An HDAC6 Inhibitor Confers Protection and Selectively Inhibits B-Cell Infiltration in DSS-Induced Colitis in Mice

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ABSTRACT

Small molecule histone deacetylase (HDAC) inhibitors with anti-inflammatory activity may be candidates for targeting intestinal inflammatory pathways in inflammatory bowel disease (IBD). This study investigated whether treatment with a potent HDAC6 inhibitor, BML-281, could protect against colonic inflammation and prevent inflammatory cell infiltration into the colon to drive disease pathology in a mouse model of acute dextran sodium sulfate (DSS) colitis. Control and acute DSS-colitis mice were treated with BML-281 (1 mg/kg per day s.c. and 10 mg/kg per day s.c.) for 8 days. Changes in disease pathology, colonic structure, function, alterations in inflammatory milieu, together with colonic inflammatory cell flux, were assessed by weight loss and disease activity index in vivo and by flow cytometry, gene expression, and histology ex vivo. Anti-inflammatory responses of BML-281 on human polymorphonuclear leukocytes were assessed in vitro. Administration of BML-281 to DSS-treated mice attenuated colitis, weight loss, and disease pathology, including changes in colon structure and function, by eliciting broad-spectrum anti-inflammatory effects and preventing infiltration and activation of key immune cells in the lamina propria of the intestinal epithelium. Among different immune cells, BML-281 particularly suppressed the infiltration of CD19^+ B-cells into the inflamed colonic lamina propria. This study supports the targeting of HDAC6 as an anti-inflammatory strategy for treating colon inflammation progressing to IBD. Some HDAC inhibitors are used in the clinic to treat cancer, and the results here for BML-281 highlight the potential for HDAC6 inhibitors to be used in a clinical setting for preventing and treating colonic inflammation and IBD in humans.

Introduction

The intestinal tract is one of the first points of contact between infectious organisms and protective immune cells of the host (Cader and Kaser, 2013). When acute inflammatory responses designed to combat intestinal infection are not terminated, inflammation persists and can lead to chronic inflammatory bowel diseases (IBD) and increased risk of colorectal cancer (CRC) (Burstein and Fearon, 2008; Cader and Kaser, 2013; Francescone et al., 2015). IBD is characterized mainly as Crohn’s disease (CD) or ulcerative colitis (UC), which share such common pathologies such as intestinal discomfort, pain and inflammation, symptoms of wasting, diarrhea, fecal occult bleeding, and often periodic disease remission (Felice et al., 2015; Francescone et al., 2015). UC primarily affects the colon and rectum, and CD affects multiple regions of the GI tract, including large and small intestine, stomach, esophagus, and even the mouth, but both conditions display characteristic patterns of inflammation and ulcerative mucosa (Lohman et al., 2012). Although the pathogenesis of IBD remains poorly understood, an overactive immune response primed by a combination of genetic, dietary, and microbiota-associated factors are crucial drivers of chronic intestinal inflammation and development of cancer (Burstein and Fearon, 2008; Felice et al., 2015). Clinical approaches to treating IBD include antibiotics, anti-inflammatory drugs, immunosuppressants, and biologics, such as anti-tumor necrosis factor (TNFα), that result in the resolution of intestinal inflammation and promotion of healing (Burstein and Fearon, 2008; Melmed and Targan, 2010). However, some human patients do not respond to biologics, and single cytokine-targeted therapies may not be effective over the long term after producing a ceiling response (Felice et al., 2015; Melmed and Targan, 2010; Taylor and Williams, 2015). There is still a very clear unmet need for new therapeutic options for human IBD and CRC. New drugs that can attenuate immune cell activation and suppress a wider range of key inflammatory cytokines and chemokines may more effectively control initiation and progression of disease (Melmed and Targan, 2010).

