

# Low Concentration of 3-Carene Stimulates the Differentiation of Mouse Osteoblastic MC3T3-E1 Subclone 4 Cells

Jong-Geun Jeong<sup>1</sup>, Young Sup Kim<sup>2</sup>, Yong Ki Min<sup>1</sup> and Seong Hwan Kim<sup>1,\*</sup>

<sup>1</sup>Laboratory of Chemical Genomics, Korea Research Institute of Chemical Technology, P.O. Box 107, Yuseong-gu, Daejeon 305-600, Korea

<sup>2</sup>Laboratory of Phytochemistry Research, Korea Research Institute of Chemical Technology, P.O. Box 107, Yuseong-gu, Daejeon 305-600, Korea

Recently, natural products have gained more interest as alternative treatments for metabolic bone disorders and for the maintenance of bone health. In this study, the anabolic activities of 89 natural compounds were evaluated by measuring the amount of newly synthesized calcium in the differentiation process of mouse osteoblastic MC3T3-E1 subclone 4 cells. Of these compounds, a low concentration (up to 5  $\mu\text{M}$ ) of 3-carene, which is a bicyclic monoterpene in essential oils extracted from pine trees, was shown to stimulate significantly the activity and expression of alkaline phosphatase, an early phase marker of osteoblastic differentiation, on differentiation day 9. On day 15, it dramatically promoted the induction of calcium in a dose-dependent manner. The stimulatory effect of 3-carene on mineralization might be associated with its potential to induce the protein expression/activation of the mitogen-activated protein kinases and the transcript levels of osteoblast mineralization-related genes such as osteopontin and type I collagen. Further studies are needed to determine the precise mechanism, but the anabolic activity of 3-carene in bone metabolism suggested that the use of natural additives to the diet including essential oils could have a beneficial effect on bone health. Copyright © 2007 John Wiley & Sons, Ltd.

*Keywords:* 3-carene; osteoblastic MC3T3-E1 subclone 4 cells; differentiation; mineralization.

## INTRODUCTION

Bone mass is maintained through the repeated cycle of destruction and rebuilding (Harada and Rodan, 2003). The process in which old bone is removed from the skeleton by multinucleated osteoclasts and new bone is added through the process of mineralization by osteoblasts is referred to as bone remodeling. Usually, a balance between osteoclastic bone resorption and osteoblastic bone formation can maintain the bone mass at its homeostatic steady state, but the imbalance that is caused by increased bone resorption over bone formation can lead to most adult skeletal diseases including osteoporosis. The development and progress of osteoporosis can increase the risk for fractures (particularly in the hip) that are a serious problem with many adverse consequences such as substantial skeletal deformity, pain and functional limitations (van der Klift *et al.*, 2005).

Until now, most therapies for osteoporosis have focused solely on the resorption side (Rodan and Martin, 2000). Therefore, treatments effective in increasing the bone mass, improving defects in bone micro-architecture and accelerating fracture healing are needed urgently.

\* Correspondence to: Dr Seong Hwan Kim, Laboratory of Chemical Genomics, Korea Research Institute of Chemical Technology, P.O. Box 107, Yuseong-gu, Daejeon 305-600, Korea.  
E-mail: hwan@kriict.re.kr

Since natural compounds and their derivatives have historically been invaluable as a source of therapeutic agents, recent approaches in the early stage of drug discovery and development include the development of therapeutic agents from natural substances, which retain the beneficial effects while minimizing the adverse side effects. Actually, a wide variety of natural compounds can influence the process of bone remodeling, particularly by inhibiting bone resorption, thus having beneficial effects on the skeleton (Putnam *et al.*, 2007).

This study carried out cell-based screening of natural compounds with potential to stimulate calcium formation in mouse osteoblastic MC3T3-E1 subclone 4 cells with the aim of developing anabolic agents.

## MATERIALS AND METHODS

**Cell culture.** All materials for cell culture were purchased from HyClone (UT). Mouse osteoblastic MC3T3-E1 subclone 4 cells with high differentiation potential (Wang *et al.*, 1999) were purchased from the American Type Culture Collection (VA) and cultured in the growth medium [GM,  $\alpha$ -minimal essential medium ( $\alpha$ MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 mg/mL streptomycin] with a change of medium every 3 days in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C.

