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Gnidimacrin, the Potent Anti-HIV Diterpene Can Eliminate Latent HIV-1 *Ex Vivo* by Activation of PKC Beta

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Abstract

HIV-1 latency-reversing agents, such as histone deacetylase inhibitors (HDACIs), were ineffective in reducing latent HIV-1 reservoirs *ex vivo* using CD4 cells from patients as a model. This deficiency poses a challenge to current pharmacological approaches for HIV-1 eradication. The results of this study indicated that gnidimacrin (GM) was able to markedly reduce the latent HIV-1 DNA level and the frequency of latently infected cells in an *ex vivo* model using patients peripheral blood mononuclear cells (PBMC). GM induced approximately 10-fold more HIV-1 production than the HDACI SAHA or romidepsin, which may be responsible for the effectiveness of GM in reducing latent HIV-1 levels. GM achieved these effects at low picomolar concentrations by selective activation of protein kinase C β I and β II. Notably, GM was able to reduce the frequency of HIV-1 latently infected cells at concentrations without global T cell activation or stimulating inflammatory cytokine production. GM merits further development as a clinical trial candidate for latent HIV-1 eradication.

TOC graphic

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Supporting Information

Antagonism of enzastaurin on GM-mediated latent viral activation and effects of high dose gnidimacrin on DC25 and CD69 expression were included in Supporting Information.

Notes

The authors declare no competing financial interest.

Keywords

Gnidimacrin; PKC agonist; HIV-1 latency; HIV-1 latency reversing agent

INTRODUCTION

Despite the advancement of antiretroviral therapy (ART), the AIDS pandemic remains a serious public health problem for many countries around the world. ART has proven successful in controlling HIV-1 replication in infected individuals by reducing plasma viral loads to undetectable levels.^{1,2} However, the viruses in these patients are suppressed rather than truly eradicated.^{3–6} Persistent HIV-1 infection, especially in latent viral reservoirs, remains a challenge for effective AIDS therapy. HIV-infected resting T cells are the major latent HIV-1 reservoir in HIV-positive patients.^{3,4} Other potential reservoirs, such as monocyte/macrophage lineages, were also reported.⁷ “Shock and kill,” which includes pharmacological activation of latent viruses, is one of the current strategies for HIV-1 eradication. Activation of the latent virus is believed to make the virus and infected cells susceptible to immune clearance and cytopathic effects (CPE) of the virus. The drug candidates for activation of a latent virus include histone deacetylase inhibitors (HDACIs), methylase inhibitors, cytokines, and protein kinase C (PKC) activators.⁸ For example, vorinostat (SAHA), an HDACI, was found to disrupt HIV-1 latency in some HIV-1–positive patients.⁹ A more potent HDACI, romidepsin, was shown to activate latent HIV-1 in patients’ CD4 cells.^{10,11} Patient-derived, HIV-1 peptide pulsed broadly specific CD8 cells, were able to clear latently infected autologous resting CD4⁺ T cells without impairing CD8 activity at physiologically relevant exposures SAHA.¹² However, it has been reported that HDACIs, including SAHA and romidepsin, were not effective in reversing HIV latency using CD4 cells from patients undergoing successful ART.^{13,14} Furthermore, a recent report

suggests that HDACIs can induce apoptosis and impair immune functions including cytotoxic T lymphocyte (CTL) activity and interferon gamma (IFN- γ) production.¹⁵

The lack of latent virus clearance following the treatment of latency-reversing agents is likely due to weak CTL activity in patients undergoing ART. Reactivation of HIV-1 specific CD8 cells appears to be required for clearance of SAHA-activated latently infected CD4 cells.^{12,16} The effectiveness of CTL responses is determined by both the quantitative and qualitative nature of antigenic peptides.¹⁷ We hypothesize that elimination of the latently infected cell can be achieved by a strong latent virus activator, which can induce robust viral replication, resulting in a more effective antigen presentation for CTL and/or viral CPE.

To test this hypothesis, we have searched for potent latency-reversing agents and discovered that several daphnane type diterpenes, including gnidimacrin (GM), are extremely potent HIV-1 regulators that activate HIV-1 replication in chronically infected cells and inhibit *de novo* infection of peripheral blood mononuclear cells (PBMCs) by HIV-1 R5 strains at low pM concentrations.^{18–20} Activation of PKC is likely responsible for the potent anti-HIV activity since GM was previously reported to exhibit potent anti-cancer cell activity through activation of PKC β II.^{21,22} The potent dichotomous anti-HIV-1 activities make GM an attractive candidate to test the possibility that strong latent virus activation could result in a reduction of the latent viral reservoir.