Small-molecule inhibitors of histone deacetylase (HDAC) enzymes are candidates for targeting intestinal inflammatory...
pathways in IBD and colorectal cancer (Halili et al., 2009; Shakespear et al., 2011; Gupta et al., 2012; Felice et al., 2015). There are 11 zinc-containing HDAC enzymes that regulate many different processes, such as immune responses, cell cycle, proliferation, and apoptosis in multiple tissue types including the intestine (Ariffin et al., 2015; Choudhary et al., 2009; Gupta et al., 2012; Iyer et al., 2012). These zinc 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). The precise role of any individual HDAC isozyme in the gut is currently unknown, but a proinflammatory influence has been characterized for a few HDACs in some murine models of colitis (Felice et al., 2015). For example, transgenic or knockout mouse studies suggest that HDACs 1, 2, 6, and 9 may play important proinflammatory roles in the initiation and progression of IBD (de Zoeten et al., 2010; de Zoeten et al., 2011; Beier et al., 2012; Alenghat et al., 2013; Turgeon et al., 2014). Most HDAC inhibitors (HDACi) tested to date inhibit all or most of the 11 zinc-containing HDACs (Felice et al., 2015), and the development of drug-like selective inhibitors of individual HDAC enzymes is not yet a reality. In some animal models of experimental colitis, broad spectrum HDACi, such as valproic acid (200 mg/kg per day), vorinostat (50 mg/kg per day), and givinostat (10 mg/kg per day) are considered anti-inflammatory at high doses and may reduce colonic inflammation and tissue damage to some extent (Glauben et al., 2006, 2008). However, their cytotoxicity leads to adverse effects that relate, at least in part, to inhibition of all or most of the 11 HDAC isozymes, and this has discouraged pursuit of HDACi as a therapy for IBD and other chronic inflammatory diseases for which long-term treatment without side effects is needed (Gupta et al., 2012; Das Gupta et al., 2016; Lohman et al., 2016). HDAC inhibitors that do not inhibit all isozymes may be more desirable and are now being sought as anti-inflammatory agents (Gupta et al., 2012; Felice et al., 2015).

Furthermore, the specific immune cell types involved in pathogenesis of human IBD remain poorly understood, but recent studies highlight the importance of innate immune cells (neutrophil, macrophage, dendritic, innate lymphoid) in the initiation phase, and Th17 and Foxp3 \( ^ {+} \) Treg cells in progression of human IBD (Sun et al., 2015). Knockout mouse studies, including in an adoptive transfer model, suggest that HDAC6 may have an important proinflammatory role in the progression of IBD, via restraining Treg function (de Zoeten et al., 2011). Since the adoptive transfer model relies on a genetically immune-compromised host, we have used the dextran sodium sulfate (DSS)-induced colitis model (Chassaing et al., 2011) to profile the anti-inflammatory efficacy of HDAC6 in the initiation of IBD-like colitis. The DSS model is advocated as a relevant model for IBD-like colitis, being sensitive to common IBD therapeutics and displaying mixed innate and adaptive inflammatory responses in the bowel, with goblet cell collapse and Endoplasmic Reticulum stress more closely resembling human UC (Heazlewood et al., 2008). Here we have examined one of the most potent reported HDAC6 inhibitors, BML-281 (Kozikowski et al., 2008; Butler et al., 2010), for its anti-inflammatory efficacy in a mouse model of acute DSS-induced colitis. A combination of histopathology, immunohistochemistry, flow cytometry, and polymerase chain reaction (PCR)–monitored inflammatory gene expression were used to profile potentially beneficial effects in this well established acute model of colonic inflammation leading to IBD-like symptoms, including colonic shortening, immune cell infiltration, release of myeloperoxidase and inflammatory cytokines into colonic tissues, colon mucin depletion, diarrhea, and intestinal bleeding.

### Materials and Methods

#### Drugs and Chemicals

BML-281 is a commercially available drug (e.g., Enzo Life Sciences, Farmingdale, NY) HDAC6 inhibitor reported to potently inhibit HDAC6 with IC\(_ {50} \) 2.4 nM (Kozikowski et al., 2008; Butler et al., 2010). For this work it was synthesized in-house, with purity determined at ≥99% as assessed by high-performance liquid chromatography and structural identity confirmed by mass spectrometry and proton nuclear magnetic resonance spectroscopy. DSS (MW ∼40 kD) was obtained from TdB Consultancy (Upsala, Sweden). Lipopolysaccharide (LPS-EK ultrapure) was obtained from Invivogen (San Diego, CA). Lipoteichoic acid (LPA) from *Staphylococcus aureus* was obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). RPMI 1640 and fetal calf serum were obtained from Invitrogen Life Technologies/ThermoFisher Scientific, VIC, Australia.

