Dihydromyricetin Activates AMP-Activated Protein Kinase and P38MAPK Exerting Antitumor Potential in Osteosarcoma

Zhiqiang Zhao1, Jun-qiang Yin1, Man-si Wu2, Guohui Song1, Xian-biao Xie1, Changye Zou1, Qinglian Tang1, Yuanzhong Wu2, Jinchang Lu1, Yongqian Wang1, Jin Wang1, Tiebang Kang2, Qiang Jia3, and Jingnan Shen1

Abstract
Numerous patients with osteosarcoma 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 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 antitumor activity both in vitro and in vivo without noticeable side effects and the underlying mechanism of action of dihydromyricetin in osteosarcoma cells. We found that dihydromyricetin 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 sphere formation capability by downregulating Sox2 expression. Mechanistic analysis showed that the antitumor potential of dihydromyricetin may be due to the activation of AMPKα and p38MAPK, as the activating AMPKα led to the inactivation of GSK3β in osteosarcoma cells. Moreover, GSK3β deletion or GSK3β inhibition by LiCl treatment resulted in increased p21 expression and reduced Sox2 expression in osteosarcoma cells. Taken together, our results strongly indicate that the antitumor potential of dihydromyricetin is correlated with P38MAPK and the AMPKα–GSK3β–Sox2 signaling pathway. Finally, immunohistochemical analysis indicated that some patients had a lower p-AMPK expression after chemotherapy, which supports that the combination of dihydromyricetin and chemotherapy drug will be beneficial for patients with osteosarcoma. In conclusion, our results are the first to suggest that dihydromyricetin may be a therapeutic candidate for the treatment of osteosarcoma.

Cancer Prev Res; 1–12. ©2014 AACR.

Introduction
Osteosarcoma is the most common primary malignant bone tumor in childhood and adolescence (1). The clinical outcome of patients with osteosarcoma can be improved with chemotherapy, and the 5-year survival rate has reached 60% to 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 patients with osteosarcoma 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 multipurpose traditional Chinese medicinal herb and has a long history of being used as food and medicine (3). Dihydromyricetin, a 2,3-dihydroflavonol compound, is the main bioactive component extracted from Ampelopsis grossedentata, is one kind of flavonoids that has many biologic effects, including antialcohol intoxication, reducing blood pressure, antibacterial, antioxidant, and antitumor properties (4–6). Recently, it has been shown in some cancer cells that dihydromyricetin possesses antitumor effects, such as antiproliferation, cell-cycle arrest, induction of apoptosis, and increased sensitivity to chemotherapeutic drugs (7, 8). Moreover, dihydromyricetin 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 dihydromyricetin’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-Aminomidazole-4-carboxamide riboside (AICAR) and metformin are pharmacologically active, potent AMPK activators 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 antitumor agent due to induction of DNA damage and apoptosis in osteosarcoma (14).

The p38MAPK and INK 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 INK 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 antitumor activity of dihydromyricetin in osteosarcoma and examined its effects on cell-cycle progression, the induction of DNA damage and apoptosis, and sphere formation. Furthermore, we have investigated the changes in AMPK/GSK3β/Sox2 and p38MAPK cell signaling in osteosarcoma cells treated with dihydromyricetin. This study is the first to demonstrate the effect of dihydromyricetin 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 dihydromyricetin.

Materials and Methods

Chemicals and reagents

Dihydromyricetin was prepared from Ampelopsis grossedentata using the chromatographic method. The variable levels for extracting dihydromyricetin were 74% ethanol consistency, a temperature of 65°C with a heating time of 94 minutes, and a 1:35 ratio of Ampelopsis grossedentata to water. The purity of the dihydromyricetin was shown to be higher than 98%, based on reversed-phase HPLC analysis. The compound was dissolved in DMSO. In addition, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Adriamycin, 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 GSK3β and SOX2/GFP plasmids were provided by Tiebang Kang (State Key Laboratory of Oncology in South China, Sun YatSen University Cancer Center, Guangzhou, China, 510060). Antibodies against GSK3β, phospho-Ser9-GSK3β, 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 dihydromyricetin for 48 hours and were subsequently collected and analyzed using a Cytomics FC 500 instrument (Beckman Coulter) equipped with CXP software after propidium iodide staining. The ModFit LT 3.1 Trial cell-cycle analysis software was used to determine the percentage of cells in the different phases of the cell cycle.

