Inhibition of p38 Mitogen-Activated Protein Kinase Prevents Inflammatory Bone Destruction

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ABSTRACT

Mitogen-activated protein kinase (MAPK) pathways are implicated in joint destruction in rheumatoid arthritis (RA) by modulating the production and functions of inflammatory cytokines. Although p38 MAPK (p38) participates in signaling cascades leading to osteolysis in arthritis, the mechanisms of its action in this process remain incompletely understood. Here, we found that the osteoclast (Ocl) precursors expressed p38α, but not p38γ, p38δ, and p38-γ isoforms. Treatment of these cells with receptor activator of nuclear factor (NF)-κB ligand (RANKL) resulted in p38 activation. Importantly, Ocl development induced by RANKL or RANKL and tumor necrosis factor (TNF) was blocked with the novel p38 inhibitor 4-(3-(4-chlorophenyl)-5-(1-methylpiperidin-4-yl)-1H-pyrazol-4-yl)piperidine (SC-409). To validate in vitro data, p38 role was further investigated in streptococcal cell wall (SCW)-induced arthritis in rats. We found that SCW-induced joint swelling and bone destruction were attenuated by SC-409. Mechanistically, the data show that SCW-stimulated DNA binding activity of the transcription factor myocyte-enhancing factor 2 C, which is downstream of p38, was inhibited by SC-409. In addition, SC-409 inhibited SCW-stimulated expression of numerous factors, including TNF-α, interleukin-1β, and RANKL. Although c-Jun NH2-termina l kinase and NF-κB pathways were activated in vitro by RANKL and in vivo by SCW, SC-409 had no significant effect on these pathways. In conclusion, our data show that p38 modulates the production and signaling of cytokines, thus providing a mechanism of the bone-sparing effect of SC-409 in rat arthritis. These data present SC-409 as a novel potent p38 inhibitor and suggest that p38-based therapies may be beneficial in preventing bone loss associated with RA.

Rheumatoid arthritis (RA) is a chronic inflammatory disease that causes progressive destruction of the joint. Although the etiology of RA is unclear, it is known that cytokines play critical roles in the inflammatory processes that lead to joint destruction (Goldring and Gravallese, 2000; Choy and Panayi, 2001). These cytokines include TNF-α and IL-1β, which are detected in synovial fluids from patients with RA and are critical in the pathogenesis of this disease. Consistent with this, clinical data on recombinant soluble TNF receptor II (sTNFRII) and IL-1 receptor antagonist, which bind to and antagonize TNF-α and IL-1β activity, respectively, indicate that these cytokine-based therapies protect joints from destruction in RA (Wolfe and Strand, 2001).

Bone resorption, per se, is the main function of osteoclasts (Ocls), which are derived from hematopoietic stem cells (Roodman, 1999). Formation and activity of these cells depend upon expression of receptor activator of nuclear factor (NF)-κB ligand (RANKL). Support for this premise is provided by evidence that deletion of RANKL gene in mice leads to osteopetrosis due to Ocl deficiency (Kong et al., 1999b). RANKL is a TNF family member, expressed within the ar-

ABBREVIATIONS: RA, rheumatoid arthritis; TNF, tumor necrosis factor; IL, interleukin; sTNFRII, soluble TNF receptor II; Ocl, osteoclast; NF, nuclear factor; RANK, receptor activator of NF-κB; RANKL, RANK ligand; OPG, osteoprotegerin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; MMP, matrix metalloprotease; SC-409, 4-(3-(4-chlorophenyl)-5-(1-methylpiperidin-4-yl)-1H-pyrazol-4-yl)piperidine; SC-448, 1-(4-(3-(4-chlorophenyl)-4-(pyrimidin-2-yl)-1H-pyrazol-4-yl)piperidin-1-yl)-2-hydroxyethanone; MEF2C, myocyte-enhancing factor 2 C; AP-1, activator protein-1; LPS, lipopolysaccharide; MEM, minimal essential medium; FBS, fetal bovine serum; TRAP, tartrate-resistant acid phosphatase; ELISA, enzyme-linked immunosorbent assay; SCW, streptococcal cell wall; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-N,N,N′,N′-tetramethylrhodamine; MGB/NFQ, minor groove binder/nonfluorescent quencher; FP, forward primer; RP, reverse primer; COX, cyclooxygenase; DTT, dithiothreitol; GST, glutathione S-transferase; MITF, microphthalmia transcription factor; M-CSF, macrophage colony-stimulating factor.
These cytokines in RA is important to stop disease progression in arthritis. It is conceivable to propose their action over that of RANKL, leading to the propagation of inflammation and bone erosion, it had no effect on joint swelling (Kong et al., 1999a), and RANK/RANKL role in cartilage degradation is still not known.

