Discovery of a Small Molecule Agonist of Phosphatidylinositol 3-Kinase p110α That Reactivates Latent HIV-1

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Abstract

Combination antiretroviral therapy (cART) can effectively suppress HIV-1 replication, but the latent viral reservoir in resting memory CD4+ T cells is impervious to cART and represents a major barrier to curing HIV-1 infection. Reactivation of latent HIV-1 represents a possible strategy for elimination of this reservoir. In this study we describe the discovery of 1,2,9,10-tetramethoxy-7H-dibenzo(de,g)quinolin-7-one (57704) which reactivates latent HIV-1 in several cell-line models of latency (J89GFP, U1 and ACH-2). 57704 also increased HIV-1 expression in 3 of 4 CD8−depleted blood mononuclear cell preparations isolated from HIV-1-infected individuals on suppressive cART. In contrast, vorinostat increased HIV-1 expression in only 1 of the 4 donors tested. Importantly, 57704 does not induce global T cell activation. Mechanistic studies revealed that 57704 reactivates latent HIV-1 via the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway. 57704 was found to be an agonist of PI3K with specificity to the p110α isoform, but not the p110δ, δ or γ isoforms. Taken together, our work suggests that 57704 could serve as a scaffold for the development of more potent activators of latent HIV-1. Furthermore, it highlights the involvement of the PI3K/Akt pathway in the maintenance of HIV-1 latency.


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Introduction

Combination antiretroviral therapy (cART) can effectively suppress HIV-1 RNA levels to below 50 copies mL−1 in patient plasma. However, interruption of cART typically results in viremia rebound. Therefore, cART does not eliminate HIV-1 infection and residual low-level viremia has been detected using ultrasensitive assays in 80% of treated patients [1–4]. Latently infected resting memory CD4+ T cells are thought to constitute the major reservoir of HIV-1 persistence [5–8]. In this reservoir, the integrated provirus remains transcriptionally silent as long as the host cell is in a resting state [9]. Upon cellular activation, HIV-1 RNA is transcribed and virus is produced. The prevailing hypothesis in the field is that molecules that reactivate latent HIV-1 infection will purge this reservoir by inducing transcription of the latent provirus (i.e. the “kick”), thereby causing cells to undergo apoptosis (the “kill”) [10]. It should be noted, however, that in a recent study by Shan et al it was shown that after reversal of HIV-1 latency in an in vitro model, infected resting CD4+ T cells survived despite viremic effects, even in the presence of autologous cytolytic T lymphocytes from most patients on cART [26]. Despite this finding, several clinical trials are directly testing the “kick and kill” hypothesis using agents including the histone deacetylase (HDAC) inhibitor vorinostat (SAHA) [ClinicalTrials.gov Identifiers: NCT01319383 and NCT01363065] or disulfiram (DSF) [ClinicalTrials.gov Identifier: NCT0047732], a drug used to treat chronic alcoholism [11]. Encouragingly, recent data from the NCT01319383 trial showed that a single 400 mg dose of SAHA induced an increase in HIV-1 RNA expression in resting CD4+ T cells in 8 HIV-infected patients on suppressive cART [12].

While the “kick and kill” concept currently represents a promising avenue that could be scaled up for treatment of HIV-infected patients, the successful implementation of this approach is limited by the paucity of potent and safe inducers of latent HIV-1 gene expression. Importantly, many of these compounds either lack potency and specificity or have unfavorable toxicity profiles. For example, several studies have demonstrated that the latent HIV-1 reactivation activity of HDAC inhibitors is sub-optimal (compared to phytohaemagglutinin or anti-CD3/CD28 antibodies) and can vary considerably in resting T cells isolated from different HIV-1-infected donors [13,14]. Additionally, HDAC inhibitors typically lack specificity and inhibit multiple HDAC isoforms (e.g. SAHA is a potent inhibitor of HDACs 1, 2, 3, 6 and 8) [16]. Similarly, the clinical utility of the PKC agonists prostratin and bryostatin may be limited by their unfavorable toxicity profiles. Prostratin induces global T cell activation [15], a phenotype that may induce clinical toxicity. In a recent phase II clinical trial for the treatment of ovarian cancer, bryostatin was found to cause severe myalgia in all study participants [17]. As such, there is an urgent need to develop potent and safe inducers of latent HIV-1 gene expression that could open new avenues to a finding a scalable and affordable cure for HIV-infected patients.
In this study, we described the discovery of 1,2,9,10-tetramethoxy-7H-dibenzo[de,g]quinolin-7-one (57704; Fig. 1). 57704 reactivates latent HIV-1 via the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway through direct binding to, and activation of, the PI3K p110α isomorph. 57704 could serve as a scaffold for the development of more potent activators of latent HIV-1.

