The Synthetic Triterpenoid TP-222 Inhibits RANKL Stimulation of Osteoclastogenesis and Matrix Metalloproteinase-9 Expression

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ABSTRACT. *Objective.* Receptor activator of nuclear factor-κB ligand (RANKL) promotes osteoclast differentiation from monocyte precursors by inducing a cohort of genes, including tartrate-resistant acid phosphatase (TRAP) and matrix metalloproteinase-9 (MMP-9). A family of synthetic triterpenoids with antiinflammatory and pro-apoptotic properties was described to modulate differentiation in monocytic cell lineages. We therefore investigated the ability of the potent and bioavailable synthetic triterpenoid TP-222 to inhibit RANKL-induced osteoclast formation and MMP-9 expression from monocytic precursor cells.

Methods. Osteoclast formation was assayed by staining for TRAP-positive multinucleated cells. MMP-9 expression was measured by quantitative RT-PCR, Western blot, immunohistochemistry, and gel zymography. *In vivo* effects of TP-222 were assessed by daily intraperitoneal injection of 4-week-old mice for 7 days followed by measurement of osteoclast number and MMP-9 expression at the cartilage/bone junction of the epiphyseal growth plate.

Results. RANKL promoted and TP-222 (300 nM) inhibited osteoclast formation in cultures of RAW264.7 cells or bone marrow-derived monocytes. RANKL also induced MMP-9 expression in RAW264.7 cells and this was reduced by concurrent or subsequent addition of TP-222. TP-222 treatment significantly reduced the mean number of osteoclasts present at the cartilage/bone interface compared to vehicle-injected control mice. Morphometric analyses of tissue sections showed that TP-222 treatment reduced the amount of immunoreactive MMP-9 present in both mononucleated pre-osteoclasts and osteoclasts.

Conclusion. Our data demonstrate that TP-222 inhibits osteoclast formation and MMP-9 expression *in vitro* and *in vivo*, and suggest that triterpenoids may be useful compounds for modulating bone resorption diseases. (First Release Mar 15 2007; J Rheumatol 2007;34:1058–68)

Key Indexing Terms:
OSTEOCLASTS   MATRIX METALLOPROTEINASES   RANKL
BONE RESORPTION   OSTEOPOROSIS

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Osteoclasts can be differentiated *in vitro* from bone marrow-derived progenitor cells of the monocytic lineage in the presence of macrophage colony-stimulating factor (M-CSF) and the ligand for receptor activator of nuclear factor-κB (NF-κB), denoted RANKL. The striking importance of M-CSF and RANKL and its receptor RANK for optimal osteoclast formation has been established *in vivo* by gene knockout studies[1]. M-CSF contributes to osteoclast generation in several ways, including driving proliferation of osteoclast progenitors and enhancing their survival[2], inducing RANK expression[3], and by inhibiting the production of osteoprotegerin[4], which is a soluble decoy receptor that negatively regulates osteoclast formation by binding RANKL. Thus, the combination of M-CSF and RANKL strongly promotes osteoclast differentiation from bone marrow progenitors[1,2].

RANKL is expressed on the surface of several cell types, including osteoblasts, and in arthritic tissues is expressed by synovial fibroblasts and activated lymphocytes[5]. Binding of RANKL to RANK on pre-osteoclasts initiates signaling events through multiple pathways including NF-κB, NFATc1,

p38-MAPKs, and PI-3-K[6]. Activation of these pathways results in a complex set of responses including transient expression of costimulatory factors that ultimately cause the fusion of osteoclast progenitors into mature multinucleated osteoclasts. These mature osteoclasts express a specific cohort of gene products, including calcitonin receptor, tartrate-resistant acid phosphatase (TRAP), cathepsin K, and matrix metalloproteinase-9 (MMP-9)[7,8].

MMP-9, expressed both in pre-osteoclasts and at higher levels in mature osteoclasts, is a key enzyme used to traverse tissue matrix during recruitment of pre-osteoclasts to regions of bone resorption[9,10]. Once a mature osteoclast arrives at a site of bone resorption, acid-mediated demineralization of calcified bone matrix is initiated by the formation of a specialized ruffled border that secretes acid and abuts the bone surface[1]. After the calcified matrix is solubilized, the enzymatic degradation of the residual matrix rich in type I collagen can take place[1]. In this regard, mature osteoclasts secrete cysteine proteases such as cathepsin K, which functions optimally within the low pH environment of resorptive pits[11]. MMP-9 is also instrumental in bone resorption, as demonstrated in mice deficient in MMP-9. MMP-9-deficient mice exhibited delayed recruitment of osteoclasts during skeletal development, due to impaired migration of pre-osteoclasts through the discontinuously mineralized hypertrophic cartilage[9]. The same study revealed that MMP-9 activity also releases a heparin-binding isoform of vascular endothelial growth factor (VEGF) that is associated with cartilage extracellular matrix, and which is important for chemotactic recruitment of pre-osteoclasts and osteoclasts. For these reasons, pharmacologic modulation of MMP-9 production by pre-osteoclasts and osteoclasts may offer a novel way to inhibit osteoclast accumulation in pathogenic bone resorption.

Studies have established that triterpenoids can inhibit MMP gene expression[12-14] by targeting specific transcription factors including Bcl-3[12] and Smads[13]. Triterpenoids are naturally occurring compounds found in foods, medicinal herbs, and plants[15]. Used in China and Japan for their hepato-protective properties, they have well characterized antitumor and antiinflammatory properties. To increase the potency of these compounds, over 300 synthetic triterpenoids have been created based on oleanolic and ursolic acids[16-18]. These compounds exert antiproliferative effects and promote differentiation[19], protect cells from oxidative stress[20,21], inhibit inducible nitric oxide synthase and inducible cyclooxygenase-2 expression in mouse macrophages[19,20], and impede NF-κB-mediated transcription[22]. It has been suggested that the antioxidant properties of synthetic triterpenoids are due to their ability to bind to Keap-1 and promote activation of the antioxidant transcription factor Nrf-2[20]. However, given the broad range of effects of these compounds, multiple cellular targets likely exist.

We examined the capacity of a novel triterpenoid denoted TP-222 to modulate osteoclast formation *in vitro* and *in vivo*.

We employed the synthetic triterpenoid TP-222 (ethyl 2-carboxy-3,12-dioxooleana-1,9(11)-dien-28-oate)[17] because of its high potency, low toxicity, and bioavailability *in vivo*. We found that TP-222 reduced osteoclast differentiation *in vitro* with an unusually high potency for a member of this family of compounds. Similar observations on *in vitro* osteoclast formation were reported for other related compounds used at considerably higher doses, and the inhibitory effects on osteoclast formation were not demonstrated *in vivo*[23]. Here we demonstrate that TP-222 reduced the numbers of mature osteoclasts and the overall level of MMP-9 protein localized at the epiphyseal growth plate when the compound was administered to mice, suggesting that its effects on osteoclast generation may not be restricted to the *in vitro* model. These studies establish a novel property of this synthetic triterpenoid, and although the precise molecular target(s) that implement its actions remain to be determined, the results suggest that TP-222 may be a useful pharmacologic modulator of bone resorption and remodeling.

