Dihydromyricetin activates AMP-activated protein kinase and P38\textsuperscript{MAPK} exerting anti-tumor potential in osteosarcoma

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Abstract

Numerous osteosarcoma patients either are not sensitive to chemotherapy or develop drug resistance to current chemotherapy regimens. Therefore, it is necessary to develop several potentially useful therapeutic agents. Dihydromyricetin (DHM) is the major flavonoid component derived from *Ampelopsis grossedentata*, which has a long history of use in food and medicine. The present study examined the anti-tumor activity both in vitro and in vivo without noticeable side effects and the underlying mechanism of action of DHM in osteosarcoma cells. We found that DHM induced increased p21 expression and G2/M cell cycle arrest, caused DNA damage, activated ATM/CHK2/H2AX signaling pathways, and induced apoptosis in osteosarcoma cells as well as decreasing the sphereformation capability by downregulating Sox2 expression. Mechanistic analysis showed that the anti-tumor potential of DHM may be due to the activation of AMPKα and p38MAPK, as the activating AMPKα led to the inactivation of GSK-3β in osteosarcoma cells. Moreover, GSK-3β deletion or GSK-3β inhibition by LiCl treatment resulted in increased p21 expression and reduced Sox2 expression in osteosarcoma cells. Taken together, our results strongly indicate that the ant-tumor potential of DHM is correlated with P38 MAPK and AMPKα/ GSK-3β/Sox2 signaling pathway. Finally, immunohistochemical analysis indicated some patients had a lower p-AMPK expression after chemotherapy which supports that the combination of DHM and chemotherapy drug will be beneficial for patients with osteosarcoma. In conclusion, our results are the first to suggest that DHM may be a therapeutic candidate for the treatment of osteosarcoma.

Key words: Dihydromyricetin, osteosarcoma, AMPK, P38MAPK, Sox2
Introduction

Osteosarcoma is the most common primary malignant bone tumors in childhood and adolescence(1). The clinical outcome of patients with osteosarcoma can be improved with chemotherapy, and the five-year survival rate has reached 60-70% (2). However, there is currently a need to identify effective agents for the treatment of this deadly disease and to develop new therapeutic strategies with less severe side effects, because numerous osteosarcoma patients are either not sensitive to chemotherapy or develop drug resistance with current chemotherapy regimens.

*Ampelopsis grossedentata*, a vine plant in South China, is a popular and multi-purpose traditional Chinese medicinal herb and has a long history being used as food and medicine (3). DHM, a 2,3-dihydroflavonol compound, is the main bioactive component extracted from *Ampelopsis grossedentata*, is one kind of flavonoids which has many biological effects, including anti-alcohol intoxication, reducing blood pressure, antibacterial, antioxidant, antitumor properties(4-6). Recently, it has been shown in some cancer cells that DHM possesses anti-tumor effects, such as anti-proliferation, cell cycle arrest, induction of apoptosis, and increased sensitivity to chemotherapeutic drugs (7, 8). Moreover, DHM has shown potential in ameliorating chemotherapy induced side effects(9). However, very little is known about its effects on osteosarcoma and the underlying mechanisms of DHM’s anticancer effects are still under investigation.

AMP-activated protein kinase (AMPK), a serine/threonine protein kinase and a member of the Snf1/AMPK protein kinase family, is a metabolic checkpoint protein downstream of the LKB1 tumor suppressor and integrates growth factor receptor signaling with cellular energy status.
AMPK is activated by metabolic stresses and xenobiotic compounds that cause a cellular energy imbalance (10). Evidence suggesting that AMPK can inhibit cell cycle progression in human hepatocellular carcinoma cells (11), and that AMPK activation requires the presence of LKB1 led us to hypothesize that AMPK activators might be useful in the prevention and/or treatment of cancer. It is possible that AMPK has many downstream targets whose phosphorylation mediates dramatic changes in cell metabolism, cell growth, and other functions.

5-Aminoimidazole-4-carboxamide riboside (AICAR) and Metformin, are pharmacologically active, potent AMPK activator and have become the focus of much research in carcinogenesis due to their regulation of various signaling pathways, such as the inhibition of mTOR signaling and blocking of the growth of glioblastoma cells that express the activated EGFR mutant, as well as their ability to control the levels of p53, p21, Cyclin D1, and caspases (12, 13). In addition, Metformin has been found to be an effective anti-tumor agent by induction DNA damage and apoptosis in osteosarcoma (14).

The p38MAPK and JNK protein kinases affect a variety of intracellular responses, such as inflammation, cell-cycle regulation, cell death, development, differentiation, senescence and tumorigenesis; as such, these kinases have been exploited for the development of therapeutics to treat a variety of different diseases, including cancer (15, 16). Constitutive activation of JNK or p38MAPK has been implicated in the induction of many forms of neuronal apoptosis in response to a variety of cellular injuries (17). Moreover, p38MAPK phosphorylation by anandamide treatment subsequently activated caspase-3 leading to apoptosis in osteosarcoma cells (18).

