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Running title: HDAC inhibition in human macrophages and rat arthritis

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Abbreviations: ATP – Adenosine Tri Phosphate; CIA – collagen induced arthritis; d – day; DAI – Disease Activity Index; DMSO – Dimethylsulfoxide; EDTA – Ethylenediaminetetraacetic acid; FBS – Foetal bovine Serum; FDA – United States Food and Drug Administration (USA); GM-CSF – Granulocyte macrophage colony stimulating factor; H&E – Haematoxylin and Eosin; HDAC – histone deacetylase; IHC – Immunohistochemistry; IL – Interleukin; LPS – Lipopolysaccharide; LDH – Lactate dehydrogenase; M-CSF – Macrophage colony stimulating factor; MTC – Masson’s Trichrome; NLR – Nod-Like Receptor; RANKL – Receptor activator of nuclear factor kappa-B ligand; TLR – Toll-Like Receptor; TNF – Tumour necrosis factor; TSA – Trichostatin; VPA – Valproic acid.

Section; Drug Discovery and Translational Medicine
Abstract

Vorinostat and other inhibitors of different histone deacetylase (HDAC) enzymes are currently being sought to modulate a variety of human conditions, including chronic inflammatory diseases. Some HDAC inhibitors are anti-inflammatory in rodent models of arthritis and colitis, usually at cytotoxic doses that may cause side effects. Here, we investigate the dose-dependent pro- and anti-inflammatory efficacy of two known inhibitors of multiple HDACs, Vorinostat and BML281, in human macrophages and in a rat model of collagen-induced arthritis by monitoring effects on disease progression, histopathology and immunohistochemistry. Both HDAC inhibitors differentially modulated lipopolysaccharide (LPS)-induced cytokine release from human macrophages, suppressing release of some inflammatory mediators (IL12p40, IL6) at low concentrations (< 3 μM) but amplifying production of others (TNF, IL1β) at higher concentrations (> 3 μM). This trend translated in vivo to rat arthritis, with anti-inflammatory activity inversely correlating with dose. Both compounds were efficacious only at a low dose (1 mg kg⁻¹ day⁻¹ s.c.), whereas a higher dose (5 mg kg⁻¹ day⁻¹) showed no positive effects on reducing pathology, even showing signs of exacerbating disease. These striking effects suggest a smaller therapeutic window than previously reported for HDAC inhibition in experimental arthritis. The findings support new investigations into repurposing HDAC inhibitors for anti-inflammatory therapeutic applications. However, HDAC inhibitors should be reinvestigated at lower, rather than higher, doses for enhanced efficacy in chronic diseases that require long-term treatment, with careful management of efficacy and long-term safety.
Introduction

Macrophages detect danger signals arising from invading pathogens, and damaged or dying host cells (Murray and Wynn, 2011; Liddiard and Taylor, 2015). They release a plethora of cytokines, chemokines and proteases to initiate and propagate inflammation, but they also have central roles in rheumatoid arthritis (RA) (Barrera et al., 2000; Kinne et al., 2000; Li et al., 2012; Davignon et al., 2013; Soler Palacios et al., 2015). Increased infiltration and polarisation of macrophages in the synovia of RA patients can lead to an imbalance of pro-inflammatory versus anti-inflammatory macrophages that contributes to pathology (Barrera et al., 2000; Kinne et al., 2000; Li et al., 2012; Davignon et al., 2013; Soler Palacios et al., 2015). RA progression can be attenuated by macrophage depletion or targeting key macrophage-derived cytokines where major clinically relevant therapies for arthritis target single cytokines such as TNFα, IL1β, IL6, GM-CSF and M-CSF (Barrera et al., 2000; Moss et al., 2008; Li et al., 2012; Davignon et al., 2013; Taylor and Williams, 2015). Novel agents that modulate macrophage functions per se may also have potential as therapeutics. Histone deacetylase (HDAC) inhibitors are candidate disease modifiers as many key inflammatory cytokines are HDAC-dependent in macrophages (Halili et al., 2009; Dinarello et al., 2011; Shakespear et al., 2011; Fairlie and Sweet, 2012; Sweet et al., 2012).

HDAC enzymes regulate reversible acetylation of lysine residues of histone and non-histone proteins (Choudhary et al., 2009; Iyer et al., 2012; Dekker et al., 2014). Eleven zinc-containing HDACs regulate many biological processes including immune responses, cell cycle, proliferation and apoptosis (Gupta et al., 2012; Falkenberg and Johnstone, 2014; West and Johnstone, 2014; West et al., 2014). These isozymes are divided into class I (HDAC1, 2, 3, 8), class IIa (HDAC4, 5, 7, 9), class IIb (HDAC6, 10)...
and class IV (HDAC11) (Gupta et al., 2012). Broad-spectrum (pan) HDAC inhibitors (HDACi) such as Vorinostat, Panobinostat and Romidepsin are FDA-approved for treatment of cutaneous T-cell lymphomas, and many are in development or clinical trials for other cancers (Falkenberg and Johnstone, 2014; West and Johnstone, 2014; West et al., 2014). Mechanisms of action of HDACi as anti-cancer agents remain uncertain, but likely involve effects on cancer cell apoptosis as well as epigenetic immunomodulatory effects (West and Johnstone, 2014; West et al., 2014). However, most HDACi also arrest growth and induce apoptosis of non-cancer cells (Gupta et al., 2012; West and Johnstone, 2014), and generally, most HDAC inhibitors have broad-spectrum activities (Gupta et al., 2012).