MATERIALS AND METHODS

Cell culture and reagents. The synthesis of TP-222 from oleanolic acid has been described[17] and this compound was used at 300 nM in all experiments unless otherwise indicated. This is a dose that does not induce apoptosis in a variety of cultured cells[12]. RANKL and M-CSF were purchased from R & D Systems (Minneapolis, MN, USA). Lipopolysaccharide (LPS) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modification of Eagle's media (DMEM) was supplemented with penicillin/streptomycin and L-glutamine (Cellgro; Mediatech, Herndon, VA, USA) and 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA). For serum-free media, FBS was replaced with 0.2% lactalbumin hydrolysate (Invitrogen, Carlsbad, CA, USA).

Cytotoxicity assays. Cell viability of TP-222-treated cultures was assessed by MTT assay (Invitrogen). In addition, cytotoxicity was assessed by staining with propidium iodide. Briefly, cells were cultured in triplicate for each condition and the media changed on Day 2 to renew the TP-222 and RANKL. After 4 days, medium was removed and replaced with phosphate buffered saline (PBS) containing 1:100 dilution of stock propidium iodide (Molecular Probes, Eugene, OR, USA). Cultures were incubated 15 min to allow propidium iodide to stain the nuclei of dead or dying cells. The cells were washed twice to remove all unbound propidium iodide, resuspended in PBS, and analyzed by flow cytometry.

Osteoclastogenesis assay. Osteoclastogenesis was assessed in 2 mouse monocyte culture systems, RAW264.7 cells and bone marrow-derived monocytes. The murine monocytic cell line RAW264.7 was purchased from American Type Culture Collection (Manassas, VA, USA). RAW264.7 cells were cultured in DMEM supplemented with 10% FBS, both with and without RANKL (50 ng/ml). Alternatively, bone marrow was flushed from the medullary space of femurs from C57 BL/6 mice into DMEM with 10% FBS and held on ice. A single-cell suspension was made by repeated passage through a small-bore pipette, and the cells washed once with DMEM with 10% FBS. Cells were cultured 24 h with DMEM supplemented with 10% FBS, after which nonadherant cells were collected, washed once with RPMI-1640 medium, and plated in 12-well dishes at one million cells per well. Cells were cultured in RPMI-1640 supplemented with 10% FBS, both with and without M-CSF (20 ng/ml) and RANKL (50 ng/ml). After 4 days (RAW264.7) or 10 days (bone marrow-derived cells), the number of osteoclasts was determined by counting multinucleated cells (at least 2 nuclei clearly visible) in 10 random fields by high magnification phase microscopy. In addition, cells were stained for expression of TRAP using a leukocyte acid phosphatase assay kit (Sigma), following the manufacturer's instructions.

Resorption assay. Osteoclast resorption was assayed on Osteologic® slides (BD Biosciences, Bedford, MA, USA) according to the manufacturer's instructions. Briefly, RAW264.7 cells were plated in the chamber slides and cultured for 4 days in the presence of RANKL (50 ng/ml). The medium was replaced with fresh RANKL-containing medium, both with and without TP-222 (300 nM). After an additional 7 days, the medium was removed and cells were removed by the addition of bleach and gentle pipetting. Following 3 rinses with deionized water, the slides were stained for 30 min with 5% silver nitrate. The slides were rinsed with deionized water and treated with 5% sodium carbonate and 25% formalin for 60 s. After rinsing with deionized water, 5% sodium thiosulfate was added. The slides were rinsed a final time and allowed to air dry.

In vivo TP-222 treatment, growth plate immunohistochemistry, and TRAP staining. TP-222 was dissolved in DMSO and mixed with one part Cremophor EL (Sigma-Aldrich, St. Louis, MO, USA) and 8 parts PBS. DBA/1J mice (4 weeks of age) were injected by intraperitoneal route daily for 7 days. After euthanasia, the skin over the knees was removed, and intact knee joints were dissected by cutting tibia and femur distal to the femoral and tibial heads. The tissue was fixed in 4% paraformaldehye in PBS overnight at 4°C and the knee joints bisected with a new single-edge razor blade. The tissue was fixed an additional 24 h in 4% paraformaldehyde/PBS and then decalcified in a 10 mM EDTA solution. After 10 days, the tissue was probed with a 30 gauge needle to verify decalcification, washed in PBS, dehydrated in 30%–100% ethanol solutions and submitted to routine paraffin embedding. The tissue blocks containing both halves of the knee joint were sectioned (7 μm sections), collecting replicate sections at 100 μm intervals through the entire block such that roughly 800 μm of knee joint tissue was represented in the tissue section series. Paraffin embedded sections were dewaxed, rehydrated, and endogenous peroxidase was blocked with 0.3% H_2O_2 in 15% methanol. Anti-MMP-9 primary antibody (goat anti-mouse MMP-9 IgG; R & D Systems) was added at 1:300 dilution in PBS for 90 min at room temperature. The sections were then washed 3 times for 10 min each and probed with a horseradish peroxidase conjugated secondary antibody (donkey anti-mouse HRP; Santa Cruz Biotechnology) for 45 min at dilution of 1:200 in PBS. To control for nonspecific binding, primary antibody was omitted. Additionally, primary antibody was preincubated with 2 μg/ml recombinant mouse MMP-9 (R & D Systems) for 2 h prior to addition to sections. MMP-9 expression was visualized using the Vector Laboratories DAB kit (Vector, Burlingame, CA, USA). Sections were also stained for TRAP for 2 min at 37°C using a leukocyte acid phosphatase assay kit (Sigma) following the manufacturer's instructions.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). For study of gene expression, RNA was harvested using TRIzol (Invitrogen, Carlsbad, CA, USA), and subjected to DNase treatment (Ambion, Austin, TX, USA) to remove genomic DNA. RNA was reverse transcribed using Oligo dT 12–18 primers (Invitrogen), and quantitative PCR carried out using a DNA Engine Opticon™ continuous fluorescence detection system (MJ Research, Waltham, MA, USA) and SYBRgreen master mix (Applied Biosystems, Foster City, CA, USA). PCR reactions consisted of a "hot start" at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Primer pairs for MMP-9 (5'-AAG CGG ACA TTG TCA TCC A-3' and 5'-AAT GGG CAT CTC CCT GAA C-3') and GAPDH (5'-CAT GGC CTT CCG TGT TCC TA-3' and 5'-GCG GCA CGT CAG ATC CA-3') were prepared by IDT (Coralville, IA, USA). To quantify gene expression, standard curves of cycle threshold versus log of picograms of DNA were generated using plasmids containing the appropriate cDNA (MMP-9 or GAPDH). These standard curves were used to calculate the amount of cDNA generated from the reverse transcribed mRNA of each sample.