Despite some discrepancies, evidence indicates that cytokines are potent inducers of bone resorption (Mundy et al., 1995). This includes TNF-α, IL-1β, and other factors (Pacifi, 1996). Recent studies that addressed this issue concluded that TNF-α, for example, promotes osteoclastogenesis in synergy with RANKL (Lam et al., 2000; Komine et al., 2001). Supporting this notion is the fact that blockade of TNF-α signaling by genetic manipulation reduced osteoclastogenesis. As other cytokines, including IL-1β, superimpose their action over that of RANKL, leading to the propagation of inflammation and bone erosion, it is conceivable to propose that blockade of the production and/or signaling pathways of these cytokines in RA is important to stop disease progression. Those pathways include NF-κB and mitogen-activated protein kinase (MAPK) pathways (Feng, 2005).

MAPKs are proline-directed kinases, which include the insulin/mitogen-regulated extracellular signal-regulated kinase (ERK), the stress-activated protein kinases/c-Jun NH₂-terminal kinases (JNKs), and the p38 kinases (p38) (Kyriakis and Avruch, 2001). MAPKs have important functions as mediators of cellular responses to variety of extracellular stimuli. Typically, the MAPK kinase/ERK family is stimulated by growth factors, whereas the JNK/p38 pathways are activated by cellular stresses, cytokines, and hypoxia. MAPKs are under active investigation in RA (Han et al., 2001) since they are known to modulate the expression of certain genes involved in joint destruction, such as matrix metalloproteases (MMPs) (Engsig et al., 2000), carbonic anhydrase II, and cathepsin K (Matsumoto et al., 2004).

Consistent with MAPK role in bone resorption, mice deficient in the c-Jun binding partner, c-fos, develop osteopetrosis (Grigoriadis et al., 1994), although mice deleted of JNK1 or JNK2 genes have normal skeletal metabolism (David et al., 2002). Furthermore, it has been shown that pharmacological blockade of JNK (Bennett et al., 2001) or p38 (Badger et al., 2000) inhibited adjuvant-induced arthritis in rats, suggesting that small molecules may be efficacious in the treatment of RA. However, the mechanisms of the joint-sparing effects of p38 inhibitors remain incompletely understood. Indeed, these inhibitors may prevent bone destruction in arthritis indirectly by altering the function of inflammatory cells without exerting direct effects on bone cells. In support of this notion, glucocorticoids are known to cause osteopenia by deregulating the activity of bone cells (Weinstein et al., 2000), while intriguingly inhibiting bone destruction in rodent models of arthritis by affecting the activity of inflammatory cells (Hom et al., 1991).

Given the key roles of MAPKs in RA, we developed a p38 inhibitor and determined the mechanisms of p38 regulation of inflammatory bone resorption in vivo and in vitro systems. Our findings indicate that the p38 inhibitor, SC-409, inhibits the production of key cytokines such as TNF-α, IL-1β, and RANKL; RANK-mediated p38 activation in Ocls precursors; and arthritis symptoms and bone destruction. Based on these findings, we propose that p38 inhibitors may be efficient in preventing bone loss associated with RA.

**Materials and Methods**

**Reagents.** Antibodies against IκB-α, phospho-IκB-α, heat shock protein 27 (HSP27), c-Jun, phospho-c-Jun, and ERK as well as oligonucleotides containing the binding sites for myocyte-enhancing factor 2 C (MEF2C), AP-1, and NF-κB were purchased from Santa Cruz (Santa Cruz, CA). Rabbit polyclonal antibodies against phospho-ERK and phospho-p38 were purchased from Cell Signaling (Beverly, CA), and Biosource International (Camarillo, CA), respectively. Anti-p38 antibody was obtained from Santa Cruz and then affinity-purified in-house. Human RANKL and M-CSF were purchased from PeproTech Inc. (Rocky Hill, NJ), and mouse RANKL and M-CSF were obtained from R&D Systems Inc. (Minneapolis, MN). Lipopolysaccharide (LPS) and salmon calcitonin were purchased from Sigma (St. Louis, MO) and Calbiochem (San Diego, CA), respectively. SC-409 and SC-448 (diarylpyrazole class of compounds) were synthesized by Pfizer (St. Louis, MO) and stored as a free base powder. All other chemicals were obtained commercially and were of the highest purity available.