In models of chronic inflammatory disease, including RA, inflammatory bowel disease, fibrosis and septic shock, HDACi are considered anti-inflammatory with effects being related to immune cell apoptosis (Halili et al., 2009; Iyer et al., 2010; Subramanian et al., 2010; Gupta et al., 2012). This cytotoxicity of pan-HDACi is expected to cause adverse effects in vivo due to non-selectivity. Such side effects have, to some extent, discouraged pursuit of HDACi as therapies for chronic inflammatory diseases (Gupta et al., 2012), where safe, long-term therapeutic regimes are required. HDACi immunomodulatory properties are complex (Bode and Dalpke, 2011; Kroesen et al., 2014) and not simply anti-inflammatory (Leoni et al., 2002; Grabiec et al., 2008; Halili et al., 2009; Fairlie and Sweet, 2012). Treatment of mouse macrophages with Trichostatin (TSA) downregulates many inflammatory genes, thus compromising host defence (Roger et al., 2011), while they can also upregulate pro-inflammatory genes, and promote cytokine secretion (Wang et al., 2011; Kroesen et al., 2014). Indeed, we previously showed that TSA differentially regulates many inflammatory and pro-arthritic genes in human and mouse macrophages in response to toll-like receptor 4 (TLR4)
stimulation (Halli et al., 2010); (Schroder et al., 2012), suggesting therapeutic effects of HDACi in arthritis, but also potentially adverse reactions.

In this study, two HDACi, Vorinostat and BML281 (Kozikowski et al., 2008), were compared for anti-inflammatory effects in human macrophages, and in a rat model of collagen-induced arthritis. A surprising inverse dose-dependent therapeutic response has been found, with the lower dose being more efficacious than the higher. These findings should stimulate new investigations into the properties of low dose HDAC inhibitors in a clinical setting, with potential for their use as effective anti-arthritis drugs without the adverse side effects observed at the higher doses normally evaluated for anti-cancer efficacy. As many HDACi are under clinical investigation for the treatment of cancers, this study supports a potential to repurpose anti-cancer HDAC inhibitors at much lower and safer doses for treating inflammatory diseases.
Materials and Methods

Animals

Female Wistar rats (250±50g, n=54) were bred at the Animal Resource Centre of Australia (WA), and transported by air and road to the University of Queensland. Animals were housed (n=3/box on corn-cobb bedding) at the Australian Institute for Bioengineering and Nanotechnology, Brisbane, QLD, Australia. Animals were maintained in a 12h light-dark cycle according to the standard of holding facility with standard chow and water provided. All experiments were approved by the Molecular Biosciences Animal Ethics Committee of The University of Queensland, and adhere to The Australian Code of Practice for Use Of Animals for Scientific Purposes (2013) and The Australian Government Guidelines to Promote the Wellbeing of Animals used For Scientific Purposes (2013). Studies involving animals are reported in accordance to the ARRIVE guidelines (Kilkenny et al., 2010).

Drugs and chemicals

Cell culture media, foetal bovine serum (FBS), penicillin, streptomycin, and L-glutamine were supplied by Invitrogen (VIC, Australia). Bovine type II collagen (nasal cartilage), Freund’s incomplete adjuvant, heparin, LPS and ATP were supplied by Sigma-Aldrich (Sydney, NSW, Australia). Vorinostat (suberoylanilide hydroxamic acid, SAHA) and BML281 (tert-butyl (4-((3-((7-(hydroxyamino)-7-oxoheptyl) carbamoyl) isoxazol-5-yl) phenyl) carbamate) (Fig. 1) were synthesized as previously described (Stowell et al., 1995; Kozikowski et al., 2008). Compounds were dissolved in 20% DMSO/saline, and administered subcutaneously (nape of neck, 1 or 5 mg kg\(^{-1}\)) daily from day 7 onwards. Chemicals and stains for histology were from Sigma-Aldrich (Sydney, NSW, Australia), unless otherwise specified.
**Macrophage culture and differentiation**

