Elimination of HIV-1 Latently Infected Cells by Gnidimacrin and a Selective HDAC Inhibitor

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Supporting Information

ABSTRACT: We have previously reported gnidimacrin (GM), a protein kinase C (PKC) agonist, significantly reduces the frequency of HIV-1 latently infected cells in peripheral blood mononuclear cells (PBMCs) from patients undergoing successful antiretroviral therapy at low picomolar concentrations *ex vivo*, which is distinct from other latency reversing agents. In this study, we demonstrate that strong viral reactivation by GM is a mechanism for elimination of latently infected cells, and a histone deacetylase inhibitor (HDACI), a thiophenyl benzamide (TPB), further potentiated the efficacy of GM against latent HIV-1. The effect of GM on latent HIV-1 activation was potentiated by TPB in cell models by 2–3-fold. The GM/TPB combination further decreased the frequency of HIV-infected cells in latently infected patient PBMCs over 3-fold when compared with GM alone, which caused a 5-fold reduction compared with the solvent control. Thus, GM/TPB is a unique combination that may reduce latent HIV-1 reservoirs at nontoxic concentrations.

KEYWORDS: Histone deacetylase inhibitor, HIV-1 latency reversal, gnidimacrin

Despite the success of combination antiretroviral therapy (cART) for HIV/AIDS, the persistence of HIV-1 latency remains an obstacle for AIDS therapy. Latent HIV reservoirs, such as those established in resting memory CD4+ T cells, are long-lasting and resistant to cART, which will cause viral rebound should cART be discontinued.^{1–3} Hence, strategies to eliminate latent HIV reservoirs are needed. “Shock and kill” is a strategy that reactivates latent viruses in reservoirs using latency-reversing agents (LRAs).⁴ Reactivation of latent HIV-1 may allow HIV-1-infected cells to be eliminated through immune clearance (CTL) and/or viral replication-induced cytopathic effects (CPE).⁵

Protein kinase C (PKC) agonists and histone deacetylase inhibitors (HDACIs) are two major classes of LRAs.⁶ HDACIs, including valproic acid, vorinostat, and romidepsin, have been tested in clinical trials in HIV-1-infected patients.^{7–9} However, it is still inconclusive regarding whether LRAs can impact the latent HIV reservoirs. Potential toxicities and side effects associated with LRAs are also of concern.^{10–13} Thus, high efficacy and selectivity are critical for LRA drug therapy, which may be achievable by combination of two synergistic LRAs.^{14,15}

We have previously reported that the diterpenoid gnidimacrin (GM) exhibits extremely potent picomolar dichotomous activity

against HIV-1.¹⁶ GM significantly decreased the frequency of HIV-1 latently infected cells in an *ex vivo* model using peripheral blood mononuclear cells (PBMCs) from patients who had been undergoing cART for many years with undetectable viral load.¹⁷ However, there is a concern that, as a PKC agonist, GM may cause NF- κ B activation-associated side effects. Thus, this study is focused on the following: (1) identify an HDACI that may synergize with GM for latent HIV-1 activation; (2) determine whether the GM/HDACI induced strong latent HIV-1 reactivation can directly result in cell death.

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Table 1. Effects of LRA on Latent HIV-1 Activation in U1 Cells

compound	target	EC ₅₀ ± SD (μM) U1 cells ^a	RMA ^b	CC ₅₀ ± SD (μM) U937 cells ^c	CC ₅₀ ± SD (μM) Jurkat cells ^d	SI ^e
vorinostat	HDAC	1.2 ± 0.25	1.0	0.78 ± 0.17		0.65
romidepsin	HDAC1,2,3	1.1 ± 0.28 nM	1.5	0.73 ± 0.17 nM		0.66
chidamide	HDAC1,2,3,10	1.4 ± 0.45	1.5	5.0 ± 2.1		3.6
1 (TPB)	HDAC1,2,3	0.93 ± 0.34	1.4	14.0 ± 1.7	12.6 ± 1.13	15.5
2 (TPyB)	HDAC1,2,3	5.7 ± 1.8	1.3	47.3 ± 25.8	44.3 ± 4.51	8.2
T247	HDAC3	0.6 ± 0.19	0.12	0.83 ± 0.26		1.4
RGFP966	HDAC3	inactive	0	nd ^f		
GM	PKC	18.0 ± 5.7 pM	12.3	4.8 ± 0.55	5.3 ± 0.45	260000
ingenol 3A	PKC	4.8 ± 1.8 nM	9.7	>200 nM		>42
GM + TPB ^g	combination	5.6 ± 2.7 pM	13.8	4.2 ± 0.38 (GM)		750000

