Histone Deacetylase Inhibitors for Purging HIV-1 from the Latent Reservoir

Shay Matalon,[1] Thomas A Rasmussen,[2] and Charles A Dinarello[1]

[1]Department of Medicine, Division of Infectious Diseases, University of Colorado Denver, Aurora, Colorado, United States of America; and [2]Department of Infectious Diseases, Aarhus University Hospital, Skejby, Denmark

A reservoir of latently infected memory $CD4^+$ T cells is believed to be the source of HIV-1 reemergence after discontinuation of antiretroviral therapy. HIV-1 eradication may depend on depletion of this reservoir. Integrated HIV-1 is inaccessible for expression, in part because of histone deacetylases (HDACs). One approach is to exploit the ability of HDAC inhibitors to induce HIV-1 expression from an integrated virus. With effective antiretroviral therapy, newly expressed HIV-1 is incapable of reinfecting naive cells. With HIV-1 expression, one assumes the infected cell dies and there is a progressive reduction in the size of the reservoir. The concept was tested using the HDAC inhibitor valproic acid. However, valproic acid is weak in inducing HIV-1 from latency *in vitro*. As such, clinical trials revealed a small or no effect on reducing the number of latently infected T cells in the peripheral blood. However, the new HDAC inhibitors vorinostat, belinostat and givinostat are more effective at targeting specific HDACs for HIV-1 expression than valproic acid. Here, we review studies on HDAC inhibitor–induced expression of latent HIV-1, with an emphasis on new and specific HDAC inhibitors. With increased potency for HIV-1 expression as well as safety and ease of oral administration, new HDAC inhibitors offer a unique opportunity to deplete the latent reservoir. An additional benefit is the antiinflammatory properties of HDAC inhibitors, including downregulation of HIV-1 coreceptor expression.

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THE LATENT POOL OF HIV-1-INFECTED CELLS

The source of latent HIV-1 infection is a long-lived pool, most likely the latently infected memory T-cell reservoir, harboring integrated HIV-1 proviral DNA (1–4). Well-established techniques are available to quantify the latent pool, and it is estimated that one in a million memory T cells of an HIV-1-positive patient bears a replication-competent integrated provirus (5,6). The latent reservoir of HIV-1 within resting $CD4^+$ T cells is established early after the acute infection, and the initiation of highly active antiretroviral therapy (HAART) during this period does not prevent its establishment (6–8). It 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 (6,9–12). With this prolonged half-life, a complete decay of the reservoir is not expected before 70 years of HAART treatment, making eradication improbable. These time frames might be somewhat shorter by starting HAART early during acute infection or by intensifying HAART, but are not sufficient as a practical method for eradication (13,14).

EARLY STUDIES ON PURGING HIV-1 FROM LATENTLY INFECTED CELLS

With the development of effective antiretroviral agents to prevent HIV-1 replication *in vivo* came the concept that eliminating the reservoir should be achievable without spreading the infection to new uninfected cells. After viral expression, latently infected cells would then be eliminated by two possible mechanisms. First, it is possible that upon HIV-1 expression, a cytopathic death of the cell would follow. Second, it is also possible that residual HIV-1–specific cytotoxic T cells would kill cells expressing HIV-1 antigens. The strategy of purging the latent pool of HIV-1-infected $CD4^+$ T cells was initially performed using interleukin (IL)-2 and other activators of T cells such as anti-CD3 antibodies (OKT3). However, although such agents caused a marked activation of the T cells, there were unacceptable toxicities and also an irreversible decrease in the amount of $CD4^+$ T cells (15,16). Other studies using IL-2 did not reduce the pool (17,18), perhaps because of a paucity of cell surface receptors for IL-2 on the resting $CD4^+$ T cells.

