Isoform-selective inhibitor of histone deacetylase 3 (HDAC3) limits pancreatic islet infiltration and protects female nonobese diabetic mice from diabetes

Preservation of insulin-secreting β-cells is an important goal for therapies aimed at restoring normoglycemia in patients with diabetes. One approach, the inhibition of histone deacetylases (HDACs), has been reported to suppress pancreatic islet inflammation and β-cell apoptosis in vitro. In this report, we demonstrate the efficacy of HDAC inhibitors (HDACi) in vivo. We show that daily administration of BRD3308, an isoform-selective HDAC3 inhibitor, for 2 weeks to female nonobese diabetic (NOD) mice, beginning at 3 weeks of age, followed by twice-weekly injections until age 25 weeks, protects the animals from diabetes. The preservation of β-cells was because of a significant decrease in islet infiltration of mononuclear cells. Moreover, the BRD3308 treatment increased basal insulin secretion from islets cultured in vitro. All metabolic tissues tested in vehicle- or BRD3308-treated groups showed virtually no sign of immune cell infiltration, except minimal infiltration in white adipose tissue in animals treated with the highest BRD3308 dose (10 mg/kg), providing additional evidence of protection from immune attack in the treated groups. Furthermore, pancreata from animals treated with 10 mg/kg BRD3308 exhibited significantly decreased numbers of apoptotic β-cells compared with those treated with vehicle or low-dose BRD3308. Finally, animals treated with 1 or 10 mg/kg BRD3308 had enhanced β-cell proliferation. These in vivo results point to the potential use of selective HDAC3 inhibitors as a therapeutic approach to suppress pancreatic islet infiltration and prevent β-cell death with the long-term goal of limiting the progression of type 1 diabetes.

Histone acetylation and deacetylation are essential posttranslational modifications in epigenetic regulation of gene expression by which the lysine residues within the N-terminal end of the histone core of the nucleosome are acetylated and deacetylated. Cleavage of acetyl groups on lysines by histone deacetylases (HDACs) usually results in chromatin condensation and repression of the target gene. Previous studies have shown that HDACs regulate inflammatory gene expression and that broad-spectrum or selective HDAC inhibitors (HDACi) possess anti-inflammatory activities (1, 2). The pursuit of HDACi as anti-inflammatory agents is particularly attractive because several nonselective inhibitors are now approved for cancer therapy in humans (3, 4).

A total of 18 different HDACs have been identified to date among which the class I members (HDAC1, 2, 3, and 8) share sequence similarities with a high identity (~80% between HDAC1 and HDAC2). Using the clinically approved HDACi CI-994 (tacedinaline) as a core chemical scaffold, we previously reported on a set of differentially subclass I–selective and highly potent HDACi (5). Subsequently, these compounds were employed to access each individual isoforms’ involvement in β-cell biology. We have shown that BRD3308, an isoform-selective HDAC3 inhibitor, suppressed β-cell apoptosis induced by inflammatory cytokines or glucolipotoxic stress, and increased insulin release in vitro. In addition, tissue-specific deletion of HDAC3 has been shown to cause improper hematopoietic stem cell maintenance (6), defects in T-cell maturation (7), and natural killer T (NKT)-cell development (8). During T-cell development, double-positive (CD4+CD8+) thymocytes interact with self-MHC molecules to ensure reactivity and specificity. This process is called “positive selection,” in which CD4+CD8+ T-cells bind MHC class I or II molecules with low affinity. Recently, Philips et al. (9) reported that HDAC3 is required for the down-regulation of retinoic acid–related orphan receptor (ROIR)γ for proper thymocyte positive selection, leading to healthy T-cell maturation. Furthermore, Patel et al. (10) showed that trichostatin A (TSA), a broad-spectrum inhibitors; NOD, nonobese diabetic; TSA, trichostatin A; SAHA, suberoylanilide hydroxamic acid.
HDAC inhibitor, effectively reduces the incidence of diabetes in nonobese diabetic (NOD) mice when administered from 18 to 24 weeks of age. In addition to its effect in autoimmune diabetes, selective inhibition of HDAC3 by BRD3308 has also been shown to improve plasma glucose levels and β-cell function in the Zucker diabetic fatty (ZDF) rat model of type 2 diabetes (T2D) (11). However, the precise mechanism(s) by which HDAC inhibitors promote improved glycemia is not fully understood. In this study, we tested the hypothesis that selective HDAC3 inhibition will suppress autoimmune infiltration, which is characterized by prominent lymphocyte invasion in pancreatic islets, and reduce β-cell death to prevent progression of type 1 diabetes in female NOD mice.

