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ORIGINAL ARTICLE

Anti-proliferative Effect of 15,16-Dihydrotanshinone I Through Cell Cycle Arrest and the Regulation of AMP-activated Protein Kinase/Akt/mTOR and Mitogen-activated Protein Kinase Signaling Pathway in Human Hepatocellular Carcinoma Cells

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Background: 15,16-dihydrotanshinone I (DHTS) is a natural abietane diterpenoid that is mainly found in the roots of *Salvia miltiorrhiza* Bunge (Labiatae). DHTS exhibits a potential anti-proliferative effect in various human cancer cells. However, the mechanisms of action of DHTS as an anti-cancer agent have not been fully elucidated. Therefore, the present study investigated the anti-cancer effect of DHTS in terms of cell cycle regulation and the regulation of the AMP-activated protein kinase (AMPK)/Akt/mTOR signaling pathway in SK-HEP-1 human hepatocellular carcinoma cells.
Methods: The anti-proliferative effects of DHTS were evaluated by the sulforhodamine B assay in SK-HEP-1 cells. Cell cycle distribution was analyzed by flow cytometry. The elucidation of mechanisms of action such as the AMPK/AKT/mTOR and mitogen-activated protein kinase (MAPK) pathway was assessed by Western blot analysis.
Results: DHTS showed a significant anti-proliferative activity against SK-HEP-1 cells. DHTS induced cell cycle arrest in the G0/G1 phase, which was mediated by downregulation of cyclin D1, cyclin A, cyclin E, CDK4, CDK2, c-Myc and p-Rb expression and with increased expression of the CDK inhibitor p21. DHTS also activated the AMPK signaling. In addition, DHTS downregulated the Akt/mTOR and MAPK signaling pathways.
Conclusions: Our results suggest that the anti-proliferative activity of DHTS might be associated with the induction of G0/G1 phase cell cycle arrest and regulation of AMPK/Akt/mTOR and MAPK signaling pathways in SK-HEP-1 cells.
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Key Words: 15,16-Dihydrotanshinone I, *Salvia miltiorrhiza*, Cell cycle arrest, AMP-activated protein kinase, Hepatocellular carcinoma

INTRODUCTION

Hepatocellular carcinoma (HCC) remains the sixth most frequently diagnosed cancer and the second most common cause of cancer-related mortality in the world.[1,2] Curative treatment, such as resection or transplantation is possible when the disease is detected at the early stage. However, most of the HCCs have been diagnosed at advanced stages when patients are not eligible for curative therapies. The advanced HCCs are resistant to most standard chemotherapy. Although novel chemotherapeutic agents such as sorafenib have been introduced for HCC therapy, the overall five-year survival rate remains to be lower than 15% in patients.[3,4] In addition, several target drugs including brivanib and sunitinib and combination therapies (sorafenib with doxorubicin, tegafur/uracil or 5-fluorouracil) have been used for HCC patients, but the efficacy was not found to be superior to the conventional drug in clinical trials.[3-5] Therefore, continuous efforts are still needed to develop new effective chemotherapeutic agents for HCC treatments.

Natural products have played an important role to provide

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potential lead compounds for the development of anticancer agents.[6] For decades, many bioactive natural compounds such as curcumin, resveratrol, silibinin, berberine, quercetin, and celastrol have been reported to have potent anti-proliferative activity on HCC.[7] The root of *Salvia miltiorrhiza* has been used as a traditional oriental medicine in the treatment of amenorrhea, coronary heart diseases, angina pectoris, inflammation, and dysmenorrhea.[8,9] Several compounds such as tanshinone I, tanshinone IIA, cryptotanshinone, dansenspiroketallactone, and dihydrotanshinone were isolated from the root of *S. miltiorrhiza*.[10] These tanshinones have been shown to possess pharmacological effects including preventing cardiovascular diseases, anti-oxidative, anti-angiogenic, and anti-inflammatory activities.[7,11] Recent studies also showed that 15,16-dihydrotanshinone I (DHTS) exhibits an anti-proliferative activity in various types of cancer cells.[12-14] However, the mechanisms underlying the growth-inhibitory activity of DHTS against human HCC cells have been not reported.