Western blotting and zymography. To assay for MMP-9 synthesis, secreted proteins present in the cell culture supernatant were incubated with an equal volume of 2× sample loading buffer (10 min, 100°C; Sigma), resolved on a 10% SDS-PAGE gel (100 v, 1 h; BioRad, Hercules, CA, USA), and transferred to a PVDF membrane. After blocking using TRIS buffered saline with 0.1% Tween 20 (TBST) and 5% (w/v) milk powder (TBST/milk), the membranes were incubated overnight with goat anti-mouse MMP-9 (1:500 dilution in TBST/milk; R & D Systems). The membranes were washed (TBST), then incubated 1 h with donkey anti-goat Ig, horseradish peroxidase linked F(ab')$_2$ fragment (1:1000 dilution in TBST/milk; Santa Cruz Biotechnology). The membrane was then washed with TBST, developed with Supersignal chemiluminesence Western blot detection reagent (Pierce, Rockland, IL, USA), and exposed to X-ray film (30 s to 5 min). For assay of MMP-9 activity in cell culture supernatant, zymography was performed as described[24] using gelatin gels (BioRad). Gels were stained using Coomassie blue R-250, the appearance of light bands indicating latent or active MMP-9 and MMP-2 activity.

Statistical analysis. Analysis of statistical significance was performed using unpaired 2-tailed Student t tests. For cultured cells this was based on data from triplicate platings and all results are representative of 2 or more experiments. For measurements of TRAP and MMP-9 at the mouse growth plate, this was based on measurements of at least 5 fields of view from each of 5 animals.

RESULTS

TP-222 inhibits RANKL-mediated osteoclast formation in vitro. We first examined the ability of the synthetic triterpenoid TP-222 (Figure 1A) to modulate the formation of osteoclasts by using the RAW264.7 cell line as precursor cells. TP-222 is a synthetic derivative of the naturally occurring triterpenoid oleanolic acid (Figure 1A). RAW264.7 cells are a unique monocytic cell line that express RANK and has the capacity to form multinucleated osteoclasts upon stimulation with RANKL and other stimuli[8]. Under our culture conditions, RAW264.7 cells cultured 4 days in the presence of 50 ng/ml RANKL formed multinucleated TRAP-positive osteoclasts, as expected. A dose-response experiment showed that TP-222 significantly reduced RANKL-induced osteoclast formation at 300 and 600 nM (Figure 1B, 1C). Further, TP-222 was significantly more potent than the naturally occurring triterpenoid oleanolic acid at both 300 and 600 nM (Figure 1B). It is well established that synthetic triterpenoids promote the differentiation of myeloid cells in culture[25]. Indeed, we observed an antiproliferative effect of TP-222 in RAW264.7 cells cultured in serum alone (data not shown). However, the effect of triterpenoids on the viability of monocytic cells differentiated with RANKL has not been examined previously. We therefore tested whether the observed reduction of osteoclast formation in TP-222-treated cells was simply a result of increased cytotoxicity. RAW264.7 cells cultured 24 h in RANKL, both with and without TP-222, showed no decrease in proliferation or cell viability as assayed by MTT assay (Figure 1D). Consistent with an earlier study[26], we found RAW264.7 cells differentiated with RANKL alone for 4 days showed significantly reduced MTT activity (Figure 1D). This is likely a result of reduced proliferation in differentiating cells. Importantly, RAW264.7 cells differentiated with RANKL for 4 days in the presence of 150 to 600 nM TP-222 did not show a decrease in proliferation or cell viability when compared to RANKL alone (Figure 1D). The MTT assay is a general index of viability and does not differentiate between reduced proliferation and increased cell death. Therefore we decided to confirm these results through propidium iodide
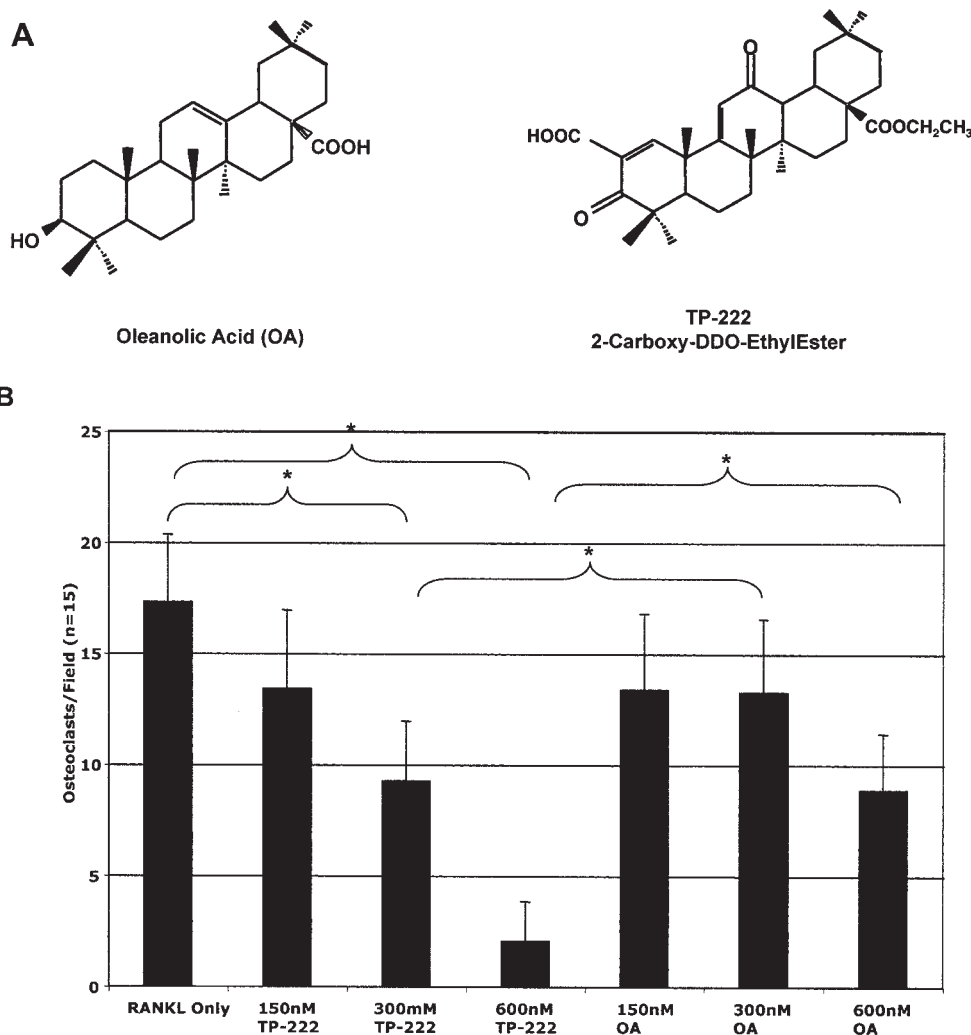
Figure 1. TP-222 inhibits RANKL-induced multinucleation of RAW264.7 macrophages. A. The chemical structures of oleanolic acid (OA) and TP-222. B. A dose-response of TP-222 inhibition of osteoclast formation in RAW264.7 cells. RAW 264.7 cells were cultured with RANKL for 4 days, with or without TP-222, then osteoclast formation was assessed by quantitation of TRAP-positive multinucleated cells. Data are shown as means plus standard deviations (*$p < 0.05$).

