Histone Deacetylase Inhibitors for Treating a Spectrum of Diseases Not Related to Cancer

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This issue of *Molecular Medicine* contains 14 original research reports and state-of-the-art reviews on histone deacetylase inhibitors (HDACi's), which are being studied in models of a broad range of diseases not related to the proapoptotic properties used to treat cancer. The spectrum of these diseases responsive to HDACi's is for the most part due to several antiinflammatory properties, often observed *in vitro* but importantly also in animal models. One unifying property is a reduction in cytokine production as well as inhibition of cytokine postreceptor signaling. Distinct from their use in cancer, the reduction in inflammation by HDACi's is consistently observed at low concentrations compared with the higher concentrations required for killing tumor cells. This characteristic makes HDACi's attractive candidates for treating chronic diseases, since low doses are well tolerated. For example, low oral doses of the HDACi givinostat have been used in children to reduce arthritis and are well tolerated. In addition to the antiinflammatory properties, HDACi's have shown promise in models of neurodegenerative disorders, and HDACi's also hold promise to drive HIV-1 out of latently infected cells. No one molecular mechanism accounts for the non–cancer-related properties of HDACi's, since there are 18 genes coding for histone deacetylases. Rather, there are mechanisms unique for the pathological process of specific cell types. In this overview, we summarize the preclinical data on HDACi's for therapy in a wide spectrum of diseases unrelated to the treatment of cancer. The data suggest the use of HDACi's in treating autoimmune as well as chronic inflammatory diseases.

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INTRODUCTION

In this issue of *Molecular Medicine*, we assembled a series of articles on the use of histone deacetylase (HDAC) inhibitors (HDACi's) in the treatment of a broad spectrum of diseases not related to cancer. Presently, the spectrum of diseases being evaluated are mostly inflammatory or autoimmune in nature. However, orally active drugs that are safe and antiinflammatory are ideal for treating chronic degenerative diseases (1). The *in vitro* and *in vivo* studies of HDACi's in models of inflammatory and autoimmune diseases have required doses considerably lower than the concentrations of HDACi's that are required to bring about the death of malignant cells *in vitro* and in tumor-bearing mice. This characteristic of an effective low dose suggests that the traditional mechanism of action of HDACi's (that is, hyperacetylation of nuclear histones with increased expression of proapoptotic genes) may not account for the antiinflammatory properties of HDACi's. Acetylation of cytoplasmic proteins may explain the unique properties of low doses of HDACi's. In this issue of *Molecular Medicine*, each contribution considers the amelioration of disease severity in a specific pathological model and proposes the likely mechanism of action of HDACi's.

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As cytokine-driven inflammation plays a fundamental role in nearly all acute as well as chronic diseases, targeting cytokines in metabolic, neurodegenerative, cardiovascular, bowel and joint diseases has become a major area of investigation (1). The importance of cytokines in the pathogenesis of these diseases has been validated by the widespread use of anticytokine monoclonal antibodies and now includes Type 1 and 2 diabetes, as reviewed by Mandrup-Poulsen *et al.* (2), Donath and Shoelson (3), and Dinarello *et al.* (4). The attractive aspect of HDACi's is that they are orally active, and low concentrations are most effective in reducing inflammation in humans (5) and animal models (6).

The success of HDACi's in the treatment of inflammatory diseases will depend on two factors: lack of organ toxicity and tolerability as well as the specificity of the inhibitor for the relevant HDAC in a particular pathological

process. Indeed, the future development of HDACi's should be focused on selective inhibitors, since there are 18 distinct HDACs (7). It is also expected that inhibition of specific HDACs will offer optimal efficacy depending on the dominant cell type in a particular disease, for example, reducing interferon (IFN)-γ production by a specific HDAC in T cells. For inflammatory diseases such as gouty arthritis or Type 2 diabetes, inhibition of interleukin (IL)-1β production or secretion would best use an HDACi that regulates caspase-1.

HISTORICAL BACKGROUND

HDACi's were initially studied for their ability to increase gene expression. Phenylbutyrate was used for many years to increase gene expression of fetal hemoglobin in patients with a genetic basis for anemia. Phenylbutyrate also has an excellent safety record. Oral phenylbutyrate was used in children and adults with sickle cell disease. Despite the increment in hemoglobin F and reticulocytes, the use of 30–40 capsules a day is not an optimal therapy (8,9). Today, however, the increasing number of orally active, synthetic HDACi's are primarily developed to treat cancer. The development of HDACi's for treatment of cancer is based on de-repression of genes that participate in endogenous proapoptotic pathways and bring about a selective death of malignant cells while sparing healthy cells. By use of this mechanism, HDACi's would avoid the toxic effects of many chemotherapeutic drugs. It was Paul Marks and his coworkers that brought the first HDACi to be approved, suberoylanilide hydroxamic acid (SAHA). SAHA (generically vorinostat) and romidepsin (FK228) are approved for the treatment of cutaneous T-cell lymphoma (CTCL). Similar to other HDACi's, SAHA and FK228 increase several genes that induce apoptosis in malignant cells and are consistent with the well-studied mechanism of all HDACi's (that is, hyperacetylation of nuclear histones).

With hyperacetylation of nuclear histones, chromatin unravels and transcription factors can now bind to DNA and initiate the synthesis of RNA coding for proapoptotic genes. For the most part, the doses of vorinostat and romidepsin used to treat humans with cancer are based on maximal tolerated doses. It is assumed that plasma levels reach those concentrations observed for increased expression of proapoptotic genes in tumor cells *in vitro*. Knowing the concentration of a particular HDACi for inducing apoptotic cell death in primary tumor cells *in vitro* is often not possible, and the success of any HDACi in the treatment of humans with cancer is thus measured by a reduction in the tumor burden. However, in terms of the antiinflammatory properties of HDACi's, the inhibitors are studied in primary cells *in vitro*. As stated, the most distinguishing property of the antiinflammatory properties of HDACi's is that significantly lower concentrations are used in models of autoimmune diseases compared with those used to reduce tumors in mice. A similar example exists for the inhibition of cytokines *in vitro*. For example, the 50% inhibition of proliferation of A549 tumor cells requires 5 μmol/L, whereas to inhibit 50% of IL-1β secretion requires 50 nmol/L in freshly obtained human peripheral blood mononuclear cells (PBMCs) (10), and similar data have been reported for ITF2357 (11). Thus, when considering the efficacy of an HDACi to treat a cytokine-driven inflammatory disease, it is possible to draw some conclusions that the *in vivo* efficacy is related to an *in vitro* concentration and, because of the lower concentrations, more likely to be achieved *in vivo*.

As discussed in the various reports in this issue, HDACi's also hyperacetylate nonhistones such as cytosolic proteins, including transcription factors themselves. Because HDACs deacetylate the highly conserved *N*-terminal lysines present on histones, it is also appropriate to use KDACi's for lysine deacetylase inhibitors. In fact, it is likely that some mechanisms by which HDACi's are effective in reducing disease severity in animal models of inflammation and immune disorders are due to acetylation of cytoplasmic proteins and not nuclear histones, although hyperacetylation of both likely take place at the same time. Complexes of HDAC with histones as well as cytoplasmic proteins in the same cell challenges the concept that targeting specific HDACs with small-molecule HDACi's may not result in the same effect or results in the same therapeutic advantage.

In general, HDACs are nonredundant. A knockdown of class I HDACs with siRNA resulted in cellular changes similar to those observed with small-molecule HDACi's (12). In mice deficient in HDAC-6, there is hyperacetylation of HDAC-6, but otherwise the mice are healthy. Mice with null mutations for HDAC-1 do not progress beyond embryonic stages (13), whereas mice deficient in HDAC-2 succumb to an early death because of hyperproliferation of cardiomyocytes (13). For example, mice deficient in HDAC-2 die with cardiomyopathy. HDAC-8-deficient mice die soon after birth because of malformations in calvarial and facial bones (14). A null mutation in HDAC-3 in the liver disrupts normal metabolic homeostasis (15). The deletion of HDAC-8 as with other HDAC deletion studies suggests that HDAC functions to suppress either the ability of particular transcription factors active during embryonic development or the expression and synthesis of other transcription factors, which serve to suppress genes. Silencing of HDAC-1, HDAC-2 or HDAC-3 by using siRNA decreased IFNγ-inducible genes, whereas overexpression of these same HDACs increased signal transducers and activators of transcription (STAT)-1-dependent genes (16).

HDACi's REDUCE THE PRODUCTION OF PROINFLAMMATORY CYTOKINES

For reducing disease severity in several immune and inflammatory conditions, synthetic glucocorticoids remain the mainstay of therapies. However, with

specific antibodies to cytokines such as tumor necrosis factor (TNF)-α, IL-1β, IL-6 receptors and others, anticytokine-based therapies have found a place in the treatment of autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, psoriasis and several others. Reducing the activity of IL-1β has had a major impact on the treatment of autoinflammatory diseases (17). Without question, neutralization of specific cytokines has canonized their causative role in inflammation and has changed the lives of millions of patients with these diseases (1). Compared with the metabolic consequences of long-term glucocorticoids, anticytokine therapies are a major improvement in treating an increasing number of diseases, but parenteral administration is a major drawback of anticytokine therapy, and decreased host defense against infection is another. Nevertheless, anticytokine therapy is nearly devoid of organ toxicity. Because inflammation plays a fundamental role in nearly all chronic inflammatory and degenerative diseases, inhibitors of proinflammatory cytokine production as well as activities has become a major area of investigation for orally active drugs. As summarized in Table 1, orally active HDACi's are effective in reducing cytokines *in vitro* and in various animal models.

SAHA was developed to treat cancer, but in studies published in 2002, we added SAHA to freshly obtained human peripheral blood mononuclear cells (PBMCs) stimulated with lipopolysaccharide (LPS) (10). There was 50% reduction in TNFα, IL-1β, IL-12 and IFNγ at 100–200 nmol/L, a 20-fold lower concentration that that required for inhibition of tumor cell proliferation *in vitro*. When stimulated with the combination of IL-12 plus IL-18, IFNγ was reduced by 85%. Steady-state mRNA levels for LPS-induced TNFα and IFNγ were markedly decreased, whereas levels for IL-8 and IL-1β mRNA were unaffected. Although the levels of the IL-1β precursor were not decreased, secretion of IL-1β was reduced at 100–200 nmol/L.

Table 1. Properties of HDACi's for treating disease not related to cancer.

