Abstract. Pristimerin is an active compound isolated from the traditional Chinese herbs *Celastraceae* and *Hippocrateaceae*. It has been reported to exert antitumor effects under experimental and clinical conditions; however, the antitumor effects and underlying mechanisms of pristimerin in oral cancer cells have not yet been identified. In the present study, the anticancer potential of pristimerin was investigated in two oral squamous cell carcinoma (OSCC) cell lines, CAL-27 and SCC-25. Results demonstrated that pristimerin was toxic against the two cell lines, and exhibited inhibitory effects against proliferation. Furthermore, pristimerin exhibited a more potent anti-proliferative activity in CAL-27 and SCC-25 cells than the common chemotherapy drugs cisplatin and 5-fluorouracil. In addition, cell cycle distribution analysis revealed that G_0/G_1 phase arrest was induced following pristimerin treatment in CAL-27 and SCC-25 cells, which was strongly associated with upregulation of p21 and p27, coupled with downregulation of cyclin D1 and cyclin E. Meanwhile, pristimerin induced significant apoptosis of CAL‑27 and SCC‑25 cells, alongside decreased levels of caspase‑3 and specific cleavage of poly(ADP-ribose) polymerase. These effects were associated with inhibition of the mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 and protein kinase B signaling pathways. These results suggest that pristimerin may be considered a potent novel active substance for the treatment of OSCC.

Introduction

Oral carcinoma is an aggressive malignant disease, with ~640,000 new cases being detected annually worldwide; in addition, it is responsible for ~145,000 cases of mortality every year (1). Five-year survival rates of patients with early oral cancer are between 55 and 60%, and decrease to 30-40% in cases of advanced oral cancer. The development of oral cancer is mainly associated with the consumption of areca nut, tobacco and alcohol (2-4). There are various types of oral cancer; however, >90% are squamous cell carcinomas. Because it is very invasive, the prognosis of oral squamous cell carcinoma (OSCC) is poor (5). Currently, surgical removal, chemotherapy and radiotherapy are the most common and effective treatments for patients with oral carcinoma. However, because of distant metastasis, chemotherapeutic resistance and poor tolerance, these treatments can fail. Therefore, the development of novel chemotherapeutics with low toxicity and high efficiency for oral carcinoma is crucial.

Interest has grown in natural compounds for the development of anticancer drugs, due to their low toxicity and higher tolerance in patients (6). Pristimerin, a quininemethide triterpenoid compound, is isolated from several plant species belonging to the *Celastraceae* and *Hippocrateaceae* families. It is commonly used as an antioxidant, anti-malarial, insecticidal, anti-inflammatory and anti-fungal agent (7-9). Pristimerin has also been reported to induce apoptosis of various human cancer cells, including in multiple myeloma (10), breast (11), liver (12), pancreatic (13) and prostate cancer (14). In addition to apoptosis induction (11), the mechanisms involved in the antitumor effects of pristimerin include stimulation of reactive oxygen species generation (15), blocking of nuclear factor-κB (16) and proteasome inhibition (10).

To the best of our knowledge, the anticancer effects of pristimerin on OSCC have rarely been reported. In the present study, the potent antitumor effects of pristimerin on OSCC cells were investigated. Pristimerin exhibited potent anti-proliferative and apoptosis-inducing effects on the OSCC cell lines CAL-27 and SCC-25. The underlying mechanisms of these effects were primarily mediated by G_1 phase cell cycle arrest and inhibition of the mitogen-activated protein kinase.
(MAPK)/extracellular signal-regulate kinase 1/2 (Erk 1/2) and protein kinase B (Akt) signaling pathways.

Materials and methods

Reagents and cell culture. Pristimerin, 5-fluorouracil, cisplatin and propidium iodide (PI) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Pristimerin (molecular structure shown in Fig. 1A) was prepared as a 20 mM stock solution in dimethyl sulfoxide. The CAL-27 and SCC-25 cells, which were kindly isolated from the epidermal tongue tissue of patients with OSCC, were kindly donated by Professor Hongzhang Huang (Department Oral & Maxillofacial Surgery, Sun Yat Sen University, Guangzhou, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) F-12 (Biological Industries, Shanghai, China) supplemented with 10% fetal bovine serum (FBS; Biological Industries), at 37°C in a humidified atmosphere containing 5% CO₂.