A goal of this study is to determine if a strong latency-reversing agent, such as GM, is capable of eliminating latently infected lymphocytes from patients with undetectable viral loads undergoing ART. The results of this study indicate that GM is indeed able to reduce latent HIV-1 DNA levels and the frequency of HIV-1 latently infected cells in an *ex vivo* model using latently infected PBMCs from HIV-1–positive individuals undergoing successful ART. The results of this study also suggest that elimination of the latently infected cells in patients' T lymphocytes may be achieved by robust viral replication induced by GM.

RESULTS

GM reduced HIV-1 DNA in latently infected CD4 cells *ex vivo*

We have previously shown that GM is able to activate latent HIV-1 in a U1 cell model at low pM concentrations.¹⁸ In the same study, the HDACI SAHA was much weaker than GM in both potency and the extent of maximal virus activation. Based on these data, we hypothesized that potent latent viral activation by GM may cause strong CTL responses and/or CPE to reduce or eliminate the latently infected cells. To determine whether GM can reduce the latent viral reservoir in resting CD4 cells, PBMCs from three HIV-1–positive patients (Pt-1, Pt-2, and Pt-3) were treated with GM (1.0 nM) or SAHA (0.5 μ M) in the presence of three antiretrovirals, TMC278 (30 ng/ml), AZT (300 ng/ml), and indinavir (300 ng/ml), for 6 days. At the time of PBMC sample collection, the patients were undergoing successful ART with undetectable viral load (Table 1).

The HIV-1 DNA level in the PBMC samples after GM or SAHA treatment was quantified with real time PCR using a pair of primers that amplified HIV-LTR. GM treatment greatly

reduced HIV-1 DNA levels seven- to eight-fold in the PBMCs from all three patients (Figure 1A). On the other hand, SAHA was ineffective in reducing HIV-1 DNA in the PBMCs from two patients. There was a small decrease in the viral DNA content, approximately 30%, in the SAHA treated PBMCs from the third patient (Pt-3). The reduction of HIV-1 DNA in the PBMCs of the three patients treated with 1 nM of GM, when compared with control, was statistically significant with $p=0.038$ in contrast to SAHA with $p=0.995$. These results suggest that a majority of latently infected cells may have been eliminated from the patients' PBMCs in the presence of GM.

GM reduced the frequency of latently infected PBMCs in a viral outgrowth assay

To further test whether GM treatment can reduce a latent HIV-1 reservoir, the frequency of latently infected cells in PBMCs was determined using a limiting dilution assay modified from a previously reported method.^{23,24} Part of the treated PBMCs from Pt-1, Pt-2, and Pt-3 described above for the HIV-1 DNA reduction study were used for the viral outgrowth assay without isolating CD4 cells to best mimic the *in vivo* condition. The GM treatment resulted in a six-fold decrease in infectious units per million PBMCs for Pt-1 (Figure 1B), while SAHA treatment resulted in a two-fold decrease in infectious units of PBMCs from the same patient. The frequency of latently infected cells was undetectable in Pt-3 after GM treatment, whereas there was no significant reduction of latently infected cells in the presence of SAHA. The infectious virus from the PBMCs of Pt-2 was below detection levels with or without drug treatment under the assay conditions. These results suggest that GM can markedly reduce the frequency of latently infected cells, and it is a much stronger latency-reversing agent than SAHA.

Low dose of GM (20 pM) reduced HIV DNA and frequency of latently infected PBMCs

We have previously shown that GM could activate HIV in the latent U1 cell model at low pM concentration.¹⁸ Using lower concentrations of GM may further reduce the possibility of toxicity and side effects. To test the effects of GM at a low dose on reducing latent HIV-1 DNA, PBMCs from Pt-3, Pt-4, and Pt-5 were treated with 20 pM of GM for 6 days in the presence of three antiretrovirals to prevent reinfection of nascent HIV-1. The data indicated that GM at 20 pM markedly reduced HIV-1 DNA in the PBMCs of all three patients (Figure 2A). Compared to the 8.5-fold reduction in HIV-1 DNA in the PBMCs of Pt-3 treated with 1 nM of GM, the low dose GM reduced the HIV-1 DNA by 5.6-fold. The low dose treatment also reduced HIV-1 DNA in the PBMCs of Pt-4 and Pt-5 by 7- and 4.4-fold, respectively.

To determine if the low dose of GM can reduce the frequency of latently infected cells, the same treated PBMCs from Pt-3, Pt-4, and Pt-5 from the experiment described above were subjected to the viral outgrowth assay. PBMCs from Pt-4 did not have detectable viruses under the assay condition (Fig. 2B). On the other hand, PBMCs from Pt-3 and Pt-5 contained relatively high frequencies of latently infected cells. GM treatment (20 pM) effectively reduced the frequency of latently infected cells in the PBMCs of Pt-3 and Pt-5 by at least 5-fold.

