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Artepillin C Inhibits Cell Proliferation by Cell Cycle Arrest at G_0/G_1 Phase Accompanied by Up-Regulation of $p27^{Kip1}$ in Human Hepatoma HepG2 Cells

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Authors' contributions

This work was carried out in collaboration among all authors. Author TH designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author WS managed the analyses of the study. Author KK managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Artepillin C, 3, 5-diprenyl-4-hydroxycinnamic acid, is one of the bioactive constituents in Brazilian propolis. In the present study, the anticarcinogenic activity of this compound was investigated in human hepatoma HepG2 cells. Artepillin C inhibited the cell proliferation in a dose- and time-dependent manner accompanied by G_0/G_1 phase arrest in the cell cycle. This compound caused a decrease in the phosphorylation levels of the retinoblastoma protein at Ser780 and Ser807/811 and a decrease in the kinase activity of the cyclinD and CDK4 complex without any change in these protein levels. Artepillin C increased the protein level of $p27^{Kip1}$, known as a CDK inhibitor. This up-

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regulation was regulated by both the transcriptional and post-transcriptional levels, *i.e.*, the treatment increased the mRNA of $p27^{Kip1}$ and decreased the proteosome activity. Thus, artepillin C induces cell cycle arrest at G_0/G_1 phase accompanied by up-regulation of $p27^{Kip1}$, resulting in the inhibition of cell proliferation in HepG2 cells. This study suggested that artepillin C will be a promising anti-cancer agent against hepatoma cancer.

Keywords: Artepilinn C; Cell cycle arrest; G_0/G_1 phase; HepG2 cells; $p27^{Kip1}$; Propolis.

1. INTRODUCTION

Propolis is a resinous substance collected by honeybees from leaf buds and cracks in the bark of various plants. It has been extensively used in folk medicine for many years [1]. The physiological functions of propolis, such as anti-bacterial [2,3,4], anti-inflammatory [5], anti-fungal [6], anti-viral [7], anti-oxidative [8,9] and anti-cancer activities [10,11], have been reported. Because Brazilian propolis is mainly collected from the Brazilian plant *Baccharis dracunculifolia* [12], this kind of propolis specifically contains 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C) as shown in Fig. 1. This compound exerts an anti-cancer activity *in vitro* and *in vivo*, *e.g.*, the induction of apoptosis in human leukemia cells [13], cell cycle arrest in colon cancer cell lines [14], and anti-renal and anti-pulmonary carcinogenesis induced by ferric nitrilotriacetate (Fe-NTA) in mice [15,16]. Our previous study indicated that artepillin C is incorporated into human hepatoma HepG2 cells, and showed an oxidative activity [17]. However, the effect of artepillin C on the proliferation of HepG2 cells is not totally understood.

Cancer is a disease characterized as cell proliferation uncontrolled by the loss of cell cycle control [18]. The G_0/G_1 phase in cell cycle is frequently associated with the carcinogenesis of many types of human cancers [19]. It has been well established that the eukaryotic cell cycle progression is regulated by the sequential activation and inactivation of a series of cyclin-dependent kinases (CDKs). CDKs are activated through binding with the corresponding cyclins [20,21]. The activated complex of CDK and cyclin phosphorylates the retinoblastoma protein (pRB), resulting in activation of the transcription factors which are required for cell proliferation [22,23]. Each complex of the CDKs and cyclins regulates the corresponding progression in cell cycle; *i.e.*, cyclin D/CDK4 or CDK6 complex for progression in G_0/G_1 phase, and cyclin E/CDK2 complex for transition through G_1 to S phases [24]. On the other hand, activation of cyclin/CDK complex is negatively regulated by CDK inhibitors (CDKIs) including the INK4, Cip and Kip families, such as $p16^{INK4a}$, $p21^{Cip1}$, $p27^{Kip1}$and $p57^{Kip2}$ [25]. Recently, research on dietary factors for anti-carcinogenic agents has focused on the ability to inhibit the proliferation of cancer cells by inducing cell cycle arrest [26,27].

Our previous study demonstrated that artepillin C caused cell cycle arrest at G_0/G_1 phase in colon cancer cell lines by the up-regulation of $p21^{Cip1}$ [14]. However, there are few studies on the effects of artepillin C on the cell cycle in liver cancer cell lines. In the present study, human hepatoma HepG2 cells were treated with artepillin C to investigate the effects of this compound on the cell proliferation and cell cycle regulatory proteins.

2. MATERIALS AND METHODS

2.1 Chemicals

Artepillin C was obtained from Wako Pure Chemical Industries (Osaka, Japan) dissolved in dimethyl sulfoxide (DMSO) and stored at -30°C. Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan), fetal bovine serum (FBS) was from ICN Biomedicals (Aurora, OH), penicillin was from Sigma Chemical (St. Louis, MO), and streptomycin was from Invitrogen (Grand Island, NY). All of the other chemicals were of the highest grade commercially available.

2.2 Cell Line and Culture

Human hepatoma HepG2 cells were cultured in DMEM supplemented with 10% FBS, 4 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C and 5% CO_2. For all experiments, the cells were seeded at a concentration of 1.0×10^5 cells/ml, pre-cultured overnight for attachment, and treated with artepillin C or the same amount of the solvent (0.1% DMSO) for the indicated time.

2.3 Cell Proliferation Assay

Cell proliferation was measured by MTS assay according to the manufacture's protocol (Promega, Madison, WI). MTS solution was

prepared as 0.2% 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS; Promega, Madison, WI) and 0.0046% 5-methylphenazinium methyl sulfate (Wako Pure Chemical Industries) dissolved in Dulbecco's phosphate-buffered saline (0.02% KCl, 0.02% KH_2PO_4, 0.8% NaCl, 0.288% $Na_2HPO_4 \cdot 12H_2O$). The cells treated with various concentrations of artepillin C in a 96-well multiplate were added 20 µl of MTS solution and incubated for 2 h at 37ºC. The absorbance of the colored product catalyzed by dehydrogenase in active cells was recorded at 492 nm and at 630 nm as the background. The data were expressed as a percentage of the 0 h-control cells.