In the present study, the anti-proliferative activities of DHTS and its underlying molecular mechanism were explored in association with cell cycle regulation and the AMP-activated protein kinase (AMPK)/mTOR and mitogen-activated protein kinase (MAPK) signaling pathways in SK-HEP-1 human HCCs.

MATERIALS AND METHODS

1. Chemicals

Bovine serum albumin, sulforhodamine B (SRB), propidium iodide (PI), anti-β-actin antibody, dimethyl sulfoxide, and ribonuclease A (RNase A) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), FBS, and antibiotic-antimycotic solution were purchased from HyClone Laboratories, Inc. (South Logan, UT, USA). Anti-cyclin A, anti-CDK4, anti-CDK2, anti-cMyc, and anti-p21 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p-Rb (S807/811), anti-Rb, anti-p-Akt (Ser473), anti-Akt, anti-p-mTOR (Ser2448), anti-mTOR, anti-p-p70S6K1 (Thr389), anti-p70S6K1, anti-p-4EBP1 (Thr37/46), anti-4EBP1, anti-p-PDK1 (Ser241), anti-p-AMPK (Thr172), and anti-AMPK were obtained from Cell Signaling (Danvers, MA, USA). Anti-cyclin D1 and anti-Cyclin E were purchased from BD Biosciences (San Jose, CA, USA). Four tanshinones (tanshinone I, tanshinone IIA, cryptotanshinone, and DHTS; Fig. 1) were isolated by Dr. Seung Ho Lee (Yeungnam University, Korea) from the roots of *S. miltiorrhiza* Bunge (Labiatae) and were provided through the Research Center for Standardization of Herbal Medicines in Korea.

2. Cell culture

The human HCC cell line (SK-HEP-1) was obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in DMEM medium supplemented with 10% FBS and antibiotics-antimycotics at 37°C humidified atmosphere containing 5% CO_2.

3. Cell proliferation assay

The cell proliferation was evaluated using SRB assay.[15] Cell suspensions were added to each well of 96-well plates and treated with various concentration of compounds for 24 to 72 hours. Cells were fixed with 10% trichloroacetic acid solution for 30 minutes at 4°C. After washing with tap water and drying in the air for 24 hours, the cells were incubated with 0.4% SRB in 1% acetic acid solution for 1 hour at room temperature. The unbound SRB was removed by washing the wells e with 1% acetic acid solution and then air dried. The stained cells were dissolved in Tris buffer (10 mm, pH 10.4), and the absorbance was measured at 515 nm.

4. Cell cycle analysis

SK-HEP-1 cells were seeded at a density of 1×10^6 cells per 100 mm culture dish. After incubation for 24 hours, cells were treated with or without DHTS for 24 hours. The cells were harvested, washed twice with PBS and fixed with 70% cold ethanol overnight

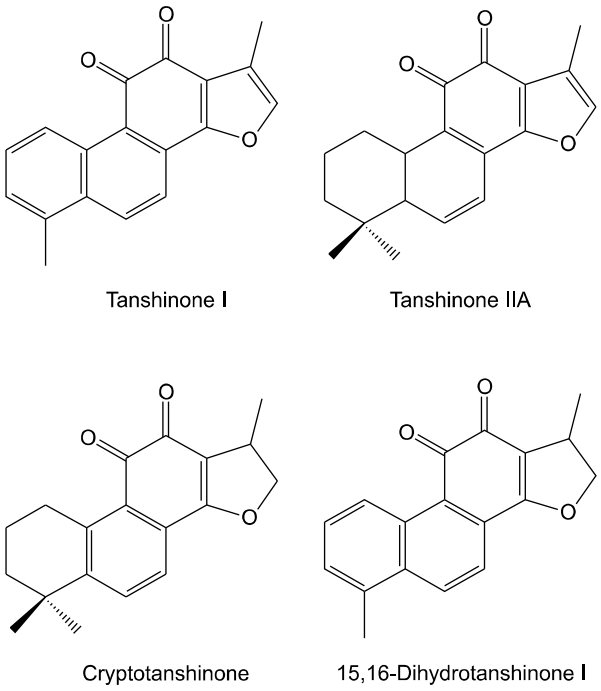

Figure 1. Chemical structures of tanshinones.

at −20°C. Fixed cells were pelleted, washed with ice-cold PBS and resuspended in staining solution containing 50 μg/mL RNase A and 50 μg/mL PI in PBS for 30 minutes at room temperature. The cellular DNA content was analyzed with a FACSCalibur flow cytometer (BD Biosciences). Approximately 10,000 cells were used for each analysis, and the results are displayed as histograms.

