Pyridone 6, A Pan-Janus-Activated Kinase Inhibitor, Suppresses Osteoclast Formation and Bone Resorption through Down-Regulation of Receptor Activator of Nuclear Factor-κB (NF-κB) Ligand (RANKL)-Induced c-Fos and Nuclear Factor of Activated T Cells (NFAT) c1 Expression

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It has been reported that Janus tyrosine kinase (JAK)-dependent signaling pathways play a critical role in the pathogenesis of numerous malignancies and immune reactions, and inhibition of JAK has been implicated in cell growth inhibition. The role which JAK has on osteoclast differentiation and anti-bone resorptive activity is not well understood. In this study, we investigated the effects of a pan-JAK inhibitor, pyridone 6, on osteoclast differentiation and bone-resorption *in vitro* and *ex vivo*. Pyridone 6 inhibited osteoclast differentiation in mouse bone marrow macrophage (BMM) cultures stimulated by the receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) and co-cultures of bone marrow cells and osteoblasts. Pyridone 6 suppressed the expression of c-Fos and nuclear factor of activated T cells (NFAT) c1 in BMMs. It also inhibited the bone resorptive activity of mature osteoclasts that was accompanied by disruption of actin rings. Pyridone 6 also suppressed I-κB degradation and extracellular signal-regulated kinase (ERK) in mature osteoclasts, suggesting that these are the key molecules that pyridone 6 targets in the inhibition of osteoclast function. These results demonstrate inhibition of JAK may be useful for the treatment of bone-resorptive diseases, such as osteoporosis.

Key words osteoclast; pyridone 6; differentiation

Osteoporosis is a disease characterized by a decrease in bone mass leading to increased bone fragility and an increased risk of fracture. Osteoporosis results from a negative balance between new bone formation and resorption, and in most cases, is associated with a relative increase in the number and activity of bone-resorbing osteoclasts.[1] Osteoclasts are of hematopoietic origin, the differentiation of which is characterized by the fusion of osteoclast precursors, creating a multinucleated giant cell. Several cytokines and hormones, including interleukin (IL)-1, IL-6, IL-11, IL-15, IL-17, tumor necrosis factor (TNF)-α, prostaglandin E_2 (PGE_2), and parathyroid hormone (PTH) increase osteoclast formation, causing bone loss and osteoporosis.[2,3] Receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) is the most important cytokine in the differentiation of osteoclasts. It activates NF-κB, c-Fos, nuclear factor of activated T cells (NFAT) c1, and several other transcription factors.[4] The NFATc1, a master regulator of the terminal differentiation of osteoclasts, can cause precursor cells to undergo proficient osteoclast differentiation in the absence of RANKL signaling and induces the expression of various osteoclast-specific genes, such as tartrate-resistant acid phosphatase (TRAP) and calcitonin receptor.[5,6]

The Janus tyrosine kinases (JAKs) are a group of tyrosine kinases that are important in mediation of cytokine signaling pathways. Currently, four mammalian JAKs have been described (JAK1, JAK2, JAK3, and tyrosine kinase 2 [Tyk2]).[7] Recently, the constitutive activation of JAK and signal transducers and activators of transcription (STAT) have been found in various malignancies and there is accumulating evidence that JAK and STAT promote abnormal cell proliferation.[8,9] There are innovative studies suggesting that inhibition of the JAK-STAT pathway may be a therapeutic target for certain malignancies.[10,11] Inhibitors of the JAK/STAT pathway can also be used as a novel immunosuppressant in solid organ transplantation, as JAK/STAT has been shown to be involved in the immune rejection of allografts.[12,13] The step which JAK/STAT occupies in osteoclast differentiation is still not clear; there are only a few studies that address this kinase and the studies are restricted to its effect on other key cytokines.[14,15]

A tetracyclic pyridine, pyridine 6 (2-*tert*-butyl-9-fluoro-3,6-dihydro-7*H*-benz[*h*]-imidaz[4,5-*f*]isoquinoline-7-one), has been shown to be a novel pan-inhibitor of JAKs.[16] Pyridone 6 is a reversible ATP inhibitor and has recently been co-crystalized with the JAK2 kinase domain.[17] It has recently been reported that pyridone 6 induces growth arrest, and in turn mediates apoptosis of multiple myeloma cells.[18] However, the effect of pyridone 6 on various cells as well as osteoclasts remains largely unknown.

