The 1,2,3-triazole derivative KP-A021 suppresses osteoclast differentiation and function by inhibiting RANKL-mediated MEK-ERK signaling pathway

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Abstract

The triazole family of compounds has been implicated in modulating various biological processes such as inflammation, tumorigenesis, and infection. To our knowledge, this is the first study to demonstrate the effects of 1,2,3-triazole substituted biarylacrylonitrile compounds, including KP-A021, on the differentiation and function of osteoclasts. KP-A021 and its triazole derivatives, at a concentration that does not cause a cytotoxic response in bone marrow macrophages (BMMs), significantly inhibited osteoclast differentiation induced by receptor activator of nuclear factor- κ B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) as assessed by tartrate-resistant acid phosphatase (TRAP) staining. KP-A021 also dramatically inhibited the expression of marker genes associated with osteoclast differentiation, such as *TRAP*, cathepsin K (*Cat K*), dendritic cell-specific transmembrane protein (*DC-STAMP*), and nuclear factor of activated T-cells, cytoplasmic 1 (*NFATc1*). Furthermore, KP-A021 inhibited actin ring formation in osteoclasts as well as resorption pit formation induced by osteoclasts. Analysis of the signaling pathway for KP-A021 indicated that this triazole compound inhibited the RANKL-induced activation of extracellular signal-regulated kinase (ERK) and its upstream signaling molecule, mitogen-activated protein kinase kinase1/2 (MEK1/2). Taken together, these results demonstrate that KP-A021 has an inhibitory effect on the differentiation and function of osteoclasts via modulation of the RANKL-induced activation of the MEK-ERK pathway.

Keywords: Triazole family, KP-A021, osteoclast, differentiation, bone resorption

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Introduction

Bone remodeling is tightly regulated by the cooperation of bone-forming osteoblasts and bone-resorbing osteoclasts. However, imbalance between bone formation and resorption can cause skeletal diseases such as osteoporosis and osteopetrosis.^{1,2} In particular, abnormal elevation of osteoclast formation and function induces inordinate bone resorption, and is one of the principal mechanisms associated with osteoporosis, rheumatoid arthritis, and Paget's disease.^{1,3,4}

Bone-resorbing osteoclasts originate from the monocyte/macrophage lineage of cells, and have unique morphologic features such as multiple nuclei and ruffled borders.^{4,5}

Differentiation of monocytes/macrophages into mature osteoclasts is mediated by macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL).^{1,6} The binding of M-CSF to its receptor activates the signaling pathways for the proliferation and survival of osteoclast progenitor cells. Signaling via RANKL/RANK primarily induces the formation and activation of osteoclasts.⁷ Binding of RANKL to RANK recruits tumor necrosis factor receptor-associated factor 6 (TRAF6) to the cytoplasmic domain of RANK. This action leads to the activation of mitogen-activated protein kinases (MAPKs) such as p38, ERK, and c-Jun N-terminal kinase (JNK) as well as transcription factors such as nuclear factor

Figure 1 Inhibitory effect of KP-A021 on RANKL-induced osteoclast differentiation. (a) Chemical structure of KP-A021. (b) Mouse BMMs were treated with M-CSF (10 ng/mL) and RANKL (20 ng/mL) in the presence or absence of 1 μM or 5 μM KP-A021. Osteoclasts were stained with TRAP. (c) TRAP-positive multinucleated cells having ≥ 3 nuclei were scored. Data are means $\pm$ SD. ** $P < 0.01$ compared with the control. (d) BMMs were cultured with M-CSF (10 ng/mL) in the presence or absence of 1 μM or 5 μM KP-A021. After 3 days, cell viability was determined using the MTT assay. The results are the representative of at least three experiments. (A color version of this figure is available in the online journal.)

kappa-light-chain-enhancer of activated B cells (NF- κ B), activator protein-1 (AP-1), and nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1).^{6,8} These transcription factors stimulate the expression of genes involved in the differentiation and activation of osteoclasts, including tartrate-resistant acid phosphatase (TRAP), cathepsin K (*Cat K*), and dendritic cell-specific transmembrane protein (DC-STAMP).^{9–11} Therefore, suppression of RANKL-induced osteoclast differentiation might be an efficient way to control enhanced osteoclast formation as well as bone resorption.