Peripheral blood mononuclear cells were isolated from buffy coats (Australian Red Cross Blood Service, Kelvin Grove, QLD, Australia) using Ficoll-Paque density centrifugation (GE Healthcare Bioscience, Uppsala, Sweden) (Hohenhaus et al., 2013; Seow et al., 2013). CD14⁺ monocytes were isolated using an antibody-tagged magnetic bead protocol (MACS; Miltenyi Biotech, Auburn, CA, USA), and differentiated to human monocyte-derived macrophages (HMDMs) in complete media, using either recombinant human granulocyte macrophage colony stimulating factor (GM-CSF; 10⁴ U ml⁻¹) or recombinant human macrophage colony stimulating factor (M-CSF; 10⁴ U ml⁻¹, PeptroTech Inc., Rocky Hill, NJ, USA) at 1.5 x 10⁶ monocytes ml⁻¹. Cells were grown to 80% confluence and seeded in 12-well plates (1 x 10⁶ cells/well, in IMDM with 10% FBS, 10 U ml⁻¹ penicillin, 10 U ml⁻¹ streptomycin, and 2 mM l-glutamine). Prior to experimentation, culture media was replaced with serum free IMDM (10 U ml⁻¹ penicillin, 10 U ml⁻¹ streptomycin, and 2 mM l-glutamine). Supernatants were removed, centrifuged and stored at -20°C until assayed.

**Lactate dehydrogenase (LDH) cytotoxicity and ELISA**

Concentration-dependent cytotoxicity of Vorinostat and BML281 was evaluated as per manufacturers instructions using a commercially available LDH release assay kit (Roche Cytotoxicity Detection Kit Plus). Inflammatory cytokines were quantified from supernatants using commercially available ELISA kits (IL12p40, IL6, TNF, IL1β; Becton Dickinson, San Jose, CA, USA), as per manufacturers instructions.

**Collagen-induced arthritis**
Arthritis was induced by collagen inoculation (collagen-induced arthritis, CIA) in rats as previously described (Lohman et al., 2012). Six groups of female Wistar rats (200–250 g) were used: CIA control group (n=12), Vorinostat (1 mg kg\(^{-1}\))-treated CIA group (n=12), Vorinostat (5 mg kg\(^{-1}\))-treated CIA group (n=6), BML281 (1 mg kg\(^{-1}\) day\(^{-1}\))-treated CIA group (n=12), BML281 (5 mg kg\(^{-1}\) day\(^{-1}\))-treated CIA group (n=6) and sham-treated group (n=6). CIA control and HDACi-treated rats were immunized on d0 with bovine nasal collagen (200 μg in 200 μL 50:50 0.05 M acetic acid/Freund’s incomplete adjuvant, s.c. tail base). Sham-treated animals received vehicle only (no collagen; 200 μL 50:50 0.05 M acetic acid/Freund’s incomplete adjuvant, s.c. tail base). Boosters of the same treatments were given on d7. Daily dosage of Vorinostat and BML281 (1 or 5 mg kg\(^{-1}\) day\(^{-1}\) s.c., in 20% DMSO/sterile saline) or vehicle (for CIA control and sham-treatment groups) began on d7. Hind paw swelling measurements were made by digital callipers (paw width and thickness), body weight and Disease Activity Index (DAI) score were measured daily from d10 to d28. Paw swelling area (mm\(^2\)) was calculated and expressed as percentage change from baseline measurements. DAI was measured by expert observation, incorporating scores representing changes in mobility, inflammation, discomfort/pain and generalised sickness behaviours (Lohman et al., 2012). Final DAI scores were the sum of these four parameters (maximal score 12).

**Histopathology**

On d28, rats were euthanized with CO\(_2\) inhalation. Hind paws were skinned, amputated, and fixed in 4% paraformaldehyde (pH 7.4) at 4° C, decalcified for 72h (10% HCl; 0.18% w/v EDTA:0.09% w/v tartrate in H\(_2\)O), then embedded in paraffin wax for histological analysis. Sagittal sections were cut at 5 μm and stained using standard protocols. All microscopic images were obtained using an Olympus BX-51 upright microscope with
Olympus DP-71 12Mp colour camera, utilizing DP Capture and DP Manager software packages (Olympus, Tokyo, Japan). For generalised bone/tissue health, sections were stained with haematoxylin and eosin (H&E). Photomicrographs (40X lens) of at least 6 sections of the tibia/talus/calcaneal joint region (both soft and hard tissue) were assessed and scored blinded as previously reported (Lohman et al., 2012). Total histopathological scores were expressed as the sum of all scores (maximal score of 12). Total collagen loss and articular cartilage capsule thickness were measured from Masson’s Trichrome (MTC)-stained sections using colour deconvolution software (FIJI/ImageJ 1.42q software, U.S. National Institutes of Health, Bethesda, MD, USA). Brightfield photomicrographs (20X lens) were made of each articular surface of each joint on 6 sections per animal stained with MTC. Images were deconvoluted into red and blue channels, then digitally analysed for pixel colour intensity per unit area using software. Data was represented as colour density per unit area. Within-animal colour/stain densities were averaged, grouped into treatments and compared between treatments. Articular cartilage thickness was also measured from the MTC stained sections. Briefly, 3 regions of at least 3 joint surfaces of each paw were measured digitally from photomicrographs, by assessing the capsule thickness from the chondral/bone junction to the articular surface. These thickness values were averaged within animals and within treatment groups, then expressed as change in thickness (in micrometre).