In this study, we investigated the effects of a pan-JAK inhibitor, pyridone 6, in osteoclast differentiation and analyzed the molecular mechanisms involved in an effort to better understand how the JAK/STAT pathway can be utilized in the treatment of osteoporosis.

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MATERIALS AND METHODS

Reagents Pyridone 6 was obtained from Calbiochem (La Jolla, CA, U.S.A.). Human soluble RANKL and macrophage-colony stimulating factor (M-CSF) were purchased from PeproTech EC (London, U.K.). 1α,25-Dihydroxyvitamin D_3 ($VitD_3$), prostaglandin E_2 (PGE_2), lipopolysaccharide (LPS) (Escherichia coli (*E. coli*) 026:B6), and anti-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). The XTT assay kit was from Roche (Indianapolis, IN, U.S.A.). Antibodies against c-Fos and NFATc1 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Antibodies against phospho-p38, p38, phospho-JNK, JNK, phospho-ERK, ERK, and I-κB were obtained from Cell Signaling Technology (Beverly, MA, U.S.A.).

Osteoclast Differentiation Bone marrow cells (BMCs) were freshly isolated from 5-week-old male ICR mice by flushing the bone marrow cavity with α-minimum essential medium (α-MEM; Welgene, Daegu, Korea) containing antibiotics (Sigma). Red blood cells (RBC) were removed in RBC lysis buffer (Sigma). Following centrifugation, cells were suspended in α-MEM supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, U.S.A.) and antibiotics, and used as BMCs. Calvarial osteoblasts were isolated from calvariae of 1-d old mice by sequential 0.1% collagenase and 0.2% disepase digestion. BMCs and osteoblasts were seeded in triplicate in 48-well plates and cultured for 5 d in α-MEM supplemented with 10% FBS, antibiotics, $VitD_3$, and PGE_2 in the presence or absence of pyridone 6. Bone marrow macrophages (BMMs) were prepared from BMCs cultured for 3 d in the presence of 30 ng/ml of M-CSF. BMMs were cultured for 4 d with 20 ng/ml of M-CSF and 50 ng/ml of RANKL in the presence or absence of pyridone 6.

Cytotoxicity Assay BMMs were plated in 96-well culture dishes and incubated for 3 d with 20 ng/ml of M-CSF in the presence or absence of pyridone 6. After 3 d, 50 μl of XTT reagent was added to each well and further incubated for 6 h. The optical density at 450 nm was analyzed using an ELISA reader.

Western Blotting Cells were lysed in lysis buffer containing 50 mM Tris–Cl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, 1% deoxycholate, and protease inhibitors. Proteins were boiled in SDS sample buffer, resolved by 10% SDS-polyacrylamide gel electrophoresis, and transferred to a PVDF membrane (Amersham Biosciences, Piscataway, NJ, U.S.A.). The membrane was probed with antibodies, as indicated, washed extensively, and then incubated with secondary antibody conjugated to horseradish peroxidase. Immune complexes were visualized with enhanced chemiluminescence.

RNA Isolation and RT-PCR Total RNA was extracted from cultured cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's instructions. One microgram of RNA was reverse-transcribed using oligo dT primers, dNTP, buffer, dithiothreitol, RNase inhibitor, and Superscript II reverse transcriptase (Invitrogen). cDNA was amplified using the following primer sets: TRAP sense, 5′-ACTTCCCCAGCCCTTACTAC-3′; TRAP antisense, 5′-TCAGCACATAGCCCACACCG-3′; c-Fos sense, 5′-CTGGTGCAGCCCACTCTGGTC-3′; c-Fos antisense, 5′-CTTTCAGCAGATTGGCAATCTC-3′; NFATc1 sense, 5′-CAACGCCCTGACCACCGATAG-3′; NFATc1 antisense, 5′-GGCTGCCTTCCGTCTCATAGT-3′; OSCAR sense, 5′-GAACACCAGAGGCTATGACTGTTC-3′; OSCAR antisense, 5′-CCGTGGAGCTGAGGAAAAGGTTG-3′; GAPDH sense, 5′-ACCACAGTCCATGCCATCAC-3′; GAPDH antisense, 5′-TCCACCACCCTGTTGCTGTA-3′. PCR products were electrophoresed by using 1% agarose gels and visualized by staining with ethidium bromide. Densitometric analysis was performed on the relative intensity of each band using the Image Pro-plus program, version 4.0 (Media Cybernetics, Carlsbad, CA, U.S.A.).