In addition, integrated provirus residing within resting regulatory $CD4^+$ T cells (T_{regs}) may also have contributed to the lack of effect. The presence of an HIV-1 reservoir within resting T_{regs} in patients on prolonged HAART has been detected; in fact, HIV-1 DNA harboring cells

Address correspondence and reprint requests to *Charles A Dinarello, University of Colorado Denver, Division of Infectious Diseases, 12700 East 19th Avenue, B168, Aurora, CO 80045. Phone: 303-724-6172; Fax: 303-724-6178; E-mail: cdinare333@aol.com.*
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appeared more abundant in this subset than in non-T_{regs}, but with a comparable estimated half-life (19). Because T_{regs} display signs of hyporesponsiveness to cell activation (19) and may inhibit IL-2 production (20), this cellular subset could have added to the viral rebound despite IL-2 treatment (21). However, the anti-inflammatory and immunosuppressive effect of some histone deacetylase (HDAC) inhibitors, such as suberoylanilide hydroxamic acid (SAHA; generic vorinostat) and ITF2357 (generic givinostat) (22), is in part owing to enhancing the production and suppressive function of T_{regs} by promoting FOXP3 acetylation (23). Therefore, it is conceivable, but yet unproven, that potent HDAC inhibitor treatment may also target the latent viral reservoir within resting T_{regs}.

ROLE OF HISTONE ACETYLATION IN HIV-1 EXPRESSION

An ideal strategy for viral purging would induce HIV-1 expression without inducing a global T-cell activation. Therefore, small molecules that can directly gain access to DNA and facilitate viral gene expression can circumvent the limitations of cell surface receptor activation. Clearly, HDAC inhibitors meet these criteria. In general, HDAC inhibitors are safe, orally active and used widely in medicine. In HIV-1 latency, maintaining histones in their deacetylated form by HDACs results in densely packed chromatin in the nucleosome with gene expression quiescent. On the other hand, hyperacetylation of specific lysine residues within nucleosomal histones disrupts chromatin binding and allows transcriptional activation of genes. Thus, adding HDAC inhibitors to cell lines with integrated HIV-1 resulted in expression of HIV-1 without any costimulation of cytokines. Table 1 lists some of the reports on HDAC inhibition *in vitro* from cell lines with integrated HIV-1. The human macrophage cell line U1, which is derived from the U937 line, served as the model for a latent virus in macrophages, whereas Ach2 cells were used as the model for integrated HIV-1 in T cells.

Table 1. Different HDAC inhibitors that have been studied *in vitro* and *ex vivo* for HIV-1 purging.

HDAC inhibitor	Assay	Reference
Sodium butyrate	TE671/RD cells	53
TSA, TPX	U1, ACH2, J49, OM10.1	54
VPA	U1, ACH2, HUT-78/IIIB/LAI	55
TSA	HeLa	56
VPA	RCT	57
SAHA	RCT	58
SAHA	J89, RCT	41
SAHA, IHI	U1, ACH2	59
SAHA, VPA	U1, J-Lat	60
Oxamflatin, VPA, SAHA, TSA	ACH2, 8E5, J-Lat	61
SAHA, VPA	24STNLESG, Hela	42
CG05, CG06	ACH2	62
ITF2357, VPA, IHI	ACH2, U1	39
TSA, oxamflatin	A7	63

TPX, trapoxin; TSA, trichostatin A; RCT, resting $CD4^+$ T cells of aviremic HIV-infected donors; IHI, investigational HDAC inhibitors.

WHICH HDAC IS BEST FOR HIV-1 EXPRESSION?

HDACs are divided into three major classes on the basis of their homolog in yeast. Class I includes HDAC-1, -2, -3 and -8. Class II is comprised of HDAC-4, -5, -6, -7, -9 and -10. HDAC-11 has properties of both class I and class II. As shown in Figure 1, the HIV-1 5′ long terminal repeat (LTR), containing the promoter and enhancer elements, has binding sites for several transcription factors and is arranged in two nucleosomes (nuc-0 and nuc-1) (24). These nucleosomes are positioned in precise locations with respect to different regulatory ele-

