# Inhibiting Histone Deacetylase 1 (HDAC 1) Suppresses both Inflammation and Bone Loss in Arthritis

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Shorty Title: Inhibiting Histone Deacetylase 1 in arthritis

Abstract

Objectives: Histone Deacetylase 1 (HDAC 1) is highly expressed in synovial tissues from rheumatoid arthritis (RA) patients. Hence, the aim was to determine if a novel HDAC inhibitor (HDACi) designed to target HDAC 1, NW-21, could inhibit the release of inflammatory cytokines and chemokines by TNF-α stimulate monocytes and osteoclastogenesis *in vitro*. Effects on inflammation and bone loss were subsequently assessed in collagen-antibody induced arthritis (CAIA) in mice.

Methods: Human peripheral blood mononuclear cells (PBMCs) stimulated with receptor activator of nuclear factor kappa B ligand (RANKL) and macrophage colony stimulating factor (M-CSF) were used to determine effects of NW-21 on both osteoclast formation and activity. Anti-inflammatory activity of NW-21 was assessed using human monocytes stimulated with either tumor necrosis factor α (TNF-α) or lipopolysaccharide (LPS) for 24 hours and treated +/- NW-21. Real time PCR was used to determine mRNA expression of monocyte chemotactic protein 1 (MCP-1), TNF-α, Macrophage Inflammatory protein 1 α (MIP-1α), IL-1 and RANTES. Effects of NW-21 *in vivo* was assessed using the CAIA model in mice with daily oral administrationat 5mg/kg/day. NW-21 was compared to Class I HDACi, MS-275, and a broad acting HDACi, 1179.4b. Effects on inflammation and bone were assessed using paw inflammation scoring, histology and live animal micro CT of the radiocarpal joints.

Results: *In vitro*, HDACi NW-21 suppressed human osteoclast formation and activity as well as significantly reducing mRNA expression of chemokines, monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein 1α (MIP-1α) in PBMCs stimulated by LPS or TNF-α.

Only the inhibitors that targeted HDAC 1, NW-21 and MS-275, reduced inflammation and bone loss *in vivo,* whereas the broad acting HDACi was not effective.

Conclusion: The results indicate that inhibitors targeting HDAC 1, such as NW-21 and MS-275, may be useful for treating RA as they reduced both inflammation and bone loss.

Key words

Histone Deacetylase 1 (HDAC 1), inflammation, osteoclasts, bone loss, collagen antibody induced arthritis

## Introduction

Soft tissue inflammation, focal bone erosions and systemic bone loss are all characteristic features of rheumatoid arthritis (RA), that currently affects approximately 1% of the world's population [1]. RA commonly affects the synovial joints, particularly the knees and fingers, resulting in joint inflammation and synovial hyperplasia associated with destruction of bone and cartilage. The bone resorbing osteoclasts are responsible for the localized bone erosions with this being demonstrated in both human RA tissues [2-5] and animal models [6, 7]. Large multinucleated osteoclastic cells resorbing the subchondral bone have been detected at sites of bone loss in the joints [8, 9].

A variety of treatments exist for RA, ranging from the basic non-steroidal anti-inflammatory drugs (NSAIDs) to the more recently developed biologic anti-rheumatic drugs (DMARDs), such as the anti-TNF agents infliximab and adalimumab. Although these treatments suppress inflammation, their actions on the focal bone erosions are variable [10]. Many of these current treatments can also have adverse side effects when taken for a long time period and it can also take a considerable time for clinicians to identify the appropriate treatment(s) for individual patients [11-17]. During this delay in finding an effective treatment regime there can be continuing erosion of the bone that strongly impacts on normal joint function both in the short and longer term. More recently a disconnect between disease activity and structural damage has also been highlighted, hence treatments that could be administered immediately that directly target the early bone erosions would be beneficial [18]. Histone deacetylase (HDAC) inhibitors might have the potential to halt damage as well as suppress in the inflammation.

HDAC enzymes are thought to play an important role in regulating gene transcription by altering the acetylation status of histone proteins resulting in regulation of gene repression. These enzymes can also deacetylate many other non-histone signal transduction proteins that are important in multiple inflammatory events [16, 17]. HDAC enzymes with a catalytic zinc ion are classified into two main classes. Class I includes HDACs 1, 2, 3 and 8 and are primarily found in the nucleus [19]. Class II includes HDACs 4, 5, 7 and 9, which belong to Class IIa, and HDACs 6 and 10, which belong to Class IIb. Class II enzymes are able to shuttle between the nucleus and cytoplasm. The inhibition of HDAC enzymes is considered to be a favourable strategy for the treatment of a wide variety of diseases, including malignancies and inflammatory diseases. Reductions in cancer cell growth have been shown by inhibiting differentiation of cells both *in vitro* and *in vivo* [20-24]. A range of HDACi are in different stages of clinical trials for treating both solid and hematological malignancies [25] with broad acting HDACi (Vorinostat (SAHA) and Romidepsin approved for clinical use to treat cutaneous T cell lymphoma. The effects of HDAC inhibitor (HDACi) treatment on inflammatory diseases appears to be related to its suppression of inflammatory cytokines, such as TNF-α and IL-1β [26]. Interestingly, the anti-inflammatory effects are observed at much lower doses (10-100x lower) than those used to treat malignancies, making them attractive therapeutic options for the treatment of inflammatory diseases [27].

A number of studies have demonstrated that various HDACi suppress disease activity in animal models of inflammatory arthritis [28-31]. In an adjuvant arthritis model, cyclic depsipeptide HDACi, FR901228 (FK228) had both prophylactic and therapeutic effects [29]. Phenylbutyrate and trichostatin A (TSA), both broad acting HDACi, have been shown to suppress joint swelling,

reduce subintimal mononuclear cell infiltration, inhibit synovial hyperplasia, suppress pannus formation, cartilage damage and bone destruction also in an adjuvant arthritis model [32]. More specific acting inhibitors, such as MS-275 which is HDAC1 selective, have also been reported to have significant anti-arthritic effects [28]. At relatively low doses (3 mg/kg/day), MS-275 inhibited inflammation and bone loss while also reducing IL-1β and IL-6 serum levels [28], however this compound is also quite toxic. MS-275 has been reported to suppress HDAC 1 at concentrations below 0.1 μM [33] and is more preferential for HDAC 1 ($IC_{50}$ 0.3 μM) compared to HDAC 3 ($IC_{50}$ 8 μM) [34]. By comparison, a broad acting HDACi, such as suberoxylanilide hydroxamic acid (SAHA), requires higher doses (10 mg/kg/day) to reduce inflammation and bone damage. SAHA was shown to suppress both inflammation and bone erosion, but could not prevent the onset of arthritis [28]. These studies demonstrate the potential of more selective HDACi for treating arthritis. Broad acting inhibitors target a range of HDAC enzymes in both classes and in many cases it is unclear exactly which HDACs are targeted and to what extent they are inhibited. The broad inhibitory action increases the possibility of side effects during long-term administration due to the widespread expression of these enzymes in a variety of tissues and organs. Research is now progressing towards using more selective compounds that target specific HDACs or HDAC classes involved in disease processes.

