Published in final edited form as:

J Allergy Clin Immunol. 2015 May ; 135(5): 1228–1239. doi:10.1016/j.jaci.2014.10.002.

Histone deacetylase 6 inhibition impairs effector CD8 T-cell functions during skin inflammation

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Abstract

Background—Broad spectrum histone deacetylase (HDAC) inhibitors are useful in the treatment of allergic and autoimmune diseases and malignancy. However, use of more specific HDAC inhibitors may limit the toxicities caused by HDAC inhibition. HDAC6, a member of the HDAC family, is highly expressed on CD8 T-cells, and it has been shown to regulate immune responses via interactions between T-cells and antigen-presenting cells; however, the mechanism by which HDAC6 inhibition affects the activation and functions of CD8 T-cell is unclear.

Objectives—We investigated the role(s) of HDAC6 in CD8 T-cell activation and functions during skin inflammation *in vitro* and *in vivo*, and examined the mechanism by which HDAC6 inhibition modifies T-cell receptor (TCR) signaling *in vitro*.

Methods—We assessed the clinical and biological effects of ACY-1215, an HDAC6-specific inhibitor, using murine CD8 T-cell-related skin disease models, including contact hypersensitivity (CHS) and experimental graft-versus-host disease (GVHD)-like disease.

Results—ACY-1215, an HDAC6 inhibitor, prevented the development of CHS and GVHD-like disease *in vivo* by modulating CD8 T-cell activation and functions; abrogated the induction of effector T-cells from naïve CD8 T-cells by anti-CD3/CD28 antibody- or antigen-specific stimulation *in vitro*; and enhanced the binding of acetylated heat shock protein 90 to lymphocyte-specific protein tyrosine kinase (LCK) *in vitro*, disrupting LCK phosphorylation leading to impairment of the mitogen-activated protein kinase pathway.

Conclusion—HDAC6, a key modifier of TCR signaling, may represent a novel target for the treatment of CD8 T-cell-related skin diseases, including contact hypersensitivity and GVHD.

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Disclosure of potential conflict of interest

The authors have no financial conflicts of interest.

Keywords

CD 8 T-cell; histone deacetylase 6; contact hypersensitivity; graft-versus-host-disease

Introduction

The utility of histone deacetylase (HDAC) inhibitors, potent epigenetic modifiers, has been demonstrated in the treatment of inflammatory and autoimmune diseases as well as in certain malignancies (1). The HDAC inhibitors are thought to exert their anti-inflammatory and immunosuppressive effects independent of their cytotoxic or cell cycle arrest actions on malignant T-cells.

Notably, suberoylanilide hydroxamic acid (SAHA: vorinostat), a broad-spectrum HDAC inhibitor for HDAC1, 2, 3, 4, 6, 8 and 10, approved for the treatment of cutaneous T-cell lymphoma, has been shown to attenuate GVHD (1) and anti-CD3 antibody (Ab)-triggered cytokine storm syndrome (2) via the modulation of T-cell functions in murine models. However, the inhibition of HDACs using member-specific HDAC inhibitors may be more favorably accepted because they may reduce adverse effects caused by the broad inhibition of HDACs.

HDAC6, a member of cytoplasmic class IIb HDACs, is a unique enzyme that deacetylates non-histone proteins such as alpha-tubulin (α -tub) and heatshock protein 90 (HSP90) leading to the regulation of multiple biological responses including cell migration, cell-cell interaction, and cell signaling (4). Especially in immune synapses (IS), cell-cell junctions of T-cells and antigen-presenting cells (APCs), HDAC6 has been shown to be a key determinant in the formation of IS and in modulating immune responses (3). In fact, HDAC6 inhibition using selective HDAC6 inhibitors such as Tubacin and Tubastatin A has been reported to result in attenuating disease activities in murine models of inflammation and autoimmunity (4, 5); however, the mechanism(s) by which HDAC6 governs activation and functions of T-cells, including T-cell receptor (TCR) signaling, is not known.

We have found that, in contrast to CD4 T cells, murine CD8 T-cells express increased HDAC6 during activation induced by anti-CD3/CD28 Ab (Supplemental Fig 1.), and therefore hypothesized that HDAC6 activity might be directly involved in the activation and functions of CD8 T-cells. We utilized two models of inflammation; 1) contact hypersensitivity (CHS), and 2) graft-versus-host disease (GVHD)-like disease (6). The onset of GVHD-like disease is dependent on CD8 T-cells (6). Since the pathogenesis of the GVHD-like disease recapitulates human diseases caused by the pathological activation of CD8 T-cells, identifying novel therapies for GVHD-like disease may facilitate development of treatments of other inflammatory and autoimmune diseases.

