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Inhibition of Osteoclast Differentiation by Tanshinones from the Root of *Salvia miltiorrhiza* Bunge

Song-Yi Lee, Doo-Youn Choi, and Eun-Rhan Woo

College of Pharmacy, Chosun University, Gwangju 501-759, Korea

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We screened natural products to find compounds with anti-osteoporotic potential using a coculture-based system by which osteoclast differentiation is effectively achieved. We found that methylene chloride soluble fraction of the root of *Salvia miltiorrhiza* Bunge (Labiatae) suppressed osteoclast differentiation. Five tanshinones, tanshinone IIA (**1**), tanshinone I (**2**), cryptotanshinone (**3**), 15,16-dihydrotanshinone I (**4**), and ferruginol (**5**) were subsequently isolated from fraction. Among the five compounds, compounds **1-4** reduced the formation of TRAP-positive multinuclear osteoclasts. These results suggest that the identified tanshinones may be useful candidates for development of therapeutic agents to treat osteoporosis and other bone-resorptive diseases.

Key words: *Salvia miltiorrhiza*, Labiatae, Tanshinones, Osteoclast differentiation

INTRODUCTION

Bone remodeling, a continual process that remove old calcified matrix and regenerate new one, is achieved by the coordinated actions of bone resorbing cells and synthesizing cells (Mundy 1999). The cell responsible for bone resorption is osteoclast and the cell generating new bone is osteoblast. An imbalance in the activity of osteoclasts and osteoblasts leads to adult skeletal defects including osteoporosis, Paget disease, and osteolysis associated with periodontal diseases and multiple myeloma. Osteoclasts originate from hematopoietic precursor cells of the monocyte/macrophage lineage and differentiate into multinucleated cells by the fusion of mononuclear progenitors (Suda *et al.*, 1999). Receptor activator of nuclear factor κB (RANK) ligand (RANKL) is a key osteoclastogenic molecule that directly binds to its cognate receptor, RANK, on osteoclast precursor cells (Anderson *et al.*, 1997; Yasuda *et al.*, 1998; Lee and Kim 2003). RANKL is required to induce the expression of genes that typify differentiated osteoclasts, including those encoding calcitonin receptor, c-src, and integrin β3 (Takeshita *et al.*, 2000; Lacey *et al.*, 2000; Miyamoto *et al.*, 2000).

Correspondence to: Eun-Rhan Woo, College of Pharmacy, Chosun University, 375 Seosuk-dong, Dong-ku, Gwangju 501-759, Korea
Tel: 82-62-230-6369, Fax: 82-62-222-5414
E-mail: wooer@.chosun.ac.kr

In an ongoing investigation into antiosteoporotic compounds from natural products, methylene chloride soluble fraction of *S. miltiorrhiza* was found to inhibit osteoclast differentiation an osteoblast/bone marrow coculture system. *S. miltiorrhiza*, which belongs to the family of Labiatae, has been traditionally used as an oriental herbal medicine and is reported to be efficacious for coronary heart disease (Tang and Eisenbrand, 1992). By means of a bioassay-directed chromatographic separation technique, five known abietane-type diterpenoids, tanshinone IIA (**1**), tanshinone I (**2**), cryptotanshinone (**3**), 15,16-dihydrotanshinone I (**4**), and ferruginol (**5**) were isolated. Among these compounds, tanshinone IIA, tanshinone I, cryptotanshinone, and 15,16-dihydrotanshinone I suppressed osteoclast generation. These results suggest potential usefulness of *S. miltiorrhiza* and the identified tanshinones in treatment of osteoporosis and other diseases involving bone loss.

MATERIALS AND METHODS

General procedure

The melting point was obtained with a Fisher Scientific melting point apparatus and uncorrected. Optical rotations were determined on a JASCO DIP-1000. UV spectra were obtained on a Shimadzu UV/Visible Spectrophotometer. IR spectra were recorded on an IMS 85 (Bruker). NMR spectra were recorded on a Varian Unity Inova 500 (500 MHz) spectrometer. ^{1}H-^{1}H COSY, DEPT, HMQC, and

HMBC NMR spectra were obtained with the usual pulse sequences. TLC and column chromatography were carried out on precoated Si Gel F_{254} plates (Merck, art. 5715), RP-18 F_{254} plates (Merck, art. 15423), and Si gel 60 (Merck, 230-400 mesh).