In this study, we have investigated the ant-tumor activity of DHM in osteosarcoma and examined its effects on cell cycle progression, the induction DNA damage and apoptosis, and
sphere formation. Furthermore, we have investigated the changes in AMPK/GSK-3β/Sox2 and p38MAPK cell signaling in osteosarcoma cells treated with DHM. This study is the first to demonstrate the effect of DHM on osteosarcoma cells and has identified the mechanism of its action, through activating AMPK and p38MAPK signaling pathways, which may help guide the clinical use of DHM.

Materials and Methods:

Chemicals and regants

DHM was prepared from Ampelopsis grossedentata using the chromatographic method. The variable levels for extracting DHM were 74% ethanol consistency, a temperature of 65°C with a heating time of 94 min, and a 1:35 ratio of Ampelopsis grossedentata to water. The purity of the DHM was shown to be higher than 98%, based on reversed-phase HPLC analysis. The compound was dissolved in DMSO. Additionally, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), adriamycin (ADM), AICAR, and Metformin were purchased from Sigma-Aldrich.

Cell Culture

The human osteosarcoma cell lines U2OS, MG63, Saos2, HOS, and 143B were obtained from the American Type Culture Collection (ATCC). U2OS/MTX cells, a methotrexate-resistant derivative of the U2OS human osteosarcoma cell line, were provided by Dr. M. Serra (Istituti Ortopedici Rizzoli, Bologna, Italy). The ZOS and ZOS-M cell lines have been described previously (19). The cells were cytogenetically tested and authenticated before being frozen. The cells were grown in DMEM supplemented with 10% fetal bovine serum (Invitrogen) at 37°C with 5% CO2. All cell-based experiments were performed on cells that were in culture for 4 weeks or less.
**Plasmids and Antibodies**

Constitutively active (CA) GSK-3β and SOX2/GFP plasmids were provided by Tiebang Kang. Antibodies against GSK-3β, phospho-Ser9-GSK-3β, Sox2, PARP, caspase-3, cleaved caspase-3, p38MAPK, phospho-p38MAPK (Thr180/Tyr182), AMPKα, phospho-AMPKα (Thr172), and the DNA Damage Antibody Sampler Kit were obtained from Cell Signaling Technology; the p65 and p21 antibodies were purchased from Santa Cruz Biotechnology.

**Cell cycle analyses**

Cells were treated with DHM for 48h and were subsequently collected and analyzed using a Cytomics FC 500 instrument (Beckman Coulter) equipped with CXP software after propidium iodide staining. The Modifit LT 3.1 trial cell cycle analysis software was used to determine the percentage of cells in the different phases of the cell cycle.

**Hoechst33258 staining**

After the cells were treated with or without DHM for 24h, the cells were washed twice with PBS, fixed with 4% paraformaldehyde for 20 min, and washed twice with ice-cold PBS. Then, the cells were stained with the DNA-specific dye Hoechst 33258 (10 mg/L), washed twice, and observed in random microscopic fields using a fluorescence microscope with the standard excitation filters (Leica Dmirb).

**Sphere formation assay**

Sphere formation assay was carried out as previously described (20). Briefly, 2000 cells were plated in triplicate in 6-well ultra-low attachment plates (Corning, Lowell, MA, USA) in DMEM/F12 (Invitrogen) supplemented with N2 medium (Invitrogen), 10 ng/mL human EGF (PeproTech, Rocky Hill, NJ), and 10 ng/mL human bFGF (PeproTech, Rocky Hill, NJ) and treated...
with or without DHM for approximately 2 weeks. Spheres were counted in each plate using an inverted phase contrast microscope.

**Caspase-3 Activity Assay**

To assess the cell viability after the indicated treatments, caspase-3 activity assays were performed according to the manufacturer’s instructions (Calbiochem, Billerica, MA). Caspase-3 activity was measured at 405 nm using a microtiter plate reader, as recommended in the manufacturer’s instructions.

**Immunofluorescence analysis**

U2OS cells were plated on culture slides (Costar, Cambridge, MA) and were treated with or without DHM. After 48h, the samples were rinsed with PBS and fixed using 4% paraformaldehyde for 15 min at room temperature. The slides were then washed with 0.1% NP40/PBS and extracted with buffer containing 0.5% Triton X-100 for 5 min. The cells were then blocked with 5% goat serum and incubated with primary antibodies overnight. After 3 washes with PBS, the samples were incubated with secondary antibody at room temperature for 1 h. Cells were then counterstained with Hoechst 33342 at room temperature for 5 min to visualize the nuclear DNA, and the slides were examined using an Olympus confocal imaging system (Olympus FV100).