Received 23 February 2004  
Accepted 24 May 2005

**Screening of natural compounds stimulating calcium formation.** Cells ( $2.5 \times 10^3$  cells/well) were plated in a 384-well plate and cultured in GM. After the cells reached confluence, they were cultured in the differentiation medium [DM, GM with 50  $\mu\text{g}/\text{mL}$  of ascorbic acid (Fluka, Germany) and 10 mM  $\beta$ -glycerophosphate (Sigma, MO)] with a change of medium every 3 days. All natural compounds used in this study were isolated as a single compound form, dissolved in dimethyl sulfoxide (DMSO; Sigma, MO), formatted in a 384-well plate and transferred into cells by using a QRep 384 Pin Replicator (Genetix, UK). On differentiation day 14, the cells were washed with 50  $\mu\text{L}$  of distilled water twice and fixed with 50  $\mu\text{L}$  of 3.5% formaldehyde in calcium and magnesium-free phosphate buffered saline (PBS) for 3 min. For decalcifying mineralized nodules, 20  $\mu\text{L}$  of 1 N HCl was added to each well and after 24 h, the calcium content in the supernatant was determined by using a Calcium C kit (Wako Pure Chemicals Industries, Japan) with modification.

**Alkaline phosphatase (ALP) staining and activity assay.** Cells ( $1.5 \times 10^4$  cells/well) were plated in a 24-well plate and cultured in GM. After the cells reached confluence, they were cultured in DM in the absence or presence of 3-carene with a change of medium every 3 day. On differentiation day 9, the cells were washed with PBS twice, fixed with 10% formalin, rinsed with deionized water and stained under protection from direct light using an Alkaline Phosphatase kit (Sigma). For measuring ALP activity, the cells were washed with PBS twice and sonicated in the lysis buffer consisting of 10 mM Tris-HCl, pH 7.5, 0.5 mM  $\text{MgCl}_2$  and 0.1% Triton X-100. After centrifugation at  $10\,000 \times g$  for 20 min at 4 °C, the supernatant was transferred and the ALP activity was measured using a LabAssay ALP kit (Wako Pure Chemicals Industries). The protein concentration of each sample was measured using a BCA Protein Assay kit (Pierce, IL). The activity assay was performed in triplicate and significance was determined by Student's *t*-test. Differences were considered significant when  $p < 0.001$ .

**Alizarin red S staining.** This staining is specific for the mineralized nodules present in the osteoblast cells. On differentiation day 15, the cells were washed with PBS twice, stained with 40 mM Alizarin red S solution (pH 4.2) for 10 min at room temperature and washed with deionized water twice.

**Western blot analysis.** The cells were homogenized in buffer consisting of 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween 20, 1 mM PMSF and one protease inhibitor cocktail tablet (Roche, Germany) at 4 °C and centrifuged at  $10\,000 \times g$  for 15 min. The BCA protein assay kit was used to determine the concentration of protein in the supernatant. Samples (10  $\mu\text{g}$ ) were mixed with sample buffer (100 mM Tris-HCl, 2% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 2% glycerol, 0.01% bromophenol blue, pH 7.6), incubated at 95 °C for 15 min, and loaded onto 10% polyacrylamide gels. Electrophoresis was performed using the Mini Protean 3 Cell (Bio-Rad, CA). Proteins separated on the gels were transferred onto nitrocellulose membrane (Schleicher & Schnell BioScience, Germany) and in order to ascertain the loading amount of proteins and the efficiency of transfer, the transferred membranes