Hoechst 33258 staining

After the cells were treated with or without dihydromyricetin for 24 hours, the cells were washed twice with PBS, fixed with 4% paraformaldehyde for 20 minutes, 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, 2,000 cells were plated in triplicate in 6-well ultra-low attachment plates (Corning) in DMEM/F12 (Invitrogen) supplemented with N2 medium (Invitrogen), 10 ng/ml human EGF (PeproTech), and 10 ng/ml human bFGF (PeproTech) and treated with or without dihydromyricetin 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). 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) and were treated with or without dihydromyricetin. After 48 hours, the samples were rinsed with PBS and fixed using 4% paraformaldehyde for 15 minutes 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 minutes. The cells were then blocked with 5% goat serum and incubated with primary antibodies overnight. After three washes with PBS, the samples were incubated with secondary antibody at room temperature for 1 hour. Cells were then counterstained with Hoechst 33342 at room temperature for 5 minutes to visualize the nuclear DNA, and the slides were examined using an Olympus confocal imaging system (Olympus FV100).

**Western blot analysis**

The procedures have been described previously (21). Equal amounts of protein were resolved on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Membranes were blocked for 1 hour with 5% non-fat dry milk (Bio-Rad) in Trisbuffered saline with Tween20 (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).

**Cell viability assay**

The osteosarcoma cell lines were seeded in 96-well plates at a density of 3,000 cells per well. The cells were treated with different concentrations of dihydromyricetin. After the indicated incubation times at 37°C, 20 μL of MTT (5 mg/mL) was added, and the plates were incubated for 4 hours at 37°C. Then, the medium was removed and 200 μL of 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 three independent experiments.

**Proteome profiler array**

The proteome profiler array (R&D Systems) 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 three times with 1× wash buffer and incubated with the diluted antibody cocktail for 2 hours at room temperature. The array membranes were then washed three times with 1× wash buffer and further incubated with streptavidin–horseradish peroxidase for 30 minutes at room temperature. Each array membrane was then washed three times with 1× 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 the Sun Yat-Sen University. Athymic nude (nu/nu) mice, 5 to 6 weeks of age, were purchased from Shanghai Slac Laboratory Animal Company Limited. U2OS/MTX and ZOS cells (1×10⁶ cells in 200 μL of 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 three groups. The first group, the control, was treated with the vehicle. The other two groups were treated with dihydromyricetin (dose1, 150 mg/kg; or dose2, 300 mg/kg) every day. After 7 days, the mice bearing the ZOS cells were separated into four groups. The first group, the control, was treated with the vehicle. Two groups of animals were treated with dihydromyricetin (150 mg/kg, every day) or adriamycin (6 mg/kg, once per week), and the last group was treated with dihydromyricetin (100 mg/kg, every day) in combination with adriamycin (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 $V = \frac{1}{2} (width^2 \times length)$. 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 were slowly injected following the procedure as described previously (23). Twelve days after injection, the mice were randomly separated into treatment groups ($n = 6$). Mice were treated with the vehicle or dihydromyricetin (dose1,150 mg/kg; or dose2, 300 mg/kg) by intraperitoneal injection every day. The length and width of the tumors ($D_1$ and $D_2$) were measured with a caliper every 3 days, and the tumor volume was calculated using the formula $V = 4/3\pi[D_1^2 + D_2^2]$. Finally, the mice were anesthetized using chloral hydrate during X-ray scans.

**Immunohistochemical staining**

Immunohistochemical (IHC) staining was performed as described previously (20). The SOX2 primary antibody
(rabbit monoclonal, clone D6D9; Cell Signaling Technology) was diluted at the ratio of 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 at the ratio of 1:100 and IHC staining was performed as described. Patient sample study was approved by the Institutional Review Board of the Sun Yat-Sen University.

Statistical analysis
An unpaired Student t test or an analysis of variance with a Bonferroni post hoc test was used for statistical significance.