Systemic inflammation
Reviewed in (79)
Improved survival after hemorrhage (89)
Decreased cytokinemia after systemic endotoxin (10,11,136)
Decreased NO production (6,10,32)
Protection against hepatitis after intravenous concanavalin A (11)
Suppression of vascular cell adhesion molecule-1 expression (137)
In vitro cytokine production
Decreased gene expression and synthesis of LPS-induced TNFα and IFNγ in human peripheral blood mononuclear cells (10,11,138)
Decreased IL-1β-induced IL-6 in synovial cells and human blood monocytes (23)
Reduced IFNγ in mouse trophoblasts (139)
Decreased production of IL-6 in PBMCs stimulated with IL-12 plus IL-18 (11)
Decreased secretion of IL-1β (10,11,52)
Decreased IL-12 and IL-17 production from monocytes and dendritic cells (10,20,50)
Decreased IL-6 from stromal cells (104)
Reduced IL-1β-induced PGE2 and NO in human chrondrocytes (140)
Decreased ischemia-induced retinal TNFα production (141)
Downregulation of IL-12 transcription in transformed lung epithelial cells (142)
Increased PGD2 production and suppression of matrix metalloproteinases (82)
Suppression of c-jun and transcription of COX-2 (83)
Models of inflammatory bowel disease
Reviewed in (74)
Reduced disease severity in dextran sodium sulfate and trinitrobenzene sulfonic acid (TNBS) colitis (143,144)
Decreased cytokine levels in colitis model (143,144)
Reduced severity of colitis in HDAC-9-deficient mice (145)
Suppression of COX-2 activation in colon cells (146)
Immunosuppressive properties
Reviewed in (43,75,76)
Survival benefit in GvHD (71,72,147)
Sparing effect on graft versus leukemia (71)
Increased $Foxp3^+$ T-regulatory cells (148,149)
Reduced incidence of diabetes in NOD mouse (36)
Induction of antigen-specific anergy in lymphocytes (150)
Improved allograft transplantation (148,151)
Induction of IDO and inhibition of dendritic cell maturation (44)
Reduced nephritis in lupus-prone mice (119,120)
Decrease disease severity in experimental allergic encephalitis (76,152)
Suppression of Th1 polarization of murine dendritic cells (153)
Inhibition of IL-2 gene expression in T cells (154,155)
Reduced cytokine production in primary human T cells stimulated with anti-CD3/CD28 (156)
Inhibition of CD154 (CD40L) expression in T cells (157)
Models of arthritis
Reviewed in (25,26)
Reduced joint destruction in collagen-induced arthritis (25,158,159)
Decreased bone and cartilage loss (25)
Lower cytokine and chemokine levels (23,24)
Decreased synovial cell proliferation (160)
Abrogation of TGFβ-1-induced fibroblast-myofibroblast differentiation (161)
Inhibition of IL-1β-induced matrix metalloproteinase expression in human articular chondrocytes (162)

Continued

Table 1. Continued.

Heart failure
Reviewed in (63)
Reduced Hop-mediated hypertrophy and heart failure (163)
Decreased left ventricular cardiac hypertrophy (164)
Reduced cardiac arrhythmias in dystrophic mice (165)
Brain and neurological systems
Reviewed in (77) and (76)
Improved neurological recovery after closed head trauma (93)
Reduced gliosis and neuronal apoptosis (93,166)
Decreased neuroinflammation in glial cells (41)
Reduced brain infarct after cerebral artery occlusion (115,167,168)
Decreased disease severity in models of Huntington disease (96,97,99)
Reduced loss of function in ALS model (169,170)
Effects in multiple sclerosis (76,152)
Models of diabetes
Reviewed in (34)
Decreased death in IL-1β-induced pancreatic insulin-producing β cells (6,32-35,171)
Decreased death in IL-1β-induced insulin-producing INS cells (32,34,35)
Protection from streptozotocin-induced diabetes (6)
Decreased islet cytokine production (6)
Inhibition of IL-1β-induced NO production by pancreatic islets (6,32,34,35)
Fibrotic diseases
Prevention of renal interstitial fibrosis after ureteral obstruction (172)
Decreased left ventricular cardiac hypertrophy (164)
Increased basic morphogenic protein 7 in the regenerative response to renal ischemia (173)
Reduced fibrotic changes in tubulointerstitial injury (174)
Inhibition of hypoxia-induced angiogenesis (175)
Decreased TGFβ-1-induced renal injury (176)
Suppression of epithelial-to-mesenchymal transition induced by TGFβ (177)
Muscular dystrophy
Reviewed in (78)
HIV-1 purging
Reviewed in (38)
In vitro studies (19,38,132,133)
Studies in humans (116,131)

A single oral dose of SAHA to mice before LPS dose-dependently reduced circulating TNFα, IL-1β, IL-6 and IFNγ. The effect of HDACi's on cytokine production was also studied with ITF2357 (generic givinostat) (11). In PBMCs, ITF2357 reduced by 50% the release of TNFα at concentrations of 10–20 nmol/L, the intracellular levels of IL-1α at 12 nmol/L, the secretion of IL-1β at 12.5–25 nmol/L and the production of IFNγ at 25 nmol/L. Similar to SAHA (10), there was no reduction in IL-8 in these same cultures. By using the combination of IL-12 plus IL-18, IFNγ and IL-6 production was reduced by 50% at 12.5–25 nmol/L. There was no evidence of cell death in LPS-stimulated PBMCs at 100 nmol/L ITF2357, by using assays for DNA degradation, annexin V and caspase-3/7. There was a 50% to 90% reduction in LPS-induced steady-state levels of TNFα and IFNγ but no reduction of IL-1β or IL-8 (11). Similar to SAHA, the secretion of IL-1β was reduced, suggesting that ITF2357 affects caspase-1 and proteins that are required for activation of caspase-1 (18). Although neither SAHA nor ITF2357 reduced the chemokine IL-8, ITF2357 did reduce surface CXCR4 and CCR5 expression on $CD4^+$ T cells and monocytes (19).

In related studies, human PBMCs were differentiated into dendritic cells, and the effect of the HDACi trichostatin A (TSA) as well as SAHA was investigated on the production of IL-12 and IL-17. There was a reduction of 86% and 83%, respectively, by SAHA. In these differentiated dendritic cells, the T-cell chemokines CXCL9, -10 and -11 were also reduced (20). Oral administration of 1.0–10 mg/kg ITF2357 to mice reduced LPS-induced serum TNFα and IFNγ by >50%. However, anti–CD3-induced cytokines were not suppressed by ITF2357 in PBMCs either *in vitro* or in the circulation in mice. In concanavalin-A–induced hepatitis, 1 mg/kg oral ITF2357 significantly reduced liver damage at the same level as 5 mg/kg. In mice subjected to streptozotocin-induced diabetes, an oral dose of 2.5 mg/kg provided protection (6). Thus, low, nonapoptotic concentrations of the HDAC inhibitor ITF2357 reduce proinflammatory cytokine production in primary cells *in vitro* and exhibit antiinflammatory effects *in vivo*. In contrast, treatment of murine macrophages with the histone deacetylase inhibitor LAQ824 induced chromatin changes in the *IL-10* gene promoter that results in recruitment of the transcriptional repressor HDAC11 (21). This result diminishes IL-10 production and induction of inflammatory cells. These results are inconsistent with the antiinflammatory properties of HDACi's.

Panobalinostat (LBH589) was tested for treatment of multiple myeloma and lymphomas. At clinically relevant concentrations added to cultured human dendritic cells (DC), LBH589 reduced the surface molecule expression on immature and mature DCs, which was associated with DC maturation, antigen presentation and T-cell costimulation (22). LBH589 also significantly reduced the production of TNFα, IL-6, IL-10, IL-12 and IL-23 stimulated with LPS. It was also reported that the RelB component of nuclear factor (NF)-κB had a critical role in the antiin-

flammatory and immunosuppressive mechanism of this HDACi (22).

Because there are 18 separate second coding for HDAC, the specificity of an HDACi for a particular HDAC or a cluster of related HDACs likely affects its mechanism of action for reducing inflammation via inhibition of cytokines. ITF2357, SAHA and generally all hydroxamate-based HDACi's do not inhibit sirtuins, 7 of the 18 known HDACs. Another consideration is the cell target being affected by HDAC inhibition. Although it can be assumed that all HDACi's enter the cell by a simple concentration gradient in that there are no specialized membrane channels required for entry, the antiinflammatory mechanism is cell specific for the disease model. For example, in rheumatoid arthritis the fibroblast-like synoviocyte plays an important role in the pathological processes of joint destruction. These cells produce several cytokines, both inflammatory as well as antiinflammatory, chemokines and prostaglandins. When stimulated with IL-1β, the fibroblast-like synoviocyte produces copious amounts of IL-6, a known growth factor for the invasive pannus of joints in rheumatoid arthritis. HDACi's reduce IL-1β-driven IL-6 production as well as reduce synovial cell survival (23). The mechanism for the reduction in IL-6 production is an accelerated degradation of IL-6 mRNA rather than inhibition of components of NFκB signaling (24). As reported in this issue, HDACi's suppress IL-1β-driven loss of bone and cartilage (25), and the targets are the chondrocyte and osteoclast, since these are key pathways for destructive joint disease in both rheumatoid arthritis as well as osteoarthritis (26).

HDACi's FOR INHIBITION OF CYTOKINE SIGNALING

Many studies on HDACi's focus on inhibition of LPS-induced cytokine production. Some of these reports are listed in Table 1. Although the LPS pathway as well as all Toll-like receptor (TLR) agonists are commonly studied with HDACi's, the signaling pathway is primarily in myeloid lineage cells such as monocytes and macrophages. These cells are, without doubt, major players in inflammation because of infections; but, most human disease is due to sterile inflammation. In the models of sterile inflammation, a hypoxic event is often the trigger and, with hypoxia, there is cell death and the release of cell contents. As such, cytokines and other intracellular components initiate the inflammatory trigger. For example, IL-1α and HMGB1 are inflammatory molecules that are released from dead cells and are highly inflammatory (27–29). In autoimmune diseases, the trigger for inflammation is not via the TLR pathway but rather via cytokines such as IL-17 (30). In Type 1 diabetes, IL-1β is highly toxic, inducing cell death of the insulin-producing β cells as well as nitric oxide (NO) (2). Similarly, IL-1β is toxic for the β cell in Type 2 diabetes (3). In bone loss, IL-1β and TNFα are the stimulators of osteoclast activation. Therefore, suppression of cytokine signaling may be the more relevant antiinflammatory mechanism for HDACi's rather than TLR-induced cytokines.