**Immunohistochemistry (IHC) for ED1 and ED2**

M1-like macrophages and giant multinucleated osteoclasts were labelled using ED1 monoclonal antibody (mouse anti-rat MCA341R Serotec, Kidlington, UK) using standard IHC techniques (DAKO pH 6.0 antigen retrieval, 1:150 2hrs at room temperature). M2-like macrophages were labelled using ED2 monoclonal antibody (mouse anti-rat
MCA342GA/CD163 Serotec, Kidlington, UK) also using standard technique (trypsin (0.1%) antigen retrieval, pH 8.0, 1:200 2hrs at room temperature). For both antibodies, the secondary antibody was a MACH1 mouse probe, followed by HRP-DAB precipitation reaction. Spleen, liver and thymus acted as positive controls. ED2 stained arthritis/paw tissue sections were generally devoid of staining, and not used for further analysis. For ED1+ cell counts, 8-10 specified regions per section (2 sections/rat) were imaged at high resolution incorporating regions of soft tissue, articular surface, trabecular and cortical bone. ED1+ cells were counted from images using software (FIJI/ImageJ). Each image was deconvoluted into brown (DAB) and background blue (Haematoxylin). The brown image was used to identify cell-like staining patterns using the particle analysing Macro supplied in FIJI software. These were counted for small cell-like staining patterns (25-70 μm²) representing macrophages, and large cell-like staining patterns (71-400 μm²) representing osteoclasts. The latter were exclusively associated with bone and cartilage. The use of an automated script for the entire process eliminated bias and counting errors. Data was presented as cells per unit area within groups and compared between groups as a marker for macrophage infiltration and osteoclasts development.

**Data analysis**

All experimental results are expressed as means ± standard error. Data were analyzed using software (GraphPad Prism v5.0a, San Diego, CA, USA). Two-way repeated measures ANOVA was used for data sets involving ≥3 groups. In the arthritis study, Vorinostat and BML281-treatment group data were compared to both CIA control and sham-treatment data, and CIA control data were compared to sham-treatment data, using 2-way repeated measures ANOVA with Bonferroni planned comparison. Small sample individual time points for histological analysis, a 1-way non-parametric Kruskal-
Wallis ANOVA as used. Student’s t-test was used for data comparing 2 data sets only. Significance was set at $P < 0.05$.

Results

*Anti-versus pro-inflammatory responses of HDACi in human macrophages*

Two known HDACi, Vorinostat and BML281 (Fig. 1A,B), were assessed for cytotoxicity on GM-CSF- and M-CSF-derived primary human macrophages using the lactate dehydrogenase (LDH) release assay. Both compounds showed little cytotoxicity at concentrations below 30 µM in either cell derivative (Fig. 1C,D). Thus, 30 µM was subsequently chosen as the upper concentration limit for evaluating dose-dependent effects *in vitro* on macrophage inflammatory responses, without causing significant cell death.

Vorinostat and BML281 dose-dependently modulated LPS-induced inflammatory cytokine production from human GM-CSF differentiated macrophages (Fig. 2A-H). Both compounds suppressed production of IL12p40 and IL6 at concentrations up to 30 µM but, surprisingly, they dose-dependently and significantly amplified production of TNF and IL1β at concentrations greater than 1-3 µM (Fig. 2A-H). These distinct differential pro- and anti-inflammatory effects for each HDACi were independent of the nature of the differentiation, being similar for both compounds on M-CSF differentiated macrophages as for GM-CSF differentiated cells (Fig. 2I-P).

*Inflammasome activation by HDACis*

The finding that co-culture of HMDM with high concentrations of Vorinostat or BML281 plus LPS stimulates increased IL1β secretion is particularly surprising. IL1β release from
cells typically requires inflammasome activation, which results in cleavage of pro-
IL1β by active caspase-1 (Schroder and Tschopp, 2010). However, there was no
inflammasome trigger (e.g. ATP, nigericin) delivered in these experiments, suggesting
that higher concentrations of HDACi may provide an independent signal for IL1β
maturation. We therefore determined whether Vorinostat potentiated IL1β released in
human GM-CSF-derived macrophages when primed with LPS (4h), followed by
triggering with ATP (0.5h) (Fig. 3). The result was a ~3 fold increase in IL1β release in
cells treated with 1 or 3 µM Vorinostat after only 4h. On the other hand, an HDAC class
I-selective HDACi (Entinostat) did not amplify IL1β release beyond what was observed
for LPS+ATP (Fig. 3). This suggests that IL1β release triggered by HDAC inhibitors is
likely to occur in response to inhibition of HDACs other than HDAC1-3, and 8. These
novel findings of differential pro- and anti-inflammatory influences of HDACi on human
macrophages raise the question as to whether such differential modulation of
inflammation translates into an in vivo inflammatory setting.

