Differential Anti-inflammatory Activity of HDAC Inhibitors in Human Macrophages and Rat Arthritis

Rink-Jan Lohman, Abishek Iyer, Thomas J. Fairlie, Adam Cotterell, Praveer Gupta, Robert C. Reid, David A. Vesey, Matthew J. Sweet, and David P. Fairlie

Centre for Inflammation and Disease Research, The Institute for Molecular Bioscience, The University of Queensland, Brisbane, Australia (R.J.L., A.I., T.J.F., A.C., P.G., R.C.R., M.J.S., D.P.F.); Centre for Kidney Disease Research, Translational Research Institute, The University of Queensland, Department of Medicine at the Princes Alexandra Hospital, Australia (D.A.V.); Received September 14, 2015; accepted December 7, 2015

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 s.c.) 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 immune responses, cell cycle, proliferation, and apoptosis (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 containing HDACs regulate many biological processes, including immune responses, cell cycle, proliferation, and apoptosis (Gupta 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, because many key inflammatory cytokines are HDAC-dependent in macrophages (Halli 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). They release a plethora of 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, because many key inflammatory cytokines are HDAC-dependent in macrophages (Halli et al., 2009; Dinarello et al., 2011; Shakespear et al., 2011; Fairlie and Sweet, 2012; Sweet et al., 2012).

Funding for this work was provided by the National Health and Medical Research Council of Australia for a Senior Principal Research Fellowship to D.F. [1027369], a Senior Research Fellowship to M.J.S. [APP1003470], and grant funding [Grants APP1047921, APP1030169, APP1074016]; the Australian Research Council for funding of a Centre of Excellence in Advanced Molecular Imaging [Grant CE140100011]; and the University of Queensland for a UQ Postdoctoral Fellowship to A.I. and a UQ Early Career Grant to R.J.L. and A.I. R.J.L. and A.I. contributed equally to this work.

ABBREVIATIONS: ANOVA, analysis of variance; ATP, adenosine triphosphate; CIA, collagen-induced arthritis; DAB, 3,3-diaminobenzidine; DAI, Disease Activity Index; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte macrophage colony stimulating factor; H&E, hematoxylin and eosin; HDAC, histone deacetylase; HDM, human monocyte-derived macrophages; HRP, horseradish peroxidase; IL, interleukin; IMDM, Iscove’s Modified Dulbecco’s Medium; LPS, lipopolysaccharide; LDH, lactate dehydrogenase; M-CSF, macrophage colony stimulating factor; MTX, Masson’s trichrome; NLR, Nod-like receptor; RA, rheumatoid arthritis; RANKL, receptor activator of nuclear factor kappa-B ligand; TNF, tumor necrosis factor.
(HDAC6, 10), and class IV (HDAC11) (Gupta et al., 2012). Broad-spectrum (pan) HDAC inhibitors (HDACi) such as vorinostat, panobinostat, and romidepsin are Food and Drug Administration-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 anticancer 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 regimens 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 downregulates many inflammatory genes, thus compromising host defense (Roger et al., 2011), whereas they can also upregulate proinflammatory genes and promote cytokine secretion (Wang et al., 2011; Kroesen et al., 2014). Indeed, we previously showed that Trichostatin differentially regulates many inflammatory and proarthritis genes in human and mouse macrophages in response to toll-like receptor 4 stimulation (Halili 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 HDACi in a clinical setting, with potential for their use as effective antiarthritic drugs without the adverse side effects observed at the higher doses normally evaluated for anticancer efficacy. Because many HDACi are under clinical investigation for the treatment of cancers, this study supports a potential to repurpose anticancer HDAC inhibitors at much lower and safer doses for treating inflammatory diseases.

Materials and Methods

Animals. Female Wistar rats (250 ± 50 g, n = 54) were bred at the Animal Resource Centre of Australia (Western Australia) and transported by air and road to the University of Queensland. Animals were housed (n = 3/box on corn-cob bedding) at the Australian Institute for Bioengineering and Nanotechnology, Brisbane, Queensland, Australia. Animals were maintained in a 12-h light-dark cycle according to the standard of the 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 with the ARRIVE guidelines (Kilkenny et al., 2010).

