The Oral Histone Deacetylase Inhibitor ITF2357 Reduces Cytokines and Protects Islet β Cells *In Vivo* and *In Vitro*

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In type 1 diabetes, inflammatory and immunocompetent cells enter the islet and produce proinflammatory cytokines such as interleukin-1β (IL-1β), IL-12, tumor necrosis factor-α (TNFα) and interferon-γ (IFNγ); each contribute to β-cell destruction, mediated in part by nitric oxide. Inhibitors of histone deacetylases (HDAC) are used commonly in humans but also possess antiinflammatory and cytokine-suppressing properties. Here we show that oral administration of the HDAC inhibitor ITF2357 to mice normalized streptozotocin (STZ)-induced hyperglycemia at the clinically relevant doses of 1.25–2.5 mg/kg. Serum nitrite levels returned to nondiabetic values, islet function improved and glucose clearance increased from 14% (STZ) to 50% (STZ + ITF2357). *In vitro*, at 25 and 250 nmol/L, ITF2357 increased islet cell viability, enhanced insulin secretion, inhibited MIP-1α and MIP-2 release, reduced nitric oxide production and decreased apoptosis rates from 14.3% (vehicle) to 2.6% (ITF2357). Inducible nitric oxide synthase (iNOS) levels decreased in association with reduced islet-derived nitrite levels. In peritoneal macrophages and splenocytes, ITF2357 inhibited the production of nitrite, as well as that of TNFα and IFNγ at an IC_{50} of 25–50 nmol/L. In the insulin-producing INS cells challenged with the combination of IL-1β plus IFNγ, apoptosis was reduced by 50% ($P < 0.01$). Thus at clinically relevant doses, the orally active HDAC inhibitor ITF2357 favors β-cell survival during inflammatory conditions.
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INTRODUCTION

To compact DNA, chromatin is wrapped tightly around nuclear histones, which are maintained in a state of deacetylation by histone deacetylases (HDAC). Histone acetylases, on the other hand, hyperacetylate histones, which then unravel DNA and permit transcription factors to bind and initiate gene expression. In humans, there are 18 distinct HDAC divided into three classes based on their dependence on zinc for enzymatic activity (1). In addition to their ability to deacetylate the highly conserved *N*-terminal lysines present on histones, other functions of each HDAC, such as acetylation of cytoplasmic proteins, also may contribute to their effects on cellular functions.

Inhibitors of HDAC are used widely in medicine. Valproic acid, the drug of choice for chronic therapy of generalized and focal epilepsy as well as obsessive disorders (2) is an HDAC inhibitor (3). Valproic acid also has been tested in patients with HIV-1 to purge the latently infected pool of memory T-cells (4). The HDAC inhibitor sodium butyrate is used to treat patients with sickle cell anemia and β-thalassemia (5,6). More potent synthetic inhibitors of HDAC also have been developed as a therapy in cancer (7). At micromolar concentrations, synthetic inhibitors of HDAC increase the expression of several proapoptotic genes that often are silenced in malignant cells, thus driving the cells toward an anticancerous phenotype. The hydroxamic acid–containing HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) (7–10) has been approved for the treatment of cutaneous T-cell leukemia (11) and appears to benefit patients with acute myeloid leukemia (12). However, SAHA also exhibits immunosuppressive and antiinflammatory properties (13).

ITF2357 is a novel hydroxamic acid–containing, orally active HDAC inhibitor that targets Class I and II HDAC. ITF2357 is effective as an antiinflammatory agent in animal models of inflammatory and autoimmune diseases (13–20). *In vitro*, the antiinflammatory properties of ITF2357 reduce production and/or activities of proinflammatory cytokines and are observed consistently

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in the low nanomolar range. As an antiinflammatory agent and cytokine-suppressing molecule, ITF2357 is 25–50-fold more effective than SAHA *in vivo* and *in vitro* (13,14,17,20–22). In endotoxin-stimulated human peripheral blood mononuclear cells (PBMC), ITF2357 inhibited the release of TNFα and IL-1β by more than 50% at 12.5 to 25 nmol/L, respectively (17). The induction of IFNγ by the combination of IL-18 plus IL-12 also was reduced by ITF2357. These data are consistent with the ability of nanomolar concentrations of ITF2357 to inhibit the enzymatic activity of Class I HDAC (23).

Safe and specific antiinflammatory agents are sought for the prevention of cytokine-induced destruction of pancreatic islet β cells. Oral ITF2357 is safe and effective in humans and is being evaluated presently in adults and children. In a Phase II study, ITF2357 reduced the constitutive proliferation of hematopoietic cells from patients with myeloproliferative neoplasms (24). In children with active systemic onset juvenile idiopathic arthritis, a daily oral dose of ITF2357 at 1.5 mg/kg for 12 weeks exhibited no organ toxicity and achieved significant reduction in parameters of systemic disease as well as the number of painful joints (25).

Since targeting IL-1β-mediated inflammation to protect islets has been demonstrated in human trials (26,27), the use of oral HDAC inhibitors to target islet inflammation should be considered. *In vitro* HDAC inhibitors reduced cytokine-induced nitric oxide formation (as measured by nitrite levels) in macrophages (13) and the decline in insulin secretion in isolated rat islets (28–30). In the present report, we describe the ameliorating properties of low doses of ITF2357 administered orally to mice in protecting islets exposed to inflammatory challenges as well as the reduction of cytokine production and increased β-cell survival. These studies suggest that oral ITF2357 would be a safe and possibly effective candidate for reducing inflammation in the islets in type 1 diabetes.

