# Honokiol stimulates osteoblastogenesis by suppressing NF-κB activation

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**Abstract.** *Magnolia officinalis*, a component of Asian herbal teas, has long been employed in traditional Japanese and Chinese medicine to treat numerous maladies. Honokiol, a biphenolic compound, is now considered to be one of the major active ingredients of *Magnolia* extract, and is under intense investigation for its anti-angiogenic, anti-inflammatory, anti-tumor and neuroprotective properties. Biochemically, honokiol has been recognized to modulate the nuclear factor κ B (NF-κB) signal transduction pathway suggesting that it possesses anti-inflammatory properties. Inflammation is intimately associated with bone turnover and skeletal deterioration and consequently, anti-inflammatory drugs may hold significant promise as bone protective agents to stem bone loss in osteoporotic conditions. We and others have demonstrated that suppression of NF-κB blunts osteoclastic bone resorption, but promotes osteoblastic bone formation. Indeed previous studies have demonstrated the anti-osteoclastogenic effects of honokiol, however, activities on osteoblast differentiation and activity have yet to be investigated. In this study, we show that honokiol is a potent inducer of *in vitro* osteoblast differentiation by virtue of its capacity to suppress basal and tumor necrosis factor alpha (TNFα)-induced NF-κB activation and to alleviate the suppressive action of TNFα on bone morphogenetic protein (BMP)-2-induced Smad activation. Our data confirm that honokiol may have considerable promise as a dual anabolic/anti-catabolic agent for the amelioration of multiple osteoporotic diseases.

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## Introduction

Skeletal renewal is a delicate balance between bone resorbing osteoclasts and bone forming osteoblasts. Osteoclasts form from precursors that circulate among the monocyte lineage and differentiate into osteoclasts under the influence of the key osteoclastogenic cytokine receptor activator of nuclear factor κ B (NF-κB) ligand (RANKL) (1,2). Activation of the NF-κB signal transduction pathway by RANKL is an essential step in osteoclast differentiation and function (3) and pharmacological antagonists of NF-κB activation repress osteoclastogenesis *in vitro* (4-7) and bone resorption *in vivo* (8,9). By contrast, activation of NF-κB signaling by inflammatory cytokines such as TNFα potently inhibits osteoblast differentiation and function *in vitro* (5,10-13) and *in vivo* (10,14,15). By contrast, suppression of NF-κB promotes osteoblast differentiation and mineralization *in vitro* (10) and bone formation at baseline (16) and in *in vivo* models of estrogen deficiency (16,17) and of fracture repair (18,19).

Honokiol is a small-molecule polyphenol isolated from the genus *Magnolia* and has been found to have anti-angiogenic, anti-inflammatory, and anti-tumor properties in preclinical models, with low toxicity (20). Biochemically, honokiol has been recognized to regulate NF-κB signaling and to block TNFα-induced NF-κB activation, IκBα phosphorylation, and IκBα degradation (20).

Consistent with this anti-NF-κB activity honokiol was found to inhibit RANKL-induced osteoclastogenesis *in vitro* (21) and to inhibit the progression of collagen-induced arthritis (an animal model of the inflammatory autoimmune disease, rheumatoid arthritis) *in vivo*, by down-regulating inflammatory cytokines, matrix metalloproteinases and blocking oxidative tissue damage (22).

Given the capacity of honokiol to antagonize NF-κB activation we examined whether honokiol could promote osteoblast differentiation and mineralization *in vitro*. Our data confirm this notion and suggest that honokiol may indeed have significant potential for use as a bone anabolic agent.

## Material and methods

*Materials*. α-minimal essential medium (α-MEM) and antibiotics (penicillin and streptomycin) were purchased from