Drugs and Chemicals. Cell culture media, fetal bovine serum, penicillin, streptomycin, and l-glutamine were supplied by Invitrogen (Victoria, Australia). Bovine type II collagen (nail cartilage), Freund’s incomplete adjuvant, heparin, LPS, and ATP were supplied by Sigma-Aldrich (Sydney, New South Wales, Australia). Vorinostat (suberoylanilide hydroxamic acid), Entinostat (Pyridin-3-ylmethyl N-[4-[(2-aminophenyl)carbamoyl]phenylimethyl]carbamate), and BML281 [tert-butyl (4-(3-((7-(hydroxyamino)-7-oxoheptyl) carbamoyl) isoxazo[2,1-b][1,3]thiazol-3-yl)phenyl)methyl]carbamate], were obtained from Tocris (Bristol, UK). Ficoll-Paque (GE Healthcare Life Sciences, Uppsala, Sweden) (Hohenhaus et al., 2013; Seow et al., 2013). Ficoll-Paque density centrifugation (GE Healthcare Bioscience, Cross Blood Service, Kelvin Grove, Queensland, Australia) using ficoll-faque 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 Biotec, Auburn, CA) 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) at 1.5 × 106 monocytes/ml-1. Cells were grown to 80% confluence and seeded in 12-well plates (1 × 105 cells/ml, well, in Iscove’s Modified Dulbecco’s Medium (IMDM) with 10% fetal bovine serum, 10 U ml−1 penicillin, 10 U ml−1 streptomycin, and 2 mM l-glutamine). Before 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 Cytotoxicity and Enzyme-Linked Immunosorbent Assay. Concentration-dependent cytotoxicity of vorinostat and BML281 was evaluated as per manufacturer’s instructions using a commercially available lactate dehydrogenase (LDH) release assay kit (Roche Cytotoxicity Detection Kit Plus). Inflammatory cytokines were quantified from supernatants using commercially available enzyme-linked immunosorbent assay enzyme-linked immunosorbent assay (ELISA) kits (IL12p40, IL6, TNF, IL1β, Becton Dickinson, San Jose, CA), as per manufacturer’s 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)-treated CIA group (n = 12), BML281 (5 mg kg−1/day)-treated CIA group (n = 6), and sham-treated group (n = 6). CIA control and HDACi-treated rats were immunized on day 0 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 day 7. Daily dosage of Vorinostat and BML281 (1 or 5 mg kg−1/day, s.c. in 20% dimethylsulfoxide/sterile saline) or vehicle (for CIA control and sham-treatment groups) began on day 7. Hind paw swelling measurements were made by digital calipers (paw width and thickness), body weight and Disease Activity Index (DAI) score were measured daily from day 10 to 28. Paw swelling area (mm2) 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 generalized sickness behaviors (Lohman et al., 2012). Final DAI scores were the sum of these four parameters (maximal score 12).

Histopathology. On day 28, rats were euthanized with CO₂ inhalation. Hind paws were skinned, amputated, and fixed in 4% paraformaldehyde (pH 7.4) at 4°C, decalcified for 72 hours (10% HCl; 0.18% w/v EDTA 0.09% w/v trtarate in H2O), and then embedded in paraffin wax for histologic 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 color camera, using DP Capture and DP Manager software packages (Olympus, Tokyo, Japan). For generalized bone/tissue health, sections were stained with hematoxylin and eosin (H&E). Photomicrographs (40× lens) of at least six 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 color deconvolution software (FIJI/ImageJ 1.42q software, U.S. National Institutes of Health, Bethesda, MD). Brightfield photomicrographs (20× lens) were made of each articular surface of each joint on six sections per animal stained with MTC. Images were deconvoluted into red and blue channels and then digitally analyzed for pixel color intensity per unit area using software. Data were represented as color density per unit area. Within-animal color/stain densities were averaged, grouped into sections and compared between treatments. Articular cartilage thickness was also measured from the MTC-stained sections. Briefly, three regions of at least three 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 and then expressed as change in thickness (micrometers).