5. Western blot analysis

Cells were treated with various concentrations of DHTS for 24 hours. Western blot analysis was carried out as described previously.[15] The blots were imaged by LAS 4000 Imager (Fuji Film Corp., Tokyo, Japan).

6. Statistical analysis

Statistical significance ($P < 0.05$) was assessed using Student's *t*-test (SigmaStat 3.1; Systat Software Inc., San Jose, CA, USA).

Table 1. Anti-proliferative activity of tanshinones in various cancer cells, IC_{50} (μM)

Compound	Cell line				
	SK-HEP-1	MDA-MB-231	T47D	SNU-638	A549
Tanshinone I	1.6	6.1	3.7	3.2	6.7
Tanshinone IIA	5.1	14.5	0.9	0.6	2.8
Cryptotanshinone	4.3	5.5	3.6	3.0	2.5
15,16-Dihydrotanshinone I	1.3	1.8	1.5	1.6	4.5

RESULTS

1. Anti-proliferative effects of dihydrotanshinone I on human cancer cell lines

The anti-proliferative activities of various tanshinones (Fig. 1) isolated from the root of *S. miltiorrhiza* were evaluated in a panel of human cancer cell lines by the SRB assay. As shown in Table 1, all tested tanshinones exhibited potent anti-proliferative effects. DHTS exhibited potential anti-proliferative activity against most of tested cell lines, and the most active in SK-HEP-1 HCC cells. Tanshinone IIA showed the potent growth-inhibitory activity in T47D and SNU-638 cells. However, the anti-proliferative activity of Tanshinone IIA, one of the major constituents of the plant, and underlying mechanisms, have already been reported in cancer cells.[16] Therefore, a further study on the potential mechanisms of action of DHTS in the downregulation of cell proliferation was conducted using SK-HEP-1 cell line. As a result, DHTS exhibited the growth inhibition of cells in a concentration- and time-dependent manners with the IC_{50} values of 7.8, 2.8, and 1.3 μM for 24, 48, and 72 hours incubation, respectively (Fig. 2A). In addition, the morphological changes of cells induced by DHTS treatment for 24 hours were observed under the inverted phase-contrast microscope (Fig. 2B). The treatment of DHTS decreased the number of cells and caused such distinct morphological changes as cell shrinkage and rounded shapes.

