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Inhibition of Histone Deacetylase 6 Improves Long-term Survival in a Lethal Septic Model

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INTRODUCTION

Sepsis, a systemic inflammatory response syndrome caused by severe infection, is still a tremendous burden for health-care systems and results in more than 225,000 deaths annually in the United States¹. No pharmacological agents have been shown to effectively change the outcomes².

Acetylation of histone is an important epigenetic mechanism that governs amplitude of the immune signaling by controlling the chromatin structure, accessibility of transcription factors to DNA, and gene transcription. Regulation of this process needs the opposing actions of two families of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). It is found that dysregulated HDAC activity is linked to the pathogenesis of inflammatory and autoimmune diseases³. So far, 18 HDAC isoforms have been identified in humans and mice and grouped into four classes⁴. Classical HDACs (class I, II and IV) are Zn²⁺ dependent hydrolases, while the class III sirtuins are NAD⁺-dependent. Class I HDACs (HDAC1, 2, 3 and 8) play a role in cell survival and proliferation. Class II HDACs, subdivided into class IIa (HDAC4, 5, 7 and 9) and IIb (HDAC6 and 10) based on domain organization⁵, may have tissue-specific roles⁶. Recently, HDAC6 has become an important target for anti-cancer drug development, and inhibition of HDAC6 was also shown to have therapeutic potential to ameliorate injury of central nervous system⁷.

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MS-275 is a HDAC class I inhibitor with selectivity for HDAC1, 2, and 3. Tubastatin A is a newly synthesized selective inhibitor of HDAC class IIb with high selectivity for HDAC6⁸. Suberoylanilide hydroxamic acid (SAHA, vorinostat) is a broad-spectrum histone deacetylase inhibitor (HDACI) with a selectivity for HDAC1, 2, 3 and 6⁹. Our laboratory has previously demonstrated that administration of SAHA improves survival in rodent models of lipopolysaccharide (LPS)-induced endotoxemia and cecal ligation and puncture (CLP)-induced septic shock^{10, 11}. However, this HDACI was also found to increase host susceptibility to bacterial infection due to cell apoptosis¹². We hypothesized that targeting different HDACs could have different effect on animal survival in a mouse model of cecal ligation and puncture (CLP)-induced sepsis. In the present study, we first determined that inhibitor of HDAC6 (Tubastatin A) rather than that of HDAC1, 2, and 3 significantly prolonged animal lives in the CLP model. We then assessed impact of HDAC6 inhibition on production of some key pro-inflammatory cytokines, organ (liver) injury, and immune cell apoptosis. Our findings suggested that selective inhibition of HDAC6 has a substantial advantage for sepsis treatment.

METHODS

Cells Culture and Reagents

Mouse primary splenocytes and RAW 264.7 murine macrophages (American Type Culture Collection, Manassas, VA) were cultured in Dubelcco's modified Eagle's medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin (Invitrogen, Grand Island, NY) at 37 °C and 5% CO₂.

Sepsis Model: Cecal Ligation and Puncture (CLP)

Male C57BL/6J mice (about 18–26 g), purchased from the Jackson Laboratory, were housed for 3 days before manipulations. The murine CLP model was used to induce fecal peritonitis as described previously¹¹. Sham-operated animals were handled in the same manner without the cecum ligation and puncture. This protocol was approved by the Animal Review Committee in our institute.

Administration of HDACI and Experimental Design

In the survival experiment, mice were randomly subjected to three groups, and received the following treatment: (1) intra-peritoneal MS-275 (70 mg/kg) dissolved in dimethyl sulfoxide (DMSO) (1 µl/g), (2) Tubastatin A (70 mg/kg) dissolved in DMSO, or (3) vehicle DMSO, 1 h after CLP (n=7–12/group). All mortality or survival was recorded for up to 10 days post-procedure.

In the non-survival experiment, animals were randomly assigned to the following three groups (n = 24/group): (a) Sham-operated animals (SHAM); (b) vehicle treated animals after CLP (CLP+DMSO), and (c) Tubastatin A treated animals after CLP (CLP + Tubastatin A). Sham-operated animals were subjected to laparotomy and intestinal manipulation, but the cecum was neither ligated nor punctured. At the time of sacrifice [3h, 24 h, 48 h, and 10 d after CLP (n= 4–7/group/time point)], abdominal cavity was opened and irrigated with 1 mL

normal saline, which was collected for analysis, and blood samples were collected by cardiac puncture. Liver tissue was harvested 24 h after CLP and fixed in 10% buffered formalin for 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.