**RNA extraction and quantitative real-time PCR**

Total cellular RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer’s instructions. RNA was reverse transcribed to produce cDNA using the Thermo Scientific Maxima First Strand cDNA Synthesis Kit (Thermo). Real-time PCR amplification was performed using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) on Hard-Shell PCR Plates (Bio-Rad).
Western blot analysis

The procedures have been described previously (21). Equal amounts of protein was resolved on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to a PVDF membrane (Millipore, USA). Membranes were blocked for one hour with 5% non-fat dry milk (BioRad) in TBST and incubated with primary antibody overnight at 4°C. Membranes were washed with TBST and incubated with horseradish peroxidase conjugated secondary antibody. Proteins were visualized using the enhanced chemiluminescence system (Pierce, USA).

Cell viability assay

The osteosarcoma cell lines were seeded in 96-well plates at a density of 3000 cells/well. The cells were treated with different concentrations of DHM. After the indicated incubation times at 37°C, 20 μL MTT (5 mg/mL) was added, and the plates were incubated for 4h at 37 °C. Then, the medium was removed, and 200μL DMSO was added and mixed thoroughly. The absorbance was subsequently measured using a Bio-Rad Microplate Reader (wavelength, 490 nm). All data are the average of 3 independent experiments.

Proteome profiler array

The proteome profiler array (R&D system, USA) was performed according to the manufacturer’s protocol. Briefly, the cell lysates were incubated with activated array membranes overnight at 4°C. Each array membrane was washed 3 times with 1X wash buffer and incubated with the diluted antibody cocktail for 2 h at room temperature. The array membranes were then washed 3 times with 1X wash buffer and further incubated with streptavidin-horseradish peroxidase for 30 min at room temperature. Each array membrane was then washed 3 times with 1X wash buffer, and finally, the membranes were exposed to X-ray film following chemiluminescent detection.

TUNEL staining assay

Apoptosis of the tumor tissues from the animal experiments was determined using a TUNEL
Assay kit (Roche Applied Science) as described previously (22). All samples were visualized using diaminobenzidine (DAB) (DAKO), and the nuclei were counterstained with hematoxylin.

**Animal experiments**

The animal study was approved by the Institutional Review Board of Sun Yat-Sen University. Athymic nude (nu/nu) mice, 5–6 weeks of age, were purchased from Shanghai Slac Laboratory Animal Company Limited. U2OS/MTX and ZOS cells (1×10^6 cells in 200 µL PBS) were subcutaneously injected near the scapula of the nude mice. After 9 days, the mice were randomly separated into the appropriate groups. The mice bearing the U2OS/MTX cells were separated into 3 groups. The first group, the control, was treated with the vehicle. The other two groups were treated with DHM (Dose1: 150 mg/kg or Dose2: 300 mg/kg) every day. After 7 days, the mice bearing the ZOS cells were separated into 4 groups. The first group, the control, was treated with the vehicle. Two groups of animals were treated with DHM (150 mg/kg, every day) or ADM (6 mg/kg, once per week), and the last group was treated with DHM (100 mg/kg, every day) in combination with ADM (6 mg/kg, once per week). All the groups received the drug through intraperitoneal injection. The resulting tumors were measured with a caliper every 2 days, and the tumor volume was calculated using the formula \( V = \frac{1}{2} \times \text{width}^2 \times \text{length} \). The weights of the mice were also recorded. At the end of the experiment, the animals were sacrificed using cervical dislocation, and the tumor weights were measured after careful resection.

For the orthotopic model of osteosarcoma, 143B cells was slowly injected following the procedure as described previously (23). 12 days after injection, the mice were randomly separated into treatment groups (n = 6). Mice were treated with the vehicle or DHM (Dose1: 150 mg/kg or Dose2: 300 mg/kg) by intraperitoneal injection every day. The length and width of the tumors (Di,
D2) were measured with a caliper every 3 days, and the tumor volume was calculated using the formula \( V = \frac{4}{3}\pi\left(\frac{1}{4}(D_1+D_2)^2\right)^3 \). Finally, the mice were anesthetized using chloral hydrate during X-ray scans.

**Immunohistochemical staining**

Immunohistochemical staining was performed, as described previously(20). The SOX2 primary antibody (rabbit monoclonal, clone D6D9; Cell Signaling Technology) was diluted 1:50 and the slides were incubated with anti-SOX2 antibody overnight at 4°C.

The clinical samples were from 24 patients with osteosarcoma, including biopsy sample (before standard neoadjuvant chemotherapy) and operation samples (after standard neoadjuvant chemotherapy). The p-AMPK primary antibody (rabbit monoclonal, Cell Signaling Technology) was diluted 1:100 and immunohistochemical staining was performed as described. Patient sample study was approved by the Institutional Review Board of Sun Yat-Sen University.