To modulate HDAC6 activity *in vivo*, we utilized ACY-1215 (ACY), an orally bioavailable HDAC6-specific inhibitor, developed for the treatment of malignant myeloma and currently being evaluated in a clinical trial (phase I/II) (7).

In this study we report that oral administration of ACY prevents and ameliorates the development of CHS and GVHD-like disease, both murine models of CD8 T-cell-mediated inflammatory disease, via novel mechanism(s).

Methods

Mice

B57CL/6 (B6) and BALB/c mice were obtained from the National Cancer Institute Animal Production Program (Frederick, MD), housed in a clean facility, and bred and used in accordance with institutional guidelines. Rag-1-knockout (KO) OT-I mice were purchased from Taconic (Germantown, NY). Keratin 14-promoter membrane ovalbumin transgenic (K14-mOVA Tg) mice have been described previously (8).

ACY-1215 administration

The reagent 2-(diphenylamino)-N-(7-(hydroxyamino)-7-oxoheptyl) pyrimidine-5-carboxamide (ACY-1215) was purchased from Chemietek (Indianapolis, IN). ACY-1215 (ACY) was first dissolved in DMSO (Sigma–Aldrich, St. Louis, MO) and then in culture medium (1–2 μ M) for cell culture. Because PEG-400 enhances the bioavailability of ACY, it was dissolved in PEG-400 (Sigma–Aldrich) and diluted with sterile water. ACY was administered to mice by gavage.

CHS

On day 0, the abdomen of each mouse was shaved and sensitized with a 3% 2,4,6-trinitrochlorobenzene (TNCB) solution in a vehicle (1:4 of acetone: olive oil) of 100 μ l (abdomen). On day 6, the right ear of the sensitized mouse was painted using a total of 20 μ l of a 1% TNCB solution in a vehicle (4:1 of acetone: olive oil). Twenty-four hours after application of the 1% TNCB, ear swelling was measured using a dial thickness gauge. Mice painted with vehicle (4:1 acetone: olive oil) alone were used as negative controls.

GVHD-like disease

Single-cell suspensions were prepared from pooled LNs (inguinal LNs, axillary LNs, and mesenteric LNs) of OT-I mice with a Rag1-KO-background. One million OT-I cells were injected i.v. into K14-mOVA mice. Weight and health status of mice were monitored daily for 14 days.

Statistical analysis

Statistical analyses were performed using Prism Graphpad (San Diego, CA). A Student's *t*-test or a Mann–Whitney *U* test was used for statistical comparisons. A *P*-value of < 0.05 was considered significant.

A more complete description of methods, and associated references, are available in this article's supplemental data.

Results

To evaluate the significance of HDAC6 inhibition in the regulation of acquired immune responses *in vivo*, we examined whether HDAC6 inhibition, using ACY, attenuated the development of TNCB-induced CHS, which is mediated by type I cytokines, including IFN- γ (11). Two different gavage doses of ACY (25 mg/kg, once daily, or 50 mg/kg, once daily) were utilized to examine the effects on CHS. While ACY administration (25 mg/kg, once daily) did not affect ear swelling, ACY administration (50 mg/kg, once daily) before elicitation and before both sensitization and elicitation decreased ear swelling (Supplemental Fig 2A and 2B). In contrast, the ACY administration before sensitization did not affect ear swelling (Fig 1A and 1B). Consistently, histology of the ears from B6 mice 24 hours after elicitation showed a dense inflammatory infiltrate in the edematous dermis; these findings were markedly attenuated by ACY administration before elicitation and before both sensitization and elicitation (Fig 1C). qRT-PCR analysis of IFN- γ expression in ear skin of B6 mice 24 hours after elicitation also showed that the upregulation of IFN- γ expression during the elicitation phase was inhibited by ACY administration before elicitation and before both sensitization and elicitation (Fig 1D). These results suggest that HDAC6 inhibition using ACY impairs the elicitation but not the sensitization phase in TNCB-induced CHS and were validated in an adoptive transfer CHS model (Supplemental Fig 3A and 3B).