CD8 may play a role in reducing HIV-1 DNA in CD8-depleted PBMCs from some but not all patients

To determine if CD8 cells play a role in the GM-mediated reduction of HIV-1 DNA, CD8 cells were depleted from the PBMCs of five patients. GM (1 nM) or SAHA (0.5 μ M) were used to treat the CD8-depleted PBMCs in the presence of three antivirals in combination for 6 days in the same manner as described above. SAHA showed minimal effect on the level of HIV-1 DNA in the CD8-depleted PBMCs from all five patients. In contrast, there was a strong reduction (> eight-fold) of HIV-1 DNA in the CD8-depleted PBMCs in three (Pt-1, Pt-6, and Pt-7) out of the five patients treated with GM (Figure 3A), and less than two-fold reduction of HIV-1 DNA in the CD8-depleted PBMCs from Pt-2 and Pt-5. These differences between treatment and control were statistically significant with GM ($p=0.011$) but not with SAHA ($p=0.644$).

The same GM- or SAHA-treated CD8-depleted cells were also subjected to the viral outgrowth assays (Figure 3B). As expected, SAHA did not reduce the frequency of HIV-1 infected cells in the CD-8 depleted PBMCs from all five patients. In fact, SAHA treatment resulted in a two-fold increase in the frequency of HIV-1 infected cells in three out of five patients. The reasons or mechanisms responsible for the increase are unclear. In contrast to SAHA, GM treatment caused a significant reduction in the frequency of HIV-1 latently infected cells in the CD8-depleted PBMCs from four out of five patients, with the exception of Pt-5 ($p=0.032$). These results suggest that GM treatment could reduce both HIV-1 DNA and those cells harboring replication competent latent HIV-1 in the majority of the patients in the absence of CD8 cells. However, CD8 activity could be important for reduction of latent HIV-1 levels upon GM treatment in some cases, such as in Pt-5.

GM is a much stronger HIV-1 activator than SAHA or romidepsin

Our results clearly showed that GM was much more effective than SAHA in reducing the HIV-1 DNA and frequency of latently infected cells using the *ex vivo* latent HIV-1 infection model described above. It is possible that the effectiveness of GM was due to its ability to induce robust HIV-1 production. To test this possibility, the potency and extent of virus induction of GM, SAHA, and romidepsin were compared using the U1 cell model. Our results showed that GM was approximately 50-fold more potent than romidepsin and was at least 10^4 -fold more potent than SAHA in latent HIV-1 activation in the U1 cells (Table 2). GM was able to potently activate latent virus at concentrations at least 1,000-fold lower than that were toxic to the HIV-free U937 cells, the parental cells of U1 cells. In contrast, the two most well studied HDACis, SAHA and romidepsin, activated the latent virus at the same concentration ranges that cause cytotoxicity. The EC_{50} of SAHA and romidepsin for latent virus activation is very close to their respective CC_{50} . This suggests that the two HDACis may have poor selectivity when used for latent virus activation.

More importantly, GM induced approximately 10-fold more HIV-1 production than SAHA or romidepsin in U1 cells (Table 2). To determine whether such a strong stimulatory activity of GM can be observed in primary lymphocytes, PBMCs from Pt-1 and Pt-6 were treated with SAHA (0.5 μ M), phytohaemagglutinin (PHA), or GM (1 nM) in the absence of other antiviral drug for 18 hours. The viruses released into culture supernatant were detected by

using a nested polymerase chain reaction. Treatment of the PBMCs from both patients with PHA or GM resulted in a significant higher level of HIV-1 RNA than that with SAHA (Figure 4). The viral RNA was neither detectable in the culture supernatant of SAHA treated PBMCs from Pt-6, nor from DMSO (solvent of GM and SAHA) treated cells. These results suggest that GM can induce much more viral antigens in HIV-1 latently infected cells, which may render the HIV-1 producing cells susceptible to CTL and/or CPE of the replicating viruses.