Actin Stain Cells were fixed with 3.7% formalin, permeabilized with 0.1% Triton X-100, and incubated with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR, U.S.A.) for 5 min. Images were photographed under a fluorescence microscope.

Resorption Assay Mature osteoclasts from co-cultures of BMCs and osteoblasts were seeded on hydroxyapatite-coated 48-well plates, and osteoblasts were detached with trypsin/EDTA treatment. The cells were incubated with the indicated concentrations of pyridone 6. After 6 h, the cells were removed from the hydroxyapatite-coated plates. The plates were placed under a light microscope, and then quantification of the resorption pit number and pit area was performed using the Pro-plus program, version 4.0 (Media Cybernetics).

Organ Culture Calvarial bones from 3-d old ICR mice were placed in 5 ml of α-MEM in 6-well plates and incubated for 7 d with the indicated treatment. The calvarial bones were fixed in 10% formalin, permeabilized in 0.1% Triton X-100, and stained with TRAP solution.

Statistical Analysis All quantitative data are presented as the mean$\pm$S.D. of 3—5 replicates. Each experiment was repeated 3—5 times, and results from 1 representative experiment are shown. Statistical differences were analyzed by Student's *t*-test. Statistically significant data (p values <0.05) are defined with an asterisk.

RESULTS

Pyridone 6 Suppresses Osteoclast Formation We determined the effects of pyridone 6 on osteoclast differentiation using co-culture systems of BMCs and osteoblasts. Through this culture-based screening, we showed that pyridone 6 reduced the number of osteoclasts in a dose-dependent manner (Fig. 1A). In the co-culture system, osteoblasts expressing RANKL play an important role in differentiation of osteoclast precursors. Thus, the possibility of pyridone 6 affecting the ability of osteoblasts to support osteoclastogenesis should be taken into account. In order to assess whether the effect of pyridone 6 on osteoclast precursors was direct, we used BMMs. In this culture system, pyridone 6 significantly reduced the formation of osteoclasts (Figs. 1B, C). To exclude the possibility of pyridone 6 exerting cytotoxicity on BMMs during the culture, the cytotoxicity assay was performed after the culture period. Pyridone 6 did not exhibit cytotoxicity against BMMs at the concentrations used in the current study (Fig. 1D), indicating that the decrease in osteoclastogenesis was not due to the toxic effects of pyridone 6

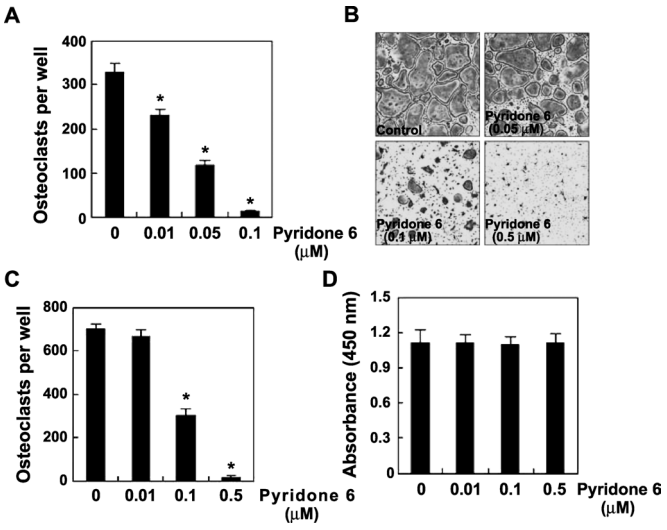

Fig. 1. Pyridone 6 Suppresses Osteoclast Formation

(A) BMCs and osteoblasts were co-cultured for 5 d with $VitD_3$ (10^{-8} M) and PGE_2 (10^{-6} M) in the presence of increasing concentrations of pyridone 6. The cells were fixed in 3.7% formalin, permeabilized in 0.1% Triton X-100, and stained with TRAP solution. TRAP-positive cells were counted as osteoclasts. (B) BMMs were cultured for 4 d with M-CSF (20 ng/ml) and RANKL (50 ng/ml) in the presence of increasing concentrations of pyridone 6. The cells were stained with TRAP solution as described above. TRAP-positive cells were photographed under a light microscope. (C) TRAP-positive cells were counted as osteoclasts. (D) BMMs were incubated for 3 d with the indicated concentrations of pyridone 6. XTT reagents were added to each well, and the cells were incubated for 6 h. The optical density at 450 nm was measured using an ELISA reader.

on BMMs.

