Abstract. Alpinetin is a type of novel plant flavonoid derived from *Alpinia katsumadai* Hayata, found to possess strong anti-hepatoma effects. However, the detailed antitumor mechanism of Alpinetin remains unclear. Mitogen-activated protein kinase kinase-7 (MKK7) can regulate cellular growth, differentiation and apoptosis. The aim of this study was to investigate the role of MKK7 in the anti-hepatoma effect mediated by Alpinetin. HepG2 cells were treated with Alpinetin at various doses and for different times, and the levels of phosphorylated MKK7 (p-MKK7) and total MKK7 were tested by RT-PCR and Western blotting. Following transient transfection with RNA interference, cell viability and cell cycle stage were determined using methyl thiazolyl tetrazolium assay and flow cytometry, in order to assess the antitumor action of Alpinetin. In addition, chemosensitization to cis-diammined dichloridoplatium (CDDP) by Alpinetin was assessed by cell counting array and the cell growth inhibitory rate was calculated. The results showed that Alpinetin suppressed HepG2 cell proliferation and arrested cells in the G0/G1 phase by up-regulating the expression levels of p-MKK7. On the contrary, inhibiting the expression of MKK7 reversed the antitumor effect of Alpinetin. Moreover, Alpinetin enhanced the sensitivity of HepG2 hepatoma cells to the chemotherapeutic agent CDDP. Taken together, our studies indicate that activation of MKK7 mediates the anti-hepatoma effect of Alpinetin. MKK7 may be a putative target for molecular therapy against hepatoma and Alpinetin could serve as a potential agent for the development of hepatoma therapy.

Introduction

Hepatocellular carcinoma (HCC) represents the fifth most prevalent cancer in terms of incidence. In addition, HCC is the third most common cause of cancer-related death in the world, resulting in more than 600,000 deaths per year. Like other solid tumors, surgical treatment is the main treatment option, but only 10-30% patients are eligible for radical treatment because of difficult early diagnosis and chronic liver disease, and it is also hard to efficiently treat live cancer by chemotherapy and radiotherapy (1-5).

*Alpinia katsumadai* Hayata, as a traditional medicine with low toxicity, has been shown to have antitumor and anti-oxidation effects (6,7). Alpinetin, (7-hydroxy-5-methoxyflavanone, molecular formula C_{16}H_{14}O_{4}, molecular weight 270.28) a kind of novel plant-derived flavonoid, is the major active ingredient of *Alpinia katsumadai* Hayata (8,9). Previous studies have proved that Alpinetin has a strong antitumor effect by suppressing proliferation of tumor cells. The anti-cancer capability of Alpinetin has also been confirmed in the treatment of various tumors, such as breast cancer, hepatoma, leukemia, carcinoma of colon and pulmonary cancer (7,10-12). However, the detailed antitumor mechanisms of Alpinetin remain largely unknown.

c-Jun N-terminal kinase (JNK) signal pathway is one of three paralleled pathways at the center of the mitogen-activated protein kinase (MAPK) pathways and plays an important role in regulating organized cellular responses, such as proliferation, differentiation or apoptosis (13-16). MKK4 and MKK7, which is also called c-jun N-terminal kinase kinase 2 (JNKK2) or stress-activated protein kinase/extracellular signal-regulated protein kinase 2 (SEK2),
are two upstream kinases of JNK pathway and directly activate the JNKs by phosphorylating the Tyr and Thr residue (17). Unlike other MAPK subfamilies, the monophosphorylation of MKK7 on the Thr residue is sufficient and specific to activate JNK pathway which, in turn, activates substrates like transcription factors or pro-apoptotic proteins (18). In addition, studies on pro-inflammatory cytokines also showed that only MKK7 is essential for JNK activation (19,20). Given its important role in JNK activity, it is necessary to illustrate the role of MKK7 in the anti-hepatoma of Alpinetin.

