6,4′-Dihydroxy-7-methoxyflavanone Inhibits Osteoclast Differentiation and Function

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6,4′-Dihydroxy-7-methoxyflavanone (DMF) is a flavonoid isolated from Heartwood Dalbergia odorifera. It has been known that DMF has antioxidant, anti-inflammatory and neuroprotective effects. DMF, however, the efficacy of bone related diseases has not been reported. In this study, we determined DMF’s efficacy on osteoclasts differentiation and function using in vitro bone marrow macrophage osteoclast differentiation culture system. DMF inhibited receptor activators of nuclear factor kappa-B ligand (RANKL) induced osteoclastogenesis dose dependently. In addition, DMF decreased osteoclast function through disruption of actin ring formation and consequently suppression of the pit-forming activity of mature osteoclasts. Mechanistically, DMF inhibited RANKL-induced expression of nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1) and c-Fos via inhibition of mitogen activated protein kinases (MAPKs) pathway. Collectively, the inhibition of osteoclasts differentiation and function by DMF suggests that DMF can be a potential therapeutic molecule for osteoclastogenic bone diseases such as osteoporosis, rheumatoid arthritis and periodontal diseases.

Key words 6,4′-dihydroxy-7-methoxyflavanone; osteoclast; bone resorption; mitogen activated protein kinase; c-Fos; nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1

Bone composed two different lineage cells like osteoblast-osteocytes and osteoclasts. Osteoclasts were multinucleated cells, resorb bone, maintained calcium homeostasis and helping normal bone remodeling. Enhanced resorptive activity by imbalanced bone remodeling causes various bone diseases such as osteoporosis, paget’s disease, hip fracture, rheumatoid and periodontal disease.15 Differentiation and maturation of osteoclasts is controlled by many factors, including macrophage colony stimulating factor (M-CSF) and receptor activators of nuclear factor κB ligand (RANKL).2,3 RANKL is members of the tumor necrosis factor (TNF) superfamily.4 RANKL interacts RANK trigger activation of tumor necrosis factor receptor-associated factor-6 (TRAF6) which subsequently induces mitogen-activated protein (MAP) kinases and transcription factors included nuclear factor-kappa B (NF-κB), activator protein 1 (AP-1), nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1).5,6 These transcription factors play an essential role in the regulation of genes involved in osteoclast differentiation and bone resorptive activity.7,8

Current treatment of osteoporosis was based on to increase bone formation (estrogen, PTH) and inhibits bone resorption (estrogen, calcitonin, calcium, bisphosphonates).9–13 Estrogen-replacement therapy (ERT) was used to be popular treatment and prevention of postmenopausal osteoporosis. However, risk of breast cancer, stroke, and heart attack increases by excessive ERT.14 Bisphosphonates are the most widely used drug to cure osteoporosis. But, it was poorly absorbed from the gastrointestinal tract and show side effects like osteonecrosis of jaw and atypical fracture.15 Furthermore, PTH cannot be given orally, expensive, limited indication and concern about osteosarcoma has led to recommendation of a 2 years maximum treatment course.16 Thus, new agents for management of osteoporosis are needed. Natural source products have been used successfully for identification and development of therapeutic agents.17,18

6,4′-Dihydroxy-7-methoxyflavanone (DMF), flavonoid, was isolated from the heartwood of Dalbergia (D.) odorifera as previous study.19 Previous phytochemical studies of D. odorifera have reported the isolation of flavonoid, quinines and phenolic constituents.20–22 Flavonoids and related plant-derived phenolic compounds are well known to have a wide range of biological activities such as anti-inflammatory, anti-cancer, anti-osteoporosis.23–27 However, the anti-osteoporotic effects of DMF have not been studied yet. In this study, we studied the effect of DMF on osteoclastogenesis in vitro.

MATERIALS AND METHODS

Reagents DMF was isolated from D. odorifera as the same method described previously.19 The structure is shown in Fig. 1. Recombinant murine macrophage colony-stimulating factor (M-CSF) and recombinant soluble receptor activators of nuclear factor kappa-B ligand (sRANKL) were purchased from PeproTech EC Ltd. (London, U.K.). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Amresco Inc. (OH, U.S.A.). Naphtol AS-MX phosphate, fast red LB salt, ρ-nitrophenylphosphate, 4,6-diamidino-2-phenylindole (DAPI) and Actin antibody were purchased Sigma-Aldrich Chemicals (St. Louis, MO, U.S.A.). Alexa Fluor 488-conjugated phalloidin was purchased from Molecular Probes Inc. (Eugene, OR, U.S.A.) and primary antibodies for phospho-e-Jun N-terminal kinase (JNK), JNK, phospho-extracellular signal-regulated kinase (ERK), ERK, phospho-p38, p38 and rabbit polyclonal antibodies from Cell Signaling Technology Inc. (Beverly, MA, U.S.A.). An

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differentiated into multinucleated mature osteoclasts (OCLs). The cells were fixed 10% formaldehyde 10 min and stained with Alexa Fluor 488-conjugated phalloidin in the dark and then washed with cold phosphate-buffered saline (PBS).