Diabetes

Because targeting IL-1β-mediated inflammation protects islets in human trials (31,32), the use of oral HDACi's to target islet inflammation should be considered for both Type 1 and Type 2 diabetes. *In vitro* HDACi's reduced cytokine-mediated NO formation and the decline in insulin secretion in isolated rat islets (32,33). Whereas the initial studies on the antiinflammatory effects of HDACi's focused on a reduction in the production of IL-1β, TNFα, IFNγ and other proinflammatory cytokines from LPS-stimulated freshly obtained human PBMCs, other investigators examined the ability of HDACi's to inhibit cytokine-induced effects. If HDACi's are to be used to treat a broad spectrum of inflammatory as well as autoimmune diseases, cytokine-driven inflammation is more relevant than inflammation driven by microbial products. Indeed, sterile inflammation is certainly the dominant cause of disease, particularly cardiovascular disease. For example, safe and specific antiinflammatory agents are sought for the prevention of cytokine-induced destruction of pancreatic islet β cells. The immune-mediated elimination of pancreatic β cells in Type 1 diabetes involves release of cytotoxic cytokines such as IL-1β and IFNγ, which induce β-cell death *in vitro* by mechanisms that are both dependent and independent of NO (2). SAHA (10) inhibits IL-1β-induced NO in mouse macrophages and also in rat primary islet cells and the INS insulinoma cell line (32). SAHA reduced the cytokine-mediated decrease in insulin secretion and increase in iNOS levels, NO formation and apoptosis (32). A similar effect of ITF2357 was observed on IL-1β-stimulated mouse and rat primary islets and the INS cell line (6). Although there was no effect of SAHA on IL-1β-mediated degradation of IκB or binding of NFκB to DNA, SAHA reduced the second phase of NFκB phosphorylation (32). As reviewed in detail, HDAC inhibition prevents cytokine-induced β-cell apoptosis and impaired β-cell function (34).

In vivo, during streptozotocin-induced β-cell destruction, ITF2357 protected the insulin-producing β cell following an oral dose of 1.25–2.5 mg/kg (6). Serum NO levels returned to nondiabetic values, islet function improved and glucose clearance increased. *In vitro*, at 25 and 250 nmol/L, ITF2357 increased islet cell viability, enhanced insulin secretion, inhibited chemokine production and reduced IL-1β-induced iNOS levels. In peritoneal macrophages and splenocytes, ITF2357 inhibited the production of NO, as well as that of TNFα and IFNγ at a 50% (median) inhibiting concentration IC_{50} of 25–50 nmol/L (6). Thus, at clinically relevant doses, the orally active HDAC inhibitor ITF2357 favors β-cell survival during inflammatory conditions.

The nonobese diabetic (NOD) mouse is often used as the model for Type 1 diabetes in humans. Because the HDACi's SAHA, ITF2357 or TSA protect the insulin-producing pancreatic β cell in primary rat islets (6,32,33,35), in primary

mouse islets (6) or the rat insulinoma cell line (6,32,35), it was not unexpected that inhibition of HDACs by TSA administered once a week was reported for reducing the incidence of diabetes that consistently develops in these mice (36). Also, not unexpectedly, there was a reduction in cellular infiltration of islets with a return of normoglycemia. Although these studies demonstrate a beneficial effect of TSA, it was reported that splenic T lymphocytes derived from protected mice resulted in enhanced expression of IFNγ mRNA and protein (36), and there were no changes in the expression of inducible NO, IL-17 or TNFα. These findings are highly inconsistent with the known beneficial effects of suppression of IFNγ activities, not only for the NOD mouse (37) but also for the property of HDACi's to reduce IFNγ production (6,10,11) and also on the property of HDACi's to suppress STAT-1 activities (16). Moreover, the combination of IL-1β plus IFNγ is highly toxic to the β cell and is prevented by SAHA and ITF2357 (6,32).

EFFECT OF HDACi's ON CHEMOKINE RECEPTORS

In several models of inflammation, infiltration of neutrophils is characteristic and the chemotactic chemokine IL-8 plays an important role. However, despite the suppression of TNFα and IFNγ gene expression in PBMCs stimulated with LPS in the presence of SAHA (10) or ITF2357 (11), there is no reduction in *IL-8* gene expression or protein levels. Rather, it appears that HDACi's reduce chemokine receptors. PBMCs from seven healthy donors were incubated in the presence of ITF2357 or valproic acid, and after 4 and 24 hours, there was a decrease in CCR4 of 47% ($P < 0.001$) and 54% ($P < 0.001$), respectively, at therapeutic concentrations (38). Consistent with the reduction of surface CXCR4 expression, steady-state mRNA levels of CXCR4 after 2 and 4 hours, as measured by RT-PCR, were reduced by 65% in cultures treated with 250 nmol/L ITF2357 compared with control cultures. There was also a marked decreased expression of CCR5 on CD14-positive blood monocytes (38). Others have reported that HDACi's decrease CXCR4 protein and mRNA levels in leukemia cell lines and lymphoblasts from patients with childhood acute leukemia (39).

MOLECULAR MECHANISMS OF ACTION OF HDACi's RELATED TO ANTIINFLAMMATORY PROPERTIES

Each of the 14 reports in this issue proposes a molecular mechanism for the ability of HDACi's to reduce the severity of disease in animal models or in cultured cells. There is no single unifying molecular mechanism. As reviewed by Christensen *et al.* (34), NFκB received a great deal of attention for its role in cytokine induction of cytokines. If one examines the effects of IL-1β on the insulin-producing pancreatic β cell, >2,000 genes are modified (34). Since HDACi's reduce the effects of NFκB activity-dependent genes, for example IL-1-induced IL-6 or inducible NO synthetase (35), it seems logical that the primary mechanism of action is inhibition of NFκB functions. However, although several reports indicate that HDACi's do reduce NFκB activity, electrophoretic mobility shift assay did not reveal such a mechanism in IL-1β-exposed cells in the presence of SAHA or TSA (32). The failure to observe a reduction in NFκB activity was also reported for primary human synovial fibroblasts stimulated with IL-1β for the induction of IL-6 (24). IL-1β induces a biphasic phosphorylation of inhibitor protein IκBα with the second peak reduced by HDACi's (32). However, no effect was observed on IκBα degradation or NFκB binding to DNA. Thus, it appears that HDAC inhibition prevents the cytokine-mediated effects by downregulating NFκB transactivating activity (40). In non–β cells, IFNγ-induced JAK activation and STAT-1 activity depends on HDAC-1, -2 and -3 activity (16).

Some studies demonstrate that HDACi's prevent the phosphorylation of IκB with downstream suppression of gene expression after TLR or cytokine stimulation. For example, IL-1β-induced phosphorylation of IκB is reduced by SAHA, suggesting that the antiinflammatory properties of HDACi's may by due to reduced NFκB activity (32,35). As discussed below, other studies suggest that HDACi's hyperacetylate NFκB. Another possible mechanism is the ability of HDACi's to acetylate STAT-3. SAHA increases histone H3 acetylation in the normal mouse brain accompanied by increased expression of the neuroprotective proteins Hsp70 and Bcl-2.

Because HDACi's increase gene expression, one possible mechanism of action is the induction of genes that are themselves inhibitors of inflammation, for example, induction of transforming growth factor (TGF)-β, IL-10 or IL-1 receptor antagonist. Suppressors of cytokine signaling-1 and -3 (SOCS1/3) have broad antiinflammatory and immunosuppressive properties, and HDACi's increase the expression of SOCS-3 (41). Other studies show that HDACi's impair the binding of c-Fos and c-Jun to DNA, thus inhibiting gene expression induced by activator protein-1 (AP-1).

Histone deacetylase 6 (HDAC-6) is a mostly cytoplasmic class II HDAC that has a unique structure with two catalytic domains and a domain-binding ubiquitin with high affinity. This HDAC acetylates α-tubulin and heat shock protein (Hsp)-90. HDAC-6 was targeted by homologous recombination, and HDAC-6-deficient mice are viable, are fertile and show hyperacetylated tubulin in most tissues. Lymphoid development is normal, but the immune response is moderately affected. Furthermore, the lack of HDAC-6 results in a small increase in cancellous bone mineral density. Thus, these data demonstrate that mice survive well without HDAC-6 and that tubulin hyperacetylation is not detrimental to normal mammalian development.

Protein lysine acetylation is a post-translational mechanism involved in all aspects of cell homeostasis. Indeed, a growing number of nonhistone proteins are targets of histone acetyltransferase (HAT) and HDAC. Acetylation affects

thousands of cellular proteins, both cytoplasmic and nuclear (42). Among these proteins, transcription factors are broadly involved in the molecular mechanisms influenced by HDACi's. An example of acetylation of a transcription factor by HDACi's is STAT-3, as reviewed by Choi and Reddy (43). Both ITF2357 and SAHA induce the indoleamine 2,3-dioxygenase (IDO), which prevents dendritic cell maturation and is regulated by STAT-3 (44). STAT-3 suppresses transcription of IFNγ and IL-17 and increases production of IL-10. Individuals with a mutation in STAT-3 suffer from hyper IgE syndrome, resulting in failure to control inflammation; disease severity in these patients is consistent with studies showing that STAT-3 suppresses cytokines. Activation of STAT-3 can take place by acetylation, and acetylated STAT-3 enhances STAT-3 dimer and thereby activity. ITF2357 and SAHA induce acetylation of STAT-3 in dendritic cells at concentrations that are achievable in humans (44).

As a posttranslational mechanism, the effects of lysine acetylations on cell functions are broad. Over 3,600 lysine acetylation sites were identified on 1,750 proteins, and the acetylations due to SAHA and MS-275 were studied (45). It was reported that lysine acetylation preferentially targets large macromolecular complexes and that acetylation impaired phosphorylation-dependent interactions (45). The reversible acetylation of key lysine residues plays a fundamental role in the function of NFκB (46). The interaction of NFκB (47,48) with acetyl transferase and deacetylase enzymes has many functional consequences. Reversible acetylation of distinct lysine residues has both positive and negative regulatory effects on nuclear NFκB activities, including transcriptional activation, DNA binding affinity, IκB association and subcellular localization. Since NFκB plays a major role in orchestrating the inflammatory response, its regulation by acetylation has been the subject of various studies aimed at elucidating the antiinflammatory mechanism of HDACi's. Chen *et al.* showed that histone deacetylase 3 deacetylates lysine 218 and 221 of p65 and promotes its high affinity binding to IκBα in the nucleus. This complex is rapidly exported from the nucleus to reestablish the latent cytoplasmic pool ready to be activated upon a new stimulation. Maintenance of lysines 218 and 221 in an acetylated state prolongs the nuclear activity of NFκB. Thus, from this study, it seems that preserving HDAC3 activity in the nucleus would lead to a blockade of NFκB activity, in apparent contradiction with the effect exerted by pan-HDACi's on proinflammatory response.