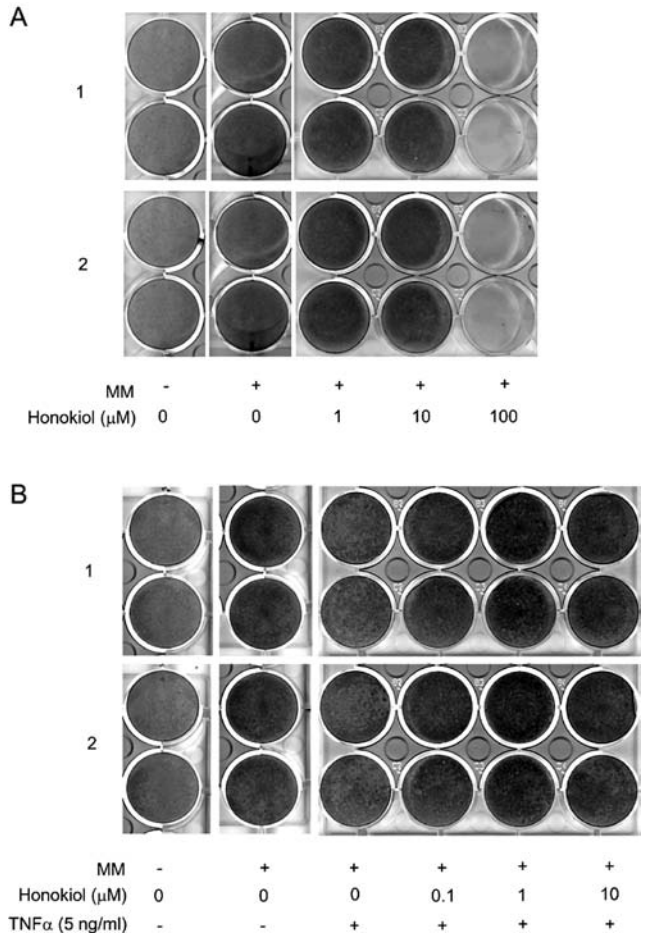


Figure 1. Honokiol promotes osteoblast differentiation and mineralization and alleviates the suppression of osteoblast mineralization induced by TNFα *in vitro*. (A) MC3T3 cells were cultured in the presence (+) or absence (-) of mineralizing medium (MM) with a dose range of honokiol (0, 1, 10 or 100 $\mu$M), and (B) with or without TNFα (5 ng/ml). Cultures were stained with Alizarin Red-S at 18 days for visualization of calcium deposition. Two sets of data-labeled (1 and 2) are shown and represent independent experiments, with each data point performed in duplicate. All wells within each experiment were form the same plate but were digitally separated and reorganized for clarity. Non-contiguous wells are separated by a white space to indicate this fact.

Invitrogen Corp. (Carlsbad, CA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). TNFα, transforming growth factor beta (TGFβ) and BMP-2 were from R&D Systems (Minneapolis, MN). Antibodies for Western blot analysis were purchased from Santa Cruz Biotechology (Santa Cruz, CA). Honokiol and all other reagents were purchased from the Sigma-Aldrich Chemical Corp., (St. Louis, MO) unless otherwise specified.

*Cell culture*. The pre-osteoblastic cell line MC3T3-E1, clone 14 (MC3T3) was purchased from the American Type Culture Collection (Manassas, VA) and cultured as previously described (10,23).

*Osteoblast differentiation assays and Alizarin Red-S staining*. MC3T3 cells, were plated and cultured for 72 h in α-MEM (1.0 ml/well) containing 10% FBS in 12-well dishes at a density of ($1.0x10^5$ cells/well). The medium was aspirated and changed to mineralization medium (α-MEM supplemented with 10% FBS, L-ascorbic acid (100 $\mu$g/ml) and 4 mM β-glycerophosphate) as previously described (5,10,24). Honokiol was added at the indicated dose and cells were replenished with fresh medium every 3 days. At 18 days, cells were rinsed with PBS and mineralization nodules were visualized by fixing the cells in 75% ethanol for 30 min at 4˚C followed by staining with Alizarin Red-S (40 mM, pH 6.2) for 30 min at room temperature. Excess stain was removed by copious washing with distilled water. Plates were imaged using a flatbed scanner (Epson Perfection V600 Photo).

*NF-κB constructs and luciferase assays*. The NF-κB responsive reporter pNF-κB-Luc (BD Biosciences) or pGL3-Smad, responsive to all R-Smads, was used as previously described by us (5,10). Briefly, reporter plasmid was transfected into MC3T3 cells ($1x10^5$ cells/well) using Lipofectamine 2000 reagent (Invitrogen) in α-MEM without FBS and antibiotics. Five hours later the medium was changed to α-MEM containing 10% FBS plus antibiotics and cells were treated with TNFα to stimulate NF-κB activity. Parallel groups received honokiol at the indicated dose. Cells were extracted with passive lysis buffer (Promega Corp., Madison, WI) 24 h later, and luciferase activity was measured using the Luciferase Assay System of Promega (Madison, WI), on a microplate luminometer (Turner Designs, Sunnyvale, CA, USA).

