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# IL-1- and TNF-Induced Bone Resorption Is Mediated by p38 Mitogen Activated Protein Kinase

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We have previously shown that p38 mitogen-activated protein kinase (MAPK) inhibitors, which block the production and action of inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), are effective in models of bone and cartilage degradation. To further investigate the role of p38 MAPK, we have studied its activation in osteoblasts and chondrocytes, following treatment with a panel of proinflammatory and osteotropic agents. In osteoblasts, significant activation of p38 MAPK was observed following treatment with IL-1 and TNF, but not parathyroid hormone, transforming growth factor-β (TGF-β), $1,25(OH)_2D_3$, insulin-like growth factor-1 (IGF-1), or IGF-II. Similar results were obtained using primary bovine chondrocytes and an SV40-immortalized human chondrocyte cell line, T/C28A4. SB 203580, a selective inhibitor of p38 MAPK, inhibited IL-1 and TNF-induced p38 MAPK activity and IL-6 production ($IC_{50}$s 0.3–0.5 μM) in osteoblasts and chondrocytes. In addition, IL-1 and TNF also activated p38 MAPK in fetal rat long bones and p38 MAPK inhibitors inhibited IL-1- and TNF-stimulated bone resorption in vitro in a dose-dependent manner ($IC_{50}$s 0.3–1 μM). These data support the contention that p38 MAPK plays a central role in regulating the production of, and responsiveness to, proinflammatory cytokines in bone and cartilage. Furthermore, the strong correlation between inhibition of kinase activity and IL-1 and TNF-stimulated biological responses indicates that selective inhibition of the p38 MAPK pathway may have therapeutic utility in joint diseases such as rheumatoid arthritis (RA). J. Cell. Physiol. 187: 294–303, 2001. © 2001 Wiley-Liss, Inc.

Mitogen-activated protein kinases (MAPK) are key intermediate enzymes in the signal transduction cascade from the extracellular environment to the nucleus (Marshall, 1995). Three groups of MAPKs have been identified in mammalian cells (Blenis, 1993; Cobb and Goldsmith, 1995). These are the extracellular signal regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 MAPKs (Cobb and Goldsmith, 1995). These enzymes are activated by dual phosphorylation on Thr and Tyr in a Thr-Xaa-Tyr (where Xaa is either a Glu in ERK, Gly in p38 or Pro in JNK) motif (Cobb and Goldsmith, 1995). In each group there appear to be multiple family members (Lee et al., 1999). In general, the ERKs are activated by growth factors and hormones whereas both JNKs and p38 MAPKs are activated by environmental stress and inflammatory cytokines (Cobb and Goldsmith, 1995; Raingeaud et al., 1995). p38 MAPK was originally identified as the target of pyridinylimidazole compounds that inhibit the production of inflammatory cytokines from monocytes (Lee et al., 1994). Subsequently, p38 MAPK has been shown to play a role in a variety of cellular processes (Lee et al., 1999, 2000).

In bone, osteoblasts and osteoclasts are the two main cell types responsible for bone formation and bone resorption, respectively. In cartilage, chondrocytes are responsible for the production and maintenance of the complex matrix present in this tissue. Cytokines are produced in both the bone and cartilage microenvironments, and cytokine regulation is critical for the local control of tissue remodeling (Lorenzo, 1991; van den Berg, 1999). Inflammatory diseases such as RA result in the destruction of cartilage and erosion of the underlying bone. In RA, elevated levels of proinflammatory cytokines such as IL-1, TNF, and IL-6 are present in

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synovial fluid of inflamed joints (MacDonald and Gowen, 1992; Kotake et al., 1996; van den Berg, 1999) and likely mediate the destruction of connective tissue.

Elevated IL-1 expression during the postmenopausal years has also been associated with osteoporosis. IL-1 receptor antagonist (IL-1ra) and soluble TNF binding protein have been shown to be effective in reducing bone resorption in in vitro models (Kitazawa et al., 1991; Manolagas, 1995). In addition, mice engineered to lack the type I IL-1R or IL-6 are protected from ovariectomy-induced bone loss (Poli et al., 1994; Lorenzo et al., 1998). These data underscore the important role of IL-1 and TNF in bone and cartilage metabolism.

IL-6 is produced by both osteoblasts and chondrocytes and can be induced by the proinflammatory cytokines IL-1 and TNF (Guerne et al., 1990; Littlewood et al., 1991; Hierl et al., 1998). In bone, IL-6 is thought to be an autocrine/paracrine factor that plays a role in osteoclast-mediated bone resorption primarily by supporting osteoclastogenesis (Ishimi et al., 1990; Roodman, 1992; Manolagas, 1998). IL-6 does not appear to have a direct effect in stimulating bone resorption mediated by mature osteoclasts (Al-Humidan et al., 1991; Bertolini et al., 1994). In cartilage, IL-6 along with several other inflammatory cytokines has been shown to be significantly upregulated in osteoarthritic vs. normal tissues (Moos et al., 1999). Thus, cytokines such as IL-1, TNF, and IL-6 appear to be involved in the catabolic response in both bone and cartilage (Lorenzo, 1991; van den Berg, 1999). Other osteotropic agents, such as parathyroid hormone (PTH), 1,25 $(OH)_2D_3$ and transforming growth factor-β (TGF-β), can have a dual effect, eliciting both catabolic and anabolic functions depending upon dose and duration of treatment. Whereas growth factors, such as insulin-like growth factor-I (IGF-I) and IGF-II are involved in the anabolic responses

While the signaling pathways for some of these growth factors have been defined, the role of specific MAPKs in the signal transduction pathways in bone and cartilage is only now beginning to be elucidated. In the present report, we have examined the activation of p38 MAPK in both osteoblasts and chondrocytes by various agents involved in bone and cartilage turnover. We show that in both osteoblasts and chondrocytes p38 MAPK is strongly activated by IL-1 and TNF. Using IL-6 production as a functional surrogate of p38 MAPK activity, we demonstrate that blocking cytokine-stimulated p38 MAPK activity with specific p38 MAPK inhibitors has functional consequences in both osteoblasts and chondrocytes. Finally, employing an in vitro fetal rat long bone (FRLB) organ culture model of bone resorption we demonstrate that IL-1and TNF mediate both the rapid activation of p38 MAPK and the subsequent induction of bone resorption and that treatment with selective inhibitors of p38 MAPK effectively prevents IL- and TNF-mediated bone resorption.