Pyridone 6 Suppresses c-Fos and NFATc1 Expression in BMMs Induced by RANKL To identify the molecules and signaling pathways involved in the inhibition of osteoclastogenesis by pyridone 6, we examined p38, JNK, extracellular signal-regulated kinase (ERK), and I-κB, which are known to play crucial roles in osteoclastogenesis-associated gene expression.[19] After stimulation of BMMs with RANKL, the difference in activation of these signal molecules with or without pyridone 6 was assessed. Pyridone 6 did not affect RANKL-induced phosphorylation of p38, JNK, ERK, and subsequent degradation of I-κB (Fig. 2A). We know from numerous previous reports that c-Fos and NFATc1 play a vital role in osteoclast differentiation.[5,6] We examined the potential effects of pyridone 6 on the expression of c-Fos and NFATc1 in response to RANKL. As shown in Fig. 2B, pyridone 6 inhibited the mRNA expression of c-Fos and NFATc1 mRNA expression in response to RANKL. Also, pyridone 6 suppressed the mRNA expression of TRAP and OSCAR, which is known as a key receptor for osteoclast differentiation. Protein levels of c-Fos and NFATc1 were increased in response to RANKL, but the expression of both c-Fos and NFATc1 was significantly inhibited by pyridone 6, hence confirming the RT-PCR results (Fig. 2C). Therefore, c-Fos and NFATc1 may be the mediator of pyridone 6 in the inhibition of osteoclast differentiation in response to RANKL.