Cell viability assay. Cell viability was measured by MTS assay (CellTiter 96 AQueous MTS Reagent; Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. Briefly, CAL-27 and SCC-25 cells (5x10⁴ cells/well) were seeded into a 96-well plate for 12 h to allow cell attachment, and were then treated with increasing concentrations of pristimerin (0, 0.15625, 0.3125, 0.625, 1.25, 2.5, 5, 10 µM), cisplatin (0, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20 µM) or 5-fluorouracil (0, 1.25, 2.5, 5, 10, 20, 40, 80, 160 µM) at 37°C for 68 h. Thereafter, 20 µl MTS/PMS (20:1 in volume) was added and incubated for an additional 4 h. Cell viability was finally determined using a microplate reader (Dette, Nanjing, China) at 490 nm. The half maximal inhibitory concentration (IC₅₀) of pristimerin was calculated.

Clonogenicity assay. CAL-27 and SCC-25 cells (2x10⁵/ml) were treated with increasing concentrations of pristimerin (0, 0.25, 0.5, 1 µM) at 37°C for 12 or 24 h. They were then collected, washed three times with PBS and seeded in a 12-well plate (10³/well) in DMEM F-12 medium containing 0.3% agar and 20% FBS. After a further 10-14 days of culture at 37°C, colonies containing >50 cells were counted under an inverted phase-contrast microscope.

Flow cytometric analysis of cell apoptosis. CAL-27 and SCC-25 cells (2x10⁵/ml) were treated with various concentrations of pristimerin (0, 0.25, 0.5, 1 µM) at 37°C for 12 or 24 h. After collection, cells were washed with PBS and stained with Annexin V-fluorescein isothiocyanate (FITC)/PI (Annexin V-FITC Apoptosis Detection kit; Sigma-Aldrich; Merck KGaA) according to the manufacturer’s protocol. The number of viable, necrotic and apoptotic cells were assessed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Assessment of cell cycle distribution. CAL-27 and SCC-25 cells (2x10⁵/ml) were treated with various concentrations of pristimerin (0, 0.25, 0.5, 1 µM) at 37°C for 12 or 24 h. After collection, they were washed twice with cold PBS and fixed with cold 75% ethanol at 4°C overnight. Ethanol was eventually discarded, cells were resuspended in PBS containing PI (50 µg/ml) and were incubated in a water bath (37°C) for 1 h. The cell cycle distribution was finally examined by flow cytometry.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following treatment with pristimerin (0, 0.25, 0.5, 1 µM for 24 h and 1 µM for 12 h), total cellular RNA was extracted from CAL-27 and SCC-25 cells with TRizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer’s protocol. RNA was reverse transcribed into cDNA (MMLV reverse transcriptase; Promega Corporation), according to the manufacturer’s protocol, and the mRNA expression levels were measured by GoTag qPCR Master Mix (Promega Corporation) using the ABI7000 cycler (Applied Biosystems; Thermo Fisher Scientific Inc.). The primers for RT-qPCR were designed as follows: p21, forward 5'-TCTGTACCTCTGTGCTCG-3', reverse 5'-GAAGATCAGCCGGCTTTG-3'; p27, forward 5'-GTCAACGTTAACAGCTCAAT-3', reverse 5'-TGCATATTGCTACATCCAG-3'; p53, forward 5'-GAGGTTCGCTCTGACTGTACC-3', reverse 5'-TCCGTCTCCAAGTAATTACAC-3'; cyclin D1, forward 5'-GTCGTCGCGAGGTGAACC-3', reverse 5'-ATCCAGTGTCGGAGATCT-3'; cyclin E, forward 5'-GGTTAAAGGAGACGCGGAGC-3', reverse 5'-TGCTCTGCTTTATCCCGTCT-3'; and GAPDH, forward 5'-CGACCCTTCTGTCAAGCTCA-3', reverse 5'-AGGGTCTACTATGCGAATCT-3'. PCR was performed at 94°C for 5 min, followed by 40 cycles at 94°C for 30 sec and 56°C for 30 sec. Relative quantification of gene expression was performed using the threshold cycle difference 2^-ΔΔCT method (17), and the geometric mean of GAPDH levels was used as an internal control to normalize the variability in expression level.