## MATERIALS AND METHODS

### Cell culture and treatments

Primary human osteoblasts (hOBs) and bovine chondrocytes were isolated as described previously (Beresford et al., 1984; Badger et al., 1998). The human osteosarcoma cell line, MG-63, was obtained from ATCC (Manassas, VA). The SV40 immortalized human chondrocyte cell line, T/C28A4, was obtained from Dr. Mary Goldring and has been characterized previously (Goldring et al., 1994). These cells were cultured in DMEM containing 10% fetal calf serum (Life Technologies Inc., Gaithersburg, MD). The proinflammatory cytokines IL-1β and TNFα were prepared at SmithKline Beecham (King of Prussia, PA). The MEK-1 inhibitor, PD098059, was purchased from New England Biolabs (Beverly, MA). The p38 MAPK inhibitors SB 203580 (Lee et al., 1994) and SB 242235 (Badger et al., 2000), were synthesized at SmithKline Beecham, dissolved in DMSO and added at the times and concentrations indicated in the Figure Legends. PTH, 1,25$(OH)_2D_3$, TGF-β and IGFs were obtained from Bachem Inc. (Torrance, CA), BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA), and Life Technologies Inc. (Gaithershurgh, MD), respectively.

### Immunoprecipitations, kinase assays, and immunoblotting

Cells were subjected to various treatments (50 ng/ml PTH, 10 nM 1,25 $(OH)_2D_3$, 20 ng/ml TGF-β, 50 ng/ml IGF-I or IGF-II, 10 ng/ml IL-1β or TNFα), washed twice in PBS and solubilized on ice in lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 25 mM β-glycerophosphate, 20 mM NaF, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 U/ml aprotinin) and centrifuged at 15,000*g* for 20 min at 4°C. Endogenous kinases were precipitated from equal amounts of cell lysates for 4 h at 4°C using anti-p38 (Lee et al., 1994) or anti-MAPK-activated protein kinase-2 (MAPKAP K-2) antibodies (1:500 dilutions) (Landry and Huot, 1995) (kindly supplied by Dr. Jacques Landry) bound to protein-A agarose. The beads were washed twice with lysis buffer and twice with kinase buffer (25 mM Hepes pH 7.4, 25 mM $MgCl_2$, 25 mM β-glycerophosphate, 100 μM sodium orthovanadate, 2 mM DTT). The immune-complex kinase assays were initiated by the addition of 25 μl of kinase buffer containing 10 μg of myelin basic protein (MBP) or 2 μg of GST-ATF2 for p38, and 3 μg of heat shock protein 27 (HSP27) for MAPKAP K-2 as substrate and 50 μM [$\gamma$-$^{32}$P] ATP (20 Ci/mmol). After 30 min at 30°C, the reaction was stopped by the addition of SDS sample buffer and the phosphorylated products were resolved by SDS-PAGE and transferred to nitrocellulose membrane and visualized by phosphorimaging (Molecular Dynamics, Sunnyvale, CA). The amount of p38 present in the immunoprecipitates and its extent of activation were determined by immunoblot using anti-p38 antibodies (1:5000) and anti-phosphotyrosine (PY20, Santa Cruz Biotechnology, 0.5 μg/ml), respectively. The immunoblots were developed using horseradish peroxidase-conjugated secondary antibodies and ECL (Pharmacia Amersham Biotech, Piscataway, NJ). Organ cultures of FRLBs were also treated with inhibitors and cytokines. The bones were harvested and homogenized in a Dounce homogenizer in the lysis buffer described above. p38 and MAPKAP K2 were immunoprecipitated from equal amounts of protein lysates and assayed as described above. The p38 inhibitors employed in these experiments were found to be non-toxic at concentrations up to 50 μM and the

effects of inhibitors were completely reversible (Kumar et al., 1999).

### Measurement of IL-6 production

MG-63 or T/C28A4 cells were plated at a density of $5 \times 10^4$ cells/ml of culture media in 48-well plates and incubated for 24 h. Cultures were washed once with PBS and replenished with fresh medium containing various concentrations of SB 203580 in the presence of either IL-1β (10 ng) or TNFα (10 ng). Cell-free supernatants were collected and analyzed for IL-6 levels by ELISA (Amersham Pharmacia Biotech).

### FRLB assay

The resorption assay was performed essentially as described previously (Raisz, 1965; Stern and Raisz, 1979; Votta and Bertolini, 1994). Briefly, timed-pregnant Sprague-Dawley rats (Taconic Farms, Germantown, NY) were injected subcutaneously with 200 μCi of $^{45}CaCl_2$ on day 18 of gestation, housed overnight, then anesthetized with Innovar-Vet (Pittman-Moore, Mundelein, IL) and sacrificed by cervical dislocation. The fetuses were removed aseptically and the radii and ulnae were dissected free of surrounding soft tissue and cartilaginous ends. The bone rudiments (n = 4) were subsequently cultured for 18–24 h in $BGJ_b$ medium (Sigma, St. Louis, MO) containing 1 mg/ml BSA, then transferred to fresh medium and cultured for an additional 48 h in the absence or presence of 1 ng/ml IL-1β or 20 ng/ml TNFα and the desired inhibitor. $^{45}Ca$ released into the medium and the residual $^{45}Ca$ in the bones (following solubilization in 5% TCA for 1 h at room temperature) were quantitated by liquid scintillation spectrometry. Data are expressed as the percent $^{45}Ca$ released from treated bones as compared with corresponding control bones. Statistical differences were assessed by a one-way analysis of variance (ANOVA). $IC_{50}$ values were based on data from three or more independent experiments. All animal experiments were conducted in accordance with guidelines established by SmithKline Beecham's animal care and use committee.