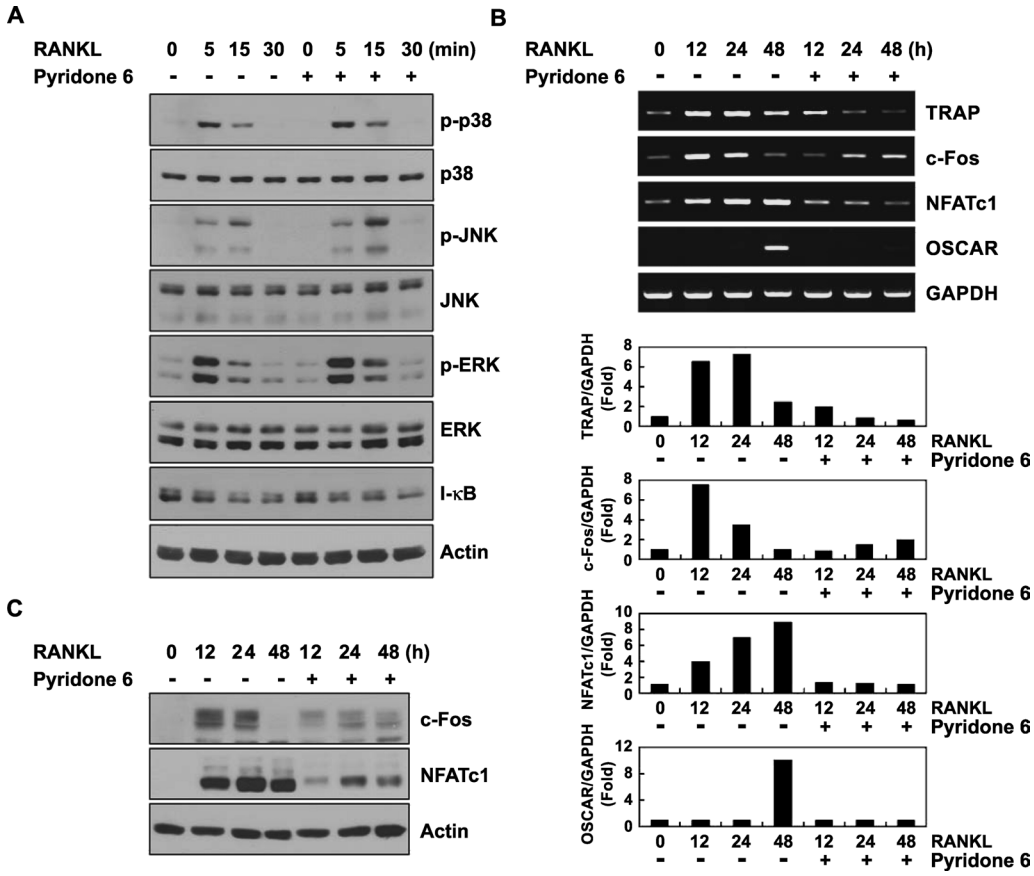

Fig. 2. Pyridone 6 Inhibits RANKL-Induced c-Fos and NFATc1 Expression

(A) BMMs were pretreated with pyridone 6 (0.5 μM) for 1 h before treatment with RANKL (100 ng/ml) for the indicated time. The cells were lysed, and the lysates were Western blotted with the indicated antibodies. (B) BMMs were pretreated with pyridone 6 (0.5 μM) for 1 h and then stimulated with RANKL (100 ng/ml) for the indicated time. Total RNA was extracted and the level of expression of the mRNA of the indicated genes was analyzed by RT-PCR (top). Relative levels of TRAP, c-Fos, NFATc1, and Oscar mRNA were quantified by densitometric analysis and were normalized to GAPDH (bottom). (C) BMMs were treated as in (B). Cell lysates were analyzed by Western blotting with antibodies for c-Fos, NFATc1, and actin.

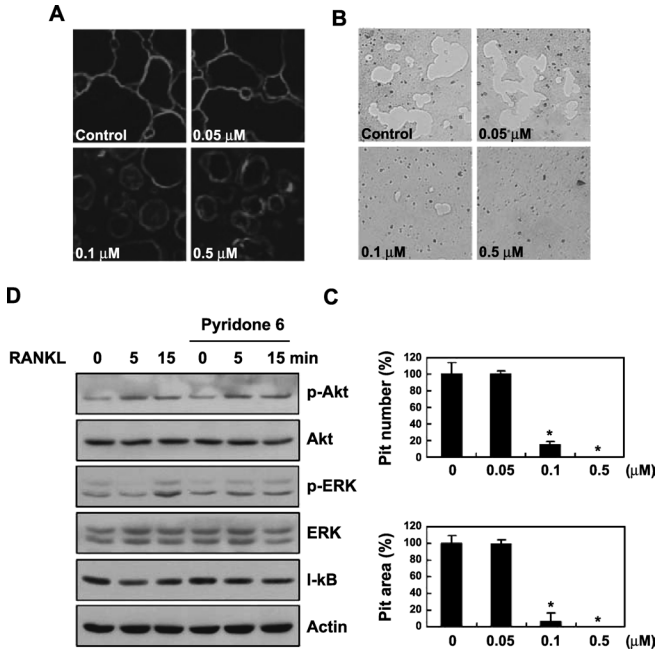

Fig. 3. Pyridone 6 Inhibits Osteoclastic Bone Resorption

Mature osteoclasts were obtained from co-cultures of BMCs and osteoblasts. (A) The cells were treated for 6 h with the indicated concentrations of pyridone 6 in the presence of RANKL (100 ng/ml). The cells were fixed, permeabilized, and stained with rhodamine-phalloidine. Actin was observed by fluorescence microscopy. (B) Mature osteoclasts were plated on hydroxyapatite-coated 48-well plates and then treated for 6 h with the indicated concentrations of pyridone 6 in the presence of RANKL (1000 ng/ml). Cells were removed from the plates and photographed under a light microscope. (C) Pit number (top) and pit area (bottom) were quantified using Image Pro-plus program, version 4.0. (D) Mature osteoclasts were pretreated with pyridone 6 (0.5 μM) for 1 h and then stimulated with RANKL (1000 ng/ml) for the indicated time. The cells were lysed, and the lysates were analyzed by Western blotting with the indicated antibodies.