The contradictory effect of HDAC inhibition on the activity of NFκB is also evident from the work of Kiernan *et al.* (49), who showed that, in a different cellular system, the acetylation of p65 took place on different lysine residues, namely Lys122 and Lys123. Moreover, the acetylation of p65 reduced its ability to bind DNA and facilitated its removal from DNA and consequently its IκB-mediated export from the nucleus. HDAC-3 was shown to be the main isoform able to deacetylate p65, but in this case, inhibition of HDAC-3 would lead to repression of NFκB activity. Inhibition of NFκB is not a necessary property for an HDACi to be antiinflammatory. In murine dendritic cells, TSA and vorinostat strongly reduced the late expression of TNFα but did not affect IL-1β production or mitogen-activated protein kinase activation (50).

Inhibition of proinflammatory responses induced by Toll-like receptor activation by HDACi's certainly relies on multiple targets, but strongly depends on cell type and stimulation. The status of the same gene may differ from one cell to another, and its activation or inhibition may take place with different kinetics. The TLR4 signaling complex activates three main intracellular pathways: the NFκB signaling pathway, interferon-related factor (IRF) signal transduction pathways and the mitogen-activated protein kinase (MAPK) cascade. Inhibition of the latter with p38 kinase inhibitors has been proven to strongly attenuate the proinflammatory innate immune response. As expected, the MAPK cascade is regulated at multiple levels, one of which relies on the deactivation of its components by specific phosphatases. MAPK phosphatases (MKPs) are dual-specificity phosphatases that inactivate MAPK members by dephosphorylating phosphotyrosine and phosphothreonine residues. In a recent report, Cao *et al.* (51) demonstrated that MKP-1 is subjected to regulation by reversible acetylation. Acetylated MKP-1 has enhanced binding to p38 and phosphatase activity, thereby reducing the level of active phosphorylated p38. The direct consequence of the increased activity of MKP-1 is the dampening of TLR-mediated inflammatory response. Inhibition of HDAC activity by TSA reduced the expression of TNFα, IL-6, and IL-1β in LPS-stimulated macrophages in an MKP-1-dependent manner (51). In the same study, the authors also showed the inhibition of NOS2 in Raw cells by TSA. NOS2 is a transcriptional target of NFκB, which was not influenced by TSA treatment.

The role of the cytoplasmic HDAC-6 in the regulation of inflammatory response was investigated on the basis of the rationale that its inhibition would lead to an altered microtubule network and reduced secretion of proinflammatory cytokines such as IL-1β (52). Although the necessity of a functional cytoskeleton and microtubule network was established in this work, no clear relationship between HDAC-6 inhibition and IL-1β secretion was found. The effect of HDAC inhibition on IL-1β secretion is relevant in that activation of caspase-1 via the inflammasome is required for the maturation of IL-1β (53). Thus, inhibition of gene expression of a critical component of the inflammasome could be envisaged as a mechanism responsible for the activity of HDACi's on IL-1β secretion. Moreover, acetylation of inflammasome components could be an important posttranslational mechanism involved in its assembly and activity.

HDAC-6 contributes to the regulation of TLRs and IL-1β-induced signaling. Once again, the activity of a deacetylase

enzyme (HDAC-6), instead of its inhibition, appears to be necessary to downmodulate the proinflammatory program induced by TLRs and the IL-1 receptor. The study showed that HDAC-6 and sequestosome 1 (SQSTM1) were required for MyD88 aggregation and lead to inhibition of TLR ligand-induced expression of IL-6 and NOS2 in RAW264.7 cells. HDAC-6 and SQSTM1 partially suppressed the activation of p38 and JNK, but they had no effect on degradation of IκB (54). According to these findings, inhibition of HDAC-6 would lead to enhanced MyD88 signal transduction activity. This result would be the logical consequence of the use of an HDAC-6 inhibitor, such as vorinostat, in a LPS-induced septic shock model. A recent report (55), showed that administration of vorinostat (50 mg/kg intraperitoneally) after a lethal dose of LPS significantly improved long-term survival and reduced the expression of TNFα and IL-6. The authors also observed that vorinostat reduced the increased expression of MyD88 caused by LPS administration and postulated that this may be the direct consequence of the decreased expression of MyD88-associated proinflammatory genes.

A large number of studies have shown that inhibition of HDAC activity leads to repression of different TLR-induced responses, mainly on the basis of analysis of the expression of proinflammatory cytokines. Two reports have analyzed the effect of HDAC inhibition using a more comprehensive approach on the basis of whole genome expression analysis. Brogdon *et al.* (56) showed that the potent pan-HDACi LAQ824 exhibited a rather selective activity on macrophages and dendritic cells stimulated with LPS. The inhibitor specifically inhibited DC-controlled T helper 1 (Th1) effector but not Th2 effector cell activation and migration. It also inhibited macrophage- and DC-mediated monocyte but not neutrophil chemotaxis.

Roger *et al.* (57) confirm the concept of HDACi's as antiinflammatory drugs through use of genome-wide microarray analyses of macrophages and DC stimulated with different TLR agonists. The study revealed a critical role for HDACs in the expression of host defense genes, including pattern-recognition receptors, costimulatory molecules, kinases, transcription regulators, complement factors, cytokines, chemokines and growth factors. The data suggest that HDACi's exert mainly an immunosuppressive effect but also possess immunostimulatory activities. Interestingly, they suggest a novel activity of HDACi's, namely the induction of the chromatin modifier Mi-2beta. Mi-2 is part of the nucleosome remodeling, histone deacetylation (NuRD) complex. The Mi-2β complex is selectively recruited along with the other complexes to the control regions of secondary response and delayed primary response inflammatory genes. In these complexes, the Mi-2β complex acts antagonistically to limit the induction of these two gene classes. Mi-2β/NuRD inhibits LPS-induced secondary and delayed primary cytokine production in macrophages and dendritic cells (58). Thus, HDACi's inhibit the expression of secondary response genes such as IL-12, IL-6 and inducible NOS, but have no effect or delayed effect on primary genes such as IL-1β and TNFα, respectively. Roger *et al.* also found that TSA increased histone H4 acetylation of IL-6 and IL-12 promoters, suggesting that there was no correlation between the status of histone acetylation and the observed downregulatory effect of TSA on gene transcription.

ARE HDACi's CONTRAINDICATED IN SOME DISEASES?

The third cause of death after cardiovascular disease and cancer is chronic obstructive pulmonary disease (COPD). Patients with COPD have a progressive, unrelenting course, and various therapies have not significantly reduced disease severity. Cytokines such as TNFα, IL-18 and IL-32 are thought to play a role in that these cytokines are elevated in lung tissue of smokers with COPD but not in smokers without COPD (59). Chronic inflammation is believed to result in the loss of elasticity of the lung to maintain the alveolar architecture. Alveolar cell death is also believed to contribute to the disease. In a large clinical trial in patients with COPD, histone HDAC expression and activity are reduced in the lung tissue (60). Mizuno *et al.* (61) explored whether HDAC inhibition causes lung cell apoptosis and emphysema in a rat model. Rats were treated with TSA. Chronic TSA treatment increased the alveolar air space area and the number of caspase-3–positive cells in rat lungs. TSA suppressed hypoxia-inducible factor-1α and vascular endothelial growth factor (VEGF) gene expression in rat lungs as well as cultured human pulmonary microvascular endothelial cells. The study suggests that administration of HDACi's may worsen the clinical course of COPD.

DOES THE USE OF HDACi's INCREASE THE RISK OF INFECTIONS?

The inhibition of a number of proinflammatory cytokines induced by microbial products through TLR's activation by HDACi's poses a question related to the effect of the use of these inhibitors on the occurrence of infections. Without a doubt, all biologics used in patients with various inflammatory diseases increase risk of infection. Anti-TNFα monoclonal antibodies (infliximab and adalimumab) and the soluble TNF receptor etanercept are associated with increased risk of opportunistic as well as routine infections and deaths (reviewed by Dinarello [1]). Blocking IL-1β with anakinra is not associated with increased opportunistic infections and is considerably safer. Some biologics are linked to progressive multifocal leukencephalopathy. At the present time, there are only two disease conditions that are being treated with HDACi's: vorinostat in graft versus host disease (GvHD) (43) and givinostat in systemic-onset juvenile idiopathic arthritis (SOJIA) (5). The GvHD study is underway and there are no data at this time, but there were no infections in the SOJIA patients treated for 12 weeks with givinostat. Moreover, these patients with

SOJIA were concomitantly treated with glucocorticoids at the same time. In the phase I trial of givinostat, the decrease in whole blood production of TNFα, IL-1β, IL-6 and IFNγ was transient after a single oral dose and returned to baseline levels within 12 hours. In contrast, antibodies to TNFα persist for several weeks and are more likely to suppress host defense than short-acting oral HDACi's (62).

HDACi's FOR HEART FAILURE

As reviewed in the report by McKinsey (63), there are several animal models that result in the physiological characteristics of human heart failure. Some are related to inflammation associated with cytokine-induced cell death and the loss of myocytes, particularly secondary to IL-1β (64,65). As a result, the ability of the heart to effectively pump blood out of its chambers is reduced, and the patients experience difficulties maintaining normal activities. Heart failure is an enormous economic burden on most societies, and with increasing numbers of patients with Type 2 diabetes and increased myocardial infarction, the incidence of heart failure increases each year. Having orally active HDACi's as antiinflammatory agents is an attractive therapeutic option. The concept that TNFα contributed to heart failure was based on two observations: levels of TNFα were elevated in the circulation of patients with stage III and IV New York Heart Association heart failure classification and treating heart failure patients with etanercept (a TNFα blocker) improved exercise tolerance (66). However, in two large studies, etanercept failed to provide improvement and infliximab actually increased mortality (67). Therefore, when there is use of HDACi's to combat heart failure, a reduction in TNFα levels is not considered beneficial.

On the other hand, studies have examined a role for IL-1β in the pathogenesis of heart failure. Serum levels of IL-1β in a large and representative population were associated with congestive heart failure ($P > 0.001$) and angina ($P = 0.02$), with Ca^{2+} serum levels ($P = 0.02$) and with a history of dyslipidemia ($P = 0.05$) (68). The data from humans as well as mice support the hypothesis from the large epidemiologic studies that IL-1β is mainly involved in the functional alterations of cardiomyocytes (69). It is unlikely that HDACi's will be used as a monotherapy in combating heart failure. As outlined by McKinsey (63), there are several drugs used to treat heart failure but few target inflammatory pathways. It is also likely that HDACi's may have an effect on cardiac remodeling independent of inflammatory cytokines.