**Cell Cultures.** CD34+ cells were immunoaffinity purified (purity >95%) from healthy human bone marrow by BioWhitaker (Walkersville, MD). Mouse RAW 264.7 cells were purchased from American Type Culture Collection (Manassas, VA). Aliquots (3 × 10⁴/cm²) of cryopreserved CD34+ cells or of RAW 264.7 cells (3 × 10⁴/cm²) were cultured in Dulbecco’s modified Eagle’s medium-high glucose or α-MEM, respectively, supplemented with 10% (v/v) fetal bovine serum (FBS), at 37°C in 95% air and 5% CO₂ atmosphere. Cells were treated with RANKL, M-CSF, and/or p38 inhibitor, SC-409. Every 3 days, half of the culture medium was gently removed to minimize loss of nonadherent cells and replaced with an equal volume of fresh medium. Cells were fixed and stained for trtartrate-resistant acid phosphatase (TRAP) activity as described below. To study the effect of TNF-α on RANKL mRNA expression, mouse bone marrow cells were isolated as follows. Bone harvest, processing, and marrow cell isolation were carried out as described previously (Mbah, 2003).

**Cell Culture and Stimulation of Cytokine Expression by Streptococcal Cell Wall or LPS.** The animal studies were approved by the St. Louis Pfizer Institutional Animal Care and Use Committee. Female Lewis rats (100–140 g) received a single i.p. injection of streptococcal cell wall or LPS.
wall (SCW) suspension (Lee Laboratories, Grayson, GA) at a dose of 15 to 60 μg rhamnose equivalents/g body weights. Treatment of rats (≥eight rats/group) daily with 15 mg/kg SC-409 b.i.d. administered orally or with 10 mg/kg recombinant soluble rat TNF was infused to the fc fragment of rat IgG (sTNFRII-Fc chimera; J. O. Polazzi and J. Portanova, unpublished data), administered i.p. twice weekly, was begun on day 10 or 18. Rats were sacrificed at day 21, 2 to 4 h after the last drug administration. The severity of arthritis was determined by measuring paw volume using plethysmometer, visual scoring, and histology. A subset of Lewis rats was injected i.v. with 1 mg/kg LPS (Sigma). The tissues were collected for electrophoretic mobility shift assay (EMSA) as described below and the serum for measurement of cytokine levels by ELISA (Biosource International).

**Computed Tomography, Histology, and Histomorphometry.** Three-dimensional images from intact rat paws were obtained from microcomputed T20 scanner (Scanco Medical, Wayne, PA) set at 380 slices, 115-ms integration time, and 34-μm resolution. Bone mineral density was determined by peripheral quantitative computed tomography using a Norland XCT Research M densitometer (Norland Medical Systems, White Plains, NY). Hind paws were removed and frozen until the analysis. The paws were laid out on the lateral side and three serial 1-mm sections were taken beginning 3 mm from the tibial-tarsal joint. Voxel size was 0.09 mm, the analysis lateral side and three serial 1-mm sections were taken beginning 3 mm from the tibial-tarsal joint. Voxel size was 0.09 mm, the analysis was performed with 10% EDTA, pH 7.0, until radiotranslucent based on radiography. Decalciﬁed paws were then dehydrated in graded alcohol, cleared with xylene, and embedded in parafﬁn. Parafﬁn blocks were sectioned longitudinally along the second digit through the metatarsal, talus, and distal tibia. Five-micron sections were then stained with either Safranin O:fast green for cartilage parameters or histochemistry for TRAP, using Osteomeasure software (Osteomeasure software, Portanova, unpublished data), administered i.p. twice weekly, was begun on day 10 or 18. Rats were sacrificed at day 21, 2 to 4 h after the last drug administration. The severity of arthritis was determined by measuring paw volume using plethysmometer, visual scoring, and histology. A subset of Lewis rats was injected i.v. with 1 mg/kg LPS (Sigma). The tissues were collected for electrophoretic mobility shift assay (EMSA) as described below and the serum for measurement of cytokine levels by ELISA (Biosource International).
mM MgCl$_2$, 0.2 mM EDTA, pH 8.0, 1 mM DTT, and Complete protease inhibitor cocktail] was added to the pellets, and the samples were rocked at 4°C for 30 min. Samples were pelleted at 4°C for 30 min in a microcentrifuge, and the supernatants were aliquoted and stored at −80°C. Protein concentrations were measured using standard BCA kit (Pierce). Aliquots (10 μg) of nuclear extracts were incubated with 5-32P-end-labeled double-stranded oligonucleotide probes. The reaction was performed at room temperature for 30 to 60 min in a total volume of 20 μl of binding buffer (20 mM HEPES, pH 7.8, 100 mM NaCl, 0.5 mM DTT, 1 μg of poly(dI-dC), and 10% glycerol). Samples (10 μl) were fractionated on a 4 to 20% gradient Tris borate-EDTA gel, and the proteins were visualized by autoradiography.