^aEC₅₀ = concentration that induced p24 production to 50% maximum calculated by using CalcuSyn (Biosoft). ^bRelative maximum activation (RMA) = peak p24 produced in the presence of a compound/peak p24 induced by vorinostat. RMA is used here as an indicator for relative robustness of latent viral reactivation of an LRA when compared to vorinostat. ^cCC₅₀ = concentration that reduced U937 viability by 50%. ^dCC₅₀ = concentration that reduced Jurkat cell viability by 50%. ^eSelectivity index = CC₅₀ (U937 cells)/EC₅₀. ^fNot determined. ^gU1 cells were treated with various concentrations of GM in the presence of TPB (0.5 μM). The data in the table were derived from three independent experiments.

Figure 1. FACS analysis of the percentage of GFP⁺ J-Lat cells. J-Lat (A2) cells were incubated with GM (80 pM), ingenol-3A (ING) (0.5 nM), TPB (0.3 μM), TPyB (1.0 μM), GM (80 pM)/TPB (0.3 μM), GM (80 pM)/TPyB (1.0 μM), ING (0.5 nM)/TPB (0.3 μM), and ING (0.5 nM)/TPyB (1.0 μM) for 72 h. (A) Frequency of GFP-expressing cells. (B) Percent of cell viability. The data were derived from two independent experiments. **p* ≤ 0.05 and ***p* = 0.005 (one-tailed *t* test).

To identify an HDACi with high selectivity that could activate latent HIV-1 without cytotoxicity, we tested a panel of HDACis of different chemical classes and isozyme selectivity profiles, including vorinostat, romidepsin, a benzamide HDAC1/2-selective inhibitor (TPB),^{18,19} and a pyridine-modified TPB derivative TPyB (analytical data are available in the [Supporting Information](#)). Other benzamide HDACis such as T247, RGFP966, and chidamide were also included.^{20–22} The results indicated that vorinostat and romidepsin activated latent HIV-1 in U1 cells with EC₅₀s at 1.2 μM and 1.1 nM, respectively, which were in their concentration ranges for cytotoxicity (CC₅₀) against U937 cells (Table 1). U937 cells, which are HIV-1-negative, are the parental cells of U1 cells. Thus, the selectivity index (SI), CC₅₀/EC₅₀, of the two compounds is low. In contrast, TPB (1) displayed much higher selectivity with an EC₅₀ at 0.9 μM and an SI of 15. TPyB (2), a pyridine analogue of TPB, was less potent but also less toxic than TPB. Chidamide was about as potent as TPB in the latent HIV-1 activation but was more toxic to U937 cells with an SI of 3.6. The HDAC3 selective inhibitor RGFP966 was inactive for latent HIV-1 reactivation in the U1 cell model. The other HDAC3 selective inhibitor T247 was active, but its capacity to elevate viral p24 production was poor as shown by a low relative maximum activation value (RMA) (Table 1). Overall, TPB exhibited the best SI among tested HDACis and was chosen to combine with GM for latent HIV-1

activation. In the presence of TPB at noncytotoxic concentration (0.5 μM), the EC₅₀ for GM was reduced more than 3-fold compared to GM alone for latent HIV-1 activation (Table 1).

The synergy between the HDACi TPB and GM was further tested in J-Lat cells (A2), which expresses GFP when activated by LRAs.²³ As shown in Figure 1A, the combination of GM and TPB significantly increased the frequency of GFP⁺ J-Lat cells (21%, *p* ≤ 0.05), whereas each compound alone induced no more than 5% of GFP⁺ J-Lat cells. GM was at least 6-fold more potency than ingenol-3A (a PKC agonist included as a comparison) since GM at 80 pM and ingenol-3A at 0.5 nM induced a similar degree of GFP expression. Moreover, GM/TPB activated more J-Lat cells than ingenol-3A/TPB. TPyB exhibited weaker effects than TPB either alone or in combination with a PKC agonist, consistent with the results using the U1 cell model. The percentage of viable cell determined by flow cytometry showed no significant differences between the compound-treated and untreated cells, suggesting the tested compounds were not cytotoxic under the assay conditions (Figure 1B).