**DAPI Staining** For DAPI staining, cells were fixed as following actin ring staining methods and added 1 µg/mL DAPI in PBS for 1h. Cells showing fragmented chromatin were considered apoptotic. The distribution of DAPI staining in mOCs was visualized under a fluorescence microscope Olympus IX71 (Olympus, Tokyo, Japan).

**Bone Resorption Pit Formation Assay** For bone resorption pit formation, BMMs were seeded at 5×10⁴ cells/well onto calcium phosphate (Ca-P) nanocrystal-coated plates and incubated with M-CSF and RANKL for 7d. After incubation of cells in bleach solution for 5min at room temperature, cells were washed twice with distilled water and dried to 3h. Resorption pits were observed using a microscope (Olympus, Tokyo, Japan). The resorption area was calculated using IMT solution FL 9.1 (Vancouver, BC, Canada) software.

**Western Blot Analysis** BMMs were washed with PBS, lysed in a Ripa buffer containing protease and phosphatase inhibitor cocktail (Thermo, U.S.A.) and centrifugation at 13000rpm for 15min. Protein concentration was determined Bradford methods using manufacturer’s kit (Bio-Rad, U.S.A.). Equal amount of each lysate was separated in sodium dodecyl sulfate (SDS)-polyacrylamide gel. After the electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membrane was blocked with 5% skim milk in TBS buffer, incubated primary antibodies (NFATc, c-Fos, phospho-JNK, JNK, phospho-ERK, ERK, phospho-p38, p38, β-actin) at 4°C overnight, washed, incubated secondary antibody, horseradish peroxidase conjugated anti-rabbit Immunoglobulin G (IgG) and anti-mouse IgG, and detected with ECL-plus (GE Healthcare Life Science). The immunoreactive bands were analyzed by LAS4000 (GE Healthcare Life Science).

**Statistical Analysis** All experiments were replicated at least 3 times. The means and standard deviations (S.D.) calculated by Sigma Plot software 10.1. Student’s t-test were used to assess the statistical significance of differences. Differences were considered significant as at p<0.05.

**RESULTS**

**Effects of DMF on RANKL-Induced Osteoclast Differentiation** As shown in Fig. 1, 6,4′-dihydroxy-7-methoxyflavanone (DMF) is one of flavonoids with methylations on hydroxyl groups (methoxy bonds) and two hydroxyl groups.19) The effect of DMF on BMM cells viability was examined by MTT assay (Fig. 2A). BMM cells were cultured 3d in various concentrations of DMF (0–30 µM). DMF did not affect cell viability at concentration of < 30 µM. To determine the effects of DMF on osteoclast differentiation, BMMs were differentiated into osteoclasts after treatment with M-CSF and RANKL. As shown in Fig. 2B, DMF inhibited TRAP activity at 3 to 30 µM significantly. This inhibition effect of DMF (10–30 µM) on osteoclastogenesis was reconfirmed by inhibition of the formation of multinuclear OCLs in dose dependent manner (Fig. 2C). Baicalein was used positive control and showed a significantly inhibit osteoclast differentiation at concentration 10–20 µM. These results indicate that DMF significantly inhibits osteoclastogenesis.

**Effect of DMF on Mature Osteoclast Actin Ring Formation and Apoptosis** Actin ring formation is essential
for bone resorption in mature osteoclasts. The actin ring was stained with Alexa-488 Flour conjugated phalloidin. The mature osteoclasts treated with DMF for 2 d were changed their morphology with disruption of actin ring formation (Fig. 3A).

To determine whether DMF induces apoptosis, nuclear condensation and fragmentation were assayed by DAPI staining. DMF did not induce nuclear condensation or fragmentation of mature osteoclasts at this condition (Fig. 3B). Baicalein was used positive control. It also induced disruption of the actin ring structure and induced apoptosis at 20 µM. In addition, these results suggest that DMF can disrupt actin ring formation of mature osteoclasts.

**Inhibitory Effects of DMF on Bone Resorption Activity**

To test the effect of DMF on osteoclast function, we measured bone resorption activity of osteoclasts. M-CSF and RANKL-induced mature osteoclasts were treated with various concentration of DMF. Resorption pits formation on Ca-P coated plate was dose dependently inhibited by DMF (Figs. 4A, 4B).
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These results indicate that DMF has inhibitory function on mature osteoclasts. In addition, baicalein also inhibited the bone resorption activity.

