Dihydromyricetin suppresses the proliferation of hepatocellular carcinoma cells by inducing G2/M arrest through the Chk1/Chk2/Cdc25C pathway

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Abstract. The aim of the present study was to evaluate the antitumor mechanism of dihydromyricetin (DHM). Results showed that DHM significantly inhibited cell viability of HepG2 and Hep3B cells in a dose-dependent manner. DHM induced G2/M cell-cycle arrest in HepG2 and Hep3B cells by altering the expression of cell cycle proteins such as cyclin A, cyclin B1, Cdk1, p53, Cdc25c, p-Cdc25c Chk1 and Chk, which are critical for G2/M transition. Knockdown of p53 and Chk1 in HepG2 cells did not affect G2/M phase arrest caused by DHM. Furthermore, G2/M arrest induced by DHM can be disrupted by Chk2 siRNA. These findings indicate that DHM inhibits the growth of hepatocellular carcinoma (HCC) cells via G2/M phase cell cycle arrest through Chk1/Chk2/Cdc25C pathway. The present study identified effects of DHM in G2/M phase arrest in HCC and described detailed mechanisms of G2/M phase arrest by this agent, which may contribute to its overall cancer preventive efficacy in HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world (1). Although numerous anticancer drugs have been used in the routine clinical treatment of HCC and result in a reduction in tumor volume at early stages, recurrence, the development of multidrug resistance, toxicity and side-effects are unfortunately common in patients. Therefore,

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there is a pressing need for new therapeutic drugs with increased efficacy and decreased toxicity.

Cell cycle deregulation is a hallmark of tumor cells and targeting the proteins that mediate critical cell cycle processes is an emerging strategy for the treatment of cancer (2). The G2/M checkpoint is the most conspicuous target for several anticancer drugs (3,4). CDK1/cyclin B1 and CDK1/cyclin A complexes play a key role in promoting the G2/M phase transition. A number of proteins are known to regulate the stepwise activation of CDK1, which controls the G2 to M transition. This process involves additional proteins, including Wee1 (5), Myt1 (6) and Cdc25C (7). The phosphatase activity of Cdc25C is inactivated by Chk1/Chk2, which are activated by ATM/ATR in response to DNA damage (8,9). Activation of ATM/ATR initiates the subsequent protein kinase cascade through both p53 dependent and independent pathways. In the p53 dependent pathways, p53 is phosphorylated on Ser15 and Ser20 and then activated downstream target genes, such as p21 (10) and 14-3-3 (7), which play an important role in G2/M checkpoint through inhibition of cyclin B1/Cdk1 (11-16). In p53 independent pathways, Chk1 and Chk2 phosphorylate Cdc25c at Ser216, which downregulate Cdc25c activity by promoting 14-3-3 protein and nuclear export. Chk/12 also phosphorylates wee1 and increases wee1 activity (8,12,17-22).

Dihydromyricetin (DHM) also known as Ampelopsin, isolated from the tender stem and leaves of the plant species *Ampelopsis grossedentata*, is one of the most common flavonoids found in grapes, berries, fruits, vegetables, herbs and other plants with certain anticancer activities. As the major bioactive constituent of *Ampelopsis grossedentata*, DHM was reported to possess numerous pharmacological activities, such as anti-inflammatory (23), antimicrobial activity, relieving cough, anti-oxidation (24), antihypertension as well as hepatoprotective (25) and anticarcinogenic effects. DHM was shown to possess certain anticancer activities. It has been reported that DHM inhibits the growth and metastasis in prostate cancer (26), lung cancer (27) and melanoma tumor (28,29). DHM also possesses anti-angiogenesis activity by inhibiting the secretion of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) from human HCC cells *in vitro* and in mice (30). DHM also reversed multidrug

Table I. Antibodies used in the present study.

Antibody	Cat. no., host		Dilution	Company
p-CDK1	4539,	rabbit polyclonal	1:300 for WB	Cell Signaling
CDK1	9116,	mouse mAb IgG1	1:300 for WB	Cell Signaling
Cyclin antibody sampler kit	9869,	rabbit polyclonal	1:300 for WB	Cell Signaling
Wee1	4936,	abbit polyclonal	1:300 for WB	Cell Signaling
p53	9282,	rabbit polyclonal	1:300 for WB	Cell Signaling
MDM2	Ab137413,	rabbit polyclonal	1:500 for WB	Abcam
p-MDM2	3521,	rabbit polyclonal	1:300 for WB	Cell Signaling
Cdc25C	4688,	rabbit polyclonal	1:300 for WB	Cell Signaling
p-Cdc25C	9528,	rabbit polyclonal	1:300 for WB	Cell Signaling
Myt1	4282,	rabbit polyclonal	1:300 for WB	Cell Signaling
p-Myt1	4281,	rabbit polyclonal	1:300 for WB	Cell Signaling
p-Chk2 (ser 33/35)	2665,	rabbit polyclonal	1:300 for WB	Cell Signaling
p-Chk2 (Thr 68)	2197,	rabbit polyclonal	1:300 for WB	Cell Signaling
p-Chk2 (ser 19)	2666,	rabbit polyclonal	1:300 for WB	Cell Signaling
Chk2	3440,	rabbit polyclonal	1:300 for WB	Cell Signaling
p-Chk1 (ser 296)	2349,	rabbit polyclonal	1:300 for WB	Cell Signaling
p-Chk1 (ser 317)	8191,	rabbit polyclonal	1:300 for WB	Cell Signaling
p-Chk1 (ser 345)	2348,	rabbit polyclonal	1:300 for WB	Cell Signaling
Chk1	2360,	mouse mAb	1:300 for WB	Cell Signaling
β-actin	8457,	rabbit polyclonal	1:1,000 for WB	Cell Signaling

WB, western blotting.

resistance in leukemia cells *in vitro* in part via decreasing the expression of p-glycoprotein (31). On the other hand, the effect of DHM on the growth and progression of HCC is rarely studied.