The potentiation of GM by TPB was also observed in an *ex vivo* model. TPB potentiated GM for latent viral reactivation using PBMCs from an HIV-1 infected patient who had undetectable viral loads under successful cART (Figure S1). TPB at 1 μM further enhanced the effect of GM on reducing

Figure 2. Elimination of U1 cells by LRA(s) from U1/U937 cell mixture. GM (26 pM), TPyB (0.57 μ M), GM (26 pM)/TPyB (0.57 μ M), and no drug control (DMSO) were incubated with latent HIV-1-infected U1 and uninfected U937 (1:4 ratio) in the presence of T20 (1 μ g/mL) for 18 days. For post-treatment reactivation, cells at day 18 were reactivated with GM (129 pM) for additional 72 h. (A) P24 level in cell culture supernatants. (B) Reduction of proviral DNA (HIV-1 pol) of U1/U937 coculture in the presence of LRAs. M, Marker; G, GM; T, TPyB; G+T, GM/TPyB combination. *The numbers in panel B are ratios of DNA quantity of compound-treated sample/DMSO-treated control, analyzed using a Kodak molecular imaging system and software. Beta-2-microglobulin level was measured as control.

HIV-1 DNA by 1.8-fold. Moreover, TPB potentiated GM for reducing the frequency of HIV-1 latently infected CD4⁺ cells by more than 3-fold, suggesting a synergy between GM and TPB. Although the results are consistent with that derived from cell line models, latently infected cells from more patients are required to demonstrate the ability of TPB in potentiation of the GM activity *ex vivo*.

The effectiveness of GM/TPB combination in eliminating latent HIV-1-infected cells may be in part due to the viral CPE following robust HIV-1 gene expression induced by GM. To investigate this possibility, TPyB, GM, and a combination of both were used to treat a mixture of latent HIV-1-infected U1 cells and uninfected parental U937 cells at 1:4 ratio for 18 days. The cytotoxicity of TPyB is the lowest among the tested HDACIs, which makes it suitable for observing viral CPE in a relatively long-term study. The fusion inhibitor T20 was added to block any potential HIV-1 infection of U937 cells. The results indicated that TPyB at 0.57 μ M had little effect on latent HIV-1 activation, whereas GM (26 pM) significantly activated latent HIV with p24 level peaking at day 9 (Figure 2A). The combination of GM/TPyB (G+T) displayed a synergy in latent HIV-1 activation, characterized by a faster rise in p24 level that peaked at day 6. After peaking, the p24 levels in GM- and GM/TPyB-treated samples decreased gradually to 49 pg/mL for GM alone and 12 pg/mL in GM/TPyB-treated sample at day 18. At day 18, a portion of cells in each group were taken for "post-treatment reactivation" in which these cells were treated with high-dose GM (129 pM) for 72 h. The results from post-treatment reactivation (Figure 2A, dashed lines) indicated that, in cells treated with GM or GM/TPyB, the high-dose GM post-treatment did not cause latent HIV-1 reactivation, as their p24 levels stayed the same as those of day 18. In contrast, cells treated with TPyB and the solvent (DMSO) control showed substantial HIV reactivation, as their p24 levels drastically increased after the high-dose GM treatment. These results suggested that the U1

cells were gradually eliminated from the GM- and GM/TPyB-treated cell mixtures.

The results of HIV-1 DNA (HIV-1 pol) levels in the cell mixtures at day 18 (Figure 2B) indicated that both the GM- and GM/TPyB-treated samples had a significant reduction in proviral DNA levels compared with the DMSO control. The proviral DNA level in the GM/TPyB combination-treated sample was 10-fold lower than that of DMSO control. In contrast, TPyB alone showed little effect on proviral DNA level. These results suggest that robust HIV-1 production in the presence of GM or GM/TPyB may result in the elimination of latently infected cells due to viral CPE.

PKC agonists may activate lymphocytes and induce inflammatory cytokine production. In contrast, some HDACIs, such as vorinostat, are known to suppress inflammatory cytokines.^{11,12} As shown in Figure 3, low-dose GM (20 pM) did not significantly induce IFN- γ production. High-dose GM

Figure 3. Effect of TPB on GM-induced IFN- γ production. Normal PBMCs were treated with GM (2000 pM, the bars in blue), GM (20 pM, the bars in red), the bars in light green) with/without 3 μ M TPB (TPB-3), 1 μ M TPB (TPB-1), or 0.3 μ M vorinostat (SAHA) for 2 days. Anti-CD3/CD28 antibodies (1 μ g/mL) were used as positive control. The culture supernatants were quantified for IFN- γ using a Qiagen ELISA kit. The data were derived from three independent experiments.