2.4 Flow Cytometry Analysis for Cell Cycle Distribution

The cell cycle distribution was analyzed by flow cytometry as previously described [28]. Briefly, the cells treated with artepilin C on a 60-mm dish were trypsinized and collected. The collected cells were washed with ice-cold PBS, then fixed with 70% ethanol at -20°C overnight. The fixed cells were washed with ice-cold PBS and stained with 50 mg/ml propidium iodide (PI; Sigma Chemical) in PBS containing 20 mg/ml ribonuclease A (RNase A; Sigma Chemical) for 1 h at room temperature in the dark. The cell cycle distribution was analyzed by a flow cytometer (Epics XL, Beckan-Coulter Miami, FL) equipped with data-analyzing Multicycle software (Phoenix Flow Systems, San Diego, CA).

2.5 Western Blotting Analysis

Cells treated with arterillin C on a 100-mm dish were washed twice with ice-cold PBS and then lysed with a RIPA buffer (150 µM NaCl, 50 mM Tris-HCl (pH 8.0), 1% Nonident P-40 (NP-40), 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 500 µM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), 5 µg/ml aprotinin, and 20 µg/ml leupeptin). After centrifugation at 11,000 *g* for 20 min at 4ºC, the supernatant was transferred to a new tube as the cell lysate. The protein concentration was determined by the Lowry method [29]. An aliquot of the cell lysate containing an equal amount of protein was heated with a SDS-treatment buffer (0.25 M Tris-HCl, pH 6.8, 4% SDS, 50% glycerol, 0.05% bromo phenol blue and 2.88 M 2-mercaptoethnol) at 100ºC for 5 min and separated by SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a poly(vinylidene difluoride) (PVDF) membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) followed by blocking with 5% non-fat milk powder (w/v) in a TBST buffer (10 mM Tris-HCl, 150 mM NaCl and 0.6% Tween 20) for 1 h at room temperature. The membrane was washed with a TBST buffer and then incubated with a primary antibody. The membrane was re-washed with a TBST buffer, and treated with a corresponding secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. The immunocomplex was visualized by an enhanced chemiluminescence using an ECL plus detection system (Amersham Pharmacia Biotech). The sources of the primary antibodies were as follows: phospho-Rb (Ser780), phospho-Rb (Ser807/Ser811), Rb, $p21^{Cip1}$, $p27^{Kip1}$ and CDK6, Cell Signaling Technology; cyclin D1, cyclin D3, Dako Japan (Kyoto, Japan); $p16^{INK4a}$, cyclin D2, cyclin E, CDK2 and CDK4, Santa Cruz Biotechnology; p53, Calbiochem; and β-actin, Sigma Chemical. The secondary antibodies were anti-mouse IgG (Nacalai Tesque) and anti-rabbit IgG (Amersham Pharmacia Biotech). Data were analyzed by the Image-J, and calculated as the ratio to control values.

2.6 Immunoprecipitation Kinase Assay

The activity of cyclin D/CDK4 complex was assayed by a previously described method [28] with a slight modification. Briefly, the cells treated with artepillin C on a 100-mm dish were washed twice with ice-cold PBS and harvested with a kinase buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$ and 1 mM PMSF). The protein concentration was determined by the Lowry method. The harvested cells containing 400 µg of proteins were pretreated with 20 µl of Protein A/G-plus Agarose (Santa Cruz Biotechnology) for 2 h at 4ºC, followed by centrifugation at 11,000 *g* for 0.5 min at 4°C. The supernatant was immunoprecipitated by an anti-CDK4 antibody (1:100) at 4°C overnight followed by incubation with 20 µl of Protein A/G-plus Agarose for 2 h at 4ºC. After centrifugation at 11,000 *g* for 0.5 min at 4°C, the pellet was washed four times with 500 µl ice-cold kinase buffer. The immunoprecipitated CDK4 was then incubated with 1µg of Rb-C fusion protein (Cell Signaling Technology) and 200 µM ATP in a kinase buffer for 30 min at 30ºC. The phosphorylated Rb-C fusion protein was detected by western blotting analysis using anti-phospho-Rb (Ser780 and Ser807/811) antibodies.

2.7 Preparation of Total RNA

The total RNA was isolated from cells with Sepasol RNA I Super (Nacalai Tesque) according to the manufacture's protocol with a slight modification. Briefly, the cells were washed twice with ice-cold PBS and harvested with 300 µl Sepasol RNA I Super preheated at 50ºC. The cell suspension was homogenized for 5 min and then incubated for 5 min at room temperature followed by adding 60 µl of chloroform. The mixture was incubated at room temperature for 3 min and centrifuged at 12,000 *g* for 15 min at 4ºC. The aqueous phase (approximately 150 µl) was transferred to a new micro-tube followed by adding 150 µl of isopropanol. The mixture was incubated at room temperature for 10 min and then centrifuged at 12,000 *g* for 10 min at 4ºC. The pellet was washed with 300 µl of ice-cold 75% ethanol, dissolved in 150 µl of diethyl pyrocarbonate (Nacalai Tesque)-treated water, and used as the total RNA solution. The absorbance of the total RNA solution was measured at 260 nm (A_{260}) and 280 nm (A_{280}). The concentration of the total RNA (µg/µl) was determined as A_{260} × 40, and the RNA purity was estimated by the ratio of A_{260} to A_{280}. The total RNA solution with the purity of ratio greater than 1.7 was used for cDNA synthesis.

2.8 cDNA Synthesis

The cDNA synthesis was performed using a 1st Strand cDNA Synthesis Kit for RT-PCR $[AMV]^+$ (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Briefly, an aliquot (1 µg) of the total RNA in 20 µl of a reaction solution containing 2.0 µl of a reaction buffer (final concentration, 1×), 4.0 µl of $MgCl_2$ (5 mM), 2.0 µl of a deoxynucleotide mix (1 mM), 2.0µl of the random primer $p(dN)_6$ (1.6 µg), 1.0 µl of the RNase inhibitor (50 U) and 0.8 µl of the AMV reverse transcriptase (20 U) and sterile water was incubated at 25°C for 10 min, incubated at 42°C for 60 min, then a subsequent heat inactivation was performed at 99°C for 5 min, followed by cooling to 4°C for 5 min. The reaction product, cDNA, was used for a real-time reverse transcriptase-polymerase chain reaction (RT-PCR).