staining, which directly measures the number of nuclei from deceased osteoclasts and osteoclast precursors. This analysis showed no significant increase in cytotoxicity with TP-222 treatment, and a slight decrease at 600 nM (Figure 1E). Together, these results demonstrate that TP-222 does not inhibit osteoclast formation through cytotoxicity of monocytic precursor cells or fused osteoclasts. Since 300 nM was the lowest concentration capable of significantly inhibiting osteoclast formation, this concentration was used in subsequent experiments. TP-222 at 300 nM also inhibited osteoclast formation in RAW264.7 cells that were treated with the combination of M-CSF and RANKL (data not shown). These data show that the synthetic triterpenoid TP-222 is more potent than the natural triterpenoid oleanolic acid in inhibiting RANKL-induced osteoclast formation in RAW264.7 cells, and that this inhibition occurs without increased cytotoxicity.

To determine if the ability of TP-222 to inhibit osteoclast formation was limited to the RAW264.7 cell line, similar experiments were performed using bone marrow cells from C57/B6 mice. Bone marrow cells were treated for 3 days with M-CSF (30 ng/ml) and then for 7 days with RANKL (50 ng/ml), with or without 300 nM TP-222. When cultured with RANKL for 7 days, TP-222 at 300 nM significantly inhibited osteoclast formation from M-CSF-pretreated bone marrow progenitor cells (Figure 2A, 2B). However, consistent with our studies in RAW264.7 cells, TP-222 at doses from 150 to 600 nM were not toxic to bone marrow-derived cells (Figure 2C). Thus the triterpenoid TP-222 is a potent inhibitor of osteoclast formation *in vitro* from either bone marrow precursor cells or the RAW264.7 cell line.

TP-222 inhibits RANKL-induced expression of MMP-9 in cultured osteoclasts. Since triterpenoids have been reported to inhibit synthesis of certain types of MMP in other systems[12-14], and MMP-9 is thought to be important in pre-osteoclast and

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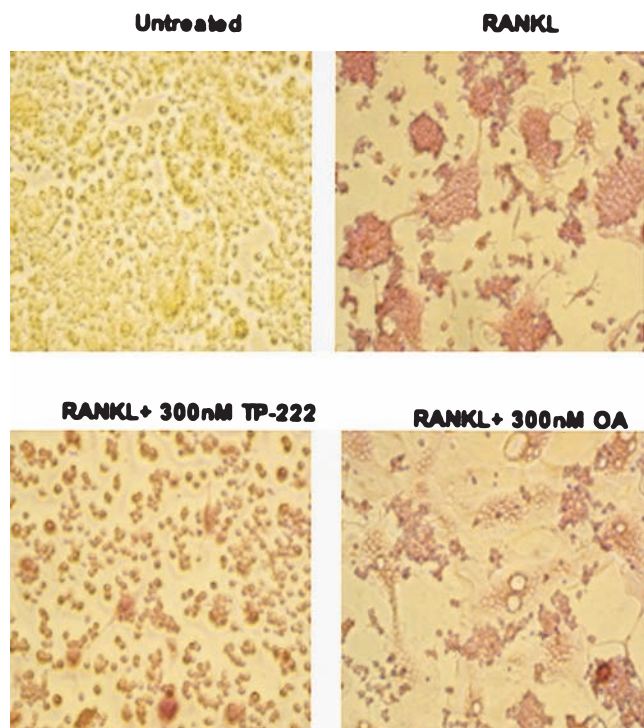
Figure 1. C. Photomicrographs of RAW264.7-derived osteoclasts. RAW-264.7 cells were cultured in growth media alone (untreated), or growth media containing 50 ng/ml RANKL, RANKL plus 300 nM TP-222, or RANKL plus 300 nM oleanolic acid. After 4 days, cultures were fixed and stained for TRAP-positive cells. D. Cytotoxicity was measured by MTT assay in RAW264.7 cultures containing RANKL alone or RANKL in the presence of increasing concentrations of TP-222. E. Cytotoxicity was also assayed by staining with propidium iodide followed by measurement of number of viable cells on a flow cytometer. Data are shown as means plus SD.

osteoclast mobility, we assayed the effect of TP-222 on MMP-9 expression both during and after osteoclast formation from RAW264.7 cells. During the formation of multinucleated osteoclasts from RAW264.7 cells, the expression of certain genes such as TRAP, calcitonin receptor, and MMP-9 is initiated and maintained[1,8]. First, RAW264.7 pre-osteoclast cells were cultured without and with RANKL and the level of expression of MMP-9 mRNA was determined by quantitative RT-PCR after 24, 48, 72, and 96 h culture. Compared to untreated cells, MMP-9 mRNA expression increased 5-fold in cells treated with RANKL within 24 h, and after 96 h of culture the expression level was increased 7.5-fold. The addition of 300 nM TP-222 counteracted the induction of MMP-9 mRNA by RANKL, such that expression of MMP-9 was reduced by 62% after 96 h culture (Figure 3).