Results
Effect of dihydromyricetin on cell-cycle progression of osteosarcoma cells
Dihydromyricetin (molecular weight, 320.25 Da), the major bioactive constituent of Rattan Tea, is an important type of flavonol, and its chemical structure is shown in Fig. 1A. To test the effect of dihydromyricetin on osteosarcoma cells, U2OS, MG63, ZOS, 143B, Saos2, HOS, and U2OS/MTX were treated with different doses of dihydromyricetin, and MTT assays were performed to determine the IC50 (Fig. 1B). Moreover, the amount of cells that survived significantly decreased after treatment with dihydromyricetin, as compared with the untreated cells (Fig. 1C). We examined the effect of dihydromyricetin on cell-cycle progression in U2OS cells, which revealed that dihydromyricetin could induce cell-cycle G2–M arrest, and the percentage of cells in G2–M increased from 13.755% to 43.287% after dihydromyricetin treatment (Fig. 1D). P21 is a potent inhibitor of cell-cycle progression; we assessed the level of P21 following dihydromyricetin treatment and found that dihydromyricetin increased the levels of p21 protein and RNA (Fig. 1E and 1F). CDKN1A is regulated by many transcription factors, including p53, a well-known tumor suppressor, and was not significantly increased by dihydromyricetin treatment (24). CDKN1A is involved in DNA damage checkpoints at different phases and ATM–CHK2–H2AX signaling pathways play important roles (24, 25). As shown in Fig. 2A, the phosphorylation levels of ataxia telangiectasia mutated (ATM; Ser1981), CHK2 (Thr68), and H2AX (Ser139) in U2OS and ZOS treated with 0 to 60 μmol/L dihydromyricetin significantly increased in a dose-dependent manner. These results indicate that dihydromyricetin 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 dihydromyricetin could induce apoptosis in osteosarcoma cells. As shown in Fig. 2B, treatment with dihydromyricetin resulted in a reduction in cell viability as measured by the MTT assay. Hoechst 33258 staining was used to detect morphologic characteristics of apoptosis after U2OS, U2OS/MTX, and ZOS cells were exposed to 0 to 60 μmol/L dihydromyricetin 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 (Fig. 2C). U2OS and ZOS cells were treated with dihydromyricetin for 24 hours, 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 (Fig. 2D). In addition, caspase-3 activity assays were also performed using in dihydromyricetin-treated U2OS and ZOS cells (Fig. 2E). Taken together, these results indicate that dihydromyricetin induces DNA damage and causes apoptosis in osteosarcoma cells.

Dihydromyricetin exerts antitumor 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 antitumor ability of dihydromyricetin was investigated by the nude mouse xenograft model using U2OS/MTX cells. The mice were randomly separated into three groups (Control, Dose 1, and Dose 2). The Dose 1 group received 150 mg/kg of dihydromyricetin every day, and the Dose 2 group received 300 mg/kg of dihydromyricetin every day. At the termination of the study, the mean volumes of the tumors were 1,957 mm3 for the control group, 1,238 mm3 for the Dose 1 group, and 834.6 mm3 for the Dose 2 group (Fig. 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 (Fig. 3B). In addition, the average body weight of the mice did not significantly differ between the two dihydromyricetin treatment groups and the control group (Fig. 3C). And no obvious side effects were observed in the important organs (heart, liver, and kidney), as detected by hematoxylin and eosin (H&E) staining (Supplementary Fig. S1). To
further evaluate the antitumor effect of dihydromyricetin, we used an orthotopic model in which the 143B osteosarcoma cells were injected. As shown in Fig. 3D, on day 33, the mean volume of the tumors in the control group was 2,317 mm³, whereas the mean volumes were 1,613 mm³ and 1,399 mm³ for the Dose 1 and Dose 2 groups, respectively. X-ray tests and H&E staining also showed that bone destruction and periosteal reactions around the tibia were more obvious in the control group (Supplementary Fig.S2). Moreover, the levels of in vivo apoptosis following dihydromyricetin treatment were analyzed using the TUNEL assay, and these results also confirmed the in vitro results that dihydromyricetin could induce apoptosis in osteosarcoma cells (Fig. 3E and 3F). Our results demonstrate that dihydromyricetin possesses anti-tumor properties and can induce apoptosis in human osteosarcoma cells in vivo.