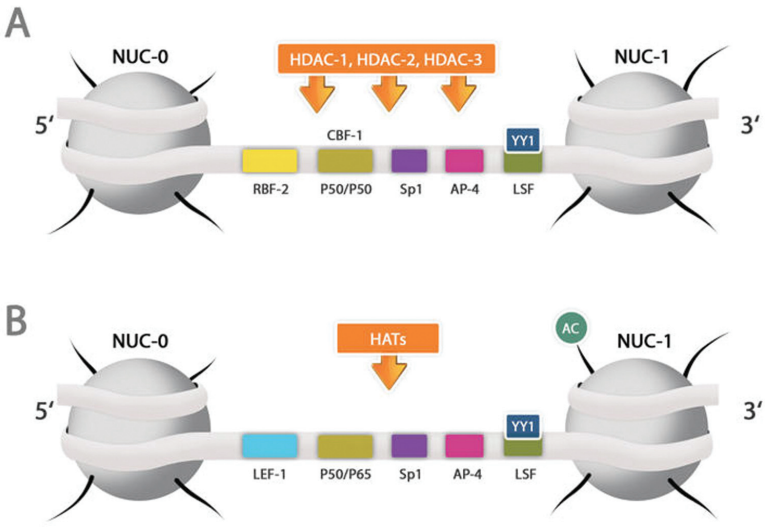

Figure 1. HDAC suppression of HIV-1 expression in latent $CD4^+$ T cells. (A) In the latent state, HDAC-1, -2 and -3 are recruited to the proviral LTR by different DNA binding proteins. Lysine residues on histones on nuc-1 are hypoacetylated and viral transcription is blocked. (B) Upon activation of the cell by exogenous stimuli, HDACs are replaced by histone acetyltransferases and nuc-1 is hyperacetylated with chromatin remodeling and viral gene transcription. Adapted from Colin and Van Lint (64).

ments. During latency, nuc-1 prevents proviral transcription, since it remains in the hypoacetylated state. Chromatin immunoprecipitation assays demonstrated that HDAC-1, -2 and -3 have a role in HIV-1 transcriptional regulation (25,26). Thus, class 1 HDACs are recruited to the proviral DNA, in proximity to nuc-1, through the action of DNA binding proteins. Nuclear factor (NF)-κB p50 homodimer, activating enhancer binding protein 4 (AP-4), retinoblastoma-family protein 2 (RBF2), as well as c-Myc and specificity protein 1 (SP-1) recruit class I HDACs to the LTR, which in turn results in deacetylation of local histones, compaction of the chromatin and prevention of RNA polymerase II binding (25,27–31). *In vitro* studies revealed that after activation by different stimuli, histone acetyltransferases are recruited to the promoter region, leading to acetylation of both H3 and H4 histones. This nuc-1 disruption eventually allows viral transcriptional activation to occur (25,32,33).

TRIALS WITH VALPROIC ACID TO REDUCE THE RESERVOIR OF LATENT VIRUS

As shown in Table 1, various HDAC inhibitors have been studied *in vitro* for HIV-1 expression. However, valproic acid (VPA), a carboxylate HDAC inhibitor that is prescribed for seizures and psychiatric disorders, is the only HDAC inhibitor that has been combined with HAART in clinical trials. VPA was administered to HIV-1-infected subjects at doses used to obtain therapeutic blood levels for seizure control while the subjects were maintained throughout the study on the HAART regimen. In the first study by Lehrman *et al.* (34), enfuvirtide was added to the HAART regimen of four HIV-1-positive patients. After 4–6 wks on intensified HAART, VPA was added to the daily regimen for 16 weeks. Plasma levels of VPA were tested and adjusted for 40–100 μg/mL (comparable to 0.25–0.6 mmol/L). The frequency of resting cell infection was measured before and after the augmented therapy and showed a significant reduction in 3 out of 4 patients. However, no measurements were performed between the addition of efuvirtide to the regimen and VPA. Therefore, an isolated effect of VPA on reducing the viral latency pool was not established.

In light of that first study, Siliciano *et al.* (35) measured the size of the HIV-1 latent reservoir in nine patients receiving both HAART and VPA for at least 3 months. Levels of latently infected cells isolated from peripheral cells were similar to those seen in patients receiving HAART alone. To exclude the possibility that the patients in the study had an exceptionally large latent pool, seven patients' latent pools were measured at least 5 months after the initial sample was performed. There was no reduction of the latent reservoir. In fact, in four patients, the frequencies of HIV-1 gene expression increased. In a French single-center study, similar frequencies of $CD4^+$ T cells with integrated viral DNA, and $CD4^+$ T cells carrying a replication-competent virus, were observed in both patients who were treated with HAART alone and patients who received HAART plus VPA (36). In 2008, a prospective study compared infection of resting $CD4^+$ T cells before and after the addition of VPA to the HAART regimen of 11 HIV-1-positive aviremic patients (37). In only 4 of 11 patients, a depletion of >50% in resting $CD4^+$ T-cells infection was observed, but this effect waned over time, as reported in a follow-up study (38). Plasma HIV-1 RNA concentrations were measured also by ultrasensitive real-time PCR to a limit of detection of 1 copy/mL (single-copy assay). There appeared to be an association between a significant decrease in the number of copies and the absence of low-level viremia. Because VPA alone failed to show the desirable effect, the researchers expanded the study and examined the effect of combing intensified antiviral therapy and the addition of VPA to the regimen of aviremic patients (38). Neither raltegravir nor enfuvirtide, combined with VPA and the baseline HAART, showed a significant decrease.