MATERIALS AND METHODS

Reagents

Recombinant mouse IL-1β, IL-12, TNFα and IFNγ were obtained from Peprotech (Rocky Hill, NJ, USA) and BD Pharmingen (Erembodegen, Belgium). Mouse IL-18 was from R&D Systems (Oxon, UK). Recombinant rat IFNγ was obtained from R&D Systems. ITF2357 was synthesized as described previously (17), reconstituted in water to 1 mg/mL, heated to 80°C and kept at room temperature. ITF2357 is stable at room temperature for 2 years. Streptozotocin (STZ) was purchased from Sigma, St. Louis, MO, USA.

Animals

Six- to 7-wk-old C57BL/6 female mice were purchased from Jackson Laboratories (Bar Harbor, ME). Three- to 6-day-old Wistar Furth rats were purchased from Charles River Laboratories (Sulzfeld, Germany). *In vivo* experiments were approved by the University of Colorado Institutional Animal Care and Use Committee.

In Vivo STZ Model

STZ was reconstituted in cold sodium-citrate buffer pH 4.3 immediately before use. Mice were injected intraperitoneally (i.p.) with STZ (225 mg/kg). ITF2357 (1.25, 2.5 and 5 mg/kg) or water (vehicle) was administered by gavage (0.1 mL), 12 h and 4 h prior to STZ, and every 12 h thereafter. Forty-eight h after STZ injection, β-cell function was assessed by glucose challenge and serum was collected for nitrite levels, as described below.

Glucose Tolerance Test (GTT) and Glucose Clearance in Mice

Mice were challenged after an overnight fast with glucose (2 mg/g), as described elsewhere (31). Blood glucose was measured prior to injection, and then at 10, 30, 60 and 90 min after challenge. Glucose clearance was calculated as percent of glucose that was removed from circulation 60 min after challenge.

Mouse Islets

Mice were anesthetized and pancreata were inflated with collagenase (1 mg/mL, type XI, Sigma), excised and incubated for 40 min at 37°C. Digested pancreata were vortexed gently, filtered through a 500-micron sieve and the pellet washed in HBSS containing 0.5% BSA (Sigma). The pellet was resuspended in RPMI 1640 supplemented with 10% FCS, 50 IU/mL penicillin and 50 μg/mL streptomycin (Cellgro, Herndon, VA, USA). Islets were collected on 100-micron cell strainer (BD Falcon, Franklin Lakes, NJ, USA) and handpicked under a microscope. Immediately after collection, islets were incubated at 37ºC for 24 h before *in vitro* assays. 100 islets per well were seeded in a 48-well plate. Islet viability was assessed using an MTT-based assay (Sigma).

Mouse Peritoneal Macrophages and Splenocytes

Thioglycollate-elicited mouse peritoneal macrophages and splenocytes were isolated as described elsewhere (32). Splenocytes were cultured in 96-well plates at 0.5×10^6 per well in triplicate.

Rat Islets

Primary neonatal rat islets were isolated and precultured for 1 wk as described previously (28) in complete medium (RPMI 1640 with 100 IU/mL penicillin and 100 μg/mL streptomycin) and supplemented with 10% newborn calf serum (Invitrogen/Gibco, Taastrup, Denmark). Five-hundred randomly picked islets per mL of complete medium containing 0.5% pooled human serum or 50 islets per 100 μL complete medium plus 0.5 % human serum were transferred to 12-well or 96-well plates (Nunc, Roskilde, Denmark), respectively. Islets were exposed to ITF2357 or vehicle for 1 h and, subsequently, cytokines were added.

Cytokine Assays

Murine IFNγ was determined using specific enzyme-linked immunosorbent assay (ELISA) (R&D Systems) given

above. Murine TNFα, macrophage inflammatory protein 1α (MIP-1α) and MIP-2 were detected by electrochemiluminescence (ECL) assay as described previously (33,34). The amount of chemiluminescence was determined using an Origen Analyzer (BioVeris, Gaithersburg, MD, USA).

Immunoblotting

Five-hundred randomly picked islets were cultured for 2–3 h and pre-exposed to ITF2357 or vehicle for 1 h. IL-1β (160 pg/mL) plus IFNγ (5 ng/mL) was added for 6 h and islets were then lysed and the protein content measured by the Bradford method. Lysates were subjected to gel electrophoresis as described (35). Anti-iNOS antibody (#610332) was purchased from BD Pharmingen (San Diego, CA, USA), anti–β-actin (ab6276) was from Abcam (Cambridge, UK) and antimouse was from Cell Signaling (Beverly, MA, USA). Immune-complexes were detected by chemiluminescence using LumiGLO (Cell Signaling) and light emission captured digitally by use of the Fuji LAS3000 (Fujifilm, Tokyo, Japan).

