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Histone Deacetylase III as a Potential Therapeutic Target for the Treatment of Lethal Sepsis

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Abstract

Background—We have recently demonstrated that inhibition of histone deacetylase (HDAC) class I, II and IV with non-specific HDAC inhibitors improves survival in a mouse model of lethal cecal ligation and puncture (CLP). However, the consequence of HDAC class III inhibition is unknown in this model. The aims of present study were to explore the effect of EX-527, a selective SIRT1 inhibitor, on survival in the lethal model of CLP-sepsis, and to assess the impact of the treatment on inflammatory cytokine production, coagulopathy and bone marrow atrophy during severe sepsis.

Methods—Experiment I: C57BL/6J mice were subjected to CLP, and 1 h later intraperitoneally injected with either EX-527 dissolved in dimethyl sulfoxide (DMSO), or DMSO only. Survival was monitored for 10 days. Experiment II: One hour after CLP animals were randomly treated with: (i) DMSO vehicle, and (ii) EX-527. Peritoneal fluid and blood samples were collected for measurement of cytokines, and blood was also used to evaluate coagulation status using Thrombelastography. In addition, long bones (femurs and tibias) were harvested from animals to determine morphological changes of bone marrow by H&E staining. Experiment III: Normal primary splenocytes were cultured, and treated with lipopolysaccharide in the presence or absence of EX-527 to assess cytokine production.

Results—EX-527 significantly improved survival, and attenuated levels of cytokines in blood and peritoneal fluid compared to the vehicle control. It also decreased TNF- α and IL-6 production

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AUTHOR CONTRIBUTION

Y.L. and H.B.A. designed this study, for which H.B.A. secured funding. T.Z. performed experiments, collected and analyzed data. R.T.Z. performed pathological examination of bone marrow. B.L. provided experimental support. I.H. performed the paper's revision. Z.L. and Y.L. wrote the manuscript, which was critically revised by Y.L., and H.B.A. All authors read and approved the final manuscript.

by splenocytes in vitro. Selective inhibition of SIRT1 was associated with dramatic improvements in fibrin cross-linkage, platelet function and clot rigidity, but without a significant impact on the clot initiation parameters. Moreover, inhibition of SIRT1 decreased bone marrow atrophy significantly.

Conclusions—Selective inhibition of Class III histone deacetylase SIRT1 significantly improves survival, attenuates “cytokine storm” and sepsis-associated coagulopathy, and decreases bone marrow atrophy in a lethal mouse septic model.

Keywords

SIRT1; EX-527; sepsis; survival

BACKGROUND

Numerous genetic and epigenetic regulatory factors modulate how immune cells react to the pathogens, which in turn shapes the inflammatory and immune responses for individuals (1). Histone acetylation is an essential epigenetic mechanism that determines the amplitude of immune signaling, by controlling the chromatin structure, accessibility of transcription factors to the DNA, and subsequent gene transcription. This process of acetylation is regulated by the opposing actions of two families of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone acetylation relaxes the chromatin structure and promotes gene transcription, whereas histone deacetylation compacts the chromatin structure favoring gene silencing. In addition, numerous non-histone proteins, such as α -tubulin, heat shock protein (HSP) 90, and steroid receptors can be reversibly modified by acetylation (2).

In humans and mice, the 18 HDAC enzymes are grouped into four classes, based on sequence homology to yeast counterparts. Classical HDACs (class I, II and IV) are Zn^{2+} dependent, while the class III sirtuins act through a nicotinamide adenine dinucleotide (NAD^+)-dependent mechanism. Class I HDACs include HDAC 1, 2, 3 and 8, with HDAC 1, 2 and 8 primarily found in the nucleus, and HDAC3 in both the nucleus and the cytoplasm. According to domain organization, the class II HDACs are subdivided into class IIa (HDAC4, 5, 7 and 9) and IIb (HDAC6 and 10) (3). The class III HDACs or sirtuins consist of seven members (Sirtuins 1–7) homologous to the yeast HDAC silent information regulator 2. Sirtuin 1 (SIRT1) is a regulator of proteins and genes involved with tumor suppressor p53 and nuclear factor-kappa B (NF- κ B) and a member of the forkhead transcription factor (FOXO) family (4, 5). SIRT1 is required for estrogen-induced breast cancer growth, and its inactivation eliminates estrogen/estrogen receptor α -induced cell growth and tumor development, triggering apoptosis (4). SIRT1 has also been shown to regulate mitochondrial functions and plays a role in neuronal plasticity and memory (6, 7).

