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Brief Article

# Discovery of Novel Benzopyranyl Tetracycles that Act as Inhibitors of Osteoclastogenesis Induced by Receptor Activator of NF-κB Ligand

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A novel benzopyran-fused molecular framework **7ai** was discovered as a specific inhibitor of RANKL-induced osteoclastogenesis using a cell-based TRAP activity assay from drug-like small-molecule libraries constructed by diversity-oriented synthesis. Its inhibitory activity was confirmed by in vitro evaluations including specific inhibition of RANKL-induced ERK phosphorylation and NF-κB transcriptional activation. **7ai** can serve as a specific small-molecule modulator for mechanistic studies of RANKL-induced osteoclast differentiation as well as a potential lead for the development of antiresorptive drugs.

## Introduction

Bones are constantly renewed by the delicate balance maintained between bone resorption by osteoclasts and bone formation by osteoblasts.[1] Osteoclasts are multinucleated cells derived from hematopoietic stem cells of monocyte/macrophage lineage and are closely related to macrophages.[2] Two critical factors supplied mainly by osteoblasts, the receptor activator of NF-κB ligand (RANKL[a]) and macrophage-colony stimulating factor (M-CSF), are essential for the formation of osteoclast precursors and for osteoclastogenesis in bones. In particular, RANKL is a key factor in the regulation process of osteoclastogenesis and maintaining the survival of mature osteoclasts.[1,3] The binding of RANKL to its receptor RANK triggers the activation of the signaling molecules involved in osteoclastogenesis and of transcription factors for the regulating genes required for the differentiation of osteoclasts and their bone resorptive activity.[4] The abnormal increase in the number and activity of osteoclasts due to aging or in the condition of diseases such as osteoporosis, rheumatoid arthritis, and cancer with bone metastasis leads to increased bone resorption.[5] Current therapeutic approaches to bone diseases mainly focus on the inhibition of bone resorption. Bisphosphonates, synthetic analogues of pyrophosphates, are currently the most important and effective antiresorptive drugs available,[6] but they have been shown to have gastrointestinal tract side effects, and long-term treatment with bisphosphonate leads to suppression of bone turnover, reducing the bone quality. It is insistent need for the development of new therapeutic agents for metabolic bone diseases. The mechanistic understanding of the differentiation, fusion, activation, and function of osteoclasts leads to the identification of various potential targets and strategies for the discovery of antiresorptive drugs. Inhibition of the osteoclastogenesis is one of the potential therapeutic approaches to bone diseases, particularly to pathological bone loss.[7] Several naturally occurring small molecules have been reported to inhibit the differentiation and functionalization of osteoclasts.[8] With the increasing study of osteoclast, the discovery of novel small-molecule osteoclastogenesis inhibitors can provide valuable insights into osteoclast biology and provide potential lead compounds for development of antiresorptive agents.

In this paper, we report a novel synthetic small-molecule inhibitor of RANKL-induced osteoclastogenesis that was identified by cell-based high-throughput screening of drug-like small-molecule libraries constructed by diversity-oriented synthesis (DOS). We previously reported a series of DOS pathways of novel heterocyclic core skeletons by the creative recombination of privileged substructural motifs such as benzopyran, pyrimidine, pyrazolopyrimidine, and benzodiazepine.[9] We have successfully constructed natural product-like small-molecule libraries with over 2000 members containing more than 30 unique core skeletons.[10] To identify small-molecule inhibitors of RANKL-induced osteoclastogenesis, we performed a cell-based tartrate-resistant acid phosphatase (TRAP) activity assay. When treated with RANKL, RAW264.7 (mouse leukemic monocyte/macrophage), a type of osteoclast progenitor cells, differentiated into TRAP-positive (TRAP$^+$) multinucleated osteoclasts.[11] By this high-throughput screening, we identified benzopyran-fused tetracyclic molecular frameworks **7**, which can effectively inhibit RANKL-induced osteoclastogenesis.