2.9 Real-time RT-PCR Analysis

Real-time RT-PCR was performed by a LightCycler (Roche Diagnosis) equipped with the Roche Molecular Biochemicals LightCycler running a LightCycler software according to the instruction manual provided by the supplier. The fluorescence was generated using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche Diagnosis). The reaction was performed with the following protocol: 10-min heat start at 95ºC, 42 cycles of denaturation at 95ºC for 15 s, annealing at 60ºC for 10 s and extension at 72ºC for 10 s. A fluorescence detection was performed at 72ºC. The sequences of primers for $p27^{Kip1}$ were as follows:

Forward primer, 5'-AGG ATG TCA GCG GGA GCC GG-3';
Reverse primer, 5'-CTT CTT GGG CGT CTG CTC CA-3'.

2.10 Analysis for the Proteasome Activity

The *In vitro* proteasome activity was measured using a peptide substrate as previously described [30] with a slight modification. The cells treated with 50 and 100 µM artepillin C or 0.1% DMSO on a 60-mm dish were harvested with a HEPES (20 mM) buffer containing 1 mM EDTA, 1 mM EGTA and 0.2% NP-40. After centrifugation at 11,000 *g* for 20 min at 4ºC, the supernatant was collected and used as the enzyme solution. The protein concentration of the enzyme solution was determined by the Lowry method. An aliquot (200 µg) of the enzyme solution was incubated with 10 µM peptide substrate, succinyl-Leu-Leu-Val-Tyr-4-methyl-coumaryl-7-amide (Peptide Institute, Osaka) in a reaction buffer (20 mM Tris-HCl, pH 8.0, 1 mM ATP and 2 mM $MgCl_2$) at 30ºC for 30 min. 7-Amino-4-methylcoumarin (AMC), a fluorescent product, was spectrofluorometrically measured at the excitation and emission wavelengths of 370 nm and 460 nm, respectively.

2.11 Statistical Analysis

Data were expressed as the mean ± standard deviation (S.D.). Statistical analyses were performed using the Student's *t* test for Fig.8B and Dunnett test for Figs. 2, 3 and 9. The results were considered to be significant at $P < 0.05$.

3. RESULTS

3.1 Artepillin C Inhibited the Proliferation in HepG2 Cells

To investigate the effects of artepillin C on the proliferation of hepatocarcinoma cells, human hepatoma HepG2 cells were treated with artepillin C. Artepillin C at 50 and 100 µM

significantly inhibited the cell proliferation by 15 and 24%, respectively, 24 h after the treatment (Fig. 2). The treatment with 50 μM continuously inhibited the growth rate up to 72 h after the treatment. On the other hand, the treatment with 100 μM decreased it from 48 h to 72 h after the treatment, probably indicating the decrease in the number of cells accompanied by cell death because the proliferation estimated by the MTS assay indicated the activity of a mitochondrial dehydrogenase in the live cells. This result indicates that artepillin C significantly inhibited the proliferation in HepG2 cells.

3.2 Artepillin C Induced Cell Cycle Arrest at G_0/G_1 Phase in HepG2 Cells

To investigate the mechanism of the inhibitory effect of artepillin C on the cell proliferation in HepG2 cells, the effect of this compound on the cell cycle progression was analyzed by flow cytometry. Consistent with the inhibition of the cell growth as shown in Fig. 2, artepillin C showed an induction of the cell cycle arrest at G_0/G_1 phase 24 h after the treatment (Fig. 3). The cell population in G_0/G_1 phase was 50, 56 and 60% in the control (0.1% DMSO), 50 μM and 100 μM artepillin C-treated cells, respectively, 24 h after the treatment. The inhibitory effect was also time-dependent (Fig. 3). Thus, artepillin C induces the inhibition of cell proliferation accompanied by cell cycle arrest at G_0/G_1 phase in HepG2 cells.

3.3 Artepillin C Inhibited the Phosphorylation of Rb Protein

Rb protein negatively regulates cell cycle during progression in G_0/G_1 phase and transition from G_0/G_1 to S phases, but the hyper-phosphorylated Rb protein loses this function [23]. To investigate the effects of artepillin C on Rb protein, the phosphorylated level of Rb protein was examined (Fig. 4). Artepillin C decreased the level of Rb protein phosphorylated at Ser780 and Ser807/811 in a dose-dependent manner 48 h after the treatment (Fig. 4A). The treatment with 50 μM artepillin C time-dependently decreased Rb protein phosphorylated at these sites (Fig. 4B). Phosphorylation levels were also reduced in the control cells treated with DMSO, but not effective as in artepillin C-treated cells. The reduction is thought to be due to a decrease in growth factors in the culture medium. Artepillin C at least decreases the phosphorylated level of Rb protein at Ser780 and Ser807/811, at which Rb protein is believed to be phospholylated by a complex of cyclin D and CDK4 [23,31]. Artepillin C at 50 and 100 μM significantly decreased the activity of cyclin D/CDK4 48 h after the treatment (Fig. 4C). These results indicate that this compound decreased the activity of cyclin D/CDK4, resulting in the decreased phospholylation of Rb protein.

3.4 Artepillin C-induced Cell Cycle Arrest is Not Related to the Protein Levels of CDK4 and cyclin D

To elucidate the mechanism of the decrease in the activity of cyclin D/CDK4, the protein levels of CDK4 and D-type cyclins were examined (Fig. 5). Artepillin C decreased CDK4 and cyclin D1 in a dose- and time-dependent manner (Fig. 5A and 5B, respectively). However, the effects at 50 μM were low 48 h after the treatment and contradicted the result of the kinase activity assay as shown in Fig. 4C. In addition to these proteins, artepillin C at 50 μM did not change the protein levels of the other CDKs and cyclins. These results suggested that the inhibition of the activity of cyclin D/CDK4 by the treatment with artepillin C for 48 h was not associated with the changes in the protein levels of CDK4 and cyclin Ds.