Since CD8 T-cells activated by antigen-specific stimulation are the major effector cells involved in CHS model (12), we assessed, *in vitro*, effector T-cell proliferation induced by antigen-specific stimulation. TNCB-sensitized T-cells were sorted from SDLNs of B6 mice 5 days after TNCB application, and incubated with APCs prepared from naïve splenocytes loaded with TNBS, a water-soluble form of TNCB, in the presence of ACY (1 and 2 μ M) or vehicle for 48 hours. A WST-1 cell proliferation assay showed that ACY inhibited TNCB-sensitized T-cell proliferation in a dose-dependent manner (Fig 2A). To further examine whether ACY inhibits antigen-specific T-cell proliferation by acting on T-cells but not APCs, CD8 T-cells sorted from splenocytes of naïve B6 mice were stimulated with an anti-CD3/CD28 Ab in the presence of ACY (1 and 2 μ M) or vehicle for 48 hours. ACY inhibited the CD8 T-cell proliferation (Fig 2B). Furthermore, qRT-PCR analysis showed that the upregulation of IL-2 and IFN- γ expression, key mediators of CHS (12), that were induced by CD8 T-cells stimulated with anti-CD3/CD28 Ab were also inhibited by ACY (Fig 2C and 2D). Proliferation and cytokine production of CD4 T-cell were also inhibited (Supplemental Fig 4A, 4B and 4C). These results indicate that HDAC6 inhibition using ACY attenuates the CHS response via modulation of cell proliferation and IFN- γ production by effector CD8 T-cells.

To investigate whether HDAC6 is directly involved in the activation and functions of CD8 T-cell, we used an experimental GVHD-like disease model. We administered vehicle or ACY (50 mg/kg, twice daily, 75 mg/kg, once daily, or 75 mg/kg, twice, daily) orally to K14-mOVA mice from the day of OT-I cell transfer (1×10^6 per mouse). Vehicle-treated control K14-mOVA mice developed mucosal lesions and erosive skin lesions; in contrast, ACY administration (75 mg/kg, twice, daily) completely prevented the development of mucocutaneous lesions 14 days after transfer of OT-I cells (Fig 3A). Histology of the ears of

vehicle-treated control mice showed marked infiltration of inflammatory cells in the dermis, vacuolar changes in the basal layer, and dyskeratosis in the epidermis; these resembled cutaneous pathological manifestations of human acute GVHD, which was attenuated in ACY-treated K14-mOVA mice (Fig 3B). GVHD-like disease activity correlated well with progressive weight loss particularly in the late phase of the disease course. The early phase of weight loss is thought to be due to decreased oral intake of food following cell transfer (10, 13). ACY-administrated mice had less weight loss [$-23.2\% \pm 1.63$ (vehicle) and $-11.2\% \pm 1.74$ (ACY) 5 days after transfer of OT-I cells; $-19.7\% \pm 2.91$ (vehicle) and $-11.7\% \pm 2.12$ (ACY) 14 days after transfer of OT-I cells (average $\pm$ SEM) ($P < 0.01$, *U*-test)] (Fig 3C). ACY administration (50 mg/kg, twice daily, or 75 mg/kg, once daily) did not prevent the weight loss caused by GVHD-like disease (Supplemental Fig 5A and 5B). To determine whether ACY administration (75 mg/kg, twice, daily) ameliorated GVHD-like disease in addition to its ability to prevent it, we administer ACY from day 8 after transfer of OT-I cells. This treatment resulted in reduced weight loss [$-20.2\% \pm 2.73$ (vehicle) and $-13.0\% \pm 2.78$ (ACY) 6 days after ACY administration (average $\pm$ SEM) ($P < 0.01$, *U*-test)] (Fig 3D). These results indicate that HDAC6 inhibition controls CD8 T-cell-mediated GVHD-like disease activity.