On the basis of this preliminary screening, we constructed a focused library containing 24 analogues for studying the structure−activity relationship of the molecular framework **7** to enhance their efficacy (Scheme 1). The analogues were

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[a]Abbreviations: RANKL, receptor activator of NF-κB ligand; M-CSF, macrophage-colony stimulating factor; DOS, diversity-oriented synthesis; TRAP, tartrate-resistant acid phosphatase; CCK-8, cell counting kit-8; BMM, bone marrow monocyte; NFATc1, nuclear factor of activated T cells.

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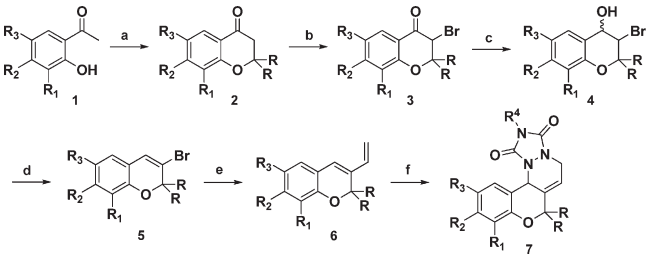
**Scheme 1.** Synthetic Scheme of the Novel Benzopyran-Fused Tetracyclic Molecular Framework **7**[a]



[a] Reagents and conditions: (a) ketone, pyrrolidine, EtOH, reflux; (b) $CuBr_2$, EtOAc/$CHCl_3$/ MeOH, reflux; (c) $NaBH_4$, EtOH, 40 °C; (d) *p*-TsOH, toluene, 80 °C; (e) vinyl boronic acid dibutyl ester, $Na_2CO_3$, $Pd(PPh_3)_4$, EtOH/toluene/$H_2O$, 70 °C; (f) 4-substituted 1,2,4-triazoline-3,5-dione derivatives, toluene, rt.

synthesized via the following series of reactions: (1) cyclization of substituted *o*-hydroxyacetophenone **1** with different ketones to afford **2**, (2) monobromination of **2** to afford **3**, (3) carbonyl reduction of **3** and subsequent dehydration under acidic condition yields vinylbromide-embedded benzopyranyl intermediates **5**, (4) palladium-mediated Suzuki cross-coupling of **5** with vinyl boronate to afford dienes **6**, (5) aza-Diels−Alder reaction of **6** with 4-substituted-1,2,4-triazoline-3,5-diones (commercial available or in-house synthesized, see Supporting Information Scheme S1) to obtain the desired molecular framework **7**. The exact structure of **7** was determined by NMR analysis as well as X-ray crystallography (Figure 1A).

After the construction of a 24-member focused library of **7**, we evaluated their inhibitory activities against RANKL-induced osteoclastogenesis. RAW264.7 cells were treated with 100 ng/mL of RANKL along with the treatment of each analogue of **7** at a concentration of 10 $\mu$M.[12] As shown in Table 1, several analogues of **7** could cause more than 50% inhibition of RANKL-induced osteoclastogenesis, which was determined by measuring the cellular TRAP activity. Their antiosteoclastogenic effects were not attributed to the cellular toxicity, which was confirmed by the cell viability test using CCK-8 assay toward RAW264.7 osteoclast progenitor cells (Table 2). We also confirmed that the olefin moiety on the tetracyclic molecular framework **7** is an essential element for the inhibitory activity of these analogues because we observed a significantly low inhibitory activity in the case of saturated analogue **7s** which was obtained by catalytic hydrogenation of **7a** with Pd/C in the presence of atmospheric hydrogen gas (Supporting Information Figure S3). In addition, the inhibitory activities of analogues of **7** with various substituents at the $R^1$, $R^2$, and $R^3$ positions on the benzene rings revealed that the phenolic hydroxyl moiety at the $R^2$ position is essential for the inhibitory activity. Further, the replacement of this hydroxyl group with other functional groups led to a significant decrease in the inhibitory activity (entries 1−8, Table 1), indicating that the inhibitory mechanism of **7a** may involve specific hydrogen-bonding interactions with the hydroxyl moiety at the $R^2$ position. The introduction of the cyclopentyl spiropyran moiety (**7i**) in place of the dimethyl moiety (**7a**) at the R position increased the inhibitory activity, but **7i** also exhibited cellular cytotoxicity against osteoclast precursor cells; therefore, **7i** was not considered for further biological evaluation (Table 2). On the other hand, the introduction of a piperidinyl moiety at the R position (**7j**, **7k**) led to a loss of their inhibitory activity. The significant reduction in the