Effects of DMF on Intracellular Signaling and the Expression Levels of Osteoclast Marker Proteins Many studies have reported that the activation of MAPKs suppressed osteoclast differentiation. To define the molecular mechanism of DMF on the inhibition of osteoclast differentiation, we investigated the effects of DMF on RANKL induced phosphorylation of MAPKs that has been known to play important roles during osteoclastogenesis. The phosphorylation of MAPKs induced by RANKL in BMM cells, reached the maximum levels at 15 min, and decreased thereafter. At a concentration of 30 µM, DMF strongly inhibited RANKL-induced phosphorylation of JNK without decrease of the phosphorylation of ERK and p38 MAPK (Fig. 5). It also suppressed c-Fos and NFATc1 expression.

DISCUSSION

DMF, a class of flavonoid, was isolated from the heartwood of D. odorifera T. CHEN (Leguminosae). The heartwood of the D. odorifera T. CHEN (Leguminosae) is used to treat ischemia, swelling and rheumatic pain in China and Korea.

Fig. 4. Effect of DMF on the Bone Resorption Activity of Osteoclasts

BMMs were cultured for 5 d with the indicated concentration of DMF in the presence of M-CSF (100 ng/mL) and RANKL (100 ng/mL). (A) A photograph of the bone-resorption activity of OCLs. The data are representative of three independent experiments. (B) The resorption area was calculated using software. The results shown are representative of three independent experiments. Results were presented as means ± S.D. (n > 3). * p > 0.05, ** p > 0.01. Baicalein (20 µM) was used positive control.

Fig. 5. Effect of DMF on RANKL-Induced MAPKs, c-Fos, NFATc1 Signaling

BMM cells were preincubated in the presence of DMF for 1 h and then treated with RANKL (100 ng/mL) for 15 min. Cell lysates were collected and separated by 10% SDS-PAGE. The levels of phosphorylated and non-phosphorylated p38 MAPK, ERK and JNK were determined by Western blotting (A). RANKL (100 ng/mL) treated 24 h, the level of NFATc1 and c-Fos were determined by Western blot (B). The results shown are representative of three independent experiments.
Previous studies showed that DMF displays anti-oxidative and anti-inflammatory heme oxygenase-1 (HO-1) inducer in mouse hippocampal HT22 cells and BV2 microglia cells. However, other biological effects of DMF have not been reported. The oriental medicine has been widely used in the prevention and the treatment of fracture and joint diseases. Recently, several studies have suggested that natural source of small molecules prevent osteoporosis by modulate osteoclastogenesis. For example, saurorsactam, sauchinone isolated from Saururus chinensis inhibited bone destruction and osteoclast formation caused by lipopolysaccharide in an animal model. Baicalein isolated from Scutellaria baicalensis inhibited osteoclast differentiation and induced apoptosis of mature osteoclasts to inhibit bone resorption. Furthermore, honokiol, a neolignan, isolated from Magnolia obovata also inhibited osteoclast differentiation and function in vitro. In this study, we further demonstrated that DMF inhibited osteoclast differentiation and modulated their function.

Osteoclasts are multi-nucleated cells that are differentiated from hematopoietic cells of the monocyte/macrophage family. The differentiation of this cell is mainly regulated by M-CSF, RANKL, or osteoprotegerin. Coincident treatment of mouse BMM cells with M-CSF and RANKL induced osteoclast differentiation. DMF did not affect the proliferation. However, DMF inhibited osteoclast differentiation induced by RANKL from BMM cells.

In osteoclast, RANKL binding to RANK prompt the activation of MAPKs signaling pathways. Also, MAPKs are important for the induction of c-Fos and NFATc1 during osteoclast differentiation. JNK has been reported to play a role in osteoclast differentiation and bone resorptive activity. In our results, DMF inhibited the JNK pathway in BMM cells. Since NFATc1 is a critical transcription factor in RANKL-induced osteoclastogenesis, the down regulation of NFATc1 by DMF effectively suppressed osteocast differentiation.

The formation and maintenance of actin rings on the bone surface are essential in order to conduct their major function of bone resorption in mature osteoclasts, so the formation of actin rings can be said to be a bone resorption factor. DMF also inhibited osteoclast bone resorption activity due to the disruption of actin ring formation. Besides, DMF directly induced disruption of the actin rings and nuclear fragmentation in mature osteoclasts and resulted in the inhibition of bone resorption. These findings suggest that DMF suppressed bone resorption through both its inhibitory effect on osteoclast differentiation and function.

In conclusion, we have demonstrated that DMF suppresses osteoclast differentiation by inhibiting RANKL induced MAPK signaling pathways and attenuates bone resorption by disrupting the actin rings in mature osteoclasts. DMF could be useful for the treatment of bone diseases associated with excessive bone resorption. In the present study, we demonstrated for first time that DMF has inhibition of osteoclast differentiation and anti-resorptive activity. Therefore, it could be good candidate to develop a therapeutic drug for osteoporosis treatment.

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