent manner (Fig. 4), as well as mobility (Fig. 5).

Western blot assay was subsequently utilized to find out the regulated protein targets related to this process. Vimentin controls the alignment of the cell traction forces to direct single-cell mesenchymal migration, and marks epithelial-mesenchymal transition (EMT) [27]. As the data showed, vimentin was dose-dependently down-regulated. Additionally, F-actin, another cytoskeleton polymers needed for migration [28], was also decreased by pristimerin. To complete cell adhesion, cellular cytoskeleton needs the assistant of integrin family to connect extracellular matrix (ECM) network [29], the decreased integrin β1 in our results indicated the inhibition of adhesive process [30]. Besides the cancer cell-cell adhesion, it also needs the ability to destroy the histological barrier of cancer cell invasion. Matrix metallopeptidase (MMP) is considered as an important member of the invasive process, which cancer cell secreted to dissolve the components of ECM or basement membrane [31]. It was previously reported that pristimerin down-regulated MMP2 and matrix metallopeptidase 9 (MMP9) to inhibit migration and invasion of esophageal squamous cell carcinoma (ESCC) [32]. Similarly, we found an obviously decreased expression of MMP2 in our study. Zuo et al. demonstrated the inhibitory action on EMT activation in prostate cancer (PCa) PC-3 cells [33], which was confirmed by EMT markers including N-cadherin, fibronectin, vimentin and ZEB1. While in our study, the activities of pristimerin to reduce the cellular cytoskeleton of vimentin and F-actin, cellular adhesion of integrin β1, as well as cellular invasion of MMP2, exerted the multiple ways of anti-cancer potential. Furthermore, the key EMT upstream protein Snail was inhibited revealed the relationship of this process (Fig. 6) [34].

To further explore whether the inhibition of these proteins was correlated with mRNA levels, RT-qPCR was applied. Consistent with the protein levels, integrin β1, MMP2 and Snail were down-regulated in a dose-dependent manner after 24 h treatment. This indicated pristimerin may regulate integrin β1, MMP2 and Snail proteins via suppressing their mRNA translation (Fig. 7). The above molecular mechanisms of pristimerin are summarized in Fig. 8.

Conclusion

In conclusion, our study demonstrated that pristimerin suppressed NSCLC cells NCI-H1299 *in vitro*, exhibited potent activities of proliferation
inhibition and apoptosis induction. Furthermore, the treatment of pristimerin decreased migration and invasion of H1299 cells, which was correlated with EMT-related proteins and mRNA.

Figure 8. Brief summary of molecular mechanisms involved in pristimerin-induced anti-proliferative, anti-migration and anti-invasive effects against human NSCLC cell line NCI-H1299.

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Authors' Contributions

JZ and YY conceived and designed the experiments; JL, QG and XL performed the experiments; LZ and CS analyzed the data; YL, WZ and HC contributed analysis tools; HW and FW revised the paper; JL and JZ wrote the paper. All authors have read and approved the manuscript.

Competing Interests

The authors have declared that no competing interest exists.

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