Pyridone 6 Also Inhibits Osteoclast Function Since we have provisional evidence that pyridone 6 inhibits osteoclast differentiation, to determine the effect of pyridine 6 on bone resorption, we first examined whether pyridine 6 has an apoptotic effect on osteoclast survival. We showed that pyridone 6 inhibits the survival of osteoclasts by RANKL (data not shown). We thus focused on the effect pyridone 6 has on osteoclast function and bone absorption capacity. Mature osteoclasts have a sealing zone by forming a ring structure made of F-actin in order to resorb bone.[4] We investigated whether pyridone 6 affects the formation of actin rings. Osteoclasts formed ringed structures of F-actin that are dense and smooth, with clear margins in the presence of RANKL. However, a gradual increase in the concentration of pyridone 6 disrupted the actin rings, which became loose and fuzzy, indicating that pyridone 6 affects the cytoskeletal organization needed for bone resorption by osteoclasts (Fig. 3A). We next looked into bone resorption to determine if pyridone 6 actually inhibited the bone-resorption activity of mature osteoclasts. Pyridone 6 significantly suppressed the pit number and pit area of osteoclastic resorption (Figs. 3B, C), indicating that pyridone 6 decreases the activity of mature osteoclasts. We thus investigated the molecules and signaling pathways involved in decreased osteoclast function. The Akt and ERK pathways are thought to be involved in osteoclast survival, while the activation of the NF-κB pathway is well-known for its role in bone resorption.[18] We examined whether pyridone 6 affected these pathways in mature osteoclasts. Pyridone 6 inhibited the phosphorylation of ERK in response to RANKL. RANKL-mediated I-κB degradation was inhibited in osteoclasts treated with pyridine 6 (Fig. 3D), indicating that pyridone 6 disrupts several signaling pathways involved in osteoclast survival and function.

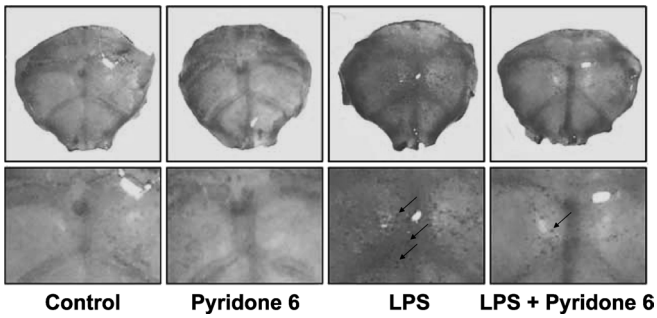

Fig. 4. Pyridone 6 Suppresses Osteoclast Formation in Calvarial Bones

Calvarial bones were incubated for 7 d with or without LPS (1 μg/ml) in the presence or absence of pyridone 6 (0.5 μM). Cultured bones were fixed, permeabilized, and stained with TRAP solution. TRAP-stained bones were photographed using a digital camera (top). Each picture presents a magnified part to view osteoclasts (bottom). Arrows indicate the osteoclasts.

Pyridone 6 Inhibits Osteoblast Formation *ex Vivo* To determine if pyridone 6 inhibits osteoclast formation in *ex vivo* conditions, we used a model using mouse calvarial bones. LPS stimulation for 7 d clearly increased TRAP-positive osteoclasts in the calvarial vertex, while the simultaneous administration of pyridone 6 and LPS decreased TRAP-positive osteoclasts (Fig. 4). These findings indicate that pyridone 6 apparently has a negative effect on osteoclast differentiation *ex vivo*.

DISCUSSION

Bone mass is the result of a balance between bone formation and bone resorption; this balance can easily be disturbed by increasing osteoclastogenic factors and systemic alterations, such as estrogen deficiency. Deregulation of osteoclast number and activity can cause bone-resorptive disorders, such as osteoporosis, rheumatoid arthritis, Paget's disease, and periodontal disease, which are to some extent associated with the immune system. The immune system and bone metabolism share a number of regulatory factors, such as cytokines, receptors, signaling molecules, and transcription factors.[20,21] The imbalance of cytokines frequently initiates and establishes inflammatory processes in many types of immune or autoimmune diseases.

Schindler and Darnell[22] highlighted that numerous factors that suppress osteoclast differentiation signals through the JAK/STAT pathway, and consequently inhibition of JAK should lead to increased osteoclast production and osteoporosis. A study performed by Lovibond *et al.*[14] supported this theory by using a negative feedback regulator of JAK/STAT signals named suppressor of cytokine stimulation (SOCS). They stated that TGF-β uses SOCS3 to suppress JAK/STAT anti-osteoclastogenic signals in order to augment TNF-α-induced osteoclast formation. We thus examined the effect of pyridone 6 in osteoclast differentiation. A low pyridone 6 concentration (0.01 μM) inhibited osteoclast differentiation in the co-cultures of BMCs and osteoblasts, but without cytotoxicity against BMMs (Fig. 1).