The objectives of the present study were to systematically evaluate DHM as a potential chemopreventive and therapeutic candidate against HCC progression, and to elucidate the underlying cellular and molecular mechanisms of DHM actions. Our results provided experimental evidence to support the future development of DHM as an effective and safe candidate agent for the prevention and/or therapy of HCC.

Materials and methods

Cell lines and cell culture. The human HCC cell lines HepG2, Hep3B and immortalized human liver cell line L02 were provided by the Cell Bank of the Institute of Biochemistry and Cell Biology at the China Academy of Sciences (Shanghai, China). All cell lines were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (HyClone) and supplemented with 100 IU/ml penicillin G and 100 μg/ml streptomycin (HyClone). All cell lines were incubated at 37˚C in a humidified atmosphere with 5% CO_2.

Drug stocks. DHM was purchased from Sigma-Aldrich and prepared at a stock concentration of 50 mM in dimethyl sulfoxide (DMSO).

MTT assay. Cell toxicity and proliferation after DHM treatment were determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, 5,000 cells/well were plated in triplicate in 96-well plates, and the cells were exposed to 2, 10, 50, 100 and 200 μM DHM for 48 h. The MTT reagent (Sigma-Aldrich) was prepared at 5 mg/ml in PBS. This MTT stock solution was then added to each well at a 1:10 dilution. Cells were incubated for 4 h and the resulting crystals were dissolved in 100 μl DMSO (Sigma-Aldrich). The absorbance at 492 nm was measured using a multiwell plate reader. The inhibition rate was calculated as follows: Inhibition rate = 1-A492 of treated cells/A492 of control cells.

Colony formation. To determine the frequency of colony formation, HepG2 and Hep3B cells were plated in 6-well plates at concentrations of $5x10^3$ cells/ml in DMEM media containing 2, 10, 50, 100 and 200 μM DHM and colonies were stained with crystal violet and counted in triplicate wells after growth for a further 2-3 weeks. DMSO was used as a negative control.

Cell cycle analysis. For cell cycle analysis, $2x10^5$ cells were plated in a 6-well culture plate and grown for 24 h. The cells were then incubated with 1 mM thymidine (Sigma-Aldrich) for 24 h to synchronize cells at the G1/S boundary. The cells were then treated with fresh media containing 2, 10, 50, 100 and 200 μM DHM for 48 h. Next, the cells were trypsinized, washed twice with cold PBS and fixed with cold 70% ethanol at -4˚C overnight. The cells were then washed twice with PBS and incubated with 10 mg/ml RNase A, 400 mg/ml propidium iodide (Sigma-Aldrich) and 0.1% Triton-X in PBS at room temperature (RT) for 30 min. Cells were subsequently analyzed by flow cytometry.

Table II. siRNA sequences.