Plant materials

The root of *Salvia miltiorrhiza* Bunge (Labiatae) was purchased from local Korean herb drug market in Gwangju, Korea, and was authenticated by Department of Pharmacognosy, Chosun University. Voucher specimens were deposited in the Herbarium of College of Pharmacy, Chosun University (853-16).

Extraction and isolation

The root (0.6 Kg) of *S. miltiorrhiza* was extracted with MeOH at room temperature to afford 70.3 g of residue. The methanol extract was suspended in water and then partitioned by dichloromethane, ethyl acetate, and *n*-butanol in turn. Two grams of the CH_2Cl_2 fraction were subjected to column chromatography over a silica gel (300 g, 4.8 × 40 cm) eluting with a Hex-EtOAc (10:1 → 8:1 → 5:1 → 3:1 → 2:1 → 1:1 → 1:2), $CHCl_3$-MeOH (3:1, MeOH only) gradient system. The fractions were combined based on their TLC pattern to yield subfractions designated as D1-D11. Subfraction D2 (222.6 mg) was further purified by column chromatography over a silica gel, and Lichroprep RP-18 to afford compounds **1**, **2**, and **4**. Subfraction D4 (64.5 mg) was purified by column chromatography over a silica gel, and Sephadex LH 20 to give compound **3**. In addition, subfraction D1 (120.8 mg) was further purified by column chromatography over a silica gel, Sephadex LH 20, and Lichroprep RP-18 to give compound **5**.

Tanshinone IIA (1)

Orange needles in EtOAc, m.p.: 205-207°C; ^{1}H-NMR (500 MHz, $CDCl_3$), ^{13}C-NMR (125 MHz, $CDCl_3$): See Table I, and II.

Tanshinone I (2)

Reddish brown needles in MeOH, m.p.: 230-232°C; ^{1}H-NMR (500 MHz, $CDCl_3$), ^{13}C-NMR (125 MHz, $CDCl_3$): See Table I and II.

Cryptotanshinone (3)

Orange needles in hexane, m.p.: 191-193°C; $[\alpha]_D^{24}$ -22.6° (*c* 0.5, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$), ^{13}C-NMR (125 MHz, $CDCl_3$): See Table I and II.

15,16-Dihydrotanshinone I (4)

Reddish brown needles in MeOH, m.p.: 201-203°C; $[\alpha]_D^{24}$ -11.6° (*c* 0.5, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$), ^{13}C-NMR (125 MHz, $CDCl_3$): See Table I and II.

Ferruginol (5)

Oil, $[\alpha]_D^{24}$ +51.7° (*c* 0.5, MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$), ^{13}C-NMR (125 MHz, $CDCl_3$): See Table I and II.

Osteoclast culture

Osteoclast differentiation was induced by coculturing

Table I. ^{1}H-NMR data of compounds **1-5** (500 MHz, $CDCl_3$)

Position	1	2	3	4	5
1	3.19(*t*, 6.5)	9.26(*d*, 9.0)	3.22(*t*, 5.1)	9.31(*d*, 8.7)	2.15(*br dd*, 12.5, 7.5), 1.37(*dt*, 13.1)
2	1.79(*m*)	7.56(*dd*, 7.0, 2.0)	1.80(*m*)	7.58(*dd*, 8.7, 6.9)	1.85(*m*), 1.66(*m*)
3	1.66(*m*)	7.36(*d*, 7.0)	1.66(*m*)	7.41(*d*, 6.9)	1.45(*m*), 1.20(*d*, 4.0)
4	-	-	-	-	-
5	-	-	-	-	1.31(*dd*, 12.5, 5.0)
6	7.63(*d*, 8.0)	8.32(*d*, 9.0)	7.64(*d*, 8.1)	8.32(*d*, 8.4)	1.72(*td*, 13.5), 1.58(*m*)
7	7.55(*d*, 8.0)	7.83(*d*, 9.0)	7.49(*d*, 8.1)	7.77(*d*, 8.7)	2.87(*dd*, 7.0, 1.5), 2.77(*dd*, 11.5, 3.5)
11	-	-	-	-	6.62(*s*)
12	-	-	-	-	-
13	-	-	-	-	-
14	-	-	-	-	6.83(*s*)
15	7.22(*d*, 1.0)	7.31(*d*, 0.5)	4.37(*dd*, 9.3, 6.0), 4.89(*t*, 9.6)	4.44(*dd*, 9.6, 4.6), 4.98(*t*, 8.1)	3.11(*q*, 7.5)
16	-	-	3.60(*m*)	3.66 (*m*)	1.24(*s*)
17	2.26(*s*)	2.30(*d*, 1.0)	1.36((*d*, 6.6)	1.42(*d*, 6.6)	1.23(*d*, 1.5)
18	1.31(*s*)	2.70(*s*)	1.31(*s*)	2.71(*s*)	0.91(*s*)
19	1.31(*s*)	-	1.31(*s*)	-	0.93(*s*)
20	-	-	-	-	1.16(*s*)