#### Mice

Animal experimental protocols were approved by the animal ethics committee of the University of Queensland, in agreement with guidelines of the National Health and Medical Research Council of Australia. Female C57BL/6 mice 6–8 weeks old were obtained from Animal Resource Centre (ARC; Canning Vale, Western Australia). The animals were housed at controlled temperature with 12-hour light-dark cycles, fed standard mice chow pellets, had access to water from bottles, and were acclimatized before being studied. Upon the end of an experimental period, mice were terminated by cervical dislocation. DSS-Induced Colitis. C57BL/6 mice (8 weeks, n = 10) were treated for 5 days with 2.5% dextran sulfate sodium (DSS) (MW ∼40 KDa; TdB Consultancy) in sterile water as the only source of drinking water. Mice were then given standard drinking water (non-DSS) for another 2 days and sacrificed on day 8. In two groups, mice were injected with BML-281 at a dose of 1 mg and 10 mg/kg body weight every day during the study protocol. Weight change during the experimental time course was calculated as change in percentages of weight from day 1. For stool consistency score, 0 points were assigned for well-formed pellets, 1 point for well-formed but soft and either very dark or lighter color than normal feces, 2 points for pasty and semi-formed stools which did not adhere to the anus, 3 points for semifirm stool that contained mucus and adhered to the anus, 4 points for liquid stool, and 5 points for excessive diarrhea with soiled rear/rectal prolapse, large solid lump in abdomen. Hemorrhoidal bleeding was scored 0 for no blood, 1 point for mild patchy blood in fecal bolus, 2 points for red stools and moderate patchy blood spots, 3 points for diarrhea with blood (not clotted), 4 points for gross continuous rectal bleeding. Mice were euthanized when either stool consistency score reached 5 points or bleeding score reached 4 points, according to ethics approvals. At the end of the experiment, the pathologic stool score was calculated as a sum of stool consistency score and bleeding score (Lohman et al., 2012). Mice were sacrificed by cervical dislocation. The entire colon from the cecum to the anus was removed, and colon length was measured as one of the parameters for disease severity.

#### Histology

Tissues were fixed in 4% paraformaldehyde at 4°C, embedded in O.C.T compound (ProSciTech, QLD, Australia) and frozen at −80°C. Formalin-fixed, 2-µm colon tissue sections were stained with hematoxylin and eosin under standard procedure. The sections were then examined under a light microscope. Histologic structure was assessed for three different ulceration parameters, severity of inflammation, depth of injury, and crypt damage (Lohman et al., 2012).

#### Mucin Staining

Sections of fixed colons were stained with Alcian blue (pH 1.0) and Safranin O using standard protocols. Sections were then examined under a light microscope and evaluated for mucin in goblet cells: 0, non-mucin; 0.5, half loss of mucin; 1, full mucin content intact. These scores were then multiplied by the percentage of tissue involvement (−25%; −50%; −75%; or ∼100%) to arrive at the mucin scores described (Lohman et al., 2012).
Esterase Staining. Sections of fixed colons were stained using Naphthol AS-D Chloroacetate (Specific Esterase) Kit ( Sigma-Aldrich Australia), according to manufacturer’s instructions.

Gut Cell Isolation. Lamina propria immune cells were isolated from segments of colon on the basis of described protocols with some modifications (Weigmann et al., 2007). In brief, after removing fat, mesenteric tissue, serosa, and muscularis externa layer, dissected colon was freed of mucus and epithelial cells by five sequential washing steps (15 minutes each) with 5 mM EDTA in calcium- and magnesium-free Hank’s balanced salt solution (HBSS-CMF, pH 7.4; Sigma-Aldrich Australia). The remaining lamina propria layer was then washed with phosphate-buffered saline (PBS; 3 times) to remove EDTA. These were then digested in RPMI medium containing collagenase D (0.5 mg/ml; Roche) and dispase II (0.3 mg/ml; Roche) for 60 minutes on an orbital shaker at 37°C. After digestion, the medium containing the single-suspension cells was filtered through a 40-μm mesh. The filtrate was collected and centrifuged at 400g for 10 minutes. After two washes in HBSS-CMF, the pellet was resuspended in cold PBS supplemented with 3% fetal calf serum (Gibco/ThermoFisher Scientific, Sunnyvale, CA) for further flow cytometry analysis.