*Western blot analysis*. Cells were plated in 35 mm wells at a density of $10^6$ cells/well in 2 ml of medium (αMEM + 10% FBS and antibiotics) and cultured for 24 h prior to addition of honokiol (10 $\mu$M) for an additional 24 h, followed by addition of TNFα (10 ng/ml) for 60 min. Cultures were subsequently lysed for preparation of cytosolic and nuclear extracts as previously described (25) for Western blotting using antibodies against p65, and IκBα. Loading controls consisted of β-actin for cytosolic proteins, or proliferating cell nuclear antigen (PCNA) for nuclear proteins.

*Statistical analysis*. Statistical significance was determined using GraphPad InStat version 3 for Windows XP (GraphPad Software, Inc., La Jolla, CA). Multiple comparisons were performed by one-way analysis of variance (ANOVA) with the Tukey-Kramer multiple comparisons post-test for parametric data. Gaussian distribution was assessed using the Kolmogorov and Smirnov test. P-value <0.05 was considered statistically significant.

## Results

*Honokiol augments the differentiation of MC3T3 cells into mineralizing osteoblasts and alleviates the suppressive effect of TNFα on osteoblast mineralization*. Honokiol has been reported to suppress NF-κB in some cell systems suggesting that it may possess bone anabolic activity. To investigate the effect of honokiol on *in vitro* osteoblast differentiation and activity we treated MC3T3 cells with honokiol (1, 10 or 100 $\mu$M) in mineralizing medium for 18 days and stained for calcium deposition with Alizarin Red-S. The data show (Fig. 1A) that at 1 and 10 $\mu$M honokiol augmented the differentiation of MC3T3 cells into mineralizing osteoblasts. At 100 $\mu$M mineralization was inhibited, although cells were still alive and attached to the plate.

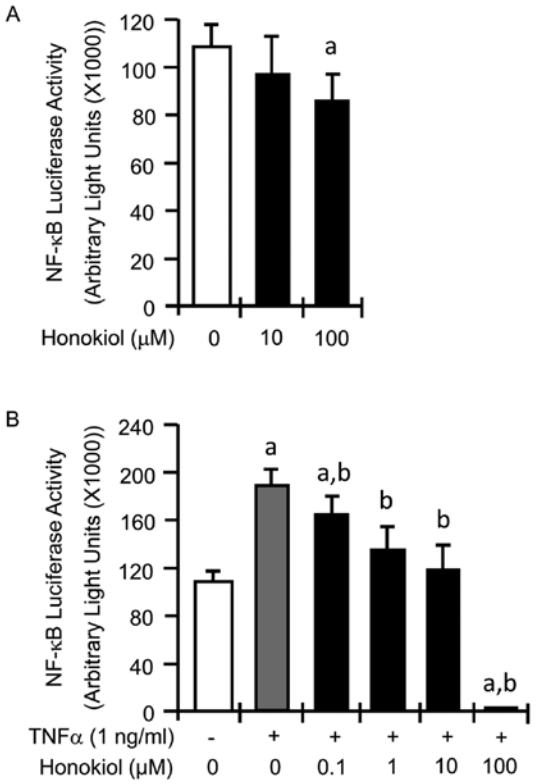


Figure 2. Honokiol suppresses NF-κB activation in osteoblast precursors. (A) MC3T3 cells were transfected with pNF-κB-Luc, an NF-κB activity reporter plasmid and cells were treated with honokiol (10 or 100 $\mu$M). [a]P<0.01 vs. control (white bar). (B) MC3T3 cells were transfected with pNF-κB-Luc, and cells were treated with honokiol (0.1, 1, 10 or 100 $\mu$M) in the presence or absence of TNFα (1 ng/ml). [a]P<0.001 vs. control (white bar); [b]P<0.001 vs. TNF only (grey bar). Data are presented as the mean ± SD of 5 replicate wells per data point and are representative of three independent experiments.