To date, only one HDACi, givinostat, has progressed to humans for the treatment of juvenile arthritis. In that study, 17 children with juvenile idiopathic arthritis (JIA) were treated with givinostat for up to 12 weeks [35]. No systemic toxicities or organ dysfunctions were observed and only mild to moderate side effects were reported. Patients treated with givinostat reported

improvements in mobility and wellbeing and there were also reductions in number of joints with active arthritis [35].

Recent studies have demonstrated elevated nuclear activity of HDACs in RA synovial tissue compared to osteoarthritis (OA) tissues [36] and HDAC 1 is highly expressed in synovial fibroblasts from RA patients [37]. High levels of HDAC 1 observed in RA tissues were found to correlate with TNF-α expression [37, 38], suggesting a link between HDAC activity and synovial inflammation. The high expression of HDAC 1 in RA along with the positive effects observed for MS-275 in animal models of arthritis suggests that targeting HDAC 1, possibly along with other class I HDACs could reduce disease activity. In this study, it was hypothesised that inhibitors targeting class I HDACs, would suppress inflammation and bone loss *in vitro* and in an animal model of inflammatory arthritis. Therefore, this study aimed to determine the effects of a novel HDACi (NW-21) on osteoclast formation and activity *in vitro*, cytokine and chemokine expression by human monocytes stimulated with TNF-α or *E. coli* LPS *in vitro* and on a collagen antibody induced arthritis (CAIA) in mice. HDACi NW-21 designed to target class I HDACs (1-3) was compared with an HAC 1-selective inhibitor, MS-275, and a novel broad-acting HDACi, 1179.4b, previously shown to suppress bone loss *in vitro* and in a mouse model of periodontitis [39]. Based upon enzyme data {REF to Gupta et al, Curr Topic Med Chem) it would appear that NW-21 targets HDAC 1, 2, 3 better than MS-275 which only targets HDAC 1. More than 1μM concentrations of MS-275 are required to inhibit HDAC 2 and 3.

In addition, expression of important factors involved in osteoclast formation and inflammation *in vitro* assays was assessed to gain further insights into mechanisms by which NW-21 inhibits

osteoclast resorption and inflammation. The results of this study indicate the importance of Class I HDACs for both anti-inflammatory and anti-resorptive activity.

## Methods

### Histone Deacetylase Inhibitors (HDACi)

HDACi NW-21 (Figure 1) is a novel compound initially developed by researchers at the University of Queensland to inhibit cancer (compound 51 in [40]) and malaria (compound ASU-13 in [41]). It was designed to specifically target Class I over Class II HDACs (e.g. inhibition of HDAC activity: $IC_{50}$ = 0.021±0.02 μM for HDAC 1 (Class I), 0.042±0.02 μM for HDAC 2 (Class I), 0.3 μM for HDAC 3 (Class I), >10 μM for HDAC 4 (Class IIa) 0.88±0.06 for HDAC 6 (Class IIb), >1 μM for HDAC 7 (Class IIa), >3 μM for HDAC 8 (Class 1) ([42] and unpublished data). NW-21, MS-275 and 1179.4b (also obtained from the University of Queensland) with concentrations used being based on previous studies *in vitro* [43] and *in vivo* [39] and upon recommendation by the developers. MS-275 is also a Class I selective inhibitor (IC50 = 0.181 μM for HDAC 1, 1.16 μM for HDAC 2, 2.31 μM for HDAC 3, >10 μM for HDAC 4, >10 μM for HDAC 6, >10 μM for HDAC 7 and >10 μM for HDAC 8 [42].

### ***In vitro O***steoclast Assay

Peripheral Blood Mononuclear cells (PBMCs) were obtained by differential centrifugation from whole blood buffy coats obtained from the Australian Red Cross Blood Service (n=9) as previously described [43]. Ethical approval was obtained from the University of Adelaide Human Ethics Committee. Briefly, cells were seeded at $2x10^6$ cells per mL on day 0 in α-

minimal essential medium (a-MEM; Invitrogen, Melbourne, Victoria, Australia) supplemented with 10% fetal calf serum (FCS; Invitrogen, Life Technologies, Carlsbad, CA), 1% penicillin–streptomycin (Invitrogen), and 1% L-glutamine (Invitrogen) with 100 nM 1a, 25(OH2)D3 (vitamin D3) (Novachem, Melbourne, Victoria, Australia), 100 nM dexamethasone (Fauldings, Adelaide, South Australia, Australia), and 25 ng/mL of recombinant human M-CSF (Chemicon International Inc, Millipore, MA). Cells were maintained at 37˚C with 5% $CO_2$ for a total of 17 days. From day 7 onwards, human recombinant receptor activator of nuclear factor kappa B (RANKL) (50 ng/mL; Chemicon International Inc.) was added to the culture medium. Cells were treated with 5 fold dilutions of the HDACi NW-21 starting at 20 nM commencing on either day 7 (coinciding with RANKL addition), day 10 or day 13, similarly to previous studies [43]. Serial dilutions of NW-21 were achieved in DMSO with a final concentration of 0.01% and hence controls were treated with 0.01% DMSO. Media was replaced every two days with the addition of RANKL and NW-21 (from day 7,10 or 13).

Cell Viability

The effect of NW-21 on cell viability was assessed over a concentration range of 0.16 nM-20 nM using a WST-1 assay (Roche Applied Science, Castle Hill, NSW, Australia). On day 14, 10 µL of cell proliferation reagent WST-1 was added to each well (100 mL media) followed by incubation for 2 h at 37˚C. A control blank consisted of culture medium with WST-1 reagent without any cells. Absorbance was measured at 450 nm following a 2 h incubation period. Absorbance in NW-21 treated wells was compared with that of controls.

Osteoclast Formation

Cells were grown in the presence of NW-21 (0.16 nM– 20 nM) commencing from either day 7, 10 or 13 in 16 well chamber slides (LabTek, Nunc International, Rochester, NY, USA). On day 14, cells were fixed with 4% glutaraldehyde in Hank's balanced salt solution (HBSS) and stained with a TRAP kit (Sigma, St. Louis, MO) according to the manufacturer's instructions and as previously described [43]. Cells were counterstained using 0.5% w/v Methyl Green. TRAP staining was imaged using a light microscope (Nikon Microphot FXA Photomicroscope, Nikon Instruments Inc., Melville, NY, USA). Three representative areas were imaged for each concentration. The number of TRAP-positive multi-nucleated (more than 3 nuclei) cells for each concentration of NW-21 was compared with the controls (0.01% DMSO) for individual donors [43].

### Osteoclast Activity

Cells for osteoclast activity assessment were grown on 5 mm sterile whale dentine slices. On day 17, cells were trypsonised and the dentine washed with milliQ water to remove cells before mounting onto SEM stubs for analysis. Dentine slices were coated using carbon gold and then imaged with the Philips XL-20 Scanning Electron Microscope (Adelaide Microscopy). Three representative images were taken for each dentine piece at 150x magnification and quantitated using Image J analysis software (National Institutes of Health, USA). The area of pit resorption for each concentration of NW-21 was expressed as a percentage of the total area of the dentine [43]. Resorption in treated wells was represented as a percentage of the average area of resorption in the control well (0.01% DMSO).