The aim of this study was to determine the action of Alpinetin in the anti-hepatoma proliferation effect and its influence on cell senescence in vitro. We also investigated whether Alpinetin can sensitize HepG2 hepatoma cells to CDDP. The possible signal transduction pathway involved in Alpinetin-induced inhibition of human hepatoma cell proliferation was also studied.

Materials and methods

Cell culture, antibodies and reagents. Human HepG2 hepatic cancer cell line and rat N1-S1 hepatic cancer cell line were purchased from American Type Culture Collection (ATCC), cultured in Iscove's modified Dulbecco's medium (IMDM), 10% fetal bovine serum (FBS) and maintained at 37˚C. HepG2 cells were grown at 48 h confluence in 6-well plates. Transfection was done for 24 h with Lipofectamine 2000 follow by treatment with Alpinetin (60 µg/ml) for 24 h. The cells were then pelleted by centrifugation and washed twice with PBS. After resuspension in 1 ml PI integration staining solution, the cells were incubated 4˚C overnight with PI (Sigma) and the fixed cells were spun by centrifugation and the cell pellets were suspended in 5 ml ice-cold 70% ethanol at 4°C. After 1 h, the fixed cells were stained with DAPI and fluorescent images were obtained using a fluorescent microscope (Leica Microsystems, Germany). The stained cells were analyzed using a FACSCalibur flow cytometer (Becton-Dickinson, USA).

Analysis of cell cycle by flow cytometry. HepG2 cells were grown at 48 h confluence in 6-well plates. Transfection was done for 24 h with Lipofectamine 2000 follow by treatment with Alpinetin (60 µg/ml) for 24 h. The cells were then pelleted by centrifugation and washed twice with PBS. Then, the cells were suspended in 5 ml ice-cold 70% ethanol at 4°C. After 1 h, the fixed cells were spun by centrifugation and the pellets were washed with PBS. After resuspension with 1 ml PI integration staining solution, the cells were incubated with RNase A (10 mg/ml), PI (50 mg/ml), 1% Triton X-100 and sodium citrate (1 g/l) shaken for 30 min at 37°C in the dark. The stained cells were analyzed using a FACSCalibur flow cytometer (Becton-Dickinson, USA).

Small interfering RNA (siRNA) transfection. Cells (5x10⁴ cells/2 ml/well) were plated at 60% confluence in a 6-well plate in RPMI-1640 without antibiotics. After 24 h, siRNA or negative control oligonucleotide was transfected into cells with Lipofectamine 2000 according to the instructions of the manufacturer’s. After 4-6 h of incubation in the CO2 incubator, the medium containing siRNA-Lipofectamine 2000 complexes was replaced with fresh RPMI-1640 containing 10% FCS and the cells were cultured for further experiment. All siRNAs were obtained from Guangzhou Ribobio Co., Ltd. and the three specific sequences for silencing were: human MKK7 siRNA-1, sense 5'-GGAGAGACAAA GUAUAAdTdT-3', and anti-sense 3'-dTdTCCUCUCUGG UUUCAUAUU-5'; siRNA-2, sense 5'-CCUACUCGGU CAGUGCUUdTdT-3', and anti-sense 3'-dTGGAUGUA GCACGUCACGAA-5'; siRNA-3, sense 5'-GCAUGAGAGU UGACCGAdTdT-3', and anti-sense 3'-dTGTCGAAACU CUACUGGC-5'. The effect of RNA interference was checked by RT-PCR and Western blot analysis.

