Plant flavonoid taxifolin inhibits the growth, migration and invasion of human osteosarcoma cells

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Abstract. The aim of the present study was to investigate the anti-cancer effects of the natural plant flavonoid, taxifolin, on human osteosarcoma cancer cells. Taxifolin was demonstrated to exhibit anti-cancer effects on U2OS and Saos-2 osteosarcoma cell lines. Treatment of cells with taxifolin inhibited proliferation and diminished colony formation in soft agar in a dose-dependent manner. In vivo, intraperitoneal administration of taxifolin in nude mice bearing U2OS xenograft tumors, significantly inhibited tumor growth. In addition, taxifolin treatment was demonstrated to promote G₁ cell cycle arrest and cell apoptosis in U2OS and Saos-2 cell lines, as demonstrated by flow cytometry analysis. Western blot analysis demonstrated that taxifolin treatment was associated with a reduction in the expression levels of AKT serine/threonine kinase 1 (AKT), phosphorylated (p-Ser473) AKT, v-myc avian myelocytomatosis viral oncogene homolog (c-myc) and S-phase kinase associated protein 2 (SKP-2) in U2OS and Saos-2 cell lines. Overexpression of AKT considerably reversed the taxifolin-induced decrease in AKT, c-myc and SKP-2 protein expression and the decrease in AKT phosphorylation, suggesting that inactivation of AKT was a mediator of taxifolin-induced inhibition of c-myc and SKP-2. Furthermore, overexpression of SKP-2 in U2OS cells partially reversed the growth inhibition mediated by taxifolin. Finally, taxifolin treatment repressed cell migration and invasion in U2OS cells and this effect was markedly reversed by SKP-2 overexpression. The results of the present study indicate that taxifolin may present a potential novel therapeutic agent for osteosarcoma treatment.

Introduction

Cancer is one of the leading causes of mortality worldwide. Among the various types of cancer, osteosarcoma is one of the most commonly diagnosed primary bone tumors, often occurring in children and adults and arising in the metaphysis (1,2). Since the systemic spread of osteosarcoma is common, the prognosis for affected patients is poor (3). Previously, combination chemotherapy was demonstrated to substantially increase the survival rates of osteosarcoma patients by 60-70% (4); however, 40% of osteosarcoma patients still exhibit a poor response. A high risk of recurrence and metastasis exists despite thorough chemotherapy treatment and curative resection of the tumor (5). Failure of chemotherapy is primarily due to drug resistance. Therefore, an improved understanding of the molecular mechanisms underlying osteosarcoma invasion, as well as the development of novel potential treatments, are necessary for osteosarcoma therapy.

Chemoprevention is the pharmacological use of synthetic compounds or phytochemicals that prevent or reverse carcinogenesis or tumor development. Flavonoids are a class of phytochemical agents that are rich in naturally occurring polyphenolic compounds. They are abundant in medicinal herbs, vegetables, fruits and nuts. Flavonoids comprise of flavones, isoflavones, flavanones, flavonols, chalcones and anthocyanins (6,7). The functions of dietary flavonoids in the prevention and treatment of cancer have been studied extensively in clinical trials and laboratory studies (7,8). The biochemical function of flavonoids and their corresponding metabolites depend on the precise chemical structure of the compound and the relative positioning of different functional groups in the molecule.

Taxifolin (Fig. 1) is a characteristic plant flavonoid, which naturally occurs in onion (8), citrus fruits, and milk thistle. Taxifolin, isolated from the barks of Cedrus brevifolia, Cedrus brevifolia (Hooker fil.), Laric siberica (Ledeb.) and Texus chinensis, has long been used in the treatment of cerebrovascular and cardiovascular disorders (9). Taxifolin, also known as dihydroquercetin, exhibits strong antioxidant abilities Taxifolin, also known as dihydroquercetin, exhibits strong antioxidant ability (10). The properties of taxifolin are similar to quercetin, which has been demonstrated to induce apoptosis in cell and animal models (10,11).

Previous studies have demonstrated that taxifolin exerts multiple biological effects, including anti-oxidative, anti-inflammatory, anti-proliferative and anti-coagulative...
effects (12,13). Rogovskii et al (14) demonstrated that taxifolin exerts anti-proliferative properties in human breast cancer cells and in murine fibroblasts. In addition, Vrba et al (15) demonstrated that mitogen activated protein kinase and protein kinase C-dependent signal transduction is regulated and maintained by taxifolin. However, to the best of the author's knowledge, no studies performed to date have assessed the in vitro and in vivo anti-cancer effects of taxifolin in osteosarcoma. Therefore, the aim of the current study was to investigate the anti-cancer properties of taxifolin in osteosarcoma. To achieve this, the effect of taxifolin on the viability and proliferation of U2OS and Saos-2 osteosarcoma cells was evaluated, and the signaling factors involved in mediating these effects were investigated.