Triazoles are heterocyclic compounds comprising three nitrogen and two carbon atoms. They can bind to various biological molecules, including enzymes and receptors. Given this fact, triazole-based compounds have been shown to have diverse biological activities, including anti-inflammatory, anti-tumor, anti-tubercular, and anti-fungal activities.^{12,13} In addition, many triazole derivatives have low toxicity, enhanced bioavailability, and few side effects.^{14,15} Therefore, researchers have attempted to use triazole derivatives as therapeutic agents against infection, cardiac disease, and seizures.¹⁶ However, few reports have reported the direct effect of triazole derivatives on the differentiation and function of osteoclasts.

Here, we report that triazole-based compounds, including KP-A021, inhibit the activity of RANKL-induced osteoclast differentiation and bone resorption. Furthermore, we demonstrated that the inhibitory effect of KP-A021 is mediated by suppression of RANKL-induced activation of MEK and ERK.

Materials and methods

Materials and antibodies

RANKL and M-CSF were obtained from R&D Systems (Minneapolis, MN). Antibodies against phospho-p38, phospho-JNK, phospho-ERK, phospho-Akt, phospho-MEK1/2, and ERK were purchased from Cell Signaling Technology (Beverly, MA). KP-A021, 3-(4-fluorophenyl)-2-{4-[1-(3-morpholino-3-oxopropyl)-1H-1,2,3-triazol-4-yl]phenyl}acrylonitrile (Figure 1a) is the compound of in-house chemical library, which consists of diverse heterocycles, and was synthesized as described previously.^{12,17}

Osteoclast differentiation

Osteoclast differentiation was induced following a previously described method.¹⁸ Briefly, murine bone marrow cells isolated from the tibiae and femora of ICR mice (Dae Han Bio Link. Co., Ltd, Chungbuk, South Korea; 6–8 weeks) were cultured for 24 h in a humidified incubator (5% CO₂ in air) at 37°C. The next day, non-adherent cells were harvested, centrifuged in a Histopaque gradient centrifuge (Sigma-Aldrich, St. Louis, MO), and cultured in α -minimal essential medium (α -MEM) supplemented with 10% fetal bovine serum (FBS) and M-CSF (30 ng/mL). After 3 days, attached cells were used as bone marrow-derived macrophages (BMMs). For osteoclastogenesis, BMMs were plated in 96-well culture plates at 2×10^4 cells/well and cultured in α -MEM containing 10% FBS, 20 ng/mL RANKL, and 10 ng/mL M-CSF in the absence or presence of 1 μM or 5 μM KP-A021. KP-A021 was dissolved in dimethyl sulfoxide

(DMSO); DMSO was used as the vehicle. Cells were cultured for 4–6 days and stained with TRAP following the manufacturer's instructions (Sigma-Aldrich). TRAP-positive multinucleated cells (MNCs) containing ≥ 3 nuclei were counted as osteoclast-like cells.

Cell viability

The effect of KP-A021 on cell viability was determined using the methyl-thiazol tetrazolium (MTT) cytotoxicity assay (Sigma-Aldrich). BMMs were seeded in 96-well culture plates and incubated with 10 ng/mL M-CSF with or without 5 μ M KP-A021. After 3 days, MTT was added and the insoluble formazan was extracted with DMSO. The absorbance of each well was measured at 570 nm by using a 96-well microplate reader (BioRad, Hercules, CA).

Analyses of gene expression

Total RNA was prepared using TRI-solution (Bioscience, Seoul, South Korea) and reverse-transcribed with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Primers and PCR conditions for *Trap*, *Cat K*, *Dcstamp*, *Nfatc1*, and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) were as described previously.^{18–20} PCR products were electrophoresed on 1.5% agarose gels and visualized by staining with ethidium bromide. Images were captured and expression levels of genes quantified using a gel documentation system (Wisdoc system, Daihan-Sci, Seoul, Korea).

Immunoblotting

Cells were lysed in RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 10% glycerol) supplemented with protease and phosphatase inhibitor cocktail. Protein samples (25 μ g) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Whatman, Florham Park, NJ). After blocking with 3% non-fat milk, membranes were incubated with primary antibodies at 4°C overnight and then with the appropriate secondary antibodies. Protein bands were detected by WesternBright ECL (Advansta, Menlo Park, CA).

Staining of actin ring

BMMs seeded on glass coverslips were cultured with M-CSF (10 ng/mL) and RANKL (20 ng/mL) with or without 5 μ M KP-A021 for 4 days. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. F-actin and nuclei were stained with rhodamine-conjugated phalloidin (Cytoskeleton, Denver, CO) and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Santa Cruz Biotechnology, Santa Cruz, CA), respectively. Images were taken using a BX51 Fluorescent Microscope (Olympus, Tokyo, Japan).