Western blot analysis. Cells were washed with cold PBS and lysed with lysis buffer (1X PBS, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) supplemented with freshly added 1 mM phenylmethylsulfonyl fluoride, 1X Roche complete Mini protease inhibitor cocktail (Roche Diagnostics, Shanghai, China), 10 mM glycerophosphate, 10 mM NaF, and 1 mM sodium orthovanadate. DNA contained in the lysate was sheared by sonication with 10-15% SDS-PAGE. Thereafter, proteins were transferred by 10-15% SDS-PAGE. Thereafter, proteins were transferred to polyvinylidene difluoride membrane and blocked with 5% non-fat milk in PBS-Tween (PBST) at 37°C for 5 min, followed by 40 cycles at 37°C for 30 sec and 56°C for 30 sec. Following quantification using the bicinchoninic acid method, proteins (60 µg) were separated by 10-15% SDS-PAGE. Thereafter, proteins were transferred to polyvinylidene difluoride membrane and blocked with 5% non-fat milk in PBS-Tween (PBST) at 37°C for 1 h. Antibodies against poly (ADP-ribose) polymerase (PARP) (cat. no. 9532), caspase-3 (cat. no. 9665), p21 (cat. no. 2947), Erk1/2 (cat. no. 4695), phosphorylated (p)-Erk1/2 (cat. no. 4370), Akt (cat. no. 4691) and p-Akt (cat. no. 4060) were purchased from CST Biological Reagents Co., Ltd. (Shanghai, China). Horseradish peroxidase-conjugated goat antibodies against mouse and rabbit (cat. no. 31430, cat. no. 31460) were purchased from Thermo Fisher Scientific Inc. Antibodies were prepared in PBS with dilutions of 1:500 (p-Erk1/2 and p-Akt), 1:8,000 (β-actin) and the rest of the antibodies were 1:1,000. Primary antibody was added to the membrane and incubated at 4°C overnight. Following washing in triplicate with PBS, the secondary antibody was added (1:5,000) and incubated at 37°C for 1 h. The Western blot was visualized using the ECL Plus Western blotting detection system (Amersham Biosciences, Buckinghamshire, UK).
room temperature for 1 h, subsequently washed 2 times with PBST and 1 time with PBS. Enhanced chemiluminescence reagent (Immobilon Western; cat. no. WBKLS0500, EMD Millipore, Billerica, MA, USA) was added and the x-ray film was exposed. Protein bands were quantified and normalized to β-actin by using Image-Pro Plus 6.0 system (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Each experiment was performed at least three times. GraphPad 5.0 Software (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis. Data are expressed as the means ± standard deviation, and differences between groups were assessed by one-way analysis of variance with post-hoc intergroup comparisons using Turkey test. P<0.05 was considered to indicate a statistically significant difference.

Results

Pristimerin inhibits OSCC cell proliferation. To investigate the possible effects of pristimerin on OSCC cells, CAL-27 and SCC-25 cells were treated with increasing concentrations of pristimerin for 68 h, prior to determining cell viability with the MTS assay. Results demonstrated that pristimerin effectively inhibited the growth of CAL-27 and SCC-25 cells in a dose-dependent manner with IC_{50} values of 0.70 and 0.73 µM, respectively (Fig. 1A). Compared with cisplatin and 5-fluorouracil, the IC_{50} values of pristimerin in oral cancer cells were lowest (~0.7 µM). Because clonogenicity is believed to better reflect the malignant behavior of tumor cells, colony formation was determined after CAL-27 and SCC-25 cells were treated either with increasing concentrations of pristimerin for 24 h, or with 1 µM pristimerin for various durations. The results demonstrated that pristimerin potently inhibited the colony formation of CAL-27 and SCC-25 cells in a dose- and time-dependent manner (Fig. 1D).