### Inflammatory Gene Expression ***In Vitro***

PBMCs were obtained from buffy coats and seeded at $2x10^6$ cells per mL in 48 well trays on day 0 in α-MEM with the supplements as described above. Cells were incubated at 37˚C with 5% $CO_2$ for 2h [44]. Following this, cells were treated with either human recombinant *E. coli* lipopolysaccharide (LPS) (100 ng/ml) (Chondrex Inc., Arthrogen-CIAs Arthritogenic Monoclonal Antibodies, Redwood WA, USA) or tumor necrosis factor α (TNF-α) (10 ng/ml) (Recombinant Human TNF-alpha, RnD Systems, Minneapolis, MN USA) with and without NW-21 (20 nM). Controls were treated with 0.01% DMSO. Cells were maintained at 37˚C with 5% $CO_2$ for a further 24h before RNA collection as described below.

## Real Time PCR to Determine mRNA Expression

RNA was extracted from cells grown in the presence of HDACi NW-21 and control wells at different time points throughout the osteoclast culture (days 0,7,10, 14 and 17) using Trizol (Invitrogen Life Technologies, Carlsbad, CA). For the inflammation assay, RNA was collected after 24 hours of incubation with either TNF-α or LPS with or without HDACi NW-21. Reverse transcription was conducted using a Corbett real time PCR machine (Corbett Research Rotor Gene RG-3000; Corbett Life Science, Mortlake, NSW, Australia). The RT reaction included 250 ng of random hexamer (Geneworks, Adelaide, SA, Australia) and 200 U of Superscript III Reverse Transcriptase according to the manufacturer's instructions to produce cDNA. Real Time PCR was carried out using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The mRNA expression of osteoclast related genes nuclear factor of activated T cells (NFATc1), TNF receptor factor-6 (TRAF-6) and osteoclast associated receptor (OSCAR) were compared between

treated and control cells. Primers were those previously used: NFATc1 [43], TRAF-6 [43] and OSCAR [45]. For the *in vitro* inflammatory assay cytokines included TNF-α, IL-1β and chemokines MCP-1, MIP-1α, IL-6 (designed using Primer3Plus), RANTES [46] Reaction mixtures consisted of 1μg cDNA, 2x supermix (containing SYBR Green 1 dye), 300 nM of forward and reverse primer and this was made up to a total volume of 15μL with diethyl pyrocarbonate (DEPC) water. PCR was performed in triplicate for each sample. mRNA expression was determined relative to endogenous reference gene human acidic ribosomal protein (hARP; [47]). The relative quantification of the mRNA expression for each of the genes was then calculated using the comparative Ct method $2^{-\Delta\Delta Ct}$ [48].

Collagen Antibody Induced Arthritis (CAIA) Model

Female Balb/c mice (29 animals) at 6-8 weeks of age were used for this study. Mice had access to food and water *ad labium*. Animal ethics approval was obtained through the University of Adelaide Animal Ethics Committee. The experiment consisted of 5 groups; group 1: controls no disease (n=6), group 2: CAIA with vehicle treatment (n = 6), group 3: CAIA with HDACi 1179.4b treatment at 5 mg/kg/day (n = 5), group 4: CAIA with HDACi treatment NW-21 at 5 mg/kg/day (n = 6) and group 5: CAIA with HDACi MS-275 treatment at 10 mg/kg/day (n = 6).

**Treatments**

HDACi NW-21, MS-275 and 1179.4b were all administered in olive oil due to their lipophilic nature. Treatments were administered daily via oral gavage [39] commencing on day 4 following the LPS injections. Groups 1 and 2 mice were treated with the olive oil vehicle.

## CAIA Induction

CAIA was induced by injecting mice intravenously via the tail vein with 150 µL (1.5 mg) of a monoclonal antibody against type II collagen (day 0) (Chondrex Inc., Arthrogen-CIAs Arthritogenic Monoclonal Antibodies, Redwood WA, USA) as previously described [49]. Control mice were injected with PBS only. Two days later mice were given an intraperitoneal (i.p.) injection of 20 µL (10 µg) of LPS (Chondrex Inc). Control animals were injected with only PBS. Mice were monitored daily by two experienced observers for a total of 14 days. Clinical record sheets were maintained throughout the study detailing changes in body weight, dull/ruffled coat, temperament, reduced food/water intake or a reluctance to move). Paws were assessed for signs of redness and swelling daily by two experienced observers. To assess inflammation each paw was given a score from 0 to 4 giving a total maximum score of 16 for each animal [49]. 0=normal paw, 1=mild but definite redness and swelling of the wrist/ankle, 2=moderates welling and redness of the wrist/ankle with digit involvement, 3=severe swelling of the wrist/ankle with multiple digit involvement and 4=maximum inflammation within the entire paw, wrist/ankle with many digits involved.

## Live Animal Micro CT Scanning

A live animal micro CT scan was initially conducted to obtain baseline measurements (4 days prior to CAIA induction) followed by a final CT scan at the completion of the study (day 14). Scans were conducted using an *in vivo* animal micro-CT scanner (Bruker formally known as SkyScan, SkyScan1076, Kontich, Belgium) situated in Adelaide Microscopy. The specifications used for scanning and machine details have been published previously [49]. Scanning was

conducted at 74 kV/136 mA with a pixel size of 18 mM, 1 mm aluminium filter and frame averaging of 1. Mice were initially anaesthetized with an i.p. injection (rat/mouse anaesthetic – 1 mL xylazine, 2 mL ketamine (100 mg/mL), 17 mL of water in the injection, 0.3 mL for a 30 g mouse). Mice were aligned using polystyrene holders with the front paws being included in the scanning region of interest. Scans were reconstructed using SkyScanN' Recon program and then realigned to allow for the same area to be analysed using SkyScan program Data Viewer. Bone volume analysis of the radiocarpal joints were conducted using SkyScan's CTAn program (SkyScan Bruker, Kontich, Belgium). The same reference point with the radiocarpal joints was utilised for all analysis with 100 slices either side of this reference point being included in the region of interest to be analysed as previously described [49].

## Histological Analysis

At the conclusion of the experiment, front paws were collected and fixed in 10% NBF for 48 h before decalcification in 10% EDTA for 8 weeks. Specimens were then processed and paraffin embedded. Sagittal sections (7 μm) of the radiocarpal joints were prepared. Routine H&E staining was conducted. Semiquantitative analysis was carried out by two blinded observers using a 4-point scale as previously described [49]. Scoring was based on the numbers of inflammatory cells with the radiocarpal joints (lymphocytes, plasma cells neutrophils or macrophages). Normal tissue (<5% inflammatory cells) was scored a 0, mild inflammation (5–20% inflammatory cells) was scored a 1, moderate inflammation (20–50% inflammatory cells) was scored a 2 and severe inflammation with a massive immune cell infiltration (>50% inflammatory cells) was scored a 3. Bone and cartilage destruction was assessed by: 0=normal, 1 =mild cartilage destruction, 2=evidence of both cartilage and bone destruction, 3= severe

cartilage and bone destruction. Pannus formation: 0=no pannus, 1=pannus formation [49]. Tartrate resistant acid phosphatase (TRAP) staining of the radiocarpal joints was conducted to detect osteoclasts on the bone surface and in the soft tissues. TRAP staining methods were adapted from [50]. Slides were stained in TRAP for 20 minutes at 37◦C before rinsing and counterstaining with haematoxylin. TRAP staining was imaged and the number of TRAP positive multinucleated cells was quantitated in the radiocarpal joint in an area of 1.92mm$^2$.