Flow Cytometry and Antibodies. Isolated cells were incubated with anti-mouse CD16/32 antibody for 30 minutes, before a 15-minute incubation with either panel of antibodies for myeloid-derived cell populations and lymphocyte populations. Anti-mouse antibodies used for myeloid-derived populations included Pacific Blue CD11b antibody, Alexa Fluor 488 CD3 antibody, and PE Ly-6G antibody. Anti-mouse antibodies used for lymphocyte populations included Alexa Fluor 488 CD3 antibody and Brilliant Violet 421 CD19 antibody. 7-Amino-actinomycin D (7-AAD) viability staining solution was added just before detection and Brilliant Violet 421 CD19 antibody. All antibodies were purchased from BioLegend (San Diego, CA). Data were collected using FACSCanto II (BD Bioscience, San Jose, CA). Data were analyzed and prepared to discriminate dead cells. 

Colonic Myeloperoxidase Activity. Myeloperoxidase (MPO) in colon tissue was analyzed using previously described methods (Lohman et al., 2012). Briefly, colons at the completion of the study were weighed and then homogenized with 50 mg/ml cold hexadecyltrimethylammonium bromide (HTAB) buffer (5% HTAB in PBS; thrice, at 4°C. To measure the MPO activity, 10 μl of the sample supernatant was collected after centrifugation at 12,500 rcf for 15 minutes then vortexed for 30 seconds and sonicated for 20 seconds on ice. Supernatant was collected after centrifugation at 12,500 rcf for 15 minutes at 4°C. To measure the MPO activity, 10 μl of the sample supernatant was mixed with 200 μl of 50 mM potassium phosphate buffer, pH 6.0, containing 0.167 mg/ml O-dianisidine HCl and 0.005% hydrogen peroxide in a clear 96-well plate. The plate was read immediately and at regular intervals (wavelength 460 nm) for 20 minutes using an imaging plate reader (PHERAstar FS; BMG LABTECH, Cary, NC). O-dianisidine HCl was purchased from Sigma-Aldrich (St. Louis, MO).

Real-Time Reverse Transcriptase–PCR Analysis. Mice colons were snap-frozen in liquid nitrogen and ground into powder with mortar and pestle. The tissue powder was transferred into an Eppendorf tube containing 1 ml of TRIzol solution (Bionole, London, UK). Chloroform (200 μl) was then added and the solution was centrifuged (10,000g, 15 minutes). The aqueous phase containing the RNA was collected and the total RNA was extracted using the Isolate RNA Mini Kit (Bionole). mRNA was isolated from extracted total RNA using Dynabead mRNA kit (Invitrogen/ThermoFisher Scientific) (Seow et al., 2013). RNA concentration was measured using the NanoDrop ND-100 Spectrophotometer v3.2.1 (NanoDrop Products/ThermoScientific, Wilmington, DE). RNA was converted to cDNA using SuperScript III Reverse Transcriptase and oligo(dT)12–18 primer (Invitrogen/ThermoFisher Scientific) in thermal cyclers (Bio-Rad, Hercules, CA) according to manufacturer’s instructions. Real-time PCR was performed using an ABI PRISM 7900HT instrument (Applied Biosystems/ThermoFisher Scientific) (Hohenhaus et al., 2013). Target genes were expressed relative to the housekeeping gene Beta-actin and fold change was calculated relative to control sample (Sham). Forward and reverse primer sequences are listed in Table 1.

Isolation of Human Polymorphonuclear Leukocytes. Sterile plastic ware and solutions were used to obtain endotoxin-free and unstimulated polymorphonuclear leukocytes (PMNs) from healthy donors. PMNs were isolated from buffy coats of anonymous healthy donors (Australian Red Cross Blood Service, Kelvin Grove, QLD, Australia) by density centrifugation using a two-layered Histopaque method (1077 and 1119; Sigma-Aldrich), following the manufacturer’s instructions. After lymphocyte and monocyte removal, the PMN-containing layer was collected and diluted to 50 ml with PBS. These cells were centrifuged at 1000g for 5 minutes at room temperature thrice, and contaminating erythrocytes were removed by osmotic lysis.