The expression of c-Fos induced by RANKL is important for initiating osteoclast differentiation and can induce the expression of NFATc1, which plays an essential role in the expression of genes required to give rise to osteoclasts.[5,6] Pyridone 6 significantly inhibited c-Fos expression in BMMs treated with RANKL (Fig. 2). The regulatory mechanism of c-Fos expression by various stimuli has been defined in detail. Mitogen-activated protein kinase (MAPK) leads to the phosphorylation of Elk-1, which binds with a serum response ternary complex, including SRF and CBP/p300. The serum response ternary complex in turn interacts with the serum response element (SRE) of the c-Fos promoter, resulting in the expression of c-Fos.[23] However, we found that pyridone 6 did not inhibit the phosphorylation of MAPK containing p38, JNK, and ERK, as well as I-κB degradation induced by RANKL treatment (Fig. 2). We thus examined the effect of pyridone 6 on c-Fos mRNA stability, but pyridone 6 inhibits c-Fos expression without taking into account the possible effect of c-Fos mRNA stability. These results suggest that pyridone 6-mediated inhibition of c-Fos expression was mediated through inhibition of other signaling pathways, except the MAPKs and NF-κB pathways.

Receptor-mediated activation of JAK/STAT leads to T-cell differentiation in the thymus, or at sites of inflammation. The role of the JAK/STAT pathway on osteoclastogenesis has rarely been studied. The receptor signals relayed by the JAKs comprise two heterosubunits which heterodimerize and autophosphorylate, followed by a subsequent phosphorylation of JAK. The corresponding JAKs create the docking sites for SH2-containing signaling proteins, including STAT proteins, and in turn cause the phosphorylation of STAT proteins on the specific tyrosine residues followed by homo- or heterodimerization. The STAT proteins translocate to the nucleus where they target the specific genes responsible for the cytokine.[24,25] There have been many studies completed by means of observing the phenotype of knock-out mice of the different JAK families with the purpose of understanding the mechanism of inhibition in physiologic and pathological conditions. JAK1$-/-$ mice are runts at birth and die perinatally, which point out that JAK1 is an obligatory and non-redundant gene in biological responses.[26] JAK2$-/-$ mice are severely deficient in erythropoiesis and die at an early stage of development from fetal anemia.[27] JAK3 knockout mice present a severe combined immune deficiency (SCID) phenotype.[28] Tyk2$-/-$ mice have enhanced T-helper (Th) cell 2-mediated antibody production and eosinophil recruitment in the airways by regulating the Th1/Th2 balance in favor of Th1. Tyk2 is necessary for the induction of IL-13-mediated goblet cell hyperplasia in the airways, suggesting that Tyk2 is responsible in the downregulation of allergic airway inflammation.[29]

In this study, we examined the role of JAK kinase blockade on osteoclast differentiation and function using two culture systems (co-culture and BMMs culture with pyridone 6). It was clear that this phenomena was not coincidental, given that pyridone 6 suppressed c-Fos and NFATc1 (Fig. 2), suggesting that these may be the molecules involved. We also proved that pyridone 6 alters cytoskeleton rearrangement for actin ring formation (Fig. 3), which is essential for osteoclast bone resorption capacity, indicating that the JAK/STAT pathway can be associated with osteoclast function. Furthermore, we found that pyridine 6 suppresses phosphorylation of ERK and I-κB degradation in mature osteoclasts but not in BMMs (Figs. 2, 3). Therefore, we asked why pyridine 6 did not inhibit ERK phosphorylation and I-κB degradation in BMMs. It is possible that the effects of pyridine 6 on RANKL-induced signal transduction depend on the cell type. For example, at a concentration of 100 ng/ml, RANKL can induce I-κB degradation in BMMs (Fig. 2). However, RANKL concentrations of at least 1000 ng/ml or more were necessary to induce I-κB degradation in mature osteoclasts (Fig. 3). This difference could be the result of cell type-specific effects of RANKL.

Finally, we examined the effects of pyridone 6 on *ex vivo* mouse calvarial bones that was activated with LPS and clearly showed the decrease in TRAP-positive osteoclasts on treatment with pyridone 6 (Fig. 4). These data can provide the basis for future understandings of the JAK/STAT in osteoclastogenesis, and possibly go on to use pyridone 6 as a novel therapeutic agent in the treatment of inflammatory and metabolic bone-resorptive disease, such as rheumatoid arthritis and osteoporosis.

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