3.5 Artepillin C-suppressed Activity of Cyclin D/CDK4 was Accompanied with an Increase in $p27^{Kip1}$

The activity of cyclin/CDK complexes is also negatively regulated by CDK inhibitors (CDKIs) including INK, Kip and Cip families [21]. To investigate the association of CDKIs with the inhibition of the activity of cyclin D/CDK4, the protein levels were examined (Fig. 6). Artepillin C increased the protein level of $p27^{Kip1}$ in a dose-dependent manner 48 h after the treatment, while this compound did not affect that of $p16^{INK4a}$, $p21^{Cip1}$ and $p57^{Kip2}$ (Fig. 6A). The treatment with 50 μM artepillin C increased the protein level of $p27^{Kip1}$ in a time-dependent manner (Fig. 6B). The increase in $p27^{Kip1}$ was observed 24 h after the treatment and continued to 72 h. This treatment also increased $p16^{INK4a}$ at 24 h, but this level then time-dependently decreased (Fig. 6B). On the other hand, $p57^{Kip2}$ never increased up to 72 h after the treatment, and $p21^{Cip1}$ slightly decreased after 72 h (Fig. 6B). In addition to CDKIs, the protein levels of p53, a main regulator of $p21^{Cip1}$ [32], was also examined, and this protein decreased after a 48 h treatment (Fig.7). These results indicated that the increase

in $p27^{Kip1}$ is involved in the decreased activity of cyclin D/CDK4 48 h after the treatment with 50 μM artepillin C, resulting in the cell cycle arrest G_0/G_1 phase and the inhibition of cell proliferation.

3.6 Artepillin C increased $p27^{Kip1}$ Levels Through Transcriptional and Post-Translational Regulations

To investigate the mechanism of the increase in $p27^{Kip1}$, the change in the protein level within 24 h after the treatment with 50 μM artepillin C was determined. The protein level of $p27^{Kip1}$ was up-regulated 4 h after the treatment, and this up-regulation remained constant for 24 h (Fig. 8A). The protein level of $p27^{Kip1}$ is regulated by the transcription and post-translation [33,34]. To examine the involvement of the transcriptional regulation in the increased $p27^{Kip1}$, the effect of artepillin C on the mRNA of $p27^{Kip1}$ was quantified by real-time RT-PCR in comparison to the control cells treated with 0.1% DMSO at 0 h. The $p27^{Kip1}$ mRNA levels significantly increased 4 h after the treatment with 50 μM artepillin C (Fig. 8B). This result suggests that artepillin C stimulates the transcriptional regulation of $p27^{Kip1}$. Regarding the post-translational regulation, the proteasome activity plays an important role in the ubiquitination-mediated degradation of $p27^{Kip1}$ [35]. To examine the association with the post-transcriptional regulation during the increase in $p27^{Kip1}$, the effect of artepillin C on the proteasome activity was determined (Fig. 9). Artepillin C slightly inhibited the proteasome activity by 6.9% and 5.3% at 24 h and 48 h, respectively, after the 50 μM treatment. These results suggest that $p27^{Kip1}$ might be up-regulated by both the transcriptional and post-translational levels.

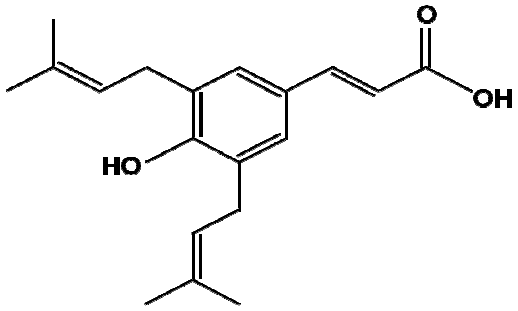

Fig. 1. Chemical structure of artepillin C

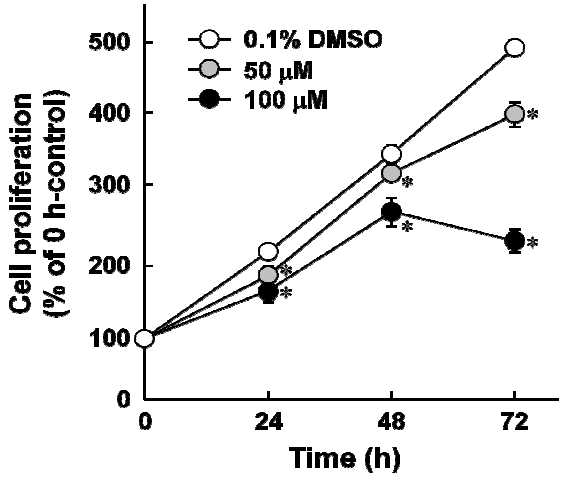

Fig. 2. Artepillin C inhibits the proliferation in HepG2 cells. HepG2 cells were cultured with 50 and 100 μM artepillin C or 0.1% DMSO as the vehicle control. Cell proliferation was determined by MTS assay as described in materials and methods and expressed as a percentage of the control value at 0 h. Data are the means ± SD, n = 6. Asterisks indicate a significant difference from the control value at the corresponding time (P<0.05, Dunnett test)