Histological Analysis

Twenty-four hours after CLP, tissue samples of liver were harvested for histological analysis as our previous study ¹¹. Briefly, the liver tissue was embedded in paraffin, sliced into 5- μ m sections and stained with hematoxylin and eosin (H&E). Hepatocellular necrosis, hemorrhage/congestion, parenchyma inflammation, sinusoidal inflammation, and degenerative changes were assessed by a blinded pathologist. Each parameter of liver injury was graded on a scale of 0–3, with 0 meaning “absent,” 1 meaning “mild,” 2 meaning “moderate,” and 3 meaning “severe.” The total injury score was expressed as the sum of the scores for all parameters ¹¹.

Bacteria Load Determination

Whole blood samples were obtained 48 h after sham operation or CLP by cardiac puncture. Samples were diluted in sterile saline, plated on tryptic soy agar (BD Difco, Franklin Lakes, NJ), and incubated at 37 °C. The number of bacterial colonies was assessed 24 hours later. Bacteria count was calculated as colony forming units per milliliter, and log transformation of these values was then used for further analysis ¹³.

Phagocytosis Assay

Mouse primary splenocytes were prepared as previously described ¹⁴. Phagocytosis was then assessed using a pHrodo™ E. coli BioParticles® Phagocytosis Kit (Invitrogen, Grand Island, NY) ^{15, 16}. Cells were seeded in a 96-well assay plate, and treated at 37 °C in the absence or presence of LPS (1 μ g/mL) or Tubastatin A (10 μ M) for 3 h before removing the medium and adding 100 μ L uptake buffer (HBSS buffered with 20mM HEPES, pH 7.4) containing 1 mg/mL pHrodo E. coli bioparticles. The assay plate was incubated for 1 h at 37 °C in a humidified chamber, and then analyzed on a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA). Net phagocytosis was calculated by subtracting the base-line fluorescence from wells containing 100 μ L of 1 mg/mL pHrodo E. coli bioparticles but no cells.

Western Blot Analysis

RAW264.7 macrophages were harvested 3 and 6 h after treatment with LPS in the absence or presence of Tubastatin A. Sham (no LPS, no Tubastatin A) macrophages served as control. Equal amounts of whole cell lysate samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes

(Bio-Rad Laboratories, Hercules, CA). Western blots were employed and quantitative analysis of detected bands was performed as described previously¹¹.

Flow Cytometry

Cell apoptosis was measured using an annexin V-fluorescein isothiocyanate (FITC) detection kit (Abcam, Cambridge, MA). RAW264.7 Macrophages were treated with LPS in the absence or presence of another HDAC6 inhibitor Tubacin for 3, 6 and 9 h. Sham (no LPS, no Tubacin) macrophages served as control. Cells were then harvested, washed in ice-cold phosphate buffered saline, resuspended in binding buffer, and incubated with annexin V-FITC for 5 min at room temperature. Cells were analyzed by flow cytometry, and apoptotic cells were defined as positive for annexin V-FITC and negative for propidium iodide (PI) staining¹⁷.

Statistical Analysis

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

RESULTS

Tubastatin A significantly improves survival in CLP-induced lethal septic model

In this CLP-induced lethal sepsis model, all mice in either DMSO vehicle group or MS-275 group died within 3 days (0% survival). However, Tubastatin A-treated animals displayed significantly higher long-term survival compared to the DMSO or MS-275 group (66.7% vs. 0% survival, $p < 0.001$; Figure 1). We previously showed that SAHA treatment significantly improved survival (40% survival)¹¹. Compared to this SAHA result, Tubastatin A treatment for an improvement in animal survival is as good as SAHA.

Tubastatin A decreases cytokine levels in peritoneal fluid and circulation

In the sham group, TNF- α levels in the peritoneal fluid and blood were low at all time points (8.7 ± 0.7 and 11.3 ± 2.3 pg/mL, respectively). Similarly, IL-6 levels in peritoneal fluid and blood were hardly detected (6.3 ± 2.0 and 14.9 ± 6.3 pg/mL, respectively). However, the levels of TNF- α at 3 and 24 h and the levels of IL-6 at 48 were increased obviously after the CLP insult.