We next examined whether TP-222 could also reduce MMP-9 expression in cultures that had already formed osteoclasts. Wittrant and colleagues reported that osteoclast formation, as assayed by calcitonin receptor and TRAP expression, is maximal in RAW264.7 cells after 4 days of culture in
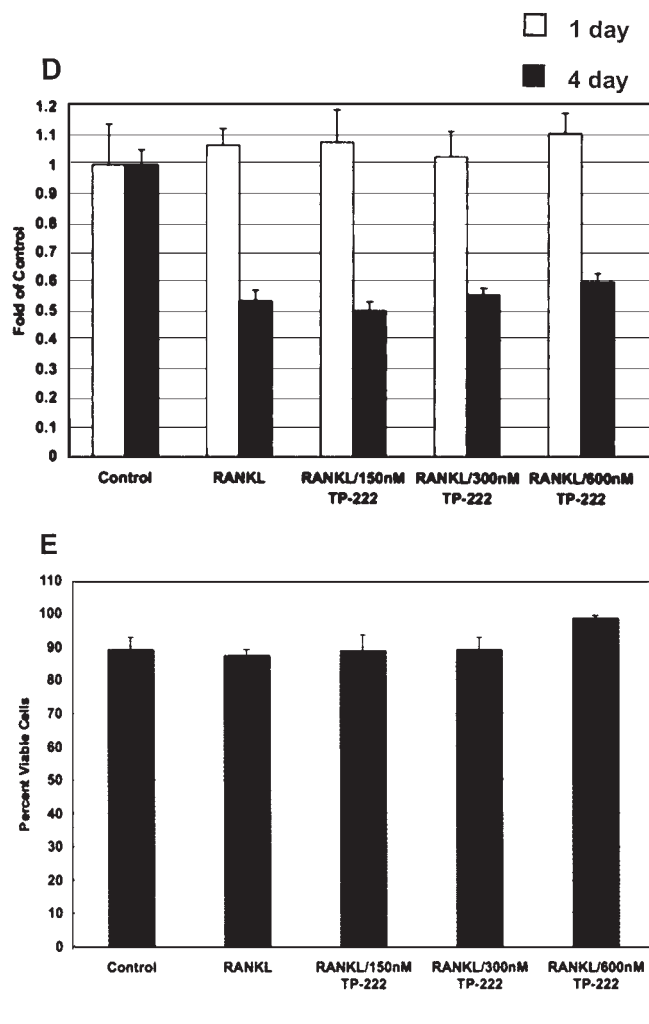
RANKL[8]. Therefore, after 4 days of culture with RANKL, serum and RANKL were washed out and RAW264.7 cells were cultured an additional 24 h in fresh serum-free media, with and without the addition of TP-222. In such osteoclast-enriched cultures, the level of MMP-9 mRNA expression was significantly reduced by the addition of TP-222 (Figure 4A). We next examined whether the inhibitory effect of TP-222 on the induction of MMP-9 mRNA by RANKL resulted in a parallel decrease in the amount and activity of secreted MMP-9 protein, as determined by Western blotting and gel zymography. RANKL treatment of RAW264.7 cells resulted in an increase of latent, but not active, MMP-9 protein (Figure 4B). The addition of TP-222 at Days 4 through 5 reduced both the amount and the activity of MMP-9 secreted by osteoclast-enriched cultures (Figure 4B). To determine if this reduction of MMP-9 expression affected the resorptive ability of osteoclast cultures, RAW264.7 cells were plated on Osteologic® slides, differentiated to osteoclasts by RANKL for 4 days, and then TP-222 was added for an additional 7 days of culture. Von Kossa staining of these slides showed that in differentiat-
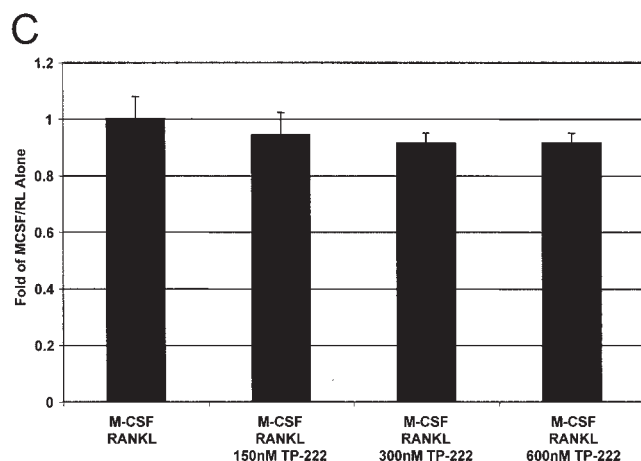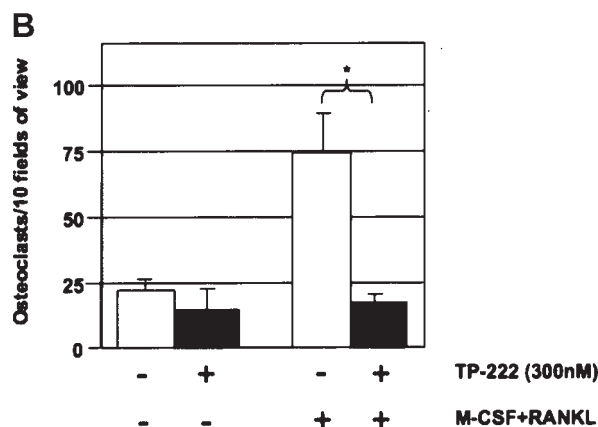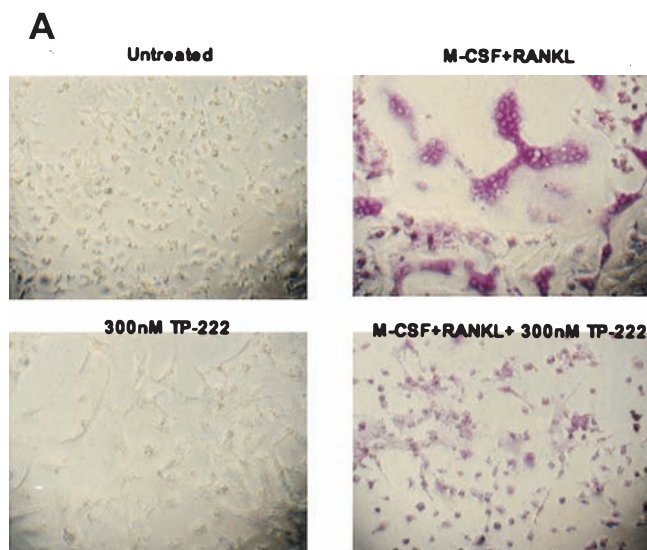
Figure 2. TP-222 inhibits RANKL-induced multinucleation of bone marrow-derived macrophages. Bone marrow-derived macrophages were cultured with M-CSF/RANKL with or without TP-222 (300 nM). After 10 days, osteoclastogenesis was assessed by quantitation of TRAP-postive multinucleated cells. A. Photomicrographs of TRAP-stained cells. B. Numerical counts of osteoclasts. Data are shown as means plus SD (*p < 0.05). C. MTT assay of bone marrow-derived monocytes cultured in M-CSF/RANKL or M-CSF/RANKL plus TP-222 for 24 h.

ed osteoclasts, the addition of 300 nM TP-222 did not significantly reduce the amount of resorption (Figure 4C). Thus, although TP-222 can inhibit MMP-9 production both during and after osteoclast formation, this does not reduce mineralized matrix resorption by these cells.

To determine if TP-222 can reduce MMP-9 expression independently of osteoclast formation, we assayed the ability of TP-222 to inhibit MMP-9 expression in response to a second agonist, LPS. Treatment of RAW264.7 cells with LPS did not increase the extent of osteoclast formation under our experimental conditions (data not shown). While others have reported LPS induction of osteoclast formation in bone marrow derived cells[27], this likely reflects the different level of monocyte differentiation in the 2 cell types. RAW264.7 cells were treated with LPS, in both the presence and absence of TP-222, for 24 h and then MMP-9 mRNA levels were assayed by quantitative RT-PCR. Similar to RANKL stimulation, TP-222 reduced LPS-induced MMP-9 expression by 50% (Figure 5A). To determine if TP-222 can inhibit MMP-9 expression in other cell types, we treated human articular chondrocytes with

interleukin 1ß (IL-1ß; 10 ng/ml), both with and without TP-222. IL-1ß caused an increase in MMP-9 mRNA in articular chondrocytes after 24 h of culture, and this was significantly reduced by the concurrent addition of TP-222 (Figure 5B). Together these data indicate that TP-222 reduces MMP-9 expression by other agonists and in other cell types, and suggests that TP-222 inhibition of MMP-9 expression and osteoclast formation are mechanistically distinct.