Results

An isoform-selective HDAC3 inhibitor, BRD3308, reverses new-onset autoimmune diabetes

Based on our previous data that the selective HDAC3 inhibitor BRD3308 suppressed pancreatic β-cell apoptosis in vitro (5), we hypothesized that inhibiting HDAC3 in vivo would limit pancreatic islet inflammation to reverse autoimmune diabetes and promote normoglycemia. To test this hypothesis, we designed an experiment wherein we followed 8-week-old female NOD mice (n = 80) until they developed diabetes (blood glucose (BG) >200 mg/dl) (Fig. 1A). Following confirmation of diabetes (C1, C2, and C3, Fig. 1A) mice were randomized into two groups receiving either vehicle or 10 mg/kg BRD3308 intraperitoneally (I.P.) daily up to 4 weeks, followed by twice per week I.P. injection for an additional 4-week period. This dose was selected based on our previous study (5) in which the I.P. injection of 10 mg/kg of BRD3308 was evaluated, with inclusion of plasma area under the curve, half-life, and C_max (which reached 30 μM). In addition to the measured pharmacokinetics in that study, we also performed simulated target engagement profiles for HDAC1, 2, and 3 and observed ~100% target engagement for up to 6 h (5). Moreover, 10 mg/kg I.P. dose was also used in another study performed on male ZDF rats (11).

To confirm the presence of BRD3308 in the bloodstream after I.P. injection, mice were administered a single dose of vehicle or BRD3308 and serum samples were obtained 1 h post injection for detection of BRD3308 (Supplemental Table 1). As expected, the HDAC3 inhibitor was below detection limits in the serum from vehicle-treated mice in contrast to clearly detectable and consistent levels of BRD3308 in treated animals. These results provided confidence that mice were exposed to BRD3308 during the experiment.

BRD3308 administration did not significantly affect body weight gain as compared with the vehicle-treated group (data not shown). Despite an increase in blood glucose (>200 mg/dl) in both groups, mice treated with BRD3308 showed significantly lower blood glucose excursion, especially within the first 4 weeks (Fig. 1B, black arrow heads T1–T4) of daily compound administration. This phenotype was lost after switching the regimen to twice a week (Fig. 1B, white arrow heads T5–T8) injection, suggesting a beneficial effect on glycemia during the daily injection period. Based on these results, we examined whether mice with different levels of glycemia responded differentially to BRD3308 administration. To this end, animals from both groups were divided into two subgroups according to diabetes onset (confirmation = C1) based on their 3-day blood glucose values (200–400 or 400–600 mg/dl). Interestingly, daily treatment of BRD3308 in mice whose blood glucose was in the range of 200–400 mg/dl (circled) showed a significant deceleration of aggressive disease progression compared with the control cohort (Fig. 1, C and D). H&E staining of pancreatic sections revealed relatively severe mononuclear immune cell infiltration in the vehicle-treated group (Fig. 1, E and F) whereas immunofluorescence staining revealed more insulin-positive cells in the BRD3308-treated group (Fig. 1E). Furthermore, despite not reaching significance (Mann-Whitney test; p = 0.5530), the number of animals that survived was higher in the BRD3308-treated group (Fig. 1G). Taken together, these results suggest that daily injection of the selective HDACi BRD3308 slows aggressive disease progression.