These studies clearly demonstrate that with or without intensified HAART, the addition of VPA is of limited value in decreasing the reservoir of infected resting $CD4^+$ T cells. Nevertheless, the potential of HDAC inhibitors to purge the virus has not been fully exploited. VPA is a weak and nonspecific HDAC inhibitor. VPA does not specifically target HDAC-1 or HDAC-2, which are thought to be the primary HDACs that prevent HIV-1 expression. Therefore, the failure to target a specific HDAC in the trials of VPA may account for the lack of an effect. In addition, safety and tolerability are essential requirements for the testing of any experimental drug in patients without a life-threatening disease. For example, we reported that VPA significantly increases HIV-1 expression in the latently infected U1 monocyte/macrophage cell line but at concentrations that would have unacceptable side effects for long-term oral dosing (39).

VORINOSTAT, GIVINOSTAT AND BELINOSTAT

Using more selective HDAC inhibitors has been studied *in vitro* and *ex vivo* for inducing HIV-1 expression. Suberoylanilide hydroxamic acid (SAHA; generic vorinostat) is a relatively selective inhibitor for class I HDACs, that is, by inhibiting HDAC-1, -2, -3 and -8, but also with some activity against class II HDAC-6, -10 and -11 (39). However, SAHA lacks activity against class II HDAC-4, -5, -7 and -8. Although SAHA is approved for the treatment of cutaneous T-cell lymphoma, the gastrointestinal side effects and particularly the fall in platelets render the anticancer dosing unacceptable for long-term use in patients with HIV-1 (40). Archin *et al.* (41) examined the ability of SAHA to induce HIV-1 expression in cell lines as well as in virus production from resting $CD4^+$ T cells from patients treated with antiretroviral agents. In those studies, SAHA induced the in-frame marker green florescence protein (GFP) mRNA from J89 cells (latently infected Jurkat cell line encoding the enhanced GFP as a marker for

Tat-driven HIV LTR expression) at nanomolar concentrations compared with millimolar concentrations for VPA. However, at clinically relevant concentrations of SAHA (340 nmol/L), there was no significant increase of GFP mRNA. When SAHA was added to $CD4^+$ T cells *in vitro* at therapeutic concentrations, there was a low level of induction of virus outgrowth in 4 out of 5 patient cells. Similarly, when used with other types of latently integrated HIV-1 cell lines, the efficacy of SAHA was no different from that of VPA (42).

Similar to SAHA, ITF2357 (generic givinostat) is a hydroxamic acid–containing HDAC inhibitor that has antiinflammatory properties *in vitro* and *in vivo* as summarized in this issue of *Molecular Medicine*. At therapeutic plasma levels of 125–250 nmol/L, there is no cell toxicity *in vitro*, and only minor thrombocytopenia occurs in patients (43). Givinostat has been administered for 12 weeks in children with systemic-onset juvenile idiopathic arthritis without side effects at a therapeutically effective dose of 1.5 mg/kg (44). The use of HDAC inhibitors in rheumatoid arthritis is reviewed in the present issue (44). We have examined the ability of ITF2357 to induce *HIV-1* gene expression *in vitro* from the latently infected cell lines ACH2 (T-cell line) and U1 (promoncytic line) (39). Givinostat increased p24 by 15-fold in ACH2 cells and, at clinically relevant concentrations, was approximately 10 times more efficient in HIV-1 stimulation than VPA. In U1 cells, VPA failed to double HIV-1 expression, as measured by p24 levels, whereas 250 nmol/L of ITF2357 induced a nine-fold increase (39).