Table II. ^{13}C-NMR data of compounds **1-5** (125 MHz, $CDCl_3$)

Position	1	2	3	4	5
1	29.89(*t*)	124.83(*d*)	29.64(*t*)	126.19(*d*)	38.87(*t*)
2	19.13(*t*)	130.70(*d*)	19.03(*t*)	130.46(*d*)	19.24(*t*)
3	37.87(*t*)	128.41(*d*)	37.76(*t*)	128.28(*d*)	41.70(*t*)
4	34.67(*s*)	135.21(*s*)	34.82(*s*)	135.00(*s*)	33.44(*s*)
5	150.14(*s*)	133.72(*s*)	143.67(*s*)	134.83(*s*)	50.36(*d*)
6	133.47(*d*)	132.96(*d*)	132.55(*d*)	132.18(*d*)	19.32(*t*)
7	119.91(*d*)	118.76(*d*)	122.48(*d*)	118.40(*d*)	29.76(*t*)
8	127.48(*s*)	129.70(*s*)	128.36(*s*)	128.89(*s*)	127.31(*s*)
9	126.52(*s*)	123.28(*s*)	126.22(*s*)	125.08(*s*)	148.68(*s*)
10	144.48(*s*)	132.83(*s*)	152.35(*s*)	131.97(*s*)	37.50(*s*)
11	183.66(*s*)	183.61(*s*)	184.00(*s*)	184.00(*s*)	110.99(*d*)
12	175.99(*s*)	175.76(*s*)	175.67(*s*)	175.99(*s*)	150.65(*s*)
13	121.16(*s*)	121.83(*s*)	118.26(*s*)	120.36(*s*)	131.39(*s*)
14	161.73(*s*)	161.26(*s*)	170.77(*s*)	170.61(*s*)	126.62(*d*)
15	141.29(*d*)	142.08(*d*)	81.43(*d*)	81.67(*d*)	26.82(*d*)
16	120.25(*s*)	120.55(*s*)	34.56(*t*)	34.75(*t*)	22.75(*q*)*
17	8.80(*q*)	8.81(*q*)	18.81(*q*)	18.86(*q*)	22.57(*q*)*
18	31.80(*q*)	19.87(*q*)	31.85(*q*)*	19.89(*q*)	21.62(*q*)
19	31.80(*q*)	-	31.89(*q*)*	-	33.31(*q*)
20	-	-	-	-	24.78(*q*)

*Assignments maybe interchanged.

mouse bone marrow cells and calvarial osteoblasts (Lee *et al.*, 2001; 2002). Primary osteoblasts were obtained by growing calvarial cells from ICR newborn mice for 1 day in α-MEM/10% FBS. Bone marrow cells were obtained by flushing tibiae from 6- to 7-week-old ICR mice with α-minimum essential medium (α-MEM; GIBCO BRL) containing 10% (v/v) fetal bovine serum (FBS; GIBCO BRL), 100 U/mL penicillin, 100 mg/mL streptomycin, and 10 ng/mL macrophage-colony stimulating factor (M-CSF; Peprotech EC). Then 1×10^7 to 2×10^7 bone marrow cells and 1×10^6 osteoblasts were seeded on a well of a 48-well plate and incubated for 6-7 days in the presence of 10^{-8} M VtD_3 and 10^{-6} M PGE_2. Osteoclasts were stained for tartarate-resistant acid phosphatase (TRAP) with the leukocyte acid phosphatase kit (Sigma, St. Louis, MO).

Statistical analysis

A paired Student's *t*-test was used to assess the significant differences among the treatment groups. The criterion for statistical significance was set at either $p<0.05$ or $p<0.01$.