## RESULTS

### Activation of p38 MAPK in human osteoblasts

To analyze the activation profile of p38 MAPK, hOBs and human osteosarcoma MG-63 cells were treated with various growth factors and cytokines (Fig. 1). Typically, these agents have been shown to have biological effects at the doses tested. Similarly, p38 MAPK is usually activated within a few minutes of stimulation (Raingeaud et al., 1995) and therefore, we selected a 15-min treatment for the initial experiments. The activation of p38 MAPK was assayed by an in vitro immune-complex kinase assay using MBP as a substrate and by anti-phosphotyrosine immunoblotting since phosphotyrosine content of MAPKs is generally believed to reflect their activation status (Cobb and Goldsmith, 1995). Using primary hOBs only IL-1 (10 ng/ml) and TNF (10 ng/ml) treatment led to activation of p38 MAPK as judged by both the kinase assay and the anti-phosphotyrosine blot (Fig. 1A, upper and middle panels, lanes 7 and 8). The anti-p38 immunoblot confirmed that equal amounts of p38 were analyzed in each sample (bottom panel). Approximately 15- and 8-fold activation of p38 MAPK was obtained in response to IL-1 and TNF, respectively. Other agents such as PTH (50 ng/ml), $1,25(OH)_2D_3$ (10 nM), TGF-β (20 ng/ml), IGF-I or IGF-II (50 ng/ml each) did not have any significant effect on p38 activation in primary hOBs. The pattern and extent of p38 MAPK induction was comparable in human osteosarcoma MG-63 cells (Fig. 1B, lane 7 and 8), and ~12- and 8-fold activation was obtained with IL-1 and TNF, respectively. However, in MG-63 cells $1,25(OH)_2D_3$, TGF-β and IGF-I & II appeared to stimulate p38 MAPK activity to a modest degree (~1.2–1.8 fold) as measured by MBP phosphorylation (Fig. 1B, upper panel). However, these effects were not confirmed by anti-phosphotyrosine immunoblotting (Fig. 1B, middle panel). Given the fact that MBP is a generic substrate of several kinases, it is possible that the apparent activation of p38 by $1,25(OH)_2D_3$, TGF-β and IGFs as measured by MBP phosphorylation reflects non-specific phosphorylation by another contaminating kinase(s). As a general rule,

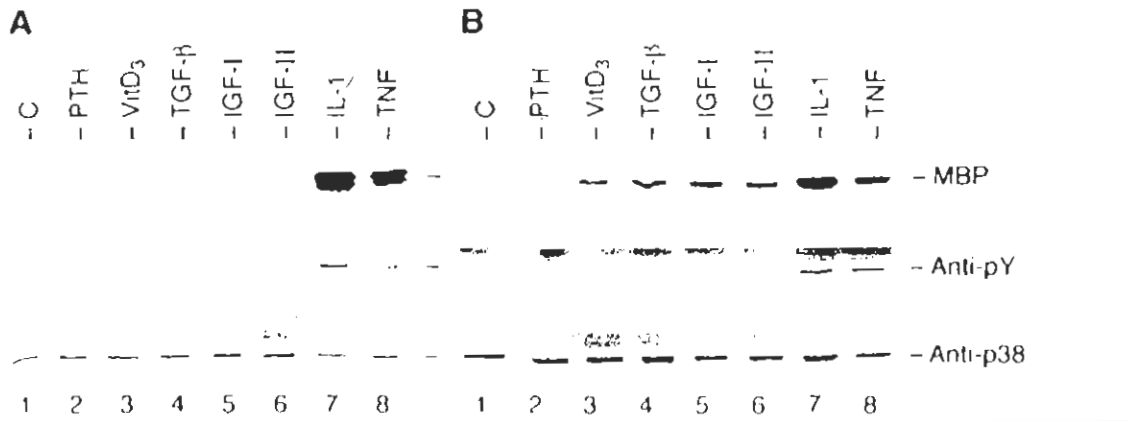


Fig. 1. Activation of p38 MAPK in human osteoblastic cells by various growth factors and cytokines. Primary hOBs (A) and MG-63 osteosarcoma cells (B) were treated with PTH (50 ng/ml), 1,25 $(OH)_2D_3$ ($VitD_3$, 10 nM), TGF β (20 ng/ml), IGF-I or IGF-II (50 ng/ml), IL-1β or TNFα (10 ng/ml) and an immune-complex kinase assay for p38 MAPK was performed as described in Materials and Methods. The top panel shows the result of a kinase assay using MBP as a substrate, whereas the middle and bottom panels show the results of an anti-phosphotyrosine (Anti-pY) and an anti-p38 immunoblot, respectively. Note the activation of p38 MAPK by IL-1 and TNF in both cell types (lanes 7 and 8).

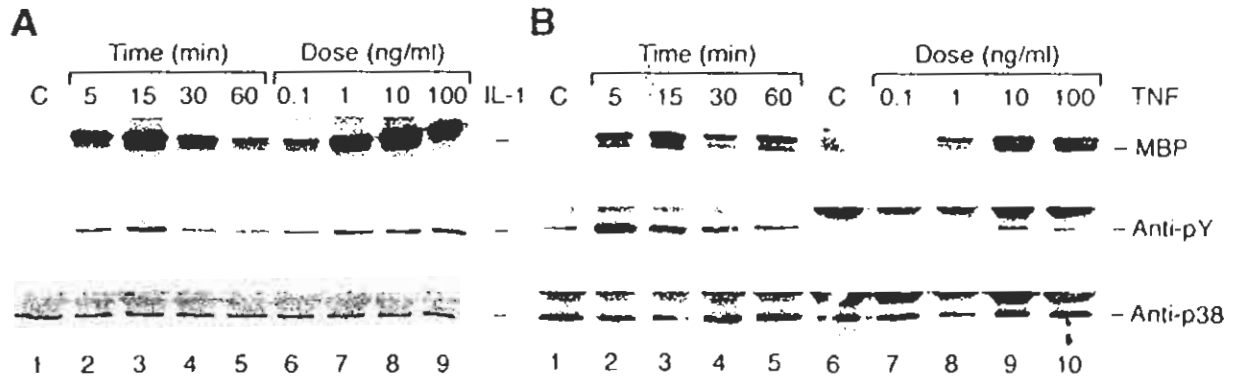


Fig. 2. Activation of p38 MAPK in MG-63 osteosarcoma cells by IL-1 and TNF. MG-63 cells were treated with 10 ng/ml of IL-1 or TNF for indicated times (A and B lanes 2–5) or for 15 min with indicated doses of IL-1 or TNF (A and B, lanes 6–9). C indicates untreated control cells. The top panel shows the result of a p38 immune-complex kinase assay using MBP as a substrate, whereas the middle and bottom panels show the results of an anti-phosphotyrosine (Anti-pY) and an anti-p38 immunoblot, respectively. Maximal activation of p38 MAPK was obtained at 5–15 min and at ~10–20 ng/ml of both IL-1 and TNF.

we interpreted data as positive only if both anti-phophotyrosine immunoblotting and the kinase assay were in agreement.