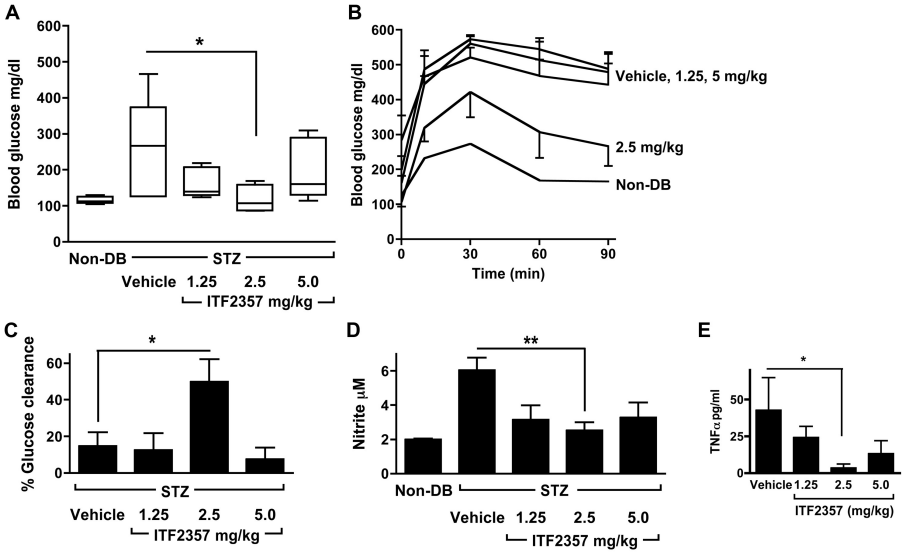

Figure 1. Effects of oral ITF2357 on STZ-induced β-cell toxicity, serum nitric oxide levels and spleen cell responses *in vivo*. C57BL/6 mice received either 100 μL vehicle (water) or ITF2357 *per os* at indicated doses, 12 and 24 h before a single STZ injection (225 mg/kg, i.p.). Mice that did not receive STZ represent the nondiabetic (non-DB) group. ITF2357 and vehicle treatment were repeated 12, 24 and 36 h after STZ (n = 5 per treatment group). (A–C) β-cell function was evaluated 48 h after STZ injection. (A) Fasting glucose levels. Percentiles ± SEM, $^*P < 0.05$; (B) Glucose tolerance test (GTT), mean ± SEM; (C) glucose clearance. Percentage of decrease in blood glucose 30 min after peak GTT-induced circulating glucose levels. Mean ± SEM, $^*P < 0.05$. (D) Mean ± SEM serum nitric oxide levels, as determined by nitrite levels. $^{**}P < 0.01$. (E) Mean ± SEM TNFα levels in 24 h supernatants from ConA (1 μg/mL) stimulated mouse spleen cells. Spleens were removed from mice 48 h after STZ. $^*P < 0.05$.

INS-1 Cells

INS-1 cells were a kind gift from C Wollheim, Geneva (28) and were maintained in RPMI 1640 supplemented with Glutamax, 10% fetal calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin and 50 μmol/L 2-mercaptoethanol (Sigma). Cells were cultured at 37°C, 5% CO_2 and subcultured weekly.

Nitrite Assay

Nitric oxide was determined by measuring nitrite levels in sera and in islet supernatants. Material was analyzed for accumulated nitrite using Griess reagent (Promega, Madison, WI, USA).

Mitochondrial Activity Assay

Cells were cultured as described for insulin assay. Following cytokine exposure, the proportion of metabolically active cells were determined by 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega, Madison, WI, USA).

TUNEL Assay

Three-hundred thousand INS-1 cells were cultured in 2-well chamber slides (Nunc) in 1.5 mL complete medium and exposed to IL-1β (250 pg/mL) and IFNγ (10 ng/mL) for 24 h. The terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay was performed according to manufacturer instructions (Chemicon Europe, Hampshire, UK). Subsequently, cells were stained with DAPI nuclear stain (1 μg/mL, Merck) for 30 min at room temperature, rinsed in PBS and the slides mounted using drops of Antifade Mounting Media (No. 002627; Dako, Carpintaria, CA, USA).

Morphological Analysis

Three-hundred thousand INS-1 cells were cultured for 2 d prior to cytokine treatment. On the day of the experiment, the medium was replaced and ITF2357 was added 30 min preceding the addition of IL-1β (250 pg/mL) and IFNγ (10 ng/mL). After 24 h at 37°C, the cells were rinsed in PBS, fixed in 1% paraformaldehyde overnight at 4°C and incubated with DAPI stain.

Statistics

The data are presented as mean ± SEM of independent experiments. Paired Student *t* test was used to analyze statistical significance.

RESULTS

ITF2357 Prevents the Onset of Hyperglycemia and Decreases Serum Nitrite Levels in a Mouse Model of STZ-Induced β-Cell Toxicity

C57BL/6 mice received an oral dose of 1.25, 2.5 or 5.0 mg/kg ITF2357 or

water (0.1 mL, by gavage), 12 and 24 h before a single injection of STZ (225 mg/kg, i.p.), and then again at 12, 24 and 36 h post-STZ. Forty-eight h after STZ injection, glucose levels were determined, glucose tolerance test (GTT) was performed and serum collected for nitrite levels. The spleens were removed for *ex vivo* splenocyte stimulation. As shown in Figure 1A, a reduction of blood glucose was observed with each of the three oral doses of ITF2357, with an optimal response at 2.5 mg/kg (from 348 ± 64 mg/dL to 120 ± 16 mg/dL, mean ± SE, $P = 0.039$). Doubling of the dose of ITF2357 to 5 mg/kg reduced glucose levels to 200 ± 37 mg/dL.