RESULTS AND DISCUSSION

Natural compounds that suppress osteoclast differentiation may have therapeutic value in treating osteoporosis and other bone erosive diseases such as rheumatoid arthritis-associated bone loss. To screen natural compounds that have inhibitory activity on osteoclast differentiation, we used a mouse osteoblast/bone marrow cell coculture system. In this coculture system, multinuclear TRAP-positive osteoclasts were efficiently generated (Fig. 1A). Using this cell-based assay system, we found that a methylene chloride soluble fraction of *S. miltiorrhiza* strongly inhibited osteoclast differentiation (data not shown).

Subsequently, the methylene chloride soluble fraction was chromatographed on columns of silica gel, Sephadex LH 20, followed by Lichroprep RP-18. This purification process afforded five compounds (**1-5**, Fig. 2). Chemical structures of compounds **1-5** were identified as tanshinone IIA (Tezuka *et al.*, 1997), tanshinone I (Ryu *et al.*, 1997), cryptotanshinone (Tezuka *et al.*, 1997), 15,16-dihydrotanshinone I (Tezuka *et al.*, 1997), and ferruginol (Harrison and Asakawa 1987), respectively, by comparing the UV, IR, ^{1}H-NMR, and ^{13}C-NMR with those of reported in literature.

The effects of these five compounds on osteoclast differentiation were then investigated. Tanshinone IIA (**1**), tanshinone I (**2**), cryptotanshinone (**3**), and 15,16-dihydrotanshinone I (**4**) showed significant inhibitory effects on osteoclast formation at all concentrations tested (Fig. 1B). In contrast, ferruginol (**5**) inhibited osteoclast differentiation only at the highest concentration tested (Fig. 1B). The effects of these compounds on viability of cells involved in the coculture were examined. The viability of bone marrow cells was moderately inhibited by compounds **2**, **3**, and **5** (Fig. 1C). Compound **4** reduced bone marrow cell viability in a greater extent whereas compound **1** did not affect bone marrow cell viability (Fig. 1C). For osteoblasts, compounds **2** and **3** moderately decreased viability while compound **4** showed a greater reduction (Fig. 1D).

Given that compound **1** inhibited osteoclast differentiation without reducing cell viability of bone marrow cells and osteoblasts, the anti-osteoclastogenic effect may lie on interference with differentiation signaling rather than reduction in osteoclast precursor number or deterioration of the differentiation-supporting ability of osteblasts. To the contrary, the suppressive effect of compounds **2**, **3**, and **4** may be partly ascribed to the decrease in osteoclast precursor cell and osteoblast numbers, which may result from cytotoxicity or blockade in proliferation.

Taken together, these results demonstrate that compounds **1-4** could be mainly responsible for the potent antiosteoporotic effect of the methylene chloride soluble fraction of *S. miltiorrhiza*, and might be suitable for further development as a leading natural antiosteoporotic agent.