### Kinetics of p38 MAPK activation by IL-1 and TNF in osteoblasts

To further characterize the IL-1 and TNF-induced p38 MAP kinase activation, we performed a detailed dose response and time course analysis using MG-63 cells. IL-1 (10 ng/ml) activated p38 MAPK within 5 min (Fig. 2A, upper panel, lane 2). The maximal activation of 11-fold was seen at 15 min with 10 ng/ml of IL-1 (lane 3), which declined to about 3-fold within 60 min (lane 5). An optimum dose of 10 ng/ml of IL-1 was required to achieve an 11-fold activation of p38 MAPK within 15 min (lane 8). An anti-phosphotyrosine blot (middle panel) confirmed the data obtained with the kinase assay and an anti-p38 immunoblot (bottom panel) confirmed that equal amounts of p38 were loaded in each lane. Similarly, TNF had a comparable activation profile in MG-63 cells where treatment with 10 ng/ml of TNF for 15 min resulted in a 4–6-fold activation of p38 MAPK (Fig. 2B, lanes 3 and 9). Higher anti-pY signal in Figure 2B, lane 1 (middle panel) is perhaps due to a greater amount of p38 loaded in this lane (see lane 1, bottom panel).

### Activation of p38 MAP kinase in chondrocytes

Primary bovine chondrocytes and the human chondrocyte cell line T/C28A4 were treated with various agents for 15 min as in Figure 1 the activity of p38 MAP kinase analyzed by the kinase assay and anti-phosphotyrosine blot. Similar to data obtained with primary hOBs, only IL-1 (Fig. 3A, lane 7, 5-fold) and TNF (lane 8, 5-fold) activated p38 MAPK in primary bovine chondrocytes. PTH (50 ng/ml), 1,25$(OH)_2D_3$ (10 nM), TGF-β

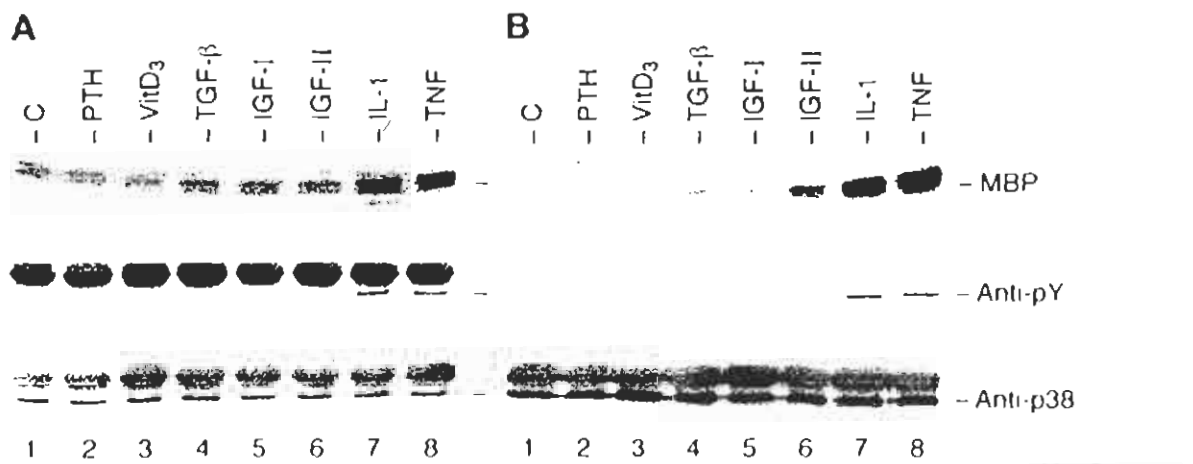


Fig. 3. Activation of p38 MAPK in chondrocytic cells by various cytokines and proinflammatory agents. Primary bovine chondrocytes (A) and the human chondrocyte T/C28A4 cells (B) were treated with PTH (50 ng/ml), 1,25 $(OH)_2D_3$ (VitD$_3$, 10 nM), TGF-β (20 ng/ml), IGF-I or IGF-II (50 ng/ml), IL-1β or TNF (10 ng/ml) and an immune-complex kinase assay for p38 MAPK was performed as described in Materials and Methods. The top panel shows the results of a kinase assay using MBP as a substrate, whereas the middle and bottom panels show the results of an anti-phosphotyrosine (Anti-pY) and an anti-p38 immunoblot, respectively. Note the activation of p38 MAPK by IL-1 and TNF in both cell types (lanes 7 and 8).