We next examined whether ACY alters the proliferation and activation of CD3⁺CD8⁺V α 2⁺V β 5⁺ OT-I cells that contribute to the onset of GVHD-like disease. We therefore analyzed OT-I cells from SDLNs of vehicle- or ACY-treated K14-mOVA mice 5 (Fig 4A) or 11 days (Fig 4B) after transfer of OT-I cells using flow cytometry. At both time points, the percentage of OT-I cells from SDLNs was reduced in ACY-treated mice compared with vehicle-treated mice. Reduced CD25 and CD44 expression and slightly enhanced CD62L expression were observed in OT-I cells of ACY-treated mice compared with those in vehicle-treated mice 5 days after transfer of OT-I cells (Fig 4C). This indicated that ACY administration interfered with the transition of naïve OT-I cells (CD25^{low}CD44^{low}CD62^{high}) into effector OT-I cells (CD25^{high}CD44^{high}CD62L^{low}). Furthermore, reduced IL-2 and IFN- γ concentration in sera was also observed in ACY-treated mice compared with vehicle-treated mice 5 days after transfer of OT-I cells (Fig 4D and 4E), indicating that ACY inhibited IL-2 and IFN- γ production *in vivo*.

To determine whether ACY administration exhibits anti-HDAC6 activity *in vivo*, we evaluated the acetylation of alpha-tubulin (α -tub) and heat shock protein (HSP) 90, which are representative proteins specifically deacetylated by HDAC6. Flow cytometric and western blot analyses showed that ACY administration enhanced the acetylation of α -tub and HSP90 of OT-I cells from OT-I/Rag-I KO mice that have only CD8 T-cells (Supplemental Fig 6C and 6E). Furthermore, ACY administration did not enhance the acetylation of histone H3 (Supplemental Fig 6H), indicating that ACY administration exhibits anti-HDAC6 activity specifically *in vivo*. ACY (1 μ M) also enhanced the acetylation of α -tub and HSP90 of Jurkat cells and OT-I cells without affecting the acetylation of histone H3 *in vitro* (Supplemental Fig 6A, 6B, 6D, 6F and 6G).

To determine the mechanism(s) by which HDAC6 inhibition using ACY impairs the proliferation and activation of OT-I cells, we performed an *in vitro* assay on the proliferation and activation of OT-I cells. Naïve OT-I cells were stimulated with an anti-CD3/CD28 Ab

in the presence of ACY (1 and 2 μ M) or vehicle for 48 hours. The WST-I cell proliferation assay showed that ACY inhibited anti-CD3/CD28 Ab-induced proliferation of OT-I cells in a dose-dependent manner (Fig 5A). To determine whether ACY also abrogates cell proliferation induced by antigen-specific stimulation, naïve OT-I cells were stimulated with APCs, SIINFEKL peptide-loaded splenocytes derived from B6 mice, in the presence of ACY (1 or 2 μ M), or vehicle for 48 hours. The WST-I cell proliferation assay (Fig 5B) and flow cytometric analysis using CFSE (Fig 5C) showed that ACY inhibited SIINFEKL-induced proliferation of OT-I cells in a dose-dependent manner. Flow cytometric analysis also showed that ACY (1 μ M) interfered with enhanced CD25 and CD44 expression induced by the stimulation of SIINFEKL peptide-loaded APCs (Fig 5D), which is consistent with the *in vivo* results shown above. In addition, flow cytometric analysis of 7-aminoactinomycin D to determine the number of nonviable cells showed that ACY (1 and 2 μ M) did not affect T-cell viability (Supplemental Fig 7A, 7B and 7C). This result is consistent with that of a previous report in which ACY displayed low cytotoxicity to normal cells and malignant T-cells (7).

Exhaustive production of IL-2 and IFN- γ is required to induce the transition of naïve CD8 T-cells into effector CD8 T-cells. We therefore examined whether ACY affected the upregulation of IL-2 and IFN- γ expression in OT-I cells *in vitro*. ACY (1 and 2 μ M) incubation for 3 hours enhanced the acetylation of α -tub of OT-I cells *in vitro* (Supplemental Fig 6B), suggesting anti-HDAC6 activity. When OT-I cells were stimulated with SIINFEKL peptide-loaded splenocytes for 3, 6, or 12 hours following incubation with ACY (1 or 2 μ M) or vehicle for 3 hours. qRT-PCR analysis showed that ACY inhibited the upregulation of IL-2 and IFN- γ expression in a dose- and time-dependent manner at the mRNA level (Fig 6A, 6B, 6C and 6D). Flow cytometric analysis using intracellular staining for IL-2 and IFN- γ also showed that ACY (1 μ M) inhibited the upregulation of IL-2 and IFN- γ expression in OT-I cells that was cocultured with SIINFEKL peptide-loaded APCs at the protein level (Fig 6E). These results indicate that ACY disrupts the production of IL-2 and IFN- γ by OT-I cells under antigen-specific stimulation that is required for the sustained activation of naïve OT-I cells leading to transformation into effector CD8 T-cells. Based on the findings that HDAC6 inhibition using ACY abrogates anti-CD3/CD28 Ab or antigen-specific stimulation-induced cell proliferation and the upregulation of IL-2 and IFN- γ expression, we determined whether HDAC6 inhibition may modify T-cell receptor (TCR) signal transduction. We analyzed the TCR signaling pathway that was stimulated with an anti-CD3 Ab in Jurkat cells, a human CD4 T-cell line. Jurkat cells were stimulated with the anti-CD3 Ab (2 μ g/ml) for 1, 2, and 5 minutes followed by incubation with ACY (1 μ M) or vehicle for 3 hours. Western blot analysis showed that the phosphorylation of LCK, ZAP70, and LAT but not FYN induced by anti-CD3 Ab stimulation were reduced in ACY-treated Jurkat cells compared with in vehicle-treated Jurkat cells (Fig 7A). These results indicate that HDAC6 inhibition using ACY interferes with LCK phosphorylation leading to the inhibition of ZAP70 and LAT phosphorylation, which are dependent on LCK phosphorylation (14).