TP-222 treatment of mice reduces osteoclast numbers and immunoreactive MMP-9 at the epiphyseal growth plate. One particularly useful characteristic of TP-222 is its bioavailability when administered to mice (M. Sporn, unpublished observations). Therefore we next determined whether TP-222 would reduce osteoclast formation *in vivo* as it did in *in vitro* models of osteoclast formation. We took advantage of the fact that the epiphyseal growth plate in mice 4–5 weeks of age is a site where osteoclasts are continually recruited and where they facilitate ongoing endochondral ossification. For this experiment, 4-week-old DBA/1J mice were given daily intraperitoneal injections of 50 µg TP-222 for 7 days, and the
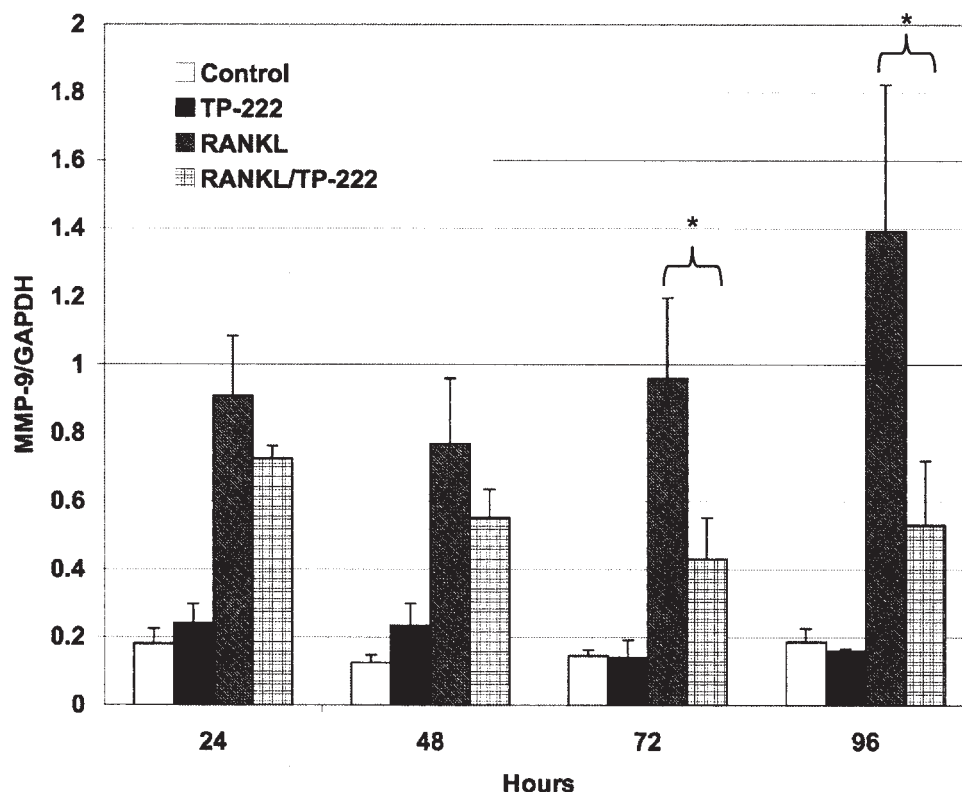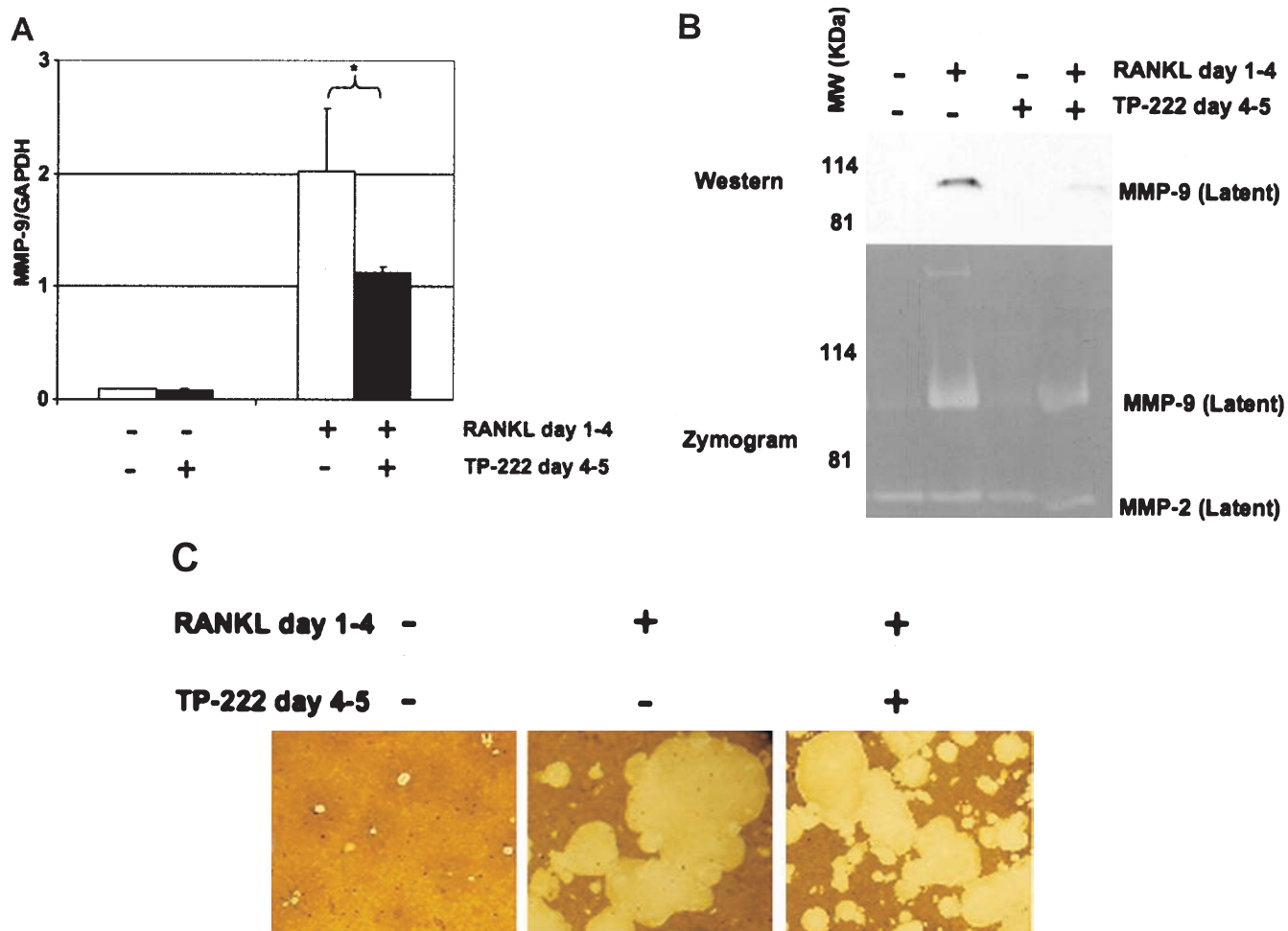
Figure 3. TP-222 inhibits expression of MMP-9 mRNA during osteoclast formation. RAW264.7 cells were cultured with RANKL with or without TP-222 (300 nM). After 24–96 h, RNA was harvested, reverse-transcribed, and subjected to quantitative real-time PCR for MMP-9. Results were normalized to GAPDH; data are shown as means plus SD of triplicate cultures (*p < 0.05).