Materials and methods

Reagents. Taxifolin was purchased from Shanghai Huicheng Technology Ltd. (Shanghai, China). MTT, cell culture dishes and Hoechst 33258 were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Matrigel was purchased from BD Biosciences (Franklin Lakes, NJ, USA). The primary antibodies, mouse monoclonal anti-Akt serine/threonine kinase 1 (Akt1; cat. no. sc-135829; 1:1,200), rabbit polyclonal anti-phosphorylated (p-Ser473) Akt (cat. no. sc-7985-R, 1:1,000), mouse monoclonal anti-v-myc avian myelocytoma-tosis viral oncogene homolog (c-myc; cat. no. sc-70469, 1:800), mouse monoclonal anti-S-phase kinase associated protein 2 [SKP-2; cat. no. sc-74477 (p45), 1:1,000] and mouse and rabbit anti-c-myc antobody, mouse monoclonal anti-β-actin (cat. no. sc-8432/sc-130656, 1:100) as well as secondary antibody, rabbit anti-mouse IgG HRP (cat. no. sc-358914, 1:10,000) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell culture. The human osteosarcoma cell lines, U2OS and Saos-2, were obtained from the American Type Cell Culture Collection (Manassas, VA, USA). These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1 mM L-glutamine (Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified incubator at 37˚C and treated with different concentrations of taxifolin (0-50 µM) for a further 48 h. A total of 50 µl MTT solution (2 mg/ml) was added to each well, and plates were incubated for 4 h. The plates were then centrifuged at 1,000 x g for 5 min at 37˚C, to remove unconverted MTT and to leave the resulting formazan crystals at the bottom of the plates. The crystals were then dissolved in 100 µl dimethyl sulfoxide (DMSO), and the absorbance was read at 570 nm using a Titertek Multiskan MCC/340 plate reader (Titertek-Berthold, Berthold Detection Systems GmbH, Pforzheim, Germany). The results were presented as a relative percentage of the untreated control cells (17).

Flow cytometry. U2OS and Saos-2 cells were seeded at 6x10⁴ cells/dish in 100 mm dishes and cultured for 24 h prior to taxifolin treatment. Cells were treated with taxifolin (50 µM) and incubated for 48 h. Cells were then fixed using 70% ice-cold ethanol diluted in phosphate-buffered saline (PBS) for ~12 h at 37˚C. Following fixation, cells were washed with PBS and incubated with PBS containing RNase A (0.1 mg/ml; Sigma-Aldrich; Merck KGaA) for 30 min at 4˚C, before they were resuspended in 50 µg/ml propidium iodide (Sigma-Aldrich; Merck KGaA). The cell cycle distribution was determined using a BD FACSscan flow cytometer (BD Biosciences) and the data were analyzed using the BD CellQuest Pro software program (version 5.1; BD Biosciences).

Soft agar assay. In order to determine soft agar colony formation, 6x10³ U2OS and Saos-2 cells were diluted in complete DMEM medium containing agar (0.3%) in 35-mm cell culture plates. The cells were cultured for 3 weeks in a 5% CO₂ incubator at 37˚C and treated with different concentrations of taxifolin (0, 25 and 50 µM). The colonies were stained with crystal violet (0.5 mg/ml) for 10 h at 37˚C.

Tumor xenografts. A total of 16 male BALB/c nude mice (age, 6-8 weeks; weight, 15-18 g) were procured from the National Cancer Institute at Frederick, Laboratory Animal Sciences Program (Frederick, MD, USA). BALB/c nude mice were maintained in a steel cage, under a 12-h dark/light cycle with free access to food and water. All the mice were subcutaneously injected with 5x10³ U2OS cells suspended in Matrigel (0.5 ml), and tumor growth was monitored weekly from 1 week post-injection. Mice were divided into control (8 mice with 14 tumors) and drug-treated (8 mice with 13 tumors) groups. At 21 days post-inoculation, mice in the drug-treated group were administered with 25 mg/kg taxifolin intraperitoneally (i.p) once every 2 days for remaining 24 days. The control mouse group was administered with an equal volume of saline (vehicle). The treatment was terminated at 45 days post-inoculation. Tumor size was measured using calipers and tumor volume was calculated using the following formula: Volume = length x width x height x 0.52 (18). Mouse body weight and tumor volumes were expressed as the mean ± standard deviation (SD). The present study was approved by the Ethics Committee of Hanzhong Centre Hospital (HCH-4665; Hanzhong, China).