As shown in Figure 1B, glucose challenge improved at the dose of 2.5 mg/kg ($P < 0.05$), but was unaffected by either lower or higher doses of ITF2357. As depicted in Figure 1C, blood glucose clearance after a glucose challenge was maximal with an oral dose of 2.5 mg/kg ($P < 0.05$).

Serum nitrite levels were reduced in treated animals (Figure 1D), resulting in a similar profile of inhibitory activity as that observed in blood glucose levels (Figure 1A). The dose of 2.5 mg/kg inhibited serum nitrite levels by 58.3 % ($P < 0.005$). The doses of 1.25 and 5 mg/kg produced a nonsignificant reduction in nitrite levels. These data are consistent with the findings presented in Figure 1A–C, confirming that the optimal response to ITF2357 is obtained at the intermediate dose of 2.5 mg/kg.

To assess the effect of oral ITF2357 on cellular responses attained at the tissue levels of the inhibitor *in vivo*, splenocytes were isolated from each of the five mice that were not injected with STZ (Figure 1D, non-DB). Cells were cultured in the presence of ConA alone (Figure 1E). Twenty-four h later, TNFα levels were measured and revealed a pattern consistent with the above observations, by which the optimal response to ITF2357 is achieved at the dose of 2.5 mg/kg.

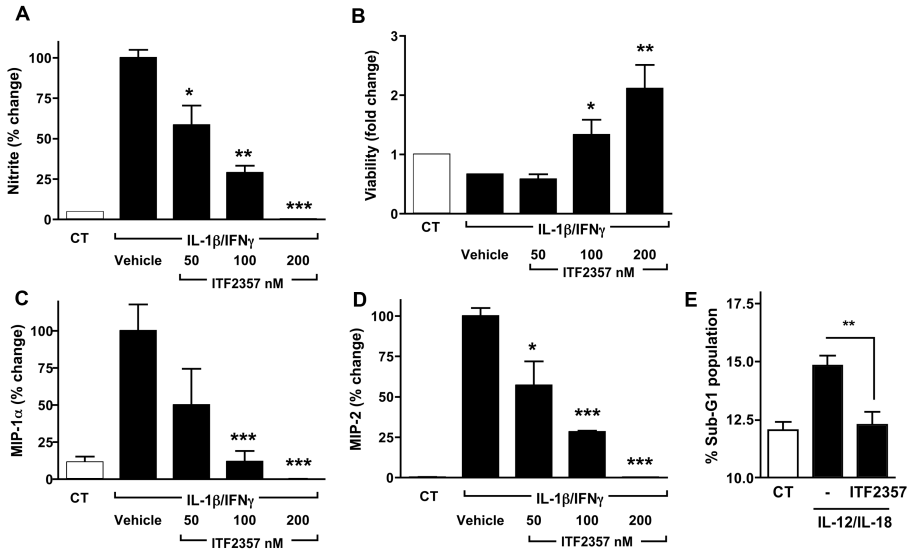

Figure 2. ITF2357 protects from cytokine-induced islet injury *in vitro*. Primary mouse islets were incubated for 1 h with increasing concentrations of ITF2357 or vehicle (water) and then IL-1β (10 ng/mL) plus IFNγ (25 ng/mL) were added. The data are derived from three independent experiments. Control (CT) islets were cultured without added cytokines. After 48 h, cell viability, supernatant nitric oxide and chemokine levels were measured and apoptosis rates were determined. Data from cytokine-stimulated islet cultures were set at 100% and the percent change was calculated for each concentration of ITF2357. (A) Mean ± SEM percent change in nitric oxide. (B) Cell viability, as determined by MTT reaction, depicted as fold-change from CT. (C and D) Mean ± SEM percent change in MIP-1α and MIP-2. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ compared with stimulated islets in the presence of vehicle. (E) Apoptosis, as determined by sub-G1 population evaluation of PI-stained islet single-cell suspension. ITF2357 (100 nmol/L) in the presence of IL-12 (2 ng) plus IL-18 (20 ng)/mL.

ITF2357 Protects Mouse Islets from Cytokine-Induced Toxicity, Nitric Oxide Generation and Chemokine Production

Islets were isolated from C57BL/6 mice and were incubated in the presence of the combination of IL-1β and IFNγ. Cell viability was assessed and secreted nitrite and chemokines were measured. As shown in Figure 2A, after 48 h in the presence of IL-1β and IFNγ, accumulated nitrite levels reached 11.2 ± 0.8 μmol/L (vehicle, set as 100%). Preincubation of islets with ITF2357 resulted in a concentration-dependent reduction in nitrite levels. Fifty and 100 nmol/L ITF2357 reduced nitrite levels by 41.6 ± 4% and 71.0 ± 12% ($P < 0.05$ and 0.01, respectively). At 200 nmol/L, ITF2357 reduced nitrite levels below detection. Consistent with the marked decrease in nitric oxide, cell viability was improved (Figure 2B) and was three-fold greater at 200 nmol/L ITF2357 than in cytokine-treated control islet cultures ($P < 0.01$).