**Materials and Methods**

**Materials**

The natural compound library was purchased from TimTec (Newark, DE). SAHA was from Enzo Life Sciences (Plymouth Meeting, PA). Wortmannin, SB203580, Go6983, and BIX-01294, cyclosporin A, DSF and prostratin were purchased from Sigma-Aldrich (St. Louis, MO). The PI3K isomorph inhibitors were from BioVision (Milpitas, CA, USA). The Akt inhibitor IV and the NF-kB activation inhibitor were obtained from EMD Biosciences (Gibbstown, NJ). Akt and phospho-AKT were obtained from Cell Signaling Technology (Boston, MA). The β-actin antibody was obtained from Abcam (Cambridge, MA). The recombinant purified HDAC isomorphs were purchased from Millipore SNAP i.d. (Billerica, MA). Cellular proteins were separated using 4–12% SDS-polyacrylamide gels from Invitrogen (Grand Island, NY). Immunodetection was performed using the Millipore SNAP i.d. (Billerica, MA).

**Cell line models of HIV latency**

Three different cell lines of HIV-1 latency were used in this study. These included J89GFP, U1 and ACH-2 cells. The J89GFP cells were a kind gift from Dr. David Levy [18]. The ACH-2 and U1 cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Dr. Thomas Folks contributor [19–21]. Reactivation of latent HIV-1 in the J89GFP cells was measured by quantifying the percentage of EGFP positive cells using a BD FACSDiva software (BD Biosciences, CA). DNA oligonucleotide primers were synthesized by Integrated DNA Technologies (San Diego, CA). The recombinant purified HDAC isomorphs were purchased from BPS Biosciences (San Diego, CA). Recombinant purified PI3K isomorphs and the PI3K activity assay kit were purchased from Millipore (Billerica, MA). Cellular proteins were separated using 4–12% SDS-polyacrylamide gels from Invitrogen (Grand Island, NY). Immunodetection was performed using the Millipore SNAP i.d. (Billerica, MA).

**Isothermal titration calorimetry**

The production of phosphatidylinositol (3,4,5)-triphosphate (PIP3) by the four class I PI3K isoenzymes (p110α, β, γ, δ) was assessed using the PI3 Kinase Activity/Inhibitor Assay Kit.
According to the manufacturer’s instructions, this assay works on the principle that the PH domain of the protein GRP-1 binds PIP3 with high affinity and specificity. This protein binds to the glutathione plate and captures either the PIP3 generated as part of the kinase reaction or the biotinylated-PIP3 tracer included in the kit. The captured biotinylated-PIP3 is detected using streptavidin-HRP conjugate and a colorimetric read out (OD 450). Because the signal in this assay is inversely proportional to the kinase activity, the activity of 57704
57704 was assessed in combination with the pan-PI3K inhibitor wortmannin.

HDAC activity assays
The lysine deacetylase activity of HDACs 1, 2, 3, and 8 was assessed using the Fluorogenic HDAC Assay (BPS Bioscience, San Diego, CA) according to the manufacturer’s instructions. The HDAC5 used in this assay was complexed with human nuclear receptor co-repressor 2 (NCOR2; amino acids 393–489), which is an activating co-factor of this HDAC isoform. All assays were carried out under steady-state conditions and the assay read-out was optimized for linearity both as a function of time and enzyme concentration. The formation of the fluorescent product was measured using a SpectraMax M2 Plate Reader (Molecular Devices, CA). The excitation and emission wavelengths were 360 nm and 450 nm, respectively. The concentrations of SAHA required to inhibit 50% of the deacetylase activity of an HDAC isoform (i.e. IC50) was calculated by regression analysis using SigmaPlot software (Systat Software, Inc., San Jose, CA).