number of osteoclasts located at the cartilage:bone interface of the growth plate was determined. The 7 days of injections of TP-222 did not cause any obvious side effects, and weights of the treated mice were not significantly different from the vehicle-injected mice (data not shown). Intact knee joints were dissected from each mouse and decalcified, and tissue sections containing both femoral and tibial growth plates were prepared and stained for TRAP. To assure that osteoclast counts were representative of the cartilage:bone interface throughout the entire growth plate, all multinucleated (> 2 nuclei) and TRAP-positive cells were counted in 10 fields (400× magnification) from sections taken from each of 5 TP-222-treated and vehicle-treated mice. The counts represent osteoclasts in an area that altogether spanned 1.1 cm linear length of growth plate, and which represented roughly 800-μm thickness of the central region of the growth plate. Compared to mice treated with vehicle alone, TP-222-treated mice had significantly fewer osteoclasts engaged in matrix resorption at the cartilage:bone interface (Figure 6A). These data indicate that TP-222 has an inhibitory effect *in vivo* on either the formation of osteoclasts from progenitor cells or the recruitment of osteoclast progenitor cells to the growth plate of cells.

Since we had found that TP-222 reduced both mRNA and secreted MMP-9 in the *in vitro* models of osteoclast forma-

tion, we next assessed if TP-222 treatment of mice affected the amount of MMP-9-immunoreactive protein associated with osteoclasts and precursor cells at the growth plate. Growth plate sections were immunostained for MMP-9 protein, and the amount of MMP-9-immunoreactive protein was quantified (Figure 6B). Most of the multinucleated osteoclasts, which were very strongly immunoreactive for MMP-9, were located at the interface between cartilage and subchondral bone or near underlying bone. To quantify the amount of immunoreactive MMP-9 in each tissue sample, we determined the extent of deposition of insoluble brown substrate (DAB) using digital images and morphometric software to determine the corresponding brown pixel count (Image J; US National Institutes of Health). The total brown pixel count included both multinucleated osteoclasts and mononuclear cells containing MMP-9. The measurements were made such that the cartilage:bone interface was included along with about 200 μm of the bone beneath. Means were calculated from the measurement of pixel areas in 8 replicate tissue sections from each of the 5 vehicle-treated and 5 TP-222-treated mice. This comparison revealed that the growth plates of TP-222-treated mice contained significantly less immunoreactive MMP-9 (Figure 6B). Thus, in addition to reducing the number of osteoclasts at the growth plate, TP-222 significantly

Figure 4. TP-222 inhibits MMP-9 mRNA expression after osteoclast formation. A. RAW264.7 cells were cultured 4 days with RANKL, then media were replaced with DMEM with or without TP-222 (300 nM). After an additional 24 h, RNA was harvested, reverse-transcribed, and subjected to quantitative real-time PCR for MMP-9. Results were normalized to GAPDH; data are shown as means of triplicate cultures, error bars represent SD of the mean (*$p < 0.05$). B. RAW264.7 cells were cultured 4 days with RANKL, then media were replaced with DMEM with or without TP-222 (300 nM). After a further 24 h, media were harvested and proteins resolved by Western blotting and by zymography. C. RAW264.7 cells were plated on Osteologic® slides and cultured 4 days in RANKL. Media were then replaced, containing RANKL (50 ng/ml) with and without TP-222 (300 nM). After 7 more days, cells were bleached off the slides and mineralized matrix was stained by the von Kossa method. Photomicrographs are representative of triplicate cultures.

reduced MMP-9 protein expression in this tissue. These data are consistent with our *in vitro* results, and demonstrate that TP-222 can act as an inhibitor of osteoclast formation and MMP-9 gene expression *in vivo*.

DISCUSSION

We observed that the synthetic triterpenoid TP-222 inhibited RANKL-induced osteoclast differentiation of RAW264.7 cells and bone marrow-derived monocytes *in vitro*, and reduced the numbers of multinucleated osteoclasts present at the mouse growth plate. We do not yet know whether the mechanisms that result in inhibition of osteoclast formation *in vitro* by TP-222 and the reduction of osteoclast numbers at the growth plate by injection of mice with TP-222 are the same or different. Since the mobility, recruitment, and formation of osteoclasts *in vivo* are very complex processes involving

chemokines, chemokine receptors, proteases, integrins, and many other factors, more work is required to identify the mechanism(s) by which TP-222 exerted its effects *in vivo*. While triterpenoids have been reported to inhibit inflammation, induce apoptosis, and promote differentiation[20], this is the first observation of triterpenoid inhibition of a specific differentiation pathway.