| Target name	Sequence
p53	5'-AAGACUCCAGUGGUAAUCUACTT-3' (sense)
	5'-GUAGAUUACCACUGGAGUCUUTT-3' (antisense)
Chk1	5'-GACUGGGACUUGGUGCAAATT-3' (sense)
	5'-UUUGCACCAAGUCCCAGUCTT-3' (antisense)
Chk2	5'-GUAAGAAAGUAGCCAUAAATT-3' (sense)
	5'-UUUAUGGCUACUUUCUUACTT-3' (antisense)
Negative control	5'-UCCUCCGAACGUGUCACGUTT-3' (sense)
	5'-ACGUGACACGUUCGGAGAATT-3' (antisense)
GAPDH positive control	5'-GUAUGACAACAGCCUCAAGTT-3' (sense)
	5'-CUUGAGGCUGUUGUCAUACTT-3' (antisense)
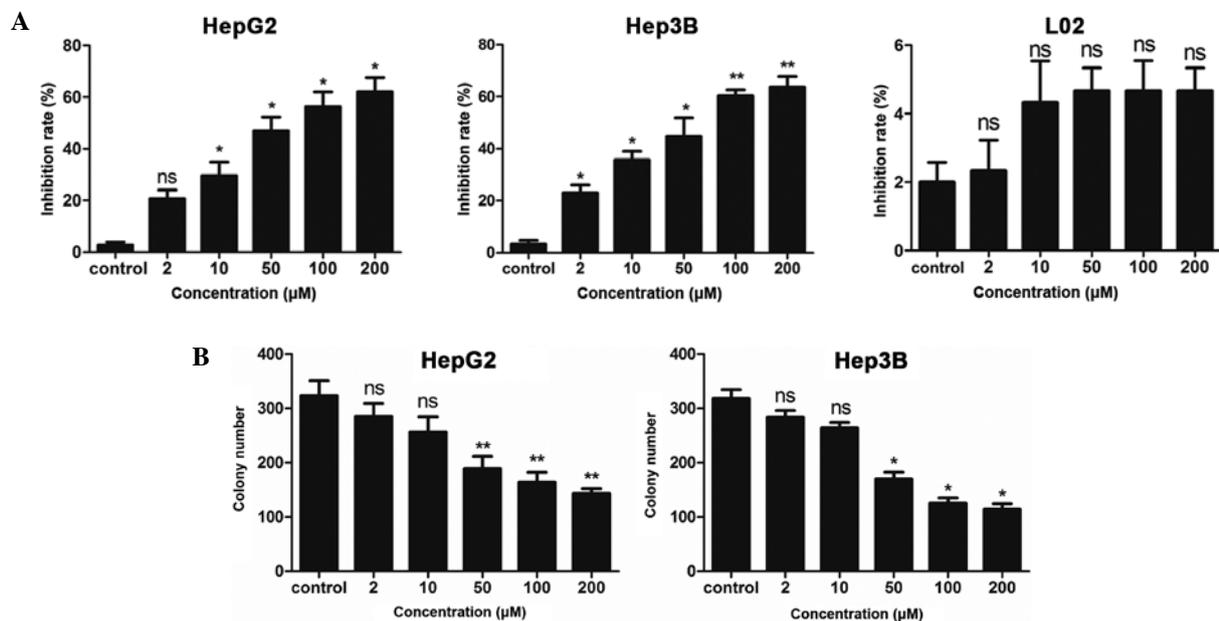
Figure 1. DHM reduces cell viability and inhibits colony formation in HCC cell lines. (A) The growth inhibition rates of HepG2, Hep3B and L02 cells resulting from treatment with DHM at various concentrations for 48 h. Cells were treated with various concentrations of DHM (2, 10, 50, 100 and 200 μM) for 48 h. DMSO was used as control. Cell viability was measured by MTT assay. (B) Colony formation of HepG2 and Hep3B cells after treatment with DHM at various concentrations. Cells were treated with various concentrations of DHM (2, 10, 50, 100 and 200 μM) and colonies were counted in triplicate wells after growth for a further 2-3 weeks. DMSO was used as control. ns, non significant. *P<0.05, **P<0.01.

Western blotting. At the end of the treatments, the HCC cells were harvested and lysed with ice-cold cell lysis solution and the homogenate was centrifuged at 10,000 x g for 15 min at 4°C. Total protein in the supernatant was quantified using a BCA protein assay kit. Total protein (30 μg) from each sample was separated by 12% SDS-PAGE and transferred to a PVDF membrane which was placed in washing buffer containing skimmed milk powder at room temperature, blocked for 2 h, and washed 3 times. The indicated primary antibodies, listed in Table I, were added, respectively, and incubated at 4°C overnight. Then, horseradish peroxidase-conjugated secondary antibody was added to incubate for 1 h. X-ray film exposure was performed and AlphaImager HP fluorescence/visible light gel imaging analyzer processing and image analysis software were used to analyze gray value.

RNA interference. Small-interfering RNA (siRNA) oligos for p53, Chk1, Chk2 and general negative control with the sequences listed in Table II, were synthesized and annealed by GenePharma (Shanghai, China). Each siRNA duplex was transfected into HepG2 using Lipofectamine® 2000 (Invitrogen) following the manufacturer's protocol. siRNA-NC, siRNA-NC-FAM and siRNA-GAPDH respectively served as negative control, transfecting control and siRNA positive control targeting GAPDH gene. Cells were exposed to 50 μM DHM after transfection and harvested for indicated analysis.