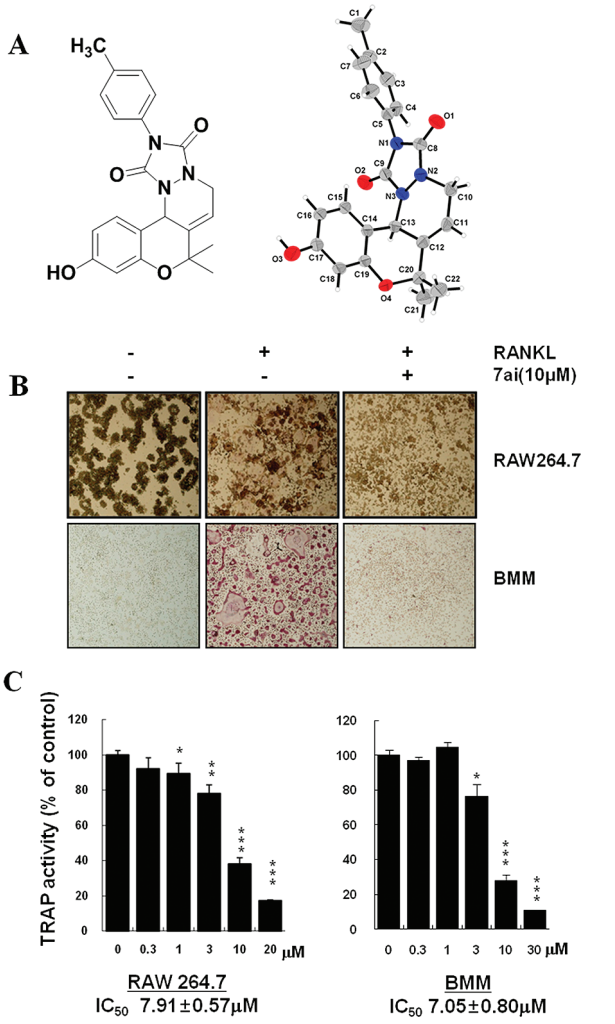



**Figure 1.** Compound **7ai** inhibit RANKL-induced osteoclastogenesis: (A) compound **7ai**, a novel inhibitor of RANKL-induced osteoclastogenesis and its X-ray crystal structure; (B) inhibitory efficacy of **7ai** on RANKL-induced osteoclastogenesis in RAW264.7 and BMM cells; (C) **7ai** reduced the TRAP activity in a dose-dependent manner. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

inhibitory activities upon the introduction of alkyl substituents at the $R^4$ position emphasized that the presence of a phenyl moiety at $R^4$ is essential for maximum inhibitory activity (entries 12−14, Table 1). The introduction of various substituents on the phenyl moiety of **7a** revealed that most of the substitutions at the $R^4$ position are not favorable to their inhibitory activity, but we confirmed that the presence of methyl and *tert*-butyl moieties at the *para* position on the phenyl ring (**7ai** and **7aii**, respectively) conserved the inhibitory activity of these compounds, similar to that of **7a**, by considering the inhibitory activity ($IC_{50}$ values) and their cytotoxicity toward osteoclast precursor cells (Table 2). On the basis of these results, we identified **7ai** as the best small-molecule inhibitor of RANKL-induced osteoclastogenesis. As shown in Figure 1B, **7ai** can significantly reduce the number of multinucleated mature osteoclasts both in RAW264.7 cells and in murine bone marrow monocyte (BMM; primary osteoclast precursor) cells as confirmed by TRAP staining. **7ai** caused a dose-dependent inhibitory activity against RANKL-induced osteoclastogenesis with an $IC_{50}$ value of approximately 7 $\mu$M in both RAW264.7 and BMM cells (Figure 1C).