GM did not significantly affect INF-r production at a concentration that activates latent virus in PBMCs

HADCIs and PKC agonists both could have immune-regulatory activity which may negatively impact immune responses, such as suppression of IFN-r production by HADCIs.^{15,25} Therefore, GM was tested for its effects on IFN-r production of PBMCs from Pts-1–3 at the end of the 6-day treatments. As described above, the patient PBMCs were treated with GM (1 nM) or SAHA (0.5 μ M) in the presence of three antiretrovirals for 6 days. Two of the patients (Pt-1 and Pt-3) had very low IFN-r levels (Figure 5), while the IFN-r level was relatively high in the culture supernatant of the PBMCs from Pt-2. As expected, SAHA potently inhibited IFN-r production at 0.5 μ M. In contrast, GM at 1 nM did not affect IFN-r production from the PBMCs of Pt-2. GM did not significantly change (up-regulate) IFN-r levels in the PBMCs from Pt-1 and Pt-3 either. It is interesting that the PBMCs of Pt-2 did not produce detectable virus levels in a viral outgrowth assay (Figure 1B). It is not clear if the high INF-r level is associated with suppression of virus production.

GM selectively activated PKC β without affecting PKC α and PKC θ

Multiple PKC isozymes are expressed in T cells. Among PKC isozymes, PKC α and PKC θ are principally relevant to the potential side effects when using PKC agonists for latent HIV-1 activation. Tumor promotion through the activation of PKC α is a major concern with PKC agonists such as phorbol 12-myristate 13-acetate (PMA).^{26,27} In addition, PKC θ is predominantly expressed in the T cells and localized in the center of immunological synapse, which controls the proliferation and differentiation of T cells.²⁸ Therefore, selective activation of PKCs without affecting PKC α and PKC θ may help to avert severe side effects including perturbation of normal T cell functions.

In order to determine the PKC isozyme specificity of GM, anti-CD3/CD28 (1 μ g/ml) activated human primary CD4 cells were cultured in the presence or absence of GM (1 nM) for 12 hours. The testing concentration (1 nM) is over 10-fold higher than that required for the dichotomous anti-HIV activity (EC_{50}) of GM.¹⁸ The cytosolic fraction of the cells was analyzed with 10% SDS PAGE followed by Western blot analysis using a panel of monoclonal antibodies against PKC isozymes (Santa Cruz Biotech). PKC activation is expected to decrease cytosolic PKC levels as a result of translocation of the enzyme from cytosol to the cell membrane. As shown in Figure 6, GM significantly reduced cytosolic PKC β I and β II levels without significantly affecting the levels of PKC α and PKC θ . Involvement of PKC β in latent HIV-1 activation by GM was also supported by the results that enzaustaurin,²⁹ a selective PKC β inhibitor, could partially antagonize GM in latent viral activation using the U1 cell model (Figure S1, Supporting Information). Enzaustaurin

at 2 μ M antagonized GM-mediated viral activation by approximately 60%. On the other hand, a non-selective PKC inhibitor, staurosporin,³⁰ did not affect the GM-mediated latent viral activation at 4 nM. Enzaustaurin and staurosporin were toxic to U1 cells at concentrations higher than 2 μ M and 4 nM, respectively, which precluded testing these compounds at higher concentrations in the U1 cell model.

GM did not induce the expression of T cell activation markers at latency-reversing concentrations

Global T cell activation through CD3 and CD28 was shown to release cytokines and chemokines, which leads to T cell depletion in HIV-infected patients.^{31,32} To test whether GM induces global T cell activation, the expression of the early and late T cell activation markers, CD69 and CD25, on GM treated normal PBMCs were measured. Anti-CD3/CD28 antibodies and prostratin were used as controls. Prostratin is a non-selective PKC agonist previously shown to activate latent HIV without significant effects on T cell proliferation.³³ However, under our experiment condition, prostratin at 0.6 μ M up-regulated the expression of both CD69 and CD25 (Figure 7). In contrast, GM at a concentration (0.3 nM) that maximally activates latent HIV-1 in a U1 cell line model¹⁸ did not significantly affect the expression of CD69 and CD25. As shown above, GM at 20 pM significantly reduced HIV-1 DNA and frequency of latent viral infected cells *ex vivo* (Figure 1–3). This suggests that GM may be able to reduce latent HIV-1 reservoirs without T cell activation.

To determine whether a higher concentration of GM will alter the expression level of CD69 and CD25, PBMCs were treated with GM at 1 and 3 nM, which is approximately 100-fold more than the concentration required for latent HIV-1 activation. Although CD25 levels were not significantly affected, CD69 levels were increased under this condition (Figure S2, Supporting Information).

GM did not significantly affect pro-inflammatory cytokine production at active latency-reversing concentrations

Excessive T cell activation could result in severe side effects including overproduction of inflammatory cytokines.³⁴ To determine whether GM might cause this adverse effect, human PBMCs were treated with GM (0.3 nM), prostratin (0.6 μ M), or anti-CD3/CD28 (1 μ g/ml) for 2 days. As expected, the levels of 6 out of 12 inflammatory cytokines were significantly increased by anti-CD3/anti-CD28 (Figure 8). The effect of prostratin on cytokine production was similar to that of anti-CD3/CD28. In contrast, GM at 0.3 nM did not significantly alter the production of the 12 cytokines.