**In Vitro Selectivity Assays.** The selectivity of SC-409 for p38α was tested against a variety of targets. Inhibition studies were carried out using ATP concentrations at the $K_m$ measured for the respective enzymes and substrate concentration 10-fold greater than the $K_m$ determined for each kinase. However, there was some difference among assays. For instance, p38 and JNK were tested for their sensitivity to SC-409, which was tested in 10-fold serial dilutions beginning at 200 μM in 10% dimethyl sulfoxide. Reactions were initiated by addition of activated p38 (20–40 nM) or JNK (150–300 nM) previously activated with GST-MKK6 or GST-MKK7b, respectively. p38 and JNK activities were determined by following the phosphorylation of epidermal growth factor receptor peptide and GST-cJun, respectively. Reaction mixtures included 50–100 μM ATP, 200 μM epidermal growth factor receptor peptide or 10 μM GST-cJun, and 0.05 to 11 μM [γ-32P] ATP at pH 7.5. Reactions were stopped by addition of 150 μl of Dowex AG 1 × 8 resin in 900 mM sodium formate buffer, pH 3.0, and enzyme activity was quantified through measurement of 33P present in an aliquot from the supernatant.

**Statistical Analysis.** All data were analyzed by a paired Student’s $t$ test. Samples were run always in triplicate, and data represent the mean ± S.E. Independent experiments were performed at least twice.

**Results**

**The p38 Inhibitor, SC-409, Blocks Ocl Formation but Not Ocl Activity.** Previous studies have suggested that p38 is involved in osteolysis. However, the mechanisms of p38 in the process of osteolysis have not been elucidated. To address this issue, we developed a selective p38 inhibitor, SC-409 (Fig. 1; Table 1), and determined its effect on Ocl differentiation from RAW 264.7 cells. Treatment of these cells with RANKL and M-CSF induced Ocl formation after 6 days of cultures (Fig. 2, A and B), and this effect was inhibited in a dose-dependent manner by SC-409 (Fig. 2, C and G), but not by a structurally similar but inactive SC-409 analog, SC-448 (p38α $IC_{50} > 100$ μM) (Fig. 2G). This dose-response effect (Fig. 2G) mimicked SC-409 potency on p38α (Table 1; data not shown). SC-409 inhibited Ocl formation in this system, even when it was present during only the first 3 days of the cultures (data not shown), suggesting that p38 is involved in the early steps of Ocl formation. SC-409 also inhibited Ocl formation induced by RANKL from human CD34+ cells (Fig. 2, D–F) and mouse and rat bone marrow cells (data not shown).

Because TNF-α has been reported to stimulate osteoclastogenesis in synergy with RANKL (Komine et al., 2001; Feng, 2005), we evaluated the effects of SC-409 on osteoclastogenesis that is induced by TNF-α and RANKL. As shown in Fig. 2H, TNF-α and RANKL synergistically induced Ocl formation, an effect that was blocked by 1 μM SC-409. We also investigated the role of p38 in Ocl activity. Treatment of bone marrow cells plated on cortical bone slices with 100 ng/ml RANKL and 10 ng/ml M-CSF for 6 days stimulated Ocl formation (data not shown). Ocls thus formed were then treated with SC-409 or calcitonin in the continuous presence of RANKL and M-CSF. We found that the release of cross-linked C-telopeptides of type I collagen from bone slice stim-

![Fig. 1. Structure of SC-409.](image-url)
ulated by RANKL and M-CSF was inhibited by calcitonin, but not SC-409, even at SC-409 concentrations higher than those required to inhibit Ocl formation (data not shown). These findings suggest that p38 is involved in Ocl formation, but not activity.

**RANKL Activates the p38 Pathway in Ocl Precursors.** To demonstrate that p38 is downstream of RANK and is functional in Ocl precursors, we determined the effect of RANKL on p38 activation. RAW 264.7 cells expressed p38α but not p38β, δ, and γ isoforms (Fig. 3A). Lack of antibody reactivity to p38β, δ, and γ isoforms from cell lysates was ruled out since the antibody reacted with recombinant p38β, δ, and γ (Fig. 3B). Treatment of RAW 264.7 cells with RANKL or LPS resulted in a rapid transient phosphorylation of p38, reaching a peak 20 min after stimulation (Fig. 3, C and D). RANKL also increased phosphorylation of ERK1/2, c-Jun, and IxB-α in RAW 264.7 cells (Fig. 3, E–G). In these cells, the degradation of phosphorylated IxB-α was obvious after 15 min of cell exposure to RANKL (Fig. 3G). These data demonstrate that RANKL activated multiple pathways, including p38, in RAW 264.7 cells.