To clarify the mechanism by which HDAC6 inhibition using ACY impairs LCK phosphorylation, we examined HSP90 binding to LCK because the chaperone function of

HSP90 is required for LCK phosphorylation (15) and because HDAC6 inhibition acetylates and disrupts the chaperone function of HSP90 (16). We examined the acetylation of HSP90 binding to LCK in Jurkat cells incubated with ACY (1 μ M) or vehicle for 3 hours. Western blot and immunoprecipitation analysis showed that acetylated HSP90 binding to LCK was enhanced in ACY-incubated Jurkat cells compared with vehicle-incubated Jurkat cells. In addition, expression of HSP90 binding to LCK and total expression of LCK were not altered by ACY incubation (Fig 7C). Taken together, these results suggest that HDAC6 inhibition using ACY disrupts LCK phosphorylation via the acetylation of HSP90. This epigenetic modulation of HSP90 via HDAC6 inhibition leads to the inhibition of ZAP70 and LAT phosphorylation. TCR signal transduction activates mitogen-activated protein kinases (MAPKs) including ERK1/2, p38, and JNK 1/2. This results in the induction of cell proliferation and the upregulation of inflammatory cytokines including IL-2 and IFN- γ (17). Western blot analysis showed that JNK1/2 and p38 phosphorylation induced by anti-CD3 Ab stimulation but not ERK were inhibited in ACY (1 μ M) -incubated Jurkat cells compared with in vehicle-incubated Jurkat cells (Fig 7B). These results indicate that HDAC6 inhibition modulates JNK1/2 and p38 activity without affecting ERK activity, thus contributing to the inhibition of cell proliferation and the upregulation of IL-2 and IFN- γ expression. Furthermore, ACY inhibited LCK and ZAP70 phosphorylation of OT-I cells stimulated with SIINFEKL peptide-loaded splenocytes *in vitro* (Fig 7D). To further examine whether ACY inhibits LCK and ZAP70 phosphorylation of OT-I cells *in vivo*, SDLNs OT-I cells from K14-mOVA mice transferred with OT-I cells 5 days earlier were examined. ACY (75 mg/kg, twice daily) was administered to the mice on day 4 after OT-I cell transfer. ACY inhibited LCK and ZAP70 phosphorylation of OT-I cells *in vivo* (Fig 7E).

Discussion

We have shown that ACY, an HDAC6-specific inhibitor, is a potent modulator of adoptive immunity using a CHS model and a GVHD-like model *in vitro* and *in vivo*. We have shown that ACY acts in the elicitation phase but not in the sensitization phase of CHS, indicating that HDAC6 inhibition may impair effector CD8 T-cells (rather than APCs of the skin, including keratinocytes, Langerhans cells, and dermal dendritic cells) during this phase of the CHS response (12). *In vitro* studies showed that ACY inhibited CD8 T-cell proliferation and IL-2 and IFN- γ upregulation induced by anti-CD3/CD28 Ab stimulation. Moreover, ACY inhibited effector T-cell proliferation induced by hapten-specific stimulation. These results indicate that HDAC6 inhibition is involved in the inhibition of effector CD8 T-cell proliferation and activation during the CHS response.