Bone resorption pit assay

BMMs were seeded on bone slices (IDS Nordic Bioscience, Herlev, Denmark) and cultured with M-CSF (10 ng/mL)

and RANKL (20 ng/mL). After 3 days, cells were treated with or without 5 μ M KP-A021 for an additional 2 days. Attached cells were then removed in 1N NaOH for 20 min, and resorption pits were identified by staining with hematoxylin for 30 s. The pit area was measured using the i-Solution image analysis program (IMT i-Solution, Daejeon, South Korea).

Statistical analyses

Data are means $\pm$ SD and statistical analyses were evaluated by the two-tailed Student's *t*-test. $P < 0.05$ or $P < 0.01$ was considered significant.

Results

KP-A021 inhibits RANKL-induced osteoclast differentiation

To examine whether KP-A021 inhibits RANKL-induced osteoclast differentiation, mouse BMMs stimulated with M-CSF and RANKL were treated with or without KP-A021. TRAP-positive MNCs were formed in response to M-CSF and RANKL in the positive control after 4–6 days of culture (Figure 1b). However, addition of KP-A021 strongly inhibited the osteoclast differentiation of BMMs (Figure 1b). In addition, the number of TRAP-positive MNCs was reduced remarkably by 62.2% at 1 μ M and 85.8% at 5 μ M KP-A021 ($P < 0.01$), respectively (Figure 1c). To determine if the inhibitory effect was caused by the toxicity of KP-A021, we examined cytotoxicity using the MTT assay. KP-A021 (≤ 5 μ M) did not exhibit cytotoxic effects on BMMs (Figure 1d). These results suggested that KP-A021 specifically inhibited the RANKL-induced osteoclast differentiation of BMMs.

Triazole family of compounds exerts inhibitory activity on osteoclast differentiation

We asked whether the triazole family of compounds, other than KP-A021, exerts inhibitory effects on osteoclast differentiation. To address this question, we synthesized structurally similar triazole derivatives of KP-A021, named as KP-A020, KPLA-1014, and KPLA-1015 (Supplementary Figure 1). These derivatives were modified with a fluoride, methyl, or ethyl group. Cytotoxicity analysis showed that KP-A020, KPLA-1014, and KPLA-1015 had cytotoxic effects at a concentration of 5 μ M (Figure 2a). At a lower concentration (1 μ M), however, both KP-A020 and KPLA-1015 did not show cytotoxicity (Figure 2a). Therefore, at a concentration that does not induce a cytotoxic response, the effect of the derivatives on osteoclast differentiation was examined. As shown in Figure 2(b) and (c), TRAP-positive osteoclast numbers were significantly reduced by triazole derivatives such as KP-A021 and KPLA-1015. These results suggest that KP-A021 and structurally related triazole derivatives have ability to suppress osteoclast differentiation of BMMs.

Figure 2 Effect of KP-A021 and other triazole derivatives on cytotoxicity and osteoclast differentiation. (a) BMMs were cultured with M-CSF (10 ng/mL) in the presence or absence of 1 μ M or 5 μ M KP-A021, KP-A020, KPLA-1014, and KPLA-1015. After 3 days, cell viability was determined using the MTT assay. * $P < 0.05$, ** $P < 0.01$ compared with the control. (b) Mouse BMMs undergoing osteoclast differentiation in response to M-CSF (10 ng/mL) and RANKL (20 ng/mL) were treated with or without 5 μ M KP-A021, 1 μ M KP-A020, 1 μ M KPLA-1014, and 1 μ M KPLA-1015. Osteoclasts were stained with TRAP. (c) TRAP-positive multinucleated cells having ≥ 3 nuclei were scored. Data are means $\pm$ SD. ** $P < 0.01$ compared with the control. The results are the representative of at least three experiments. (A color version of this figure is available in the online journal.)

KP-A021 inhibits expression of osteoclast marker genes

To confirm the inhibitory effect of KP-A021, expression of the osteoclast marker genes, including *Trap*, *Cat K*, *Dcstamp*, and *Nfatc1* was analyzed. Consistent with TRAP staining, expression of the osteoclast marker genes was induced markedly after 4–6 days in response to M-CSF and RANKL (Figure 3). However, the increased expression of marker genes was suppressed considerably by treatment with KP-A021 (Figure 3). In particular, mRNA expression of *Dcstamp* (the key molecule involved in fusion of osteoclast progenitors) and *Nfatc1* (the master transcription factor for osteoclast differentiation) was decreased dramatically by KP-A021 (Figure 3). Quantitative densitometry

also showed a significant down-regulation of the marker genes. The expression of *Trap* was inhibited by approximately 50%, and those of *Dcstamp* and *Nfatc1* were inhibited by >80% by KP-A021 compared to the positive control (Figure 3 right). These results suggest that KP-A021 significantly inhibits the expression of genes involved in the differentiation, fusion, and function of osteoclasts.