**Statistical analysis**

An unpaired Student’s t test or an analysis of variance with a Bonferroni post hoc test was used for statistical significance.

**Results**

**Effect of DHM on cell cycle progression of osteosarcoma cells**

DHM (molecular weight, 320.25Da), the major bioactive constituent of Rattan Tea, is an important type of flavonol, and its chemical structure is shown in Figure 1A. To test the effect of DHM on osteosarcoma cells, U2OS, MG63, ZOS, 143B, Saos2, HOS and U2OS/MTX were treated with different doses of DHM, and MTT assays were performed to determine the IC50
Moreover, the amount of cells that survived significantly decreased after treatment with DHM, as compared to the untreated cells (Figure 1C). We examined the effect of DHM on cell cycle progression in U2OS cells, which revealed that DHM could induce cell cycle G2/M arrest, and the percentage of cells in G2/M increased from 13.755% to 43.287% after DHM treatment (Figure 1D). P21 is a potent inhibitor of cell cycle progression, we assessed the level of P21 following DHM treatment and found that DHM increased the levels of p21 protein and RNA (Figure 1E and 1F). CDKN1A is regulated by many transcription factors, including p53, a well-known tumor suppressor, was not significantly increased by DHM exposure, suggesting that P21 increase was P53-independent mechanism (data not shown). These results indicate that DHM could induce G2/M arrest in human osteosarcoma cells.

**DHM induces DNA damage and apoptosis in osteosarcoma cells**

Our study has showed that DHM can induce a G2 arrest and increased P21 expression in a p53-independent manner in human osteosarcoma cells. To further investigate the molecular mechanisms behind this arrest, we examined the expression of P-ATM, P-CHK2, γH2AX in U2OS and ZOS cells; Previous studies have demonstrated that a G2 cell cycle arrest is frequently the result of DNA damage. Moreover, different molecular mechanisms involved in DNA damage checkpoints at different phases and ATM/CHK2/H2AX signaling pathways play important roles (24, 25). As shown in Figure 2A, the phosphorylation levels of ATM (Ser-1981), CHK2 (Thr68), and H2AX (Ser139) in U2OS and ZOS treated with 0 to 60 μM DHM significantly increased in a dose-dependent manner. These results indicate that DHM treatment induced not only a G2/M arrest in osteosarcoma cells but also DNA damage.

Cell cycle arrest allows cells to repair the damaged DNA to maintain genomic stability in
eukaryotic cells; a failure to repair the DNA can result in cell death or apoptosis (26). Therefore, we sought to determine whether treatment with DHM could induce apoptosis in osteosarcoma cells. As shown in Figure 2B, treatment with DHM resulted in reduction in cell viability as measured by MTT assay. Hoechst 33258 staining was used to detect morphological characteristics of apoptosis after U2OS, U2OS/MTX and ZOS cells were exposed to 0 to 60uM DHM for 24 hours. The number of apoptotic cells increased gradually in a dose-dependent manner, and the cells displayed a reduction of the cellular volume; brightly stained, condensed or fragmented nuclei; and the appearance of apoptotic bodies (Figure 2C). U2OS and ZOS cells were treated with DHM for 24 h, and immunoblot analysis was used to measure the cleavage of caspase-3 cleavage, a marker of apoptosis, which results in the cleavage of another protein, PARP (Figure 2D). In addition, caspase-3 activity assays were also performed using in DHM-treated U2OS and ZOS cells (Figure 2E). Taken together, these results indicate that DHM induces DNA damage and causes apoptosis in osteosarcoma cells.

**DHM exerts anti-tumor activity in vivo**

The study of DNA damage and apoptosis in tumor cells will not only help us to understand regulatory mechanisms but also provide a potential avenue for the development of tumor therapies. Thus, the *in vivo* anti-tumor ability of DHM was investigated by the nude mouse xenograft model using U2OS/MTX cells. The mice were randomly separated into 3 groups (Control, Dose 1, and Dose 2). The Dose 1 group received 150 mg/kg DHM every day, and the Dose 2 group received 300 mg/kg DHM every day. At the termination of the study, the mean volumes of the tumors were 1957 mm$^3$ for the control group, 1238 mm$^3$ for the Dose 1 group, and 834.6 mm$^3$ for the Dose 2
group (Figure 3A). The average tumor weights were 1.73 g for the control group, 1.15 g for the Dose 1 group, and 0.816 g for the Dose 2 group (Figure 3B). Additionally, the average body weight of the mice did not significantly differ between the two DHM treatment groups and the control group (Figure 3C), and no obvious side effects were observed in the important organs (heart, livers and kidney), as detected by HE staining (Supplemental Figure 1). To further evaluate the antitumor effect of DHM, we used an orthotopic model in which the 143B osteosarcoma cells were injected. As shown in Figure 3D, on day 33, the mean volume of the tumors in the control group was 2317 mm$^3$, whereas the mean volume was 1613 mm$^3$ and 1399 mm$^3$ for the Dose 1 and Dose 2 groups, respectively. X-ray tests and HE staining also showed that bone destruction and periosteal reactions around the tibia were more obvious in the control group (Supplemental Figure 2). Moreover, the levels of in vivo apoptosis following DHM treatment were analyzed using the TUNEL assay, and these results also confirmed the in vitro results that DHM could induce apoptosis in osteosarcoma cells (Figure 3E&3F). Our results demonstrate that DHM possesses anti-tumor properties and can induce apoptosis in human osteosarcoma cells in vivo.