Results

Discovery of 57704
To identify novel activators of latent HIV-1 expression, we screened 640 pure natural products from the TimTec Natural Compound Library at a concentration of 10 ng/mL for 24 hrs in a Jurkat cell [J89GFP] model of latency for their cytotoxicity and ability to reactivate HIV-1 expression. J89GFP cells contain a stably integrated, full-length HIV-1 provirus with an enhanced GFP (EGFP) reporter incorporated into the viral genome [18]. The viral genome in these cells is transcriptionally silent. However, upon stimulation viral transcription is activated and viral expression can be measured by EGFP production. Seven compounds (1.1%) were identified that reactivated latent HIV-1 expression, none of which showed significant cytotoxicity at the single concentration tested (data not shown). Following extensive analyses in different cell line models of HIV-1 latency we found that 57704 (Fig. 1) was one of the more robust analogs identified in the screen. The effective concentration of compound that reactivated 50% of latent HIV-1 gene expression (i.e. EC50) ranged from ~5 to 9 µM in the ACH2 and U1 cell lines of virus latency (Fig. 2B, C, D). By comparison, in J89GFP cells the EC50 was calculated to be 10.3 ± 1.2 µM (Fig. 2A). The EC50 values for SAHA ranged from ~1 to 5 µM in the same cell lines (data not shown). Importantly, 57704 was significantly less cytotoxic than SAHA in several different cell lines and in CD8+-depleted MNC (Table 1). Of note, the latent HIV-1 reactivation activity of 57704 was increased in an additive manner when combined with other inducing agents including prostratin (Fig. 2E), SAHA (Fig. 2F), DSF (Fig. 2G) and the histone methyltransferase inhibitor BIX-01294 (Fig. 2H).

Table 1. Cytotoxicity of 57704 and SAHA in different cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Jurkat1 CC50 (µM)</th>
<th>HeLa1 CC50 (µM)</th>
<th>293T1 CC50 (µM)</th>
<th>CD8+-depleted MNC2 CC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>57704</td>
<td>92.9 ± 7.5</td>
<td>293.2 ± 56.42</td>
<td>105.5 ± 31.4</td>
<td>80</td>
</tr>
<tr>
<td>SAHA</td>
<td>10.0 ± 0.1</td>
<td>11.3 ± 0.3</td>
<td>14.1 ± 0.2</td>
<td>0.7</td>
</tr>
</tbody>
</table>

1Data represent the mean ± standard deviation from 3 replicate experiments.
2Data represent the mean from 2 independent replicate experiments.

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57704 increases HIV-1 expression in CD8+-depleted MNC from HIV-1-infected donors on suppressive cART without inducing global T cell activation

We compared the activity of 57704 to reactivate latent HIV-1 from CD8+-depleted blood MNC from 4 different cART-treated individuals with plasma HIV-1 RNA<50 copies/mL (Table 2, Fig. 3A). SAHA was included as a control. 5 µM 57704 increased HIV-1 RNA in culture supernatants ~4-fold compared to the control (Fig. 3A) although significant variability was observed between donors (Table 2). Specifically, increases in HIV-1 RNA levels in culture supernatant compared to the vehicle control were detected in the CD8+-depleted blood MNC from donors 1 and 2 but not in donors 3 and 4. Although the mean supernatant HIV-1 RNA copy number determined for the 4 donors after treatment with 1 µM 57704 was comparable to that of controls, higher levels were detected in donors 3 and 4. By contrast, SAHA exhibited a very limited capacity to increase HIV-1 expression in the CD8+-depleted MNC across all four donors (Table 2). Since agents that induce latent HIV-1 through global T cell activation are toxic, we next assessed the 57704’s capacity to induce this phenotype. Purified T cells were treated with 10 µg/mL PHA (positive control), 5 µM SAHA or different concentrations of 57704 for up to 5 days, and then stained for surface expression of CD69, CD38 and HLA-DR (Fig. 3B, C). 57704 did not up-regulate the surface expression of any of these markers, even after 5 days of exposure to drug (Fig. 3C). Taken together, these data indicate that 57704 increases HIV-1 expression in CD8+-depleted MNC from HIV-1-infected donors without inducing global T cell activation.