JOINT DISEASES

In addition to cytokine-driven loss of the insulin-producing β cell, the chondrocyte is important in the production of proteoglycans for maintenance of cartilage integrity, but is a target for cytokine-driven destruction (70). Overexpression of IL-1β results in irreversible cartilage destruction. ITF2357 is protective on cartilage catabolism in a mouse model of nonautoimmune inflammatory arthritis induced by *Streptococcus pyogenes* cell walls. Administered orally at a dose of 1 and 10 mg/kg, ITF2357 suppressed joint swelling (25). As reviewed by Joosten *et al.* (25), there was an inhibition of chondrocyte proteoglycan synthesis of 44% with 1 and 10 mg/kg ITF2357. Thus, HDACi's may be used to preserve cartilage to protect against osteoarthritis. It is likely that modulation of IL-1β by HDACi's, as shown in several reports, results in reduced inhibition of chondrocyte proteoglycan synthesis and hence in a protective effect against cartilage catabolism during arthritis. As reviewed by Joosten *et al.* (25), ITF2357 also protects bone loss induced by IL-1β.

The proof of principle study to test the hypothesis that HDACi's would benefit joint disease can be found in the observations of the trial of givinostat (ITF2357) in children with SOJIA (5). ITF2357 is a hydroxamic acid–containing, orally active inhibitor targeting class I and II HDAC. The children received a daily dose of 1.5 mg/kg in two divided doses. At a dose of 1.5 mg/kg, the peak blood levels of ITF2357 were in the range of 125–200 nmol/L. There was no organ toxicity, and the study achieved significant ($P < 0.01$) reduction in parameters of systemic disease as well as the number of painful joints in over 75% of patients. At week 4, the American College of Rheumatology (ACR Pediatric) 30, 50 or 70 improvement was 77.8%, 55.6% and 22.2% and increased further to 77.8%, 77.8% and 66.7%, respectively, at week 12. The most consistent finding was the reduction in the number of active joints and/or joints with limited range of motion (5). Also, in that trial, there was a reduction in the white blood cell counts, the percentage of neutrophils, the levels of IL-1α and CD40 ligand as well as sedimentation rate and CRP (26). After 12 weeks, givinostat resulted in significant safety and benefits.

GRAFT VERSUS HOST DISEASE

After allogeneic bone marrow transplantation in mice, treatment with HDACi's provides a survival benefit in models of GvHD. Administration of SAHA after bone marrow transplantation reduced intestinal histopathology, clinical severity and mortality from acute GvHD (71). Moreover, in mice receiving lethal doses of tumor cells, administration of SAHA did not impair graft versus tumor activity and resulted in significantly improved tumor-free survival. Similar observations were reported for other HDACi's such as ITF2357 (44). The mechanism for these effects in GvHD may be due to decreased cytokine production, as was shown in those studies (71). An early step in models of GvHD is antigen maturation of dendritic cells. Dendritic cells exposed to either SAHA or ITF2357 increase the expression of IDO, a suppressor of dendritic cell functions (44). As reviewed by Choi and Reddy (43) in this issue, acetylation of STAT-3 activates its functions, and indeed HDACi's activate STAT-3 by acetylation (72). With the acetylation of STAT-3, there is downregulation of dendritic cells. Therefore, HDACi's likely serve several functions in the prevention of

GvHD. STAT-3 also promotes the transcription of IDO. Thus, acetylation of nonnuclear proteins such as STATs provides mechanistic insight into HDAC inhibition of cytokine production and immune cell functions (72). In terms of cellular mechanisms, inhibitors of HDAC increase Fox-p3–expressing T-regulatory cells, which contribute to the reduction of disease in autoimmune models. SAHA is presently in trials to prevent or reduce disease severity in GvHD in patients with bone marrow transplants. The topic of HDACi's in GvHD is reviewed in detail in this issue (43).

WHERE DO HDACi's FIT INTO THE CURRENT TREATMENT OF CHRONIC INFLAMMATORY DISEASES?

Joint, Bowel and Metabolic Diseases

As stated above, the antiinflammatory properties of HDACi's are based primarily on a reduction in either the production or the activity of inflammatory cytokines in animals as well as in primary cells at significantly lower concentrations that those used in tumor cells. If HDACi's are to be used for treating chronic inflammatory conditions, HDACi's should also inhibit the production or activities of mediators such as matrix metalloproteinases, cyclooxygenases, adhesion molecules and chemokines. As of this writing, the only clinical trial to support the concept that a low dose of HDACi's exhibits antiinflammatory properties is the trial of ITF2357 in children with SOJIA (5). ITF2357 (generic givinostat) is a hydroxamic acid–containing, orally active inhibitor targeting zinc-dependent HDAC isoform class I and II HDAC. On the basis of a reduction in the secretion of IL-1β, givinostat was also used to treat Schnitzler syndrome, as reported in this issue of *Molecular Medicine* (73). Vorinostat is presently in clinical trials in patients with GvHD (43), on the basis of animal models (44,71,72).

In addition to inhibition of the production of inflammatory cytokines, HDACi's also inhibit cytokine post-receptor signaling. For example, SAHA and ITF2357 reduce IL-1β-driven death of the insulin-producing β cell in primary rat and mouse islets and cell lines (6,32,35) as reviewed in this issue of *Molecular Medicine* (34). Similar studies were reported for inhibition disease in animal models of rheumatoid arthritis and osteoarthritis, as reviewed in this issue (25). At first glance, these are seemingly unrelated models. But on closer examination, each has inflammatory cytokines as part of the pathological mechanism. For example, there is a cytokine pathway in established models of inflammatory bowel disease (74), lupus nephritis (75), chronic heart failure (63), rheumatoid arthritis and osteoarthritis (25), multiple sclerosis (76) and GvHD (43). There is also a cytokine component in traumatic brain injury (77). However, it is unlikely that inflammation affects disease in muscular dystrophy (78).

Atherosclerosis

More than any other chronic inflammatory disease, atherosclerosis is the major cause of death in the developed world because of overnutrition and Type 2 diabetes; overnutrition is also a cause of Type 2 diabetes in developing countries. Because of their safety and ease of oral administration, most patients at high risk of a cardiovascular event are treated with a "statin," since these drugs have reduced the incidence of cardiovascular events and deaths. The beneficial view of statins is thought to be because of a combination of their property to reduce low-density lipoproteins (LDLs) as well as antiinflammatory agents. By comparison, HDACi's could be ideal for reducing inflammation in the atherosclerotic plaque. As pointed out in the review by Halili *et al.* (79), little is known about the effects of HDACi's on the atherosclerotic process. There are two mouse models of atherosclerosis: the apoenzyme E (APOE) and the LDL receptor–deficient mouse. To our knowledge, there is one study in which HDACi's was used to treat these mice and evaluate the effects.

LDL-deficient mice fed a high-fat diet for 4 and 8 weeks and treated with TSA, a specific histone deacetylase inhibitor for HDAC-1 and HDAC-2, revealed no reductions in atherosclerotic lesions (80). In fact, treatment with TSA worsened the disease. TSA was injected intraperitoneally every other day at 0.5 and 1.0 mg/kg and, as stated by the authors, at "doses used to treat tumors in mice" (80). The 50% inhibitory concentration of TSA for HDAC-1 is 2 nmol/L and for HDAC-2 is 3 nmol/L (7). Thus, these doses are likely to be considerably higher than those required to suppress inflammation. *In vitro*, acetylation of H4 in mouse macrophages was observed at 10 ng/mL (33 nmol/L), which is 10-fold greater than the IC_{50} for HDAC-2 (80).

Cyclooxygenase-2 (COX-2) is considered proatherogenic, and COX-2 expression is in increased bone marrow–derived mouse macrophages treated with SAHA or TSA. Similarly, plasminogen activator inhibitor-1 (PAI-1) is also proatherogenic, and its expression is increased in the same macrophages treated with SAHA or TSA (81). However, the concentration of SAHA added to these cultures was 10 μmol/L, which is 100-fold higher than the IC_{50} for HDAC-1 and 75-fold higher than that for HDAC-2 (7). In mouse macrophages stimulated with LPS, PAI-1 protein levels increase over 10-fold when incubated with TSA; however, the concentration of TSA used in those studies was 500 nmol/L, which is a 250-fold higher concentration than the IC_{50} for HDAC-1 (81). Similarly, COX-2 increased over 40-fold in the presence of 500 nmol/L TSA, which is 250-fold over the IC_{50} for TSA on HDAC-1. Importantly, at 10 nmol/L TSA, which is only three-fold greater than the IC_{50}, there was no increase in COX-2 expression in LPS-stimulated macrophages (81). In addition, an increase in PGD2 by HDACi inhibits the production of IL-1-induced metalloproteinases (82). In contrast, Yamaguchi *et al.* (83) reported that HDACi's block the induction of c-jun transcription by inhibiting the recruitment of the complex to the c-jun promoter. This results in reduced expression of several activator protein-1–dependent genes, including *COX-2*.

Similar data can be found in an earlier report. Bone marrow–derived mouse macrophages were stimulated with LPS and at various time points, HDAC-1, HDAC-4, HDAC-5, HDAC-7, HDAC-8 and HDAC-9 were elevated (84). Of these, HDAC-1 exhibited the greatest increase and duration. TSA at a concentration of 500 nmol/L was examined for its effects on LPS-induced genes. With use of microarray analysis of LPS-inducible genes, one group was unaffected, another was superinduced and a third group was suppressed. The genes that were suppressed were not revealed in the report. Of those genes that were superinduced, COX-2 mRNA and protein increased. A similar increase was observed for the chemokine CXCL2 and endothelin (84). The authors concluded that HDACs act as negative regulators of LPS-induced COX-2 (84). In support of this concept, mouse macrophages were transfected with the proximal promoter of COX-2, and LPS increased the activity of the promoter five-fold. In the presence of TSA (500 nmol/L), the increase was 18-fold (84).