Synthetic triterpenoids have been reported to promote monocytic, adipogenic, and neuronal differentiation *in vitro*[19]. Thus, our data, together with previous reports[19] that triterpenoids can promote differentiation of the LCDB myelogenous leukemia cell line, suggest that TP-222 may reduce the formation of osteoclasts from monocytic precursor cells by promoting changes in precursor cells toward a monocytic phenotype that is less responsive to RANKL stimulation or downstream responses. We found that TP-222 reduced osteoclast
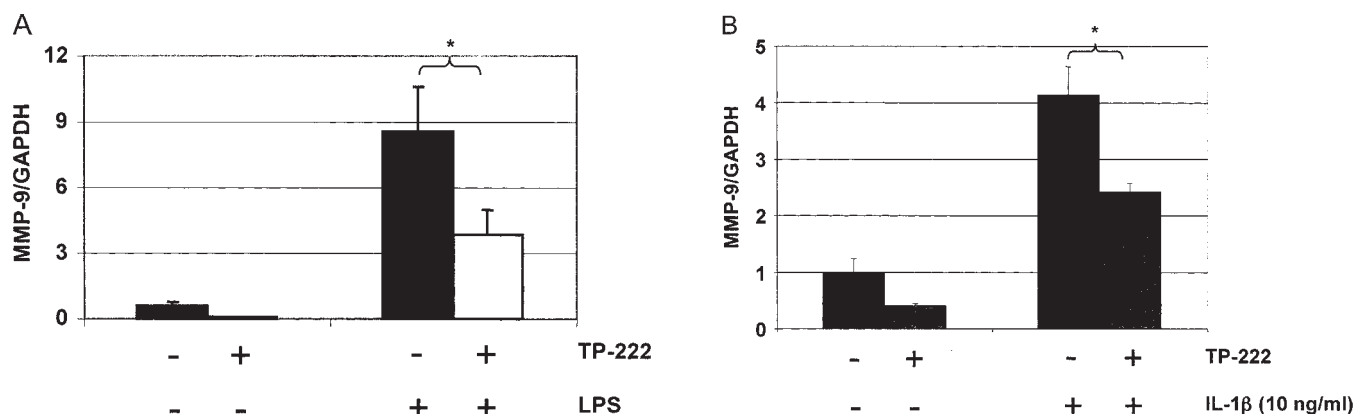
Figure 5. TP-222 inhibits LPS-induced MMP-9 expression in macrophages and IL-1ß-induced MMP-9 expression in chondrocytes. A. RAW264.7 cells were cultured with LPS (100 ng/ml) with or without TP-222 (300 nM) for 24 h prior to harvest. B. Human articular chondrocytes were cultured in IL-1ß (10 ng/ml) with and without TP-222 (300 nM). Total RNA was harvested and MMP-9 and GAPDH mRNA levels were assayed by quantitative RT-PCR. MMP-9 levels were normalized to GAPDH; data are shown as means plus SD of triplicate cultures ($p < 0.05$).
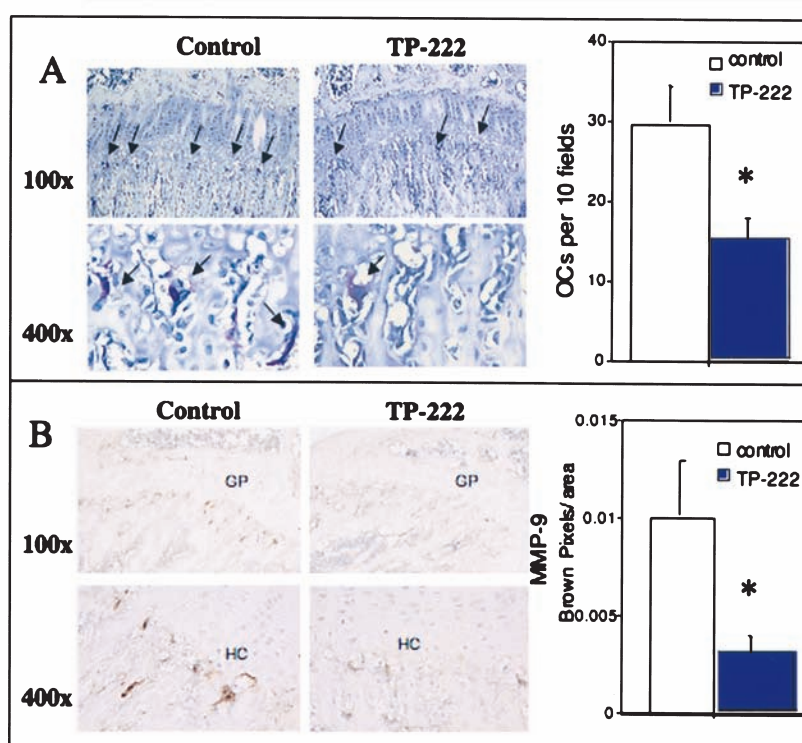
Figure 6. *In vivo* administration of TP-222 reduced osteoclast numbers and MMP-9 expression at the developing mouse growth plate. Young mice (n = 5) were given daily intraperitoneal injections of vehicle or TP-222 for 7 days; growth plates were dissected from the knees, fixed, and subjected to TRAP staining (A) and MMP-9 immunolocalization (B). Osteoclasts were counted as TRAP-positive cells containing > 2 nuclei. MMP-9 expression was measured by pixel density. Data are shown as means plus SD of 10 fields (400×) from growth plates of 5 mice ($p < 0.05$). GP: growth plate, HC: hypertrophic chondrocytes, OC: osteoclasts.

formation *in vitro* with an unusually high potency for a member of this family of compounds. Similar observations on *in vitro* osteoclast formation were reported for other related compounds used at a dose of 10 μM; however, the inhibitory effects on osteoclast formation were not demonstrated *in vivo*[23]. Clearly, the chemical modifications of TP-222 have increased its potency *in vitro*, since TP-222 was significantly more effective at inhibiting osteoclast formation than was the

naturally occurring triterpenoid and precursor molecule, oleanolic acid. We were not able to compare the relative efficacies of TP-222 and oleanolic acid *in vivo*, due to the vastly different bioavailabilities of these compounds (M. Sporn, unpublished observations). Nonetheless, TP-222 and related compounds may represent a novel approach to inhibiting bone destruction in disease.

In addition to inhibiting differentiation, we found that TP-222 effectively reduced RANKL, LPS, and IL-1ß-induced MMP-9 gene expression. Because MMP-9 is abundant in mature osteoclasts, it can be regarded as an indicator gene for osteoclasts, and there is strong evidence of its importance for recruitment of these cells to sites of bone resorption[9]. We do not know if reduced MMP-9 expression in TP-222-treated cells contributes to the observed reduction in osteoclast formation. However, that TP-222 inhibited both RANKL and LPS-dependent induction of MMP-9 in monocytes and IL-1ß-induced MMP-9 expression in chondrocytes suggests that TP-222 targets the MMP-9 gene directly and does not simply reduce the number of MMP-9-expressing cells *in vitro* and *in vivo*. This contention is further supported by our *in vitro* study, which demonstrates that TP-222 reduces MMP-9 expression even after osteoclast formation is allowed to occur. Interestingly, we did not observe an effect of TP-222 at the level of resorption by mature osteoclasts. This result argues that MMP-9 expression by osteoclasts may not be essential for degradation of mineralized matrix.

There is accumulating evidence that MMP-9 is very important in diverse aspects of osteoclast recruitment, formation, and function in bone biology[9,28]. Engsig and colleagues reported that mice deficient in MMP-9 have reduced recruitment of osteoclasts to the growth plate, due to impaired migration through discontinuously mineralized matrix and reduced activation of the chemoattractant VEGF[9]. This link between osteoclast recruitment and MMP-9 expression is supported by our findings here. Mice treated with TP-222 had significantly fewer osteoclasts present at the developing growth plate, and this was mirrored by reduced immunoreactive MMP-9 in this tissue. Surprisingly, we did not observe thickening of the mineralized zone at the growth plate. This may be explained by our *in vitro* results, which show that TP-222 reduction of MMP-9 expression does not impede bone resorption in mature osteoclasts. Further, cartilage resorption still occurs during endochondral ossification in mice lacking functional osteoclasts[29]. Thus, during the short duration of our *in vivo* experiments, other cell types may have compensated for the reduced number of osteoclasts present in TP-222-treated mice. Nevertheless, these findings present the interesting possibility that TP-222 can repress osteoclast formation and/or recruitment, and that this may involve MMP-9. First, as shown in the *in vitro* work, TP-222 inhibits the formation of mature osteoclasts. Second, as osteoclasts express MMP-9 to promote migration and recruitment to areas of bone resorption, TP-222 inhibits this signal as well. This ability of TP-222, and perhaps other oleanolic-derived triterpenoids, to interfere with osteoclast function at multiple levels presents a potentially useful method of inhibiting bone degradation in diseases such as rheumatoid arthritis and osteoporosis.

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