**Table 1.** Inhibitory Effects of Synthetic Analogues with Molecular Framework **7** on RANKL-Induced Osteoclastogenesis Measured by %TRAP Activity

| entry | compd | R | $R^1$ | $R^2$ | $R^3$ | $R^4$ | %TRAP activity[a] |
|---|---|---|---|---|---|---|---|
| 1 | **7a** | $CH_3$ | hydro | hydroxy | hydro | phenyl | 43 ± 1.5 |
| 2 | **7b** | $CH_3$ | hydro | hydro | hydro | phenyl | 104 ± 2.2 |
| 3 | **7c** | $CH_3$ | hydro | methoxy | hydro | phenyl | 108 ± 5.2 |
| 4 | **7d** | $CH_3$ | hydro | methoxy | methoxy | phenyl | 99 ± 4.1 |
| 5 | **7e** | $CH_3$ | hydro | hydro | methoxy | phenyl | 100 ± 1.4 |
| 6 | **7f** | $CH_3$ | hydroxy | methoxy | hydro | phenyl | 69 ± 7.3 |
| 7 | **7g** | $CH_3$ | hydro | fluoro | hydro | phenyl | 121 ± 7.8 |
| 8 | **7h** | $CH_3$ | hydro | methylamino | hydro | phenyl | 107 ± 2.7 |
| 9 | **7i** | cyclopentyl[b] | hydro | hydroxy | hydro | phenyl | 30 ± 2.8 |
| 10 | **7j** | *N*-acetyl-4′-piperidyl[b] | hydro | hydroxy | hydro | phenyl | 113 ± 0.3 |
| 11 | **7k** | *N*-propyl-4′-piperidyl[b] | hydro | hydroxy | hydro | phenyl | 99 ± 5.2 |
| 12 | **7l** | $CH_3$ | hydro | hydroxy | hydro | cyclohexyl | 113 ± 4.1 |
| 13 | **7m** | $CH_3$ | hydro | hydroxy | hydro | methyl | 95 ± 4.7 |
| 14 | **7n** | $CH_3$ | hydro | hydroxy | hydro | benzyl | 98 ± 4.8 |
| 15 | **7ai** | $CH_3$ | hydro | hydroxy | hydro | *p*-methylphenyl | 50 ± 3.7 |
| 16 | **7aii** | $CH_3$ | hydro | hydroxy | hydro | *p*-*tert*-butylphenyl | 53 ± 4.8 |
| 17 | **7aiii** | $CH_3$ | hydro | hydroxy | hydro | *p*-methoxyphenyl | 93 ± 5.3 |
| 18 | **7aiv** | $CH_3$ | hydro | hydroxy | hydro | *m*-methoxyphenyl | 86 ± 3.5 |
| 19 | **7av** | $CH_3$ | hydro | hydroxy | hydro | *p*-fluorophenyl | 68 ± 5.6 |
| 20 | **7avi** | $CH_3$ | hydro | hydroxy | hydro | *o*-iodophenyl | 101 ± 0.7 |
| 21 | **7avii** | $CH_3$ | hydro | hydroxy | hydro | *m*-nitrophenyl | 105 ± 4.3 |
| 22 | **7aviii** | $CH_3$ | hydro | hydroxy | hydro | *p*-chlorophenyl | 112 ± 6.0 |
| 23 | **7aix** | $CH_3$ | hydro | hydroxy | hydro | *o*-chlorohenyl | 95 ± 3.6 |
| 24 | **7ax** | $CH_3$ | hydro | hydroxy | hydro | *p*-bromohenyl | 83 ± 3.2 |

[a] Relative TRAP activity of individual compounds at 10 μM in the presence of 100 ng/mL RANKL; 100% refers the difference of TRAP activity between the treatment of 100 ng/mL RANKL and vehicle. The screening was carried out in triplicate and the number here were the mean value containing standard derivations. [b] R is connected to the quaternary carbon through spiropyranyl connection.