KP-A021 suppresses cytoskeletal organization and bone resorption

Bone resorption takes place after the actin ring (a unique cellular structure of mature osteoclasts and essential for the

Figure 3 Effect of KP-A021 on the mRNA expression of genes associated with osteoclast differentiation. mRNA expression of the indicated osteoclastic marker genes (*Trap*, *CatK*, *Dcstamp*, and *Nfatc1*) was analyzed using RT-PCR, and shown as relative fold compared with the RANKL group. * $P < 0.05$, ** $P < 0.01$ compared with the control. The results are the representative of at least three experiments

formation of the sealing zone) is formed.^{21,22} Thus, we asked whether KP-A021 also modulates cytoskeletal reorganization during osteoclast differentiation. As shown in Figure 4(a), KP-A021 treatment suppressed the formation of actin rings as well as the cell-cell fusion of osteoclast progenitors (Figure 4a). These results strongly suggest that KP-A021 suppresses bone resorption possibly by impairment of appropriate actin ring formation. Examination of the effect of KP-A021 on the bone-resorption activity of osteoclasts indicated that KP-A021 remarkably inhibited pit formation by osteoclasts (93% reduction) compared to the control (Figure 4b). Taken together, these data strongly suggest that KP-A021 suppresses the bone-resorption activity of osteoclasts by inhibiting cytoskeletal reorganization.

KP-A021 inhibits osteoclast differentiation by suppressing RANKL-induced MEK-ERK phosphorylation cascade

RANKL activates a variety of signaling pathways and transcription factors, including MAPKs, Akt, AP-1, NFATc1, and NF- κ B in osteoclasts. To examine how KP-A021 inhibits RANKL-induced osteoclast differentiation, the phosphorylation of MAPKs and Akt in response to RANKL was examined. RANKL stimulation strongly induced the phosphorylation of p38, ERK, JNK, and Akt at 15 min (Figure 5a). Interestingly, treatment with KP-A021 suppressed only RANKL-induced phosphorylation of ERK (Figure 5a). Phosphorylation of other signaling molecules such as p38, JNK, and Akt was not changed by KP-A021 (Figure 5a). Furthermore, phosphorylation of MEK1/2 was inhibited by KP-A021, suggesting that KP-A021 may target the MEK-ERK signaling pathway (Figure 5b). Taken together, these results imply that KP-A021 suppresses activation of the MEK-ERK signaling pathway, leading to inhibition of osteoclast differentiation.

Discussion

Numerous reports have shown that the enhanced differentiation and activation of osteoclasts are primarily associated

with bone loss.^{4,23,24} Thus, managing osteoclast formation and bone resorption is considered the most efficient way to prevent and treat pathologic bone loss. In this study, we demonstrated that KP-A021 and structurally related triazole derivatives strongly inhibit the RANKL-induced differentiation and function of osteoclasts through suppression of RANK signaling pathways (Figure 6).

Stimulation of RANK signaling pathways ultimately leads to the activation of transcription factors such as AP-1 (c-Fos and c-Jun), NF- κ B, and NFATc1, which in turn induce the expression of genes essential for the fusion of osteoclast progenitors into multinucleated osteoclasts, and for bone resorption by mature osteoclasts.^{25,26} We found that KP-A021 considerably suppressed the expression of RANKL-induced NFATc1 (Figure 3). Down-regulation of *Nfatc1* expression was accompanied with the suppression of its target genes, including *Trap*, *Cat K*, and *Dcstamp* (Figure 3). These results suggest that KP-A021 targets the expression of *Nfatc1* in osteoclast precursors.

NFATc1 expression can be regulated by RANKL-mediated signaling pathways, including calcium/calmodulin-dependent protein kinase (CaMK)-MEK-ERK pathway.²⁷ An analysis of RANKL-mediated signaling pathways showed that RANKL-induced phosphorylation of ERK was dramatically inhibited by KP-A021 (Figure 5a). ERK is one of the key intracellular signaling molecules in osteoclast differentiation. ERK activation contributes to the induction of the adhesion protein required for cell-cell fusion during RANKL-induced osteoclast differentiation.²⁸ Recently, He *et al.*²⁹ reported that genetic deletion of ERK leads to decreased osteoclast formation and impaired bone resorption, suggesting that MEK-ERK signaling pathway has critical roles in osteoclastogenesis and bone-resorbing activity. In this way, inhibition of MEK-ERK in RANK signaling pathways by KP-A021 might be the major mechanism contributing to the suppression of differentiation and function of osteoclasts (Figure 6).