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using repeated ice-cold sterile water. Isolated PMNs were seeded at $1 \times 10^6$ cells/ml and were rested for 1 hour in RPMI supplemented with 1% fetal bovine serum, 100 IU/ml penicillin, 100 mg/ml streptomycin at 37°C in presence of 5% CO₂ before experiments.

Statistical Analysis. All data were presented as mean ± S.E.M. from at least triplicate measurements and analyzed using GraphPad Prism version 6.0 for Mac OS X (GraphPad Software, San Diego, CA). One-tailed unpaired Student’s t-test was conducted when comparing each marker between two groups of animals. One-way analysis of variance (ANOVA) with multiple comparison was applied to assess significant differences between sham, disease control, and drug-treated groups. Two-way ANOVA with Bonferroni post-test was used for multiple comparisons between multiple groups at different time points such as daily body weight changes and pathologic stool core. For all data, the significance level was set to $P$ value < 0.05.

Results

BML-281, a Potent HDAC6 Inhibitor Protects against Acute DSS-Colitis. Oral administration of 2.5% aqueous dextran sulfate sodium to mice over an 8-day protocol (Fig. 1A) induced a well-characterized inflammatory response manifesting as colitis, and this model has been used extensively to study colon inflammation (Kim et al., 2010b; Wang et al., 2015), which can lead to IBD and ultimately to colorectal cancer. Some disease symptoms were evident just 1–2 days after commencing administration of 2.5% DSS, notably changes in stool consistency including semiformed stools with mucus. After 5 days, animals displayed wasting, measured as loss in body weight (Fig. 1B). In the acute colitis model, the immune response contributes to characteristic pathologic signs that include intestinal inflammation and mucosal damage, followed by symptoms of wasting, diarrhea, and focal occult bleeding (Fig. 1C) (Felice et al., 2015). Further, at the day of sacrifice (day 8), all experimental groups receiving DSS also displayed reduced colon lengths (Fig. 1D).

BML-281, a previously reported (Kozikowski et al., 2008; Butler et al., 2010) potent inhibitor of human HDAC6 (IC₅₀ 2.4 nM), was administered daily for 8 days at 1 mg/kg s.c. and

Fig. 1. BML-281 is efficacious in acute DSS colitis. (A) Treatment schedule for BML281 during 2.5% DSS administration; (B–D) Mice receiving DSS plus BML-281 (1 mg/kg per day or 10 mg/kg per day s.c.) were monitored over days 1–8 for weight loss (B), pathologic and stool score (C) and shortening of colon length at day 8 (D); (E, F) Representative histologic images and score for mucin depletion in colons from mice receiving DSS plus BML-281 at day 8. For all experiments, n = 6. Data are presented as mean ± S.E.M.. Statistical comparisons between sham versus disease controls represented as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Comparisons between disease control versus drug-treated represented as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. 

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10 mg/kg s.c. to mice given 2.5% DSS on day 1. BML-281 was found at day 8 to protect mice from DSS-induced weight loss (Fig. 1B), and dose dependently attenuated pathologic symptoms such as diarrhea, mucosal damage, fecal occult bleeding, and colon shortening (Fig. 1, C and D). Moreover, the inhibitor also affected the intestinal mucus layer, which is produced by goblet cells and coats the villi and crypts of the intestinal tract to play vital protective roles in regulating gut immunity (Kim and Ho, 2010a). Whereas DSS treatment promoted depletion of goblet cells and mucin (Fig. 1, E and F), treatment with BML-281 at either 1 mg/kg per day or 10 mg/kg per day decreased susceptibility to colitis, although it failed to prevent DSS-induced goblet cell depletion from transverse or distal regions of the colon (Fig. 1, E and F).

Histologic evaluation at day 8 showed that DSS treatment distorted colonic crypts and resulted in a swollen submucosal layer that was most severe in the distal colon region (Fig. 2). These changes were accompanied by increased infiltration of immune cells over the 8-day DSS treatment (Fig. 2). Treatment with BML-281 at either 1 mg/kg per day or 10 mg/kg per day prevented some of these structural changes in different colon regions compared with DSS-alone mice (Fig. 2). Collectively, these data suggest that BML-281 preserves the structural integrity of the colon and attenuates acute clinical manifestations of acute DSS colitis.