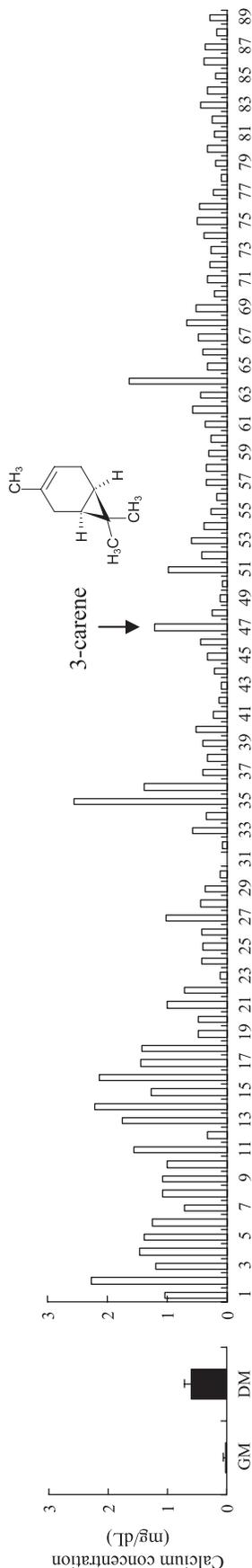
were stained with Ponceau S staining solution. The stained membranes were washed and incubated in blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 3% nonfat dry milk). The membrane was incubated for 2 h at room temperature with 1:1000 diluted primary antibody. Antibodies used in this study were purchased from Santa Cruz Biotechnology Inc. (CA). After washing with blocking buffer three times for 15 min, the membranes were probed with 1:2000 diluted secondary antibody for 1 h. The membrane was washed three times for 15 min and developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) using a LAS-3000 luminescent image analyser (Fuji Photo Film Co., Ltd, Japan).

**Evaluation of mRNA expression level.** Primers were chosen with an on-line primer design program (Rozen and Skaletsky, 2000). The sequences of the primers used were: osteopontin sense, 5'-CCC GGT GAA AGT GAC TGA TT-3', antisense, 5'-TCT CCT GGC TCT CTT TGG AA-3'; type I collagen sense, 5'-AGG CAT AAA GGG TCA TCG TG-3', antisense, 5'-GTT CGG GCT GAT GTA CCA GT-3'; GAPDH sense, 5'-ACT CCA CTC ACG GCA AAT TC-3', antisense, 5'-GTC ATG AGC CCT TCC ACA AT-3'. Total RNA was isolated with TRIzol reagent (Life Technologies, MD) according to the manufacturer's protocol. The concentration and purity of total RNA were calculated with absorbance at 260 and 280 nm. First strand cDNA was synthesized with 1  $\mu\text{g}$  of total RNA and 1  $\mu\text{M}$  of oligo-dT<sub>15</sub> primer using Omniscript Reverse Transcriptase (Qiagen, CA). SYBR green-based quantitative PCR amplification was performed using the Stratagene Mx3000P Real-Time PCR system and Brilliant SYBR Green Master Mix (Stratagene, CA) with first-strand cDNA diluted 1:50 and 20 pmol of primers according to the manufacturer's protocols. The PCR reaction consisted of initial denaturation at 94 °C for 3 min, 3-step cycling (40 cycles) at 94 °C for 40 s, 60 °C for 40 s, and 72 °C for 1 min, and final extension at 72 °C for 5 min. All reactions were run in triplicate, and data were analysed by the  $2^{-\Delta\Delta\text{C}_T}$  method (Livak and Schmittgen, 2001). GAPDH was used as the control gene. Significance was determined by Student's *t*-test with GAPDH-normalized  $2^{-\Delta\Delta\text{C}_T}$  values. Differences were considered significant when  $p < 0.05$ .