### Statistical analysis

Statistical analysis was conducted using GraphPad Prism 6 (GraphPad Software Inc.) For TRAP stain quantitation and pit resorption analysis a one-way analysis of variance, followed by a Bonferoni's Post Hoc Test was conducted. Students T-Test was used to compare between treated and controls for the PCR results at each time point relative to expression at day 7. In the arthritis model a one way analysis of variance was used to compare between different groups for paw scoring, CT analysis and histological scoring.

## Results

### Osteoclast Formation and Activity

Cells treated for 24 h with HDACi NW-21 showed no significant changes in viability at all concentrations tested (0.16– 20.0 nM) (data not shown). RANKL stimulation resulted in the formation of TRAP positive multinucleated cells detected at day 14 (Figure 2A and these cells resorbed pits in the mineralised substrate by day 17 (Figure 2B). Treatment of the cells with HDACi NW-21, commencing from day 7 coinciding with RANKL addition, resulted in a significant reduction in the number of multinucleated TRAP positive cells for the highest

concentrations (20 nM ($p<0.001$) and 4 nM ($p<0.01$)) (Figure 3A). This reduction was associated with a similar reduction in resorption activity (Figure 2B). At all concentrations above 0.16 nM there was a significant reduction in the area of pit resorption ($p<0.01$) (Figure 3). There was a marked reduction with increasing concentrations, 1.1% of the control area resorbed with 20 nM treatment and 21% with 4 nM NW-21 treatment. Experiments were carried out with treatments commencing on day 10 and day 13 to assess effects on the later stages of osteoclast differentiation. Interestingly, treatment from either day 10 or day 13 did not significantly reduce either the formation of TRAP multinucleated cells or resorption (Figure 3A and B).

## Gene Expression in Osteoclasts

Effects of NW-21 treatment commencing from day 7 on osteoclast related genes were assessed using real time PCR (Figure 3C). NW-21 treatment resulted in a 1.4 fold reduction in the mRNA expression of TRAF-6 ($p<0.001$) at day 10. NFATc1 mRNA expression was also significantly decreased at day 10 ($p<0.05$) and at day 17 however at this time point it was not significant ($p>0.05$). OSCAR mRNA expression was significantly reduced by 1.3 fold at day 10. At day 10, there was a 2.3 fold increase in MCP-1 mRNA expression relative to day 7, and this expression was reduced throughout osteoclast differentiation. At day 10, HDACi NW-21 resulted in a 1.7 fold reduction in the MCP-1 mRNA expression ($p<0.05$).

## Inflammatory Cytokines and Chemokine Gene Expression

Monocytes stimulated with either LPS or TNF-α were treated with NW-21 (20 nM). This treatment resulted in a significant ($p<0.05$) reduction in mRNA expression of inflammatory

chemokines MIP-1α and MCP-1. For TNF-α treated cells, there was a 1.7 fold reduction in the relative mRNA expression and in LPS stimulated cells there was a 4.6 fold reduction. In both cases there was no significant change in the mRNA expression of cytokines TNF-α, IL-1β or RANTES as shown in Figure 5 ($p>0.05$). There was a 2.2 fold reduction in the relative mRNA expression of RANTES in both TNF- stimulated and 1.7 fold reduction in LPS stimulated monocytes although this was not significant. NW-21 treatment caused a 4.1 fold decrease in IL-1β mRNA expression in LPS stimulated cells and a 2.7 fold decrease in IL-1β mRNA in TNF-α stimulated cells ($p>0.05$). In TNF-α stimulated cells there was a 2.3 fold reduction in IL-6 mRNA expression, whilst unexpectedly in NW-21 treated LPS stimulated cells there was an increase in IL-6 expression although not shown to be significant ($p>0.05$).

## Collagen Induced Arthritis

The effects of NW-21 (5 mg/kg/day) daily oral treatment commencing following LPS administration on day 4 were assessed in a CAIA mouse model. The effects of NW-21 were compared to broad acting inhibitor 1179.4b and Class I selective inhibitor MS-275.

## Weight Changes

Mice were monitored daily for changes in weight and all the disease groups did lose weight (9%) at day 3 and this is likely associated with the LPS injection (data not shown). Although there was no statistically significant difference, there was less weight loss by all HDACi treatment groups.

## Inflammation

Induction of inflammatory arthritis resulted in significant swelling and redness in the paws as shown in Figure 4A. Paw inflammation was first noted from day 4 following the LPS injections, which peaked at day 10 and began to reduce over the following 4 days (Figure 4I). There was no significant effect of 1179.4b treatment on the paw scoring at any of the time points ($p>0.05$). Both NW-21 and MS-275 treated groups had significantly reduced paw scores compared to untreated mice, as depicted in the macroscopic images in Figure 4B and D. The histology (Figure 5A) of the tissues was consistent with paw inflammation seen macroscopically with evidence of extensive inflammatory cell infiltration in both the vehicle treated (oil) (and 1179.4b treated mice. There was very little inflammatory cell infiltration in the paws of mice treated with either NW-21 or MS-275 (Figure 5B). There was also evidence of pannus formation in the mice given the vehicle or 1179.4b that was mostly absent in the NW-21 and MS-275 treated groups (Figure 5C). In the NW-21 treated group, 1 of the 6 mice failed to respond to NW-21 treatment in any way and this caused a lack of statistical significance other than in paw scoring on days 10 and 11.

Bone Loss

Analysis using live animal CT also revealed evidence of bone loss throughout the 14 days in the diseased mice (Figure 4J). There was an expected normal growth of the mice with a 8.9% increase in bone volume in the controls over the experimental period. In diseased (oil) mice there was on average a 1% decrease in bone volume. In 1179.4b treated mice there was greater bone loss than the oil treated mice with an average 4.4% reduction in bone volume overtime although not significant. There was an average 8.7% increase in bone volume in NW-21 treated mice and a 12% increase in MS-275 treated mice as shown in Figure 4J. This did not however reach

statistical significance when compared to untreated mice. There was a marked reduction in bone and cartilage destruction following NW-21 and MS-275 treatments as assessed on H&E stained radiocarpal joints. In both the diseased group and the 1179.4b treated mice there was evidence of large numbers of TRAP positive cells on the bone surfaces and in the inflamed soft tissues as depicted in Figure 6. Very few TRAP positive cells were found in the joints from mice treated with either NW-21 or MS-275 (Figure 5A and E).