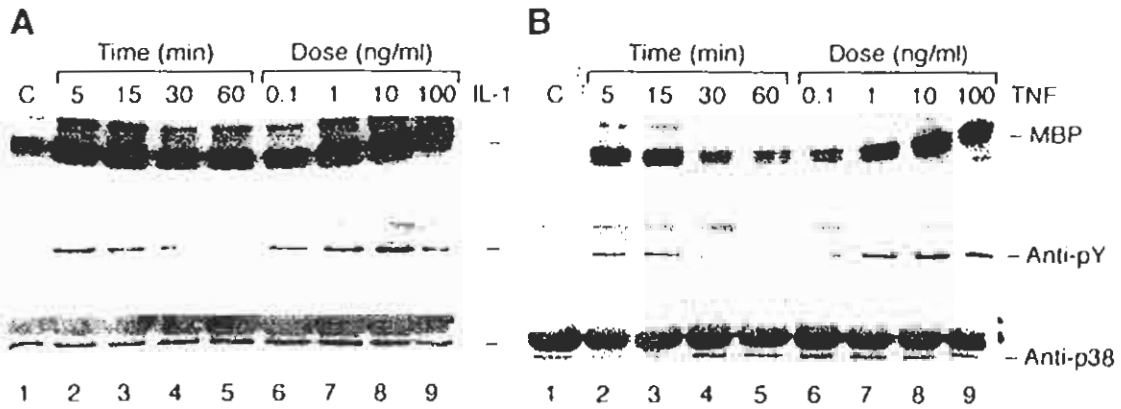


Fig. 4. Activation of p38 MAPK in T/C28A4 human chondrocyte cells by IL-1 and TNF. T/C28A4 cells were treated with 10 ng/ml of IL-1 or TNF for indicated times (A and B, lanes 2–5) or for 15 min with indicated doses of IL-1 or TNF (A and B, lanes 6–9). C indicates untreated control cells. The top panel shows the result of a p38 immune-complex kinase assay using MBP as a substrate, whereas the middle and bottom panels show the results of an anti-phosphotyrosine (Anti-pY) and an anti-p38 immunoblot, respectively. Maximal activation of p38 MAPK was obtained at 5–15 min and at ~10–20 ng/ml of both IL-1 and TNF.

(20 ng/ml), IGF-I or IGF-II (50 ng/ml each) did not have any apparent effect on the activation of p38 MAP kinase. IL-1 and TNF activated p38 MAPK in T/C28A4 cells to a similar extent. (Fig. 3B, lanes 7 and 8). However, analogous to the data obtained with the osteoblastic cell line MG-63, a modest increase in phosphorylation of MBP (but not with respect to anti-phosphotyrosine immunoblotting) was observed in T/C28A4 cells in response to TGF-β and IGF-I Fig. 3B, lane 4–6). In contrast, both IGF-I and IGF-II strongly activated ERK MAPK (data not shown).

### Kinetics of p38 MAPK activation by IL-1 and TNF in chondrocytes

Since the activation of p38 MAPK was comparable for primary bovine chondrocytes and the T/C28A4 human chondrocyte cell line, we further characterized the activation of p38 MAPK by IL-1 and TNF by performing a detailed dose response and time course study of p38 MAPK activation in the human chondrocyte T/C28A4 cells. As shown in Figure 4A, ~3–5-fold activation of p38 MAPK was achieved within 5–15 min of treatment with 10 ng/ml of IL-1 (lanes 2, 3, and 8). Similarly, 10 ng/ml of TNF also activated p38 MAPK within 5–15 min (~5–6-fold) (Fig. 4B, lanes 2, 3, and 8). As little as 0.1 ng/ml of IL-1 or TNF was sufficient to activate the p38 MAP kinase and optimal activation was seen at 10 ng/ml of either IL-1 or TNF. In all cases the kinase activity of p38 correlated with the phosphorylation of tyrosine in p38 MAPK as judged by the anti-phosphotyrosine blot (middle panels).

### Inhibition of p38 MAP kinase activity by a selective p38 MAPK inhibitor

SB 203580 has been shown to be a selective p38 MAPK inhibitor. To determine if SB 203580 also inhibits p38 MAPK activity in bone and cartilage cells, MG63 and T/C28A4 cells were treated with different amounts of SB 203580 and then stimulated with TNF to activate p38 MAPK and MAPKAP K2. The effect of SB 203580 on p38 activity was measured by assaying the activity of MAPKAP K2 using HSP27 as a substrate. MAPKAP K2 is an in vivo substrate of p38 and is widely used to demonstrate the inhibition of p38 in cells (Cuenda et al., 1995; Kumar et al., 1999). As shown in Figure 5, in both MG63 (upper panel) and T/C28A4 cells (lower panel), TNF-activated MAPKAP K2 activity (lane 2) was inhibited by SB 203580 in a dose-dependent manner ($IC_{50}$ of ~0.3–0.5 μM, lanes 2–7). This is the same dose-range as reported previously for other cells including primary bovine chondrocytes (Badger et al., 1998). Similar results were obtained when IL-1 was used as a stimulating agent (data not shown).

### Inhibition of IL-1- and TNF-induced IL-6 production by p38 MAPK inhibitor

Osteoblasts and chondrocytes are known to produce low levels of IL-6 that can be further induced in response to IL-1 and TNF (Guerne et al., 1990; Roodman, 1992). In human monocytes IL-1-induced IL-6 production has been demonstrated to be mediated by p38 MAPK activation (Kotlyarov et al., 1999). Therefore, we used

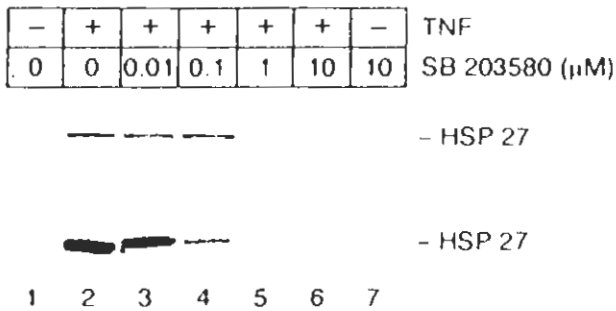


Fig. 5. Inhibition of p38 MAPK activity by SB 203580 in MG63 and T/C28A4 cells. MG63 (upper panel) and T/C28A4 (lower panel) cells were pretreated for 10 min with various concentrations of SB 203580 as indicated. The p38 MAPK cascade was then activated by addition of TNF for 30 min. Cell lysates were prepared and MAPKAP K2 activity was assayed using equal amounts of protein by an immune-complex kinase assay using HSP27 as a substrate. The $IC_{50}$ was calculated to be about 0.3 μM for MG-63 and 0.5 μM for T/C28A4 cells.