**Table 2.** $IC_{50}$ of Compounds on RANKL-Induced Osteoclastogenesis and Cell Viability

| | $IC_{50}$ (μM) | | cell viability[a] | |
|---|---|---|---|---|
| compd | RAW264.7 | BMM | day 1 | day 4 |
| **7a** | 7.07 ± 0.30 | 6.13 ± 1.13 | 85.88 | 73.97 |
| **7i** | 5.09 ± 0.97 | 6.05 ± 1.28 | 77.79 | 45.96 |
| **7ai** | 7.97 ± 0.57 | 7.05 ± 0.80 | 100.33 | 86.86 |
| **7aii** | 6.94 ± 0.10 | 6.77 ± 0.61 | 79.67 | 73.43 |
| **7av** | 8.08 ± 0.30 | 11.94 ± 5.06 | 99.02 | 80.72 |
| **7f** | N/D | 22.20 ± 1.18 | N/D | N/D |
| **7b** | N/D | N/D | N/D | N/D |

[a] Cell viability was measured by CCK-8 assay in RAW264.7 cells upon treatment with individual compounds at 30 μM final concentrations for 1 day and 4 days. Cell viability represents the percentage of the control. N/D: Not determined due to its low inhibitory activity.

After identifying novel small-molecule inhibitors of RANKL-induced osteoclastogenesis, we examined whether **7ai** and **7a** could induce any perturbations in the signaling cascades that were monitored by immunoblot analysis. Upon treatment with RANKL, the intracellular receptor RANK recruits adaptor molecules such as TRAF6 and activates multiple downstream signaling pathways.[13] NF-κB and MAPKs (JNK, p38, and ERK) are involved in the early signaling event induced by RANKL.[1,14] In this study, RAW264.7 cells were treated with RANKL in the presence of three analogues of **7** (**7a**, **7ai**, and **7b**), and the cell lysates were analyzed by Western blotting. We selected **7b** as a negative control because it is structurally almost identical to active compound **7a** except for the absence of hydroxyl moiety at $R^2$ position. As shown in Figure 2A, our novel antiosteoclastic agents **7ai** and **7a** can selectively inhibit the RANKL-induced activation of ERK phosphorylation but not in the case of the inactive analogue **7b**. However, the RANKL-induced phosphorylation of p38 was inhibited by these three analogues, suggesting that the p38 phosphorylation can be blocked by the treatment with analogues of **7** due to its structural similarity, but the inhibitory effect of **7b** on p38 phosphorylation is insufficient to obstruct the RANKL-induced formation of TRAP-positive multinucleated osteoclasts. This led us to conclude that the interference with ERK activation may be responsible for the inhibitory effects of **7ai** and **7a**; therefore, the potential target protein of our antiosteoclastic agents may lie the upstream of ERK phosphorylation. Moreover, NF-κB is activated and translocated into the nucleus upon treatment with RANKL, which results in the expression of target genes for osteoclast differentiation. The involvement of NF-κB in the inhibitory effect of **7a** or **7ai** on the osteoclastogenesis was confirmed by NF-κB luciferase activity assay (Figure 2B); the RANKL-induced transcriptional activation of NF-κB was dramatically inhibited by the treatment of either **7a** or **7ai** in a dose-dependent manner but not by **7b**.

We then monitored the expression levels of the biomarkers and specific transcription factors involved in osteoclastogenesis. The key biomarkers of osteoclastogenesis are the following: MMP-9, one of main matrix metalloproteases involved in the invasive activity of osteoclast,[15] c-Src, intracellular signal transduction molecule required for osteoclast activation,[16] as well as TRAP.[17] As shown in Figure 3A, the expression levels of these osteoclastogenesis biomarkers were significantly reduced upon treatment with our antiosteoclastic agents **7ai** and **7a** at 10 μM. However, the treatment with structurally similar inactive analogue **7b** at the same concentration resulted in a drastic difference in the expression levels. Transcription factors also play a critical role in RANKL-induced osteoclast development. For example, NFATc1 (nuclear factor of activated T cells, calcineurin-dependent 1, also known as NFAT2) and c-fos (a member of the AP-1 protein family,