Mechanism of action studies
To gain insight into the mechanism by which 57704 reactivates latent HIV-1, we evaluated its activity in combination with inhibitors of different signaling pathways or kinases in J89GFP cells (Fig. 4A, B). We found that the activity of 57704 was significantly attenuated by the PI3K inhibitor wortmannin, by the Akt Inhibitor IV, and by the NF-kB inhibitor 6-amino-4-(4-phenoxyphenylethylamino) quinazoline. By contrast, its activity was unaffected by the PKC inhibitor Go6983, by the c-Jun N-terminal kinase (JNK) kinase inhibitor SP600125, by the p38 mitogen-activated protein kinase inhibitor SB203580, and by the NFAT inhibitor cyclosporin A. Consistent with its mechanism of action, the latent HIV-1 reactivation activity of prostratin was attenuated when combined with the PKC inhibitor Go6983, but remained unaffected by inhibitors of calcineurin, JNK, PI3K and Akt (Fig. 4A). Taken together, these data suggested that 57704 reactivated latent HIV-1 via the PI3K/Akt signaling pathway. To determine whether 57704 directly activated the PI3K/Akt signaling pathway, we next assessed the levels of Akt phosphorylation in the J89GFP cells after exposure to 5 µM 57704. Western blot analysis clearly demonstrated that 57704 increased Akt phosphorylation (Fig. 4C).
The class I PI3Ks are heterodimeric enzymes formed by association of a p110 catalytic subunit with a p85 regulatory subunit. Table 2. Latent HIV-1 reactivation activity of 57704 in CD8+ depleted MNC from HIV-1-infected donors on suppressive cART.

<table>
<thead>
<tr>
<th>Donor</th>
<th>CD4+ Count</th>
<th>Viral Load (RNA/mL)</th>
<th>ART Regimen</th>
<th>Copies HIV RNA/mL control</th>
<th>Copies HIV RNA/mL 1 μM 57704</th>
<th>Copies HIV RNA/mL 5 μM 57704</th>
<th>Copies HIV RNA/mL 0.5 μM SAHA</th>
<th>Copies HIV RNA/mL 2 μM SAHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>484</td>
<td>not detected</td>
<td>Reyataz, Epzicom, Norvir</td>
<td>96</td>
<td>0</td>
<td>389</td>
<td>0</td>
<td>51</td>
</tr>
<tr>
<td>2</td>
<td>777</td>
<td>&lt;40</td>
<td>Reyataz, Truvada, Norvir</td>
<td>79</td>
<td>35</td>
<td>0</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>713</td>
<td>&lt;20</td>
<td>Reyataz, Truvada, Norvir</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>571</td>
<td>not detected</td>
<td>Atripla</td>
<td>20</td>
<td>20</td>
<td>27</td>
<td>0</td>
<td>23</td>
</tr>
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</table>

doi:10.1371/journal.pone.0084964.t002

Figure 3. Latent HIV-1 reactivation activity of 57704 and T cell activation in CD8+ depleted MNC.

(A) Quantitation of cell-free HIV-1 RNA in the culture supernatants of CD8+ depleted blood MNC incubated with 57704 or SAHA. (B) Surface expression of CD69, CD38 and HLA-DR after 24 h in purified T cells following exposure to 10 μg/mL PHA, 57704 or SAHA. (C) Surface expression of CD69, CD38 and HLA-DR after 5 days in purified T cells following exposure to 10 μg/mL PHA, 57704 or SAHA.

doi:10.1371/journal.pone.0084964.g003
There are 4 variants of the p110 catalytic subunit designated p110α, β, δ or γ [22]. To determine whether 57704 displayed PI3K isoform specificity, we first assessed its activity in J89GFP cells in combination with inhibitors specific to each PI3K isoform (Fig. 4D). We found that 57704’s activity was significantly attenuated by the pan PI3K inhibitor LY 294002 and by the PI3K p110α specific inhibitor PI-103. By contrast, its activity was not reduced by inhibitors of PI3K p110β, p110δ or p110γ. Of note, AS-605240 and TGX-221 which target PI3K p110γ and p110β, respectively, moderately enhanced the latent HIV-1 reactivation activity of 57704. To determine whether 57704 directly impacted the gene expression levels of the PI3K isoforms, we assessed RNA transcript levels in J89GFP cells treated with either 57704 or SAHA.

Table 3. In vitro activity of 57704 and SAHA against class I HDAC isoforms.