The findings of increased IFNγ and TNFα production in the study in NOD mice (36), the exacerbation of atherosclerosis in the LDL-deficient mouse (80), the increases in VCAM-1 and scavenger receptor CD36 (80) in COX-2 (81, 84) and elevated PAI-1 (81) by TSA and SAHA in mouse macrophages are inconsistent with the overall portfolio of antiinflammatory properties of HDACi's. How does one explain the discrepancies? Using *in vitro* concentrations of TSA of 500 nmol/L is certainly inconsistent with the low concentration of this HDACi with an IC_{50} for HDAC-1 and HDAC-2 of 2 and 3 nmol/L, respectively (7). LDL receptor–deficient mice were treated with 1 or 0.5 mg/kg of TSA every other day, the dose used to treat tumors in mice. In the case of studies with TSA, one cannot predict the relevance for human disease, since there are no studies on the blood levels of TSA after intraperitoneal injections, and the toxicity of TSA in humans will likely never be known. It is interesting to note that the beneficial effect of TSA in the NOD model was achieved at a dose of 0.5 mg/kg once weekly. However, the reduction in diabetes was only observed when the treatment was limited to the interval of 18–24 weeks of age (36), whereas early treatment at 5–18 weeks of age was without effect. In the LDL receptor–deficient mouse, for inducing atherosclerosis, the dose was 1.0 mg/kg every other day. One may conclude that the antiinflammatory properties of HDACi's are best observed at low dosing schedules and that few conclusions can be drawn by using antitumor doses in mice. However, one may also interpret from these data that treating rodents with HDACi's in models of disease requires higher doses than in humans.

Unlike TSA, there are ample data in studies with SAHA, FK228 or ITF2357 in humans. Blood levels and tolerability have been established. In the case of SAHA, the maximal tolerated dose of 800 mg/day in two divided doses induces fatigue, nausea and some vomiting (85,86). In the report by Halili *et al.* (81), SAHA increased gene expression of COX-2, PAI-1 and IL-12 in mouse macrophages at 10 μmol/L, a concentration 100-fold greater than the IC_{50} for HDAC-1 (7), which is clinically irrelevant. *In vitro*, SAHA reduces LPS-induced cytokines in primary human PBMCs at concentrations between 50 and 200 nmol/L, far lower than the 10 μmol/L used by Halili *et al.* The concentration of 10 μmol/L *in vitro* is used to induce proapoptotic genes in tumors. For ITF2357, there is tolerability in healthy humans (87), in patients with autoinflammatory diseases (73) and in children with SOJIA (5). At an oral dose of 1.5 mg/kg, ITF2357 (givinostat) is well tolerated. At this dose, there is a transient reduction in LPS-induced cytokines in whole blood cultures during a phase I trial in healthy humans, and this reduction occurs at a blood level of 125–250 nmol/L, concentrations known to inhibit LPS-induced cytokines in PBMCs (11,87). In patients with SOJIA, clinical and hematological improvement is observed at this low dose (5,26) and similarly in patients with Schnitzler syndrome (73). It appears that HDACi's such as ITF2357 (givinostat) may be candidates for treating chronic inflammatory diseases including atherosclerosis.

TREATING ACUTE DISEASES WITH HDACi's

Studies have focused on mouse models of sepsis, but it is unlikely that HDACi's will be tested in clinical trials for human sepsis. HDACi's have also been tested in models of ischemia/reperfusion (88,89). Ischemia/reperfusion is essentially sterile inflammation, and, here, cytokines such as IL-1α and TNFα play a significant role. But the testing of HDACi's in humans with hemorrhagic shock, acute stroke or myocardial infarction is doubtful. One recurrent acute disease is gout and, here, HDACi's may provide an additional benefit to colchicine, as studies demonstrate that gout is highly responsive to IL-1β blockade (90–92).

NEURODEGENERATIVE DISEASES

Neurodegeneration includes the slow progressive forms characteristic of atherosclerotic brain disease as well as Alzheimer disease but also that following acute brain trauma of a stroke or blunt injury. Histone deacetylation is often altered in the brains of animals used to study neurodegeneration. In this issue, Shein and Shohami (77) review the evidence for the use of HDACi's in treating either the acute or slowly progressive course of neurodegeneration. The efficacy of HDACi's in acute brain injury was reported by Shein *et al.* (93) as well as in models of amyotrophic lateral sclerosis (ALS), Alzheimer disease, spinal muscular atrophy and experimental autoimmune encephalomyelitis. Although many neurodegenerative diseases include a cytokine-driven inflammatory component, Huntington disease remains a uniquely noninflammatory loss of neurons. Nevertheless, several models of

neurodegeneration are due to inflammatory processes. In this regard, the antiinflammatory properties of HDACi's appear to be neuroprotective. In animal models of Alzheimer disease, the most studied neurodegenerative disease, HDAC inhibition decreased β-amyloid production and effective neuroprotection was associated with increased acetylation of histone 4 (94). In a model of closed head trauma, the antiinflammatory properties of HDACi's prevented the deterioration of neurological impairment, even when administered 24 hours after the injury (93).

Huntington Disease

Although the role of inflammation in Huntington disease is presently unclear, inhibition of caspase-1 delays the onset of motor abnormalities in a mouse model of the disease (95) and treatment with oral SAHA also reduced disease severity (96). Because the effect of valproic acid on the central nervous system is well documented, clinical trials with oral HDACi's could be initiated in patients with early indications of dementia. Although currently available HDACi's are "pan HDAC" inhibitors, HDACi's with greater specificity will be the future direction for this class of drugs.

Huntington disease is an autosomal-dominant genetic disease. The pathological lesions occur in the striatum and cortex and the disease is fatal approximately 20 years after the onset of symptoms. There is an expanded CAG/polyglutamine repeat in "huntingtin," a protein of unknown function. The disease is progressive with loss of motor function, and there is no treatment to slow the progress of the disease. In 2003, there was a report that SAHA added to the drinking water of R6/2 HD mice, the model for this disease, resulted in marked improvement in motor function (96). Transgenic mice expressing human huntingtin with an expanded CAG/polyglutamine repeat develop a progressive syndrome with many of the characteristics of human Huntington disease (95). Indeed, SAHA crosses the blood-brain barrier and increases histone acetylation in the brain (96). The administration of SAHA has consistently shown this therapeutic benefit in the mouse model; nevertheless, which HDAC is affected by SAHA to improve motor function in the R6/2 mouse model remains unclear. Brain levels of HDAC-7 are reduced in both wild-type animals and in animals with SAHA (97). However, there was no improvement in a number of physiological or behavioral phenotypes in R6/2 mice deficient in HDAC-7 (97). Thus, the SAHA-mediated amelioration or disease severity in the mouse model for Huntington disease is not via HDAC-7, and the ability of SAHA to reduce brain levels of HDAC-7 appears to be unrelated.

Intracerebroventricular administration of a caspase inhibitor delays disease progression and mortality in the transgenic mouse (95). In a rat cerebral artery occlusion model of stroke, post-insult treatment with the HDACi valproic acid reduced ischemia-induced brain infarction, caspase-3 activation and neurological deficits (98). In a rat excitotoxic model of Huntington disease, treatment protected against DNA damage, caspase activation and apoptosis of striatal neurons (98). Thus, HDACi's are potential drugs for treating some forms of neurodegenerative diseases, and whether the benefit of HDACi's is via a cytokine-mediated process such as caspase-1 remains to be demonstrated. In neurons, acetylation at lysine 40 of α-tubulin increased by TSA results in the release of the brain-derived neurotrophic factor (99). Tubulin acetylation is reduced in the brains of animals with Huntington disease, and TSA compensates for the transport- and release-defect phenotypes that are observed in disease (99).

Amyotrophic Lateral Sclerosis

ALS is another disease in which there is progressive loss of brain neurons. A study in mice with mutations in superoxide dismutase revealed a role for caspase-1 processing of IL-1β. The mouse model for ALS is G93A-SOD1, a transgenic mouse expressing mutant superoxide dismutase 1 (SOD1). Mutant SOD1 activates caspase-1 and IL-1β in microglia (100) and supports the concept that neuroinflammation contributes to ALS disease progression (101). Mutant SOD1-induced IL-1β correlated with amyloid-like misfolding and was independent of dismutase activity. ALS-linked cytoplasmic accumulation of mutant SOD1 was sensed by an apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC)-containing inflammasome and antagonized by autophagy, limiting caspase-1–mediated inflammation (100). A deficiency in caspase-1 reduced IL-1β levels, extended the lifespan of G93A-SOD1 transgenic mice and attenuated inflammatory pathology. Similar results were observed with treatment with recombinant IL-1Ra (100). These findings identify microglial IL-1β as a causative event of neuroinflammation and suggest IL-1 as a potential therapeutic target in ALS (101).

IS THERE A ROLE FOR CYTOKINE INHIBITION IN MULTIPLE MYELOMA?

The cytokine IL-6 is the primary growth factor for myeloma, and anti–IL-6 monoclonal antibodies as well as anti–IL-6 receptor monoclonal antibodies are used to treat Castleman disease. However, in patients with a premyeloma condition called smoldering or indolent myeloma, blocking IL-1β to reduce IL-6 production significantly prevents progression to full-blown multiple myeloma (102,103). HDACi's inhibit the production of IL-6 by LPS as well as IL-1β-driven IL-6 (10,11,23,24). Therefore, although multiple myeloma is cancer, the use of HDACi's in the precancer condition of smoldering/indolent myeloma is a likely therapy compared with the daily injection of anakinra that was used in the trial (102).

The activity of ITF2357 was studied on multiple myeloma and acute myelogenous leukemia cells *in vitro* and *in vivo*. ITF2357 induced apoptosis in freshly obtained multiple myeloma and acute myelogenous leukemia cells at an IC_{50} of 200 nmol/L (104). ITF2357 induced hy-

peracetylation of histone H3, H4 and tubulin. ITF2357 was also cytotoxic for the IL-6–dependent multiple myeloma cell line CMA-03. Of particular relevance, ITF2357 inhibited the production of IL-6, vascular endothelial growth factor and IFNγ by mesenchymal stromal cells by 80–95% (104). *In vivo* in mice, ITF2357 significantly prolonged survival of severe combined immunodeficient mice inoculated with acute myelogenous leukemia cells at a dose of 10 mg/kg and inhibited the production of growth and angiogenic factors by bone marrow stromal cells, in particular IL-6 and VEGF (104). These results suggest that part of the mechanism of action of HDACi's in multiple myeloma and acute myelogenous leukemia may be due to a reduction in cytokine production, since these hematopoietic malignancies are driven by IL-1β and IL-6 as growth factors (102,105,106). A phase II, multiple-dose clinical trial in 19 patients with relapsed or progressive multiple myeloma was carried out (107). The first six patients received 150 mg ITF2357 twice daily, but because of toxicity, the dose was reduced to 100 mg ITF2357 twice daily. This was the maximal tolerated dose. Although these patients had advanced disease, a dose of 100 mg twice daily alone or combined with dexamethasone, ITF2357 proved tolerable and showed a modest clinical benefit (107). It appears that the optimal use of HDACi's in multiple myeloma would be in smoldering myeloma, where the combination of IL-1β blockade plus dexamethasone significantly delays or even prevents the progression to full-blown multiple myeloma (102).