(2000 pM) could induce IFN- γ release; however, the level was 50-fold lower than that induced by anti-CD3/CD28 antibodies (CD3/CD28). TPB at a noncytotoxic concentration (3 μ M) did not affect IFN- γ production, but it markedly antagonized the effect of high-dose GM on IFN- γ induction. After correction with background IFN- γ levels in the PBMC culture, TPB at 3 μ M reduced high-dose GM-induced IFN- γ productions by approximately 90%. The effects of vorinostat (SAHA) and TPB were comparable on GM-induced IFN- γ production. However, vorinostat was effective at a concentration very close to cytotoxic concentrations, whereas TPB was effective at a concentration 14-fold lower than its CC₅₀.

TPyB, the isosteric isomer of TPB, and vorinostat were also tested for their effect on high-dose GM-induced T cell activation. Both of the HDACIs exhibited minimal effect on high dose (3 nM) GM-induced T cell activation indicated by the expression of the early T cell activation marker, CD69 (Figure S2).

It has been suggested that class I HDACs, including HDAC1, 2, and 3, are recruited to the HIV promoter and participated in establishing and maintaining HIV-1 latency.^{24–26} To test if HDAC1/2 selectivity is associated with the high SI of TPB, the effect of TPB on HDAC1, 2, and 3 was determined. The results of HDAC isozyme inhibition showed that TPB was equally potent in inhibiting HDAC1 and 2 with an IC₅₀ at 0.07 μ M, but was 20-fold less potent in inhibiting HDAC3 under the assay conditions (Table S1). TPyB was approximately 2-fold less potent than TPB against HDAC1 and 2, which is consistent with their relatively lower potency for latent HIV-1 reactivation. The known HDAC3 selective inhibitor T247 inhibited both HDAC2 and 3 in this assay, whereas RGFP966 only inhibited HDAC3. Thus, selective inhibition of HDAC1 and 2 may be responsible for the relatively high SI of TPB and TPyB.

Despite evidence of LRAs in latent HIV-1 activation, there are few data showing that LRAs by themselves reduce the latent viral reservoirs. Our previous study indicated that GM can reduce the frequency of HIV-1 latently infected cells 5- to 10-fold in all tested PBMC samples from patients *ex vivo*, which is unique among LRAs.¹⁷ It is encouraging that TPB can further augment the effects of GM by 2- to 3-fold in this study. The effectiveness of GM may result from its ability to induce a robust viral gene expression, which may lead to the clearance of HIV infected cell through (1) stimulating the virus-specific host immune responses (CTL) or (2) cytopathic effect (CPE) after viral reactivation. In this study, we tested if CPE induced by GM may play a role in reduction of HIV-1 latently infected cells. The results showed that GM or GM/TPyB is able to effectively eliminate U1 cells from the U1/U937 cell mixture (Figure 2). Thus, the strong viral reactivation-induced CPE by GM is at least in part contributing to GM-induced reduction of the frequency of HIV-1 latently infected cells. In addition to potentiation of GM for antilatenacy activity, the results also suggest that TPB or TPyB may antagonize GM-induced inflammatory cytokine production but is unable to suppress the effect of high-dose GM on T cell activation.

In summary, GM and the selective HDACI TPB represent a novel combination of LRAs that may synergize for reduction of latent HIV reservoirs. TPB not only can further reduce the concentration of the already extremely potent latency reversing agent GM but also may mitigate the potential adverse effects of GM.

■ EXPERIMENTAL PROCEDURES

Compounds. GM was isolated with over 98% purity from *Stellera chamaejasme* L. (Thymelaeaceae).²⁷ TPB and TPyB were synthesized according to Moradei et al.¹⁹ T247 was kindly provided by Dr. N. Miyata (Nagoya City University, Nagoya, Japan). T20 (Fuzeon) was generously provided by Trimeris (Durham, NC). RGFP966 (APEXBIO, Boston, MA), chidamide (Santa Cruz Biotechnology), ingenol-3-angelate (AdipoGen, San Diego, CA), and romidepsin (MedChem Express, Monmouth Junction, NJ) were purchased as indicated. AZT, vorinostat, and phytohemagglutinin (PHA) were obtained from Sigma-Aldrich (St. Louis, MO). Indinavir was obtained from the NIH AIDS Reagent Program.