As depicted in Figure 2C and D, cytokine-induced chemokine production by cultured islets was inhibited by ITF2357 in a concentration-dependent manner. A 50% reduction in MIP-1α (Figure 2C) was observed at 50 nmol/L, whereas a nearly 100% reduction was achieved at 100 nmol/L ITF2357 ($P < 0.001$). At 200 nmol/L ITF2357, MIP-1α levels were below constitutive MIP-1α production levels ($P < 0.001$). Another CXC chemokine, MIP-2, was reduced by 50% at 50 nmol/L ITF2357 (Figure 2D), and was inhibited maximally at 200 nmol/L ITF2357 ($P < 0.001$). Cytokine-induced apoptosis was determined in mouse islets with the combination of IL-12 plus IL-18 added 1 h after pretreatment with ITF2357. The average apoptotic rate

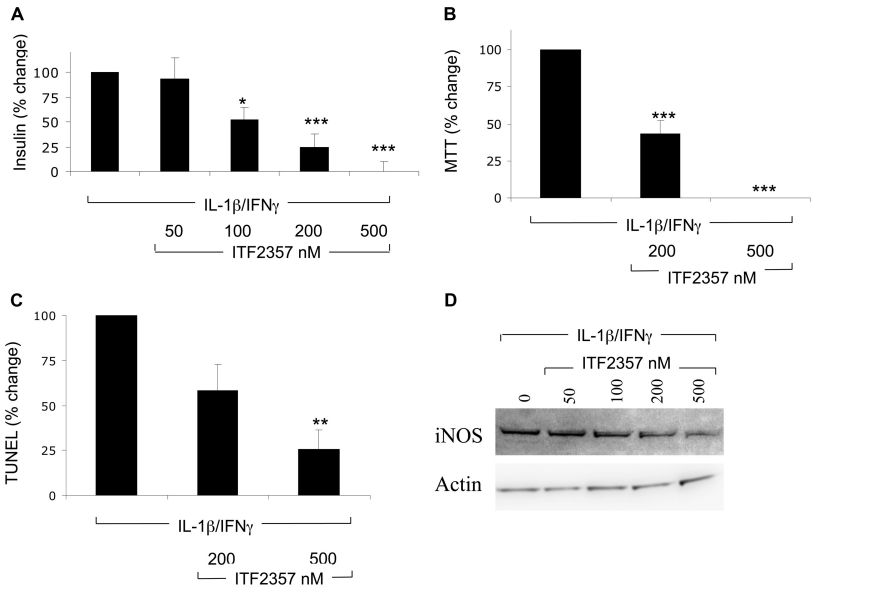

Figure 3. Effect of HDAC inhibition on insulin secretion, apoptosis, viability and iNOS expression in rat islets. Rat islets were precultured with increasing concentrations of ITF2357 for 1 h and then IL-1β (160 pg/mL) and IFNγ (5 ng/mL) were added and the islets were cultured for 48 h. The data are derived from 4–11 independent experiments. (A) Mean ± SEM percent change in supernatant insulin levels. $^{*}P < 0.05$, $^{***}P < 0.001$, *t*-test. (B) Mean ± SEM percent change in islet viability by MTT. $^{***}P < 0.001$, *t*-test. (C) Mean ± SEM percent change in islet apoptosis as assessed by the TUNEL assay (n = 4 $^{**}P < 0.01$). (D) iNOS expression. Western blot analysis of 6 h lysates. Actin control is shown. One blot representative of four.

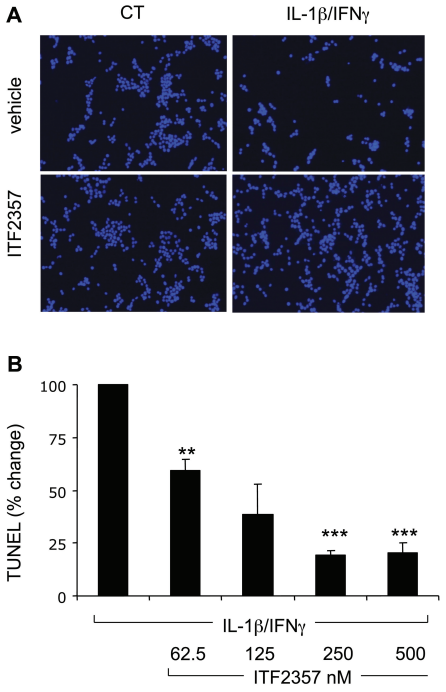

Figure 4. Effect of HDAC inhibition on cytokine-induced INS-1 cell death. INS-1 cells were precultured with vehicle or ITF2357 (250 nmol/L) and IL-1β (150 pg/mL) and IFNγ (2 ng/mL) were then added for 24 h. CT, unstimulated cells. (A) DAPI staining of cell nuclei. (B) Cytokine-induced apoptosis, assessed by the TUNEL assay. Mean ± SEM, percent change set at 100% for cytokine-induced apoptosis, n = 4. $^{**}P < 0.01$, $^{***}P < 0.001$.

of islets 24 h after cytokine stimulation returned to control levels in the presence of 100 nmol/L ITF2357 (Figure 2E).