We have recently demonstrated that suberoylanilide hydroxamic acid (SAHA), a non-selective HDAC inhibitor (HDACI) that inhibits classes I, II and IV HDAC, improves survival in a mouse model of lethal cecal ligation and puncture (CLP) (8). However, SAHA does not inhibit the NAD^+ -dependent class III sirtuins (2, 9), and the consequence of class III HDAC (distinct from other HDAC) inhibition is unknown in this model. The aims of this

study were to explore the effect of EX-527, a selective inhibitor of SIRT1, on survival in a lethal model of CLP-sepsis, and to assess the impact of the treatment on inflammatory cytokine production, coagulopathy and bone marrow atrophy during severe sepsis.

METHODS

Cells and Reagents

Mice primary splenocytes were prepared as previously described (10). In brief, mice spleen was harvested and homogenized through a 70 μ m-nylon mesh, after which erythrocyte lysis was performed using red blood cell Lysis Buffer (Sigma Aldrich, St. Louis, MO). Mice primary splenocytes were cultured in Dubelcco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (Invitrogen, Grand Island, NY) at 37 °C and 5% CO₂. All drugs were added at the same starting point. Where indicated, cells were incubated with 1 μ g/mL LPS (Sigma Chemical Co, St. Louis, MO) or 10 μ M EX-527 (Selleck Chemicals, Houston, TX) (11, 12).

Sepsis Model: Cecal Ligation and Puncture (CLP)

Male C57BL/6J mice (18–26 gm) were purchased from The Jackson Laboratory and housed for 3 days before manipulations. The CLP murine model (13), modified by our laboratory, was used to induce fecal peritonitis. In brief, the peritoneal cavity was opened under inhaled isoflurane anesthesia. The cecum was eviscerated, ligated below the ileocecal valve using a 5-0 suture, and punctured through and through (2 holes) with a 20 gauge needle. The punctured cecum was squeezed to expel a small amount of fecal material and returned to the peritoneal cavity. The abdominal incision was closed in two layers with 4-0 silk suture. Animals were resuscitated by subcutaneous injection of 1 mL of saline. Sham-operated animals were handled in the same manner, except that the cecum was not ligated or punctured. This protocol was approved by the Animal Review Committee at the Massachusetts General Hospital.

Administration of EX-527 and Experimental Design

In the survival experiment, mice received intra-peritoneal EX-527 dissolved in DMSO (47 mg/kg) or vehicle DMSO 1 h after CLP (n=10/group). Survival was monitored for up to 10 days post-procedure.

In the non-survival experiment, animals were randomly assigned to the following three groups (n=10–15/group): (a) Sham-operated animals (SHAM); (b) vehicle treated animals after CLP (CLP+DMSO), and (c) EX-527 treated animals after CLP (CLP+ EX-527). Sham-operated animals were subjected to laparotomy and intestinal manipulation, but the cecum was neither ligated nor punctured. At the time of sacrifice [24 and 48 h after CLP (n=4–7/group/time point)], a peritoneal lavage was performed with 1 mL normal saline, which was collected for analysis, and blood samples were collected by cardiac puncture. Blood at 48 h was also used to evaluate coagulation status using Thrombelastography. In addition, long bones (femurs and tibias) from animals were harvested at 48 h and fixed in 10% buffered formalin for later histological analysis.

Cytokine Measurements

Concentrations of tumor necrosis factor- α (TNF- α) and interleukin (IL)-6 in the peritoneal fluid, plasma, or cell culture supernatant were measured using the Quantikine Enzyme-Linked Immunosorbent Assay (ELISA) Kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

Thromboelastography (TEG)

The whole blood samples were collected into heparin-rinsed tubes by cardiac puncture 48 h after CLP. Three hundred and forty μ l of each blood sample was added to a disposable heparinase-coated cuvette and analyzed by the TEG[®] 5000 Thrombelastograph[®] Hemostasis Analyzer System (Haemonetics Corporation, Braintree, MA). The following parameters were evaluated: SP (Split Point, time from sample placement to first divergence of the trace; denotes the initial fibrin formation rate), R (Reaction time, time from sample placement until TEG[®] tracing amplitude reaches 2 mm; denotes the initial fibrin formation rate), K (measures the time from clotting factor initiation (R) until clot formation reaches amplitude of 20 mm; represents the time for development of fixed degree of viscoelasticity during clot formation), Angle (angle formed by the slope of the initial TEG[®] tracing; reflects speed at which solid clot forms), and MA (Maximum Amplitude, the greatest amplitude of the TEG[®] tracing; reflects platelet function and absolute strength of the fibrin clot) (14).