**Acute DSS-Colitis Alters Myeloid and Lymphoid Cell Populations in Colonic Lamina Propria.** Abnormal immune cell infiltration and activation in early colonic inflammation are key drivers of the pathology associated with chronic IBD (Cader and Kaser, 2013). The model of acute DSS colitis is characterized by marked immune cell infiltration and activation as well as ulceration of the colonic mucosa. However, it is not clear what roles each immune cell type plays in DSS colitis, perhaps owing to the varying concentrations of immune cell populations, which are all important in mucosal immune function and activation in early colonic inflammation, attenuating the expression of most of these inflammatory markers measured at day 8 (Fig. 5, A–D). The inflammatory genes that were upregulated in DSS-treated mice included 11 cytokines (Fig. 5A), five CC-chemokines (Fig. 5B), six CXC-chemokines (Fig. 5C), and two colony-stimulating growth factors (Fig. 5D). The profile of BML-281 (1 mg/kg per day) was broadly anti-inflammatory, attenuating the expression of most of these inflammatory markers measured at day 8 (Fig. 5, A–D).

HDAC6 inhibition has been shown to suppress innate immune cytokine production in a mouse sepsis model (Li et al., 2015). The present study shows that BML-281 did not substantially affect DSS-induced colonic neutrophil infiltration (Fig. 6A), consistent with findings from flow cytometry studies (Fig. 3, A and D). However, it did selectively attenuate the activation of these cells as measured by MPO activity (Fig. 6B). To confirm these anti-inflammatory responses of HDAC6 inhibition in neutrophil or granulocyte-like cells, we profiled the dose-dependent response of BML-281 in activated human PMNs. Toll-like receptor (TLR) activation has an important role in colon homeostasis and colitis (Ungaro et al., 2009). Here we show that agonists of TLR2 and TLR4 induce secretion from human PMNs of key inflammatory cytokines, such as TNFα (Fig. 6C), interleukin-6 (Fig. 6D), and interleukin-8 (Fig. 6E). On the other hand, BML-281 (0.1–10 μM) dose-dependently suppressed the secretion of these cytokines in these cells (Fig. 6, C–E). Collectively, these studies support the idea that HDAC6 signaling modulates infiltration of B-cells in particular and plays a key role in neutrophil activation and expression of inflammatory cytokines and chemokines during the induction of colitis.

**BML-281 Attenuates Neutrophil Activation and Colonic Inflammation in Acute DSS Colitis.** HDAC6 signaling has been reported to promote inflammation and immune cell activation by constraining T-regulatory cell function (de Zoeten et al., 2011) and by mediating innate immune cytokine production and stress responses (Zhao et al., 2014; Li et al., 2015). Here, we have investigated whether BML-281 attenuates the proinflammatory milieu associated with acute DSS colitis. Together with immune cells, colonic epithelial cells may also contribute to the inflammatory microenvironment in colitis (Felice et al., 2015). Hence, we profiled inflammatory gene expression in colon tissue after mice were given DSS ± BML-281. Compared with sham mice, DSS mice at day 8 displayed increased expression of nearly all 24 key inflammatory chemokine and cytokine genes that were examined (Fig. 5, A–D). The inflammatory genes that were upregulated in DSS-treated mice included 11 cytokines (Fig. 5A), five CC-chemokines (Fig. 5B), six CXC-chemokines (Fig. 5C), and two colony-stimulating growth factors (Fig. 5D). The profile of BML-281 (1 mg/kg per day) was broadly anti-inflammatory, attenuating the expression of most of these inflammatory markers measured at day 8 (Fig. 5, A–D).

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**Discussion**

Although immune cells have important host protective functions, uncontrolled or aberrant immune cell infiltration and activation are key drivers of chronic inflammation, tissue destruction, and pathology in many inflammatory diseases, including IBD (Burstein and Fearon, 2008; Cader and Kaser, 2013; Francescone et al., 2015). Current anti-inflammatory
Fig. 2. BML-281 treatment prevents acute DSS colitis induced structural changes in colon epithelia. Representative histologic images comparing distorted colonic crypts, goblet cell integrity and swollen sub-muscularis layers in proximal, transverse and distal colons on day 8 from mice receiving DSS plus BML-281 (1 mg/kg per day or 10 mg/kg per day) for 8 days. Inset pictures show representative histologic images for immune cell infiltration in swollen submuscularis layer in the colons with BML-281 (1 mg/kg per day or 10 mg/kg per day) treatment at day 8. White arrows, immune cell infiltration into swollen submuscularis layer; black arrows, distorted crypts; blue arrows, complete loss of goblet cell and crypt structure.
therapeutic strategies for IBD include the blockade of single cytokines such as TNFα (Kim et al., 2010b; Melmed and Targan, 2010; Felice et al., 2015). However, such treatments are very expensive, out of reach for most patients, and ineffective in patients who either do not respond or rapidly reach a threshold response beyond which further treatment has no effect (Melmed and Targan, 2010; Felice et al., 2015; Taylor and Williams, 2015). New drugs that modulate a wider spectrum of inflammatory mediators could have potential as adjunct therapies in combination drug treatments.