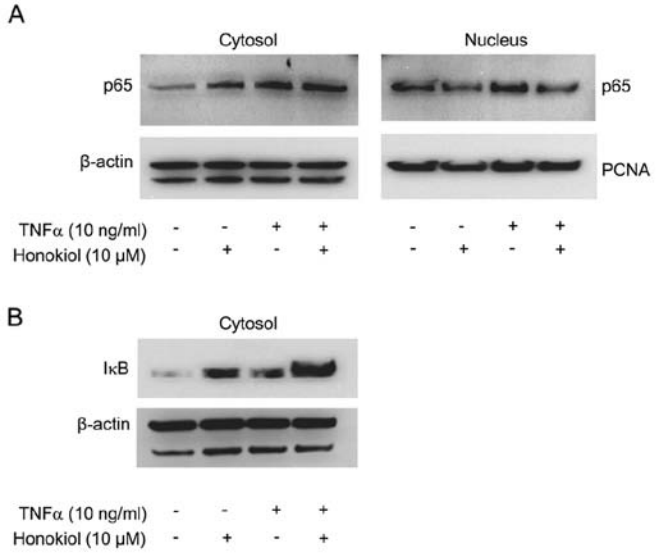


Figure 3. Honokiol suppresses nuclear accumulation of p65 in the nucleus and promotes IκB retention in the cytosol of osteoblast precursors. MC3T3 cells were treated with honokiol (10 $\mu$M) for 18 h followed by addition of TNFα (10 ng/ml) for 60 min. Cells were lysed and cytosolic and nuclear proteins extracted and Western blot analyses performed for (A) the NF-κB subunit p65 or (B) IκBα. β-actin was used as a cytosolic loading control and PCNA as a nuclear loading control. Data are representative of two independent experiments.

TNFα is an established inhibitor of osteoblastic differentiation both *in vivo* and *in vitro* (10,26). Consistent with these known actions, TNFα (5 ng/ml) suppressed mineralization of MC3T3 cells. Addition of honokiol at 0.1 $\mu$M and above alleviated the repressive effect of TNFα and potentiated mineralization (Fig. 1B).

*Honokiol suppresses basal and TNFα-induced NF-κB activity in MC3T3 cells*. Because honokiol is reported to antagonize NF-κB, a pathway inhibitory to osteoblast differentiation and a key mechanism of TNFα signaling we next investigated whether honokiol antagonizes basal and/or TNFα-induced NF-κB activation. We transiently transfected MC3T3 cells with an NF-κB luciferase reporter and quantitated luciferase activity in the presence or absence of honokiol. Honokiol weakly, but significantly, suppressed basal NF-κB activity (Fig. 2A) and potently and dose-dependently (0.1 to 100 $\mu$M) suppressed TNFα-induced NF-κB activity (Fig. 2B).

*Honokiol antagonizes the nuclear accumulation of p65 NF-κB subunit by TNFα and promotes cytosolic retention of IκBα*. As the NF-κB subunit p65 is considered to be a major species in osteoblasts (26) we performed Western blot analyses for p65 on nuclear and cytosolic extracts from MC3T3 osteoblast precursors, in the presence or absence of TNFα and/or honokiol.

TNFα-induced a pooling of p65 in the nucleus (Fig. 3A) while honokiol markedly reduced basal and TNFα-induced p65 nuclear translocation. Consistent with these data honokiol promoted cytosolic retention of IκBα under basal conditions as well as following TNFα stimulation (Fig. 3B).

*Honokiol alleviates the suppressive actions of TNFα on BMP-2- and TGFβ-induced Smad activation in MC3T3 cells*. TNFα suppresses osteoblast differentiation in part by antagonizing Smad activation by bone morphogenetic proteins (BMP) including BMP-2 and early osteoblast commitment factor TGFβ. We thus examined the effect of honokiol on basal, BMP-2-induced and TGFβ-induced Smad activation and the capacity of honokiol to reverse the suppressive action of TNFα on Smad induction by BMP-2 and TGFβ using a Smad-luciferase reporter responsive to all Smad species (10). While honokiol had no direct effect on basal, BMP-2- (Fig. 4A), or TGFβ-induced (Fig. 5A) Smad-activation, it significantly reversed the suppressive activity of TNFα on BMP-2 (Fig. 4B) and TGFβ (Fig. 5B) induced Smad activation.

## Discussion

Honokiol has been recognized as an antagonist of NF-κB activation in a number of cell types, and consistent with such action has been reported to inhibit osteoclast differentiation (21), an NF-κB-dependent process (7,8,27,28). By contrast, we have previously demonstrated that TNFα, a potent inducer of NF-κB, is a suppressor of bone formation *in vivo* in mice and even at physiological concentrations significantly lowers peak attainable bone mineral density (BMD) (10).