Selective inhibition of HDAC3 by BRD3308 prevents diabetes onset in female NOD mice

Because it is well-known that female NOD mice exhibit pancreatic islet inflammation as early as 4 weeks of age (12), we next hypothesized that inhibiting HDAC3 would protect β-cells from inflammation and apoptosis and therefore prevent the onset of diabetes. To test this possibility, we performed daily I.P. injections of either vehicle or BRD3308 beginning at 3 weeks of age, prior to onset of islet inflammation (12), for 2 weeks (i.e. up to 5 weeks of age) and then followed with a regimen of twice per week injection until 25 weeks of age (Fig. 2A) in female NOD mice (n = 72) divided into four groups (animals injected either with vehicle or 0.1, 1, or 10 mg/kg BRD3308).

Although administration of vehicle or BRD3308 immediately after weaning did not show differences in body weights between groups up to 12 weeks of age, the group injected with 10 mg/kg continued to gain weight until the follow-up period, compared with the other groups (Fig. 2B). As expected, the mice started to develop diabetes (blood glucose > 200 mg/dl) at ~12 weeks of age and the cumulative number of diabetic animals in the vehicle- and 0.1 mg/kg-treated groups increased compared with those in the 1- and 10 mg/kg BRD3308-administered groups, suggesting protective effects in animals receiving the higher doses of the HDAC3 inhibitor (Fig. 2C). The impact of the protective effects on β-cells was evident in the improved glycemia. Thus, although 8-week-old mice did not show major differences in glucose tolerance between groups, at 16 weeks the groups receiving 1 or 10 mg/kg of BRD3308 showed improved tolerance compared with mice treated with vehicle or the 0.1 mg/kg dose (Fig. 2, D and E). Moreover, whereas fed and fasting blood glucose values were within the normal range within all groups, fed blood glucose values were significantly lower in the 1 mg/kg (16 weeks) and 10 mg/kg (8 and 16 weeks) treated groups, suggesting no adverse effects of BRD3308 treatment and supporting the findings of improved glucose tolerance (Fig. 2F). The somewhat low glucose levels observed in the NOD mice, especially in the fasting state, are consistent with reports from other groups (32, 33).

4 R. Mirmira, personal communication.
values using an independent colorimetric assay. Previously, we showed that BRD3308 treatment improved insulin secretion, content, and gene expression in INS-1E cells in the presence of excess nutrients (5). Therefore, we sought to determine the effect of BRD3308 on both mouse and human islets. Interestingly, we observed that treatment of mouse islets with 10 \( \mu \text{M} \) BRD3308 increased insulin secretion only in basal conditions. (Fig. 2G). It is possible that a prolonged incubation with...
BRD3308 is necessary to unmask beneficial effects at high glucose concentrations by reducing inflammation and allowing β-cells to regenerate. Moreover, treatment of human islets obtained from a single donor demonstrated higher basal levels of insulin secretion, which was further increased during glucose stimulation (data not shown). Finally, the diabetic profile in the vehicle- or 0.1- or 10 mg/kg-treated groups showed 30, 30, 40, or 78% (p = 0.0477 versus vehicle) diabetes-free animals, respectively (Fig. 2G). Considering the cumulative incidence of diabetes onset in female NOD mice is ~80% (13), these results suggest that administration of BRD3308 early in life prevents diabetes onset in female NOD mice.