ITF2357 Protects Rat Islets from Cytokine-Induced Toxicity and Reduces iNOS Expression

Islets were preincubated with increasing concentrations of ITF2357 and then exposed to the combination of IL-1β plus IFNγ. After 48 h, insulin levels in the supernatants were measured and islet viability was evaluated. The mean insulin release in control islets was 1027 ± 83 nmol/L. Following cytokine exposure, the mean level fell to 230 ± 25 nmol/L; for each experiment, the level of cytokine-induced suppression of insulin was set at 100% (Figure 3A). The percent change due to ITF2357 was determined for each experiment and the mean values were calculated. ITF2357 reversed the cytokine-driven reduction of accumulated insulin in a concentration-dependent manner. At 100 nmol/L, ITF2357 resulted in 50% less cytokine-mediated suppression of insulin release and, at 500 nmol/L, the HDAC inhibitor allowed for near-normal insulin secretion (781 ± 90 nmol/L). Similarly, as shown in Figure 3B, loss of islet viability in the presence of IL-1β plus IFNγ, as assessed by the MTT assay, was reduced at 200 nmol/L (45%, $P < 0.001$) and absent at 500 nmol/L of ITF2357. In addtion, 500 nmol/L of ITF2357 reduced cytokine-induced apoptosis by 75% ($P < 0.01$) (Figure 3C). Finally, the combination of IL-1β and IFNγ induced iNOS production in rat islet cells (Figure 3D). As depicted by Western blot analysis, there was a progressive reduction in iNOS protein levels in the presence of 100 to 500 nmol/L ITF2357.

ITF2357 Prevents Cytokine-Induced Cell Death in Insulin-Secreting Rat INS-1 Cells

INS-1 cells were preincubated with ITF2357 (250 nmol/L) and exposed to the combination of IL-1β and IFNγ. Cytokine stimulation for 24 h induced a profound reduction in cell density (Figure 4A, upper right panel), an effect that was abrogated by ITF2357 (lower right panel). Notably, cell population density in cytokine-stimulated, ITF2357-treated cells (lower right panel) was greater than in nonstimulated cells (upper left panel), suggesting that ITF2357 also prevents spontaneous apoptosis that is related to endogenous β-cell–derived injurious cytokines under routine culture conditions. In support of this concept, ITF2357 appeared to increase cell density in untreated control cells (upper left *versus* lower left panels). As shown in Figure 4B, cytokine-induced apoptosis in INS-1 cells as assessed by the TUNEL assay was reduced by ITF2357. At the lowest concentration tested (62.5 nmol/L), apoptosis

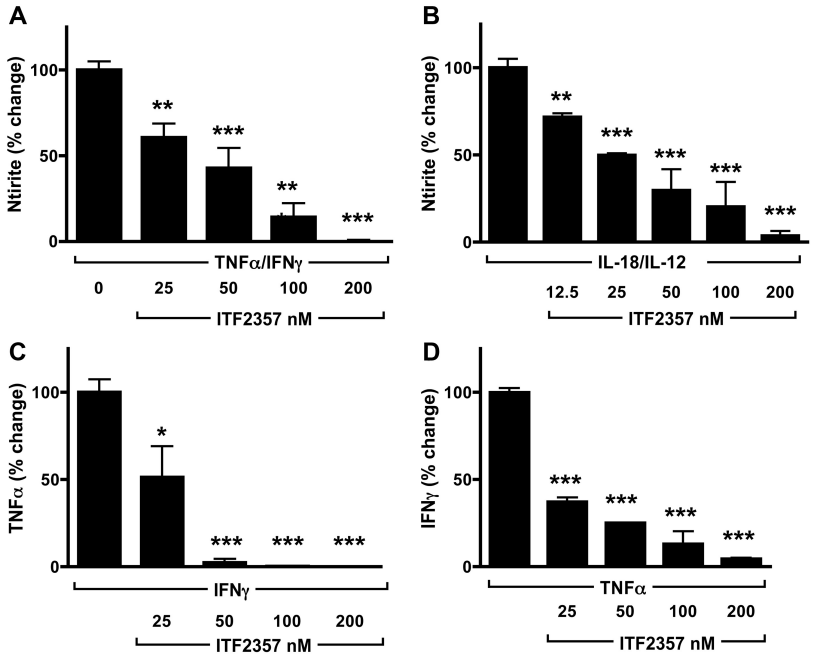

Figure 5. Effect of ITF2357 on cytokine and NO production in peritoneal macrophages. Thioglycollate-elicited mouse peritoneal macrophages were cultured in the presence of increasing concentrations of ITF2357 1 h before the addition of cytokines as indicated under the horizontal axes. After 48 h, levels of nitrite and released cytokines were determined in the supernatants. (A) Nitric oxide (as determined by nitrite) induced by TNFα and IFNγ, (B) Nitric oxide (nitrite) induced by IL-12 and IL-18, (C) TNFα induced by IFNγ and (C) IFNγ induced by TNFα. The data are expressed as the mean ± SEM percent change in the absence of ITF2357. The data represent macrophage cultures from five separate mice assayed in triplicate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ compared with stimulated cells without ITF2357.

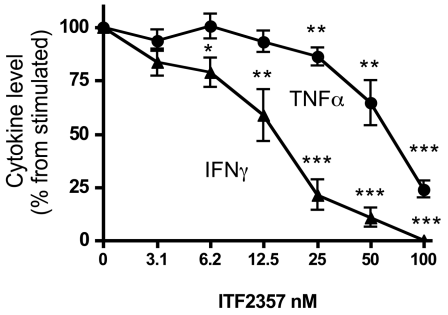

Figure 6. Effect of ITF2357 on cytokine production by mouse splenocytes. Mean ± SEM percent change of TNFα and IFNγ from mouse splenocytes. Splenocytes were cultured in triplicate in round bottom 96-well plates in the presence of ITF2357 present for 1 h before the addition of ConA (1 μg/mL). After 24 h, supernatants were collected and cytokine levels determined. Data are the combined results from five spleens. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

was reduced by 35% ($P < 0.01$). Maximal protection from apoptosis was obtained at 250 nmol/L ITF2357 ($P < 0.001$).