Pristimerin induces G_{0}/G_{1} phase arrest and modulates the expression levels of cell cycle-associated molecules. To explore the underlying mechanism of pristimerin-induced inhibition of cell proliferation, the effects of pristimerin on cell cycle distribution were examined by flow cytometry (Fig. 2A). The results revealed that the proportion of G_{0}/G_{1} phase CAL-27 cells treated with 0, 0.25, 0.5 and 1 µM pristimerin for 24 h were 51.92, 55.53, 66.32 and 74.80%, respectively. Similarly, in SCC-25 cells, G_{0}/G_{1} phase distributions were 60.63, 71.63, 85.73 and 89.63% following treatment with 0, 0.25, 0.5 and 1 µM pristimerin, respectively, for 24 h. In addition, the proportion of G_{2}/M phase CAL-27 cells was 65.08% and of G_{2}/M phase SCC-25 cells was 76.50% following treatment with 1 µM pristimerin for 12 h (Fig. 2A). In parallel, the S and G_{2}/M phase distributions were reduced. These data revealed that pristimerin significantly increased the proportion of G_{0}/G_{1} phase CAL-27 and SCC-25 cells, thus suggesting that G_{0}/G_{1} phase arrest may be induced following pristimerin treatment. Furthermore, the expression levels of molecules involved in the cell cycle process, including cyclin D1, cyclin E, cyclin-dependent kinase (CDK) inhibitors (CDKIs) p21 and p27, and p53, were assessed by RT-qPCR.
Results demonstrated that p21, p27 and p53 expression levels were significantly increased following pristimerin treatment (Fig. 3A-C), whereas cyclin D1 and cyclin E expression levels were significantly decreased under the same conditions.
These modulating effects of pristimerin were observed in a dose- and time-dependent manner in both cell lines. Moreover, western blot analysis exhibited increased levels of p21 protein in a dose- and time-dependent manner in both cell lines (Fig. 5A and B). These results were consistent with the ones obtained from RT-qPCR analysis of p21 expression (Fig. 3).

**Pristimerin induces apoptosis of CAL‑27 and SCC‑25 cells.** The effects of pristimerin on cancer cell apoptosis were assessed by flow cytometry. CAL‑27 and SCC‑25 cells were treated with various concentrations of pristimerin for various durations (Fig. 6), and were double-stained with Annexin V-FITC/PI. Results demonstrated that apoptotic cells were significantly increased by pristimerin in a dose- and time-dependent manner (Fig. 6A and B). The mean apoptotic cell rates from three independent experiments were 1.29, 18.81, 39.1 and 47.7% in CAL‑27 cells, and were 2.1, 21.1, 30.05 and 50.23% in SCC‑25 cells following treatment with pristimerin at 0, 0.25, 0.5 and 1 µM, respectively for 24 h. Following cell treatment with 1 µM pristimerin for 12 h, the mean apoptotic cell rates were 13.61% in CAL‑27 cell and 16.67% in SCC‑25 cells. Additionally, PARP and caspase-3, the hallmark proteins in apoptosis, were examined by western blot analysis. As presented in Fig. 7, caspase-3 expression levels were significantly reduced in the two cell lines in a dose- and time-dependent manner following pristimerin treatment. In addition, cleaved-PARP (85 kDa) levels were substantially increased in a dose- and time-dependent manner following pristimerin treatment in CAL‑27 and SCC‑25 cells (Fig. 7). The upregulated cleaved-PARP level and downregulated caspase-3 level further suggested that apoptosis was induced following pristimerin treatment in OSCC cells (Fig. 7).

**Pristimerin inhibits the MAPK/Erk1/2 and phosphoinositide 3-kinase (PI3K)/Akt signaling pathways.** To determine whether the MAPK/Erk1/2 and PI3K/Akt pathways were involved in the antitumor effects of pristimerin in OSCC cells, p-Akt, Akt, p-Erk1/2 and Erk1/2 levels were detected by western blotting (Fig. 8). Results demonstrated that p-Akt and p-Erk1/2 expression levels were decreased in a dose- and time-dependent manner; however, there were no significant differences in the expression levels of total Akt and Erk1/2.

**Discussion**

There has been growing interest in the last 30 years in natural products derived from plants for the development of novel anticancer therapies (6). Pristimerin is a quinine methyltriterpenoid extracted from Chinese plants; it has been reported to possess potential anticancer effects in various types of cancer (9). To the best of our knowledge, the present study is the first to determine the cytotoxic potency of pristimerin against two OSCC cell lines, CAL‑27 and SCC‑25, in a time- and dose-dependent manner. Furthermore, the effects of pristimerin on cell proliferation were greater than those observed by the conventional drugs cisplatin and 5-fluorouracil. In the present study, the antitumor activities of pristimerin were only detected in CAL‑27 and SCC‑25 cells; our future studies aim to test the antitumor activities of pristimerin in other OSCC cells.