**HDAC3 treatment in vivo prevents pancreatic islet infiltration and protects β-cells from apoptosis**

BRD3308 has been shown to decrease caspase-3 activity in INS-1E cells induced by either palmitate, glucose, or their combination (5). This protective effect was also observed in rat and human islets in vitro. In addition, we reported that INS-1E cells treated for 24 h with several HDAC inhibitors, including BRD3308, increased acetylation by Western blot and a structural analog that lacks HDAC activity had no effect on H3 acetylation (5). To explore whether early administration (3 weeks of age) of HDAC3 inhibitor protects pancreatic islets from mononuclear cell infiltration, we harvested pancreata from vehicle- or BRD3308-treated mice at 5 weeks of age (end of daily treatment), 8 weeks of age, and greater than 8 weeks of age (Fig. 1A). H&E staining of pancreas sections of 5-week-old mice showed a similar level of infiltration (15–20%) in the islets in all groups (Fig. 3, A and B, upper panels). However, the infiltration appeared more severe (~40% of pancreatic islets) at 8 weeks of age in the vehicle- or 0.1- or 1 mg/kg-treated animals. On the other hand, 8-week-old mice treated with 10 mg/kg of BRD3308 demonstrated minimal infiltration (~10% of islets), suggesting significant protection (Fig. 3, A and B, middle panels). Because some animals, whether vehicle or BRD3308 treated, develop diabetes and die before reaching 25 weeks of age, we also analyzed pancreas sections from these mice (labeled as >8 weeks). The groups treated with or without BRD3308 at >8 weeks showed severe mononuclear immune cell infiltration in vehicle- or 0.1- or 1 mg/kg-administered groups. On the contrary, only ~15% of animals in the group treated with 10 mg/kg of BRD3308 exhibited infiltration, which was mostly in the periphery of the islets (insulitis score 1), clearly indicating protection from development of diabetes (Fig. 2G).

We next determined the impact of BRD3308 on β-cell apoptosis by using the TUNEL assay to detect β-cell DNA fragmentation. We observed no significant differences between vehicle- or 0.1- or 1 mg/kg-treated mice at 5 weeks of age (Fig. 4, A and B, upper panels). On the other hand, insulin/TUNEL-positive β-cells were significantly decreased in the 10 mg/kg-treated group compared with all other groups (absolute numbers of analyzed β-cells and TUNEL-positive β-cells are listed in supplemental Table 2). Although this phenotype was largely maintained at 8 weeks of age (Fig. 4, A and B, middle panels), TUNEL analysis of pancreas sections from mice >8 weeks of age demonstrated low levels of apoptotic β-cells when compared with other groups (Fig. 4, A and B, bottom panels). Taken together, these results point to a role for BRD3308 in protecting β-cells from apoptosis by preventing pancreatic islets from mononuclear immune cell infiltration.

**β-cell proliferation is increased in animals treated with BRD3308**

One of the proposed strategies to cure type 1 diabetes is to prevent immune cell infiltration in islets and simultaneously promote regeneration of functional β-cells to maintain normoglycemia. Because pancreatic islet infiltration and β-cell apoptosis in BRD3308-treated mice was significantly reduced, we next evaluated β-cell proliferation in all groups. β-cell mitosis, detected by BrdU incorporation, showed an increase in the BRD3308-treated group but reached statistical significance (absolute numbers of analyzed β-cells and BrdU-positive β-cells are listed in supplemental Table 3) only in the 1 mg/kg group at 5 weeks of age compared with controls (Fig. 5, A and B, upper panels). However, the difference was significant for both 1- and 10 mg/kg BRD3308-treated groups at the later time of 8 weeks (Fig. 5, A and B, middle panels). Finally, the significance of enhanced proliferation of β-cells in BRD3308-treated mice was not preserved in mice >8 weeks of age when compared with controls, suggesting that the daily regimen of BRD3308 for 2 weeks induces improved β-cell regeneration (Fig. 5, A and B, bottom panels). Taken together, these results suggest that daily treatment of BRD3308 leads to a reduced inflammation phenotype, allowing β-cells to proliferate in compensation.