**Anti-inflammatory and pro-inflammatory responses of HDACi in CIA**

There are numerous reports of anti-inflammatory activity for HDACi, although relatively
few on rodent models of arthritic disease (Lin et al., 2007; Grabiec et al., 2008; Saouaf et
al., 2009; Joosten et al., 2011; Li et al., 2013; Hsieh et al., 2014; Cantley et al., 2015).
Most in vivo studies have used extremely high doses of HDACi (VPA, 400 mg kg⁻¹
(Saouaf et al., 2009); Vorinostat, 200 mg kg⁻¹ (Hsieh et al., 2014) or 50 mg kg⁻¹ (Lin et
al., 2007) or 30 mg kg⁻¹ (Li et al., 2013)), which are liable to cause serious side effects
(cardiac T-wave flattening, anemia, neutropenia, thrombocytopenia, weight loss,
anorexia, etc) (Subramanian et al., 2010). We sought to determine whether the
differential inflammatory responses observed above for human macrophages were
mirrored in an *in vivo* model of chronic inflammatory disease such as collagen-induced arthritis (CIA) in rats.

Collagen inoculation induced progressive arthritic paw swelling particularly in hind limbs and caused a progressively deteriorating DAI in Wistar rats (Fig. 4). Treatment with either Vorinostat (Fig. 4A) or BML281 (Fig. 4C) at 1 mg kg\(^{-1}\) day\(^{-1}\) significantly reduced paw swelling by d28 (p<0.05 vs CIA control, but not vs sham, (p>0.05), rmANOVA), and significantly reduced development of arthritis-like or other DAI symptoms, with only mild DAI scores recorded at d28 (Fig. 4B,D). In marked contrast to the effects of low dose HDACi, both HDACi at 5 mg kg\(^{-1}\) day\(^{-1}\) s.c. exacerbated paw swelling (Fig. 4A,C) and DAI (Fig. 4B,D), as compared to CIA controls. Indeed, 5 mg kg\(^{-1}\) day\(^{-1}\) Vorinostat- and BML281-treated animals had to be euthanised on d20 and d22, respectively, due to illness severity. Thus, neither compound at the higher dose (5 mg kg\(^{-1}\) day\(^{-1}\)) provided any therapeutic benefit, instead causing apparent disease exacerbating effects.

**Histopathology of arthritic joints in HDACi-treated rats**

Histological examination of H&E-stained tibia-talus joints confirmed CIA control animals, but not sham controls, had severe cardinal histopathologies of arthritic disease, such as inflammatory cell invasion, synovial hyperplasia, pannus formation, and cartilage and bone erosion (not shown). Daily administration of low dose HDACi significantly reduced all histopathological changes (p<0.05, ANOVA; Fig. 5C,E). Conversely, 5 mg kg\(^{-1}\) day\(^{-1}\) HDACi treatment did not alleviate any of the histopathological-disease features described above (Fig. 5D,F).

Masson’s trichrome-stained tissue showed significantly reduced collagen loss in 1 mg kg\(^{-1}\) day\(^{-1}\) HDACi-treated rats compared to CIA controls, having a greater proportion of
Aniline Blue staining representative of collagen (Fig. 5H). Likewise, the articular cartilage thickness was unchanged from sham in the two groups treated with HDACi at 1 mg kg\(^{-1}\) day\(^{-1}\), whereas CIA controls showed significant loss of articular thickness (p<0.05, ANOVA; Fig. 5I), confirming the reduced arthritic conditions of rats treated with HDAC inhibitors at the low dose. Significant losses in collagen and articular cartilage thickness were observed in 5 mg kg\(^{-1}\) day\(^{-1}\) HDACi-treated rats (p<0.05, ANOVA), which were similar to that of CIA controls (Fig. 5H-I).