**DHM decreases the osteosphere formation by downregulating Sox2 in human osteosarcoma cells**

Osteosphere culture was used to isolate and expand osteosarcoma stem cells in a serum-free suspension. Recent studies demonstrated that some inhibitors had the potential of targeting osteosarcoma stem cells, such as salinomycin (20). Thus, to test whether DHM could target osteosarcoma stem cells, we determined the ability of the control and DHM treated cells to form osteospheres. As shown in Figure 4A, U2OS, U2OS/MTX and 143B cells treated with DHM as indicated have reduced ability to form osteospheres. Sox2 is a transcription factor of the highly
mobility group (HMG) domain family that has a critical role in embryonic development and in maintaining pluripotency and self-renewal of osteosarcoma stem cells (20, 27). Therefore, we investigated whether DHM could affect the expression of Sox2. We found that Sox2 mRNA and protein were down-regulated in DHM-treated cells (Figure 4B&C). As expected, the expression of Sox2 in the nuclei was also reduced as detected by immunofluorescence (Figure 4D). To further confirm the role of Sox2 in the DHM-induced suppression of osteosphere formation, U2OS cells were transfected with a Sox2-expressing plasmid or an empty vector, and 48 h later, the cells were treated with or without DHM. As shown in Figure 4E&F, treatment with DHM led to a downregulation of endogenous and exogenous Sox2 expression, and overexpressed Sox2 enhanced the ability of osteospheres in U2OS cells, which was partly recovered, when combined with DHM. Additionally, we performed immunohistochemical (IHC) analysis for Sox2 on tumor samples, and the results showed that tumor treated with DHM had a decrease in the expression of Sox2 (Figure 4G). The above findings indicated that DHM reduced the ability of cells to form osteospheres due to the depletion of Sox2, which may be responsible for maintaining stem cell characteristics in osteosarcoma stem cells.

**Activation of AMPKα and p38MAPK may play a role in the anti-tumor potential of DHM**

To identify the upstream signaling kinases responsible for DHM induced cell cycle arrest, apoptosis, and reduction of Sox2 expression in osteosarcoma cells, we performed the proteome profiler antibody array. As shown in Figure 5A and supplemental Table1, the phosphorylation levels of multiple kinases were altered after treatment of DHM, including AMPKα, p38MAPK, GSK-3β, JNK pan, and MSK whose expression have increased 10–40 folds. Recently, many studies have
indicated that activation of AMPK is related to apoptosis in various cancer cell lines (28, 29). Metformin and AICAR treatments activate the AMPKα signaling pathway in various cancer cell types, including osteosarcoma cells, and these drugs have been shown to have anti-tumor properties (12, 30, 31). It has also been reported that the activation of the MAPK signaling pathway, which includes p38MAPK, JNK pan, and the downstream target MSK1/2, could induce cancer cell death (15, 32). In osteosarcoma cells, the P38 MAPK phosphorylation subsequently activated caspase-3 leading to apoptosis or regulated Eag channel functions (33, 34). Therefore, we further investigated the expression of the AMPKα and p38MAPK signaling pathways in U2OS and ZOS cells treated with the indicated doses of DHM. Our results were consistent with the proteome profiler antibody array analysis (Figure 5B). Because of the role of the AMPK and p38MAPK signaling pathway in the development of osteosarcomas, these results indicated that activation of AMPK and p38MAPK may play a role in the anti-tumor potential of DHM.

Our recent data have demonstrated that GSK-3β plays a key oncogenic role in osteosarcoma growth by regulating NF-κB signaling (23). In Figure 5A&B, we also showed that DHM could suppress the activity of GSK-3β. Therefore, we investigated whether the activation of AMPK was correlated with the inactivation of GSK-3β. Next, we used Metformin and AICAR, which activate AMPKα, to treat U2OS and MG63 cells, and we detected an increasing level of phosphorylated GSK-3β, which suggested that DHM could inactive GSK-3β through the activation of AMPKα (Figure 5C). Additionally, we demonstrated that DHM treatment resulted in the downregulation of Sox2 and reduced the formation of osteospheres. We investigated the levels of Sox2 and p21 expression after the depletion of GSK-3β or the inhibition of GSK-3β by LiCl treatment in U2OS and MG63 cells. We found that Sox2 expression was downregulated and that p21 expression
increased; these results were similar to the results obtained from DHM-treated cells (Figure 5D).