**HDAC3 treatment in vivo prevents white adipose tissue infiltration in NOD mice**

In addition to β-cell destruction, type 1 diabetes is characterized by inflammation and loss of white adipose tissue (14). To explore whether BRD3308 has beneficial extrapancreatic effects, we analyzed mononuclear immune cell infiltration in multiple metabolic tissues in vehicle- or BRD3308-treated groups. An immune cell-free morphology was evident in all tissues tested (e.g. heart, liver, skeletal muscle, kidney, lung, and
spleen) in the vehicle- or BRD3308-treated groups. However, whereas the subcutaneous and visceral adipose tissues showed immune cell infiltration in the vehicle- and the 0.1- or 1 mg/kg BRD3308-administered animals (Fig. 6A–H), the group of mice receiving 10 mg/kg of BRD3308 did not show detectable infiltration, suggesting that the highest dose of BRD3308 has beneficial anti-inflammatory properties both in pancreatic islets and in white adipose tissue (Fig. 6, C and D).

**HDAC3 inhibition protects NOD mice from autoimmune diabetes**

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Figure 2. HDAC3 inhibition protects female NOD mice from developing autoimmune diabetes. A, experimental design showing daily administration of vehicle or BRD3308 (0.1, 1, and 10 mg/kg) to female NOD mice (n = 18 mice/group) starting post weaning for 2 weeks followed by twice per week injection regimen up to 25 weeks of age. Pancreas and other tissues (e.g., heart, liver, skeletal muscle, kidney, lung, and spleen) were harvested from four mice in each group at 5, 8, and >8 weeks of age depending on diabetes development. B, average of body weight gain between groups. C, weekly blood glucose measurements of each animal up to 25 weeks of age. Vertical arrowheads show starting time of daily or twice a week vehicle and BRD3308 injection. Horizontal arrows indicate threshold for blood glucose for diabetes development. D and E, glucose tolerance test (D) and area under the curve analysis (E) in D, performed for 16-week-old vehicle- and BRD3308-treated groups. F, fed and fasting blood glucose values for vehicle- or 0.1- and 1- and 10 mg/kg BRD3308-treated mice at 8 and 16 weeks of age. G, effects of BRD3308 (10 μM) treatment on glucose-stimulated insulin secretion in isolated mouse islets. H, Kaplan-Meier plot of mice treated with vehicle or 0.1, 1, and 10 mg/kg BRD3308 (n = 24) (Mann-Whitney test; p = 0.0477 versus vehicle). Data are expressed as mean ± S.E. *, versus vehicle; #, versus 0.1 mg; δ, versus 1 mg. *, δ, p < 0.05; **, ##, δδ, p < 0.01; and $$$, p < 0.001 (Student’s t test).

Figure 3. HDAC3 inhibition suppresses pancreatic islet infiltration. A, representative pancreas sections obtained from vehicle- or BRD3308-treated mice at 5 (upper panel), 8 (middle panel), and >8 weeks (bottom panel) of age. B, quantification of A for mononuclear cell infiltration. n = minimum of 4 mice/group.

Discussion

Table 1. Summary of HDAC inhibitors in NOD mice

| HDAC Inhibitor | Effects | Limitations |
|---------------|---------|-------------|
| TSA          | Promotes β-cell survival | Off-target effects |
| BRD3308      | Suppresses islet infiltration | Requires daily administration |

Historically, expression levels of HDACs have been used as biomarkers in tumor tissues (15). Knock down of individual HDACs, especially HDAC1, 2, 3, and 6, in different tumor types such as colon (16), breast (17), or lung (18) has been shown to induce apoptosis and cell cycle arrest. Previous studies have reported the impact of broad-spectrum HDACi, such as trichostatin A, suberoylanilide hydroxamic acid (SAHA), and ITF2357 (givinostat), to reduce cell death and increase insulin secretion from INS-1E β-cells exposed to pro-inflammatory cytokines (19). These broad-spectrum HDACi have also been shown to protect rodent islets from cytokine-induced cell death (20). Moreover, Patel et al. (10) highlighted the importance of TSA against autoimmune diabetes development in NOD mice.