HDAC inhibitors that target intestinal inflammatory pathways may represent a potential new therapeutic drug class for human colitis, especially for refractory IBD and for preventing progression to colorectal cancer (Felice et al., 2015). Anti-inflammatory activity, including suppression of key cytokines and chemokines that might initiate and perpetuate IBD, has been reported for some broad-spectrum HDAC inhibitors, such as vorinostat and givinostat (Glauben et al., 2006, 2008). There are very few selective inhibitors of individual HDAC enzymes known to date, with most HDACi acting on all or most HDACs.
Compounds that do not inhibit all isoforms are being sought as more selective drugs to deliver anti-inflammatory efficacy, at the same time avoiding undesirable adverse effects that accompany long-term administration of pan-HDACi (Dinarello et al., 2011; Gupta et al., 2012). Less toxic HDAC inhibitors with higher efficacy in IBD, such as BML-281 demonstrated here, may have a faster track to the clinic than other drug classes because there are already some HDACi as FDA-approved anticancer agents, and many others are in clinical development for cancer (Gupta et al., 2012).

In the present study, we have examined a reported but little studied inhibitor of the class IIb HDAC enzyme, HDAC6, for its capacity to curtail colonic inflammation and attenuate immune cell infiltration and activation in acute DSS-induced colitis as a model of initiation of IBD. Although the precise role of each HDAC enzyme in the gut is currently unknown, knockout mouse studies suggest that, by virtue of its capacity to constrain T-regulatory cell function, HDAC6 may have an important proinflammatory role in the initiation and progression of IBD (de Zoeten et al., 2011; Beier et al., 2012). The present study offers further insights into possible beneficial effects of HDAC6 inhibition in the context of colon inflammation that may lead to IBD and progress to CRC. We found that the potent HDAC6 inhibitor BML-281 offers protection against acute DSS colitis by eliciting a broad-spectrum anti-inflammatory effect, including prevention of infiltration and activation of certain immune cells into the lamina propria of the colonic epithelium. Administration of DSS in the drinking water for 5 days led to colonic inflammation that was characterized on day 8 by increased infiltration and activation of neutrophils, macrophages, T-cells, and B-cells. Interestingly, daily administration of BML-281 selectively suppressed the infiltration of CD19+ B-cells into colonic lamina propria but did not inhibit the influx of neutrophils, monocytes/macrophages, or T cells. BML-281 did however attenuate neutrophil activation in the colonic epithelium, as demonstrated by attenuation of MPO activity. BML-281 exhibited a broad anti-inflammatory profile in attenuating the expression of 24 key inflammatory cytokines and chemokines in colon tissue of mice given the inflammmagen DSS. The results support the inhibition of HDAC6 expression or function as a potential therapeutic strategy for inflammatory bowel conditions.