DISCUSSION and CONCLUSIONS

Despite some previous reports showed that latency-reversing agents used alone or in combination could activate latent HIV-1 *ex vivo*,^{9–11,35,36} it is generally believed that a single latency-reversing agent is not sufficient to significantly reduce the size of the HIV-1 reservoir without enhanced CTL or other immune mechanisms.^{12,16} In this study, we showed that GM alone could markedly reduce latently infected cells at a low pM concentration in an *ex vivo* model. The PBMCs used in the study were collected from

HIV-1–positive individuals who had been under continuous ART for 6 to 21 years (Table 1). We found that GM treatment significantly decreased the proviral DNA levels in patient PBMCs. There was an average of 7.7-fold decrease in proviral DNA when PBMCs from 3 patients, Pts-1–3, were treated with 1 nM of GM.

A significant reduction of HIV-1 DNA levels could also be observed at a GM concentration as low as 20 pM. There was an average of 5.7-fold decrease in proviral DNA when PBMCs from 3 patients, Pts-3–5, were treated with 20 pM of GM. Regardless of high or low dose GM treatment, the decrease in HIV-1 DNA was observed in all tested PBMCs from five HIV-1–positive individuals, ranging from 4.4- to 8.4-fold reduction. These results strongly suggest that GM can eliminate cells that harbor HIV-1 DNA. In contrast, SAHA did not significantly reduce HIV-1 DNA levels in the tested PBMCs from these patients.

Many quantitative PCRs are used to quantify HIV-1 latently infected cells.^{37,38} However, there appeared to be no significant correlation between the frequency of cells harboring replication-competent viruses and proviral DNA levels in PBMC samples from patients undergoing ART.³⁸ Since GM is expected to have similar activation effects on both defective and replication-competent viral DNA, it is likely that a markedly decreased HIV-1 DNA will be accompanied by a reduced frequency of latently infected cells harboring replication-competent HIV-1. Our results are consistent with this notion in that GM treatment could eliminate CD4 cells that harbor replication-competent HIV. The GM-treated PBMCs from 2 out of the 5 patients did not have detectable virus levels in the absence of latency-reversing agents. It is possible that low frequency of latently infected cells and/or high non-lytic CD8 suppressor activity of the PBMC samples are responsible for the undetectable virus replication. On the other hand, the rest of the GM treated PBMCs from Pt-1, Pt-3, and Pt-5 had at least a five-fold reduction in the frequency of latently infected cells. In contrast to GM, SAHA only showed a two-fold reduction in one patient (Pt-1). This is consistent with recent reports that HDAC inhibitors are ineffective in latent HIV-1 activation *ex vivo*.^{13,14}

Global T cell activation has been one of the main concerns regarding the use of PKC activators as latency-reversing agents. We have previously shown that GM can activate latent HIV-1 at pM concentrations using ACH-2 and U1 cell line models.¹⁸ This study further demonstrated that GM can effectively reduce the frequency of HIV-1 latently infected cells from HIV-1–positive individuals at a concentration as low as 20 pM. Such a low concentration is unlikely to have effect on global T cell activation since GM at 0.3 nM did not significantly affect the expression of the T cell activation markers CD25 and CD69 (Figure 7). The lack of effect on CD25 and CD69 expression is consistent with the results that GM at 0.3 nM did not significantly alter a panel of inflammatory cytokine production from normal human PBMCs (Figure 8). It is interesting that GM at a higher concentration showed differential effects on the surface expression of CD25 and CD69 (Figure S2). GM at as high as 3 nM did not significantly affect CD25 levels, but it did induce the surface expression of CD69. One possible explanation of this differential effect is that GM at 3 nM is effective in translocating CD69 from an existing cytoplasmic pool, but not sufficient to induce the gene expression of the T cell activation markers, CD25 and CD69. It is known that surface expression of CD69 does not initially require new RNA or protein synthesis,

while the expression of CD25 requires gene transcription.^{39,40} The lack of global T cell activation of GM at latency-reversing concentrations is consistent with the results that GM at 0.3 nM did not significantly alter inflammatory cytokine production of PBMCs (Figure 8).

The results of this study clearly indicate that GM is able to activate latent HIV and reduce latent HIV-1 reservoirs from all the tested patient cells. GM treatment resulted in reduction of both HIV-1 DNA and frequency of HIV-1 latently infected cells. This effect was less prominent in a small part of patients when CD8 cells were depleted from PBMCs (Figure 3). However, elimination of latently infected cells from majority of patients by GM was comparable to that without CD8 depletion. These results suggest that the GM-mediated latent HIV elimination may involve multiple mechanisms including both viral CPE and CD8-mediated elimination of latently infected cells in the presence of GM.