<table>
<thead>
<tr>
<th>HDAC Isoform</th>
<th>SAHA IC50 (nM)</th>
<th>57704 IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC1</td>
<td>13.7±0.15</td>
<td>&gt;50</td>
</tr>
<tr>
<td>HDAC2</td>
<td>62.0±0.15</td>
<td>&gt;50</td>
</tr>
<tr>
<td>HDAC3</td>
<td>869±0.15</td>
<td>&gt;50</td>
</tr>
<tr>
<td>HDAC8</td>
<td>6.8±0.15</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

Data represent the mean ± standard deviation from 3 replicate experiments. doi:10.1371/journal.pone.0084964.t003
p110α. Furthermore, our data validate prototype of a new mechanistic class of agents that could help Importantly, 57704 was found to reactivate latent HIV-1 without its potential to reduce or eliminate the latent HIV-1 reservoir. That of SAHA, which is currently being tested in clinical trials for regard, its activity in CD8

Discussion

The latent reservoir in resting memory CD4+ T cells represents a major obstacle towards curing HIV-1 infection. The mechanisms that lead to HIV-1 latency in CD4+ T cells are still not completely understood. Indeed, there is ample evidence that multiple restrictions prevent the emergence of virus from latency, including both cellular and viral factors [24]. Therefore, a better understanding of the establishment and maintenance of HIV-1 latency and identification of pharmacologic targets will open avenues to rational therapeutic approaches for clearing infection.

In this study, we show that 57704 – a novel agonist of PI3K p110α – can reactivate latent HIV-1 in different cell lines of virus latency and increase HIV-1 expression in CD8+-depleted blood MNC from infected individuals on suppressive cART. In this regard, its activity in CD8+-depleted MNC was more robust than that of SAHA, which is currently being tested in clinical trials for its potential to reduce or eliminate the latent HIV-1 reservoir. Importantly, 57704 was found to reactivate latent HIV-1 without inducing global T cell activation. As such, 57704 serves as the prototype of a new mechanistic class of agents that could help purge the latent HIV-1 reservoir. Furthermore, our data validate PI3K p110α as a target for the discovery of more potent activators of latent HIV-1 expression.

The PI3K/Akt signaling pathway appears to play a key role in the maintenance of HIV-1 latency. Indeed, previous studies have demonstrated that hexamethylene bisacetamide (HMBA) transiently activates this pathway, which leads to phosphorylation of HEXIM1 and the subsequent release of active positive transcription elongation factor b (P-TEFb) from its transcriptionally inactive complex with HEXIM1 and 7S small nuclear RNA (snRNA). As a result, P-TEFb is recruited to the HIV-1 promoter to stimulate transcription elongation and viral production [25]. Similarly, SAHA and other HDAC inhibitors have been shown to activate this pathway in several subpopulations of T cells, including memory T cells [16,23]. Importantly, inhibition of PI3K significantly reduces the latent reactivation activity of HDAC inhibitors [16,23]. The mechanism by which HMBA activates the PI3K/Akt pathway is not known. However, in this study we show that SAHA, like 57704, acts as an agonist of PI3K p110α. However, unlike 57704, SAHA also shows agonistic activity toward the other PI3K isoforms. Therefore, 57704 and SAHA activate the PI3K/Akt signaling pathway via a similar mechanism of action. However, it should be noted that 57704 is not an HDAC inhibitor.

The class I PI3Ks include four isoforms consisting of two subdivisions, namely, class IA (PI3K p110α, β and δ) activated by tyrosine kinases, antigen and cytokine receptors, and class IB (PI3K p110γ) activated by G-protein-coupled receptors [22]. The class IA isoforms share 42–58% amino acid sequence. PI3K p110γ shares 36% identity with p110α. Despite largely conserved active sites (they all catalyze the same reaction), isoform specific inhibitors have been developed to each of the PI3K isoforms [22]. Interestingly, we found that 57704 shows unique specificity to PI3K p110α. Its interaction with this isoform and the mechanism by which it enhances kinase activity are not known, but warrant further investigation. Similarly, the contribution of each PI3K isoform in the establishment and maintenance of HIV-1 latency is not known. In this regard, additional research is required to determine whether an agonist with broader specificity to other PI3K isoforms may elicit a more pronounced effect in reactivating latent HIV-1 expression.

In conclusion, we have discovered that 57704, a novel small molecule agonist of PI3K p110α, can reactivate latent HIV-1. The 57704 pharmacophore could serve as a scaffold for the development of more potent activators of latent HIV-1 that could be used, possibly in combination with other agents, to induce expression of the latent reservoir in HIV-1-infected individuals.

Author Contributions

Conceived and designed the experiments: NSC JWJM. Performed the experiments: GD MDS KH. Analyzed the data: GD MDS JWJM NSC. Contributed reagents/materials/analysis tools: DM. Wrote the paper: NSC.

References