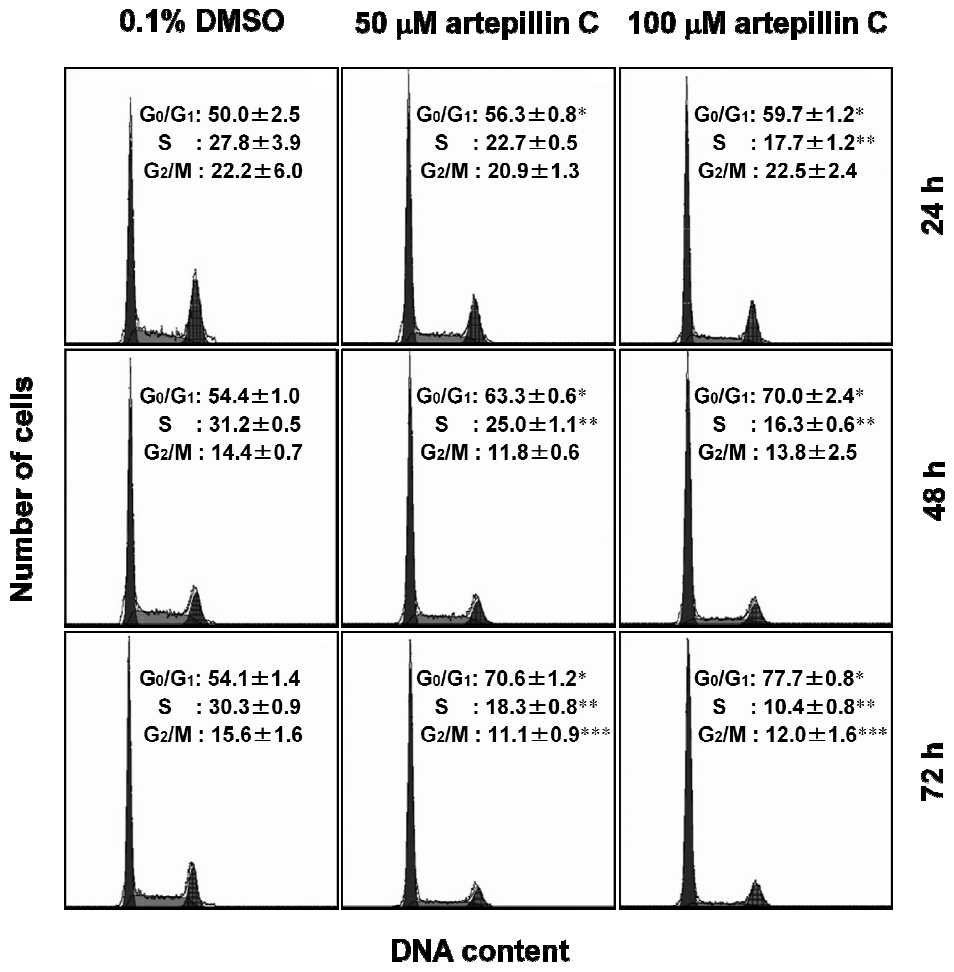

Fig. 3. Artepillin C induces cell cycle arrest at G0/G1 phase in HepG2 cells. HepG2 cells were treated with 50 and 100 μM artepillin C or 0.1% DMSO as the vehicle control for 24, 48 and 72 h. The cell cycle distribution was analyzed by flow cytometry as described in materials and methods. Data are presented as means ± SD (n = 3) of percentage of cell cycle distribution. Asterisks indicate significant differences from the control values (0.1% DMSO) at the corresponding time by Dunnett test (P<0.05): * for G0/G1 phase, ** for S phase, and * for G2/M phase**

4. DISCUSSION

Cell cycle regulation is associated with the development of cancer, and is a potential target for cancer treatment [36,37]. The present study showed that artepillin C in Brazilian propolis inhibited the proliferation of human hepatoma HepG2 cells in a dose-dependent manner through inducing cell cycle arrest at G_0/G_1 phase. This effect is involved with the up-regulation of $p27^{Kip1}$ followed by the decrease in the activity of cyclin D/CDK4 complex and in the phosphorylation level of Rb protein. A schematic of the artepillin C-induced cell cycle arrest at G_0/G_1 phase is shown in Fig. 10. Thus, the present study demonstrated that artepillin C is one of the candidates for an anti-cancer agent against hepatoma.

Rb protein is phosphorylated by cyclin/CDK complexes, whose activity is regulated at multiple levels such as an expression of these proteins and binding with CDKIs [38]. D-type cyclins; *i.e.*, cyclin D1, cyclin D2 and cyclin D3 are essential for cell proliferation [39,40]. Cyclin D1 is overexpressed in many cancers including hepatoma [19] and prostate [41], breast [42] and colon cancers [43]. A previous study showed that

11-13% of hepatoma is caused by the overexpression of cyclin D1 [19]. In the present study, cyclin D1, cyclin D2 and cyclin D3 only slightly decreased 48 h after the treatment with artepillin C. Therefore, it is unexpected that artepillin C prevents carcinogenesis through the inhibition of the cyclin expression.

On the other hand, CDKIs including INK4, Kip, and Cip families bind to the complexes of cyclin/CDK and inhibit the kinase activity [20], resulting in the cancer growth suppression. Most cancer cells express non-functional CDKIs or have a low expression of CDKIs, suggesting that the induction of the normal CDKI expression is also important for the approach to cancer prevention [36,44]. In the present study, artepillin C increased $p16^{INK4a}$ and $p27^{Kip1}$, but not $p21^{Cip1}$ and $p57^{Kip2}$ (Fig. 6). Previous studies showed that a change in the expression of $p16^{INK4a}$ or $p27^{Kip1}$, *e.g.*, their low expression and mutants, contributed to the carcinogenesis of the majority of hepatomas [19]. It is reported that $p16^{INK4a}$ is inactivated in approximately 34% of the hepatomas [45]. The expression of $p27^{Kip1}$ decreases in more than half of the hepatomas, and patients with the higher expression of $p27^{Kip1}$ survived longer than those with the lower expression [46,47]. In the present study, the expression of $p27^{Kip1}$ was observed 4 h after the treatment with artepillin C (Fig. 8), and continued at high levels up to 72 h (Fig. 6B). Therefore, artepillin C may exert an anti-carcinogenic activity in hepatomas accompanied by G_0/G_1 cell cycle arrest resulting from the accumulation of $p27^{Kip1}$ in hepatoma HepG2 cells. Though the increase in $p16^{INK4a}$ in the protein level was transient (Fig. 6B), it may also partly contribute to the cell cycle arrest.