Statistical analysis. The data are presented as the mean ± standard deviation (SD). Statistical analyses (two group comparisons) were performed using the Student's t-test. P<0.05 was considered to indicate a statistically significant difference.
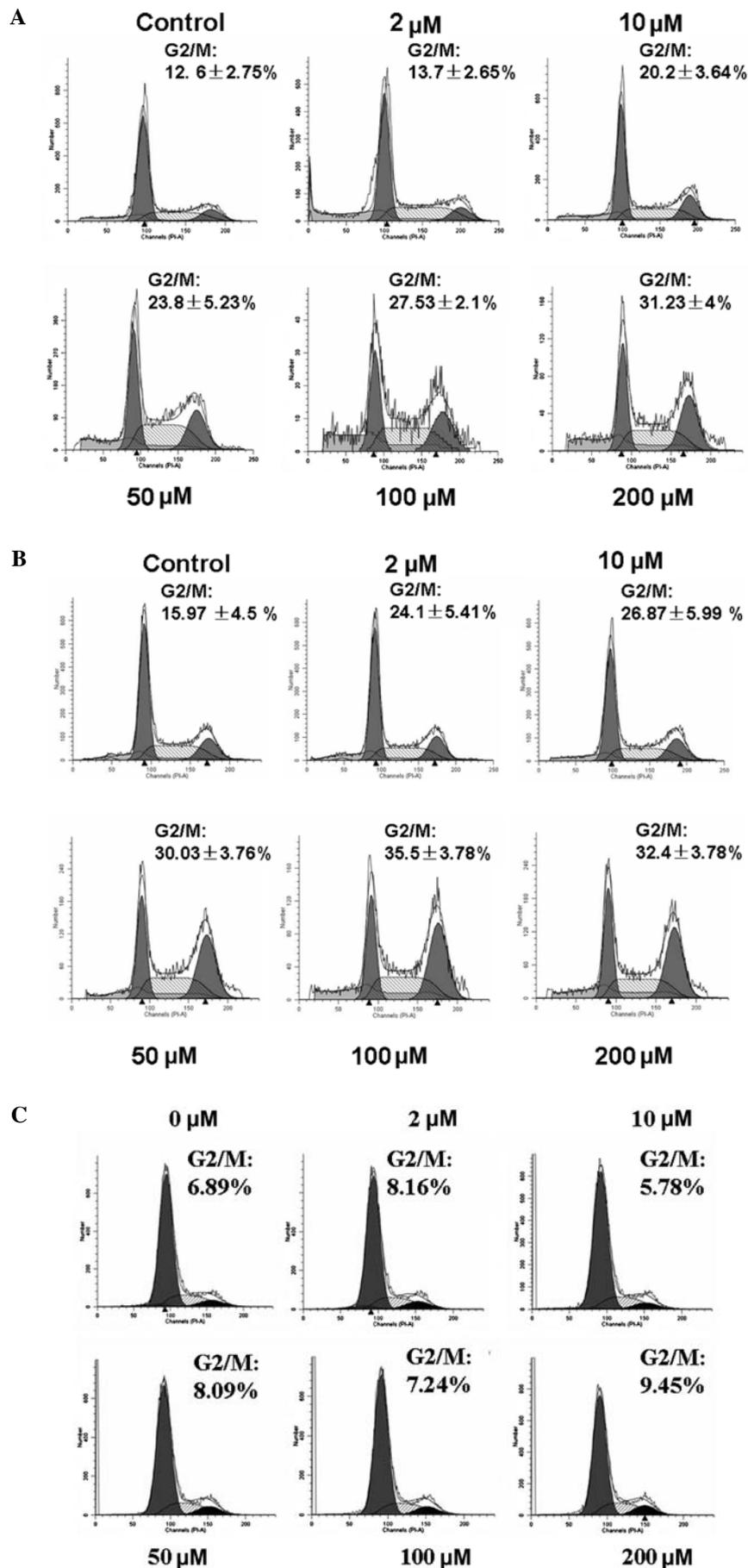
Figure 2. DHM induces G2/M phase cell cycle arrest in HepG2 and Hep3B cells. (A) HepG2, (B) Hep3B and (C) L02 cells were treated with DHM at various concentrations (2, 10, 50, 100 and 200 μM) for 48 h. The cell cycle distribution was analyzed by flow cytometry using PI staining method. The histograms (bottom) represent the percentage of HepG2 cells in G2 phase following treatment with DHM for various times.
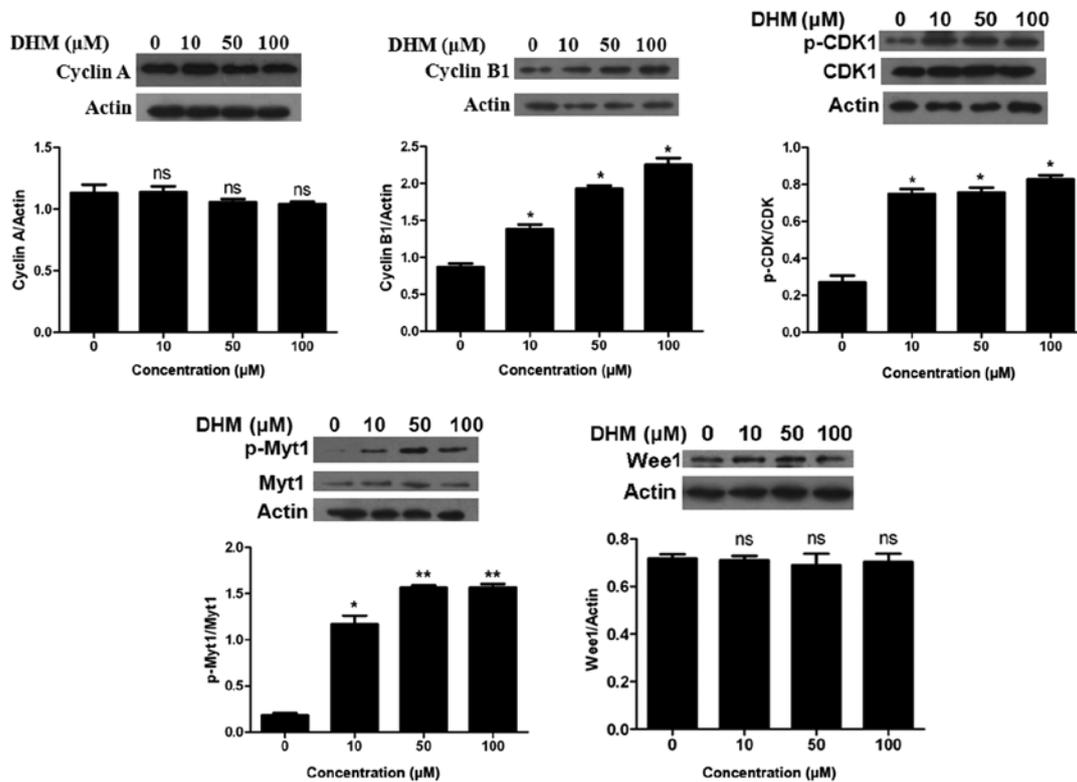
Figure 3. DHM induces G2/M cell cycle arrest by decreasing the activity of CDK1. Cells were treated with DHM at various concentrations (10, 50 and 100 μM) for 48 h. The expression levels of cyclin A, cyclin B1, CDK1, Myt1 and Wee1 were measured by western blotting using specific antibodies. β-actin was used as a loading control. The histograms (bottom) represent the protein levels expressed as densitometry after normalization to β-actin. Values are means ± SEM of three independent experiments. Ns, non significant. *P<0.05, **P<0.01.