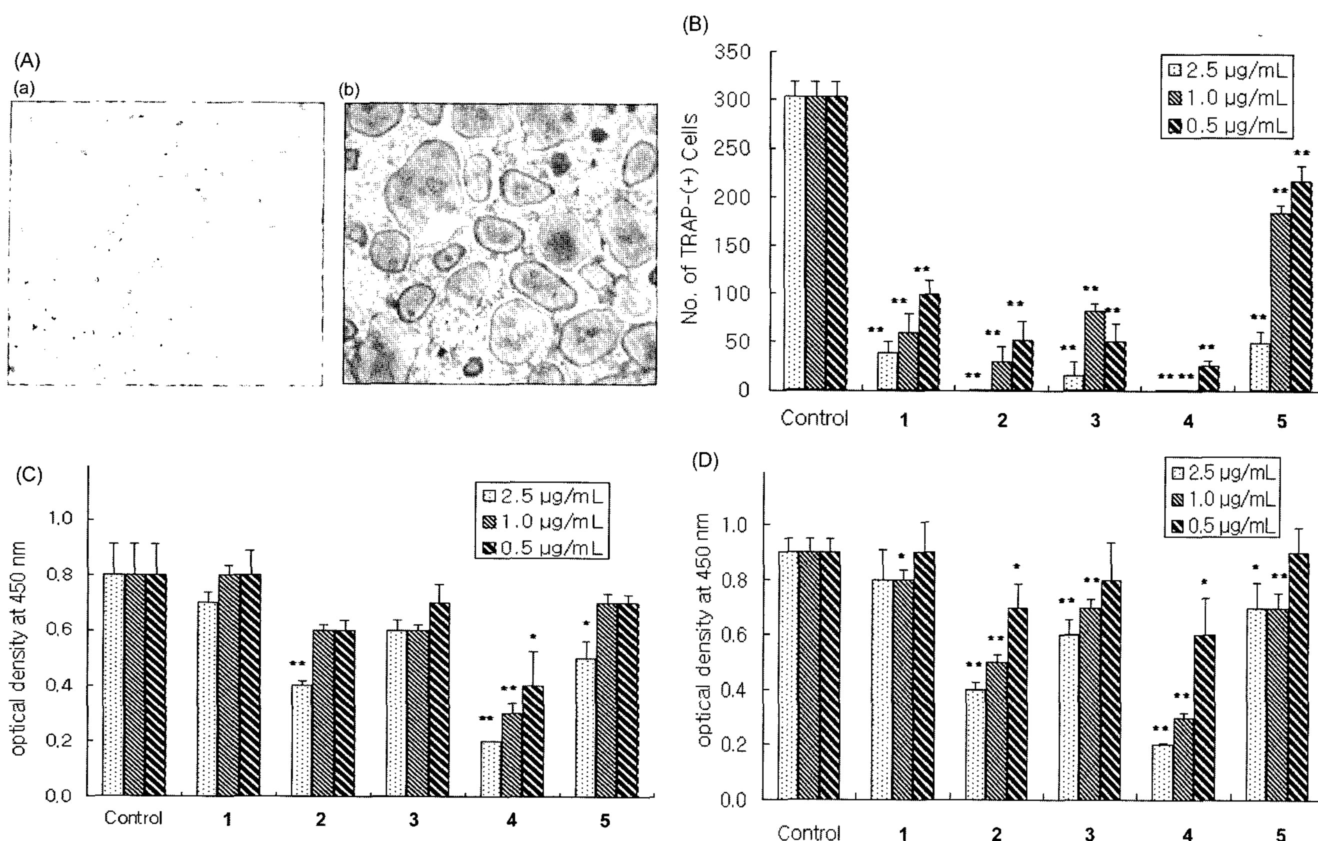

Fig. 1. Effects of compounds **1-5** on osteoclast differtiation. (A) Bone marrow cells and calvarial osteoblasts were cocultured in the absence (a) or presence (b) of VtD_3 and PGE_2 as described in materials and methods. Photographs of TRAP-stained cells are shown. (B) Bone marrow-derived osteoclast precursor cells were cultured with calvarial osteoblasts in the presence of VtD_3 and PGE_2 for 7 days. The indicated concentration of compounds **1-5** was added to the culture. Cells were fixed and stained for TRAP. TRAP-positive multinuclear cells containing three or more nuclei were counted as osteoclasts. Data from three experiments are presented as mean ± S.D. **P* <0.05; ***P*<0.01, significant differences from the control (n=3). (C) Bone marrow-derived osteoclast precursor cells were cultured in the presence of M-CSF and indicated concentrations of compounds **1-5** for 3 days. The MTT assay was performed and the absorbance was read at 450 nm. (D) Osteoblast cells were cultured in the presence of indicated concentrations of compounds **1-5** for 3 days. The MTT assay was performed and the absorbance was read at 450 nm.

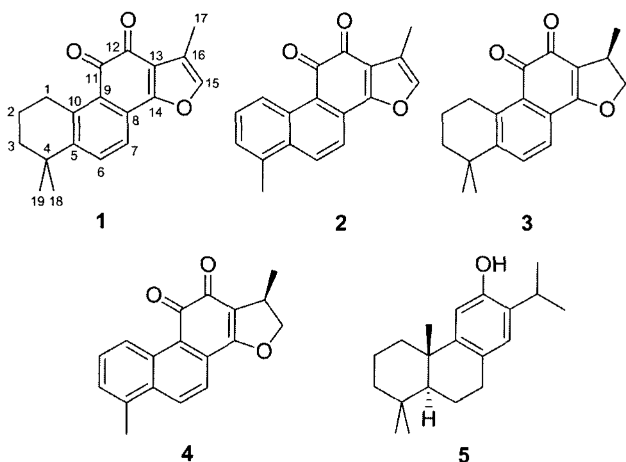

Fig. 2. Chemical structures of compounds **1-5** isolated from the root of *S. miltiorrhiza*

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