Gene expression profiling of the KMS18 multiple myeloma cell line was performed with ITF2357 (108). The modulation of several genes and their biological consequence were verified in a panel of multiple myeloma cell lines and cells freshly isolated from patients by using polymerase chain reaction analysis and Western blotting. Out of 38,500 human genes, several were downregulated and, in particular, ITF2357 down-modulated the IL-6 receptor α transcript and protein in both cell lines and freshly isolated patient cells (108). The decrease in the IL-6 receptor expression was accompanied by decreased signaling, as measured by STAT-3 phosphorylation. These data support the previous studies that ITF2357 inhibits cytokine-mediated myeloma cell growth and survival.

IS THERE A ROLE FOR CYTOKINE INHIBITION IN MYELOPROLIFERATIVE DISORDERS?

The efficacy of givinostat in 12 patients with polycythemia vera and in 16 patients with myelofibrosis, bearing the JAK2V617F mutation, has been reported (109). Givinostat was given orally for 24 weeks at a dose of 50 mg twice daily; however, a dose reduction was applied in 10 patients. The median treatment duration was 20 weeks. The troubling pruritus disappeared in most patients, and reduction of splenomegaly was observed in 75% of polycythemia vera patients and 38% of myelofibrosis patients. Reverse transcription polymerase chain reaction identified a trend to reduction of the JAK2V617F allele burden. Givinostat was well tolerated and could induce hematological response in most polycythemia vera patients (109). *In vitro*, the effect of ITF2357 was evaluated on carrying the JAK2 (V617F) mutation obtained from polycythemia vera and essential thrombocythemia patients (110). The clonogenic activity of JAK2 (V617F) mutated cells was reduced by low concentrations of 1–10 nmol/L of ITF2357, which is 100- to 250-fold lower than concentrations that are traditionally required to inhibit the growth of tumor cells lacking this mutation. By Western blotting, ITF2357 resulted in the disappearance of total and phosphorylated JAK2 (V617F) as well as pSTAT5 and pSTAT3 (110).

Together, these studies provide evidence that, in some hematopoietic disorders, the efficacy of HDACi's is in part due to a reduction in either cytokine production or cytokine signaling. Clinically, acute myelogenous leukemia, polycythemia vera and multiple myeloma are associated with elevated CRP and markers of systemic inflammation such as recurrent fevers. The low concentrations *in vitro* and *in vivo* also support the concept that the cytokine-reducing properties of HDACi's contribute to controlling disease activity rather than direct tumor cell death. To accomplish tumor cell death, dexamethasone or another proapoptotic regimen is required.

CUTANEOUS T-CELL LYMPHOMA

Two HDACi's, vorinostat and romidepsin, are approved for the treatment of cutaneous T-cell lymphoma (CTCL). It is likely that there is a role for cytokines in this disease. The levels of IL-1α in epidermis of patients with CTCL was investigated by using immunohistochemistry and specific enzyme-link immunosorbent assays. Significant increases of IL-1α were released from the involved CTCL epidermis compared with the normal epidermis from healthy individuals (111). Both keratinocytes and leukocytes could release IL-1α, but the majority was derived from the keratinocytes. The IL-1α was biologically active (111). Extracorporeal photopheresis is used to treat CTLC. Following this therapy, lymphocytes become apoptotic and monocytes, exposed to post-extracorporeal photopheresis (post-ECP), reduce spontaneous proinflammatory cytokine secretion including IL-1α (112). IFNγ is commonly used to treat CTLC. The mechanism of action of IFNγ in CTLC may include the induction of IL-1Ra (113). SAHA as well as givinostat do not suppress IL-1Ra (11) and, in some studies, increase the production of IL-1Ra (87).

SAFETY AND TOLERABILITY REQUIREMENTS OF HDACi's FOR TREATING CHRONIC DISEASES

When considering the property of HDACi's in the treatment of chronic diseases characterized by inflammatory, autoimmune or degenerative processes, use of HDACi's to treat such diseases is safe and well tolerated. In general, HDACi's are safe in humans. For example, valproic acid, a carboxylate HDACi, is the drug of choice for chronic therapy of

generalized and focal epilepsy as well as obsessive disorders (114,115). Valproic acid has also been used to increase latent *HIV-1* gene expression in patients while being treated with anti–HIV-1 drugs without interference (116). Butyrates are used to treat patients with sickle cell anemia and β-thalassemia (117). In fact, valproic acid is used chronically by millions of people. SAHA (vorinostat) is presently being evaluated for treatment in GvHD (71). ITF2357 (givinostat) has been used in children with SOJIA for 12 weeks without adverse effects (5,26). Givinostat has also been tested in patients with the autoinflammatory disease Schnitzler syndrome, and the observations are reported in this issue (118). In both the SOJIA and Schnitzler syndrome studies, there were clear indications of the desired antiinflammatory effects in that clinical scores of disease severity as well as a decrease in the circulating number of white blood cells occurred. In both studies, the dose of 1.5 mg/kg was well tolerated.

Chronic diseases that are likely to be treated with HDACi's are rheumatoid arthritis, osteoarthritis, inflammatory bowel disease and Type 2 diabetes and, being orally effective, HDACi's have considerable advantages over the parenterally administered anticytokines. These diseases are dramatically affected by anticytokine antibodies and soluble receptors, which have no organ toxicities other than the pain of the injection needle; indeed, they are remarkably well tolerated. In contrast, HDACi's do have organ toxicities and, at doses used to treat cancer, all HDACi's cause gastrointestinal disturbances and other constitutional symptoms. These constitutional symptoms would not be tolerated in patients without life-threatening cancer. If clinical trials of HDACi's are proven to reduce activity in chronic diseases, they will need to be well tolerated. Therefore, the dose required to reach an effective tissue level should be without significant constitutional symptoms. It also remains unclear whether HDACi's will be used as a monotherapy or in combination with other antiinflammatory agents such as glucocorticoids. In the children with SOJIA, givinostat was used in combination with steroids.

In assessing the effects of various HDACi's in animal models of disease, one must establish that any beneficial effect in the model is consistent with a dose that is likely to be tolerated in humans. In many models, TSA is used to assess a role for HDACi's in the amelioration of disease activity. For example, TSA in models of lupus nephritis TSA was administered for 5 weeks at a dose of 0.5 mg/kg/day with a clear beneficial effect (119). However, it is impossible to draw any conclusions as to whether the tissue concentrations of TSA required for the effect of TSA are in the range of clinically relevant HDACi's. In this model of lupus nephritis, mice were also treated with SAHA at a dose of 25 and 50 mg/kg intraperitoneally for 10 weeks (120). In humans with leukemia, the maximum tolerated dose for SAHA was 200 mg twice daily (1.5 mg/kg twice a day) (85). Dose-limiting toxicities were fatigue, nausea, vomiting and diarrhea. Even assuming that the mouse requires a 10-fold greater amount of SAHA than humans to reach the effective concentration *in vivo*, SAHA at doses of 2.5 mg/kg was not well tolerated.

On the other hand, the use of HDACi's gained considerable validity when the effective concentration *in vitro* was attainable in humans after oral administration. In the phase I trial of givinostat in healthy male subjects, a single oral dose of 100 mg resulted in a peak plasma level of 207 nmol/L at 2 hours and an area under the curve (AUC) of 1,410 nmol/L/h with a half-life of 6 hours (87). *In vitro*, 100 nmol/L of ITF2357 (givinostat) suppressed by 90% IL-1β-driven loss of bone in the rat calvarium model for osteoclast activation and similarly for TNFα-driven bone loss (25). The oral dose of 100 mg also suppressed LPS-induced TNFα, IL-1β and IFNγ 4 hours after dosing. In freshly obtained human PBMCs, concentrations of 12.5 to 25 nmol/L of ITF2357 were observed for IC_{50} of LPS-induced TNFα, IL-1β and IFNγ.

MUSCULAR DYSTROPHY

As reviewed in this issue of *Molecular Medicine* (78), HDACs regulate muscle-specific gene transcription via control of histone acetylation. Thus, the balance between acetylation and deacetylation is a critical determinant of muscle gene transcription and is emerging as a possible intervention for the regenerative potential of muscle stem cells. For muscular dystrophy, Consalvi *et al.* (78) showed that the mechanism responsible for the activity of HDACi's in the animal model of muscular dystrophy goes beyond follistatin induction. There is the known potential of dystrophic muscles to activate mechanisms of compensatory regeneration. This evidence supports the current view that HDACi's are emerging candidate drugs for pharmacological interventions in muscular dystrophies and reveals unexpected common beneficial outcomes of pharmacological treatment. The activation of a differentiating program in the stem cells resident in the muscle is a further key mechanism that contributes to the activity of these compounds. Differentiation of stem cells toward a specific lineage through epigenetic modulation has a potential in many field of medicine. With safety on its side, it is possible that a clinical trial of HDACi's may result in activating stem cells to differentiate into muscle cells, but the specificity of HDAC and cofactors for differentiation are not presently established.

AUTOPHAGY AND HDACi's

Autophagy

In general, autophagy is an adaptive mechanism protecting the loss of cells by reusing intracellular components that have been generated by pathological processes. Autophagy is often studied *in vitro* in cells deprived of nutrients, but *in vivo*, autophagy replenishes cells after an ischemic or stressed event and prolongs their function. Autophagy is also observed in yeasts, and the weak HDACi valproic acid increases autophagy in yeasts undergoing the stress of DNA breaks (121).

Without this mechanism, cells die with intracellular debris and organs fail. In some disease processes, autophagy can be maladaptive for the host, and this is certainly the case in malignant cells. HDACi's function in the treatment of cancer by increasing genes that inhibit autophagy.

In diabetes, autophagy is a protective process (122,123). Endogenous proteins that are trapped in the endoplasmic reticulum (ER) result in ER stress. In the insulin-producing pancreatic β cell, ER stress can occur because of a failure to release insulin into the extracellular compartment after glucose load and die an apoptotic death. Cells producing IL-1β such as the β cell itself have an additional threat related to caspase-1, that is, the processing of the IL-1β precursor and release of active IL-1β. Active IL-1β can then trigger the IL-1 receptor type I (IL-1RI) leading to NO production and cell death. HDACi's reduce IL-1β processing and release and therefore are of potential use in protecting the β cell and thus are a treatment possibility in diabetes.