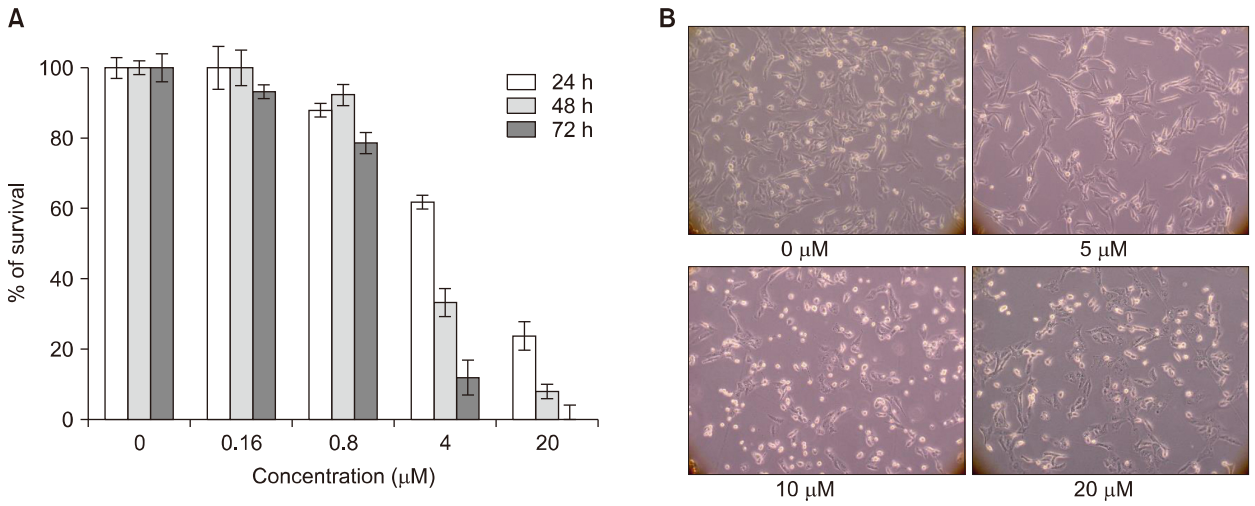

Figure 2. Effect of dihydrotanshinone I (DHTS) on the proliferation of human hepatocellular carcinoma SK-HEP-1 cells. (A) Cells were treated with or without various concentrations of DHTS for 24 to 72 hours. Anti-proliferative activity was determined using the sulforhodamine B assay. Data are represented as the mean ± standard deviation (n = 3). (B) Morphological changes caused by the DHA treatment for 24 hours were observed under the phase-contrast microscope (×100).

2. Effect of dihydrotanshinone I on cell cycle distribution

To verify the effects of DHTS on cell cycle progression, SK-HEP-1 cells were treated with DHTS for 24 hours, and flow cytometry analysis using PI staining was performed. As a result, treatment with DHTS markedly resulted in increases in the number of cells in the G0/G1 phase (Fig. 3). However, the sub-G1 peak, an indicator of cellular apoptosis, was not significantly elevated even at 20 μM DHTS. These finding suggest that DHTS inhibited the cell growth through the G0/G1 phase cell-cycle arrest without the induction of apoptotic cell death. Based on these data, protein expression involved in the G0/G1 cell cycle progression was determined by Western blot analysis. DHTS suppressed the expression of the G1 cell cycle checkpoint proteins, such as cyclin D1, CDK4 and CDK2, as well as, G1/S checkpoint biomarkers, such as cyclin A, cyclin E, cMyc, Rb in a concentration-dependent manner (Fig. 4). In addition, the induction of the cyclin-dependent kinase inhibitor p21 and downregulation of cMyc was also observed following DHTS treatment. These results suggest that DHTS induced G0/G1 phase cell cycle arrest by regulating the expression of cell cycle regulators in SK-HEP-1 cells.

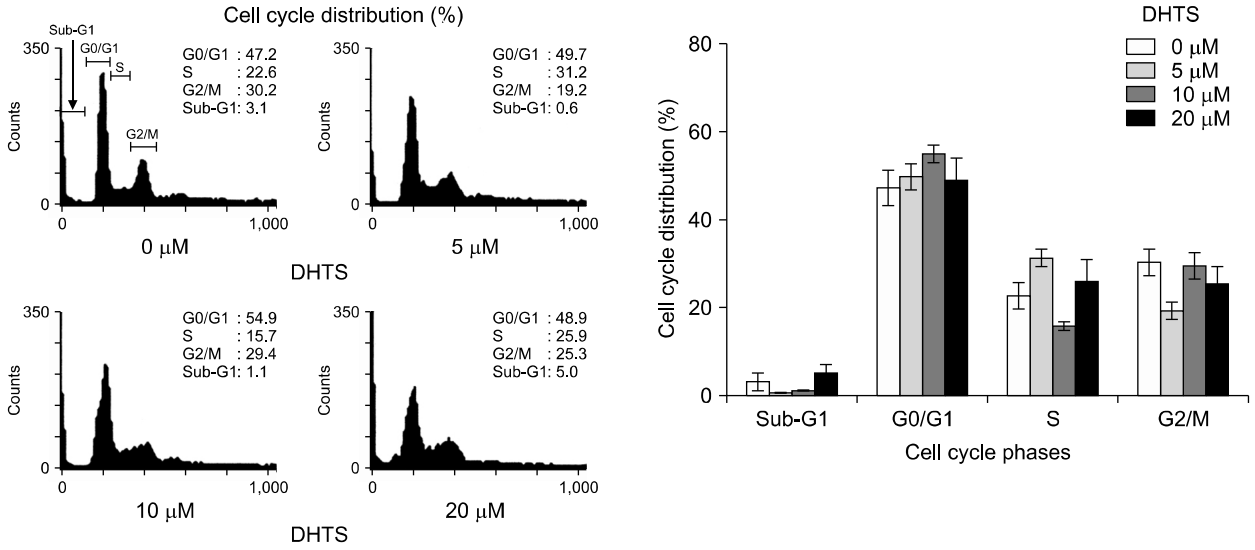

Figure 3. Effect of dihydrotanshinone I (DHTS) on the cell cycle progression in the SK-HEP-1 cells. Cells were treated with or without various concentrations of DHTS for 24 hours. The cell cycle distribution was analyzed by flow cytometry. The data are representative of three independent experiments.