Results

DHM suppresses proliferation and colony formation of HepG2 and Hep3B cells. To investigate the suppressive growth effect of DHM, the HCC cell lines HepG2 and Hep3B were incubated with 2, 10, 50, 100 and 200 μM DHM for 48 h. Cell proliferation was subsequently measured by the MTT assay. Our results show that DHM inhibited the growth of HepG2 and Hep3B cells in a dose-dependent manner (Fig. 1A). To exclude the possibility that cell death was due to drug toxicity, the effect of DHM on the immortalized human liver cell line L02 was also investigated. L02 cells were found to have a low sensitivity to DHM treatment (Fig. 1A).

We also investigated whether DHM inhibited the ability of HCC cells to initiate colonies on plastic. HepG2 and Hep3B cells were treated with 2, 10, 50, 100 and 200 μM DHM. DMSO was used as a negative control. HepG2 and Hep3B cells treated with DHM showed a reduction in colony formation compared to those treated with DMSO (Fig. 1B). These results indicated DHM inhibited colony formation of HCC cell lines.

DHM induces G2/M cell cycle arrest in HepG2 and Hep3B cells. To further elucidate the inhibitory effects of DHM on HCC cell growth, the cell cycle distributions of HepG2 and Hep3B cells were determined by flow cytometry. Following treatment with 1 mM thymidine for 24 h to synchronize cells at the G1/S border, the cells were incubated with 2, 10, 50, 100 and 200 μM DHM for 48 h. A dose-dependent G2/M arrest in the cell cycle was observed in HepG2 and Hep3B cells

after treatment with DHM. By contrast, G2/M arrest was not observed in L02 cells treated with DHM for 48 h (Fig. 2).

DHM induces G2/M cell cycle arrest by decreasing the activity of CDK1. We next examined the expression of the key molecules that promote the G2/M phase transition. The activation of CDK1 is essential for cells to correctly enter the M phase (32). This process involves the formation of a complex between CDK1 and cyclin B1 or cyclin A. Western blot analysis showed that the expression level of cyclin A was not influenced by DHM treatment. DHM increased the expression of cyclin B1 and the inhibitory phosphorylation status of CDK1 (Tyr15) in a dose-dependent manner (Fig. 3). Since the accumulation of p-CDK1 (Tyr15) indicated the presence of an inactive complex, our data suggested DHM inactivated the CDK1/cyclin B1 complex.

Phosphorylation of CDK1 at Tyr15 and Thr14 sites is known to be performed by the Wee1 and Myt1 protein kinases. We observed an upregulation in the expression level of p-Myt1 protein following DHM treatment in HepG2 cells; however, DHM did not affect the expression level of Wee1 (Fig. 3).