PXD101 (belinostat), another hydroxamic acid–containing HDAC inhibitor, has activity against class I and II HDACs at nanomolar concentrations, but is slightly less potent than ITF2357 (45). However, because PXD101 has primarily been administered through the intravenous route for treatment of various cancers (46), information on safety, tolerability and pharmacokinetics using oral administration is still limited (47). Certainly, the side effects from the dosing used in patients with refractory cancer diseases will be unacceptable for HIV-1-infected patients on suppressive HAART. As shown in Figure 2, we compared the ability of VPA, SAHA, ITF2357 and PXD101 to induce HIV-1 expression in the latently infected cell line U1. ITF2357 and PXD101 stimulate HIV-1 expression with a similar potency, and both have activity within the low 125–500 nmol/L range. Higher concentrations of SAHA were needed to achieve the same virus production, and, as expected, millimolar concentrations of VPA were needed for HIV-1 expression in this system (Figure 2).

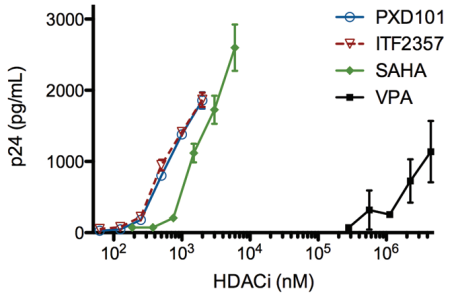

Figure 2. HIV-1 expression in U1 cells stimulated by ITF2357, PXD101, SAHA or VPA. Mean ± SEM p24 levels of two separate experiments for HDAC inhibitors (HDACi) are indicated under the horizontal axis. Phorbol-12-myristate-13-acetate (PMA) is used as the positive control (omitted from figure). ITF2357 was supplied by Italfarmaco, SpA, Cinisello Balsamo, Italy; PXD101 and SAHA were purchased through Selleck Chemicals LLC (Houston, TX, USA).

THE IMPORTANCE OF HIV-1 SELECTIVITY

As reviewed above, class 1 HDACs and specifically HDAC-1, -2 and -3 are considered the major HDACs involved in HIV-1 silencing. Therefore, the use of selective HDAC inhibitors would be a reasonable approach to increase its efficacy and at the same time decrease any adverse effects. Specific enzymatic activity assays have been used to compare the selectivity of vorinostat, VPA and givinostat (39). Analogs of givinostat with a different selectivity profile were also examined. The class 1 HDAC selectivity profile correlated directly with HIV-1 stimulation from the latently infected U1 and Ach2 cell lines. Whereas VPA showed a weak and nonselective HDAC inhibition, the most selective givinostat analog showed a 30-fold increase in HIV-1 expression from ACH2 cells. This effect was not due to cell stress. Interestingly, in Ach2 cells, the degree of HDAC-2 inhibition was directly correlated with the induction of *HIV-1* gene expression (39). These observations correlate well with the molecular association between the HIV-1 LTR and HDAC-1, -2 and -3.

A recent study by the Margolis group demonstrated that HDAC inhibitors, which specifically targeted class I HDACs, induced *HIV-1* gene expression from cell lines as well as in resting $CD4^+$ T cells of aviremic patients (48). An additive effect was seen with inhibition of HDAC-6 as well. These findings support data regarding the selectivity of class I HDAC-1, -2 and -3 for induction of *HIV-1* gene expression. However, preserving the deacetylated status of HDAC-6 prevents HIV-1 infected $CD4^+$ T cells from *Env*-mediated syncytia formation (49). Therefore, analogs that have inhibitory properties targeting HDAC-6 may not be optimal.

EFFECT OF HDAC INHIBITION ON HIV-1 CORECEPTORS

Despite the fact that HDAC inhibitors often increase gene expression, ITF2357 did not increase CCR5 surface expression on $CD4^+$ T cells (39). It would be counterproductive if agents stimulating the latent virus upregulated CCR5 and CXCR4 surface expression. In fact, surface expression of CXCR4 on $CD4^+$ T cells and CCR5 on monocytes was reduced by 50% by ITF2357 (39). Consistent with decreasing coreceptor surface expression, ITF2357 reduced steady-state mRNA levels of CXCR4 and CCR5 in peripheral blood mononuclear cells (PBMCs), as measured by reverse transcriptase–polymerase chain reaction (RT-PCR) and by Affimetrix gene expression, respectively. Thus, in a clini-

cal trial of givinostat in HIV-1-infected patients, this property of givinostat may also reduce any reinfection of naive $CD4^+$ T cells.