**SC-409 Selectively Blocks p38 Signaling in Pre-Ocls without Interfering with JNK and NF-κB Pathways.** Although SC-409 is a selective inhibitor of p38α (Table 1; Fig. 2. SC-409 inhibits Ocl formation. Mouse RAW 264.7 cells (A–C and G) or human CD34+ cells (D–F and H) were treated with RANKL and M-CSF in the absence or presence of SC-409 or a structurally similar but inactive RANKL analog, SC-448, for the entire culture period. Cells (A–F) or culture plates (G) were photographed, and Ocls (TRAP+ cells with ≥ three nuclei, arrows) were quantified. The red color in G indicates TRAP staining. Although SC-409 had no inhibitory effect on cell growth up to 10 μM (C and F), cytotoxicity was observed with SC-448 at 10 μM (G). The synergism between RANKL and TNF-α on osteoclastogenesis was blocked by 1 μM SC-409 (H). Cell density and morphology are shown at different times of the cultures. The asterisks indicate statistically significant differences, ***, p < 0.001.

Fig. 3. The Ocl precursors express only the p38α isoform, which is downstream of RANK. Proteins were extracted from untreated RAW 264.7 cells and then analyzed by Western immunoblot (A). p38α was the only isoform detected by its specific antibody, although specific antibodies to either p38β or p38δ or p38γ did detect the corresponding recombinant proteins (10 ng/lane) that were used as positive controls (B). The isoform-selective detection limit for each antibody was <2 ng of the corresponding p38 isoform (data not shown). RAW 264.7 cells were also treated with 100 ng/ml RANKL for different times (C), and the degree of p38 phosphorylation (p-p38) after 20 min of exposure to RANKL, analyzed by Western immunoblot, was compared with that of 100 ng/ml LPS, used as a positive control (D). Optimal stimulation of p38 phosphorylation was reached after 20 min of exposure to RANKL. Protein extracts from untreated RAW 264.7 cells or RAW 264.7 cells treated for 10 or 20 min with 100 ng/ml RANKL or 100 ng/ml LPS were also analyzed by Western immunoblot for p-ERK (E), p-c-Jun (F), or p-IxB-α (G). RANKL stimulated ERK, c-Jun, and IxB-α phosphorylation with no change in total ERK and c-Jun. Degradation of phosphorylated IxB-α was obvious after 15 min of cell exposure to RANKL.
J. Monahan, unpublished data), the selectivity of its effects on p38 signaling induced by RANKL in RAW 264.7 cells was analyzed. These cells were preincubated with SC-409 for 30 min before treatment with RANKL for 20 min. No inhibition by SC-409 of RANKL-induced c-Jun phosphorylation (Fig. 4A) and IκB-α degradation (Fig. 4B) was observed even at 10 μM SC-409, a concentration higher than those required to block osteoclastogenesis (Fig. 2G). Since SC-409 has no effect on p38 phosphorylation (data not shown), HSP-27, a protein downstream of p38 and selectively phosphorylated by the p38 pathway, was used to study the specificity of SC-409. Since HSP-27 was undetectable in RAW 264.7 cells (data not shown), CD34+ cells were used to test the specificity of SC-409 effects in cells. RANKL failed to induce c-Jun and IκB-α phosphorylation in CD34+ cells, despite the fact that these proteins were expressed in these cells (data not shown). This observation was consistent with very low to undetectable levels of RANK on these cells (data not shown). Next, CD34+ cells were treated with M-CSF and RANKL for 7 days to induce cell commitment to the Ocl lineage. Like RAW 264.7 cells, only p38α was expressed in human Ocls (Fig. 4C). In these cells, RANKL induction of HSP27 phosphorylation, but not c-Jun and IκB-α phosphorylation, was inhibited by SC-409 at concentrations of 2 and 10 μM (Fig. 4, D–F). These data suggest that SC-409 inhibited Ocl development in vitro by selectively inhibiting p38α signaling.