DHM inactivates CDK1 independent of the p53 pathway. It is well known that CDK1/cyclin B1 complex can be inactivated by the p53 pathways. Therefore, to elucidate whether the p53 pathway is involved in the phosphorylation of CDK1 observed in our experiments, we determined the levels of p53 by western blotting. Our results indicated that DHM increased the protein level of p53 and decreased p-MDM2 expression
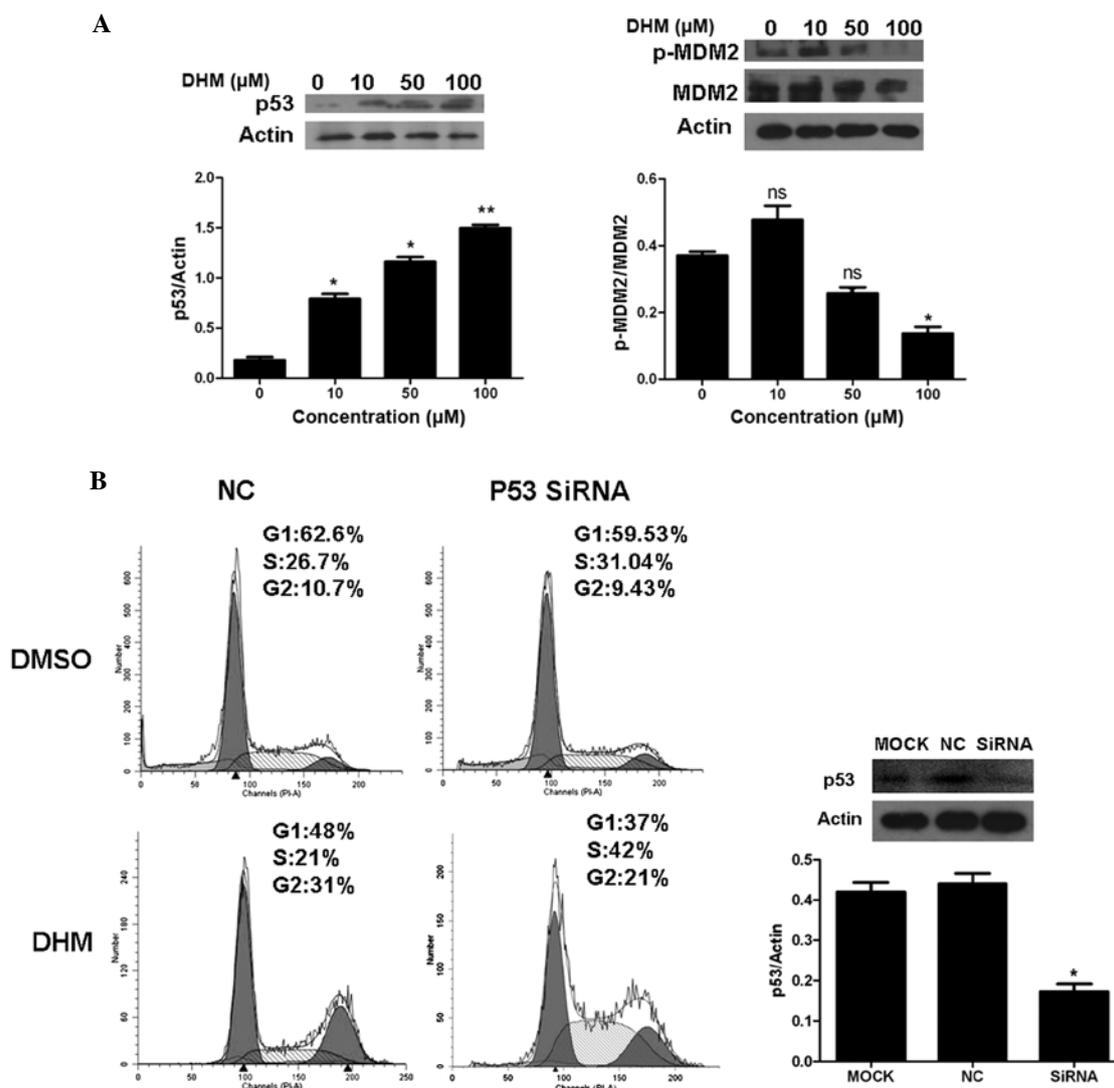
Figure 4. DHM inactivates CDK1 independent of the p53 pathway. (A) Western blot analysis of the expression of p53 and MDM2 in HepG2 cells after DHM treatment for 48 h. (B) Western blot analysis of the silencing effect of p53 expression level and the cell cycle distribution of HepG2 cells after 50 μM DHM treatment for 48 h following p53 siRNA transfection. The histograms (bottom) represent the protein levels expressed as densitometry after normalization to β-actin. Values are means ± SEM of three independent experiments. Ns, non significant. *P<0.05, **P<0.01.

level; however, DHM did not affect the expression level of MDM2 (Fig. 4A). To further determine the relative contribution of p53 to DHM-induced G2/M arrest, HepG2 cells were treated with DHM after transfection with either p53 siRNA or a negative control. The efficiency of p53 siRNA was confirmed by western blot analysis (Fig. 4B). Cell cycle analysis showed that in the p53 knockdown HepG2 cells, the G2/M percentage of negative control (NC), p53 siRNA was 31 and 21%, respectively, after DHM treatment (Fig 4B). The results suggest that p53 siRNA does not disrupt the G2/M cell cycle arrest induced by DHM.

DHM inactivates CDK1 through the Chk1/Chk2/Cdc25C pathway. The CDK1/cyclin B1 complex can be inactivated by the Chk1/Chk2/Cdc25C pathways. The Cdc25C protein activates the cyclin B1/CDK1 complex by dephosphorylating these inhibitory residues on CDK1. Inactivated phosphatase activity of Cdc25C can contribute to CDK inactivation. The phosphatase activity of Cdc25C is inactivated by Chk1/

Chk2, which are activated by ATM/ATR in response to DNA damage. We found that DHM treatment resulted in an increase in the p-Cdc25C protein level and did not affect the total level of Cdc25C (Fig. 5A). Therefore, these data indicate that DHM may inactivate Cdc25C which leads to inactivation of the CDK1/cyclin B1 complex.

In DHM-treated HepG2 cells, we observed an upregulation of the phosphorylation of Chk1 (at Ser317) and increased phosphorylation of Chk2 at Ser33/35, but it had no effect on other phosphorylation sites within this protein (Fig. 5A). These results suggest that the inactivation of CDK1 observed with DHM treatment is mainly induced by Chk1- and Chk2-mediated phosphorylation of Cdc25C.