We also found that Tubacin, another specific HDAC6 inhibitor, attenuated CHS in the elicitation phase (Supplemental Fig 8A). Tubacin inhibited CD8 T-cell proliferation induced by anti-CD3/28 Ab stimulation without affecting cell viability *in vitro* (Supplemental Fig 8B). However, 4 μ M Tubacin was required to inhibit CD8 T-cell proliferation induced by a-CD3/28 stimulation, while 1 μ M ACY inhibited the CD8 T-cell proliferation (Fig 2B), indicating that the bioavailability of ACY may be greater than that of Tubacin. Furthermore, Tubacin is not optimized for oral delivery and cannot be tested in clinical trials.

We also utilized a murine model of CD8 T-cell-mediated GVHD-like disease. We previously showed that the transition to effector OT-I cells from naïve OT-I cells is required for the development of GVHD-like disease that is governed by IFN-regulatory factor (IRF)-8 (6). ACY administration to K14-mOVA mice transferred with OT-I cells 1) prevented the onset and 2) attenuated the progression of GVHD-like disease. Flow cytometric analysis revealed that ACY administration reduced the percentage of OT-I cells proliferating in SDLNs of K14-mOVA mice and modulated expression of activation markers on the OT-I cells, indicating that HDAC6 inhibition interferes with the transition of effector OT-I cells from naïve OT-I cells *in vivo*. These results suggest that HDAC6 inhibition act on antigen-specific proliferative CD8 T-cells expressing high levels of HDAC6 *in vivo*.

Since low-dose vorinostat (SAHA) (35 mg/kg or 40 mg/kg, once daily, i.p.) has also been shown to exert immuno-suppressive effects with anti-pan HDAC activity resulting in ameliorating GVHD and anti-CD3 antibody-induced cytokine storm in murine models (1, 2), we examined whether the SAHA administration affected the development of CHS and GVHD-like disease. Interestingly, we found that the SAHA administration did not prevent the development of CHS (Supplemental Fig 2C and 2D) or GVHD-like disease (Supplemental Fig 5C and 5D). According to Reddy et al (1), SAHA administration does not inhibit donor T-cell proliferative and cytotoxic responses to host antigens in their murine GVHD model, implying that the epigenetic modulation via the broad HDACs (including HDAC6) inhibition by SAHA may preserve the T-cell functions. In contrast, we have shown that the specific inhibition of HDAC6 using ACY-1215 ameliorates the development of CHS and GVHD-like disease via impairment of CD8 T-cell functions. Therefore, our data suggest that HDAC6 may have a critical role in the activation and functions of effector CD8 T-cells and the specific inhibition of HDAC6 using ACY-1215 may be more useful in the treatment of CD8 T-cell-mediated diseases compared with broad-spectrum HDAC inhibitors including SAHA.

In examining the molecular basis for the impairment of T-cell activation and functions caused by HDAC6 inhibition, we found that HDAC6 inhibition by ACY abrogated LCK, ZAP70, and LAT phosphorylation induced by anti-CD3/CD28 Ab stimulation. ZAP70 and LAT phosphorylation are dependent on LCK (14); therefore, we focused on how LCK was modified by HDAC6 inhibition. Western blotting and immunoprecipitation analysis revealed that HDAC6 inhibition by ACY enhanced acetylated HSP90 binding to LCK. Because it has been reported previously s that (i) chaperone function of HSP90 is required for LCK phosphorylation (15), (ii) pharmacological inhibition of HSP90 functions using geldanamycin, an inhibitor against HSP90 activity, prevents LCK phosphorylation in T-cells (18), and (iii) HDAC6 inhibition increases acetylation of HSP90 and disrupts HSP90 chaperone function (16), our results suggest that HDAC6 inhibition by ACY may disrupt LCK phosphorylation via acetylation of HSP90 (i.e., epigenetic modification of HSP90 via HDAC6 inhibition), leading to TCR signaling pathway impairment. To our knowledge, the blockade of LCK–ZAP70–LAT activity via specific HDAC6 inhibition has not been previously identified.

Taken together, we have shown that ACY, an HDAC6-specific inhibitor, is useful in the treatment of CD8 T-cell-mediated murine models of skin diseases such as CHS and GVHD-like disease. Clinical trials for the treatment of multiple myeloma using ACY are ongoing. To date, adverse events have been limited to grades 1 and 2 (19).