**SC-409 Prevents Joint Swelling and Bone Destruction in SCW-Induced Arthritis in Rats.** To further demonstrate a role for p38 in osteoclastogenesis, we examined the effect of SC-409 on bone destruction in rat model of SCW-induced arthritis. The course of arthritis in this model is biphasic, with an acute inflammatory phase developing within 1 to 3 days followed by a chronic joint erosive phase developing 10 to 28 days after SCW injection. Treatment of SCW-inoculated rats with SC-409 starting on day 10 decreases paw swelling and arthritis severity (G. Anderson and J. Portanova, unpublished data). Doses of SC-409 were selected based on mouse pharmacokinetic/pharmacodynamic data (data not shown), resulting in free blood levels of compound that are consistent with a p38-selective effect. Microcomputed tomography confirmed that SC-409 attenuates bone destruction (Fig. 5, A–C). Bone mineral density measured by peripheral quantitative computed tomography indicated that it was normalized by SC-409 (Fig. 5D). Histologic evaluation showed that SC-409 also decreased joint infiltration by inflammatory cells was also decreased by SC-409 (Fig. 5, E–G). Although SC-409 partially protected the growth plate (Fig. 5J), it significantly inhibited the total numbers of Ocls/marrow area (Ocls/marrow area, control, 0.17 ± 0.125 Ocls/mm²; arthritis, 61.2 ± 18.95 Ocls/mm²; arthritis + SC-409, 12.36 ± 8.62 Ocls/mm²; Fig. 5, H–J). These findings demonstrate that p38 signaling is critical in the bone destruction in SCW-induced arthritis.

**SC-409 Inhibits the Expression of Numerous Inflammatory Mediators.** The data suggest that inhibition of p38 in Ocl precursors by SC-409 may account for its mechanism of action into the bone-sparing effects in vivo. Since a variety of genes are induced in states of arthritis, we examined the role of p38 on the expression of genes that are commonly increased in arthritis. Since elevation of cytokines levels is not readily measured in blood during SCW-induced arthritis, we used real-time reverse transcriptase-PCR (TaqMan) analysis of paw mRNA to determine change in gene expression associated with paw inflammation. Rats were treated with SC-409 from day 18 to 21, late after the onset of the disease, to determine the effects of SC-409 on gene expression by cells that have already infiltrated the joints. RANKL, IL-6, IL-1β, TNF-α, TRAP, MMP-3, -9, -13, and COX-2 mRNA expression was induced in paws from SCW-injected animals (day 21), whereas OPG mRNA expression was decreased slightly in the same paws (Fig. 6A). Treatment with SC-409 normalized SCW-induced RANKL, IL-6, IL-1β, TRAP, MMP-9, -13, and COX-2, but not TNF-α, OPG, and MMP-3 mRNA expression. Consistent with its inhibitory effect on COX-2 mRNA expression, SC-409 completely blocked prostaglandin E₂ production by human fibroblasts at concentrations as low as 2 μM (data not shown). To further understand the mechanisms of SC-409 inhibition of gene expression, we analyzed RANKL mRNA expression by primary rat bone marrow stromal cells treated in vitro with TNF-α. We found that RANKL mRNA expression was only partially inhibited by SC-409 (Fig. 6B) in this cell system compared with SCW samples, suggesting that this compound may inhibit the expression of certain cytokines in the joint via prevention of cell influx. Although SC-409 did not decrease the levels of TNF-α mRNA induced by SCW, it did inhibit LPS-induced both serum TNF-α and IL-6 levels by ≥90% (Fig. 6C), suggesting post-transcriptional regulation of TNF-α expression by p38.

**SCW-Induced Arthritis Activates the p38 Pathway.** Given that SC-409 is a selective inhibitor of p38, we asked whether this compound affected the DNA binding activity of the transcription factor MEF2C, a downstream substrate of p38. Using EMSA, we demonstrated that MEF2C exhibits higher DNA binding activity if nuclear extracts are from arthritic paws compared with normal paws (Fig. 7A). Next, we examined the effects of SC-409 on SCW-induced MEF2C DNA binding activity to show that this activity was inhibited by SC-409 (Fig. 7B).

Prominent mediators of arthritis, other than p38, have been described. These include AP-1/c-Jun (downstream of JNK) and the transcription factor NF-κB (Han et al., 1998;
Garg and Aggarwal, 2002). To assert that SC-409 inhibits arthritis through direct blockade of the p38 pathway, the effect of this compound on the activity of AP-1 and NF-κB was tested. Our data indicate that AP-1 and NF-κB exhibits higher DNA binding activity in nuclear extracts from arthritic paws compared with normal paws (Fig. 7, C and E). More importantly, administration of SC-409 only partially reduced NF-κB and AP-1 activity (Fig. 7, D and F). These findings indicate that SC-409 specifically blocks p38-mediated events. The marginal reduction in AP-1 activity may be
genes induced by SCW (Fig. 6). To elucidate the role of TNF-α-induced arthritis. AP-1, and NF-κB signaling modulates the expression of many genes in-duced by SCW, including RANKL.