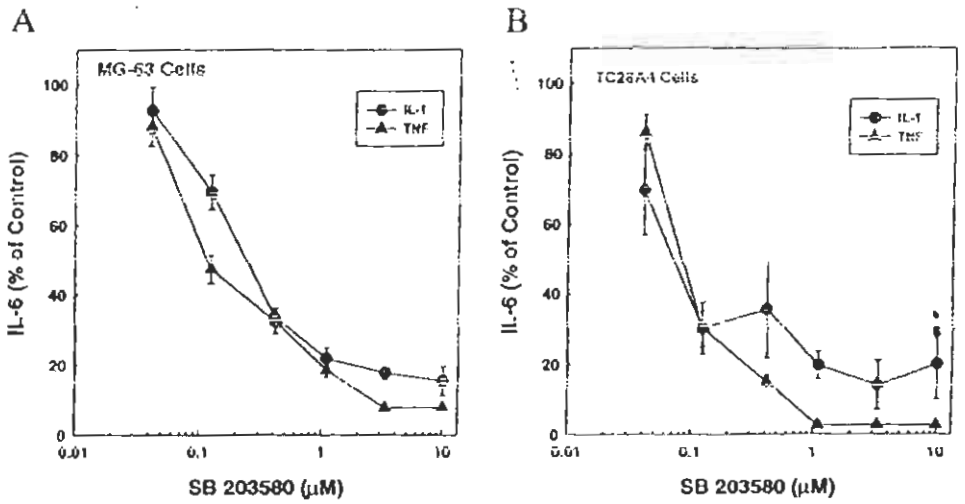


Fig. 6. Inhibition of IL-1- and TNF-induced IL-6 production. MG-63 osteosarcoma (A) and T/C28A4 chondrocyte cells (B) were pretreated with SB 203580 and then stimulated with IL-1β (●) or TNFα (▲). The level of IL-6 in cell culture supernatant was measured by ELISA and the data expressed as percent inhibition of IL-6 produced in the absence of inhibitor. Each treatment was performed in triplicate.

IL-6 production as a functional readout for p38 MAPK activation in osteoblasts and chondrocytes. Osteosarcoma MG-63 and chondrocyte T/C28A4 cells were stimulated with IL-1 and TNF and the production of IL-6 in cell culture supernatants was measured by ELISA. Approximately 3–5 ng/ml of IL-6 is produced in unstimulated cells which is induced to 51–110 ng/ml of IL-6 for MG-63 and 73–150 ng/ml of IL-6 for T/C28A4 cells in response to TNF (10 ng/ml) and IL-1 (10 ng/ml), respectively. Thus, IL-1 and TNF caused a 20–30-fold increase in IL-6 production after 24 h in both osteoblasts and chondrocytes. We next determined whether the p38 MAP kinase inhibitor, SB 203580, would inhibit the IL-1- and TNF-induced production of IL-6. MG63 and T/C28A4 cells were treated with SB 203580 and then stimulated with IL-1 or TNF. A dose-dependent inhibition ($IC_{50}$ ~0.1–0.3 μM) of IL-1- and TNF-induced IL-6 production was observed with SB 203580 treatment in both MG63 (Fig. 6A) and T/C28A4 cells (Fig. 6B). Similar results were obtained with primary hOBs (data not shown).

### Inhibition of bone resorption by a p38 MAPK inhibitor

IL-1 and TNF mediate a catabolic effect in bone and cartilage and have been shown to be involved in bone resorption (Kotake et al., 1996; van den Berg, 1999). We have established that both IL-1 and TNF signal through p38 MAPK. Therefore, selective inhibition of the p38 MAPK pathway should inhibit IL-1 and TNF-induced bone resorption. In order to test this hypothesis we assessed the effect of selective p38 MAPK inhibitors (SB 203580, SB 242235) on IL-1-stimulated bone resorption in the FRLB assay (Fig. 7). IL-1-stimulated bone resorption, as measured by the release of $^{45}Ca$ into the culture medium, was inhibited by both SB 203580 ($IC_{50}$ ~0.8 μM) and SB 242235 ($IC_{50}$ ~0.5 μM). This inhibition was consistent with the inhibition of p38 MAPK activity and IL-1-induced IL-6 production. Similar data was obtained when TNF was used to stimulate bone resorption (data not shown). In contrast the ERK selective inhibitor (PD 098059) was ~100-fold less effective (Fig. 7). Inhibition at higher concentrations of the MEK inhibitor may reflect toxicity.

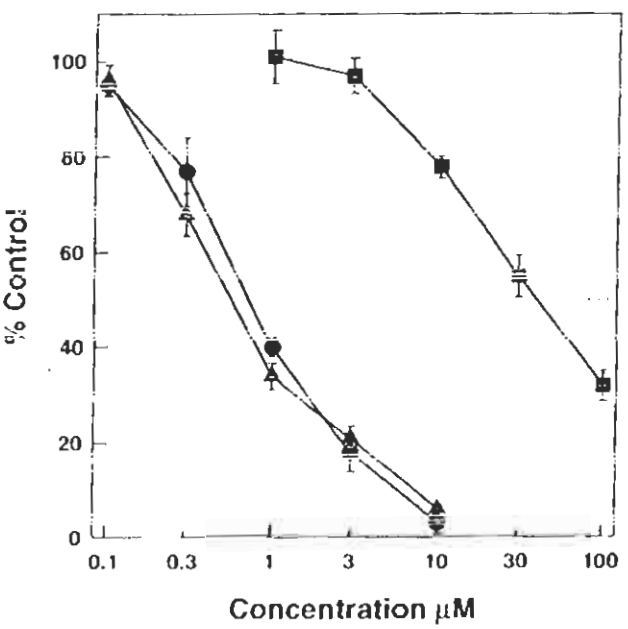


Fig. 7. Inhibition of fetal rat bone resorption. $^{45}Ca$-labeled fetal bone rudiments were cultured for 48 h in the presence of IL-1β and the indicated concentrations of either SB 203580 (■), SB 242235 (▲), or the MEK-1 inhibitor, PD98059 (■) and the resorption assay was performed as described in Materials and Methods. The data is expressed as percent of control resorption in the absence of inhibitors. Each data point represents the mean ± S.D. of four bones. These data are representative of three independent experiments.

### Inhibition of p38 MAPK activity in FRLBs

In order to relate the SB 203580- and SB 242235-mediated inhibition of resorption in FRLB cultures to its effect on p38 MAPK activity, we measured p38 MAPK activity directly in IL-1 and TNF-stimulated FRLBs in both the absence and presence of inhibitors. Figure 8A demonstrates that the kinetics of p38 activation induced by either IL-1 (10 ng/ml) or TNF (10 ng/ml) is comparable to the kinetics of activation obtained with primary osteoblasts, chondrocytes and related cell lines as described above. A 4–6-fold stimulation of p38 activity was observed within 15 min for TNF and IL-1, respectively, which diminished over time. The p38 MAPK selective inhibitors, SB 203580 and SB 242235, inhibited IL-1-induced p38 MAPK activity as evidenced by the concentration-dependent inhibition of MAPKAP K2-mediated phosphorylation of HSP27 (Fig. 8B). The $IC_{50}$ for SB 203580 and SB 242235 was calculated to be 0.5 and 0.3 μM, respectively and complete inhibition was obtained at 10 and 1 μM of SB 203580 and SB 242235, respectively. As expected, treatment with the MEK-1 inhibitor PD 098059 was ineffective and only marginal (less than 30%) inhibition was observed at the highest concentration of 20 μM. Identical results were obtained when TNF was used as a stimulus (data not shown).