Cells. U937, U1, and J-Lat (A2) cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID/NIH. Human PBMCs were prepared from whole blood from American Red Cross (Charlotte, NC). The PBMC samples used in the *ex vivo* study were from HIV-1-positive patients as described previously.¹⁷

Latent HIV-1 Activation Assay in U1 Cells by HIV-1 p24 Quantification. U1 cells (2×10^5 cells/mL) were incubated in the presence of various concentrations of LRAs at 37 °C for 48 h. The culture supernatant was assayed for p24 with an HIV p24 ELISA kit (ZeptoMetrix) following the manufacturer's protocol.

Cytotoxicity Assay in U937 Cells. U937 cells (2×10^5 cells/mL) were cultured in the presence of various concentrations of LRAs for 48 h at 37 °C. Cell viability was determined using a CellTiter-Glo Luminescent Cell Viability Assay kit (Promega) following the manufacturer's instructions. CC₅₀ and SD were derived for each LRA.

Fluorescence-Activated Cell Sorting (FACS) Analysis of GFP-Expressing J-Lat Cells. J-Lat (A2) cells (1×10^6 cells/well) were incubated in the presence of various concentrations of LRA(s) at 37 °C for 72 h. The GFP-expressing cells and percent of cell viability were analyzed by using a BD LSRII/Fortessa cell analyzer (Becton-Dickinson).

Elimination of U1 Cells from U1/U937 Cell Mixture. Mixtures of U1/U937 cells at a 1:4 ratio (1×10^5 cells/mL, 5 mL) were incubated with LRA(s) and DMSO (control) in the presence of T20 (1 μ g/mL). The supernatants were analyzed for p24 levels by using a HIV-1 p24 Antigen 2.0 ELISA kit (ZeptoMetrix) every 3 days, and the cells were replenished with fresh medium containing corresponding compound(s) with cell concentration readjusted to 1×10^5 cells/mL. At day 18, a portion of cells was incubated in a new medium containing 129 pM GM for additional 72 h before being analyzed for p24 for post-treatment latent HIV-1 reactivation (day 21 data).

Proviral DNA Quantification on U1/U937 Cell Mixture. LRA-treated cells (18 days post-treatment) described above were quantified for proviral DNA by using a PCR with primer sequences from HIV-1 *pol* [a forward primer 5'-GGGGGAATTGGAGGTTTATCA-3' (2394–2415) and a reverse primer 5'-CATTCTGGCTTTAATTTTACTGTACAGT-3' (2568–2597)], which produced a 200 bp PCR product. PCR was performed on a Bio-Rad T-100 thermal cycler at 95 °C for 5 min, then 30 cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. Beta-2-microglobulin (B2M) DNA (779 bp DNA fragment) was used as internal control. The primers used for B2M measurement were the following: forward primer 5'-AGAATGTGTACCTAGAGGGC-3' and reverse primer 5'-TGCTGTCAGCTTCAGGAATG-3', which produced a 779 bp DNA fragment. The cycling parameters for the B2M PCR were similar to those used for amplification of HIV-1 *pol* DNA described above, except that the PCR was performed for 25 cycles at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The PCR products were analyzed by electrophoresis using 2% agarose gels. The relative quantities of DNA on gel were analyzed with the KODAK IMAGE STATION 4000 MM and Carestream Molecular Imaging Software.

IFN- γ Quantification in PBMCs. Normal PBMCs were treated with GM alone or in combination with TPB for 2 days. Anti-CD3/CD28 antibodies (1 μ g/mL each) were used as a positive control for IFN- γ expression. The culture supernatants were quantified for IFN- γ level by using a human IFN- γ single analyte ELISArray kit (QIAGEN) following the manufacturer's instructions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchem-lett.8b00012.

Figure S1, Figure S2, Table S1, experimental protocols, and analytical data of TPbY (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Conceived project (C.H.C.), performed experiments (W.H.L., L.H., L.Z.), provided key materials (L.X., W.L., L.W., K.H.L.), analyzed data (C.H.C., W.H.L., L.H.), and prepared manuscript (L.H., C.H.C.). All authors reviewed the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

cART, combination antiretroviral therapy; CTL, cytotoxic T-lymphocytes; CPE, cytopathic effect; GM, gnidimacrin; HDACi, histone deacetylase inhibitor; LRA, latency reversing agent; RMA, relative maximum activity; TPB, thiophenyl benzamide (4-(acetylamino)-N-[2-amino-5-(2-thienyl)phenyl]-benzamide); TPbY, thiophenyl pyridyl benzamide, (4-(acetylamino)-N-[3-amino-6-(2-thienyl)pyridyl]-benzamide)

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