Cell proliferation assay. Cell viability was determined using methyl thiazolyl terazolium (Sigma) assay. Cells in logarithmic phase were seeded in the 96-well plate and then treated with Alpinetin. MTT (20 µl) (0.5 mg/ml) was added to each well and the cells were incubated at 37°C for 4 h to allow the yellow dye to be transformed into blue crystals. The medium was removed and 200 µl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the dark blue crystals. The cell pellets were washed with PBS. After resuspension with 1 ml PI integration staining solution, the cells were incubated with PI (Sigma), and the fixed cells were spun by centrifugation and the pellets were washed with PBS. After resuspension with 1 ml PI integration staining solution, the cells were incubated with RNase A (10 mg/ml), PI (50 mg/ml), 1% Triton X-100 and sodium citrate (1 g/l) shaken for 30 min at 37°C in the dark. The stained cells were analyzed using a FACSCalibur flow cytometer (Becton-Dickinson, USA).

Western blot assay. Cells were washed once with ice-cold phosphate-buffered saline (PBS) containing 100 mM sodium orthovanadate and solubilized in lysis buffer [50 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 100 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1% Nonidet P-40, 5 mM cocktail; pH 7.4]. After centrifugation at 12,000 x g for 20 min, the supernatant was collected. After determination of the protein concentration using BCA kit assay (Pierce, USA), β-mercaptoethanol and bromophenol blue were added to the sample buffer for electrophoresis. The protein was separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and trans-blotted to polyvinylidene difluoride membranes (Bio-Rad Laboratories, USA). The blots were incubated at 4°C overnight with antibodies, and the resulting bands were detected using enhanced chemiluminescence. Intensities of the bands were semi-quantified using an image-analysis system.

RNA extraction and RT-PCR assay. Total RNA from hepatic cancer cells was prepared using RNAiso™ Plus (Takara) according to the routine method. The concentration of total RNA samples was evaluated with spectrophotometer (Beckman Coulter, Inc., USA). The specific primers for GAPDH and MKK7 were designed and synthesized by Guangzhou Ribobio Co., Ltd. (China). The primers for amplification were as follows: GAPDH, forward primer, 5'-GAACGGGAAGCT CACTGG-3', reverse primer, 5'-GCCTGCTTACCACCT TCT-3'; MKK7, forward primer, 5'-CCCCGTAAATACAC AAAGAAAATCC-3', reverse primer, 5'-GGCGGACACA CACTCATAAACAGA-3'. The RT-PCR was performed using an RT-PCR kit according to the protocols of the manufacturer.
Statistical analysis. SPSS 16.0 statistical software was used for statistical analysis. Values were shown as mean ± SD. Statistical analysis was carried out using Student’s t-test. Differences between groups were identified as statistically significant at p<0.05.

Results

Alpinetin inhibits growth of both human HepG2 and rat N1-S1 hepatic cancer cells. To investigate the anti-hepatoma effect, the two cell lines were treated with Alpinetin for different doses and times and MTT assay was performed to determine cell viability. Results showed that the viability of Alpinetin-treated cells greatly decreased with increased drug dose or treatment time (Fig. 1). Furthermore, the effect of inhibition in hepatoma cells increased proportionately when treated with Alpinetin at a range from 20-80 µg/ml and the effective dosage of inhibition is 60 µg/ml. Our data indicate that proliferation of hepatoma cells were suppressed in a dose- and time-dependent manner by Alpinetin.

Alpinetin increases phosphorylation of MKK7 in human hepatic cancer cells. To explore the role of MKK7 in the anti-hepatoma effect of Alpinetin, we checked the levels of MKK7 and p-MKK7 in HepG2 hepatoma cells treated with different concentrations of Alpinetin for 24 h by RT-PCR and Western blot assay (Fig. 2A and B). Furthermore, as MKK4 is also able to regulate the JNK pathway, the expressions of MKK4 and p-MKK4 were examined by Western blot assay simultaneously (Fig. 2B). Then the results of Western blot assay were further semi-quantitatively estimated using Gel-Pro Analyzer 4.0 software. The changes in the expression level of p-MKK7 and MKK7 were estimated by a polygram.