Histological Analysis

Tissue samples of long bones (femur and tibia) were harvested to determine morphological changes of bone marrow by Hematoxylin and Eosin (H&E) staining at 48 h after CLP. Each sample was fixed by immersion in 10% buffered formalin. The sample was then embedded in paraffin, sliced into 5- μ m sections and stained with H&E. The atrophy of bone marrow was graded by a pathologist blinded to the group allocation of samples. The degree of bone marrow atrophy was graded on a scale of 0%–100%, with 0% reflecting “no atrophy,” and 100% reflecting “complete atrophy.” Bone marrow atrophy was evaluated according to the diameter proportion of veins to bone marrow cells (15).

Statistical Analysis

Results are shown as mean $\pm$ SEM. Kaplan-Meier method was used for survival, and differences were analyzed using log-rank test. Differences between 3 or more groups were assessed using one way analysis of variance (ANOVA) followed by Bonferroni post hoc testing for multiple comparisons. Student's t-test was used to compare the differences between two groups. All analyses were performed using GraphPad Prism. *P* values of 0.05 or less were considered significant.

RESULTS

EX-527 improves survival significantly in a CLP-induced lethal septic model

In this CLP-induced lethal septic model, all mice in the DMSO vehicle group died in less than 3 days. However, EX-527-treated animals displayed significantly higher long-term survival compared to the DMSO vehicle group (50% vs. 0% survival, *P*=0.0007; Figure 1).

EX-527 attenuates cytokine levels markedly in blood and peritoneal fluid in vivo

EX-527 attenuated levels of cytokines in blood (TNF- α : 298.3 ± 24.6 vs. 55.3 ± 8.0 pg/ml, $P=0.0049$; IL-6: 583.8 ± 83.8 vs. 216.1 ± 135.6 pg/ml, $P=0.0398$; Figure 2 and 3) and peritoneal fluid (IL-6: 704.8 ± 67.7 vs. 378.4 ± 128.4 pg/ml, $P=0.0388$; Figure 3) compared to the DMSO vehicle control.

EX-527 decreases cytokine levels in culture supernatant of mice primary splenocytes in vitro

EX-527 significantly suppressed TNF- α and IL-6 production (induced by TLR4 ligand LPS) in normal primary splenocytes, as measured in the supernatant at 6 h (TNF- α : 68.1 ± 6.4 vs. 40.5 ± 5.1 pg/ml, $P=0.0152$; IL-6: 73.1 ± 4.2 vs. 45.8 ± 4.8 pg/ml; $P=0.0091$; Figure 4).

EX-527 restores fibrin cross-linkage, clot formation speed, platelet function and clot rigidity in the lethal septic model

Animals subjected to CLP displayed prolonged fibrin formation compared to sham animals (SP: 3.6 ± 0.6 vs. 8.1 ± 1.1 min; R: 5.1 ± 0.8 vs. 11.5 ± 2.2 min; $P<0.05$) and fibrin cross-linkage time (K: 3.2 ± 0.7 vs. 18.6 ± 3.8 min, $P=0.0261$), and decreased clot formation speed (Angle: 40.5 ± 8.3 vs. 10.3 ± 2.5 deg, $P=0.0112$), platelet function and clot rigidity (MA: 59.3 ± 3.8 vs. 28.7 ± 6.0 mm, $P=0.0051$; Figure 5). Selective inhibition of SIRT1 was associated with dramatic improvements in fibrin cross-linkage compared to animals receiving DMSO vehicle (K: 18.6 ± 3.8 vs. 5.5 ± 0.7 min, $P=0.041$), clot formation speed (Angle: 10.3 ± 2.5 vs. 36.2 ± 3.9 deg, $P=0.001$), platelet function and clot rigidity (MA: 28.7 ± 6.0 vs. 60.6 ± 2.9 mm, $P=0.0092$; Figure 5), but without a significant impact on the clot initiation parameters (SP: 8.1 ± 1.1 vs. 8.6 ± 1.0 min; R: 11.5 ± 2.2 vs. 9.4 ± 1.3 min).