**Macrophage and osteoclast numbers in HDACi-treated rats**

CIA tissue showed significant increases in ED1-positive (+) (Fig. 6A,B,G) but not ED2-positive (+) (data not shown) immune cell populations, as compared to sham-treated animals (p<0.05, ANOVA), particularly within the synovium, pannus, and lining the articular surface. Using size exclusion software algorithms, we identified that CIA progression induces the infiltration of ED1+ macrophage-like cells and promoted formation of ED1+ osteoclast-like giant multinucleated cells in both soft tissue and bone in control animals compared to sham animals (Fig. 6H,I). In agreement with improved disease symptoms, both HDACi at 1 mg kg\(^{-1}\) day\(^{-1}\) generally significantly reduced populations of ED1+ immune cells, as compared to CIA diseased controls (p<0.05, ANOVA, Fig. 6C,E,G-I). Conversely, HDACi at 5 mg kg\(^{-1}\) day\(^{-1}\) significantly increased numbers of both macrophages in and around the synovium, and osteoclast-like cells in bone and synovium compared to sham animals and low-dose HDAC treatments (p<0.05, ANOVA, Fig. 6D,F,G-I). Most strikingly, both macrophage and osteoclast-like cell (ED1+) populations were almost doubled compared to CIA control in high dose BML281-treated rats (p<0.05, ANOVA). Vorinostat (1 mg kg\(^{-1}\) day\(^{-1}\)) had a modest but significant effect in reducing macrophage numbers, as compared to CIA control, whilst the effect on osteoclast-like giant cells was more pronounced (Fig. 6H-I).
higher dose of Vorinostat did not have significant effects on numbers of either ED1+ macrophages or osteoclasts. Thus, only at the low dose did either compound have any positive effect in reducing numbers of pathogenic macrophage/osteoclast populations, a finding that correlated with the effects observed for disease pathology, cartilage erosion and histopathology.
Discussion

Macrophage activation is a key driver of arthritis pathology (Davignon et al., 2013; Liddiard and Taylor, 2015). Here, we have evaluated the viability of targeting epigenetic and post-translational mechanisms by inhibiting HDACs (Grabiec and Reedquist, 2013) to curb macrophage-related inflammation in arthritis. At high doses, HDACi have shown a degree of efficacy in animal models of arthritis (Halili et al., 2009) as well as in clinical trials (Furlan et al., 2011; Joosten et al., 2011; Vojinovic and Damjanov, 2011). However, their uses in chronic inflammatory diseases are considered to be quite limited because of significant adverse effects, such as thrombocytopenia, neutropenia and cardiac abnormalities, making them unsuitable for long-term treatment regimes (Subramanian et al., 2010). These can be attributed, in part, to non-selective pan-inhibition of HDACs and associated proteins (Gupta et al., 2012).

This study has demonstrated two unexpected observations, both of which support expanding the clinical horizons of HDACi beyond cancer (Dinarello et al., 2011; Fairlie and Sweet, 2012). Firstly, two HDACi, Vorinostat and BML281 were shown to exert anti-inflammatory effects on human macrophages at low concentrations (<3 µM), but have pro-inflammatory effects at higher, still non-cytotoxic, concentrations (>3 µM). Both Vorinostat and BML281 enhanced the LPS-induced secretion of TNFα and IL1β, key arthritic disease-causing cytokines (Taylor and Williams, 2015). Secondly, this phenomenon was mirrored in vivo in a rat model of collagen-induced arthritis where Vorinostat and BML281 were both more efficacious at lower doses, either attenuating (at 1 mg kg⁻¹ day⁻¹) or exacerbating (at 5 mg kg⁻¹ day⁻¹) arthritis, without causing the common hematological side effects (neutropenia, leukopenia, thrombocytopenia, creatinine etc. data not shown) often associated with these drugs at still higher doses (Subramanian et al., 2010). Also, TNF and IL1β could not be detected using standard
ELISA in plasma at d28 in any group (data not shown), so it is possible that these were amplified in earlier phases of disease. These differential effects of HDACi on both human macrophages and in collagen-induced rat arthritis, suggest there is a small therapeutic window for the treatment of chronic inflammatory arthritis using clinically approved HDACi. This surprising observation in both *in vitro* and *in vivo* settings challenges the paradigm that increased efficacy correlates with higher doses of drugs, at least with respect to HDACi that have often been evaluated at impractically high, cytotoxic doses (30-200 mg kg$^{-1}$) in many studies of rodent models of inflammatory diseases (Leoni et al., 2005; Lin et al., 2007; Halili et al., 2009; Joosten et al., 2011; Roger et al., 2011; Shakespear et al., 2011; Alias et al., 2012; Falkenberg and Johnstone, 2014). The divergent inflammatory responses at different HDACi concentrations on inflammatory cytokine secretion in human GM-CSF- and M-CSF-derived macrophages may reflect distinct functions of individual human HDAC enzymes and their pan-suppression with increasing concentrations. These findings support the possibility of repurposing anti-cancer HDACi drugs for acute and chronic inflammatory diseases in humans (Dinarello et al., 2011; Fairlie and Sweet, 2012), if given at lower doses. Most importantly, since one of the HDACi in this study is used for the treatment of human cancers, and others are in clinical trials (West and Johnstone, 2014), these compounds could be revisited in a clinical setting as effective anti-inflammatory agents at lower doses, thus potentially with fewer adverse effects than previously reported (Vojinovic and Damjanov, 2011).