Immunohistochemistry for ED1 and ED2. M1-like macrophages and giant multinucleated osteoclasts were labeled using ED1 monoclonal antibody (mouse anti-rat MCA341R Serotec, Kidlington, UK) using standard immunohistochemistry techniques (DAKO pH 6.0 antigen retrieval, 1:150 2 hours at room temperature). M2-like macrophages were labeled using ED2 monoclonal antibody (mouse anti-rat MCA342GA/CD163 Serotec) also using standard technique (trypsin (0.1%) antigen retrieval, pH 8.0, 1:200 2 hours at room temperature). For both antibodies, the secondary antibody was an MACH1 mouse probe followed by Horseradish peroxidase conjugated 3,3-diaminobenzidine (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/ paw) were imaged at high resolution incorporating regions of soft tissue, articular surface, and trabecular and cortical bone. ED1⁺ cells were counted from images using software (FIJI/ImageJ). Each image was deconvoluted into brown 3,3-Diaminobenzidine (DAB) and background blue (hematoxylin). The brown image was used to identify cell-like staining patterns using the particle analyzing 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 were 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 v5.0α, San Diego, CA). Two-way repeated measures analysis of variance (ANOVA) was used for data sets involving ≥3 groups. In the arthritis study, Vorinostat and BML281 treatment group data were compared with both CIA control and sham-treatment data, and CIA control data were compared with sham-treatment data, using two-way repeated measures ANOVA with Bonferroni planned comparison. For small sample individual time points, such as histological analysis, a 1-way non-parametric Kruskal-Wallis ANOVA as used. Student’s t test was used for data comparing two 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. 1, A and 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. 1, C and 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. 2, A–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. 2, A–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. 2, I–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 (Schröder 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 (4 hours), followed by triggering with ATP (0.5 hours) (Fig. 3). The result was an approximately three fold increase in IL1β release in cells treated with 1 or 3 μM Vorinostat after only 4 hours. 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 [valproic acid, 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 day 28 \(P < 0.05\) versus CIA control, but not versus sham \((P > 0.05)\), rmANOVA and significantly reduced development of arthritis-like or other DAI symptoms, with only mild DAI scores recorded at day 28 (Fig. 4, B and 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. 4, A and C) and DAI (Fig. 4, B and D) compared with CIA controls. Indeed, 5 mg kg\(^{-1}\) day\(^{-1}\) Vorinostat- and BML281-treated animals had to be euthanized on day 20 and day 22, respectively because of 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. Histologic 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

Fig. 3. Inflammasome activation by HDACi in cultured GM-CSF-derived HMDMs. IL1\(\beta\) release from HMDM treated for 4 hours with LPS \pm\ vorinostat or entinostat followed by triggering with ATP (0.5 hour) as quantified by cytokine ELISA \((n = 3 \pm \text{S.E.M.}; \ast P < 0.05\) versus LPS + ATP).

Fig. 4. Low- versus high-dose HDACi in rat collagen-induced arthritis. Lower dose Vorinostat (A and B) and BML281 (C and D) are efficacious, whereas a higher dose exacerbates disease symptoms in collagen-induced arthritis in rats. (A and C) Rear paw swelling; (B and D) disease activity index; Data shown are from two independent experiments \((n = 12\text{/group, except 5 mg kg}\(^{-1}\) dose, }n = 6, \text{mean }\pm \text{S.E.M.}; **** \(P < 0.05\) versus CIA rats).
Fig. 5. Low- versus high-dose HDACi in CIA histopathology. Lower dose Vorinostat and BML281 are efficacious, whereas 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 ± S.E.M.; *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 ± S.E.M.; *P < 0.05 from sham). (I) Quantitation of articular cartilage thickness in micrometer (n = 6/group, mean ± S.E.M.; *P < 0.05 from sham).
administration of low-dose HDACi significantly reduced all histopathological changes ($P < 0.05$, ANOVA; Fig. 5, C and E). Conversely, 5 mg kg$^{-1}$ day$^{-1}$ HDACi treatment did not alleviate any of the histopathological disease features described above (Fig. 5, D and F).