In summary, GM is a potent HIV-1 latency-reversing agent that can eliminate HIV-1 latently infected cells at low pM concentrations. GM achieved this anti-HIV latency activity through activation of PKC β I and β II without affecting PKC α and PKC θ . This is critically important because activation of PKC α is linked to tumor promotion and PKC θ controls the proliferation and differentiation of T cells.^{26–28} Furthermore, GM alone was able to reduce HIV-1 DNA and the frequency of HIV-1 infected cells in an *ex vivo* latent HIV-1 model without global T cell activation or stimulating inflammatory cytokine production. The anti-latent HIV activity is likely associated with the ability of GM to induce robust virus replication. A future animal model study is warranted to determine the *in vivo* efficacy of this class of specific PKC activators in reduction of latent HIV-1 reservoirs.

EXPERIMENTAL SECTION

Antivirals and reagents

Gnidimacrin used in this study was isolated from *Stellera chamaejasme* L. as previously described.¹⁹ The purity of gnidimacrin (>98%) was confirmed by quantitative high-performance liquid chromatography photo-diode array (HPLC-PDA) analysis and ¹H NMR spectroscopic analysis. Prostratin, staurosporin, and enzastaurin were purchased from LC Laboratories, Woburn, MA. Romidepsin is obtained from MedChem Express, Monmouth Junction, NJ. AZT, SAHA, and phytohemagglutinin (PHA) were purchased from SigmaAldrich, St. Louis, MO. Indinavir was obtained from NIH AIDS Reagent Program. TMC278 was provided by Dr. Lan Xie of Beijing Institute of Toxicology, Beijing, China. Anti-CD3 and antiCD28 antibodies were purchased from BD Biosciences, San Jose, CA.

Patient profiles

The patient profiles, including CD4 count, plasma, and duration on ART at the time of sample collection, are shown in Table 1. Due to the limited cell quantity available from the 7 patients, the PBMC samples were divided into four groups for different experimental conditions: low dose GM, high dose GM, high dose GM on CD8-depleted PBMCs, and effects on virus production (Table 1).

Quantitative measurement of HIV-1 proviral DNA by real-time PCR (RT-PCR)

PBMCs from HIV-1-positive patients were treated with GM or SAHA (0.5 μ M) for 6 days in the presence of three antiretrovirals, TMC278 (30 ng/ml), AZT (300 ng/ml), and indinavir (300 ng/ml). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units of penicillin/streptomycin, and 5% T cell growth factor (TCGF) (ZeptoMetrix, Buffalo, NY). The culture medium and antivirals were refreshed every two days. Genomic DNA was extracted from the cells after the 6-day treatment. A part of the treated cells was set aside for a limiting dilution viral outgrowth assay, described below. The rest of the treated cells were used for HIV-1 DNA quantification. DNA levels in each sample was quantified in triplicate by RT PCR using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and HIV-1 5' LTR specific primers (HIVshortF and HIVshortR) described by Overman et al.⁴¹ The two primers used in the RT-PCR were 5'-CCTGGGAGCTCTCTGGCTAA-3' and 5'-AACAGACGGGCACACACTACTTT-3. RT-PCR was performed in an ABI 7500 Real-time PCR System with the following cycling profile: one cycle of 50°C for 2 min, one cycle of 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Dissociation curves were performed on all PCR reactions to determine the specificity of each PCR reaction. The absolute quantification data was analyzed using Sequence Detection Software v1.2.2 (Applied Biosystems).

Detection of extracellular HIV-1 RNA with a nested reverse transcription polymerase chain reaction (rt-PCR)