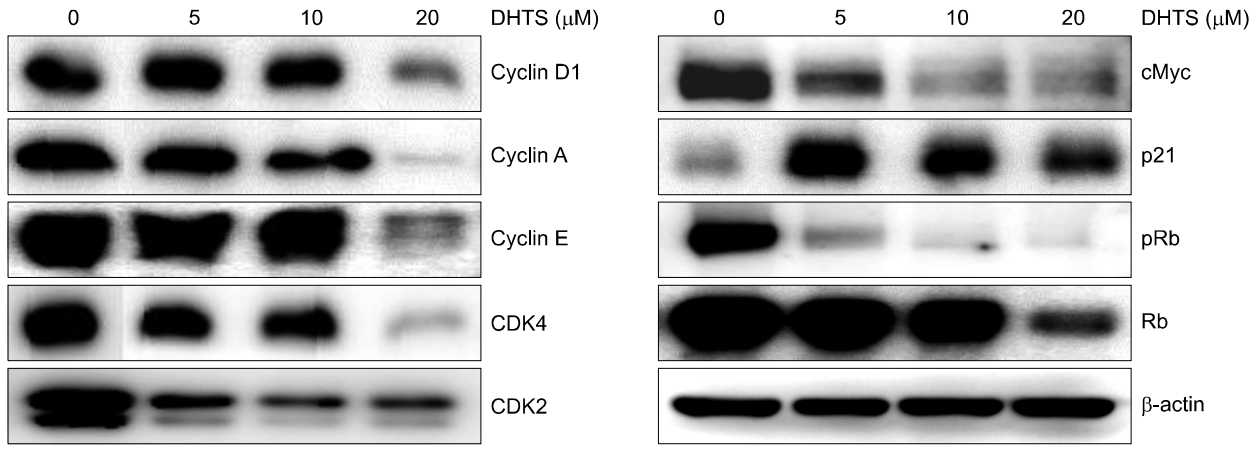

Figure 4. Effect of dihydrotanshinone I (DHTS) on the expression of cell cycle regulatory proteins in SK-HEP-1 cells. Cells were treated with or without various concentrations of DHTS for 24 hours. The expression level of proteins was analyzed by Western blot.

3. Effect of dihydrotanshinone I on the AMP-activated protein kinase/Akt/mTOR pathway

In order to elucidate the antiproliferative mechanism of DHTS, the regulation of the cell signaling pathway was further confirmed. The AMPK/Akt/mTOR pathway regulates cell survival, cell proliferation, and cell cycle regulation in HCC cells.[17] Western blot analysis showed that the levels of p-AMPK were increased in SK-HEP-1 cells treated with DHTS in a concentration-dependent manner (Fig. 5). Concomitantly, the protein expression levels of mTOR and p-mTOR (Ser2448) were reduced by DHTS. mTOR can bind to Raptor to form a mTORC1 complex, which controls phosphorylation of ribosomal p70S6 kinase (S6K1) and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), mTOR also binds to Rictor and forms mTORC2, which in turn modulates phosphorylation of Akt (Ser473).[18] Therefore, the effect of DHTS on the expression of downstream of mTOR was examined in SK-HEP-1 cells. DHTS suppressed the expression of p-p70S6K (Thr389) and p-4EBP1 (Thr37/46). DHTS also suppressed the phosphorylation of phosphoinositide-dependent kinase-1 (PDK1), upstream of Akt. However, DHTS did not increase the level of phosphorylation of acetyl-CoA carboxylase (ACC), a marker of AMPKα signaling (data not shown). These data suggest that anti-proliferative activity of DHTS is partly associated with the suppression of the activated AMPK/Akt/mTOR signaling pathway in hepatoma cells.