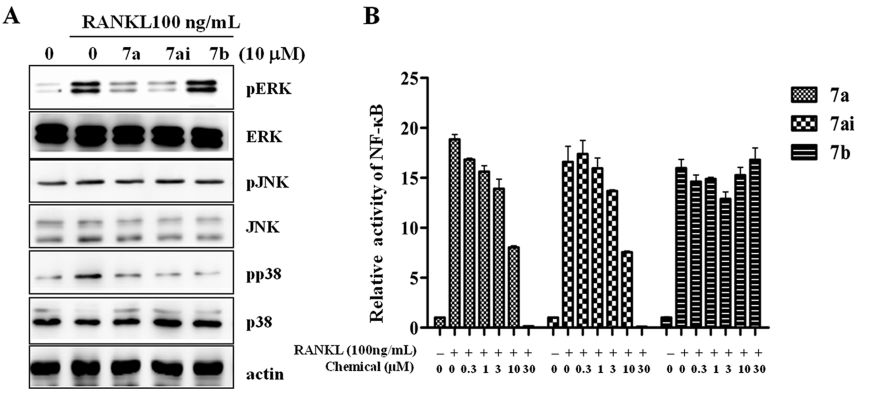



**Figure 2.** Inhibitory effects on RANKL-induced early signaling pathways upon treatment with analogues of **7** in RAW264.7 cells. (A) Western blot analysis of RANKL-induced activation of MAPK signaling pathways in RAW264.7 cells; actin was used as the internal control for cytosolic fractions. (B) NF-$\kappa$B luciferase activity assay data to confirm the inhibitory effect of RANKL-induced NF-$\kappa$B activation upon treatment with analogues of **7** in RAW264.7 cells.

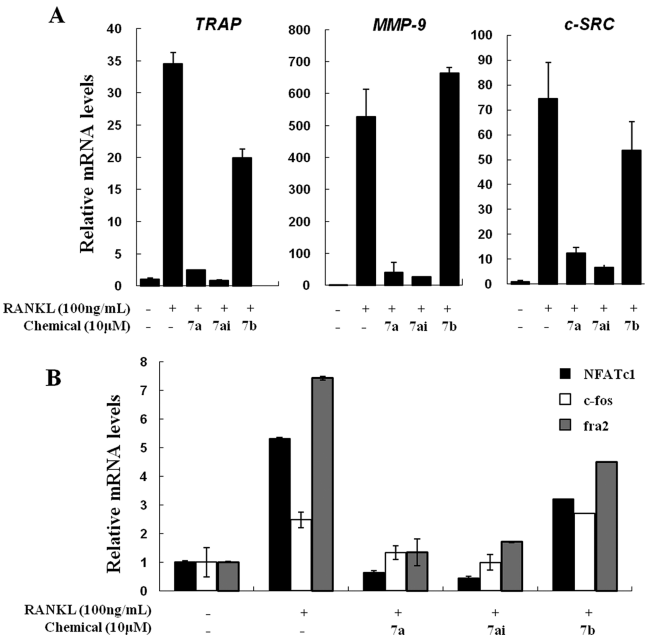



**Figure 3.** In vitro confirmation of the inhibitory efficacy of **7a** and **7ai**. (A) Real-time PCR analysis of mRNA expression levels of specific biomarkers for osteoclastogenesis, such as TRAP, MMP-9, c-Src, in RAW264.7 cells. (B) Real-time PCR analysis of mRNA expression level of key transcription factors in osteoclastogenesis, such as NFATc1, c-fos, and fra2.

such as c-fos, fosB, fra1, fra2) activate gene transcription in osteoclast differentiation, which can be strongly induced by RANKL.[8] In particular, NFATc1 is supposed to be one of the key target genes of NF-$\kappa$B in the early phase of osteoclastogenesis. By real-time PCR analysis, we confirmed that the RANKL-induced mRNA expression of c-fos, NFATc1, and fra2 was dramatically down-regulated upon treatment with **7ai** and **7a** but not upon treatment with inactive analogue **7b** (Figure 3B).