## Discussion

The *in vitro* results of this study support the hypothesis that inhibitors targeting HDAC 1 would suppress both inflammation and bone loss. Novel HDACi NW-21 reduced the formation of TRAP positive osteoclasts as well as the osteoclasts resorptive activity in a concentration-dependent manner. Some previous studies have demonstrated that HDACi can suppress osteoclast differentiation and activity i*n vitro*, but these studies used broader acting inhibitors of HDACs, such as, trichostatin A (TSA) [51, 52], suberanilohydroxamic acid (SAHA) [43], ITF2357[29] and 1179.4b [43]. We have previously shown that MS-275, which inhibits HDAC 1 and to a much lesser extent other Class I HDACs [42] was able to suppress human osteoclast formation and activity [43]. However, MS-275 was only effective at relatively high concentrations (100 nM) and hence this could suggest that other class I HDACs may be important such as HDAC 2. The suppression of osteoclasts with NW-21 was surprising given that HDAC 1 expression was not shown to be significantly increased during osteoclast development, while HDACs 5 and 8 were highly expressed as osteoclasts formed [43]. These results could be related to the potency of NW-21 or could suggest that although HDAC 1 expression is not markedly increased during osteoclastogenesis, its activity is required for

osteoclast differentiation. It is clear that HDAC 2 may also be important which is not surprising given that HDAC 1 and 2 function together in a protein complex and are thought to be redundant [53]. NW-21 was more effective at suppressing osteoclasts at lower concentrations than MS-275, possibly supporting a role for both HDAC 1 and 2. Studies by others have also focused on the roles of HDAC 3 and HDAC 7, with suppression of HDAC 7 accelerating osteoclast differentiation and suppressing HDAC 3 shown to inhibit differentiation [54]. While NW-21 inhibits HDAC1 ($IC_{50}$=0.021 μM) and also HDAC 2 ($IC_{50}$=0.042±0.02 μ M) at low concentrations it can also weakly inhibit HDAC 3 ($IC_{50}$ = 0.3μM), so further studies may be needed to determine if NW-21 inhibits osteoclastogenesis by inhibition of HDAC 1 and 2 in vivo. Over-expression of HDAC 5 has been shown to reduce RANKL-mediated acetylation of NFATc1 (NFATc1), indicating HDACs can regulate NFATc1 activity [55]. While these studies show the importance of HDACs in osteoclast differentiation, it is still unclear which HDACs are the most important. It may well be that it is a combination of HDACs that influence these processes, some being more important than others.

An interesting observation, when commencing with NW21 treatment later than day 10, was that this did not result in a decrease in osteoclast activity. This is similar to the effect of HDACi 1179.4b, which suppressed both osteoclast formation and activity when treatment was commenced from day 7, but not at later times [43]. In order to better understand the mechanisms involved, mRNA analysis was conducted. NW-21 treatment from day 7 resulted in a reduction in the mRNA expression of TRAF-6 and NFATc1, similar to that for 1179.4b. The reduction in TRAF6 at day 10 may be responsible for the reduced NFATc1 expression seen later as TRAF-6 signalling, initiated soon after RANK/RANKL interaction, is up stream of NFATc1 elevation

essential during the terminal stages of osteoclast formation [56]. This is consistent with the lack of effect of NW-21 after day 10 (3 days after RANKL administration).

NW-21 caused a significant reduction in expression of chemokines MCP-1 (also referred to as Chemokine (C-C motif) ligand 2 (CCL2)) and MIP-1α (also referred to as Chemokine (C-C motif) ligand 3 (CCL3). MCP-1 is known to recruit monocytes, memory T cells, and dendritic cells to sites of inflammation. It is also reportedly produced by synovial cells and infiltrating monocytes in RA [57]. RA synovial tissue macrophages were found to constitutively express MCP-1 [57]. TNF-α and IL-1 stimulation of synovial cells resulted in increased MCP-1 mRNA expression[58]. The anti-TNF drug, etanercept, was shown to significantly reduce serum levels of MCP-1 in RA patients[59]. These levels correlated with disease activity, confirming a role for MCP-1 in the pathogenesis of RA inflammation [59]. MCP-1 was also reported to be expressed by mature osteoclasts and shown to promote human osteoclast formation in absence of RANKL, however, these cells were unable to resorb bone [60]. Macrophage inflammatory protein 1α (MIP-1 α) is known to be produced by macrophages following stimulation by bacterial endotoxins [61]. It is known to be chemotactic for PMNs, macrophages and T cells [61]. Significantly higher levels of MIP-1α have been shown in synovial fluids from patients with RA compared to OA. Mononuclear cells from synovial fluid produce MIP-1α along with synovial fibroblasts and this can be stimulated by TNF-α or LPS [62]. MIP-1α has also been shown to induce osteoclast formation [63]. Both MCP-1 and MIP-1α were significantly reduced by NW-21 treatment in this study. A lack of MCP-1 in osteoclasts has been shown to result in a down-regulation of NFATc1 and osteoclast suppression [64]. This is consistent with the notion that

inhibition of NFATc1 and osteoclast formation occurs downstream of MCP-1 suppression initiated by inhibition of HDAC 1. Studies by others have also shown that HDACi can suppress NFATc1 [29] in osteoclasts *in vitro* whilst others have shown that broad acting HDACi (TSA and sodium butyrate) can suppress TNF induced NF-Kappa B activation in RAW264 cells [51]. These results in this study have revealed a role for HDACs in MCP-1 expression and hence osteoclast differentiation. It is clear that HDACi may work via different mechanisms in osteoclasts and this could be dependent on what specific HDACs are targeted. Further studies are clearly needed to elucidate which specific HDACs are involved in the osteoclast signaling pathways.

The second part of the study hypothesis, that HDACi (NW-21 and MS-275) which target HDAC 1 would suppress inflammation and bone loss, was also supported by our findings in the CAIA mouse model. Arthritis was induced in mice using the CAIA model which induced features including inflammation, cartilage and bone destruction. There was some variation in levels of inflammation and bone destruction observed in untreated CAIA mice although this is a normal variation observed with this model. Both NW-21 and MS-275 suppressed inflammation as demonstrated by both reduced clinical paw scoring and histologically with scoring for inflammatory infiltrate reduced in treated mice. In this model, 1179.4b did not reduce inflammation in paws. Surprisingly, the effects of both 1179.4b and MS-275 were opposing in a model of periodontal disease, with 1179.4b suppressing bone loss despite having no effect on inflammation and MS-275 reducing inflammation but having no effect on bone loss [39]. Given the similarities between periodontitis and RA and the relationship between the two pathologies [49], it was expected that 1179.4b would also be able to suppress bone loss in RA. However, this

lack of effect is most likely due to differences in HDACs involved in the two diseases, the capacity of 1179.4b to also inhibit all other HDACs may lead to detrimental effects that overwhelm HDAC1-mediated pathology. Several studies point to the importance of HDAC 1 in RA with increased expression in disease [37] and its suppression inhibits inflammation and bone resorption. MS-275 selectively targets HDAC 1 ($IC_{50}$=181 nM) over HDAC 2 ($IC_{50}$=1155 nM) and HDAC 3 ($IC_{50}$=2311nM) [42]. In regards to effects on bone loss both MS-275 and NW-21 suppressed radiocarpal joint erosion. MS-275 although not significant was slightly more effective in suppressing both inflammation and bone resorption in the CAIA model. NW-21 targets HDAC 1 and 2 and, given that MS-275 predominately targets HDAC 1, confirms the importance of HDAC 1. This importance of targeting HDAC 1 in RA is also consistent with a report showing that MS-275 has greater anti-arthritic activity than the broader acting HDACi SAHA [28]. In this CIA mouse model MS-275 was shown to reduce serum IL-6 and IL-1beta levels [28]. Recent studies have also shown that MS-275 was able to suppress cytokine-induced MMP1 and MMP13 in human articular chondrocytes [65]. A recent study demonstrated that a novel HDACi MPT0G009, which targets class I HDACs (1,2,3 and 8) as well as class II HDAC 6, was able to suppress paw swelling and bone destruction in a rat adjuvant arthritis model. MPT0G009 was compared to SAHA *in vitro* and shown to reduce cytokine release by LPS stimulated macrophages with overexpression of HDAC 1 shown to reduce the effects of MPT0G009 [66]. These studies highlight the importance of targeting HDAC 1 although the effects of specific HDAC 1 inhibition in arthritis are not known and with the development of even more selective inhibitors this will become possible. Assessment of the effects of these inhibitors on acetylation levels both histone and non-histone proteins in vivo is warranted in the future.