Effector CD8 T-cells play an important role in the development of human diseases such as GVHD (20), vitiligo (21), alopecia areata (22); therefore, HDAC6 may be a novel target to treat patients with CD8 T-cell-mediated diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

We thank Jay T. Linton (NCI, NIH) for his outstanding technical support, and Hong Zhang (NCI, NIH) for advice.

A declaration of all sources of funding for the research

This work was supported by the Center for Cancer Research of the National Cancer Institute, NIH.

Abbreviations

α-tub	α -tubulin
CHS	Contact hypersensitivity
CFSE	Carboxyfluorescein succinimidyl ester
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FITC	Fluorescein isothiocyanate
FYN	Proto-oncogene tyrosine-protein kinase
JNK	c-Jun amino terminal kinase
LAT	Linker-for-activation of T-cells
LCK	Lymphocyte-specific protein tyrosine kinase
LN s	Lymph nodes
MAPK	Mitogen-activated protein kinase
PEG	Polyethylene glycol
SAHA	Suberoylanilide hydroxamic acid
WT	Wild type
ZAP70	Zeta-chain-associated protein kinase 70

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Clinical implications

Histone deacetylase 6 may be a novel target to treat GVHD and contact hypersensitivity. Its inhibition by ACY-1215 may be a useful therapeutic agent for patients with CD8 T-cell-mediated diseases.

Fig 1. HDAC6 inhibition using ACY attenuates the CHS response in the elicitation phase Increase in ear thickness (A and B). B6 (A) or Balb/c (B) mice were sensitized and elicited by TNCB with or without ACY as indicated. Histology (C) and IFN- γ mRNA expression (D) of ears of the B6 mice treated with TNCB. ** $P < .01$. (n = 5)

Fig 2. ACY inhibits proliferation induced by hapten-specific stimulation and IL-2 and IFN- γ upregulation induced by anti-CD3/28 Ab *in vitro*

Hapten-sensitized T-cells stimulated by hapten-loaded splenocytes for 48 hours (A). Naïve CD8 T-cells stimulated by anti-CD3/28 Ab for 48 hours (B) or 3 hours for IL-2 and IFN- γ mRNA fold changes (C and D) ***P < .01. (n = 3)

Fig 3. ACY prevents and ameliorates GVHD-like disease

Photographs (A) and histology of ears (B) of K14-mOVA mice on day 14 after OT-I cell transfer. The curve shows percentages of weight loss. ACY was started on the day of transfer (C), or 8 days after transfer of OT-I cells (D). (n = 5). **P < .01 and *P < .05.

Fig 4. ACY inhibits proliferation and activation of OT-I cells *in vivo*

Proportion of OT-I cells in SDLNs of K14-mOVA mice 5 days (A) and 11 days (B) after OT-I cell transfer. Activation markers of OT-I cells in SDLNs (C) and serum IL-2 and IFN- γ concentration (D) of K14-mOVA mice 5 days after OT-I cell transfer. (n = 6). **P < .01.

Fig 5. ACY abrogates OT-I cell proliferation and activation induced by anti-CD3/CD28 Ab and antigen-specific stimulation *in vitro*

OT-I cells were stimulated by anti-CD3/CD28 Ab (A) or SIINFEKL peptide-loaded splenocytes (B) for 48 hours. The bars present optical densities (OD). $**P < .01$. CFSE staining (C) and activation markers (D) of OT-I cells stimulated with SIINFEKL for 48 hours ($n = 3$).

Fig 6. ACY inhibits IL-2 and IFN-γ upregulation induced by antigen-specific stimulation in OT-I cells *in vitro*

IL-2 and IFN-γ mRNA fold changes in OT-I cells stimulated with SIINFEKL-loaded splenocytes for 3 hours (A and C) or indicated times (B and D). ** $P < .01$. (E) IL-2 and IFN-γ production of OT-I cells stimulated (24 hours) by SIINFEKL peptide-loaded splenocytes. (n = 3)

Fig 7. ACY impairs LCK phosphorylation and enhances acetylated-HSP90 binding to LCK
 Phosphorylation of TCR molecules (A), or MAPKs (B) in Jurkat cells. Enhanced acetylated-HSP90 binding to LCK in Jurkat cells (C). LCK and ZAP70 phosphorylation of OT-I cells stimulated (24 hours) by SIINFEKL *in vitro* (D) or of OT-I cells in SDLNs from OT-I cell transferred K14-mOVA mice (n =3). I.P., immunoprecipitation; W.B., Western blotting