IL1$\beta$ and TNF$\alpha$ are key macrophage-derived cytokines involved in the pathogenesis of human arthritis (Taylor and Williams, 2015). Anti-IL1$\beta$ and anti-TNF$\alpha$ biologics (e.g. canakinumab, infliximab, adalimumab, etanercept) have been trialled both individually as
well as in combination to treat human arthritis with some success (Taylor and Williams, 2015). Our studies in human macrophages indicate that high Vorinostat and BML281 concentrations amplify LPS-induced production of TNFα and IL1β. IL1β release usually requires inflammasome activation, which results in cleavage of pro-IL1β by active caspase-1 (Schroder and Tschopp, 2010). Thus, our novel finding, that macrophages exposed to high Vorinostat and BML281 concentrations with LPS triggers IL1β secretion is surprising given that no specific known inflammasome trigger was delivered. It is not clear if HDACi may induce IL1β maturation independently of the inflammasome (Hildebrand et al., 2014; Netea et al., 2015). Given the important role of this cytokine in many inflammatory disease processes, more detailed future studies will be required to investigate this phenomenon and whether characterized inflammasomes (e.g. NLRP3) are affected by HDAC activity. Moreover, the finding that suppression of IL12p40 and IL6 (as previously reported (Bode et al., 2007; Grabiec et al., 2008; Halili et al., 2010)) but amplification of TNFα and IL1β, is clearly distinguishable over a Vorinostat and BML281 concentration range, suggests that modulation of transcriptional versus post-translational or secretory mechanisms by these HDACi should be carefully investigated in future studies.

Macrophages are precursors to bone-resorbing osteoclasts that play key pathological roles in arthritis (Vignery, 2005; Yagi et al., 2005). In response to specific stimuli (eg. RANKL, TNFα, IL1β, IL6), macrophages fuse to form giant multinucleated cells that control bone reabsorption, remodelling and homeostasis (Vignery, 2005; Wei et al., 2005; Yagi et al., 2005). TNFα induces RANKL gene expression in bone marrow stromal cells, which appears to be mediated in part by IL1 receptor activation (Wei et al., 2005; Kim et al., 2009). On the other hand, TNFα and IL1β also induce osteoclastogenesis that...
is independent of RANKL (Wei et al., 2005; Kim et al., 2009). Thus, exacerbation of
disease symptoms with Vorinostat and BML281 at 5 mg kg\(^{-1}\) may relate to both
increased macrophage infiltration and release of TNF\(\alpha\) and IL1\(\beta\). These cytokines may
subsequently mediate macrophage differentiation and fusion to giant multinucleated
osteoclasts that ultimately contribute to the heightened disease pathologies observed
(Schroeder and Westendorf, 2005; McGee-Lawrence and Westendorf, 2011). These
require further clarified in future \textit{in vitro} studies.

Given the high doses of HDACi (10-200 mg kg\(^{-1}\)) that have been used to suppress
collagen-induced arthritis in rodents (Chung et al., 2003; Lin et al., 2007; Nasu et al.,
2008; Saouaf et al., 2009; Joosten et al., 2011; Li et al., 2013; Hsieh et al., 2014), it is by
no means certain that therapeutic effects of such high doses are associated with HDAC
enzyme inhibition. Moreover, long-term treatment is not feasible using such doses due to
development of severe side effects, as demonstrated here. Givinostat, for instance,
inhibits joint inflammation and destruction in a rodent arthritis model, but phase I/II
clinical data indicate unacceptable adverse effects in humans (Furlan et al., 2011;
Joosten et al., 2011). Nonetheless, givinostat given at a low dose is apparently
efficacious to some extent in children treated for idiopathic arthritis (Vojinovic and
Damjanov, 2011).

There is a perception that cytotoxic and anti-cancer effects of HDAC inhibitors primarily
relate to inhibition of class I HDACs. However, the adverse effects of HDACi at higher
doses could relate to inhibition of all or multiple HDAC isoforms (Gupta et al., 2012).
Selective inhibitors targeting class II HDACs may deliver anti-inflammatory efficacy,
without the undesirable side effects of class I inhibition during long-term administration of
these compounds (Gupta et al., 2012), as required for treatment of chronic inflammatory
diseases. Selective HDAC class II inhibitors may therefore provide new opportunities for HDAC inhibitor-based anti-inflammatory therapies beyond cancer treatment, but few such selective compounds have been developed to date. In the meantime, it may be possible to repurpose Vorinostat and other clinically advanced anti-cancer HDAC inhibitors as anti-inflammatory drugs if much lower doses are used to minimise adverse effects of treatment.

By virtue of their capacity to inhibit multiple inflammatory cytokines, HDACi are prime candidates for treating chronic inflammatory diseases such as degenerative arthritis. The novel findings presented herein support new opportunities for repurposing anti-cancer HDACi drugs for new clinical uses in human inflammatory diseases. However, our novel evidence suggests that HDACi provide significant anti-inflammatory relief in a rat model of collagen-induced arthritis only at a lower, rather than higher dose. Anti-cancer pan-HDACi therapeutics such as Vorinostat and BML281 may therefore have potential to be repurposed as anti-inflammatory and anti-arthritic medications if carefully monitored by clinicians for dosage and therapeutic benefit, in the long-term treatment regimes needed to combat chronic inflammatory diseases.
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Authorship contributions

*Participated in research design*: Lohman, Iyer, Sweet and D. Fairlie.