The disruption of cell cycle regulation is a main cause for the proliferation of tumor cells. Therefore, modulation of cell cycle progression in cancer cells is considered a target for the treatment of human malignancies (18-21). Cyclins, CDKs and CDKIs are three important modulators of cell cycle progression. Abnormal expression of these molecules leads to aberrant cell proliferation that can stimulate tumor development. Cyclins bind to CDKs and promote cell cycle progression, whereas CDKIs inhibit CDK activity by binding to cyclin-CDKs, cyclins or CDKs, ultimately blocking cell proliferation (22-25). In this study, the upregulation of p21 and p27, and downregulation of cyclin D1 and cyclin E expression levels were observed and responsible for the pristimerin-induced G0/G1 phase arrest, eventually leading to...
cell proliferation suppression in CAL-27 and SCC-25 cells. Previous studies reported likewise, that pristimerin can induce G₁ phase arrest, which is mediated by the upregulation of p21 and downregulation of cyclin D1 (10,26,27). Additionally, the tumor suppressor protein p53, which is known as a cell cycle regulator and a guardian of genetic integrity, was increased following pristimerin treatment in CAL-27 and SCC-25 cells.

Most anticancer drugs mediate their effects via cell apoptosis induction, which is the major mechanism involved in the treatment of cancer, including oral cancer (28). Apoptosis can be initiated either by the mitochondria (the intrinsic pathway) or through cell death receptors (the extrinsic pathway), leading to the activation of caspase cascades and resulting in apoptosis. Previous studies have reported that pristimerin may induce cell apoptosis via both the mitochondria and the death receptor-mediated extrinsic pathways in U87 glioma cells (29) and cervical cancer cells (30). Pristimerin likewise modulates the levels of B-cell lymphoma 2 (Bcl-2) family proteins, which are known to be involved in mitochondria-mediated apoptosis, in pancreatic cancer cells (31). In the present study, pristimerin induced significant apoptosis of CAL‑27 and SCC‑25 cells, which was mediated by PARP specific cleavage and caspase-3 downregulation. Lee et al (26) and Yousef et al (27) also reported that pristimerin induces PARP cleavage and caspase-3 downregulation. Lee et al (26) and Yousef et al (27) also reported that pristimerin induces PARP cleavage and caspase-3 downregulation. Lee et al (26) and Yousef et al (27) also reported that pristimerin induces PARP cleavage and caspase-3 downregulation. Lee et al (26) and Yousef et al (27) also reported that pristimerin induces PARP cleavage and caspase-3 downregulation. Lee et al (26) and Yousef et al (27) also reported that pristimerin induces PARP cleavage and caspase-3 downregulation.

Figure 5. Pristimerin increases p21 protein expression levels. (A) CAL-27 and SCC-25 cells were treated with the indicated concentrations of pristimerin for 24 h or with 1 µM for various durations, and p21 protein expression was detected by western blot analysis. (B) Statistical analysis of p21 protein density normalized to the internal control β-actin. *P<0.05; **P<0.01 and ***P<0.001 vs. control.

Figure 6. Pristimerin induces apoptosis of oral squamous cell carcinoma. (A) CAL-27 and SCC-25 cells were exposed to the indicated concentrations of pristimerin for 24 h or with 1 µM for 12 and 24 h, and cell death was assessed after Annexin V/PI double staining. (B) Statistical analysis of flow cytometry analysis in CAL-27 and SCC-25 cells (n=3). FITC, fluorescein isothiocyanate; PI, propidium iodide. *P<0.05 and **P<0.01 vs. control.
itself ultimately induces cell apoptosis (32,33). The effects of
pristimerin against apoptosis-associated Bcl-2 family proteins,
and whether the intrinsic and/or extrinsic pathway(s) are
involved in the apoptotic process in CAL-27 and SCC-25 cells
requires further investigation.

The MAPK/Erk1/2 and PI3K/Akt pathways are
important signaling pathways associated with the regulation
of cell proliferation, differentiation, apoptosis and
tumor pathogenesis (34-36). The downregulation of
MAPK/Erk1/2 and PI3K/Akt signaling demonstrated in this
study may account for the proliferation-suppressing and apop-
tosis-inducing effects of pristimerin in CAL-27 and SCC-25
cells. Furthermore, previous studies have reported that pris-
timerin inhibits MAPK/Erk1/2 and PI3K/Akt signaling in
breast (26,37), colorectal (27) and pancreatic cancer cells (13).
However, the complete regulatory mechanisms of pristimerin
on MAPK/Erk1/2 and PI3K/Akt signaling pathways in OSCC cells requires further elucidation.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Author’s contributions

HW and LL conceived and designed the study. HW, LL, ZA and JY performed the experiments. HW and LL wrote the manuscript. LC reviewed and edited the manuscript and was also involved in the conception of the study. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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