Alpinetin inhibits growth of both human HepG2 and rat N1-S1 hepatic cancer cells. To investigate the anti-hepatoma effect, the two cell lines were treated with Alpinetin at different dosage (0, 20, 40, 60 and 80 µg/ml) for 24 h, and then MTT assay was done to determine cell viability. (B) HepG2 and N1-S1 cells were treated with Alpinetin (60 µg/ml) for 0, 12, 24, 36 and 48 h. The treated cell viability was examined by MTT assay.

Figure 2. Alpinetin increases phosphorylation level of MKK7 in human hepatic cancer cells. (A) HepG2 cells were treated with different concentrations of Alpinetin for 24 h, and then the level of MKK7 mRNA was assessed using RT-PCR assay. (B) p-MKK4/7 and total MKK4/7 levels were determined respectively by Western blot analysis after treatment with various concentrations of Alpinetin for 24 h. (C) The protein expressions of p-MKK4/7 and MKK4/7 were further analyzed using Gel-Pro Analyzer 4.0 software. The changes in the expression level of p-MKK7 and MKK7 were estimated by a polygram.

Figure 3. After transfection with siRNA, HepG2 cells were stained with DAPI. A high efficiency of transfection was verified using a fluorescence microscope.
MKK7 siRNA-3 is optimal for silencing the expression of MKK7. Three siRNAs (siRNA-1, -2 and -3) were planned to silence the expression of MKK7 in HepG2 hepatoma cells. Three siRNAs and FAM-negative control oligonucleotide were transfected into HepG2 cells at 57 nM for 24 h and transfection efficiency was examined by fluorescence microscopy (Fig. 3), RT-PCR and Western blot assays (Fig. 4). To explore the optimal interference conditions, a variety of doses of siRNA-3 were transfected into HepG2 cells for various durations. The results of our study showed that MKK7 siRNA-3 was more efficient in silencing the expression of MKK7 than others (Fig. 4A). Furthermore, we found that transfection with 57 nM siRNA-3 for 24 h notably declined the expression of MKK7, and this silencing effect lasted no less than 72 h (Fig. 4B and C). The result suggested that transfecting with 57 nM siRNA-3 for 24 h was the most favorable condition for MKK7 silencing.

Inhibition of MKK7 reduced the ability of Alpinetin to anti-proliferation in vitro. To further confirm the role of MKK7 in the anti-hepatoma effect of Alpinetin, we transfected HepG2 cells with siRNA-3 for 24 h to downregulate the expression of MKK7. After treating with Alpinetin (60 µg/ml) for 24 h, HepG2 cell viability was confirmed using the MTT assay. Cell viability in the MKK7 siRNA-3 + Alpinetin group was higher than that in the control siRNA + Alpinetin group (*P<0.05).

Figure 4. MKK7 siRNA-3 is optimal in silencing the expression level of MKK7. (A) siRNA-1, -2 and -3 were transfected into HepG2 cells at 57 nM for 24 h, and then the mRNA level of MKK7 was determined by RT-PCR. Results show that siRNA-3 was more efficient in inhibiting the expression of MKK7 than siRNA-1 or -2. (B) HepG2 cells were transfected with siRNA-3 at various doses for different durations. RT-PCR assays were done to determine the interference efficiency. Transfection with 57 nM siRNA-3 for 24 h remarkably decreased the mRNA expression of MKK7, and this effect of interference lasted at least 72 h. (C) Western blot analysis was performed to further verify the interference efficiency. The protein expression of MKK7 was effectively inhibited by treatment with siRNA-3 at 57 nM for 24 h.

Figure 5. MKK7 siRNA-3 inhibits the increased phosphorylation level of MKK7 induced by Alpinetin. (A) The changes in the expression of MKK7 and p-MKK7 were determined using Western blot assay. (B) The results of (A) were analyzed with Gel-Pro Analyzer 4.0 software. The level of p-MKK7 in control siRNA + Alpinetin treated group is higher than that in control siRNA group (*P<0.05); the expression of MKK7 and p-MKK7 were lower in MKK7 siRNA-3 + Alpinetin treated group than that in control siRNA + Alpinetin treated group (**P<0.05).