4. Effect of dihydrotanshinone I on mitogen-activated protein kinase

The MAPK signaling pathway is also considered to be involved in AMPK activation, and mTOR can be activated downstream of the MAPK signaling pathway.[19] To examine whether DHTS is able to affect the activation of the MAPK signaling pathway, cells were treated with DHTS and the expression of MAPK proteins was determined by Western blot analysis. As shown in Figure 6, DHTS

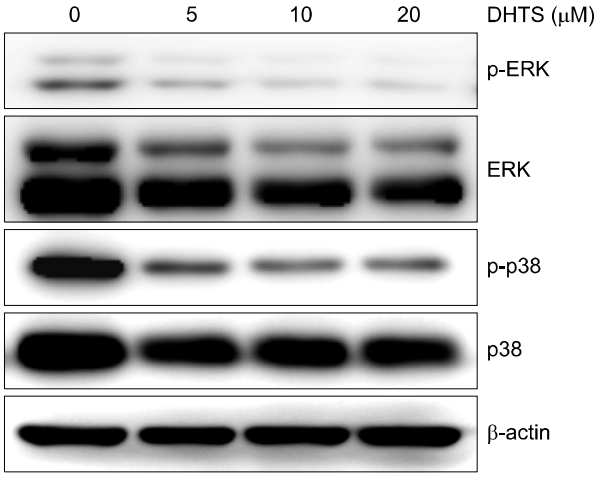

Figure 6. Effect of dihydrotanshinone I (DHTS) on the expression of ERK/p38 proteins in SK-HEP-1 cells. Cells were treated with or without various concentrations of DHTS for 24 hours. The expression levels of mitogen-activated protein kinase-related proteins were determined by Western blot analysis.

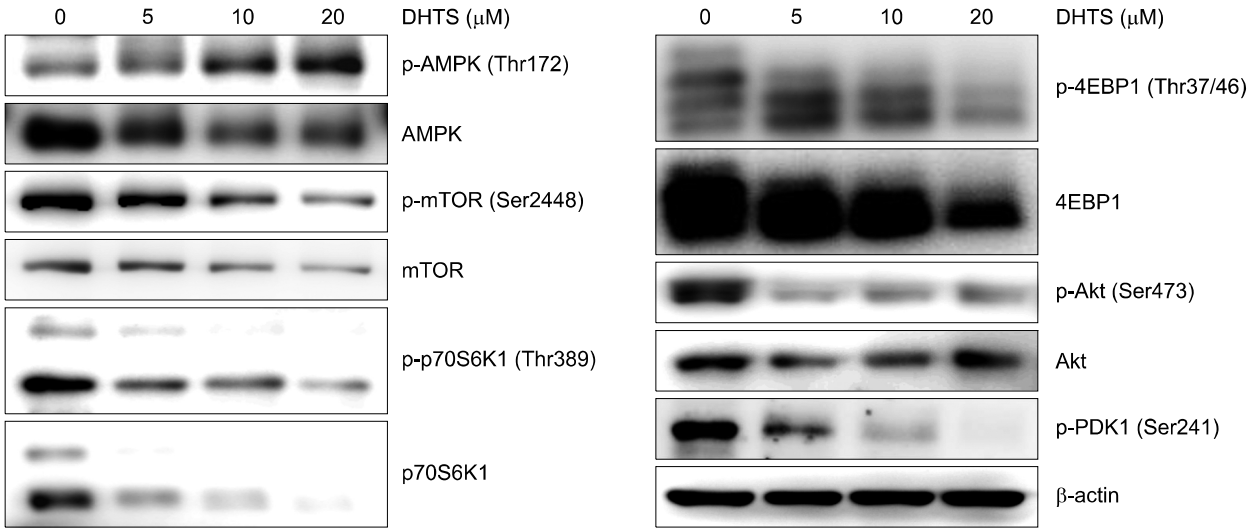

Figure 5. Effect of dihydrotanshinone I (DHTS) on the expression of AMP-activated protein kinase (AMPK)/Akt/mTOR signaling proteins in SK-HEP-1 cells. Cells were treated with or without various concentrations of DHTS for 24 hours. The expression levels of proteins were determined by Western blot analysis.

suppressed the expressions of the phosphorylation of ERK1/2 and p38 MAPK, but the expression of SAPK/JNK was not affected by DHTS (data not shown). These results indicate that DHTS partially inhibits the proliferation of SK-HEP-1 cells through the inhibition of MAPK signaling pathways.