The results of this study highlight the importance of HDAC 1 in the pathogenesis of RA. The HDACi, NW-21, targets both HDAC 1 and HDAC 2 (both class I HDACs) was able to suppress osteoclast resorption and inflammation *in vitro* and *in vivo*. This may be due to the ability of NW21 to suppress chemokines, MCP-1 and MIP-1α, as these chemokines are important in osteoclast formation as well as inflammation. In addition, the inhibitory effects of HDACi NW-21 on osteoclasts occurs upstream of NFATc1 expression and are likely to be due to suppression of TRAF6 and/or chemokines that are elevated following RANKL/RANK interaction. Overall, the results demonstrate that a safely tolerated inhibitor of HDAC 1, such as NW-21, might be very useful for the treatment of RA as such a drug has the capacity to simultaneously target both inflammation and bone resorption.

Key messages

1. HDACi NW-21 designed to target HDAC 1 suppressed human osteoclast bone loss *in vitro*.
2. NW-21 suppressed mRNA expression of MCP-1 and MIP-1α in TNF-α or LPS stimulated monocytes.
3. NW-21 and MS-275 that both target HDAC 1 suppressed inflammation and bone loss in a collagen antibody induced murine arthritis.

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References ????

Figure 1.
Chemical structure of HDACi NW-21developed at the Institute for Molecular Bioscience at the University of Queensland. Chemical formula: $C_{26}H_{31}N_5O_4$. Molecular Weight: 477.56

Figure 2.
Representative TRAP stained sections (10x mag) (A,C, E, G, I)and scanning electron microscope images of dentine pieces (imaged using the XL-20 at 150x mag) (B, D. F, H, J). (A, B): Control (no HDACi treatment 0.01% DMSO). HDACi NW21 treatment commencing from day 7 at (C, D): 20 nM; (E, F): 4nM; (G, H): 0.8nM; and (I, J): 0.16nM.

Figure 3.
A) Average fold change in the number of TRAP positive multinucleated (>3 nuclei) cells relative to control (0.01% DMSO) Controls =1 (n=9). NW-21 treatment commencing from day 7 (n=9), day 10 (n=4) and day 13 (n=4).
B) Percentage area of pit resorption on dentine expressed as a percentage of the control area (0.01% DMSO). NW-21 treatment commencing from day 7 (n=9), day 10 (n=4),13 (n=4).
Bars represent mean±SEM. ** $p<0.01$ and *** $p<0.001$ compared to control.
C) Fold change in the relative mRNA expression assessed using real time PCR analysis. Relative to endogenous gene hARP. Fold change at days 10, 14 and 17 relative to day 7. n=3 healthy donors. Bars represent mean±SEM. * $p<0.05$ *** $p<0.001$ compared to control (0.01% DMSO).

Figure 4.
Representative macroscopic images of the front paws (A) CAIA +Oil B) CAIA+NW-21 C) CAIA+1179.4b D) CAIA+MS-275.
Representative images of the front left-hand side paws. 3D models created from micro CT analysis conducted at day 14 (final day) using SkyScan program ANT. (E) CAIA +Oil F) CAIA+NW-21 G) CAIA+1179.4b H) CAIA+MS-275.
I) Average daily (0-14) clinical paw scoring out of a total of 16. * $p<0.05$ NW-21 compared to oil. # $p<0.05$ MS-275 compared to oil. To assess inflammation each paw was given a score from 0 to 4 giving a total maximum score of 16 for each animal [49].
J) Average percentage change in bone volume (BV) from scan 1 (4 days prior to mAb injection) to scan 3 (day 14) as assessed using micro CT with the SkyScan 1076.

Figure 5.
A) H&E stained sections of the corresponding radiocarpal joint imaged at 20x and 4x magnification. TRAP stained images of the corresponding radiocarpal joints at 20x and 4x magnification.
B) Average inflammation score of H&E stained sections out of a total of 3. Scoring was based on the numbers of inflammatory cells with the radiocarpal joints (lymphocytes, plasma cells neutrophils or macrophages).
C) Presence of pannus on H&E stained sections. Scoring for pannus formation: 0=no pannus, 1=pannus formation.
D) Average Bone and Cartilage destruction score of H&E stained sections total score /3 Bone and cartilage destruction was assessed by: 0=normal, 1 =mild cartilage destruction, 2=evidence of both cartilage and bone destruction, 3= severe cartilage and bone destruction.
E) Average number of TRAP+ multinucleated cells on bone surface in $1.92mm^2$ area of the radiocarpal joint.