100/H11003 activity (F). Nuclear extracts from arthritic paws were incubated with the distal tibiotalar junction of the paw. This is associated including lymphocytes, neutrophils, and monocytes, occurs at destruction. A massive infiltration by inflammatory cells, chronic phase of joint swelling and erosion and massive bone destruction in the SCW-induced arthritis model due to its osteolysis (Merrell et al., 2003), other studies have suggested that p38 is involved in osteoclastogenesis (Matsumoto et al., 2000; Lee et al., 2002) and tumor-induced osteolysis (Merrell et al., 2003), other studies have suggested that p38 pathway regulates bone resorption through its action in osteoblasts (Li et al., 2002). In this study, we demonstrate that SC-409 is efficient in protecting bone from destruction in the SCW-induced arthritis model due to its ability to inhibit the expression of key inflammatory mediators, including MMPs, which are involved in the breakdown of the extracellular matrix and are regulated by p38 (Wada et al., 2006). Our data point out that SC-409 spared bone in arthritis likely by inhibiting the expression of TNF-α by monocytes or macrophages. Subsequently, it may inhibit the expression of MMPs, RANKL, IL-1β, IL-6, and others by various cells, including synovial fibroblasts, endothelial cells, and chondrocytes. This is consistent with our data showing that TNF-α stimulated RANKL expression in bone marrow due to in vivo cross-talk between the various inflammatory pathways or may be secondary to the resolution of inflammation following the treatment with SC-409.

**TNF-α Production or Signaling Is Critical in SCW-Induced Arthritis.** SC-409 inhibits the expression of many genes induced by SCW (Fig. 6). To elucidate the role of TNF-α in SCW-induced arthritis, we compared the effects of SC-409 and the recombinant soluble decoy TNF receptor II, sTNFRII-Fc chimera, on RNA expression in this model. Interestingly, the profile of inhibition of cytokine expression by SC-409 was similar to that of sTNFRII-Fc chimera. Total RNA were isolated and subjected to TaqMan analysis. The profile of inhibition of cytokine expression by SC-409 was similar to that of sTNFRII-Fc chimera.

**Discussion**

In this study, we examined the role of p38 during joint destruction in the SCW-induced rat model of human rheumatoid arthritis (Brahm, 1991). The hallmark features of this model are a transient acute inflammatory phase preceding a chronic phase of joint swelling and erosion and massive bone destruction. A massive infiltration by inflammatory cells, including lymphocytes, neutrophils, and monocytes, occurs at the distal tibiotalar junction of the paw. This is associated with a significant increase in Ocl numbers, cortical bone erosion and destruction of the growth plates, and replacement of subchondral and lamellar trabecular bone by woven bone. Although Ocls are generally associated with existing bone surface, in this model, due to profound bone destruction, significant numbers of Ocls are observed in the marrow spaces where they are not attached to bone structures. Despite the aggressiveness of arthritis in this model, the p38 inhibitor, SC-409, at doses chosen to selectively inhibit p38, attenuated arthritis symptoms, bone destruction, and Ocl numbers. Since tissue destruction in this disease starts within 4 days after antigen inoculation, it is likely that prophylactic treatment with SC-409 may have been more efficient in sparing joint structures. Histological analysis of specimens showed that bone from SC-409-treated rats was mostly woven with islands of lamellar bone. This was expected because longer time was needed for the newly formed bone to acquire normal bone architecture (Rauch et al., 2000). Minimal loss of proteoglycan staining and erosion of the articular cartilage surface was observed at the joint of the tibiotalar junction, reflecting the relatively minor cartilage erosion in SCW-induced rat arthritis compared with bone destruction.