DISCUSSION

DHTS, a constituent of *S. miltiorrhiza*, has been reported to exert several pharmacological effects including anti-inflammatory activity.[13] DHTS also showed inhibition of cell proliferation in various cancer cells such as colon, breast and liver cancers.[13,14,20] Previous studies reported that DHTS induces the apoptosis of HepG2 HCC cells through production of reactive oxygen species and activation of the p38 signaling pathway.[21] However, the precise mechanism of action in the anti-proliferative activity of DHTS against hepatoma cells is still unclear in association with the cell cycle regulation and cellular signal transduction pathways. Therefore, we investigated for the first time the underlying mechanism of DHTS in the anti-proliferative activity via induction of cell cycle arrest and the modulation of the AMPK/Akt/mTOR signaling pathway in human HCC SK-HEP-1 cells.

DHTS exhibits a potent growth-inhibitory activity against a panel of cancer cell lines (Table 1). The anti-proliferative activity of DHTS was in part associated with the induction of the G0/G1 cell cycle arrest. Interestingly, DHTS did not show significant induction of the apoptotic cell death at the test concentration of 20 μM for 24 hours in SK-HEP-1 cells. Previous studies reported that DHTS may induce apoptosis in several human cancer cell lines including 143B (osteosarcoma),[22] HL-60 (leukemia),[12] SW480 (colon),[13] DU145 (prostate),[23] MDA-MB-231 (breast),[14] and HCT-116 (colon) cells.[20] These results might be in part related to the different cell types or incubation times for the cells. A previous study also suggested that three HCC cell lines such as Huh-7, Hep3B, and SK-HEP-1 showed tumor necrosis factor-related apoptosis-inducing ligand resistance and bortezomib was able to overcome the resistance through the inhibition of the PI3K/Akt pathway.[8] In addition, You et al.[24] have reported that SK-HEP-1 cells have Fas-positive characteristic and more resistance to apoptosis induced by N-(4-hydroxyphenyl-retinamide (4HPR), a chemopreventive and chemotherapeutic agent, than Fas-negative (Hep3B and PLC/PRF/5) hepatoma cells. Therefore, the mechanisms for DHTS-mediated anti-proliferative activity may be different depending on cell types with different genetic backgrounds.

The expression of p-AMPK (Thr172) is downregulated in the majority of the patients with HCC, and the lower p-AMPK level is considered to be correlated with poor prognosis of HCC.[25] AMPK is one of the major signaling molecules activated by many phytochemicals.[26] The activation of AMPK suppresses the expression of cyclin D1, CDK4 and CDK6, thereby inhibiting cancer cell proliferation.[27] Tsai et al.[14] have reported that DHTS suppresses cyclin D1, cyclin E, and CDK4 expression and induces the G0/G1 phase cell cycle arrest in breast cancer cell lines. In this study, we also found that DHTS induced the G0/G1 cell cycle arrest through downregulation of cyclin D, cyclin A, cylcin E, CDK2, CDK4, cMyc, and p-Rb, and upregulation of p21 (Fig. 4). Therefore, the present findings, along with previous reports, suggest that the activation of AMPK by DHTS may induce the G0/G1 phase arrest via modulation of cell cycle checkpoint proteins, thereby mediating the anti-proliferation of SK-HEP-1 cells.

AMPK is regulated by activation of tumor suppressor STK11, also known as the liver kinase B1 (LKB1), a serine/threonine kinase. In addition, AMPK could also be regulated by other additional upstream kinases (i.e., calmodulin-dependent protein kinase kinase β [CAMKKβ], TGF-β-activated kinase-1 [TAK1]), therefore acting independently of LKB1.[28] In the present study, however, we found that the p-LKB1 expression was suppressed by DHTS, suggesting that DHTS activated AMPK in a LKB1-independent manner. Detailed mechanisms of how to activate the AMPK need to be further clarified.

In conclusion, the present study demonstrates the anti-proliferative activity of DHTS, an abietane diterpenoid from the root of *S. miltiorrhiza*, in human HCC cells. A plausible mechanism of action responsible for the anti-proliferative activity of DHTS was associated with the induction of the G0/G1 cell cycle arrest and modulation of AMPK/Akt/mTOR signaling pathways. Taken together, these findings suggest that DHTS might be a potential lead candidate for developing chemotherapeutic agents against HCC cells.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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