EX-527 decreases bone marrow atrophy significantly in the lethal septic model (H&E, magnification 40 $\times$)

Sham-operated animals displayed normal bone marrow histology and cell composition. In vehicle treatment group, the proportion of veins to bone marrow cells increased at 48 h after CLP, with venous dilation and bone marrow cell depletion (0 ± 0 vs. 64.0 ± 4.0 %). The bone marrow depletion and atrophy were markedly decreased by inhibition of SIRT1 (64.0 ± 4.0 vs. 35.0 ± 12.6 %, $P=0.0456$; Figure 6).

DISCUSSION

We have explored the therapeutic effect of a selective inhibitor of SIRT1, EX-527, in a CLP-induced lethal septic model and revealed several important discoveries: (1) selective inhibition of Class III HDAC SIRT1 significantly improves long-term survival; (2) significantly attenuates “cytokine storm” and sepsis-associated coagulopathy; and (3) decreases bone marrow atrophy markedly.

SIRT1 is primarily a nuclear deacetylase, which contains at least two nuclear localization signals and two nuclear export signals, shuttling between the nucleus and cytoplasm under certain conditions (16, 17). SIRT1 removes the acetyl group from the amino group of lysine residues in histones and non-histone proteins, and regulates target gene expression and

protein activities that control various cellular processes, including cell proliferation, differentiation, apoptosis, metabolism, DNA damage and stress response, genome stability, and cell survival in complex matters (18–20).

Unopposed early proinflammatory responses and “cytokine storm” occur in severe sepsis and septic shock, which impairs cellular function, induces cell apoptosis, and leads to end organ damage. Cytokines attract immune cells such as macrophage and T lymphocytes to the site of infection, while more cytokines are produced by these cells to perpetuate the response. The feedback loop is normally tightly regulated; however, in severe sepsis and septic shock there is often an overly exaggerated immune responses can get out of control in severe sepsis and septic shock (21). “Cytokine storm” has the potential to cause severe cellular damage as it potentiates the destructive ability of macrophages, plasma cells, complement system and T lymphocytes, and retard the healing effects of other cell types, such as M2 macrophages, regulatory T lymphocytes, and Th2 lymphocytes. We discovered that EX-527 remarkably attenuated local and systemic proinflammatory cytokines in the peritoneal fluid and the circulation following CLP in vivo. It also suppressed LPS-stimulated cytokine production from primary splenocytes in vitro. These findings provide evidence supporting the anti-inflammatory properties of EX-527 in lethal sepsis, and explaining in part the survival advantages rendered by the inhibition of SIRT1.

In addition, inhibition of SIRT1 may protect cells from cytokine and inflammatory mediator-induced apoptosis in severe sepsis. SIRT1 is shown to increase the sensitization of cells to TNF- α -induced apoptosis, by deacetylating the RelA/p65 subunit of NF- κ B at lysine 310, inhibiting its transcriptional activity, and decreasing pro-survival gene products that are responsible for overcoming TNF-induced apoptosis (19). TNF- α -induced apoptosis occurs through TNF- α receptor-mediated activation of the Fas-associated death domain (FADD) protein, resulting in the activation of caspase-8 (22). Treatment of neuroblastoma cell lines with resveratrol, an agonist of sirtuin activity, sensitizes cells to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis (23). It is likely that inhibition of SIRT1 may be protective towards cytokine and inflammatory mediator-induced cell apoptosis, reducing organ damage, and preserving function of immune cells to fight against foreign pathogens and clear apoptotic cell debris.

In our study, we detected a progressive hypocoagulable state in the lethal septic model, which was attenuated by inhibition of SIRT1. Complex interactions between coagulation and inflammatory responses are involved in the pathogenesis of sepsis. Overwhelming host responses to microorganisms and their derivatives, and to the over-expressed inflammatory mediators and complement activation products, are now believed to be responsible for massive thrombin formation and fibrin deposition, resulting in DIC, multiple organ dysfunction syndromes (MODS), leading to consumption of coagulation factors and platelets, and increases bleeding tendency (24–26). In addition, extracellular nuclear materials released from apoptotic or necrotic cells, such as High Mobility Group Box-1 (HMGB-1) and histones, are endowed with cell toxicity, proinflammatory and clot-promoting properties, and may be late mediators that propagate further inflammatory responses, consumption of coagulation components, cell death and MODS (27, 28). Therefore, the attenuation of coagulation imbalance by inhibition of SIRT1 in the lethal

septic model may be in part due to its anti-inflammatory and anti-apoptotic properties. Whether the inhibition of SIRT1 affects platelet function and other coagulation components directly need to be explored.