Masson’s trichrome-stained tissue showed significantly reduced collagen loss in 1 mg kg$^{-1}$ day$^{-1}$ HDACi-treated rats compared with 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. 5, H and I).

**Macrophenge and Osteoclast Numbers in HDACi-Treated Rats.** CIA tissue showed significant increases in ED1-positive (+) (Fig. 6, A, B, and G) but not ED2-positive (+) (data not shown) immune cell populations, compared with 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 with sham animals (Fig. 6, H and I). In agreement with improved disease symptoms, both HDACi at...
1 mg kg\(^{-1}\) day\(^{-1}\) generally significantly reduced populations of ED1\(^+\) immune cells compared with CIA diseased controls (\(P < 0.05\), ANOVA, Fig. 6, C, E, and 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 with sham animals and low-dose HDACi treatments (\(P < 0.05\), ANOVA, Fig. 6 D, F, G–I). Most strikingly, both macrophage and osteoclast-like cell (ED1\(^+\)) populations were almost doubled compared with 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 compared with CIA control, whereas the effect on osteoclast-like giant cells was more pronounced (Fig. 6, H and I). In contrast, the 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 evaluated the viability of targeting epigenetic and posttranslational 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 regimens (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 demonstrated two unexpected observations, both of which support expanding the clinical horizons of HDACi beyond cancer (Dinarello et al., 2011; Fairlie and Sweet, 2012). First, two HDACi, Vorinostat and BML281, were shown to exert anti-inflammatory effects on human macrophages at low concentrations (<3 \(\mu\)M) but have pro-inflammatory effects at higher, still non-cytotoxic concentrations (>3 \(\mu\)M). Both Vorinostat and BML281 enhanced the LPS-induced secretion of TNF\(\alpha\) and IL1\(\beta\), key arthritic disease-causing cytokines (Taylor and Williams, 2015). Second, 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 hematologic 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 IL1\(\beta\) could not be detected using standard ELISA in plasma at day 28 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 anticancer 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, because one of the HDACi in this study is used for the treatment of human cancers and others are in clinical trials (West and Johnstone, 2015), 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 trialed 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 TNF\(\alpha\) and IL1\(\beta\). IL1\(\beta\) release usually requires inflammasome activation, which results in cleavage of pro-IL1\(\beta\) by active caspase-1 (Schroder and Tschopp, 2010). Thus, our novel finding that macrophages exposed to high vorinostat and BML281 concentrations with LPS trigger IL1\(\beta\) secretion is surprising given that no specific known inflammasome trigger was delivered. It is not clear if HDACi may induce IL1\(\beta\) 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 TNF\(\alpha\) and IL1\(\beta\) is clearly distinguishable over a vorinostat and BML281 concentration range, suggests that modulation of transcriptional versus posttranslational or secretory mechanisms by these HDACi should be carefully investigated in future studies.

Macrophages are precursors to bone-resorbing osteoclasts that play key pathologic roles in arthritis (Vignery, 2005; Yagi et al., 2005). In response to specific stimuli (e.g., RANKL, TNF\(\alpha\), IL1\(\beta\), IL6), macrophages fuse to form giant multinucleated cells that control bone reabsorption, remodeling, and homeostasis (Vignery, 2005; Wei et al., 2005; Yagi et al., 2005). TNF\(\alpha\) 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 TNFα and IL1β. 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 clarification in future in vitro studies.


Lohman, Iyer, T. Fairlie, Cotterell, and D. Fairlie.

Conducted experiments: Lohman, Iyer, T. Fairlie, Cotterell, and D. Fairlie.

Wrote or contributed to the writing of the manuscript: Lohman, Iyer, T. Fairlie, Sweet, and D. Fairlie.

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.

References


Address correspondence to: Professor David Fairlie, Institute for Molecular Bioscience, The University of Queensland, 306 Carmondy Road, St Lucia, QLD 4072, Australia. E-mail: d.fairlie@imb.uq.edu.au