## Conclusion

In summary, we identified a novel benzopyran-fused molecular framework **7** that acts as an inhibitor of RANKL-induced osteoclastogenesis; this was done by using a cell-based TRAP activity assay from drug-like small molecule libraries constructed by diversity-oriented synthesis. The subsequent systematic structure−activity relationship study established **7ai** as a promising antiosteoclastic agent. Its inhibitory activity toward RANKL-induced osteoclast differentiation was confirmed by in vitro evaluations including specific inhibitions of ERK phosphorylation, NF-$\kappa$B activation, and mRNA expression of key osteoclast biomarkers and transcription factors involved in RANKL-induced osteoclastogenesis. Even though the further in vivo evaluation of **7ai** is currently underway to confirm the specific inhibition of RANKL-induced osteoclastic bone resorption, this novel molecular framework can be used as a specific small-molecule modulator for mechanistic studies of RANKL-induced osteoclastogenesis as well as potential lead compounds for the development of antiresorptive drugs. Since we determined that the inhibition of signaling process on ERK phosphorylation and NF-$\kappa$B activation is the major cause of the inhibitory activity toward RANKL-induced osteoclastogenesis, it is important to identify the molecular target of this antiosteoclastic agent; studies for target identification will be reported in due course.

## Experimental Section

**Cell Culture and RANKL-Induced Osteoclast Differentiation.** All the materials for the cell culture were purchased from HyClone (UT, USA). Osteoclast generation was achieved using either RAW264.7 cells or primary cultures of mouse BMMs. Mouse monocyte/macrophage RAW264.7 cells were purchased from the American Type Culture Collection (VA, USA) and maintained at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1× antibiotic−antimycotic (Gibco, CA, USA) in a humidified atmosphere with 5% $CO_2$. The medium was changed every 3 days. For osteoclast differentiation, RAW264.7 cells were suspended in α-minimal essential medium (MEM) supplemented with 10% FBS in the presence of 100 ng/mL RANKL (R&D Systems, MN, USA) and plated at a density of $1 \times 10^3$ cells/well in a 96-well plate (on differentiation day 0). Multinucleated osteoclasts were observed on differentiation day 4. For the generation of BMM-derived osteoclasts, monocytes were isolated from the femurs and tibiae of BALB/c mice (Central Lab Animal, Korea), seeded and cultured in α-MEM with 10% FBS and 10 ng/mL macrophage colony stimulating factor (M-CSF; R&D Systems) for 1 day. The suspended cells were then transferred into new plates and cultured in α-MEM with 10% FBS and 30 ng/mL M-CSF for 3 days; adherent cells at this stage were considered to be M-CSF-dependent BMMs and were used as osteoclast precursors. Differentiation of BMMs into osteoclasts was achieved after plating the former into 96-well plates at

a density of $3 \times 10^5$ cells/well in α-MEM with 10% FBS, 100 ng/mL RANKL, and 30 ng/mL M-CSF (on differentiation day 0). Multinucleated osteoclasts were observed from differentiation day 6 onward.

**TRAP Staining and Activity Assay.** To identify compound with the potential to inhibit the RANKL-induced osteoclastogenesis, RAW264.7 cells were suspended in α-MEM with 10% FBS and 100 ng/mL RANKL and then plated in a 96-well plate at a density of $1 \times 10^3$ cells/well. After 24 h, cells were treated with compound or vehicle (DMSO). On differentiation day 4, cells were fixed with 10% formalin for 10 min and ethanol/acetone (1:1) for 1 min and then stained with a leukocyte acid phosphatase kit 387-A (Sigma, MO, USA). Images of the cells were captured by a microscope with DP Controller (Olympus Optical, Japan). To measure the TRAP activity, osteoclasts were fixed with 10% formalin for 10 min and 95% ethanol for 1 min, and then 100 $\mu$L of citrate buffer (50 mM, pH 4.6) containing 10 mM sodium tartrate and 5 mM *p*-nitrophenylphosphate (Sigma, MO, USA) was added to the fixed cells-containing wells of a 96-well plate. After incubation for 1 h, the enzyme reaction mixtures in the wells were transferred to new plates containing 100 $\mu$L of 0.1 N NaOH. Absorbance was measured at 410 nm using a Wallac EnVision microplate reader (PerkinElmer, Finland). The experiment was performed in triplicate, and the significance was determined by using the Student's *t* test and the differences were considered to be significant at $P < 0.05$.

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**Supporting Information Available:** All experimental procedures, spectroscopic characterization data of all new compounds, and procedures for biological studies (pdf and cif). This material is available free of charge via the Internet at http://pubs.acs.org.

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