*Conducted experiments*: Lohman, Iyer, T. Fairlie, Cotterell and Vesey.

*Contributed new reagents or analytic tools*: Gupta and Reid.

*Performed data analysis*: Lohman, Iyer, T. Fairlie and D. Fairlie.

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Footnotes

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Figure legends

Figure 1. Chemical structures and cell viability of HDAC inhibitors. (A) Vorinostat (suberoylanilide hydroxamic acid, SAHA) and (B) BML281 (tert-butyl (4-(3-((7-(hydroxyamino)-7-oxoheptyl) carbamoyl) isoxazol-5-yl) phenyl) carbamate). (C,D) Concentration-dependent LDH release by Vorinostat and BML281 in (C): GM-CSF differentiated HMDM and (D): M-CSF differentiated HMDM. Data are from 3 independent healthy human donors (n = 3 ± SEM).

Figure 2. HDACi have differential inflammatory responses at increasing doses in GM-CSF and M-CSF HMDMs. Vorinostat (A-D, I-L) and BML281 (E-H, M-P) have both anti- & pro- inflammatory effects on LPS-stimulated GM-CSF-derived (A-H) and M-CSF-derived (I-P) HMDM at 24h post-LPS stimulation, detected and quantified by cytokine ELISA (IL12p40, IL6, TNFα, IL1β). Data are from 8-10 independent healthy donors (n = 8-10 ± SEM; * p < 0.05 vs LPS).

Figure 3. Inflammasome activation by HDACi in cultured GM-CSF-derived HMDMs. IL1β release from HMDM treated for 4h with LPS ± Vorinostat or entinostat, followed by triggering with ATP (0.5h), as quantified by cytokine ELISA (n=3±SEM; * p < 0.05 vs LPS + ATP).

Figure 4. Low versus high dose HDACi in rat collagen-induced arthritis. Lower dose Vorinostat (A,B) & BML281 (C, D) are efficacious, whilst a higher dose exacerbates disease symptoms in collagen-induced arthritis in rats. A,C; Rear paw swelling B,D; Disease activity index; Data shown are from two independent experiments (n=12/group, except 5 mg kg⁻¹ dose, n=6, mean ± SEM; **** p < 0.05 vs CIA rats).
Figure 5. Low versus high dose HDACi in CIA histopathology. Lower dose Vorinostat & BML281 are efficacious, whilst a higher dose exacerbates histopathological symptoms of collagen-induced arthritis in rats. Representative Masson’s Trichrome staining for collagen-loss. A, sham B, CIA control. C, Vorinostat 1 mg kg\(^{-1}\) day\(^{-1}\). D, Vorinostat 5 mg kg\(^{-1}\) day\(^{-1}\). E, BML281 1 mg kg\(^{-1}\) day\(^{-1}\). F, BML281 5 mg kg\(^{-1}\) day\(^{-1}\). Quantitation of histopathology; G, Arthritic disease score based on pathology of H&E stained tissue (photomicrographs not shown) (n = 6 / group, mean ± SEM; * p<0.05 from sham). H, Digital quantitation of collagen loss, as measured by loss of blue-collagen-like staining from Masson’s Trichrome images (% loss from normal/sham, n = 6, mean ± SEM; * p < 0.05 from sham). I, Quantitation of articular cartilage thickness in micrometer (n = 6/group, mean ± SEM; * p < 0.05 from sham).

Figure 6. Low versus high dose HDACi in macrophage and osteoclastlike cells in CIA. (A-F) Representative photomicrographs for ED1\(^{+}\) cell infiltration in collagen-induced arthritis in rats treated with low & high dose Vorinostat & BML281. Left column represent regions of larger osteoclast-like ED1 cell staining patterns, and the right represents regions of smaller macrophage-like ED1 cell staining patterns (25-70 μm\(^{2}\)). A, sham B, CIA control. C, Vorinostat 1 mg kg\(^{-1}\) day\(^{-1}\). D, Vorinostat 5 mg kg\(^{-1}\) day\(^{-1}\). E, BML281 1 mg kg\(^{-1}\) day\(^{-1}\). F, BML281 5 mg kg\(^{-1}\) day\(^{-1}\). G-I, Cell count quantitation taken from ED1-stained tissue for G, total cell counts. H, large osteoclast-like cells only (cell area 71-400 μm\(^{2}\)) and I, small macrophage-like cells (25-70 μm\(^{2}\)). Data expressed as cells/mm\(^{2}\). ** p < 0.01 ANOVA. (n = 6, mean ± SEM; ** p < 0.01).
Figure 1
Figure 2
Figure 4
Figure 5
Figure 6