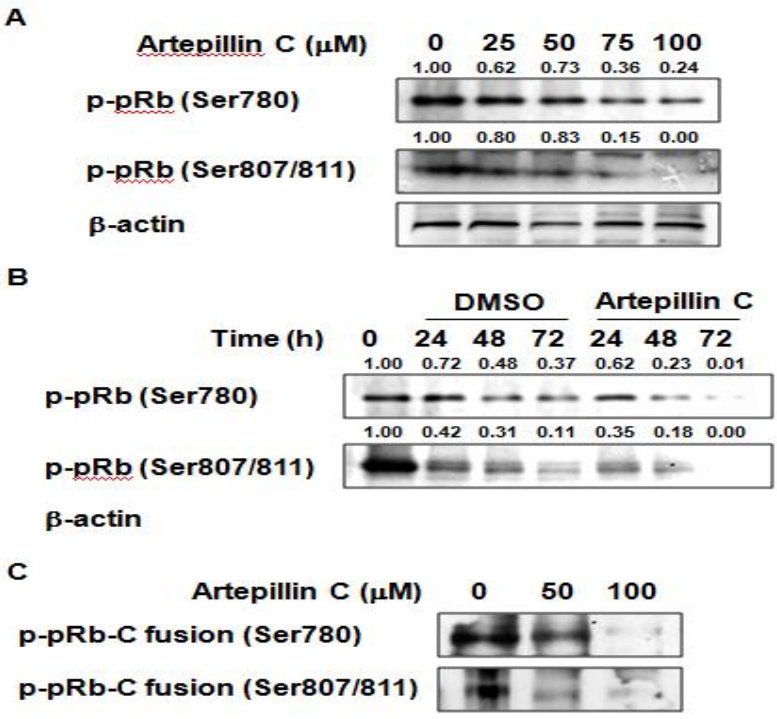

Fig. 4. Artepillin C inhibits phosphorylation of Rb protein (pRb). (A) HepG2 cells were treated with the indicated concentrations of artepillin C for 48 h or 0.1% DMSO as the vehicle control for 0 μM artepillin C. (B) Cells were treated with 50 μM artepillin C or 0.1% DMSO for the indicated time. The phosphorylation level of Rb protein (pRb) was examined by Western blotting analysis as described in Materials and methods. β-Actin protein was used as the loading control. Data shown are representative of three independent experiments. The values upper the data of p-pRb (Ser780) and p-pRb (Ser807/811) represents each ratio of p-pRb/β-actin to control values. (C) Cells were treated with 50 or 100 μM artepillin C for 48 h. The immunoprecipitation kinase assay for cyclin D/CDK4 was performed as described in Materials and methods. The phosphorylation of Rb-C fusion protein (pRb-C fusion) was detected by the Western blotting analysis. Data shown are representative of three independent experiments

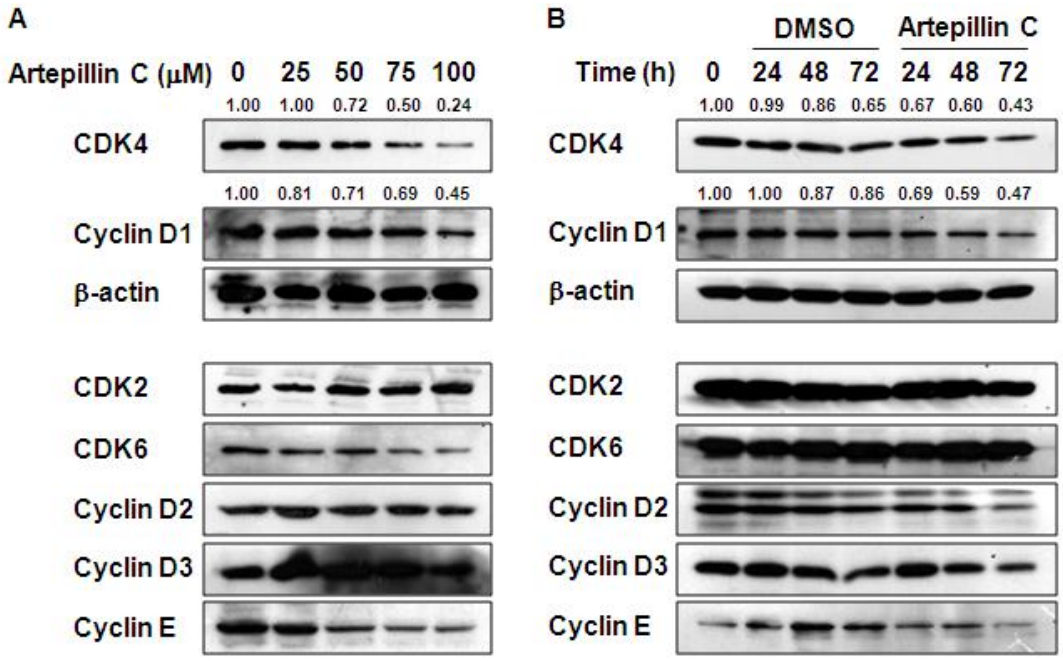

Fig. 5. Artepillin C only slightly affects the protein levels of CDKs and cyclins. (A) HepG2 cells were treated with the indicated concentrations of artepillin C for 48 h or with 0.1% DMSO as the vehicle control for 0 μM artepillin C. (B) Cells were treated with 50 μM artepillin C or 0.1% DMSO for the indicated times. The protein level of CDKs and cyclins was examined by a Western blotting analysis as described in Materials and methods. β-Actin protein was used as the loading control. Data shown are representative of three independent experiments. The values upper the data of CDK4 and cyclin D1 represents each ratio of corresponding protein/β-actin to control values