ITF2357 Reduces Proinflammatory Cytokine and Nitric Oxide Production in Mouse Peritoneal Macrophages

Peritoneal macrophages were exposed to ITF2357 1 h before addition of the combination of IL-18/IL-12 or TNFα/IFNγ. As depicted in Figure 5A and B, production of nitrite by peritoneal macrophages was reduced in a concentration-dependent manner. IFNγ induction of TNFα (Figure 5C) was similarly reduced by ITF2357, and, at a concentration as low as 25 nmol/L, was reduced by 50%. At 50 nmol/L ITF2357, TNFα production was nearly completely abolished. Similarly, TNFα induction of IFNγ (Figure 5D) exhibited an 86% reduction at 100 nmol/L ITF2357.

ITF2357 Reduces Proinflammatory Cytokine Production in Cultured Mouse Splenocytes

The effects of ITF2357 were also assessed in ConA-stimulated mouse splenocytes. Basal levels of TNFα and IFNγ were 11 and 30 pg/mL and increased to 556 ± 132 and 1230 ± 460 pg/mL following exposure to the stimulant. As shown in Figure 6, ConA-induced IFNγ and TNFα productions were reduced in mouse splenocytes in a concentration-dependent manner. IFNγ was reduced to 50% at 12.5 nmol/L ITF2357 and TNFα was reduced to 50% at 50–100 nmol/L ITF2357 ($P < 0.05$).

DISCUSSION

We began these studies with oral dosing of ITF2357 in mice and observed a significant reduction in the development of hyperglycemia induced by a single administration of STZ. In this acute model, circulating nitrite levels increase and there is death of the β cell. We observed that the optimal efficacy of ITF2357 in protecting islets from STZ challenge was at 1.25 and 2.5 mg/kg. Although the single dose STZ model is clearly not a model for cytokine-mediated loss of the β-cell function in type 1 diabetes, the rationale for using this model was based on our earlier studies demonstrating that SAHA inhibited the production of nitrite from mouse macrophages stimulated by IL-1β (13). In the present studies, we used the single dose STZ model to establish the dose of oral ITF2357 to inhibit nitrite production in a whole animal model. The dose of 1.25–2.5 mg/kg was optimal and consistent with blood levels; these levels were comparable to the nanomolar concentrations of ITF2357 *in vitro* that protect β cells. In a Phase I pharmacokinetic trial in healthy humans, the mean maximal plasma concentration was 104 nmol/L 2 h after a single oral dose of 50 mg; after 100mg, the level was 199 nmol/L (36). In those studies, the production of IL-1β, TNFα and IFNγ was reduced significantly in cultures of whole blood from

the subjects (36). Thus, the effective oral doses of ITF2357 in mice in the STZ model are consistent with the blood levels obtained in humans and also consistent with the effective concentrations of ITF2357 *in vitro* on mouse and rat islets as well as on primary spleen and peritoneal cells in the present studies. Once oral dosing was established, the study focused on *in vitro* effects of ITF2357 relevant to β-cell loss.

The antiinflammatory effects of oral ITF2357 have been studied in children. Seventeen children with active systemic onset juvenile idiopathic arthritis were treated for 12 weeks with a daily dose of 1.5 mg/kg of ITF2357. ITF2357 was safe and after 12 weeks of daily dosing, there was a 70% improvement in the juvenile idiopathic arthritis score in 63% of patients, providing the proof-of-concept that oral ITF2357 is a safe candidate for treating inflammation (25). The observations of oral ITF2357 in healthy humans and in children with arthritis are consistent with the present studies in that low concentrations of ITF2357 are antiinflammatory and protect the islet β cells.

In vitro, ITF2357 suppressed the β-cell toxic effects of the combination of IL-1β and IFNγ as well as reduced production of islet-derived TNFα, IFNγ and nitrite. Whereas gene expression and synthesis of TNFα and IFNγ are effectively suppressed by ITF2357 and SAHA in LPS-stimulated human monocytes, gene expression of IL-1β and biosynthesis of the IL-1β precursor are not affected (13,17). Rather the effect of ITF2357 or SAHA is primarily to reduce the secretion of IL-1β. In fact, after reaching an optimal suppression of IL-1β release, the inhibitory activity of SAHA is diminished (13), and we have observed a similar effect on IL-1β secretion by ITF2357. Thus, the efficacy of protecting islets following STZ using 1.25 and 2.5 mg/kg ITF2357, rather than 5 mg/kg, may reflect, in part, what the optimal dose is for inhibiting the secretion of IL-1β. There is a protective effect of specific IL-1 blockade in multiple low-dose STZ-induced diabetes (37). Although we did not use multiple low-dose STZ, we believe that inhibition of IL-1β may be part of the protective mechanism of ITF2357 in the single dose STZ model. The optimal *in vivo* dose of 2.5 mg/kg is consistent with the dose-response of ITF2357 in inhibiting IL-1β secretion in human PBMC (17) and consistent with the blood levels achieved with ITF2357 in humans. The inhibitory effects of ITF2357 on IL-1β secretion may involve tubulin hyperacetylation; ITF2357 and SAHA hyperacetylate HDAC-6, which participates in tubulin assembly. Hyperacetylation of tubulin by HDAC inhibitors may therefore interfere with the secretion of IL-1β by affecting the release of processed IL-1β via the secretory lysosome (21).