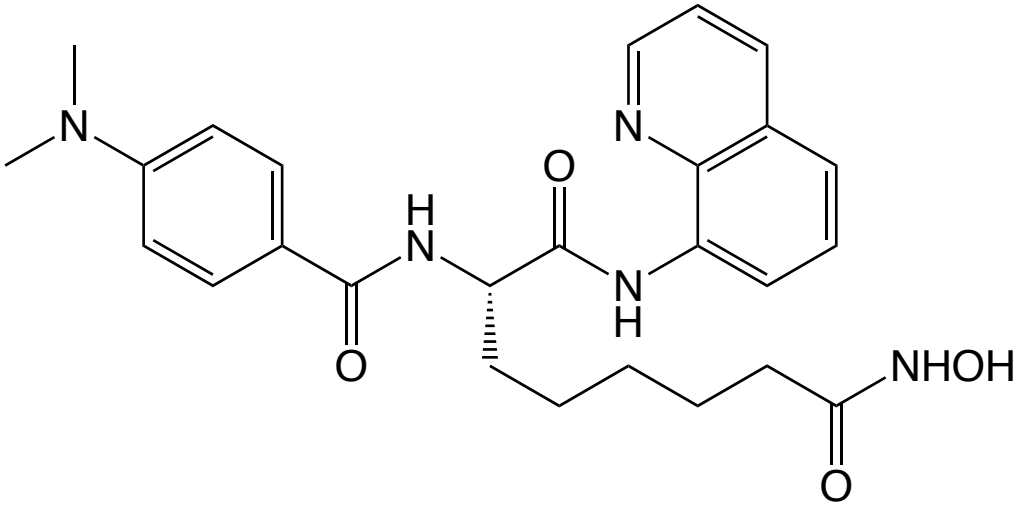


Figure 1

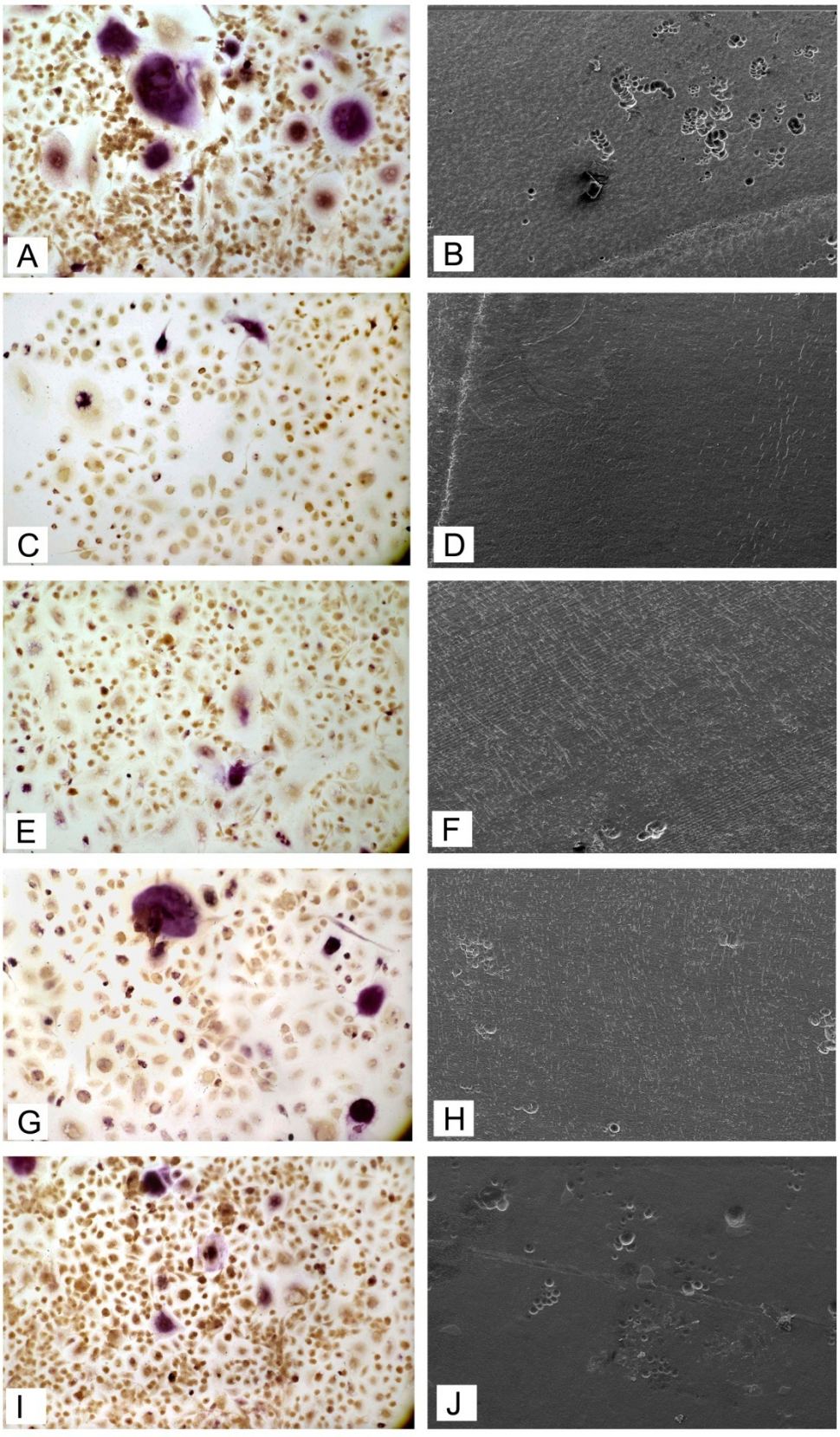


Figure 2

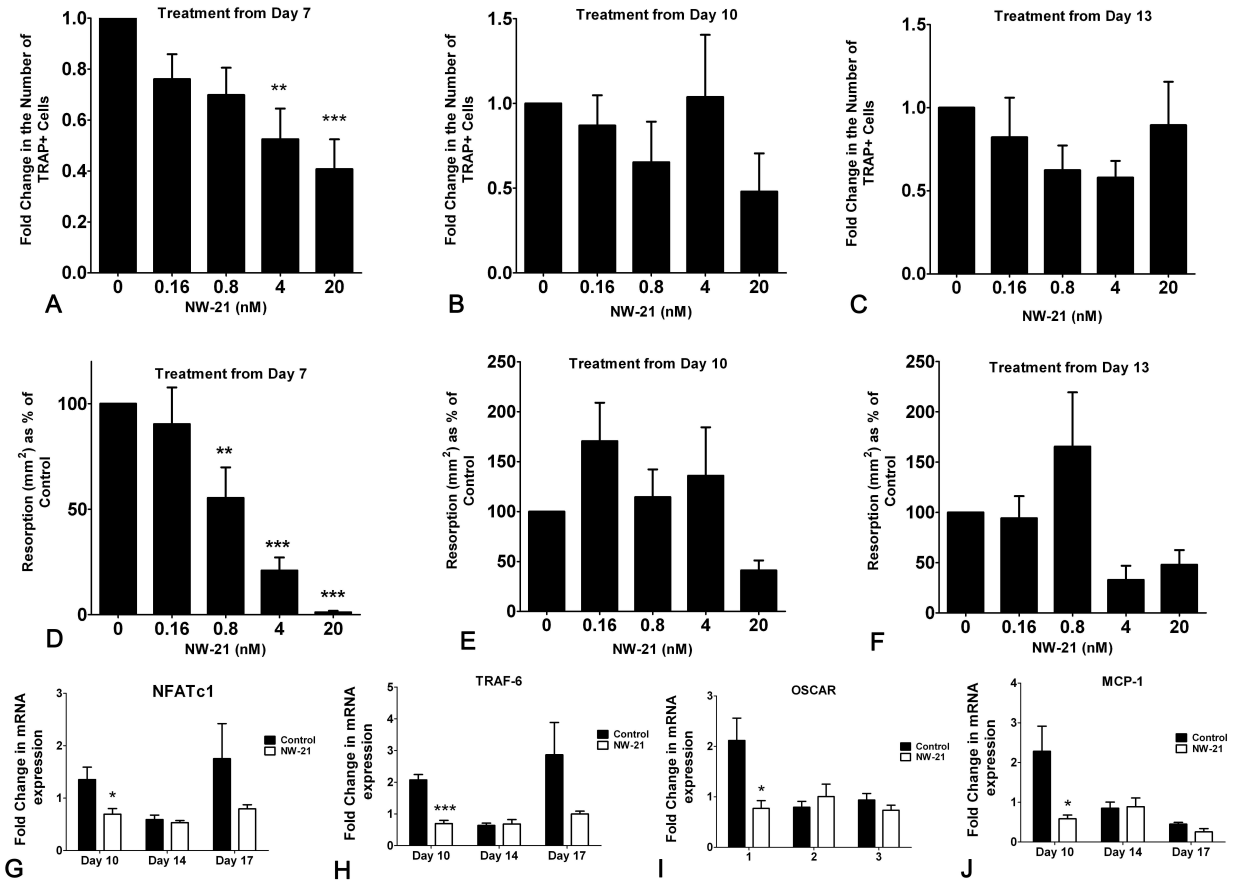
Treatment from Day 7
Fold Change in the Number of TRAP+ Cells
NW-21 (nM)
A
Treatment from Day 10
B
Treatment from Day 13
C
Resorption (mm2) as % of Control
D
E
F
NFATc1
Fold Change in mRNA expression
Control
NW-21
Day 10
Day 14
Day 17
G
TRAF-6
H
OSCAR
I
MCP-1
J