Taken together, these findings strongly suggest that the anti-tumor potential of DHM in osteosarcoma cells is correlated with the p38MAPK and AMPK/ GSK-3β/Sox2 signaling pathways.

**Combined effect of DHM with chemotherapeutic drugs on osteosarcoma cells**

The combined treatment of cisplatin, doxorubicin, and methotrexate was established as the standard treatment regimen for osteosarcoma 30 years ago; however, improving the survival of patients with osteosarcoma has proven to be an enormously difficult challenge (35). Recent research has demonstrated that the activation of AMPK contributes to doxorubicin-induced cancer cell death and apoptosis and that Metformin could sensitize cancer cells to the chemotherapeutic drugs, which also is significantly associated with increased survival among the patients (36, 37). Additionally, the expression of p-AMPK in 25 pairs of biopsy and operation osteosarcoma samples was evaluated by immunohistochemical staining. We compared the change of p-AMPK expression between biopsy and operation samples from the same patient. In this cohort, 16 (64%) cases had a higher expression after chemotherapy, 7 (28%) cases were lower, and 2 (8%) cases almost had no change (Figure 6A). Therefore, we hypothesize whether combining DHM with these chemotherapeutic drugs would have a synergistic effect in killing osteosarcoma cells. For the experiment, ZOS cells were injected into nude mice, and 7 days later, the mice were randomly divided into four groups and drugs were injected intraperitoneally. As single therapeutic modalities, DHM or ADM induced similar effects on tumor growth (the mean volume of tumors: 1381 mm³ VS 1075 mm³; the mean weight: 1.38g VS 1.24g), with a small advantage for ADM. Importantly, the combination of DHM with ADM worked additively to inhibit tumor growth from osteosarcoma cells in nude mice (Figure 6, B, C and D). Finally, we examined the body weight of
all the nude mice and found that DHM had a slight potential of reducing ADM-induced weight loss side effect (Figure 6E). These data suggest that the combination of DHM and ADM may be an attractive therapeutic option for osteosarcoma.

**Discussion**

Different chemotherapy regimens have been compared in osteosarcoma patients, but the survival rates never significantly improve (38). Additionally, chemotherapy resistance is a common problem that can significantly diminish clinical outcomes (39). Therefore, it is necessary to develop several potentially useful therapeutic agents for overcoming the challenge, particularly those from natural origins (40, 41).

Recent studies have shown that DHM has many biological effects, including anti-alcohol intoxication, reducing blood pressure, antibacterial, antioxidant, antitumor properties (4-6, 42, 43). This study demonstrated that DHM exhibits anti-tumor activity in osteosarcoma both *in vitro* and *in vivo*. We found that DHM treatment could inhibit the viability of human osteosarcoma cells, including the MTX resistant cell line U2OS/MTX. Cell cycle arrest is an essential early event in the inhibition of cell proliferation, and our result of cell cycle assay showed that DHM could induce a G2/M cell cycle arrest. The CDKI p21 regulates many cellular processes, such as cell cycle arrest, DNA replication and repair, cell proliferation and differentiation, senescence and apoptosis (44, 45). One potential consequence of DHM induced cell cycle arrest would be an increase in p21 levels in osteosarcoma cells. Indeed, p21 was increased in osteosarcoma cells, including U2OS, MG63, U2OS/MTX and ZOS cells. Meanwhile, the tumor suppressor P53 did not increase in response to DHM treatment, suggesting that increased P21 was p53 independent. The DNA damage checkpoints are biochemical pathways that delay or arrest cell cycle in response
to genomic DNA damage, and cell would activate ataxia-telangiectasia mutated (ATM), one of the sensor kinases. In turn, ATM phosphorylates multiple downstream substrates, including the effector kinase CHK2 and histone H2AX, which is the gold standard for early detection of DNA damage, resulting in cell cycle arrest and/or apoptosis (46). In this study, we found that DHM treatment not only caused significant DNA damage but also induced apoptosis of osteosarcoma cells in dose dependent, as detected by PRAP and Caspase3 activity. Further, we also reported that DHM had anti-tumor potential in nude mice, including orthotopic model, and the toxicity of combination was reduced compared with ADM treatment alone. This study is the first to show that DHM exhibits strong anti-tumor effects against human osteosarcoma cells both in vitro and in vivo without noticeable side effects.