Tubastatin A significantly attenuated the CLP-induced increase of TNF- α at 3 and 24 h in the peritoneal fluid (6.3 ± 7.7 pg/mL vs. 79.5 ± 19.0 at 3 h, $p < 0.05$; 53.5 ± 19.6 pg/mL vs. 463.3 ± 48.4 at 24 h, $p < 0.01$; Figure 2A) and plasma (7.3 ± 2.0 pg/mL vs. 9.9 ± 3.5 at 3 h, $p < 0.05$; 24.5 ± 5.0 pg/mL vs. 298.3 ± 24.6 at 24 h, $p < 0.001$; Figure 2A). Ten days after Tubastatin A treatment, TNF- α levels in the peritoneal fluid and blood returned to nearly normal (2.1 ± 1.2 and 7.3 ± 2.1 pg/mL, respectively; Figure 2A). In addition, Tubastatin A significantly suppressed an increase of IL-6 levels in the peritoneal fluid (71.7 ± 3.7 pg/mL

vs. 646.4 ± 81.8 , $p < 0.01$; Figure 2B) and plasma (88.1 ± 65.0 pg/mL vs. 521.5 ± 94.3 , $p < 0.05$; Figure 2B) 48 h after CLP.

Tubastatin A decreases TNF- α and IL-6 levels in culture supernatant of mouse primary splenocytes in presence of LPS ex vivo

In Sham group, only low levels of TNF- α and IL-6 were detected in cell culture supernatant of the primary splenocytes. Treatment of the cells with LPS for 6 h markedly increased secretion of these cytokines. However, Tubastatin A significantly attenuated the LPS-induced production of TNF- α and IL-6 (15.1 ± 1.4 pg/mL vs. 68.1 ± 6.4 , 4.6 ± 1.4 pg/mL vs. 61.3 ± 12.1 , respectively, $p < 0.01$; Figure 3).

Tubastatin A decreases acute liver injury

Sham-operated animals had normal liver histology, but the liver at 24 h after CLP showed increased parenchyma inflammation and degenerative changes. These pathological alterations were attenuated by Tubastatin A treatment, with significantly lower acute liver injury scores (2.6 ± 0.3 vs. 5.1 ± 0.8 , $p < 0.05$; Figure 4).

Tubastatin A increases bacteria clearance in circulation in vivo and phagocytosis of mouse primary splenocytes ex vivo

Compared with vehicle treated animals, Tubastatin A treated animals had remarkably lower bacteria load in circulation 48 h after CLP (2.5 ± 0.9 vs. 6.2 ± 0.3 log₁₀ CFU/mL, $p < 0.01$; Figure 5A). Moreover, Tubastatin A treatment increased phagocytosis of splenocytes markedly (28.8 ± 1.4 RFU vs. 18.5 ± 2.2 , $p < 0.01$; Figure 5B).

HDAC6 Inhibitors inhibits cell apoptosis of RAW264.7 macrophages in vitro

Tubastatin A decreases cleaved caspase-3 expression in macrophages at 3 and 6 h (23.8 ± 5.2 vs. 97.2 ± 4.2 and 52.6 ± 6.0 vs. 172.4 ± 9.6 , respectively, $p < 0.001$; Figure 6).

DISCUSSION

In the present study, we investigated the effects of selective HDAC inhibitors with distinct isoform selectivity, and found: (1) The HDAC6-selective inhibitor Tubastatin A significantly improves long-term survival, while MS-275, an inhibitor of HDAC1, 2, and 3, is not protective against septic shock; (2) Tubastatin A significantly attenuates local and systemic proinflammatory cytokines *in vivo*, as well as the LPS-stimulated cytokines production from primary splenocytes *in vitro*; (3) Tubastatin A treated animals display attenuated acute liver injury; (4) the HDAC6 inhibitor increases blood bacteria clearance and phagocytosis of immune cells, and (5) Tubastatin A decreases macrophage apoptosis (see Figure 6B for proposed mechanism).

It has been known that HDAC6 is a unique HDAC and predominantly localized in the cytoplasm, where it associates with non-histone substrates, such as heat shock protein (HSP) 90, α -tubulin and cortactin⁷. Overexpression of HDAC6 leads to tubulin deacetylation and increased cell motility¹⁸. Inhibition of HDAC6 activity increases acetylation of α -tubulin and HSP90, reduces cellular motility and induces degradation of client proteins of

HSP90¹⁹. So far, several client proteins of HSP90 have been reported, such as Bcr-Abl, Raf-1, Akt, HER2/Neu (ErbB2), interleukin-1 receptor associated kinase 1 (IRAK1), and hypoxia-inducible factor-1 α ²⁰. Our laboratory has recently shown that SAHA treatment increases acetylation of HSP90 in LPS-stimulated macrophages, and results in dissociation and degradation of IRAK1. The IRAK1 degradation then decreases nuclear translocation of Nuclear Factor- κ B (NF- κ B), leading to attenuation of key pro-inflammatory cytokines²¹. Triantafyllou in 2004 reported that HSPs are involved in the innate recognition of bacterial products²². The association of TLR4 with HSP70 and HSP90 following LPS stimulation was found both on the cell surface and in the cells. HSP70 and HSP90 form a cluster with TLR4 within lipid micro-domains. In addition, HSP70 and HSP90 seem to be involved in TLR4/LPS trafficking and targeting to the Golgi apparatus²². Therefore, instability of HSP90 after acetylation modulated by HDAC6 inhibitors may impair pathogen recognition as well.