## DISCUSSION

IL-1 and TNF are two pleiotropic pro-inflammatory cytokines shown to exhibit potent bone resorbing and cartilage destructive properties (MacDonald and Gowen, 1992; Kimble et al., 1994; Kitazawa et al., 1994; Manolagas, 1995; Ammann et al., 1997; van den Berg, 1999). Therapies designed to either reduce the availability or inhibit the activity of TNF and IL-1 by administration of soluble TNF receptors and IL-1ra, respectively, are being used in the clinic for RA underscoring the importance of these cytokines. It is therefore important to study the signaling pathways initiated by the proinflammatory cytokines in these highly specialized tissues.

Pyridinylimidazole compounds were originally identified as agents that inhibited the production of inflammatory cytokines such as IL-1 and TNF (Lee et al., 1993). These effects were subsequently shown to be due to selective inhibition of p38 MAPK (Lee et al., 1994). Furthermore, these inhibitors have been shown to block the production of other cytokines such as IL-6 and IL-8, in a wide range of cell types (Lee et al., 1999, 2000). One such inhibitor SB 203580, has been shown to be effective in several in vivo models where IL-1 and TNF have been implicated, including arthritis, bone resorption, endotoxic shock, and immune function (Badger et al., 1996). Recently, another highly selective p38 MAPK inhibitor SB242235 was shown to have disease-modifying properties in a rat model of adjuvant-induced arthritis. This inhibitor exerted anti-inflammatory activity and resulted in the prevention of cartilage loss and bone erosion in the diseased joints (Badger et al., 2000). To further explore the role of p38 MAPK in cartilage and bone, we examined the effect of various agents known to influence osteoblast and chondrocyte function on p38 MAPK activity. SB 203580, a representative pyridinylimidazole compound,

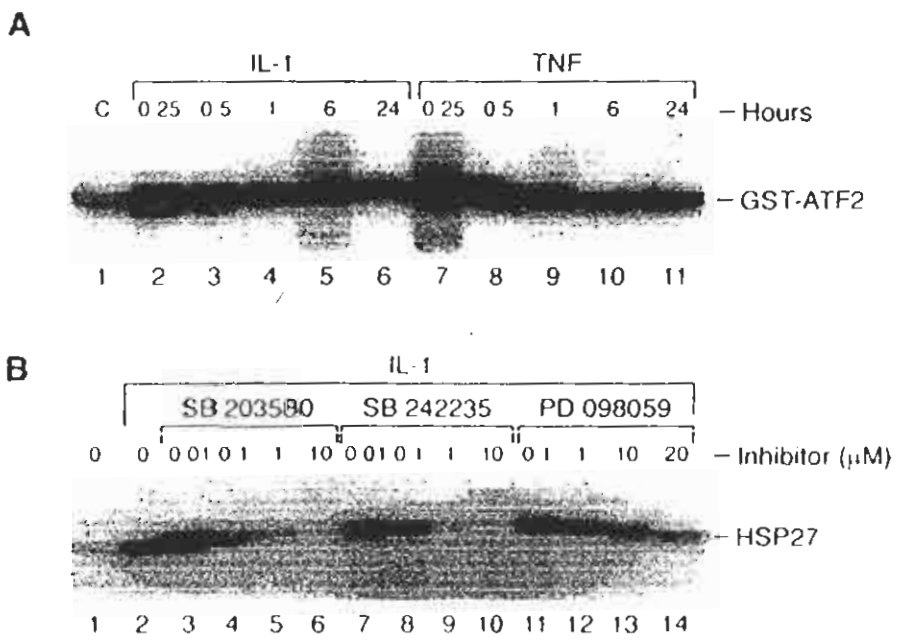


Fig. 8. p38 MAPK activity in FRLBs. A: Fetal bone rudiments were cultured for various times as indicated in the presence of either IL-1α (10 ng/ml; lanes 2–6) or TNFα (10 ng/ml; lanes 7–11). p38 MAPK was immunoprecipitated from equal amounts of bone homogenates and an immune complex kinase assay was performed using GST-ATF2 as a substrate. Lane 1 represents untreated control (C) bones. B: Fetal rat bone rudiments were precultured with various concentrations of different inhibitors as indicated for 30 min and then exposed to IL-1α (10 ng/ml) for 10 min. MAPKAP K2 was immunoprecipitated from equal amounts of bone homogenates and an immune complex kinase assay was performed using HSP27 as a substrate.

and SB 242235, a highly selective p38 inhibitor were then used to demonstrate that inhibition of p38 MAPK activation correlated with functional activity.

The major findings of this study are: (i) among the various osteotropic and proinflammatory agents tested, IL-1 and TNF activate p38 MAP kinase to the greatest extent, (ii) this occurs in primary osteoblasts, chondrocytes, related cell lines, and in FRLBs, (iii) selective inhibitors of p38 MAPK activity inhibit downstream functional consequences (e.g. IL-6 production) of cytokine stimulation in both cell types, (iv) SB 203580 and SB 242235 inhibit both IL-1- and TNF-stimulated p38 MAPK activity and bone resorption in FRLB cultures, and (v) the concentration of inhibitor needed to inhibit the p38 MAPK activity correlates closely with the concentration required to inhibit IL-1- and TNF-induced functional responses.