Since both Chk1 and Chk2 were phosphorylated after DHM treatment, to further determine the relative contribution of Chk1 and Chk2 to DHM-induced G2/M arrest, HepG2 cells were treated with DHM after transfection with either Chk1/Chk2 siRNA or a negative control. The efficiency of Chk1/Chk2 siRNA was confirmed by western blot analysis
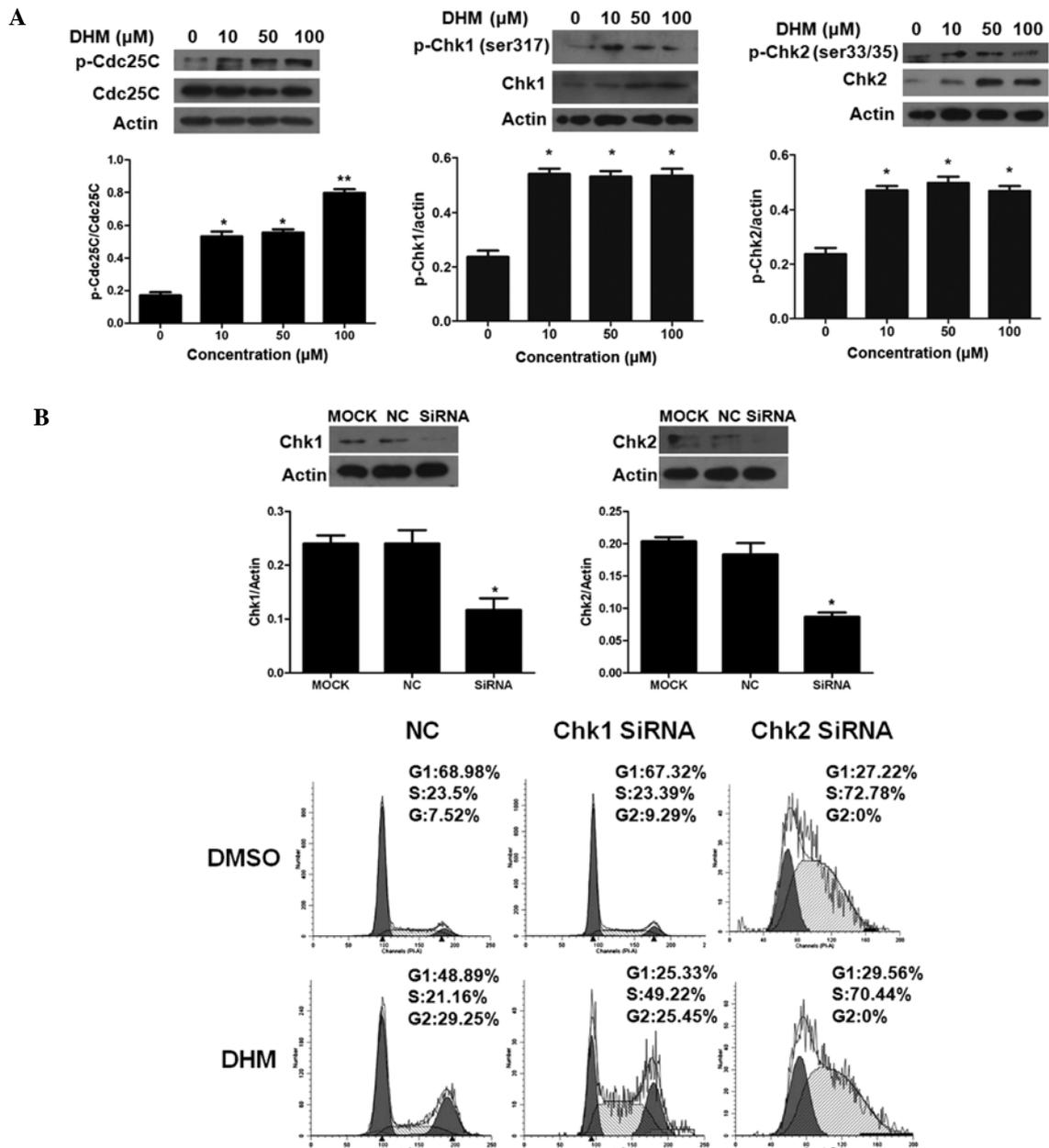
Figure 5. DHM inactivates CDK1 through the Chk1/Chk2/Cdc25C pathway. (A) Western blot analysis of Cdc25C, Chk1 and Chk2 in HepG2 cells after DHM treatment for 48 h. (B) Western blot analysis of the silencing effect of Chk1 or Chk2 expression levels and the cell cycle distribution of HepG2 cells after 50 μM DHM treatment for 48 h following Chk1 or Chk2 siRNA transfection. The histograms (bottom) represent the protein levels expressed as densitometry after normalization to β-actin. Values are means ± SEM of three independent experiments. *P<0.05, **P<0.01.

(Fig. 5B). Cell cycle analysis showed that in the Chk1 and Chk2 knockdown HepG2 cells, the G2/M percentage of negative control (NC), Chk1 siRNA and Chk2 siRNA was 29.25, 25.45 and 0%, respectively, after DHM treatment (Fig. 5B). The results suggest that Chk2 siRNA disrupts the G2/M cell cycle arrest, while the negative control or Chk1 do not.

Discussion

The Chinese herb *Ampelopsis grossedentata* is widely distributed in South China and is used to treat cold and tinea corporis. It contains a rich resource of phytochemicals with dihydromyricetin (DHM), a naturally occurring flavonoid found in grapes, berries, fruits, vegetables, herbs and other

plants with certain anticancer activities. As the major bioactive constituent of *Ampelopsis grossedentata*, DHM has been shown to be mainly responsible for the reported biological activities, including hypoglycemic (33), anti-oxidative (24) and hepatoprotective activities (25). DHM also enhanced the chemokinesis and chemotaxis effects of neutrophilic granulocytes and monocytes (23).