Figure 6. Silencing of MKK7 by siRNA-3 blocks the anti-proliferative effect of Alpinetin. siRNA-3 was transfected into HepG2 cells for 24 h to downregulate the expression of MKK7. After treating with Alpinetin (60 µg/ml) for 24 h, HepG2 cell viability was confirmed using the MTT assay. Cell viability in the MKK7 siRNA-3 + Alpinetin group was higher than that in the control siRNA + Alpinetin group (*P<0.05).
group. This result revealed that down-regulation of MKK7 by siRNA-3 attenuated the anti-proliferative effect of Alpinetin in vitro.

**Down-regulation of MKK7 by siRNA suppresses Alpinetin-induced G0/G1-phase arrest in human hepatoma cells.** To further investigate the mechanism by which Alpinetin suppressed hepatoma cells proliferation, HepG2 cells were transfected with siRNA or siRNA negative control and treated with Alpinetin (60 µg/ml) for 24 h, before cell cycle progression was assessed using flow cytometry. The percentage of different treatment groups in G0/G1 phase are shown by histograms (Fig. 7E). The percentage of hepatoma cells in the G0/G1 phase was higher in Alpinetin-treated group than the untreated cells (Fig. 7A and B). The fraction of hepatoma cells in the G0/G1 phase were lower in siRNA transfected group treated by Alpinetin than in Alpinetin-treated group (Fig. 7B and D). Our data imply that the anti-proliferation effect induced by Alpinetin is possibly through the activation of MKK7 pathway, thereby causing G0/G1 phase arrest.

**Alpinetin enhances chemosensitivity of HepG2 hepatoma cells to cis-diammined dichloridoplatium (CDDP).** Previous study has reported that activation of JNK and P38/MARK...
pathway was associated with enhanced chemosensitivity to CDDP in HepG2 hepatoma cells (21). To investigate whether treatment with Alpinetin sensitized HepG2 cells to CDDP, cells were plated in 6-well plates (5x10^3/well) and cultured for 24 h. Cells were treated in the following groups: control group (untreated, Fig. 8Aa), Alpinetin group (treated with 60 µg/ml Alpinetin and 20 µg/ml CDDP for 24 h, Fig. 8Ab), CDDP group (treated with 20 µg/ml CDDP for 24 h, Fig. 8Ac) and Alpinetin + CDDP group (treated with 60 µg/ml Alpinetin and 20 µg/ml CDDP for 24 h, Fig. 8Ad). After above treatment, surviving cells were measured by cell counting (Beckman Coulter, Inc., USA). The cell growth inhibitory rate (GIR) was calculated as the ratio of (number of cells in the control group - number of cells in the treated group) to (number of cells in the control group) x 100% (Fig. 8B). The results demonstrated that the GIR was higher in Alpinetin + CDDP group than that in CDDP group and Alpinetin group (Fig. 8B). In addition, the effect of combined treatment was stronger than the presumed additive effect of Alpinetin and CDDP treatments. Our result indicated that Alpinetin enhances chemosensitivity of HepG2 hepatoma cells to CDDP.

Discussion

In vitro, Alpinetin exerts anti-proliferative activity against various types of tumors such as hepatoma, breast carcinoma and leukemia. Some studies have reported that the antitumor effect of Alpinetin is connected to inhibition of NF-kappaB (11). Our present study also found that Alpinetin showed strong antitumor activity in hepatoma cell lines from both human and rat. However, less is known regarding defined signaling pathways involved in these processes.

The mitogen-activated protein kinase (MAPK) signaling pathways are composed of a large family of protein kinases which allow the cells to respond to exogenous and endogenous stimulus (22-24). These protein kinases are part of cascade reaction of a three-tiered signaling module which consist of MAPKKKs (MAPKKs)-MAPKs (MKKs)-MAPks. JNK, p38 MAPK and extracellular signal-regulated kinase (ERK) are three major MAPks and play important roles in regulating organized cellular responses. JNK1 and JNK2 are widely expressed in the tissues and are connected with the development of various cancers (25,26).