Sox2, as a transcription factor, marked and maintained a distinct cell population in osteosarcomas that had stem cell properties and was responsible for their tumorigenic potential. A recent study reported overexpression of Sox2 in sphere-forming cells from human sarcomas and Sox2 downregulation decreased the stem cell population in murine and human osteosarcomas (27, 47). Our previous work also demonstrated that overexpressed Sox2 could increase the capability of sphereformation in osteosarcoma cells (20). In the present study, we demonstrated that DHM could decrease the capability of sphereformation in osteosarcoma cells. Furthermore, we investigated the expression of Sox2 in osteosarcoma cells treated with DHM, and found that Sox2 was downregulated through transcriptional level in dose-dependent. Exogenous transfection of Sox2 enhanced the ability of osteospheres in U2OS cells, and when combined with DHM, the enhanced osteosphere was partly recovered. Overall, these results suggest that Sox2 is a key player in osteosphere and DHM could suppress osteosphere through downregulation of Sox2 and
increase the stem cell population in human osteosarcomas.

To determine the potential molecular mechanism of action of DHM in osteosarcoma, we used a proteome profiler array and found that the phosphorylation levels of multiple kinases were affected by DHM treatment. Specifically, we observed the DHM-mediated activation of AMPK and p38MAPK in osteosarcoma cells. Activation of AMPK usually occurs under conditions of metabolic stress or when the ATP:AMP ratio decreases (48). Activation of AMPK in response to metabolic stress results in the silencing of intracellular energy-consuming anabolic processes and activates energy-producing catabolic processes (10). The energy status of the cell is a crucial factor in all aspects of cell function; it is possible that AMPK has many downstream targets whose phosphorylation mediates dramatic changes in cell metabolism, cell growth, and other functions.

The potent AMPK activator, Metformin, has been demonstrated as an effective anti-tumor agent through induction DNA damage and apoptosis in osteosarcoma (14). GSK-3β, a serine/threonine protein kinase, also plays key roles in multiple pathways. While GSK-3β is generally recognized as a tumor suppressor that is frequently inactivated in a variety of tumors, we demonstrated that GSK-3β activity may promote osteosarcoma tumor growth and induce apoptosis in osteosarcoma cells (23). In this study, we demonstrated that cells treated with AMPKα activators resulted in the increased phosphorylation of GSK-3β, suggesting that DHM treatment could inactive GSK-3β through the activation of AMPKα. We further investigated the expression of Sox2 and p21 after GSK-3β depletion or GSK-3β inhibition by LiCl treatment in osteosarcoma cells and found that Sox2 expression was downregulated and that p21 was upregulated; these results were consistent with the results of the DHM treatment. Based on our results, we suggest that DHM possesses anti-tumor activity due to its ability to affect the AMPK/GSK-3β/Sox2 signaling pathway. Another
important observation was that DHM induces the activation of p38MAPK and the JNK protein kinases. The MAPK signaling pathway has been exploited in cancer treatment because of its key roles in inflammation, cell-cycle regulation, cell death, development, differentiation, senescence, and tumorigenesis (15, 16). A recent study also demonstrated that anandamide activated caspase-3 through an increase in p38MAPK phosphorylation in osteosarcoma cells (18). However, the further studies are needed to clearly understand how DHM induces AMPK activation, which can be caused by generating more reactive oxygen species (ROS) or other metabolic stress.

In conclusion, we have demonstrated that DHM possess strong anti-tumor effects against human osteosarcoma cells without noticeable side effects, and that DHM can reduce the toxicity of the chemotherapeutic ADM when the two agents are used in combination. Molecular study revealed that DHM activated AMPK and p38MAPK in osteosarcoma cells and induced strong apoptotic response. Moreover, DHM also decreases the population of stem cells and sphereformation capability in osteosarcoma through downregulation of Sox2. Considering the current clinical treatment outcome, these results suggest that DHM may be a promising agent for the treatment of osteosarcoma.

Reference


**Figure 1.** Effect of DHM on cell cycle progression of osteosarcoma cells

A. Chemical structure of DHM. B. Cells were seeded in 96 well plates and after 24 hours were treated with a range of concentrations of DHM for 24 hours. The viable cells were measured by the MTT assay and the IC50 were calculated. C. Cells were seeded in 6 well plates and were subsequently treated with DMSO or DHM as indicated. The panels show colony assays stained with crystal violet 4 days later. D. Representative cell cycle analysis by flow cytometry of U2OS cells treated with DMSO or DHM as indicated for 24 hours and the length of each cell-cycle phase was calculated. E. The graph shows levels of P21 mRNA relative to GAPDH determined by quantitative RT-PCR. The indicated cell lines were incubated with DMSO or DHM for 24 hours before RNA was prepared. F. Western blot analysis for P21 in the indicated cell lines incubated
with DMSO or DHM for 24 hours before protein lysates were prepared.