STUDY DESIGN OF NEW HDAC INHIBITORS IN HIV-1 PURGING

Safety is always a consideration when evaluating a drug to treat a disease with no immediate danger to the patient. In testing the hypothesis that HDAC inhibition will purge the latent pool of HIV-1, VPA falls short because of a low level of induced expression compared with ITF2357. Givostat is safe and effective in humans. In healthy human subjects in a phase I trial, a single dose of givinostat of 1.5 mg/kg resulted in a peak plasma level of 200 nmol/L (43). In a phase II trial in children with active systemic-onset juvenile idiopathic arthritis, a daily oral dose of givinostat at 1.5 mg/kg for 12 weeks exhibited no organ toxicity and achieved significant ($P < 0.01$) reduction in parameters of systemic disease as well as the number of painful joints (44). Doses of VPA, on the other hand, when used to reach concentrations >110 μg/mL in females or >135 μg/mL in males, carries significant side effects and especially severe thrombocytopenia (50). Thus, higher concentrations of VPA would be impractical to induce more viral expression.

In light of the *in vitro* data suggesting its potency in inducing HIV-1 expression as well as the clinical data suggesting that givinostat is safe, a phase II clinical trial of givinostat would be worthwhile. Administration of givinostat at 1.5 mg/kg or possibly 1.0 mg/kg in two divided doses may induce a limited number of $CD4^+$ T cells to express HIV-1. One end point of such a study is whether after a 12-week course there is a reduction in the inducible pool of latent virus. In addition, it would also be important to determine whether anti–HIV-1 cytotoxic T cells increased after these courses of givinostat. Although some patients may be reluctant to stop antiretroviral therapy after courses of HDAC inhibitors, the *ex vivo* testing of the reservoir would provide evidence that there has been an objective reduction in the latent pool. Surely, the test of the hypothesis that HDAC inhibitor can reduce the reservoir comes with cessation of antiretroviral therapy and the duration of time before circulating mRNA levels reappear. Ideally, there would be no return of viral load. Nevertheless, a significant delay in the return of viral load would provide encouragement for the development of specific targeting of HDACs that assist in maintaining HIV-1 latency.

At another level, givinostat or a similar well-tolerated HDAC inhibitor may provide an additional benefit to HIV-1-infected patients. There are patients being treated with antiretroviral therapy with <50 copies of HIV-1 mRNA but who succumb to all-cause mortality, particularly cardiovascular and renal events (51). In these patients, biomarkers of inflammation, C-reactive peptide (CRP), IL-6 and D-dimer are elevated. The relative risk for death associated with elevated CRP in these patients was 2.0: for elevated IL-6, it was 8.3, and for D-dimer, the increased risk was 12.4 (51). In patients who delay antiretroviral therapy, there was an expected rise in $CD4^+$ T cells on initiation of antiretroviral therapy. Although there was also a decrease in the level of D-dimer, there was no change in elevated IL-6 or CRP (52). It is presently unclear whether the decrease in D-dimer will reduce clinical disease; however, the continued elevated IL-6 and CRP levels indicate a continued inflammatory state, even in patients being successfully treated with antiretroviral therapy and who sustain an increased risk for non–HIV-1-associated death. Given the antiinflammatory properties of HDAC inhibitors, the markers of systemic inflammation may decrease in a study of HIV-1 purging. On the other hand, it is possible that markers of inflammation may increase as a result of HIV-1 expression and inadequate prevention of reinfection by antiretroviral therapy.

CONCLUSION

Purging the latent reservoir of HIV-1 in resting $CD4^+$ T cells by HDAC inhibitors is under active clinical investigation. Use of well-tolerated and selective HDAC inhibitors for HIV-1 purging should be tested *in vitro* and provide a rationale for clinical studies in aviremic HIV-1 patients on antiretroviral therapy.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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