Western blot analysis. U2OS and Saos-2 cells (1x10⁴) were treated with different concentrations of taxifolin (0, 25 and
50 µM) for 72 h at 37˚C and lysed using lysis buffer containing 0.5% sodium deoxycholate, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% nonidet P-40, 0.1% SDS, 1 mM dithiothreitol, 5 mM EDTA, 50 mM sodium fluoride, and 10 µg/ml aprotinin by incubating for 1 h at 37˚C. The resultant cell lysates were centrifuged at 3,000 x g for 10 min at 37˚C and total cellular protein concentration was determined using a BCA protein assay kit (BioVision, Inc., Milpitas, CA, USA). Protein extracts (∼60 µg) were separated by 10% SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes. The membrane was blocked with TBS containing Tween-20 and 5% skimmed milk for 2 h at 37˚C and incubated with primary antibodies against Akt, p-S473-Akt, c-myc, SKP-2 and β-actin at 4˚C overnight, followed by probing with secondary antibody for 2 h at 37˚C. Detection of bands was performed using an enhanced chemiluminescence detection kit (GE Healthcare Life Sciences, Chalfont, UK), and band densities were measured using Scion Image software (version 4.0) from Scion Co. (Frederick, MD, USA).

**Overexpression of AKT and SKP-2.** U2OS cells (5x10⁵) were plated in 6-well plates for 24 h prior transfection and then U2OS cells were transfected with SKP-2 and AKT overexpression plasmids (1 µM) like pcDNA3.1-SKP2, pcDNA3.1-AKT (designed by Nanjing Keygen Biotech Co., Ltd., Nanjing, China) and an empty vector (pcDNA3.1) using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.), for 24 h. Finally the transfected cells were treated with 0 or 25 µM of taxifolin for 48 h. Transfection of U2OS cells with the empty pcDNA3.1 vector served as a negative control (19).

**Transwell migration assay.** Briefly, U2OS cells (1x10⁴) were plated in 6-well plates for 24 h prior to transfection. Cells were treated with 0, 25 and 50 µM taxifolin for 48 h, then trypsinized (centrifuged at 600 x g for 10 min at 37˚C) and washed with PBS to remove adherent cells. A total of 1x10⁴ treated cells diluted in 200 µl serum-free DMEM were added to the upper compartment of transwell migration chambers (BD Biosciences), and DMEM containing 10% FBS was added to the lower compartment. The chambers were then incubated at 37˚C overnight. Cells that had migrated to the underside of the transwell filters were fixed with 70% methanol and stained with 10% Giemsa solution at room temperature for 1 h. Filters were washed with water and quantification was performed by analyzing 6 random fields and counting the number of cells on the filter (lower side) under phase contrast microscopy at a magnification of x200.

**Transwell invasion assay.** For the invasion assays, Matrigel-coated transwell chambers were used (BD Biosciences). A total of U2OS cells (1x10⁵) were plated in 6-well plates for 24 h prior to transfection. Cells were then treated with 0, 25, and 50 µM taxifolin for 48 h, and trypsinized (centrifuging at 600 x g for 10 min at 37˚C) to remove adherent cells and washed with PBS. Then the 1x10⁴ taxifolin-treated U2OS cells were diluted in 200 µl serum-free DMEM and added to the upper compartment of Matrigel-coated transwell chambers (BD Biosciences). Matrigel containing 10% FBS was added to the lower chamber and the plates were incubated overnight. The non-invaded cells were removed and the invaded cells on the underside of the filters were fixed with 70% methanol at 37˚C for 1 h. Filters were washed with water and stained with 10% Giemsa solution for 1 h at 37˚C.
The viability and proliferation of taxifolin-treated human U2OS and Saos-2 osteosarcoma cells were examined using MTT and Hoechst 33258 assays, respectively. Results from the MTT assay demonstrated that the viability of U2OS and Saos-2 cells was inhibited by taxifolin treatment in a dose-dependent manner at concentrations ≥25 µM compared with the untreated cells (Fig. 2A). In addition, taxifolin treatment suppressed the proliferation of U2OS and Saos-2 cells in a dose-dependent manner at concentrations ≥25 µM when compared with the untreated cells (Fig. 2B). The half maximal effective concentration of taxifolin was similar for cell viability and proliferation (data not shown), suggesting that the observed decrease in cell viability may have been due to the suppression of cell proliferation.