To our knowledge, this is the first detailed report of an analysis of p38 MAPK activation in primary osteoblasts and chondrocytes and in FRLBs in response to various osteotropic agents. Chaudhary and Avioli, (1997) have previously reported the activation of ERKs in response to growth factors such as IGF, FGF and PDGF. An analysis of MAPK activation in chondrocytes has been reported by Geng et al., 1996. Similar to our data, they reported that only TNF and IL-1 treatment led to the activation of all three MAPKs, p38, JNK, and ERK, whereas IGF-I, PDGF, and IL-6 activated only ERK (Geng et al., 1996). PTH has been shown to both activate and inhibit ERK depending upon cell type, dose, and time of treatment (Verheijen and Defize, 1995; Verbeijen and Defize, 1997). However, these studies did not relate the activation of MAPKs to any functional consequences in either osteoblasts or chondrocytes.

We have further dissected the molecular basis of IL-1- and TNF-induced bone and cartilage destruction often associated with immune-mediated inflammatory diseases, such as RA. Of the agents tested, those known to have anabolic activity, including PTH (50 ng/ml), IGFs (50 ng/ml), TGF-β (20 ng/ml), or $1,25(OH)_2D_3$ (10 nM) did not directly activate p38 MAPK in either primary osteoblasts or chondrocytes. However, both PTH (50 ng/ml) and $1,25(OH)_2D_3$ (10 nM) have been shown to stimulate bone resorption in the FRLB cultures. This resorption was inhibited by SB 203580 with an $IC_{50}$ comparable to its inhibition of IL-1- and TNF-stimulated resorption (Badger et al., 1996). PTH- and $1,25(OH)_2D_3$-mediated bone resorption is thought to be mediated via its effect on osteoblasts. It is likely that in the organ culture system employed, secondary mediators including cytokines are produced in response to PTH or $1,25(OH)_2D_3$ treatment. Indeed specific neutralizing anti-rat IL-1 antibodies inhibit both PTH- and $1,25(OH)_2D_3$-stimulated bone resorption, even though neither PTH nor $1,25(OH)_2D_3$ directly activates p38 MAPK (BJV, unpublished communications).

Because of the complex and heterogeneous nature of the tissue employed in the FRLB organ cultures, which contain various cell types in addition to osteoblasts and osteoclasts, it is not possible to conclude that inhibition of p38 kinase activation/activity in any particular cell is responsible for the observed inhibition of resorption. While it is likely that in this model IL-1 and/or TNF are directly acting on both osteoblasts and osteoclasts, it is possible that other cell types may also be involved in this process. In any event, it is clear that IL-1 and, therefore, p38 MAPK signaling are most likely involved in mediating the observed resorptive response irrespective of the cell type(s) involved.

It has been well documented in other systems that p38 MAPK signaling is involved in mediating the production of multiple cytokines in response to a variety of stimuli. In this study we have measured IL-6 production as a convenient functional surrogate for p38 MAPK activation. We further demonstrated that specific inhibitors of p38 MAPK are effective in inhibiting IL-1-and TNF-induced IL-6 production by osteoblasts and chondrocytes at doses consistent with inhibition of p38 MAPK activity. However, it has been reported that IL-6 does not activate the p38 MAPK pathway directly implying that the catabolic effect of IL-6 may be mediated via an independent mechanism (Geng et al., 1996). While it is unlikely that IL-6 is mediating the catabolic effects of IL-1 or TNF in the resorption model employed in these studies, a role for IL-6 in bone resorption has been described. For example, Kurihara et al., (1990) have reported that in long term human marrow cultures IL-6 stimulates osteoclastogenesis by inducing IL-1 release. In a recent report, bFGF was shown to induce IL-6 production in the osteoblastic MC3T3-E1 cell line via a p38 MAPK-dependent pathway as SB 203580 inhibited this process (Kozawa et al., 1999). In addition, co-administration of IL-6 along with its soluble receptor has been shown to induce collagenase 3 expression in primary osteoblasts isolated from fetal rat calvariae (Franchimont et al., 1997)

Recently, receptor activator of NF-kB ligand (RANK-L) has been shown to be an important osteoclastogenic and osteoclast-inducing factor secreted by osteoblasts and stromal cells (Kong et al., 2000). It is possible that IL-1 and TNF induce the production of RANK-L by osteoblasts which in turn stimulate bone resorption by inducing osteoclasts. Indeed, the induction of RANK-L by TNF and IL-1 have been recently published while this manuscript was under review (Nakashima et al., 2000). Alternatively, RANK-L itself may activate p38 MAPK activity in osteoclasts. In preliminary experiments, we have observed weak but measurable activation of p38 MAPK by RANK-L in osteoclasts (SK, unpublished communication). Recently, the effect of SB 203580 on RANK-L-mediated osteoclastogenesis has been published while this manuscript was under review (Matsumoto et al., 2000). Similarly, TNF and IL-1 have been shown to directly activate both mature osteoclasts and their progenitors (Fox et al., 2000; Kobayashi et al., 2000) and therefore a possible effect of TNF and IL-1 on osteoclasts in our system cannot be ruled out. Thus, IL-1 and TNF may activate p38 MAPK and induce bone resorption via osteoblast or stromal cell production of RANK-L (or other factors) or directly via activation of p38 MAPK in osteoclasts.

We have clearly demonstrated that inhibition of p38 MAPK activity effectively prevents IL-1- and TNF-induced bone resorption. SB 203580, while a potent and selective inhibitor of p38 MAPK activity, has recently been shown to inhibit JNK2β1 and c-Raf at high doses (Delaszlo et al., 1998; Lee et al., 1999). Therefore, we also used SB 242235 which is a highly selective inhibitor of

p38 MAPK without any effect on several other kinases including JNK2β1 and c-Raf (Badger et al., 2000). The results obtained with SB 242235 for both inhibition of p38 MAPK activity and bone resorption in the FRLB assay were comparable to those obtained with SB 203580. This strongly suggests that it is the selective inhibition of p38 MAPK activity that results in the inhibition of bone resorption.

These data strongly implicate p38 MAPK as a downstream signal transduction molecule involved in IL-1 and TNF signaling in both osteoblasts and chondrocytes. p38 MAPK inhibitors appear to act at two levels. They not only inhibit the signaling and production of IL-1 and TNF by blocking p38 MAPK activity, but also block the local production of additional inflammatory cytokines that can further contribute to the destruction of bone and cartilage. Therefore, p38 MAPK inhibitors may have therapeutic potential in inflammatory diseases, such as RA where IL-1, TNF, and IL-6 play major roles.

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