In isolated rat islets, ITF2357 prevented IL-1β/IFNγ-induced suppression of insulin production, cell death and iNOS expression. ITF2357 also afforded protection against cytokine-induced apoptosis in the rat clonal β-cell line, INS-1. Moreover, in the absence of IL-1β/IFNγ, control INS-1 cells exposed to ITF2357 (see Figure 4A, lower left) exhibited higher cell density than cells without ITF2357. This unexpected finding suggests that there is intrinsic cell death in INS-1 cells during standard incubation conditions which may be similar to the loss of β-cell function observed in pathological conditions (38) and which is reduced by HDAC inhibition. It remains unclear why ITF2357 appears to protect β cells from intrinsic cell death, but similar protection was observed *in vitro* using SAHA (28) and TSA (29). However, unlike TSA, ITF2357 is orally active and compared with SAHA provides longer hyperacetylation of histones in primary cells (17).

There was modest inhibition of cytokine-induced iNOS protein in rat islets at 200 nmol/L ITF2357, but complete inhibition of nitrite was observed in mouse islets at this concentration. Adult mouse islets are less sensitive to cytokine-mediated death than neonatal rat islets (28,39). Within but not between species, there is consistent dose-dependent reduction in nitric oxide (NO) by HDAC inhibition (see Figures 2A and 5) (28,30).

In diabetes, islets are assaulted initially by infiltrating immune cells with the subsequent development of insulitis. As shown in Figure 2C and D, the combination of IL-1β and IFNγ induced the chemokines MIP-1α and MIP-2, both promoting the recruitment of macrophages. In addition, these chemokines induce the generation of reactive oxygen species (40). Disruption of this inflammatory cascade, such as that which occurs in MIP-1α knockout mice (41), results in reduced insulitis and protection from diabetes. In a recent report, ITF2357 decreased surface expression of the chemokine receptors CXCR4 and CCR5 on CD4+ T-cells and monocytes from healthy humans (23). Similar to reducing the production of chemokines in mouse islets, clinically achievable nanomolar concentrations of ITF2357 suppress constitutive expression of the receptors to these chemokines, and hence would be an additional mechanism for HDAC inhibition of insulitis.

There is clearly more than one mechanism for the reduction in inflammation by HDAC inhibitors relevant to type 1 diabetes. In humans, the expression of the stimulatory CD154 molecule on T cells from patients with various autoimmune diseases has been reported (reviewed in [42]) and HDAC inhibitors reduce both CD154 and CD40 expression on effector T cells (43). Inhibition of dendritic cell (DC) function is another property of HDAC inhibitors. Pretreatment of DC with ITF2357 significantly decreased Toll-like receptor–induced secretion of proinflammatory cytokines, suppressed CD40 and CD80 expression and reduced the *in vitro* and *in vivo* allostimulatory responses induced by the DC (44). In addition, injection of DCs treated *ex vivo* with ITF2357 inhibited graft versus host disease (GVHD) during murine allogeneic bone marrow transplantation (44). Increased expression of indoleamine 2,3-dioxygenase, a known suppressor of DC function, appears to be a property of ITF2357 (44). Acetylation of the nonhis-

tone protein signal transducer and activator-3 (STAT-3) by ITF2357 implicates another mechanism for ITF2357 regulation of DC functions (45).

The suppression of IL-1β/IFNγ-driven iNOS expression and NO production by ITF2357 protected mouse and rat islet β cells as well as INS-1 cells from death. Since IFNγ, an inducer of NO, also potentiates the β-cell toxicity of IL-1β, inhibition of IFNγ production (Figure 5D) is highly relevant to islet survival. Both IL-12 and IL-18 are required for the production of IFNγ and the combination of IL-12 plus IL-18 induction of NO (Figure 5B) is likely mediated by IFNγ. At 100 nmol/L, ITF2357 inhibited the activity of IL-12/IL-18 in human PBMC (17). In the present studies, ITF2357 reduced ConA-induced IFNγ in mouse splenocytes as well as TNFα-induced IFNγ in peritoneal macrophages. The suppression of IFNγ by ITF2357 may be due to reduced IL-12. In fact, SAHA inhibits the production of IL-12 in human blood monocytes stimulated with LPS (13), an observation confirmed in murine cells (46). IFNγ activity is dependent on STAT1. In a study of GVHD, SAHA inhibited LPS-induced phosphorylation of STAT1, which is essential for IFNγ-induced NO (18). Inhibition of JAK kinases that phosphorylate STAT-1 prevented NO production in LPS-treated macrophages (18).

In islets, glucose stimulates IL-1β production (47,48); therefore, inhibition of IL-1β secretion by HDAC inhibitors may protect β cells from self-inflicted damage driven by endogenous cytokines during hyperglycemic states. As islet β-cell–derived cytokines contribute to the progression of insulitis leading to diabetes, treatment with low doses of orally active HDAC inhibitors, such as ITF2357, provides an attractive approach for protecting the β cell. Thus, HDAC inhibitors may serve as safe therapeutic target for cytokine-mediated β-cell loss.

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DISCLOSURE

P Mascagni is an employee of Italfarmaco, the manufacturer of ITF2357.

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