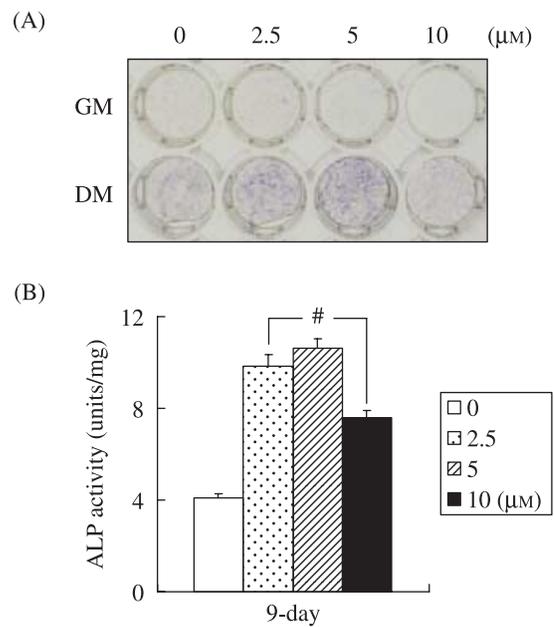
## RESULTS

The effect of natural compounds on calcium formation was evaluated in MC3T3-E1 subclone 4 cells. As shown in Fig. 1, the concentration of newly synthesized calcium in cells cultured in DM for 14 days was 30-fold higher than that in GM, and interestingly, several compounds were shown to stimulate calcium formation. Of these compounds, the effect of 3-carene on osteoblast differentiation and mineralization was focused on in this study.

To ascertain the anabolic activity of 3-carene in bone metabolism, first, the effect of 3-carene on the expression and activity of ALP, an early phase marker of osteoblastic differentiation was evaluated. 3-Carene was shown to stimulate significantly both expression and activity of ALP on differentiation day 9 when compared with



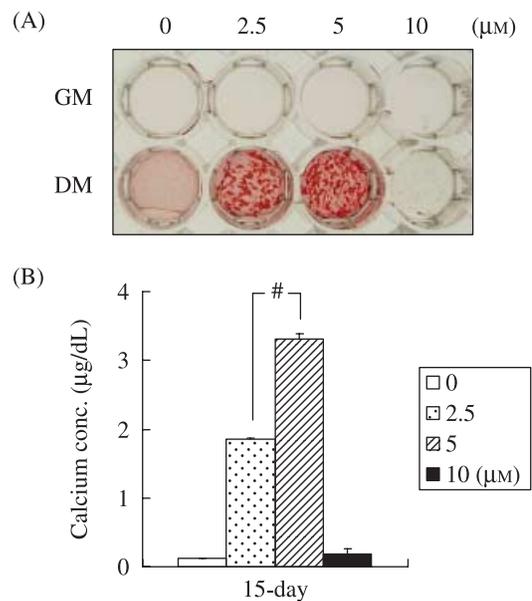
**Figure 1.** Screening of natural compounds with a potential to stimulate the calcium formation in the differentiation of MC3T3-E1 subclone 4 cells. Cells were differentiated as described in 'Materials and Methods' and the calcium contents were measured on differentiation day 14. The calcium contents in cells cultured with GM or DM were used as a negative or positive control and natural compounds were added in cells cultured with DM. One of the compounds stimulating the calcium formation, 3-carene, is indicated by an arrow and its structure presented.



**Figure 2.** Effect of 3-carene on the ALP protein expression and its activity on differentiation day 9. The differentiation of cells in 24-well plates was induced in the absence or presence of 3-carene and on day 9, the ALP protein expression was observed by the ALP staining (A) and its ALP activity (units/mg) was measured in triplicate as described in 'Materials and Methods' (B). The data are expressed as mean  $\pm$  SD. #  $p < 0.001$  (compared with vehicle control).

the controls (Fig. 2). However, its stimulatory activity at 10 μM was shown to be lower than that at 5 μM.

Next, on differentiation day 15, the effect of 3-carene on mineral deposition was monitored by staining with Alizarin red S and measuring the amount of newly synthesized calcium (Fig. 3). Continuous treatment with 3-carene (up to 5 μM) was shown to accelerate strongly



**Figure 3.** Effect of 3-carene on the calcium formation on differentiation day 15. Alizarin red S staining was performed for the demonstration of mineralized nodule formation (A) and the amount of newly synthesized calcium was measured in triplicate as described in 'Materials and Methods' (B). The data are expressed as mean  $\pm$  SD. #,  $p < 0.001$  (compared with vehicle control).

calcium formation in the process of osteoblastic cell differentiation. The deposition in DM controls was observed from differentiation day 18 (data not shown).