HIV-1 RNA in PBMC culture supernatant was prepared using a protocol modified from that described by Bullen et al.¹³ Five million PBMCs from HIV-1-infected individuals were treated with GM (1 nM), SAHA (500 nM), or PHA (0.5 μ g/mL) in 1 ml of RPMI 1640 with 10% FBS for 18 hours. HIV-1 RNA was extracted from 0.25 ml of the culture supernatants with a Qiagen QIAamp viral RNA kit according to the manufacturer's protocol. Five μ L of the mRNA sample was used for a nested rt-PCR described below. The sequences of the forward and reverse rt-PCR primers were 5'-CAGATGCTGCATATAAGCAGCTG-3' (9501-9523) and 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTGAAGCAC-3' (9629-poly A) (13). The primers for subsequent nested forward and reverse PCR primers were 5'-TGCCTGTACTGGGTCTCTCTG-3' (9529-9549) and 5'-TGAAGCACTCAAGGCAAGCT-3' (9617-9636), respectively. Nucleotide coordinates are indicated relative to HXB2 consensus sequence. The primary rt-PCR was performed using a QIAGEN OneStep RT-PCR kit according to the manufacturer's protocol. The PCR was performed using a Bio-Rad T-100 thermal cycler with the following cycling profile: 50°C for 30 min; 95°C for 15 min followed by 35 cycles of 45 sec at 94°C, 45 sec at 55°C, and 1 min at 72°C. The rt-PCR was concluded with a final extension of 10 min at 72°C. The rt-PCR product (2.5 μ L) was used for the nested PCR: 5 min at 95°C; 20 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, which was followed by a final extension at 72°C for 10 min.

Limiting dilution viral outgrowth assay

A portion of GM or SAHA treated PBMCs from the above described RT-PCR experiment was used to determine the frequency of latently infected cells using a limiting dilution viral

outgrowth assay method similar to that described by Siliciano et al.²³ The medium with compounds was completely removed by washing the PBMCs with culture medium. The cells were then activated with 0.5 µg/mL of PHA for 48 hours. A series of five-fold dilutions of the activated patient PBMCs, starting at 5×10^6 , were co-cultured with PHA-activated uninfected donors' PBMCs at a ratio of 1 to 2 (patient to normal PBMCs). The cell mixtures were cultured for 10 days and the culture medium was refreshed every 2 days. The culture supernatants were tested for HIV-1 p24 protein on day 10 using a Perkin Elmer P24 ELISA kit. The frequency of latently infected cells among the input patient's PBMCs was calculated by a maximum likelihood method as previously described and is expressed as infectious units per million.²³

Depletion of CD8+ lymphocytes

Patient PBMCs were washed and re-suspended in PBS supplemented with 0.5% bovine serum albumin. CD8 cells were depleted using MACS human CD8 Microbeads (Miltenyi Biotec, San Diego, CA) following the protocol provided by the manufacturer. CD8-depleted PBMCs were then cultured in RPMI 1640 containing 10% fetal bovine serum and 5% (v/v) Natural Human Interleukin-2 (IL-2)/T-Cell Growth Factor (TCGF) (ZeptoMetrix, Buffalo, NY).

IFN-γ ELISA

Patient PBMCs were treated with 1 nM GM or 500 nM SAHA combined with antiretroviral agents (30 ng/mL TMC278, 300 ng/mL AZT, 300 ng/mL indinavir) in RPMI 1640 media supplemented with 10% fetal calf serum and 5% (v/v) IL-2/TCGF for 6 days. The culture medium with compounds was refreshed every 2 days. The culture supernatants were harvested on day 6 for IFN-γ measurement. The detection of IFN-γ level in culture supernatant was performed using a human IFN-γ single analyte ELISArray kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions.

Statistical Analysis

Statistical comparisons between groups were analyzed using the two-sided Welch Two Sample t-test, with the mean of 0 and the limit of detection being substituted for left-censored data. Sample sizes were n=3 per group for the experiments shown in Figure 1 and Figure 2, and n=5 per group for the experiments shown in Figure 3. All comparisons made were between treatment (GM or SAHA) and the negative control. A nominal value of p=0.05 was used to determine statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

AIDS	acquired immunodeficiency syndrome
ART	antiretroviral therapy
CPE	cytopathic effects of virus
CTL	cytotoxic T lymphocyte
GA	gnidimacrin
HDACI	histone deacetylase inhibitors
HIV-1	human immunodeficiency virus type 1
PBMC	peripheral blood mononuclear cells
PHA	phytohaemagglutinin
SAHA (vorinostat)	suberoylanilide hydroxamic acid
TCGF	T-Cell growth factor

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Figure 1.

GM reduced latent HIV in patient PBMCs. Patient PBMCs were treated with 1 nM GM or 0.5 μ M SAHA for 6 days in the presence of antiretrovirals. (A) The level of viral DNA in PBMCs was quantified using real time PCR. (B) The frequency of latently infected cells was determined using a limiting dilution viral outgrowth assay. The number “0” in the figure denotes undetectable viral outgrowth; “Ctr” represents control samples in the absence of compounds. “IUPM” denotes infectious units per million PBMCs from the patients.

Figure 2.

Low dose GM reduced latent HIV in patient PBMCs. Patient PBMCs were treated with 20 pM GM for 6 days in the presence of antiretrovirals. (A) The level of viral DNA in PBMCs was quantified using real time PCR. (B) The frequency of latently infected cells was determined using a limiting dilution viral outgrowth assay. The number "0" in the figure denotes undetectable viral outgrowth; "Ctr" represents control samples in the absence of compounds.