The pathogenesis of IBD and colorectal cancer remains poorly understood (Felice et al., 2015; Wang et al., 2015). In general, the immune system orchestrates a local controlled inflammatory response designed to identify and remove infection and damaged cells, and heal the wound (Nathan, 2002). However, if the acute response fails, inflammation can become chronic and ultimately induce disease pathology...
In the case of colonic inflammation, the current paradigm is that an overactive immune response, primed by a genetic predisposition and environmental factors such as diet and microbiota, is a fundamental driver of a prolonged intestinal inflammation that leads to IBD (Burstein and Fearon, 2008; Francescone et al., 2015). Apart from IBD, this persistent inflammatory microenvironment in the gut is now thought to initiate and perpetuate the formation of epithelial dysplasia, inducing tumorigenesis, malignancy, and metastasis (Burstein and Fearon, 2008; Secher et al., 2010). Thus, studies on colon inflammation can provide important mechanistic insights into how inflammatory bowel diseases and colorectal cancer develop and how to diagnose and treat these conditions (Secher et al., 2010). A better understanding of disease-initiating events, roles for different immune cell populations, and key changes during disease initiation and progression may lead to better diagnostic, preventive and treatment strategies, particularly against development of malignancy in long-term IBD (Burstein and Fearon, 2008; Cader and Kaser, 2013). Precise roles for different immune cell types in the early inflammatory stages of colitis are currently unknown (Cader and Kaser, 2013). Neutrophils, mast cells, macrophages, dendritic cells, T-cells, B-cells, and the newly discovered innate lymphoid cells such as mucosal-associated invariant T (MAIT) cells, all appear to have characteristic features in mucosal immune function (Noronha et al., 2009; Cader and Kaser, 2013; Noronha Serriari et al., 2014; Schulz et al., 2015; Wang et al., 2015). Pharmacological or genetic depletion of some of the key innate immune cell types, such as neutrophils, macrophages, and cytotoxic T-cells, in the early inflammatory phase have been shown to protect against...
acute DSS-induced colitis (Cader and Kaser, 2013; Serriari et al., 2014; Schulz et al., 2015; Wang et al., 2015). On the other hand, regulatory T-cells and B-cells may play protective roles since both have been implicated in the resolution of DSS colitis (Wang et al., 2015). In the context of human IBD, neutrophils and other polynuclear myeloid cells are known to be activated and to secrete a range of inflammatory cytokines and proteins after being recruited to local sites of inflammation (Kvedaraite et al., 2016). Interestingly, BML-281 treatment prevents activation of neutrophils without affecting their infiltration into the lamina propria in DSS colitis mice. Furthermore, BML-281 was shown here to selectively suppress the infiltration of CD19+ B-cells into colonic lamina propria. Although some of the pharmacological responses of BML-281 may relate to suppression of immune cell activation, without affecting their infiltration, it is interesting to note that B-cells associated with a protective phenotype are suppressed by BML-281 treatment in our study. In support of this observation, a very recent study demonstrated manipulation and reduction of CD19+ B-cell responses with nonselective HDAC inhibitors such as vorinostat and panobinostat in a mouse model of autoimmune lupus (Waibel et al., 2015). Further studies tracking the proliferation,
recruitment, and drainage of B-cells and T-cells into or from gut-associated lymphoid and other tissues with HDAC6-inhibitor treatment seem warranted. These studies could help elucidate whether B-cell recruitment or proliferation has a causal role in IBD and also validate mechanisms of action of HDAC6 and its inhibitors. HDACs seem to be important in both gut homeostasis and colonic inflammation, and therefore selective inhibition of proinflammatory HDAC enzymes may be worthy of further investigation in relation to colitis, IBD, and colorectal cancer.

In summary, our results have shown that a potent inhibitor of HDAC6 can suppress colonic inflammation and protect against DSS-induced acute colitis in mice. Although the precise role of HDAC6 in the gut is currently unknown, we attribute the pharmacological effects of BML-281 to anti-inflammatory activity that includes the capacity to prevent infiltration and/or activation of key immune cells into the lamina propria of the intestinal epithelium. As some HDAC inhibitors are already clinically validated for the treatment of some cancers, there may be a rapid pathway to development of HDAC6 inhibitors as human therapeutics for treating colonic inflammation, in particular refractory IBD in humans.

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Authorship Contributions

Participated in research design: Do, Fairlie, Iyer.
Conducted experiments: Do, Lohman, Iyer.
 Contributed new reagents or analytic tools: Reid.
 Performed data analysis: Do, Lohman, Fairlie, Iyer.
 Wrote or contributed to the writing of the manuscript: Do, Sweet, Fairlie, Iyer.

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Alerghat T, Osborne LC, Kapetanovic R, Iyer A, Reid RC, Fairlie DP, and Sweet MJ (2012) Histone deacetylases 6 and 9 and sirtuin-1 control Foxp3+ T cells in gut homeostasis and colonic inflammation, and therefore selective inhibition of proinflammatory HDAC enzymes may be worthy of further investigation in relation to colitis, IBD, and colorectal cancer.


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