Effect of taxifolin on the viability and proliferation of osteosarcoma cells. The viability and proliferation of taxifolin-treated human U2OS and Saos-2 osteosarcoma cells were examined using MTT and Hoechst 33258 assays, respectively. Results from the MTT assay demonstrated that the viability of U2OS and Saos-2 cells was inhibited by taxifolin treatment in a dose-dependent manner at concentrations ≥25 µM compared with the untreated cells (Fig. 2A). In addition, taxifolin treatment suppressed the proliferation of U2OS and Saos-2 cells in a dose-dependent manner at concentrations ≥25 µM when compared with the untreated cells (Fig. 2B). The half maximal effective concentration of taxifolin was similar for cell viability and proliferation (data not shown), suggesting that the observed decrease in cell viability may have been due to the suppression of cell proliferation.

Effect of taxifolin on soft agar colony formation. The anchorage-independent growth of U2OS and Saos-2 cells was examined using a soft agar colony formation assay. The colony formation results demonstrated that treatment with 25 and 50 µM taxifolin markedly suppressed the ability of U2OS and Saos-2 cells to form colonies when compared with the untreated cells (Fig. 3). These results suggest a potential anti-tumor effect of taxifolin.
Effect of taxifolin treatment on osteosarcoma tumor growth in vivo. In order to determine whether taxifolin treatment inhibits tumor growth in vivo, U2OS cells were used to generate tumor xenograft models in nude mice. As demonstrated in Fig. 4A, intraperitoneal administration of taxifolin (25 mg/kg once every 2 for 24 days) was associated with a 55% reduction (P<0.01) in the mean volume of U2OS xenograft tumors at the end of the experiment (45 days). The body weight of untreated and taxifolin-treated mouse groups was gradually reduced but not significantly altered (Fig. 4B). These results suggest that taxifolin treatment inhibited osteosarcoma tumor growth in vivo.

Effect of taxifolin treatment on osteosarcoma cell cycle distribution and apoptosis. The effect of taxifolin on the cell cycle was examined in human osteosarcoma U2OS and Saos-2 cells by flow cytometry analysis. Treatment of U2OS and Saos-2 cells with 25 µM taxifolin for 48 h significantly increased the percentage of cells in the G1 phase, and significantly reduced the percentage of cells in the S and G2/M phases when compared with untreated cells (Fig. 5). In addition, taxifolin treatment increased the percentage of U2OS and Saos-2 cells in the sub-G1 fraction (cells undergoing apoptosis) when compared with untreated cells (Fig. 5). These results demonstrated that taxifolin treatment induced G1 cell cycle arrest and increased apoptosis in osteosarcoma U2OS and Saos-2 cells.

Effect of taxifolin on cell cycle-associated proteins. Western blot analysis was performed to determine whether taxifolin treatment altered the expression of proteins involved in cell cycle regulation. Taxifolin treatment markedly reduced the protein expression levels of AKT, p-Ser473-AKT, c-myc, SKP-2 and β-actin when compared with untreated cells (Fig. 6A). Transfection of U2OS cells with empty pcDNA3.1 plasmids or the pcDNA3.1-AKT vector was performed in order to overexpress AKT and investigate its association with c-myc and SKP-2. As demonstrated in Fig. 6B, overexpression of AKT markedly reversed the taxifolin-induced repression of p-AKT, c-myc and SKP-2 protein expression levels, indicating that decreased AKT expression may mediate taxifolin-induced c-myc and SKP-2 inhibition. These results revealed that taxifolin reduced the expression levels of signaling factors implicated in cell cycle regulation, potentially via the AKT signaling pathway.