Figure 3

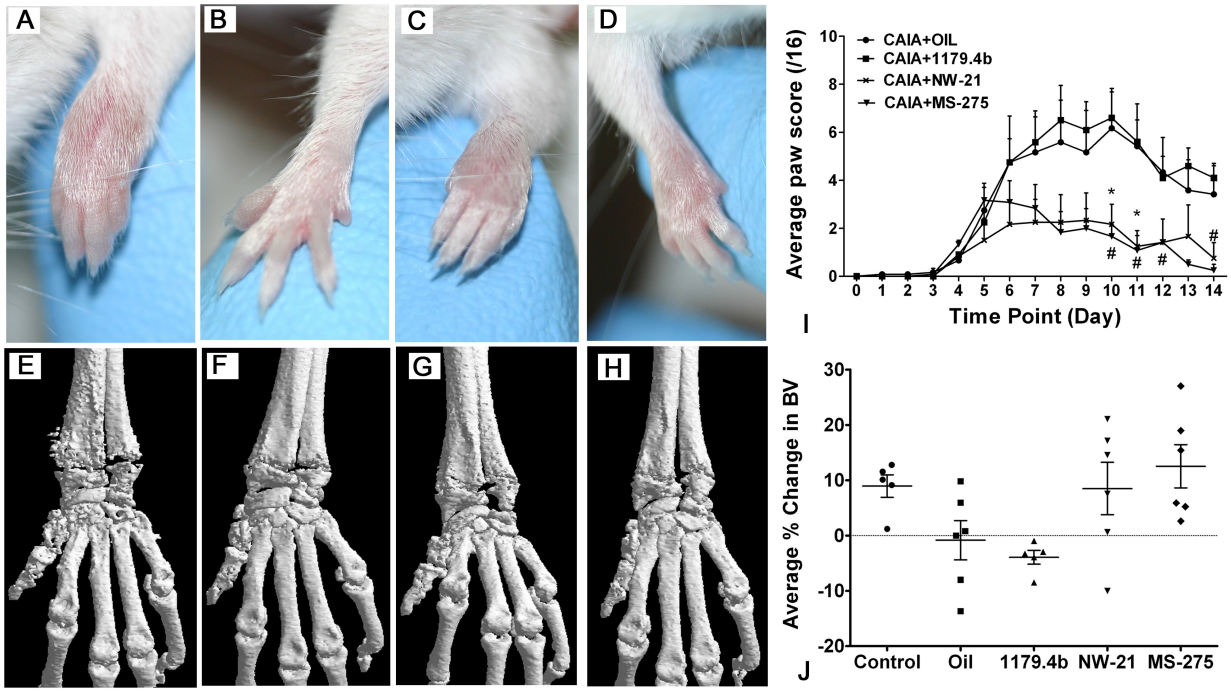
A
B
C
D
E
F
G
H
CAIA+OIL
CAIA+1179.4b
CAIA+NW-21
CAIA+MS-275
Average paw score (/16)
Time Point (Day)
I
Average % Change in BV
Control
Oil
1179.4b
NW-21
MS-275
J

Figure 4

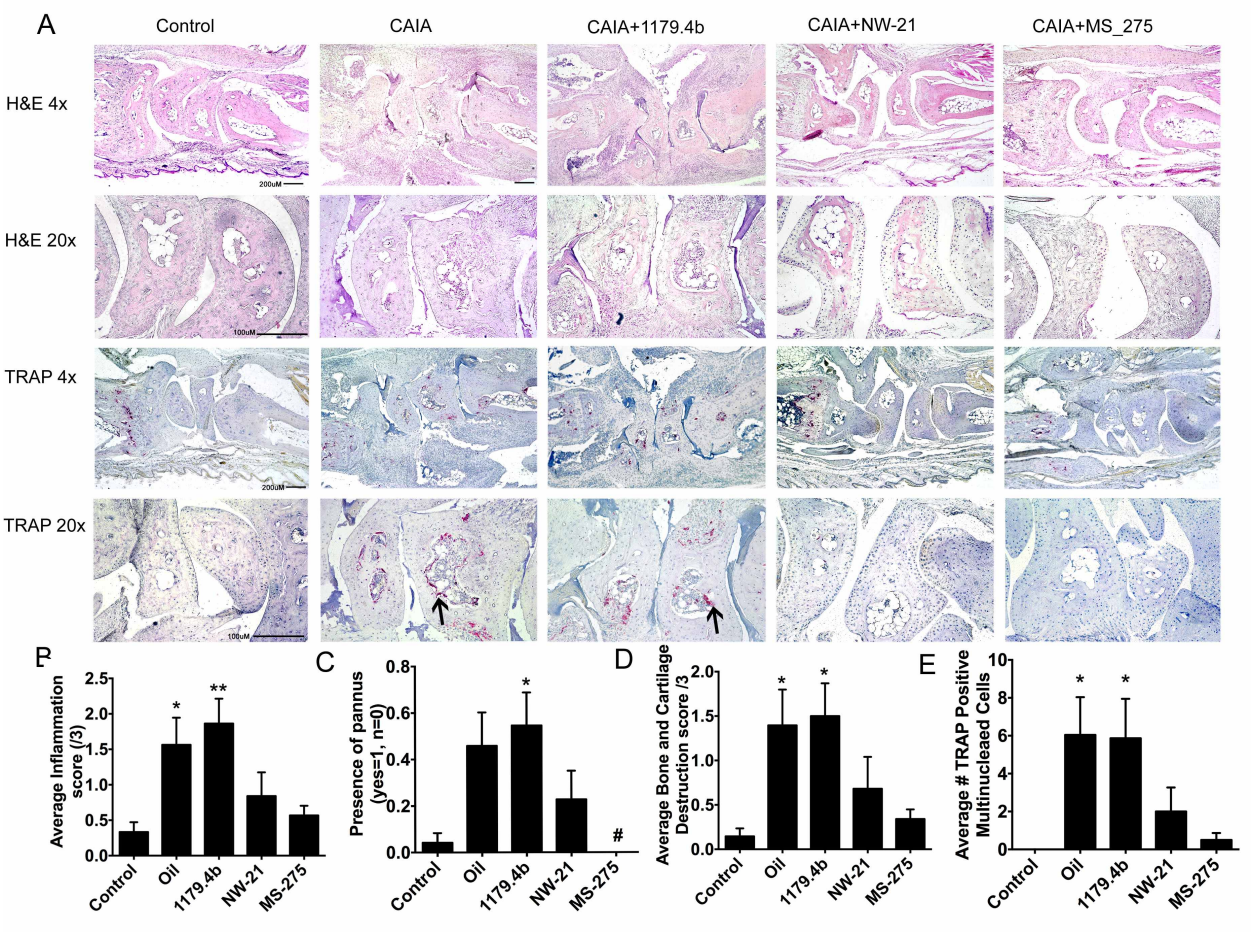
A
Control
CAIA
CAIA+1179.4b
CAIA+NW-21
CAIA+MS_275
H&E 4x
H&E 20x
TRAP 4x
TRAP 20x
200uM
100uM
B
Average Inflammation score (/3)
C
Presence of pannus (yes=1, n=0)
D
Average Bone and Cartilage Destruction score /3
E
Average # TRAP Positive Multinucleaed Cells
Control
Oil
1179.4b
NW-21
MS-275

Figure 5

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