The reverse process however, NF-κB suppression, promotes osteoblast differentiation *in vitro* by pharmaco-

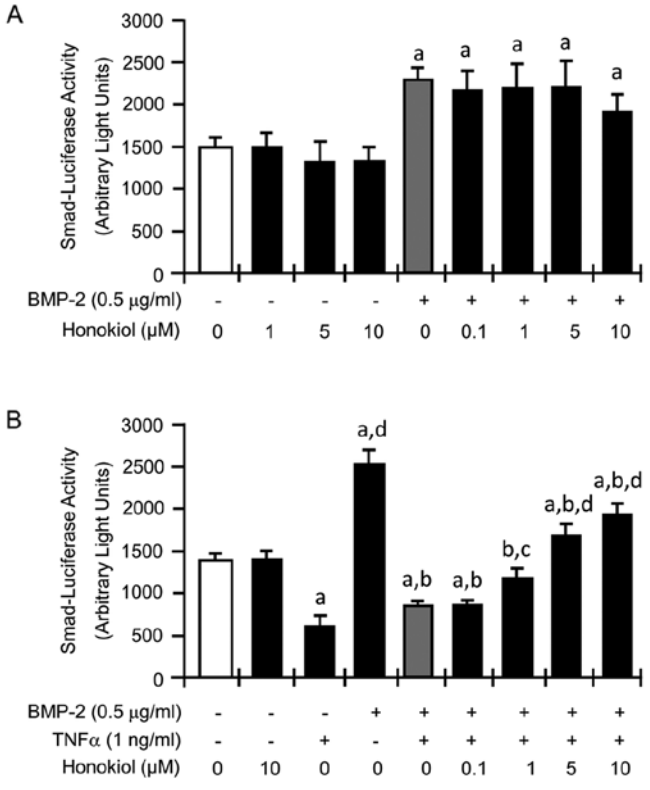


Figure 4. Honokiol alleviates TNFα-induced suppression of BMP-2-induced Smad activation. (A) MC3T3 cells were transfected with pGL3-Smad luciferase reporter plasmid, and the cells were treated with BMP-2 (0.5 $\mu$g/ml) or vehicle in the presence or absence of honokiol (0.1, 1, 5 or 10 $\mu$M). [a]P<0.001 vs. control (white bar). (B) MC3T3 cells were transfected with pGL3-Smad reporter plasmid, and the cells were treated with BMP-2 (0.5 $\mu$g/ml) or vehicle in the presence or absence of honokiol (0.1, 1, 5 or 10 $\mu$M) and/or TNFα (1 ng/ml). [a]P<0.001 vs. control (white bar); [b]P<0.001 vs. BMP-2 only; [c]P<0.01 vs. BMP-2 + TNFα (grey bar) and [d]P<0.001 vs. BMP-2 + TNFα (grey bar). Data are presented as the mean ± SD of 5 replicate wells per data point and are representative of two independent experiments.