Although the beneficial effects of HDAC inhibitors (which are potent inhibitors of class I, II, and IV HDACs) are recognized, their limitations include off-target effects because they also regulate more than 50 transcription factors (21). Major adverse effects include thrombocytopenia and anemia (22). Therefore, isomorph-selective HDACi are considered more suitable for therapeutics.

In a recent evaluation of 11 differentially selective HDACi we observed that selective inhibition of HDAC3 protects INS-1E β-cells from cytokine-induced apoptosis (23). Other studies have reported the relevance of HDAC3 in regulating the inflammatory gene expression program in rheumatoid arthritis fibroblast-like synoviocytes, and that it is required for inflammatory progress in macrophages (24, 25). These collective observations provided us with a strong rationale to use inhibitors directed to individual HDAC isoforms to modulate autoimmune diseases such as type 1 diabetes.

To specifically address the relevance of HDACi in vivo, we used BRD3308, a selective HDAC3i (5), to examine its ability to reverse new-onset disease in NOD mice which spontaneously develop diabetes. Although the inhibitor did not “reverse” the disease phenotype at the doses administered, we detected significantly lower blood glucose values within the first 4 weeks of their use.
daily injection in animals. Considering HDAC3 is linked to inflammation (10, 23, 26), it is possible that its daily administration limited progression of the disease to preserve the remaining β-cells to secrete insulin. Indeed, immunofluorescence staining of the pancreas revealed relatively larger numbers of insulin-positive β-cells in BRD3308-treated animals, which gains significance, especially in the end stages of the disease process, when more than 80% of islets are already destroyed.

The data in the “reversal study” prompted us to undertake experiments to explore whether administration of BRD3308 prior to transition from the prediabetic to the diabetic state prevents or delays onset of autoimmune diabetes. Indeed, daily injection of 10 mg/kg BRD3308 resulted in a significantly larger number of diabetes-free animals (>75%). A majority of the treated animals also showed virtually absent mononuclear immune cell infiltration in the pancreatic islets at 5 and 8 weeks, suggesting a strong suppression on immune infiltration. This gains significance because monocyte chemoattractant protein-1 (MCP-1), an important chemokine regulating migration and infiltration of monocytes/macrophages in response to inflammation (26), is regulated by HDAC3 (26). Together with dramatically reduced infiltration, β-cell proliferation was increased, suggesting a correlation because of the loss of recurrent immune attack against β-cells. The significance of reduced infiltration in adipose tissue at the higher dose of treatment with the HDAC inhibitor and how this contributes to the improved phenotype requires further investigation (14).

Although previous studies have focused their studies on different HDACi and different models including protection against effects of palmitate and glucolipotoxicity in β-cells (11, 26), our results provide evidence for a significant effect of the HDAC inhibitor in vivo in mice. The highest dose of BRD3308 administered early in life (10 mg/kg) led to a significant decrease in the numbers of apoptotic β-cells. This effect was maintained to >8 weeks of age. It has been indicated that mice treated with TSA could increase the number and function of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T-regulatory cells (27), and Patel <i>et al.</i> (10) showed that the transcription factor <i>Tbet/Tbx21</i>, which is involved in <i>Ifng</i> transcription and required for IL4 and IL17 transcription, was up-regulated in activated splenocytes of protected mice. Recently, Marino <i>et al.</i> (28) reported protection from type 1 diabetes in NOD mice by microbiota-metabolite–induced alterations on cytotoxic T-cells acting via B-cells, raising the possibility that the effects of HDAC inhibitors in vivo require a secondary factor. They show that decreased HDAC3 expression in splenic B-cells correlates with altered MHC-I and costimulatory molecule expression resulting in a tolerogenic state. A possible explanation for the protection and especially minimal inflammation in islets in our studies is the potential role of an increase in protective CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T-cells over cytotoxic T-cell subsets (27). These results might also explain the increase in proliferating β-cells and improved glycemia we observed at higher doses of BRD3308-treated animals.