In order to determine the anabolic mechanism of 3-carene, the effects of 3-carene on the protein expression/activation of the mitogen-activated protein (MAP) kinases and the transcript expression levels of osteoblast mineralization-related genes such as osteopontin and type I collagen were evaluated by Western blot analysis and real-time PCR, respectively. As shown in Fig. 4A, the protein expression levels of c-Jun *N*-terminal kinase (JNK) and p38 were induced and the activations of extracellular-signal regulated kinase (ERK) and JNK were also induced by 3-carene on day 9. On day 15, the protein expression level of JNK was dose-dependently induced by 3-carene and at 2.5  $\mu\text{M}$ , it was likely that 3-carene slightly induced the JNK activation. The transcript level of osteopontin was dramatically induced by 3-carene at a range of 2.5–5  $\mu\text{M}$  on day 15, and that of type I collagen was significantly induced from the

early stage of osteoblast differentiation and maintained to the stage of mineralization with higher fold change.

## DISCUSSION

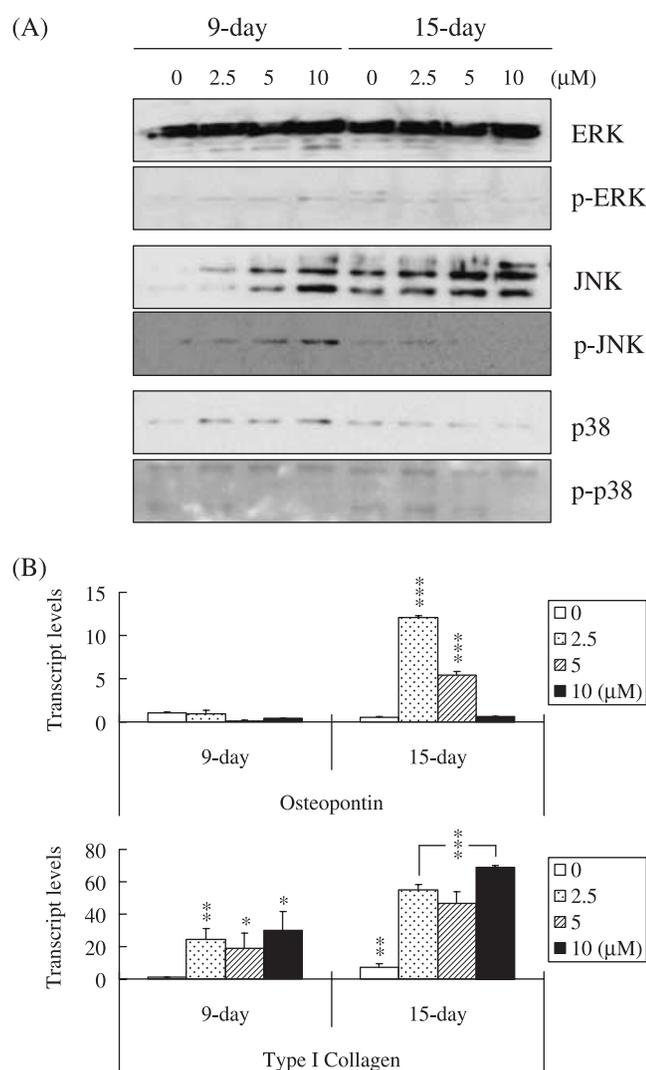
Recently, the development of anabolic agents has gained more interest in the field of osteoporosis. Therefore, natural compounds with an anabolic activity were screened by measuring the amount of newly synthesized calcium in MC3T3-E1 subclone 4 cells and it was found that low concentration of 3-carene could stimulate mineralization by promoting the protein expression and activation of MAP kinases and the gene expression of osteoblast mineralization-related genes such as osteopontin and type I collagen.

3-Carene is a bicyclic monoterpene which occurs naturally in pine trees. Monoterpenes, the major components of essential oils, belong to the group of isoprenoids containing 10 C-atoms, and as essential oils and monoterpenes have a pleasant odor and taste when used at a low concentration, they have been extracted since ancient times from many plants, both edible and inedible, and are used today as food additives. 3-Carene has several biological effects such as antiinflammatory activity (Ocete *et al.*, 1989), antibacterial activity (Pichette *et al.*, 2006), antifungal activity (Cavaleiro *et al.*, 2006) and inhibitory activity for acetylcholinesterase (Miyazawa and Yamafuji, 2005).