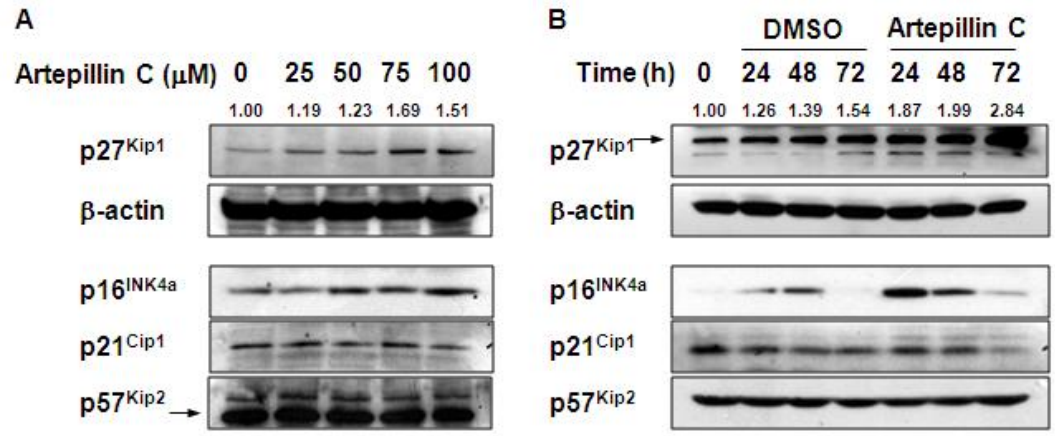

Fig. 6. Artepillin C increases the protein levels of p27Kip1. (A) HepG2 cells were treated with the indicated concentrations of artepillin C for 48 h or with 0.1% DMSO as the vehicle control for 0 μM artepillin C. (B) Cells were treated with 50 μM artepillin C or 0.1% DMSO for the indicated times. The protein levels of CDK inhibitors; p16INK4a, p21Cip1, p27Kip1 and p57Kip2, were examined by Western blotting analysis as described in Materials and methods. β-Actin protein was used as the loading control. Data shown are representative of three independent experiments. The values upper the data of p27Kip1 represents each ratio of p27Kip1/β-actin to control values

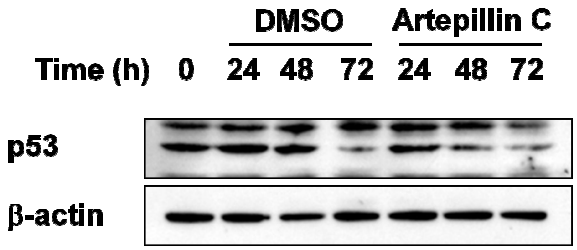

Fig. 7. Artepillin C down-regulates the protein levels of p53. HepG2 cells were treated with 50 μM artepillin C or 0.1% DMSO as the vehicle control for the indicated time. The protein level of p53 was examined by a Western blotting analysis as described in Materials and methods. β-Actin protein was used as the loading control. Data shown are representative of three independent experiments. The values upper the data of p53 represents each ratio of p53/β-actin to control values

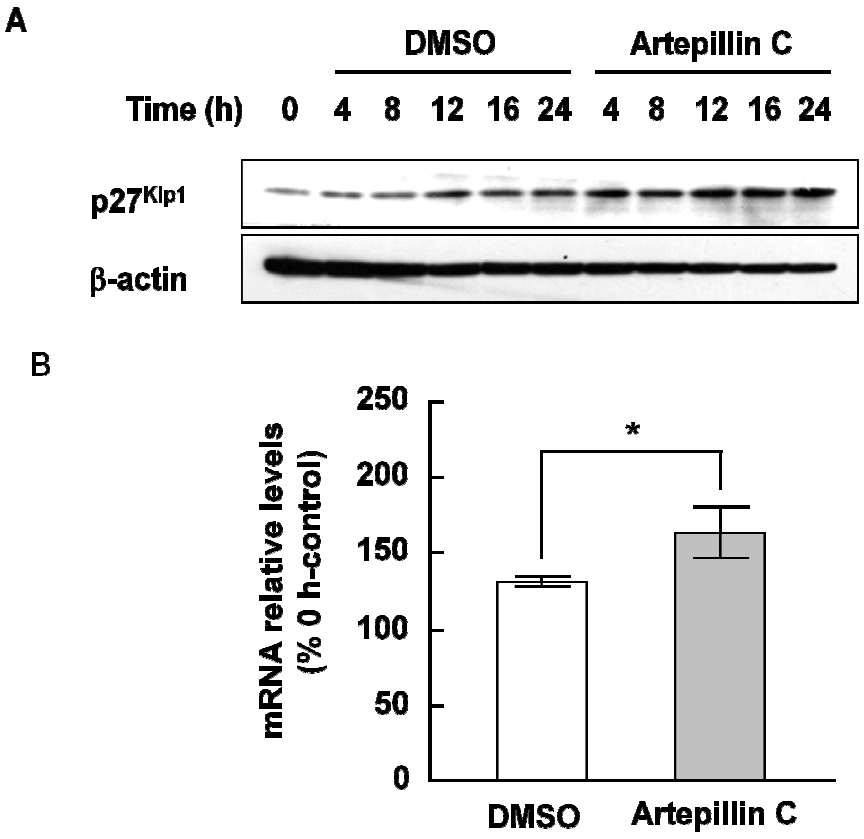

Fig. 8. Artepillin C increases the p27Kip1 mRNA levels. (A) HepG2 cells were treated with 50 μM artepillin C or 0.1% DMSO as the vehicle control for the indicated time. The protein level of p27Kip1 was examined by a Western blotting analysis as described in Materials and methods. β-Actin protein was used as the loading control. Data shown are representative of three independent experiments. The values upper the data of p27Kip1 represents each ratio of p27Kip1/β-actin to control values. (B) Cells were treated with 50 μM artepillin C for 4 h. The p27Kip1 mRNA levels was quantified by real-time RT-PCR as described in Materials and methods, and expressed as a percentage of the value at 0 h i.e., that in untreated cells. Data shown are the means ± S.D. of triplicate experiments. Asterisk indicate a significant difference (P<0.05, Student's t test)