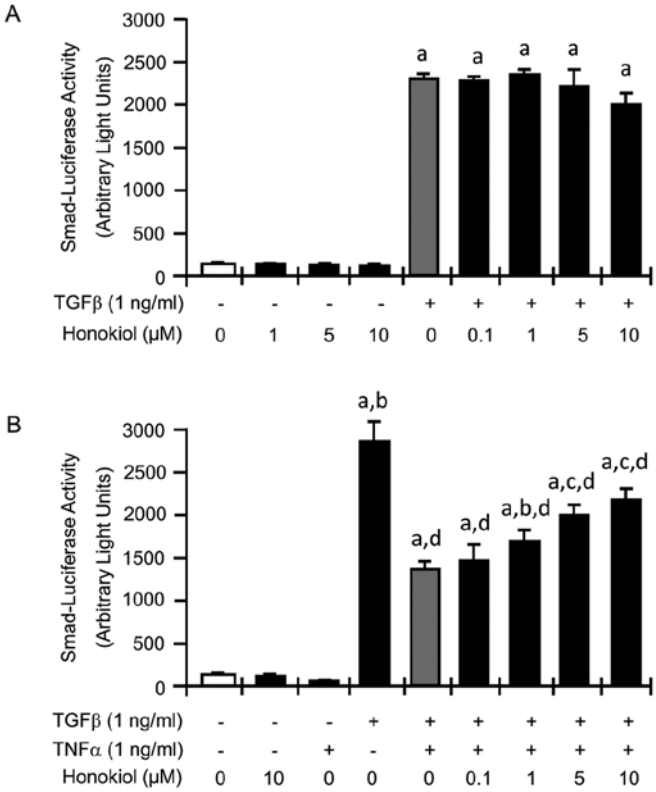


Figure 5. Honokiol alleviates TNFα-induced suppression of TGFβ-induced Smad activation. (A) MC3T3 cells were transfected with pGL3-Smad luciferase reporter plasmid, and cells treated with TGFβ (1 ng/ml) or vehicle, in the presence or absence of honokiol (0.1, 1, 5 or 10 $\mu$M). [a]P<0.001 vs. control (white bar). (B) MC3T3 cells were transfected with pGL3-Smad reporter plasmid, and cells were treated with TGFβ (1 ng/ml) or vehicle, in the presence or absence of honokiol (0.1, 1, 5 or 10 $\mu$M) and/or TNFα (1 ng/ml). [a]P<0.001 vs. control (white bar); [b]P<0.01; [c]P<0.001 vs. TGFβ + TNFα (grey bar); [d]P<0.001 vs. BMP-2 only. Data are presented as the mean ± SD of 5 replicate wells per data point and are representative of two independent experiments.

logical antagonists (10) as well as by nutritional factors with bone anabolic properties including vitamin K2 (5), and the carotenoids p-hydroxycinnamic acid and β-cryptoxanthin (11). Based on the actions of these other NF-κB antagonists, we hypothesized that honokiol by virtue of its anti-NF-κB activity may also possess bone anabolic activity. We examined this notion *in vitro*, and found that honokiol does indeed up-regulate mineralization in the pre-osteoblastic cell line MC3T3, as well as reverses the suppressive action of TNFα on mineralization. Honokiol suppressed NF-κB activation in the context of a biological assay (the NF-κB-driven reporter system) demonstrating a potent and sustained effect. Consistent with the bio-assay we found that nuclear translocation of the NF-κB subunit p65 was suppressed by honokiol and that the cytosolic concentration of the physiological NF-κB activation inhibitor IκB, was intensified following treatment by honokiol. Our data are consistent with previous findings showing that honokiol inhibits TNF-induced NF-κB activation, and IκBα degradation (20).

TGFβ is an early osteoblast commitment factor (29) and induces migration of bone mesenchymal stem cells (osteoblast progenitors) to sites of bone turnover (30). BMPs by contrast, are potent inducers of osteoblast differentiation (31). Both TGFβ and BMPs are potent inducers of Smad signaling, although TGFβ uses predominantly R-Smads 2 and 3, while BMPs promote mainly R-Smads 1, 5 and 8. We previously reported that one mechanism by which TNFα suppresses osteoblast differentiation is through NF-κB-mediated antagonism of TGFβ- and BMP-2-induced Smad signaling in differentiating osteoblasts (10). Other studies have further reported that NF-κB signaling in osteoblasts up-regulates the inhibitor of Smad-signaling, Smad7 (32), and that TNFα promotes proteasomal degradation of BMP signaling proteins by up-regulating Smad ubiquitination regulatory factor 1 (Smurf1), a process that leads to systemic bone loss (15). We recently demonstrated that NF-κB antagonists including a pharmacological peptide based on the Nemo Binding Domain (10) and vitamin K2 (5) can alleviate this repressive action of TNFα on Smad signaling. Our data now reveal that honokiol also possesses this ability.

Although, anti-resorptive agents have long been the preferred standard of care for the amelioration of bone loss, because the processes of bone resorption and bone formation are tightly 'coupled', anti-resorptive therapies are also observed clinically to simultaneously suppress bone formation (33) leading to a 'low bone turnover state'. Although in general anti-resorptive agents do an excellent job of preventing additional bone loss, they do not allow for adequate regeneration of lost

bone mass. In recent years an intensive effort has begun to identify or develop anabolic agents capable of rebuilding lost bone mineral density. At present teriparatide, a fragment of the human parathyroid hormone is the only Food and Drug Administration (FDA)-approved anabolic agent currently available. This agent represents a significant leap forward, but as a biologic-based agent its use is limited by high cost and the need for daily injection. Furthermore, therapy is not recommended for more than 2 years due to the potential for osteosarcoma (34). As a consequence there is intense interest for the identification of additional anabolic agents. We have previously suggested that NF-κB antagonists may represent novel dual anabolic and anti-catabolic drugs (10). Our data suggest that honokiol may represent one such agent. A significant advantage of honokiol over biologics that require injection is its demonstrated bioavailability following oral delivery in animal models (20). Furthermore, as honokiol is a simple polyphenol compound, it can be synthesized in large concentrations at low cost.

In conclusion, our data suggest that honokiol may have significant potential for development into a bone anabolic agent for amelioration of bone loss in multiple metabolic disease states. Studies in animal models are now needed to demonstrate bone anabolic activity of honokiol *in vivo*.

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