Effect of SKP-2 overexpression. As demonstrated in Fig. 6A, taxifolin treatment resulted in a gradual reduction in SKP-2 expression levels in U2OS and Saos-2 cells. Therefore the effect of SKP-2 overexpression on taxifolin-induced anti-cancer properties was examined further. SKP-2 overexpression in U2OS cells (Fig. 7A) partially inhibited the taxifolin-induced decrease in cell viability when compared with cells transfected with empty vectors (Fig. 7B). The migration and invasion
of U2OS cells transfected with empty vector plasmids were markedly reduced by taxifolin treatment when compared with untreated cells (Fig. 7C and D). By contrast, overexpression of SKP-2 significantly attenuated the taxifolin-mediated reduction in U2OS cell migration and invasion when compared with empty vector-transfected cells (Fig. 7C and D). As overexpression of SKP-2 was not observed to eliminate the inhibitory effects taxifolin completely, it is possible that additional mechanisms or signaling pathways, other than AKT/SKP-2, may be triggered by taxifolin in osteosarcoma cell lines.

Discussion

Flavonoids exhibit a broad range of pharmacological and biological effects, with chemoprevention as one of their most characterized effects (6,8). In the present study, the anti-cancer properties of taxifolin were investigated in human U2OS and Saos-2 osteosarcoma cell lines. Treatment of osteosarcoma cells with taxifolin resulted in G1 cell cycle arrest and increased apoptosis. Results from the MTT assay, and transwell migration and invasion assays revealed that taxifolin treatment inhibited cell viability, as well as the migration and invasion abilities of human osteosarcoma cells. In addition, the soft agar colony formation assay revealed that taxifolin decreased the anchorage-independent growth of osteosarcoma cells. Furthermore, taxifolin treatment suppressed the expression of c-myc and SKP-2, and the inactivation of AKT may have been responsible for these taxifolin-mediated alterations in c-myc and SKP-2 expression. In vivo studies demonstrated that taxifolin administration significantly inhibited tumor growth in mice bearing U2OS cell-derived xenograft tumors. These results indicate that taxifolin may present an effective agent for the treatment of osteosarcoma.

In order to examine the mechanisms underlying the anti-tumor effects of taxifolin further, western blotting analysis was performed. The results indicated that the protein expression levels of AKT, p-AKT (Ser473), c-myc and SKP-2 were markedly reduced in U2OS and Saos-2 osteosarcoma cells treated with taxifolin when compared to untreated cells. Phosphatidylinositol 3-kinase (PI3K)/AKT signaling activation is important during cell proliferation and the progression of osteosarcoma (20). In addition, AKT is an important regulator of cell survival pathways by inhibiting apoptosis, and its kinase activation is mediated by Ser473 phosphorylation. Overexpression of proteins in the PI3K/AKT signaling pathway is associated with a poor clinical prognosis (21-23).

C-myc is a major transcriptional regulator. It is a multi-functional proto-oncogene and a nuclear phosphoprotein that mediates cell proliferation, cell cycle arrest and apoptosis (24). Abnormal c-myc mRNA expression promotes the invasion of osteosarcoma cells (25) and leads to cancer development (26,27), whereas c-myc protein attenuation leads to diminished cell proliferation and tumor growth in osteosarcoma (28). Abnormal c-myc function has been identified in stomach, breast, colon and lung cancers (29). Therefore, c-myc is considered to be a promising anti-cancer drug target (30). In the present study, taxifolin treatment suppressed AKT, p-AKT (Ser473) and c-myc protein expression, suggesting that c-myc repression may mediate the inhibitory effects of taxifolin on cell growth.

SKP-2 serves an important role in regulating the activation of cyclin-dependent kinase inhibitor 1B (CDKN1B; also known as p27kip1), as well as in regulating ubiquitin-dependent processes (31,32). CDKN1B inhibits cell proliferation by inducing cell cycle arrest at the G1 phase (33). In the present study, taxifolin treatment of U2OS and Saos-2 cells reduced
SKP-2 expression, promoted G1 cell cycle arrest and induced apoptosis. SKP-2 overexpression partially inhibited the taxifolin-induced reduction in U2OS cell viability, migration and invasion, suggesting that SKP-2 suppression may be a major factor in taxifolin-mediated growth inhibition of osteosarcoma cell lines. As overexpression of SKP-2 did not completely reverse taxifolin-mediated cell growth inhibition, it is possible that additional signaling pathways may be induced by taxifolin to inhibit osteosarcoma cell growth.

In conclusion, the results of the present study revealed that taxifolin treatment inhibited the proliferation, migration and invasion of U2OS and Saos-2 human osteosarcoma cell lines, potentially via AKT and SKP-2 signaling pathways. The results suggest that taxifolin may present an effective therapeutic agent for the treatment of osteosarcoma.

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References