Inhibition of HDAC6 by Tubastatin A could reduce expression of hypoxia-inducible factor-1 α (HIF-1 α). We have recently demonstrated that SAHA attenuates HIF-1 α -mediated inflammatory pathway in macrophages, and suppresses hypoxia-induced release of pro-inflammatory nitric oxide and TNF- α ²³. HIF-1 α is a key pro-inflammatory transcription factor in TLR4-dependent inflammatory responses in macrophages, and is essential for myeloid-dependent inflammation in vivo²⁴. HDAC6 has been shown to interact with HIF-1 α and regulate its function. Inhibition of HDAC4 and HDAC6 by small interfering RNA can reduce protein expression and transcriptional activity of HIF-1 α in a renal cell carcinoma cell line²⁵.

The inhibition of macrophage apoptosis may be another explanation for the improved survival outcomes after treatment with HDAC6 inhibitors in our lethal septic model. Macrophages, as the most efficient pathogen scavengers and the predominant source of inflammatory cytokines, are critical effector cells contributing to the altered innate immune response against infection. Severe sepsis has been found to be associated with progressing macrophage dysfunction and cell death²⁶. It is not clear how HDAC6 affects phagocytosis and bacteria clearance. Inhibition of HDAC6 likely reduces the apoptosis of macrophages, improves their ability to phagocytize foreign pathogens and decreases blood bacteria load. HDAC6 inhibitors might also decrease bacteria translocation from ischemia/necrosis sites, by decreasing systemic pro-inflammatory chemo-attractants.

During sepsis, excessive production of pro-inflammatory cytokines promotes migration of leukocytes, lymphocytes, and platelets to the infected areas, leading to endothelial damage, increased micro-vascular permeability, platelet aggregation, local blood flow reduction, and ischemia/reperfusion (I/R) injury, finally results in multiple organ damage. We previously found that SAHA treatment attenuates acute liver injury during severe sepsis¹¹. We also demonstrated that SAHA significantly decreases LPS-induced expression of the pro-inflammatory MAPK, phosphorylated p38, phosphorylated extracellular signal-regulated kinase, myeloperoxidase, and IL-6 in the liver, while increasing levels of the anti-inflammatory IL-10^{27,28}. The attenuation of activation of MAPK and phosphorylated extracellular signal-regulated kinase, subsequent decrease in NF- κ B-dependent gene

transcription, and alteration of inflammatory markers might account for the protective role of HDAC6 inhibition in reducing acute liver injury in the lethal septic model.

Notably, enthusiasm for pan-HDAC inhibition has been tempered by its toxicity toward host ¹². For example, HDAC1 and HDAC2 can promote B cell proliferation ²⁹. When HDAC1 and HDAC2 are ablated, B cell development is blocked. The pre-B cells are then stopped in G1 phase accompanied by the induction of apoptosis ²⁹. In addition, HDAC1 and HDAC2 are essential for normal T cell development and genomic stability in mice. Conditional deletion of HDAC1 in T cells results in increased Th2 cytokine production as well as enhanced airway inflammation ³⁰. Meanwhile, Class I HDACs repress TNF-induced NF- κ B-dependent gene expression ³¹, and promote interferon signaling. Therefore, inhibitors of Class I HDAC may have cytotoxicity for immune cells, impair lymphocyte development, amplify production of TLR/NF- κ B-inducible inflammatory mediators, and compromise anti-microbial responses. In contrast, HDAC6-deficient mice are viable and develop normally ³¹. Lymphoid development is normal after HDAC6 deletion, with moderately-affected immune responses. HDAC6-deficient mouse embryonic fibroblasts have apparently normal microtubule organization and stability, and the acetylation of HSP90 is increased correlating with its impaired function. Accordingly, this data demonstrate that HDAC6 deletion is not detrimental to normal mammalian development. Although the mechanism how HDAC6 inhibitors prevent immune cell apoptosis is not clear, antiapoptotic property of these inhibitors may render them advantageous to treat severe sepsis and septic shock, compared to the first generation non-selective HDACI.