**Figure 4.** Treatment with HDAC3 inhibitor reduces the number of apoptotic β-cells in NOD mice. <i>A</i>, immunofluorescence staining for apoptotic β-cells in pancreatic sections harvested from vehicle- or BRD3308-treated mice at 5 (upper panel), 8 (middle panel), and >8 weeks (bottom panel) of age showing insulin (red), TUNEL (green), and DAPI (blue). Scale bar = 20 μm. White arrows point to insulin/TUNEL double-positive β-cells. Representative experiment from n = 4–8. <i>B</i>, quantification of TUNEL-positive β-cells in <i>A</i>. n = minimum of 4 mice/group. (Absolute numbers for analyzed sections are given in supplemental Table 2.) Data are expressed as mean ± S.E. * versus vehicle; #, versus 0.1 mg; δ, versus 1 mg. *, #, δ, p < 0.05 (Student’s t test).
It is likely that the age when the HDACi is injected makes a significant difference, because insulitis is first observed at 4–5 weeks of age, when few β-cell autoantigens are targeted by effector T-cells (29). This may also explain, in part, the observation that TSA did not protect NOD mice when treated weekly between 5 and 10 weeks of age (10), in contrast to our study in which the selective HDAC3 inhibitor BRD3308 was injected at 3 weeks of age. The larger number of diabetes-free animals (>75%) in our study might be because of an action of the HDAC inhibitor prior to establishment of T-cell compartmentation in the NOD mice.

A limitation with moving forward clinically with broad-spectrum HDAC inhibitors is that they also exhibit some cytotoxic properties in β-cells. For instance, TSA and SAHA, which restored insulin release and decreased death in INS-1E cells in the presence of IL-1β and IFN-γ, also reduced the levels of these parameters on their own (19), suggesting that the specificity of these compounds plays a major role. A major advantage of our approach is that we use a recently identified chemical highly selective for the target we want to study. Thus BRD3308 is highly selective for HDAC3 over HDAC1 and HDAC2 and has no activity on any other HDAC isoform. Although HDAC inhibitors are tolerated by humans, as shown in cancer studies, an isoform-selective inhibitor, BRD3308, will be safer. This is critically important to support the potential development of HDAC inhibitors in nononcology indications.

Taken together, treatment with the selective inhibitor BRD3308, especially daily early in life, potentially promotes chromatin remodeling and resetting of the immune system from a destructive to a protective phenotype and delays the progression of autoimmune type 1 diabetes.

**Experimental procedures**

**Mice**

Female NOD/ShiLTJ (NOD) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in pathogen-free animal care facilities at Joslin Diabetes Center. Animals were housed on a 12 h light/12 h dark cycle with ad libitum access to water and food (PMI Nutrition International). Eighty and 72 female NOD mice were used in the "reversal from new onset of diabetes" or "prevention from diabetes" studies, respectively, described below.

**Treatment of mice and diabetes monitoring**

Mice were randomly divided into treatment groups receiving different doses (0.1, 1, and 10 mg/kg) of BRD3308 or vehicle (10% DMSO, 45% PEG-400, and 45% saline). In the reversal from new onset of diabetes study new-onset diabetic NOD...
mice were treated with daily intraperitoneal injections for 4 weeks followed by an additional 4 weeks of twice a week injection regimen. In the prevention from diabetes study, 3-week-old mice were administered either vehicle or different doses of BRD3308 compound daily by I.P. injection for 2 weeks followed by twice a week injection for up to 25 weeks of age. Tail vein blood was collected weekly to measure glucose using a hand-held glucometer (Bayer). The blood glucose levels measured by the glucometer were validated using a colorimetric assay (Crystal Chem, catalog no. 81692). A blood glucose reading of >200 mg/dl on three consecutive days was considered diabetic (30).