Dihydromyricetin 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 dihydromyricetin could target osteosarcoma stem cells, we determined the ability of the control and dihydromyricetin-treated cells to form osteospheres. As shown in Fig. 4A, U2OS, U2OS/MTX, and 143B cells treated with dihydromyricetin as indicated have reduced ability to form osteospheres. Sox2 is a transcription factor of the high-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 dihydromyricetin could affect the expression of Sox2. We found that Sox2 mRNA and protein were downregulated in dihydromyricetin-treated cells (Fig. 4B and C). As expected, the expression of Sox2 in the nuclei was also reduced as detected by immunofluorescence (Fig. 4D). To further confirm the role of Sox2 in the dihydromyricetin-induced suppression of osteosphere formation, U2OS cells were transfected with a Sox2-expressing plasmid or an empty vector, and 48 hours later, the cells were treated with or without dihydromyricetin. As shown in Fig. 4E and F, treatment with dihydromyricetin 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 dihydromyricetin. In addition, we performed IHC analysis for Sox2 on tumor samples, and the results showed that the tumor treated with dihydromyricetin had a decrease in the expression of Sox2 (Fig. 4G). The above findings indicated that dihydromyricetin 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 antitumor potential of dihydromyricetin

To identify the upstream signaling kinases responsible for dihydromyricetin-induced cell-cycle arrest, apoptosis, and reduction of Sox2 expression in osteosarcoma cells, we performed the proteome profiler antibody array. As shown in Fig. 5A and Supplementary Table S1, the phosphorylation levels of multiple kinases were altered after treatment of dihydromyricetin, including AMPKα, p38MAPK, GSK3β, JNK pan, and MSK whose expression have increased 10 to 40 fold. 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 antitumor 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.
In osteosarcoma cells, the P38MAPK phosphorylation subsequently activated caspase-3, leading to apoptosis or regulated Eag channel functions (33, 34). Therefore, we further investigated the expression of the AMPKa and p38MAPK signaling pathways in U2OS and ZOS cells treated with the indicated doses of dihydromyricetin. Our results were consistent with the proteome profiler antibody array analysis (Fig. 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 antitumor potential of dihydromyricetin.

Our recent data have demonstrated that GSK3β plays a key oncogenic role in osteosarcoma growth by regulating NF-kB signaling (23). In Fig. 5A and B, we also showed that dihydromyricetin could suppress the activity of GSK3β. Therefore, we investigated whether the activation of AMPK was correlated with the inactivation of GSK3β. Next, we used metformin and AICAR, which activate AMPKa, to treat U2OS and MG63 cells, and we detected an increasing level of phosphorylated GSK3β, which suggested that dihydromyricetin could inactivate GSK3β through the activation of AMPKa (Fig. 5C). In addition, we demonstrated that dihydromyricetin 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 GSK3β or the inhibition of GSK3β 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 dihydromyricetin-treated cells (Fig. 5D). Taken together, these findings strongly suggest that the antitumor potential of dihydromyricetin in osteosarcoma cells is correlated with the p38MAPK and AMPK–GSK3β–Sox2 signaling pathways.

Combined effect of dihydromyricetin 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 proved 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). In addition, the expression of p-AMPK in 25 pairs of biopsy and operation osteosarcoma samples was evaluated by IHC staining. We compared the change of p-AMPK expression between biopsy and operation samples from the same
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 (Fig. 6A). Therefore, we hypothesize whether combining dihydromyricetin 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, dihydromyricetin or adriamycin induced similar effects on tumor growth (mean volume of tumors, 1,381 vs. 1,075 mm³; mean weight, 1.38 vs. 1.24 g), with a small advantage for adriamycin. Importantly, the combination of dihydromyricetin with adriamycin worked additively to inhibit tumor growth from osteosarcoma cells in nude mice (Fig. 6B, C, and D). Finally, we examined the body weight of all the nude mice and found that dihydromyricetin had a slight potential of reducing adriamycin-induced weight loss side effect (Fig. 6E). These data suggest that the combination of dihydromyricetin and adriamycin may be an attractive therapeutic option for osteosarcoma.