Bone marrow plays an important role in B cell lymphopoiesis and myelopoiesis, and minimally involved in T-cell maturation (29–31). There is a significant decline of about four-fold in the percentage of Gr1⁺-myeloid cells in bone marrow in CLP-sepsis, accounting for 80% of the decrease in viable cell yield in bone marrow tissue, and the deceased cell number is not associated with an increase in cell apoptosis (32). It is likely that myeloid cells are recruited to inflammatory sites outside the bone marrow in severe sepsis and septic shock, and EX-527 may prevent bone marrow from depletion and exhaustion by decreasing systemic cytokines and cell apoptosis, and reducing chemotaxis and recruitment of bone marrow cells to the circulation and inflammatory sites. In addition, SIRT1 plays an important role in the regulation of hematopoietic stem cell activity. SIRT1 inhibitor nicotinamide has been shown facilitate expansion of hematopoietic progenitor cells with enhanced bone marrow homing and engraftment (33). Additionally, hematopoietic cells derived from SIRT1-deficient mice (SIRT1^{-/-}) display increased in vitro proliferation activity (34). Therefore, EX-527 may prevents bone marrow atrophy in this lethal septic model by stimulating expansion of hematopoietic progenitor cells, which may potentially alleviate depletion of phagocytes released into circulation, providing another possible explanation for the survival advantages rendered by inhibition of SIRT1 in the lethal septic model.

The study has some limitations to be acknowledged. For logistical reasons, we only measured selected cytokines and explored limited pathways. Many more mechanisms are likely to be influenced by the inhibition of SIRT1. Furthermore, whether the inhibition of SIRT1 directly affects platelet function and other coagulation components need further investigations.

In conclusion, we have demonstrated that selective inhibition of Class III histone deacetylase SIRT1 significantly improves survival, attenuates “cytokine storm” and sepsis-associated coagulopathy, and decreases bone marrow atrophy in a lethal mouse septic model. Although the underlying molecular and cellular mechanisms still need further investigation, Class III HDACs may represent a potential therapeutic target for the treatment of lethal sepsis.

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Figure 1. EX-527 improves survival significantly in CLP-induced lethal septic model
Mice were intraperitoneally administered 47 mg/kg EX-527 or vehicle DMSO 1 h after CLP (n=10 animals/group). Treatment with EX-527 significantly improved long-term survival compared to DMSO vehicle group (50% versus 0% survival, $P < 0.001$).

Figure 2. EX-527 attenuates TNF- α levels markedly in blood in vivo
Blood samples were collected at 24 h after CLP and assayed for TNF- α levels by ELISA
(means $\pm$ SEM, n = 4–7 animals/group).

Figure 3. EX-527 attenuates IL-6 levels markedly in peritoneal fluid and blood in vivo
Peritoneal fluid and blood were collected at 48 h after CLP and assayed for IL-6 levels by ELISA (means $\pm$ SEM, n = 6–7 animals/group).

Figure 4. EX-527 decreases TNF-α and IL-6 levels in culture supernatant of mice primary splenocytes in vitro

Concentrations of TNF-α and IL-6 in culture supernatant of mice primary splenocytes were determined by ELISA at 6 h after LPS treatment in the absence or presence of EX-527.

Untreated primary splenocytes served as control (means ± SEM, n = 4/group).

Figure 5. EX-527 restores fibrin cross-linkage, clot formation speed, platelet function and clot rigidity in the lethal septic model

K is the time from clotting factor initiation until clot formation reaches an amplitude of 20 mm, which indicates the time for development of fixed degree of viscoelasticity during clot formation and fibrin cross-linkage; Angle is formed by the slope of the initial TEG[®] tracing, which represents speed at which solid clot forms; MA is the greatest amplitude of the TEG[®] tracing that reflects platelet function and absolute strength of the fibrin clot. The K, Angle, and MA values were recorded in TEG[®] 5000 Thrombelastograph[®] Hemostasis Analyzer System and compared between three experimental groups (means $\pm$ SEM, n = 5–8 animals/group).

Figure 6. EX-527 decreases bone marrow atrophy significantly in the lethal septic model (H&E, magnification 40×)

Tissue samples of long bones (femur and tibia) were harvested at 48 h after CLP. Samples were processed and stained with H&E, and representative images were chosen from different experimental groups. Semiquantitative pathology scores for bone marrow atrophy were graded according to the diameter proportion of veins to bone marrow cells (means $\pm$ SEM, n = 5–6 animals/group).