To investigate the antitumor effect of DHM in hepatocellular carcinoma (HCC), the HCC cell lines HepG2, Hep3B and the human liver cell line L02 were exposed to DHM for 48 h. In the present study, DHM treatment resulted in a clear inhibition of proliferation at a relatively low concentration in HCC cell lines (Fig. 1). By contrast, L02 cells were found to be markedly resistant to this compound. In L02 cells, the observed inhibi-

tory rate was less than 5% (Fig. 1), indicating that DHM may be less toxic to L02 cells than to cancer cells. Therefore, DHM may not exhibit toxicity in experimental animals.

To investigate the mechanism behind the antitumoral properties of DHM, cell cycle analysis was performed. DHM induced G2/M phase arrest in HepG2 and Hep3B cells but not in L02 cells (Fig. 2). Cell cycle deregulation is an important mechanism to modulate HCC cell proliferation. Cell cycle progression is tightly regulated by cyclin/cyclin-dependent kinase (Cdks) complexes. For instance, cyclin D/Cdk4 and Cdk6 drive the sequential progression from G1 to S phase (34,35); cyclin A/Cdk2 and Cdc2 (Cdk1) complexes control the S and G2 phases (36); and cyclin B/Cdk1 complex drives the G2/M transition as well as processes during mitosis (32). The G2/M checkpoint allows the cell to repair DNA damage before entering mitosis. The stepwise activation of CDK1 is essential for cells to correctly enter the M phase (32). This process involves the formation of a complex between CDK1 and cyclin B1 or cyclin A. According to our results, DHM treatment increased expression levels of cyclin B1 and Cdk1. The expression level of cyclin A was not influenced by DHM treatment. These findings indicate DHM might induce G2/M cell cycle arrest by increasing the level of inactive cyclin B1/Cdk1 complex (Fig. 3).

Numerous proteins are known to regulate the activation of CDK1 including Wee1 (5), Myt1 (6) and Cdc25C. CDK1 is subsequently activated via a Cdk-activating enzyme, which phosphorylates the activating residues on CDK1. Inhibitory phosphorylation can also be performed at Thr160/161, Thr14 and Tyr15 by Wee1 (5) and Myt1 (6). The phosphatase Cdc25C, by contrast, can dephosphorylate Thr14 and Tyr15 (37). According to our results, DHM treatment induced a pronounced G2/M phase arrest by increasing the level of cyclin B1 as well as the accumulation of Thr14/Tyr15-phosphorylated CDK1. DHM induced Myt 1 upregulation may contribute to the accumulation of Thr14/Tyr15-phosphorylated CDK1 (Fig. 3).

It is known that the initial activation of cyclin B1/Cdk1 also involves Cdk1 dephosphorylation at Thr14 and Tyr15 by Cdc25C (38). Decreased Cdc25C phosphatase activity can lead to inactivation of cyclin B1/Cdk1 (19). In the present study, DHM treatment led to an upregulation of Ser216-phosphorylated Cdc25C (Ser216) which downregulates Cdc25c activity and leads to accumulation of Thr14/Tyr15-phosphorylated CDK1. Our data, therefore, suggest that DHM inactivates the CDK1/cyclin B1 complex by inactivating Cdc25C.

The phosphatase activity of Cdc25C is inactivated by Chk1/Chk2, which are activated by ATM/ATR in response to DNA damage (13,15,16,19). These kinases are activated upon DNA damage, which results in the inactivation of Cdc25C. Chk1 is activated by phosphorylation at Ser317, Ser345 and Ser296, while Chk2 is activated at Ser33/35, Ser516, Ser296 and Thr68 (39,40). In the present study, p-Chk1 (Ser317 and Ser345) and p-Chk2 (Ser33/35) were upregulated after DHM treatment. To determine the relative contributions of these proteins on the DHM-induced cell cycle arrest, we analyzed the cell cycle distribution of siRNA-mediated Chk1 or Chk2 knockdown HepG2 cells after treatment with DHM or DMSO (Fig. 5). The results showed that Chk2 knockdown disrupts the G2/M arrest compared with NC or Chk1. These results indi-

cate that DHM treatment activates Chk1 and Chk2, allowing these kinases to inactivate Cdc25C. Cdc25C, Wee1 and Myt1 then decrease the activity of the CDK1/cyclin B1 complex, resulting in an arrest of the cell cycle at the G2/M phase.

The activation of CDK1/cyclin B1 can also be prevented by p53. Our results indicate that DHM increased the protein level of p53 and decreased p-MDM2 expression level; however, DHM did not affect the expression level of MDM2 (Fig. 4). To further determine the relative contribution of p53 to DHM-induced G2/M arrest, HepG2 cells were treated with DHM after transfection with either p53 siRNA or a negative control. The efficiency of p53 siRNA was confirmed by western blot analysis (Fig. 4B). Cell cycle analysis showed that in the p53 knockdown HepG2 cells, the G2/M percentage of negative control (NC), p53 siRNA was 31 and 21%, respectively, after DHM treatment (Fig. 4B). The results suggest that p53 siRNA does not disrupt the G2/M cell cycle arrest induced by DHM.

In conclusion, our present study demonstrated that DHM inhibited HCC cell growth through G2/M phase cell cycle arrest dependent on the Chk1/Chk2/Cdc25C pathway. Based on the present study, DHM could be a potential agent against HCC development. Future studies on the effects of DHM on detailed mechanisms of cell cycle arrest and other signal pathways for *in vitro* cell lines and *in vivo* animal models are required to further elucidate the detailed mechanism(s) of action of DHM on HCC chemoprevention.

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