Although it has been reported that p38 compounds inhibited adjuvant-induced arthritis (Badger et al., 2000) and more recently that p38 is involved in osteoclastogenesis (Matsumoto et al., 2000; Lee et al., 2002) and tumor-induced osteolysis (Merrell et al., 2003), other studies have suggested that p38 pathway regulates bone resorption through its action in osteoblasts (Li et al., 2002). In this study, we demonstrate that SC-409 is efficient in sparing bone from destruction in the SCW-induced arthritis model due to its ability to inhibit the expression of key inflammatory mediators, including MMPs, which are involved in the breakdown of the extracellular matrix and are regulated by p38 (Wada et al., 2006). Our data point out that SC-409 spared bone in arthritis likely by inhibiting the expression of TNF-α by monocytes or macrophages. Subsequently, it may inhibit the expression of MMPs, RANKL, IL-1β, IL-6, and others by various cells, including synovial fibroblasts, endothelial cells, and chondrocytes. This is consistent with our data showing that TNF-α stimulated RANKL expression in bone marrow...
stromal cell cultures and SC-409 blocked by ≥90% LPS-induced TNF-α protein production. Furthermore, rats that were treated with SC-409 were indistinguishable from those treated with sTNFRII-Fc chimera in that paws from both treatment groups contained lower levels of inflammatory mediators and were equally protected from developing arthritis (G. Anderson, G. Mbalaviele, and J. Monahan, unpublished data). Thus, prevention of TNF-α production or signaling is sufficient to protect the bone from destruction in SCW arthritis. Consistent with this, TNF-α not only regulates RANKL expression (Kumar et al., 2001), but both cytokines also act in synergy to stimulate Ocl development (Komine et al., 2001; Feng, 2005). SC-409 has no effect on p38 phosphorylation, implying that SC-409 binding to p38 does not prevent proximal upstream MAPK kinases, such as MKK3 and MKK6, from phosphorylating this enzyme but inhibits the activity of p38 itself. In addition to p38, inhibition by SC-409 of casein kinase 1, which phosphorylates p80 TNF receptor (Darney et al., 1997), may contribute to its tissue protective effects. This mechanism is however unlikely since our other selective p38α inhibitors from different chemical series that spare casein kinase 1 by >200× are, if anything, more efficacious than SC-409 in inhibiting osteoclastogenesis and experiments in arthritis in rats. In addition to inhibiting cytokine expression, SC-409 may also directly inhibit bone resorption in vivo by antagonizing cytokine signaling inside the Ocl precursors. In support of this notion, in vitro data demonstrate that these cells expressed p38α, which could be activated by RANKL. Furthermore, SC-409 blocked Ocl formation that was induced by exogenous recombinant RANKL, by inhibiting p38 activity, demonstrating that this pathway is involved in downstream RANKL/RANK signaling. The reason that multiple pathways, including p38, ERK, JNK, and NF-κB (Matsumoto et al., 2000, 2004; Miyazaki et al., 2000; David et al., 2002; Xing et al., 2002) are inducible in Ocls is poorly elucidated. These pathways may be differentially triggered in physiological and pathological bone conditions or during the many steps involved in Ocl development, including precursor cell proliferation, differentiation, fusion, activation, and survival. Concomitant activation of all of these pathways by RANKL, similar to that seen in vitro, may be due to high levels of this protein needed to achieve desired effects. It is possible that JNK and p38 pathways, which are classically activated by stresses and inflammatory cytokines (Kyrkiakis and Avruch, 2001), are critical in bone loss associated with inflammation. This is consistent with the finding that mice deleted of JNK1 or JNK2 genes have normal skeletal metabolism (David et al., 2002). Future experiments are required to determine the role of p38 pathway in bone loss in other pathological conditions such as osteoporosis. The mechanisms of p38 signaling in Ocl precursors are still not clear. Our unpublished data indicate that SC-409 has no effect on cell growth or TRAP expression by mononuclear cells. However, this compound is able to block osteoclastogenesis even when added to cultures for only a short-term period of time when Ocl precursors fuse to form multinucleated Ocls. Recent data indicate that the microphthalmia transcription factor (MITF) is directly phosphorylated by p38 (Mansky et al., 2002b). Interestingly, MITF binding sites have been found in the promoter sequences of TRAP and E-cadherin genes, and expression of the latter was signifi-

cantly decreased in Ocls from mice with null mutation for MITF (Mansky et al., 2002a). We previously showed that E-cadherin is involved in the fusion of murine Ocl precursors (Mbalaviele et al., 1995), and in this study, we found that SC-409 decreased E-cadherin protein expression in Ocl precursors (data not shown). In addition, E-cadherin was found in Ocl ruffled borders, although its role in Ocl activity has not been shown. It is tempting to suggest that SC-409 inhibits osteoclastogenesis at least in part by inhibiting E-cadherin expression.

In conclusion, our p38 inhibitor, SC-409, was shown in this study to block the expression of inflammatory genes and p38 signaling in Ocl precursors. As a result, this inhibitor inhibited Ocl development and attenuated SCW-induced arthritis symptoms and bone destruction in rats.

References


phosphatidylinositol 3-kinase, p38 and extracellular signal-regulated kinase pathways are involved in osteoclast differentiation. Bone (NY) 30:71–77.