HDACi's and the Failing Heart

Although autophagy benefits the insulin-producing β cell, autophagy is maladaptive in left-side ventricular hypertrophic heart disease (124). In the stressed ischemic heart, the autophagic process attempts to prevent loss of the myocyte and reuse the intracellular debris generated during the ischemic event. Inhibition of autophagy would seem counterproductive for an ischemic heart. HDACi's do, in fact, inhibit autophagy, and therefore their use would be counterproductive in rescuing cells from death. However, in hypertrophic left ventricle heart failure, preventing autophagy is beneficial, since it reduces the proliferation. In many cases of heart failure, there is a constriction of the aortic outflow, and the hypertrophic left ventricle undergoes a proliferative response, which is necessary to compensate for the restriction. The benefit of HDACi's in this form of heart failure is not the case in right side heart failure due to pressure overload. Suppression of histone deacetylases worsens right ventricular dysfunction after pulmonary artery banding in rats via a mechanism of decreased angiogenesis and eNOS expression (125). Whereas HDAC inhibition reduces left ventricular hypertrophy, fibrosis and apoptosis, the opposite effects of HDACi treatment were observed with pressure overload of the right ventricle (125). It is unclear from that study of pulmonary banding–induced right ventricular failure if lower doses of TSA would have yielded the same data *in vivo*. *In vitro*, 300 nmol/L TSA was required to reduce eNOS and VEGF in cultured human cardiac microvessel endothelial cells, a concentration 100-fold greater for the IC_{50} of TSA for HDAC-2 and 150-fold greater for HDAC-1 (7). However, these authors are correct in that HDACi's do reduce angiogenesis, as we have observed blood vessel formation in Matrigel plugs embedded with 10 ng IL-1β in mice treated with 2.5 mg/kg oral ITF235 (unpublished data).

Left ventricular hypertrophy takes place as a compensatory mechanism to restore functionality in conditions of increased afterload or wall stress (that is, after an ischemic event). Left ventricular hypertrophy is an unfavorable prognostic factor and represents a major clinical problem affecting millions of patients worldwide. Indeed, the concept has been tested in mouse models of left ventricular hypertrophy. The study used phenylephrine-induced hypertrophic growth and was prevented by treatment with TSA (124). Other HDACi's reduced hypertrophic growth in cultured cardiomyocytes. Hypertrophic agonist–induced autophagy is suppressed in neonatal cardiomyocytes in culture when treated with the hypertrophic agonists in the presence of TSA (66 nmol/L) or valproic acid (6 mmol/L) (124). To demonstrate the role of autophagy in the hypertrophic cardiomyocyte growth, expression of endogenous ATF5, the gene that is essential for autophagic protection, was suppressed with inhibitory RNA and resulted in reduced cardiomyocyte growth *in vitro*. The ability of HDACi's to inhibit autophagy appeared to target HDAC-1 and HDAC-2 (124); thus, HDAC-1 and -2 contribute to maintaining autophagy. To show the utility of these studies, the authors treated mice with established left ventricular hypertrophy with TSA (1.0 mg/kg/day) and observed that ventricular function returned to normal levels (124). Similar protective results of HDACi's were reported in animal models of ischemic and nonischemic (doxorubicin) myocardial injury (126,127). These studies and the role of HDACi's in heart failure are reviewed by McKinsey (63) in this issue. The data suggest that oral HDACi's may have a role in treating left ventricular hypertrophy, while caution should be used in cases of right ventricular hypertrophy.

HDACi's AS A METHOD FOR HIV-1 ERADICATION

Since the introduction of highly active antiretroviral therapy (ART) more than a decade ago, HIV-1 infection can be well controlled, and in nearly all treated patients, HIV-1 viremia remains below detectable levels as long as therapy is sustained. Indeed, ART has changed the face of HIV-1. For example, after the initiation of ART, markedly reduced $CD4^+$ T cells rose to near-normal levels, and death from opportunistic infection was rarely observed. As such, this formally fatal disease has been transformed into a chronic disease. Nevertheless, lifespan, even in patients well controlled on ART, remains below that of the general population. Despite the effectiveness of ART in suppressing HIV-1 replication, after discontinuation of ART, there is a rebound viremia, which occurs typically after 2 weeks (128). The source of viral reemergence is a long-lived pool, most likely the latently infected memory $CD4^+$ T-cell reservoir, harboring integrated HIV-1 proviral DNA (129). Attacking this latent pool remains *a priori*ty in combating HIV-1 infections (130).

The latent reservoir of HIV-1 within resting $CD4^+$ cells is established early after acute infection and is an extremely stable reservoir, having a half-life of 6–44 months, even in treated patients who are continuously aviremic for long periods of time (129). The integrated proviral DNA is present in densely orga-

nized nucleosomes; for viral replication, transcription factors such as NFκB bind to the long terminal repeat of HIV-1 and drives expression of HIV-1 with release of competent virus. During the process of viral replication, the $CD4^+$ T cells die, but new $CD4^+$ T cells are infected after the release of a competent virus. However, in patients on ART, any newly released virus cannot be integrated into the DNA of naïve $CD4^+$ T cells, since the activity of reverse transcriptase and intergrases are inhibited by the antiretroviral drugs. The use of the chemokine/HIV-1 receptor antagonists also prevents viral entry. To deplete the latent pool, activating the $CD4^+$ T cell with IL-2 therapy in patients on ART was tested in large clinical trials but without a significant reduction. Another approach is to force viral expression, bypassing activation of the $CD4^+$ T cells.

In latently infected $CD4^+$ T cells, HDAC-1 and HDAC-2 deacetylate local histones, compact the chromatin and prevent the binding of NFκB and other transcription factors, and RNA polymerase II binding does not occur. *In vitro* studies demonstrated that activation of a latent viral by NFκB reverses the repressive effect of the p50 homodimer HDAC-1 complex by the binding of cytosolic NFκB p50-RelA heterodimer. This step would enable the recruitment of the histone acetyltransferase, acetylation of the local histones, relaxation of the chromatin and initiation of viral transcription. Inhibition of the enzymatic activity of HDAC-1 and possibly other HDACs by HDACi's results in activation of the HIV-1 long terminal repeat, and viral expression is observed in latently infected cells *in vitro*.

Because the HDACi valproic acid stimulates HIV-1 expression *in vitro*, valproic acid was tested in trials of HIV-1 in infected patients while on continuous ART. However, those trials did not yield the desirable decrease in the latent reservoir (116,131,132). It is believed that valproic acid failed to have a significant impact because it is a nonspecific and weak HDACi. Thus, whether the use of HDACi's can purge the virus from the latent reservoir requires more potent agents. SAHA (vorinostat) is more potent than valproic acid, has greater specificity for HDAC-1 and HDAC-2 and is in clinical trials in HIV-1-infected patients while taking ART (131,133). As reported in this issue of *Molecular Medicine*, ITF2357 (givinostat) appears to be more potent and specific than vorinostat (19,38). We believe givinostat should be considered for a clinical trial to force HIV-1 out of its latency and gradually eliminate the infection.

The hypothesis assumes that, with expression of HIV-1 from it latency, the infected $CD4^+$ T cell dies of a cytopathic process and that the newly released virus is incapable of being integrated into naïve $CD4^+$ T cells. As reported by Matalon *et al.* (19) and reviewed in this issue (38), givinostat induces HIV-1 from latently infected human macrophages and T-cell lines at concentrations achievable in humans and at doses that are well tolerated. By comparison, to achieve levels of vorinostat *in vivo* comparable to the concentrations required to induce HIV-1 expression in latently infected human macrophage and T-cell lines, the drug would likely not be well tolerated. Similarly, to obtain the same level of HIV-1 expression from these cell lines *in vitro*, valproic acid concentrations *in vivo* would not be well tolerated. Similar to other treatment options of HDACi's in chronic diseases, as summarized in this issue of *Molecular Medicine*, any disease-modifying benefit of an HDACi must be at a dose that is well tolerated.

THROMBOCYTOPENIA

HDACi's used to treat malignant disease consistently induce thrombocytopenia. In fact, in addition to gastrointestinal disturbances such as nausea and diarrhea, a low platelet count appears to be the major dose-limiting toxicity (134). A fall in platelets has also been observed in healthy subjects during a phase 1 study of givinostat in healthy subjects (87). In that study, there was no concomitant use of chemotherapy or radiation, as is often the case with cancer patients receiving HDACi's. In subjects treated with daily dosing of givinostat for 7 days, a reduction in platelet count was observed across all doses. The effect was evident from day 5 onward, reaching the lowest value on day 9, that is, 48 hours after last dosing. On day 9, the mean platelet counts decreased by 17%, 25% and 35% in groups receiving givinostat at 50, 100 and 200 mg each day, respectively. For the most part, the effect on platelets recovered on day 14 (7 days after last dosing). In children treated with givinostat for 12 weeks at a dose of 1.5 mg/kg in two divided doses, there was a fall in platelets; however, disease activity in SOJIA includes thromocytosis, and fall in platelets is interpreted as beneficial. Nevertheless, at a dose of 1.5 mg/kg, platelet count remained in the normal range.

For givinostat, a dose between 1.0 and 1.5 mg/kg in two divided doses is well tolerated and unlikely to reduce platelets to below the normal range. If a particular HDACi is well tolerated in terms of not inducing constitutional symptoms related to gastrointestinal disturbances or decreasing daily functions, a fall in platelets in the range of 15–20% is without clinical significance. Nevertheless, regardless of the dosing schedule, any use of HDACi's for treating a chronic disease will require monitoring of platelet counts.

The fall in platelets is not due to bone marrow suppression. The HDACi romidepsin (FK228) is approved for the treatment of cutaneous T-cell lymphoma and is selective for HDAC-1 and -2 (135). In examining the mechanism for the reduction in platelets, it was concluded that romidepsin in mice is not myelosuppressive and does not reduce the lifespan of platelets (134). Rather, studies with romidepsin demonstrated that HDACi's are more likely to cause a reduction in platelets because of the impaired release of platelets from the megakaryocytes. In the romidepsin study, primary megakaryocytes from mice showed reductions in proplatelet extensions with an increase in the phosphorylation of myosin light chain 2 (134). Of particular interest in that study was the observation that that the HDACi-induced thrombocytopenia in

mice was prevented by coadministration of a thrombopoietin mimetic (134).

SUMMARY

Studies in animal models have revealed the unexpected observations that HDACi's can be used to reduce severity in a broad range of diseases. For the most part, the spectrum of diseases is inflammatory; and hence HDACi's exhibit antiinflammatory properties owing to a reduction in cytokine production as well as inhibition of cytokine effects. Distinct from their use in cancer, the effects of HDACi's are consistently observed at low concentrations compared to higher concentrations required for killing tumor cells. HDACi's are therefore attractive as a therapeutic option for suppressing inflammation.

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DISCLOSURE

CA Dinarello is a consultant to Italfarmaco. G Fossati and P Mascagni are employees of Italfarmaco.

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