Several natural compounds have been reported to enhance osteogenic differentiation; one flavonoid from the herb *Epimedium*, icariin stimulated the proliferation of bone marrow stromal cells and also increased ALP activity and calcium deposition during osteogenic induction (Chen *et al.*, 2005). Menaquinone-7 (in the fermented soybean), genistein, daidzein (in soybean) and epigallocatechin-3-gallate (in green tea) have been also shown to stimulate the osteoblastic bone formation (Yamaguchi and Gao, 1998; Sugimoto and Yamaguchi, 2000; Yamaguchi *et al.*, 2001; Pan *et al.*, 2005; Vali *et al.*, 2007). Resveratrol, which is a phenolic compound found in the berry skins of most grape cultivars, directly stimulated cell proliferation and differentiation of osteoblasts (Mizutani *et al.*, 1998).

Interestingly, essential oils and monoterpenes present in common herbs have been shown to inhibit bone resorption when added to the food of rats (Muhlbauer *et al.*, 2003), but this is the first report that a low concentration of 3-carene, a monoterpene, stimulates the mineralization in osteoblastic cells and its activity could result from its potential to promote the activation of MAP kinase signaling pathways.

The MAP kinase signaling pathways are essential for regulating the response of osteoblasts to a variety of extracellular stimuli. Subsequently, the activation of these pathways can regulate the induction of genes such as osteopontin and type I collagen, of which products play an important role in the process of osteoblastic mineralization (Hipskind and Bilbe, 1998). Recently, the involvement of ERK and JNK activation in the induction of ALP activity and the matrix mineralization have been reported in MC3T3-E1 cells (Hanai *et al.*, 2006; Suzuki *et al.*, 2006). However, considering that the ERK and JNK cascades target an overlapping but not identical set of genes (Hipskind and Bilbe, 1998),



**Figure 4.** Effect of 3-carene on the activation of MAP kinases and the transcript expression levels of osteopontin and type I collagen. The activation of MAP kinases (ERK, JNK and p38) by 3-carene was evaluated by Western blot analysis (A) and the gene induction of osteopontin and type I collagen by 3-carene was evaluated by real-time PCR (B). The GAPDH-normalized fold changes are expressed as mean  $\pm$  SD. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  (compared with vehicle control on differentiation day 9).

the degree of cross-talk between MAP kinase pathways might play an important role in controlling the complexity of osteoblastic gene expression. This could suggest that the activation of MAP kinases by 3-carene was not consistent with its potential to promote the ALP activity/expression, the calcium formation and the transcript induction of osteopontin and type I collagen. Other signaling pathways and their cross-talk with MAP kinase pathways might be highly linked to the action mechanism of 3-carene.

In conclusion, 3-carene can stimulate mineralization by the induction of osteoblast mineralization-related genes such as osteopontin and type I collagen these genes via the induction/activation of MAP kinases in part. Further studies are required to determine the precise mechanism of its action, but with these results, it is suggested that the use of natural additives to the diet

including essential oils could have a beneficial effect on bone mineralization. Finally, it is hoped that this kind of study will elucidate the pharmacological and nutritional roles of natural compounds in the prevention of osteoporosis and the use of an improved cell-based screening system based on this system for identifying anabolic agents from natural/chemical substances will provide the initiative in early drug discovery and development for osteoporosis.

### Acknowledgements

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST) No. M10526020001-06N2602-00110 to JGJ, YKM and SHK, and by No. M10510010001-05N1001-00110 to YSK.