**Figure 2.** DHM induces DNA damage and apoptosis in osteosarcoma cells.

A. Western blot analysis of expression of P-ATM, P-CHK2, γH2AX in U2OS and ZOS cells. B. Inhibitory effect of DHM on viability of U2OS and ZOS cells. C. Hoechst staining showed typical apoptotic morphology changes after DHM treatment in U2OS, U2OS/MTX and ZOS cells. D. Western blot for apoptosis related proteins PARP and cleaved caspase3 in DHM treated U2OS and ZOS cells. E. caspase-3 activity assays were performed in U2OS and ZOS cells after treatment with DHM.

**Figure 3.** DHM exerts anti-tumor potential in vivo.

A and B, Examination of tumor volumes and weight to evaluate the effect of DHM on U2OS/MTX cells in a xenograft model. The mice bearing U2OS/MTX cells were treated as described in "Materials and Methods". The tumor volumes were monitored as indicated, and the xenografts were excised and weighed on the day 23. C. Effect of DHM treatments on mouse body weights in A were measured every 2 days as indicated. D. Use of DHM to treat 143B cells in an orthotopic mouse model. The mice bearing 143B cells were treated as described in "Materials and Methods". The tumor volumes were monitored every 3 days, as indicated. E and F. Tunel staining analysis of DHM-treated tumors (U2OS/MTX and 143B). (*, P<0.05; **, P<0.01; ***, P<0.001.)

**Figure 4.** DHM decreases the sphereformation through downregulation Sox2 in human osteosarcoma.

A, Osteosphere assay of osteosarcoma cells treated with DHM. Results from a representative experiment are shown. Similar results were obtained with the U2OS, U2OS/MTX and 143B cells.
B and C, Sox2 expression were analyzed by real-time PCR and western blot in U2OS, U2OS/MTX and 143B cells treated with DHM. D, Subcellular localization of Sox2 in the U2OS treated with DHM. E, PcDNA3.1 and SOX2/GFP plasmid were transfected into U2OS treated with or without DHM, and Sox2 expression were detected by western blot. F, Osteosphere assay of U2OS as in E. was performed and spheres were counted after 2 weeks. G. Sox2 expression on animal samples was detected by IHC.

Figure5. DHM regulates activity of AMPKα and p38MAPK in osteosarcoma cells.

A, Cell lysates of control and DHM treated U2OS cells were applied to the proteome profiler antibody array analysis showing the phosphorylation of 45 kinases. The antibody array was composed of duplicate spots for each kinase on the single membrane. B, Cell lysates of control and DHM treated U2OS and ZOS cells were analyzed by Western blot with various antibodies, as indicated. C, Effects of Metformin and AICAR on the phosphorylation of GSK-3β in U2OS and MG63 cells were confirmed by Western blot. D, The levels of Sox2 and p21 expression after the depletion of GSK-3β or the inhibition of GSK-3β by LiCl treatment in U2OS and MG63 cells were assayed by Western blot.

Figure6. Combined effect of DHM with chemotherapeutic drugs on osteosarcoma cells.

A, p-AMPK staining was observed in osteosarcoma specimens between biopsy and operation samples B, Effect of DHM and/or ADM combination treatments on osteosarcoma xenografts in nude mice. ZOS cells were injected subcutaneously near the scapula of the nude mice. The mice bearing ZOS cells were treated as described in "Materials and Methods". The tumor volumes were
monitored every 2 days, as indicated. C, Xenografts excised from the tumor bearing mice in A at day 27. D, Weights of the xenografts from B at day 27. E, The body weight of tumor bearing mice in B at day 27. (**, P<0.01; ***, P<0.001.)
**Figure 1**

(A) Chemical structure

(B) Graph showing IC50 values for different cell lines.

(C) Images of cell cultures treated with different concentrations of DHM.

(D) Flow cytometry histograms showing cell cycle distribution for different concentrations of DHM.

(E) Expression levels of P21 mRNA in U2OS and ZOS cells treated with different concentrations of DHM.

(F) Western blot analysis showing expression levels of P21 and GAPDH in U2OS, MG63, and ZOS cells treated with DHM.
Figure 3
Figure 4
Figure 5
Figure 6

A. Biopsy images of Case 1, Case 2, and Case 3.

B. Graph showing tumor volume over time for ZOS Xenograft experiments with Ctrl, DHM, ADM, and Combination treatments.

C. Image showing samples of Ctrl, DHM, ADM, and Combination treatments.

D. Scatter plot showing tumor weight with significance levels.

E. Bar graph showing body weight with significance levels.
Dihydromyricetin activates AMP-activated protein kinase and P38MAPK exerting anti-tumor potential in osteosarcoma

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