Detection of BRD3308 in serum samples

To ensure that BRD3308 could be detected in the circulation, vehicle (n = 9) or 10 mg/kg BRD3308 (n = 7) was administered I.P. as a single dose to mice and blood samples were collected from each animal 1 h post dose and placed in EDTA-treated vacutainer tubes. Samples were kept at room temperature for

| A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|
| Liver | Muscle | Subcutaneous adipose | Visceral adipose | Kidney | Spleen | Heart | Lung |
| Vehicle | 0.1 mg/kg | 1 mg/kg | 10 mg/kg | | | | |

Figure 6. HDAC3 inhibitor treatment prevents infiltration status in white adipose tissue. A–H, representative images of H&E staining for liver (A), muscle (B), subcutaneous adipose (C), visceral adipose (D), kidney (E), spleen (F), heart (G), and lung (H) in vehicle or 0.1- and 1- and 10 mg/kg BRD3308-treated animals.
25 min, and serum was separated by centrifugation at 9000 rpm for 10 min and stored at −80 °C until analysis. The compound level was measured using the research grade assay level 2 bioanalysis of mouse plasma samples at Agilux Laboratories (Worcester, MA).

Glucose tolerance test and insulin assay

Glucose tolerance tests (GTTs) were performed after an overnight (16-h) fast by intraperitoneal injection of glucose (2 mg/kg body weight) and was followed by blood glucose measurements before 0, 15, 30, 60, and 120 min after glucose injection. For insulin assay, healthy noninfilitrated islets were isolated from NOD-scid IL2Rγnull (The Jackson Laboratory, stock no. 005557) mice followed by overnight recovery. Thirty-five size-matched islets were preincubated in Krebs-Ringer Bicarbonate (KRb) buffer in the absence or presence of 10 μM BRD3308 or 60 μM GLP-1 for 1 h at low glucose (3.3 mM) and the media were switched to high glucose (16.7) to stimulate islets for another 1 h. Media were collected for insulin ELISA, and insulin content was measured by acid ethanol extraction followed by ELISA.

Immunohistochemistry

Pancreata were harvested 6 h after an I.P. injection of BrdU (100 mg/kg) and fixed and embedded in paraffin as described earlier (31). Five-micron sections were stained with H&E or using antibodies directed against insulin (Linco Research, Inc., guinea pig, 1:100) or BrdU (Sigma, 1:100) followed by appropriate secondary antibodies and counterstained with DAPI. At least, 2000 β-cell nuclei were counted per animal and data were expressed as percent BrdU + β-cells. Cell death was detected by enzymatic in situ labeling for DNA strand breaks by using TUNEL and by using an in situ cell death detection kit for pancreas sections (ApopTag, Chemicon, no. S7100). Images were analyzed with an Olympus microscope (Olympus BX-61, Olympus America, Melville, NY), equipped with a DP72 digital camera and software (DP Manager).

Insulitis scoring

Insulitis was evaluated on H&E-stained paraffin-embedded sections using a standardized scoring system (30). Briefly, the scoring was based on observing normal islet architecture, devoid of immune cells (score 0), peri-insulitis only (score 1), insulitis involving <50% of the islet (score 2), or insulitis involving >50% of the islet (score 3) (30).

Statistics

Statistical significance was assessed using Student’s t test. In case of nonparametric data, significance was confirmed by non-parametric t-tests (Mann-Whitney test). All data are expressed as mean ± S.E. A p value ≤ 0.05 was considered to be statistically significant.

Study approval

All animal experiments were conducted after approval by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center in accordance with National Institutes of Health (NIH) guidelines.

Author contributions—E. D. conceived the idea, designed the experiments, performed all experiments, analyzed the data, and wrote the manuscript. R. N. and R. M. contributed to animal maintenance and assisted in intraperitoneal injections. J. H. assisted in the immunohistochemical experiments. E. B. H., B. K. W., and F. F. W. contributed to designing the experiments, troubleshooting, and providing the BRD3308 compound. R. N. K. and B. K. W. conceived the idea, designed the experiments, supervised the project, and wrote the manuscript. R. N. K., B. K. W., and E. D. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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