The actin ring is a peculiar cytoskeletal structure of osteoclasts. It is formed by cell-cell fusion and is required for efficient bone resorption. Yagi *et al.*³⁰ reported that DC-STAMP-deficient mice exhibit considerable reduction of

Figure 4 Inhibition of formation of the actin rings and resorption pits by KP-A021. (a) BMMs were seeded on glass coverslips and cultured with M-CSF and RANKL in the presence or absence of KP-A021 (5 μ M). After 4 days, cells were fixed and stained with rhodamine-conjugated phalloidin and DAPI to identify actin rings and nuclei. Actin rings were counted under a fluorescence microscope. ** $P < 0.01$ compared with control. (b) Mouse BMMs were plated onto bone slices and cultured with M-CSF and RANKL for 3 days to induce osteoclast differentiation. Cells were then treated with or without KP-A021 (5 μ M) for an additional 48 h. Resorption pits were identified by staining with hematoxylin, and the resorbed area was measured using an image analysis program. ** $P < 0.01$. The results are the representative of two independent experiments. (A color version of this figure is available in the online journal.)

Figure 5 Effect of KP-A021 on the RANK signaling pathways. (a) Mouse BMMs were cultured in serum-free medium for 5 h, pretreated with KP-A021 (5 μ M) or vehicle for 1 h, and then stimulated with RANKL (50 ng/mL) for the indicated times. Phosphorylation of ERK, JNK, p38, and Akt was evaluated by immunoblotting. Expression of ERK was used as loading controls. (b) Phosphorylation of MEK1/2 was examined in the mouse BMMs stimulated with RANKL for 15 min in the presence or absence of KP-A021. The results are the representative of at least three experiments

bone-resorbing activity due to impaired multinucleation, implying that cell-cell fusion is vital for resorption of osteoclasts and that DC-STAMP has a role in bone resorption. KP-A021 significantly suppressed DC-STAMP expression (Figure 3). Consistent with the above study, down-regulation of DC-STAMP was accompanied with reduced formation of multinucleated cells (Figure 1c) and actin rings (Figure 4a), clearly indicating that KP-A021 impairs cell fusion. Since the formation of multinucleated osteoclasts greatly contributes to bone resorption, inhibition of formation of multinucleated osteoclasts can influence bone resorption. As expected, the lack of multinucleated cells reduced the formation of resorption pits following KP-A021 treatment (Figure 4b). Therefore, KP-A021 apparently suppresses fusion, actin ring formation, and bone resorption of osteoclasts.

Triazoles and their derivatives possess various biological properties.^{12,13,31,32} α -1,2,3-triazoloamides inhibit lymphoid tyrosine phosphatase, which is a negative modulator of T-cell receptor signaling, causing rheumatoid arthritis and autoimmunity.³³ 1,4-Disubstituted 1,2,3-triazoles exhibit modest inhibition of the Src kinases implicated in cell proliferation, migration, and growth.¹² Furthermore, Src kinases have been shown to play an important role in the cytoskeletal rearrangement and bone resorption of

Figure 6 Proposed model for the action of KP-A021 on the RANK signaling pathways in osteoclast differentiation, bone resorption, and cytoskeletal rearrangement. Schematic drawing was modified from the KEGG schema (hsa04380)

mature osteoclasts.³⁴ Therefore, we examined the effect of KP-A021 on phosphorylation/activation of Src kinase, and found that KP-A021 did not block the RANKL-induced activation of Src kinase (data not shown). In addition, phosphorylation of Akt, a downstream signaling molecule, was not inhibited by KP-A021 (Figure 5a), suggesting that KP-A021 may control cytoskeletal rearrangement by targeting downstream of Akt (Figure 6).

Taken together, our findings suggest that the triazole-based compound KP-A021 and its derivatives effectively suppress osteoclast differentiation of BMMs through the suppression of MEK-ERK signaling pathway. In addition, KP-A021 significantly reduced the formation of actin rings and bone-resorbing activity of mature osteoclasts. Therefore, KP-A021 could be a candidate agent for the prevention and treatment of bone-related diseases caused by the increased formation of osteoclasts, and the subsequent increase in bone resorption.

Authors' contributions: HJI designed and performed the experiments and wrote the manuscript. DL and TL carried out biochemical studies. H-IS and YCB discussed the results and analyzed the statistical data. S-HK and EKP administered the experiments and revised the manuscript. All authors read and approved the final manuscript.

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