### REFERENCES

- Cavaleiro C, Pinto E, Goncalves MJ, Salgueiro L. 2006. Antifungal activity of Juniperus essential oils against dermatophyte, Aspergillus and Candida strains. *J Appl Microbiol* **100**: 1333–1338.
- Chen KM, Ge BF, Ma HP, Liu XY, Bai MH, Wang Y. 2005. Icarin, a flavonoid from the herb Epimedium enhances the osteogenic differentiation of rat primary bone marrow stromal cells. *Pharmazie* **60**: 939–942.
- Hanai Y, Tokuda H, Ishisaki A *et al.* 2006. The involvement of p44/p42 MAP kinase in insulin-like growth factor-I-induced alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells. *Mol Cell Endocrinol* **251**: 42–48.
- Harada S, Rodan GA. 2003. Control of osteoblast function and regulation of bone mass. *Nature* **423**: 349–355.
- Hipskind RA, Bilbe G. 1998. MAP kinase signaling cascades and gene expression in osteoblasts. *Front Biosci* **3**: d804–d816.
- Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the  $2^{-\Delta\Delta C_T}$  method. *Methods* **25**: 402–408.
- Miyazawa M, Yamafuji C. 2005. Inhibition of acetylcholinesterase activity by bicyclic monoterpenoids. *J Agric Food Chem* **53**: 1765–1768.
- Mizutani K, Ikeda K, Kawai Y, Yamori Y. 1998. Resveratrol stimulates the proliferation and differentiation of osteoblastic MC3T3-E1 cells. *Biochem Biophys Res Commun* **253**: 859–863.
- Muhlbauer RC, Lozano A, Palacio S, Reinli A, Felix R. 2003. Common herbs, essential oils, and monoterpenes potently modulate bone metabolism. *Bone* **32**: 372–380.
- Ocete MA, Risco S, Zarzuelo A, Jimenez J. 1989. Pharmacological activity of the essential oil of *Bupleurum gibraltarium*: anti-inflammatory activity and effects on isolated rat uteri. *J Ethnopharmacol* **25**: 305–313.
- Pan W, Quarles LD, Song LH *et al.* 2005. Genistein stimulates the osteoblastic differentiation via NO/cGMP in bone marrow culture. *J Cell Biochem* **94**: 307–316.
- Pichette A, Laurouche PL, Lebrun M, Legault J. 2006. Composition and antibacterial activity of *Abies balsamea* essential oil. *Phytother Res* **20**: 371–373.
- Putnam SE, Scutt AM, Bicknell K, Priestley CM, Williamson EM. 2007. Natural products as alternative treatments for metabolic bone disorders and for maintenance of bone health. *Phytother Res* **21**: 99–112.
- Rodan GA, Martin TJ. 2000. Therapeutic approaches to bone diseases. *Science* **289**: 1508–1514.
- Rozen S, Skaletsky HJ. 2000. Primer3 on the WWW for general users and for biologist programmers. In *Bioinformatics Methods and Protocols: Methods in Molecular Biology*, Krawetz S, Misener S (eds). Humana Press: Totowa, 365–386.
- Sugimoto E, Yamaguchi M. 2000. Anabolic effect of genistein in osteoblastic MC3T3-E1 cells. *Int J Mol Med* **5**: 515–520.
- Suzuki A, Ghayor C, Guicheux J *et al.* 2006. Enhanced expression of the inorganic phosphate transporter Pit-1 is involved in BMP-2-induced matrix mineralization in osteoblast-like cells. *J Bone Miner Res* **21**: 674–683.
- Vali B, Rao LG, El-Sohemy A. 2007. Epigallocatechin-3-gallate increases the formation of mineralized bone nodules by human osteoblast-like cells. *J Nutr Biochem* **18**: 341–347.
- van der Klift M, de Laet CD, Pols HA. 2005. Assessment of fracture risk: who should be treated for osteoporosis? *Best Pract Res Clin Rheumatol* **19**: 937–950.
- Wang D, Christensen K, Chawla K, Xiao G, Krebsbach PH, Franceschi RT. 1999. Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct *in vitro* and *in vivo* differentiation/mineralization potential. *J Bone Miner Res* **14**: 893–903.
- Yamaguchi M, Gao YH. 1998. Anabolic effect of genistein and genistin on bone metabolism in the femoral-metaphyseal tissues of elderly rats: the genistein effect is enhanced by zinc. *Mol Cell Biochem* **178**: 377–382.
- Yamaguchi M, Sugimoto E, Hachiya S. 2001. Stimulatory effect of menaquinone-7 (vitamin K2) on osteoblastic bone formation *in vitro*. *Mol Cell Biochem* **223**: 131–137.