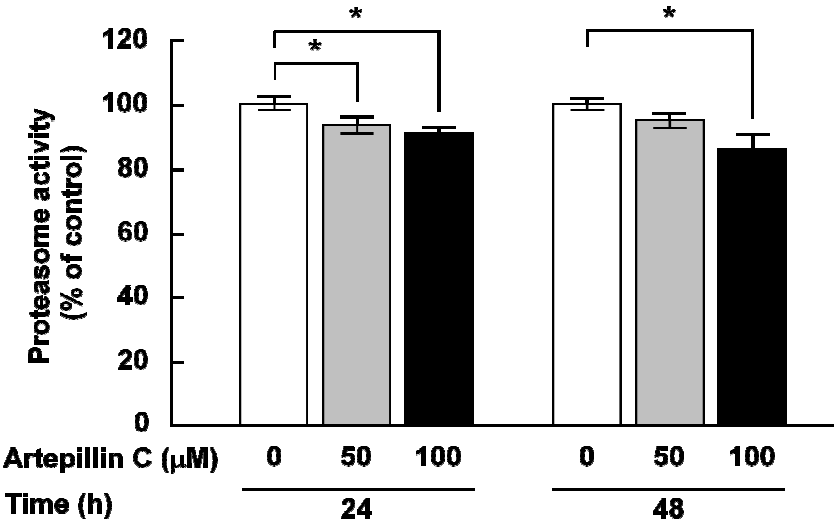

Fig. 9. Artepillin C represses the proteasome activity. HepG2 cells were treated for 24 and 48 h with 50 and 100 μM artepillin C or 0.1% DMSO as a vehicle control for 0 μM artepillin C. The proteasome activity was analyzed as described in Materials and methods and expressed as a percentage of the control value at each time point. Data shown are the means ± S.D. of triplicate experiments. Asterisks indicated a significant difference from the vehicle control at the corresponding time (P<0.05, Dunnett test)

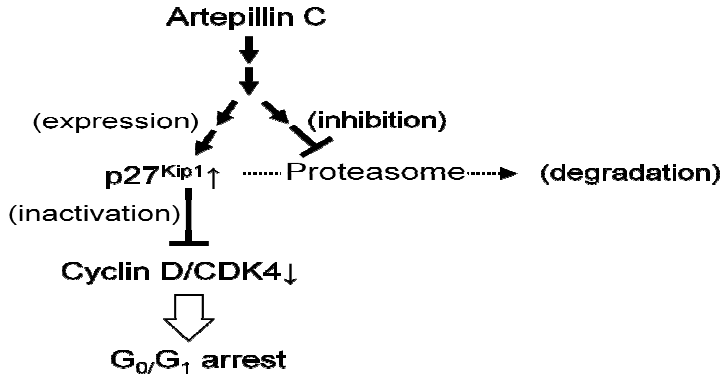

Fig. 10. A schematic of artepillin C-induced cell cycle arrest at G0/G1 phase in HepG2 cells

The extracellular signal-regulated kinase (ERK)-signaling pathway is a major determinant in the control of cell growth through regulation of the downstream regulators including $p27^{Kip1}$ [48,49]. Phosphorylation of ERKs, *i.e.*, the activation of the ERK pathway, shows the inhibition of cancer cell growth [50]. However, artepillin C did not promote the phosphorylation of ERKs in HepG2 cells (data not shown), suggesting that the up-regulation of $p27^{Kip1}$ was independent of the ERK pathway in HepG2 cells. A further study is needed to elucidate the mechanism of the up-regulation of $p27^{Kip1}$.

In a previous study, artepillin C induced cell cycle arrest at G_0/G_1 phase through the up-regulation of $p21^{Cip1}$ in colon cancer WiDr and HCT116 cells [14]. The up-regulation of $p21^{Cip1}$ is through a p53-dependent [51] or p53-independent pathway [19]. Since WiDr cells possess the mutated p53, the artepillin C-increased $p21^{Cip1}$ in the previous study was independent of p53. Contrary to the previous study, this study demonstrated that $p21^{Cip1}$ decreased in HepG2 cells 72 h after the treatment (Fig. 6B), and p53 decreased 48 h after the treatment (Fig. 7). These results indicated that the decrease in $p21^{Cip1}$ might be p53-dependent in the artepillin C-treated HepG2

cells. The difference between HepG2 and WiDr cells may be due to the original tissue; *i.e.*, liver or colon, and to the character of the individual cancer cell lines. Thus, artepillin C induces cell cycle arrest at G_0/G_1 phase via different mechanisms in the different cell lines, although the key factor to the bifurcate pathways; *i.e.*, $p27^{Kip1}$-dependent and $p21^{Cip1}$-dependent ones, is still unclear.

Most phenolic compounds show a low bioavailability, because they are conjugated with sulfate and/or glucuronidate in the small intestine and liver in the absorptional and metabolic processes [52-54]. Since conjugations convert these compounds to inactive forms [55], the absorption of aglycones without conjugations is very important for the phenolic and polyphenolic compounds to express their activities in the body. As shown in Fig. 1, artepillin C has a chemical structure with two prenyl groups neighboring a hydroxyl group, which makes conjugation difficult [17], because the two prenyl groups can shield the hydroxyl group from the approach of the conjugating enzyme. Artepillin C was shown to be incorporated into the body and HepG2 cells without any conjugation in a previous study [17] and induce cell cycle arrest at G_0/G_1 phase in HepG2 cells in the present study. Taken together, artepillin C is a promising candidate as an anti-cancer agent against hepatoma. However, it is necessary as future research to investigate the comprehensive molecular mechanism action of artepillin C on antiproliferation of HepG2 cells and the *In vivo* test.

5. CONCLUSION

Artepillin C induces cell cycle arrest at G_0/G_1 phase accompanied by up-regulation of p27, resulting in the inhibition of cell proliferation in HepG2 cells. This study suggested that artepillin C will be a promising anti-cancer agent against hepatoma cancer.

DISCLAIMER

The company name used for this research is commonly and predominantly selected in our area of research and country. There is absolutely no conflict of interest between the authors and company because we do not intend to use this company as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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