**Discussion**

Different chemotherapy regimens have been compared in patients with osteosarcoma, but the survival rates never significantly improve (38). In addition, 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 dihydromyricetin has many biologic effects, including antialcohol intoxication, reducing blood pressure, antibacterial, antioxidant, and antitumor properties (4–6, 42, 43). This study demonstrated that dihydromyricetin exhibits antitumor activity in osteosarcoma both *in vitro* and *in vivo*. We found that dihydromyricetin 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 dihydromyricetin could induce a G2-M cell-cycle arrest. The CDK1 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 dihydromyricetin-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 dihydromyricetin 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 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 dihydromyricetin treatment not only caused significant DNA damage but also induced apoptosis of osteosarcoma cells in dose dependent, as detected by PARP and caspase-3 activity. Further, we also reported that dihydromyricetin had antitumor potential in nude mice, including orthotopic model, and the toxicity of combination was reduced compared with adriamycin treatment alone. This study is the first to show that dihydromyricetin exhibits strong antitumor 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–like 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 sphere formation in osteosarcoma cells (20). In the present study, we demonstrated that dihydromyricetin could decrease the capability of sphere formation in osteosarcoma cells. Furthermore, we investigated the expression of Sox2 in osteosarcoma cells treated with dihydromyricetin, 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 dihydromyricetin, the enhanced osteosphere was partly recovered. Overall, these results suggest that Sox2 is a key player in osteosphere and dihydromyricetin could suppress osteosphere through downregulation of Sox2 and decrease the stem cell-population in human osteosarcomas.
To determine the potential molecular mechanism of action of dihydromyricetin in osteosarcoma, we used a proteome profiler array and found that the phosphorylation levels of multiple kinases were affected by dihydromyricetin treatment. Specifically, we observed the dihydromyricetin-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 antitumor agent through induction DNA damage and apoptosis in osteosarcoma (14). GSK3β, a serine/threonine protein kinase, also plays key roles in multiple pathways. Although GSK3β is generally recognized as a tumor suppressor that is frequently inactivated in a variety of tumors, we demonstrated that GSK3β activity may promote osteosarcoma tumor growth and induce apoptosis in osteosarcoma cells (23). In this study, we demonstrated that cells treated with AMPK activators resulted in the increased phosphorylation of GSK3β, suggesting that dihydromyricetin treatment could inactivate GSK3β through the activation of AMPKα. We further investigated the expression of Sox2 and p21 after GSK3β depletion or GSK3β 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 dihydromyricetin treatment. On the basis of our results, we suggest that dihydromyricetin possesses antitumor activity due to its ability to affect the AMPK–GSK3β–Sox2 signaling pathway. Another important observation was that dihydromyricetin induces the activation of p38MAPK and the INK 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, further studies are needed to clearly understand how dihydromyricetin induces AMPK activation, which can be caused by generating more reactive oxygen species (ROS) or other metabolic stress.

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

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Z. Zhao, J. Yin, M. Wu, X. Xie, Q. Jia, J. Shen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Zhao, J. Yin, M. Wu, C. Zhou, J. Lu, Y. Wang, Q. Jia
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Zhao, J. Yin, M. Wu, Y. Wu, J. Lu, Q. Jia
Writing, review, and/or revision of the manuscript: Z. Zhao, J. Yin, I. Wang, Q. Jia, J. Shen

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Zhao, J. Yin, C. Song, X. Xie, C. Zhou, Q. Yang, W. Wang, Q. Jia

Study supervision: Z. Zhao, J. Yin, I. Wang, T. Kang, Q. Jia, J. Shen

Acknowledgments

The authors thank the graduate students in Prof. Tiebang Kang's laboratory in Sun Yat-Sen University Cancer Center, Guangzhou, China.

Grant Support

This work was supported by the Natural Science Foundation of China (No. 81272939, to J.N. Shen and No. 8110 2040, to J.Q. Yin); Sun Yat-Sen University Clinical Research 5010 Program (No. 200709, to J.N. Shen), and the Ministry of Science and Technology of Guizhou Province, China (2012 7006, to Q. Jia). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 24, 2014; revised May 12, 2014; accepted May 20, 2014; published OnlineFirst June 3, 2014.

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