The present study has demonstrated several possible mechanisms of Tubastatin A action (see Figure 6B for summary). Treatment with Tubastatin A could down-regulate key pro-inflammatory cytokines, prevent liver damage, and decrease macrophage apoptosis. Moreover, Tubastatin A can also enhance bacterial clearance and splenocyte phagocytosis. We must acknowledge that the study has certain limitations. For logistical reasons, we only measured selected cytokines and explored limited pathways. Many more mechanisms are likely to be influenced by Tubastatin A treatment. Similarly, we only studied liver injury although there are other organs which are likely affected by this treatment. Mechanism of action of Tubastatin A needs to be explored.

In conclusion, we have demonstrated that Tubastatin A, an inhibitor of HDAC6, can improve survival significantly in a lethal polymicrobial sepsis model. The survival advantage is associated with an attenuation of local and systemic proinflammatory cytokines, protection against distant organ injury, enhancement in bacterial clearance and phagocytosis, and inhibition of immune cell apoptosis. Although the fundamental molecular and cellular signaling events still require further investigation, HDAC6 may represent a novel and promising therapeutic target for septic shock.

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Figure 1. Tubastatin A protects mice against severe sepsis and septic shock-induced lethality
Mice were intraperitoneally given Tubastatin A (70mg/kg), MS-275 (70mg/kg), or vehicle (DMSO) 1 h after CLP (n=7–12 animals/group). Treatment with Tubastatin A significantly improved long-term survival compared to DMSO vehicle group (66.7% vs. 0% survival, $p < 0.001$), while MS-275 did not show protective effects against sepsis-induced mortality. CLP: cecal ligation and puncture; Tub.A: Tubastatin A.

Figure 2. Tubastatin A decreases levels of TNF- α and IL-6 in peritoneal fluid and plasma during severe sepsis and septic shock.

The peritoneal fluid and blood were collected at different time points to measure concentrations of TNF- α (Figure 2A; at 3 h, 24 h, and 10 d after CLP) and IL-6 (Figure 2B; at 48 h after CLP) using ELISA kits (means $\pm$ SEM, n = 4 animals/group). Tub.A: Tubastatin A

Figure 3. Tubastatin A decreases TNF- α and IL-6 production in cell culture supernatant of primary splenocytes insulted by LPS

Concentrations of TNF- α and IL-6 in culture supernatant of primary splenocytes were determined by ELISA at 6 h after LPS treatment in the absence or presence of Tubastatin A. Untreated macrophages served as control (means $\pm$ SEM, n = 4/group). Tub.A: Tubastatin A

Figure 4. Tubastatin A protects animals from acute liver injury 24 h after CLP (H&E, magnification 40×)

Mice were intraperitoneally administered 70mg/kg Tubastatin A, or vehicle (DMSO) 1 h after CLP. Twenty-four hours after operation, liver tissues were obtained. Representative images were chosen from different experimental groups. Semi-quantitative pathology scores were determined by a scoring system as described in Materials and Methods (means ± SEM, n = 4–6 animals/group). Tub.A: Tubastatin A

Figure 5. Tubastatin A increases blood bacteria clearance *in vivo* and enhances phagocytosis of mouse primary splenocytes *ex vivo*

(A) Whole blood samples were obtained 48 h after operation by cardiac puncture. Samples were diluted in sterile saline, plated on tryptic soy agar, and incubated at 37 °C. The number of bacterial colonies was assessed 24 hours later. Bacteria number was calculated as colony forming units per milliliter, and data were log transformed for presentation (Means ± SEM, n = 6 animals/group). CFU: colony forming units. (B) Mouse primary splenocytes were treated as described in material and methods. Cells were then incubated with 1 mg/ml pHrodo *E. coli* bioparticles and the fluorescence analyzed on a plate reader. Mean values are relative fluorescence units (RFU), calculated from four wells per group subtracting the baseline fluorescence from wells containing pHrodo *E. coli* bioparticles but no cells. (Means ± SEM, n = 4/group). Tub.A: Tubastatin A

Figure 6.

(A) Tubastatin A decreases cleaved caspase-3 protein in macrophages after LPS insult.

Macrophages were harvested at 3 and 6 h after treatment with LPS in the absence or presence of Tubastatin A. Sham (no LPS, no Tubastatin A) macrophages served as a control. Whole cell lysates were subjected to Western Blot with anti-cleaved Caspase-3 and anti-actin antibodies. Specific bands were then quantified by densitometry (means $\pm$ SEM, n = 4/group). Tub.A: Tubastatin A. **(B) Schematic depiction of proposed mechanism in the present study.** Treatment with Tubastatin A, on one hand, could decrease production of key pro-inflammatory cytokines (TNF- α and IL-6), liver injury, and microphage apoptosis; on the other hand, it could increase bacterial clearance and splenocyte phagocytosis.