Figure 3.

GM reduced latent HIV DNA in CD8-depleted patient PBMCs. CD8 cells were depleted from patient PBMCs and treated with 1 nM GM for 6 days in the presence of antiretrovirals. (A) The level of viral DNA in PBMCs was quantified using real time PCR. (B) The frequency of latently infected cells was determined using a limiting dilution viral outgrowth assay. The number “0” in the figure denotes undetectable viral outgrowth; “Ctr” represents control samples in the absence of compounds.

Figure 4.

GM activated latent HIV-1 in PBMCs. PBMCs from Pt-1 and Pt-6 were treated with GM (1 nM), SAHA (0.5 uM), or PHA (0.5 ug/mL). The PCR products from supernatants were analyzed with 2% agarose gel electrophoresis and documented with a Kodak molecular imager. *RG denotes relative quantity of the HIV-1 DNA of a given sample over that of the PHA treated sample analyzed using a Kodak molecular imaging software.

Figure 5.

GM did not affect IFN-r production. Patient PBMCs were treated with GM (1nM) or SAHA (0.5 uM) in the presence of three antiretrovirals TMC278 (30 ng/ml), AZT (300 ng/ml), and indinavir (300 ng/ml) for 6 days. The culture supernatants were quantified for IFN-r using a Qiagen ELISA kit. "Ctr" in the figure represents control samples in the absence of compounds.

Figure 6.

GM activated PKC- β_I and - β_{II} . CD4 cells were treated with GM (1 nM). Cytosolic fraction was analyzed in 10% SDS PAGE. Monoclonal antibodies to PKC isozymes were used in a Western blot. A horse radish peroxidase labeled goat anti-mouse antibody and Amersham ECL reagents were used to detect the PKC isozyme with a Kodak molecular imager. *RQ (relative quantity) is the ratio of GM treated PKC level after normalized with β -actin over that of control (no GM) for each PKC isozyme analyzed using a Kodak molecular imaging software.

Figure 7.

GM did not activate primary human PBMCs. Human PBMCs were treated with GM (0.3 nM), prostratin (0.6 uM), or anti-CD3/anti-CD28 (1 ug/ml) for 1 day. The expression of the T cell activation markers, CD25 and CD69, were measured by FACS analysis. The color assignment to each assay condition is the same for both FACS panels. The control in the figure denotes PBMCs without compound or antibody treatment.

Figure 8.

GM did not induce inflammatory cytokines. Human PBMCs were treated with GM (0.3 nM), prostratin (0.6 uM), or anti-CD3/anti-CD28 (1 ug/ml each) for 2 day. The culture supernatants were quantified for inflammatory cytokines using a Qiagen ELISA kit.

Patient virological and treatment profiles

Table 1

Patient	Viral load (Copies/mL) ^a	CD4 (Counts/ul) ^b	ART (yr) ^c	Designated experimental groups		
				GM high ^d	GM low ^e	-CD8 ^f HIV-RNA ^g
Pt-1	< 48	419	16			
Pt-2	ND	679	6			
Pt-3	ND	592	7			
Pt-4	ND	693	21			
Pt-5	< 48	835	>8			
Pt-6	< 48	802	15			
Pt-7	< 48	1033	16			

^aND = Not detectable.

^bCD4 counts/ul in blood sample.

^cYears on ART.

^dPBMCs with high dose (1 nM) GM treatment.

^ePBMCs with low dose (20 pM) GM treatment.

^fPBMCs with GM (1 nM) treatment under CD8 cells depletion.

^gPBMCs used in experiments that measure latent HIV activation.

Table 2

Effect of GM or HDACIs on Latent HIV-1 Activation

Compound	U1 (EC ₅₀ ± SD) ^a	RMA ^b	U937 (CC ₅₀ ± SD) ^c
SAHA	1.2 ± 0.25 uM	1.0	0.78 ± 0.17 uM
Romidepsin	1.1 ± 0.28 nM	1.54	0.73 ± 0.17 nM
GM	18 ± 5.7 pM	12.3	> 10 nM

^aU1 cells at 2×10^5 cells/ml were treated with various concentrations of the compounds for two days. The culture supernatant was then collected for P24 quantification. The numbers in the table are the average of 3 experiments. EC₅₀ = Concentration that induced P24 production to 50% maximum.

^bRelative Maximum Activation (RMA) = peak [P24] produced in the presence of a compound/peak [P24] induced by SAHA.

^cCC₅₀ = Concentration that reduced U937 cell viability by 50%.