As two important members of a three-tiered cascade reaction, MKK4 and MKK7 can phosphorylate distinct JNK activation sites to activate the JNK pathway and regulate cellular growth, differentiation and apoptosis (27). MKK4/7 can be activated through phosphorylation by MKKks. MKK4/7 form complexes with their upstream kinases via the DVD domain specificity. For instance, mixed lineage kinase 3 (MLK3), MEK1 and TAK1 can interact with MKK4 and MKK7, while DLK specifically binds to MKK7 and MEKK4 to MKK4 (28-32). Apart from this, MKK7 is independent and specific to trigger JNK signal pathway activity while the additional phosphorylation by MKK4 ensures optimal JNK activation (18). It has been reported that MKK7 frequently mediates the antitumor effects of various agents, such as Withanolide D, and Phenethyl isothiocyanate (33,34). Thus, MKK7 is the pivotal factor in our study of the anti-hepatoma mechanisms of Alpinetin. Previous studies have found that Alpinetin suppress the activity of NF-kappaB in various malignant tumors (7). Meanwhile, inhibition of NF-kappaB activity can induce MKK7/JNK1-dependent apoptosis in human acute myeloid leukaemia cells (35). These studies indicate that the antitumor effect of Alpinetin is related to the activation of MKK7-JNK signaling pathway. The present study showed that Alpinetin suppresses the proliferation of hepatoma cells through the activation of the MKK7-JNK signaling pathway. In addition, a down-regulation of MKK7 expression by RNA interference reduced the phosphorylation level of MKK7 and reversed the anti-hepatoma effect of Alpinetin. Therefore, in view of its key function in inhibiting the proliferation of human hepatoma cells, activation of MKK7 by Alpinetin offers a significant strategy for molecular therapy against hepatoma.

Direct phosphorylation of target proteins by p38 arrests the cells in a G0/G1 phase while ERK1/2 activation has the reverse effect (36). Accumulating evidence suggests that JNK pathway is also a physiologic activator of p38 under certain conditions, resulting in cell cycle arrest (37). In our study, we found that Alpinetin arrested hepatoma cells in G0/G1 through activating MKK7 phosphorlyation. In addition, when MKK7 level was down-regulated by siRNA, the inhibitory effect of Alpinetin decreased. Most likely the activation of JNK pathway in our study leads to p38 activation, thereby arresting the cell cycle, but this mechanism needs to be validated with further experiments.

CDDP is a common clinical chemotherapeutic agent, used to treat many malignant solid tumors including hepatocellular carcinoma. Current study has found that the chemosensitivity to CDDP in HepG2 cells can be improved by JNK signal pathway (21). Therefore, we tested the sensitivity of Alpinetin-treated HepG2 cells to CDDP-induced cytotoxicity. Our data indicate that Alpinetin and CDDP have a synergistic inhibitory effect on HepG2 cell growth and proliferation. Our research suggests that the augmentation of CDDP’s efficacy by Alpinetin is connected with the activation of the MKK7-JNK signaling pathway. Furthermore, as either a promising chemosensitizer or adjuvant, Alpinetin is worth further investigation, which may bring about the development of a therapeutic regimen combining Alpinetin with CDDP or other chemotherapeutic drugs to treat malignant tumors.

In summary, we have found that activation of MKK7, a specific upstream regulator of JNK signal pathway, mediates the anti-proliferative effect of Alpinetin. Furthermore, the antitumor effect of Alpinetin is found to be responsible for the arrest of hepatoma cell cycle. Taken together, our study suggests that MKK7 is a novel molecular target and combination chemotherapy in hepatoma, while Alpinetin may be a potential traditional Chinese medicine for the future development of hepatoma therapy.

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