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Protein Phosphatase-1 –targeted Small Molecules, Iron Chelators and Curcumin Analogs as HIV-1 Antivirals

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Abstract

Background—Despite efficient suppression of HIV-1 replication, current antiviral drugs are not able to eradicate HIV-1 infection. Permanent HIV-1 suppression or complete eradication requires novel biological approaches and therapeutic strategies. Our previous studies showed that HIV-1 transcription is regulated by host cell protein phosphatase-1. We also showed that HIV-1 transcription is sensitive to the reduction of intracellular iron that affects cell cycle-dependent kinase 2. We developed protein phosphatase 1-targeting small molecules that inhibited HIV-1 transcription. We also found an additional class of protein phosphatase-1-targeting molecules that activated HIV-1 transcription and reported HIV-1 inhibitory iron chelators and novel curcumin analogs that inhibit HIV-1. Here, we review HIV-1 transcription and replication with focus on its regulation by protein phosphatase 1 and cell cycle dependent kinase 2 and describe novel small molecules that can serve as future leads for anti-HIV drug development.

Results—Our review describes in a non-exhaustive manner studies in which HIV-1 transcription and replication are targeted with small molecules. Previously, published studies show that HIV-1 can be inhibited with protein phosphatase-1-targeting and iron chelating compounds and curcumin analogs. These results are significant in light of the current efforts to eradicate HIV-1 through permanent inhibition. Also, HIV-1 activating compounds can be useful for “kick and kill” therapy in which the virus is reactivated prior to its inhibition by the combination antiretroviral therapy.

Conclusion—The studies described in our review point to protein phosphatase-1 as a new drug target, intracellular iron as subject for iron chelation and novel curcumin analogs that can be developed for novel HIV-1 transcription-targeting therapeutics.

Keywords

HIV-1; Tat; CDK9; CDK2; protein phosphatase-1; iron chelators; curcumin

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CONFLICT OF INTEREST

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1. INTRODUCTION

Despite efficient antiretroviral therapy, eradication of human immunodeficiency virus type 1 (HIV-1) infection is challenging and requires novel biological insights and therapeutic strategies. Eradication of latent HIV-1 provirus is especially challenging as integrated HIV-1 is not affected by the existing combination antiretroviral therapy (cART) unless viral transcription is activated [1]. cART reduces plasma viremia to undetectable levels, but the viremia rapidly rebounds when cART is interrupted [2, 3]. Resting memory CD4⁺ T cells are the best studied reservoir of latent HIV-1 [4], however additional reservoirs exist where HIV-1 undergoes low-level replication in subjects on cART [5]. To achieve a functional cure, HIV-1 transcription needs to be permanently silenced to avoid low-level HIV-1 replication and prevent the release of viral particles from the viral reservoirs. Thus, there is a need for new classes of drugs that can target HIV-1 transcription.

2. REGULATION OF HIV-1 TRANSCRIPTION

Efficient HIV-1 transcription from HIV-1 long terminal repeat (LTR) requires both host cell factors and HIV-1 Tat protein [6]. HIV-1 Tat recruits the positive transcription elongation factor b (P-TEFb), a heterodimeric complex containing cell cycle-dependent kinase (CDK) 9 and cyclin T1, to the transactivation response (TAR) RNA [7] (see Fig. 1 for general schematics of HIV-1 transcription). Tat also recruits histone acetyl transferases (HATs) [8–10] and SWI/SNF remodeling complex [11] to induce transcription from the integrated HIV-1 promoter. P-TEFb activity is repressed by the chicken ovalbumin upstream promoter transcription factor (COUP-TF) interacting protein 2 (STIP2) which also represses HIV-1 promoter and blocks HIV-1 transcription in microglial cells [12]. STIP2-repressed P-TEFb is recruited to HIV-1 and cellular promoters by high mobility group AT-hook 1 (HMGA1) protein [13]. P-TEFb triggers HIV-1 transcriptional elongation via the phosphorylation of the C-terminal domain (CTD) of RNA polymerase II (RNAPII), the negative elongation factor (NELF) and the DRB-sensitivity inducing complex (DSIF/Spt4/Spt5) [1, 14] (Fig. 1). P-TEFb exists in a form of distinct molecular weight complexes [15]. A low molecular weight, functionally active kinase consists of CDK9 and cyclin T1 subunits [14]. However, the enzymatically inactive, high molecular weight complex, carries other additional factors including 7SK RNA, HEXIM1 protein, La-related LARP7 protein [16–18] and the methylphosphate capping enzyme MePCE [19, 20] (see Fig. 1). The high molecular weight complex serves as a source of P-TEFb, from which HIV-1 Tat extracts P-TEFb and recruits it to HIV-1 LTR [21]. Subsequently, Tat facilitates the formation of super-elongation complex (SEC) at HIV-1 LTR, which, in addition to P-TEFb, also carries additional elongation factors including AFF1, AFF4, ELL2 and ENL/AF9 [23, 24]. Enzymatic activity of P-TEFb and its interaction with Tat is regulated by phosphorylation of CDK serine/threonine residues located in the regulatory T-loop [15]. Phosphorylation of CDK9 Thr-186 is required for its enzymatic activity and also facilitates the association of CDK9/cyclin T1 with 7SK RNA snRNP [25, 26]. CDK9 Thr-186 is phosphorylated by CDK7/cyclin H [27]. CDK7/cyclin H has emerged as a universal CDK-activating kinase that phosphorylates and regulates cell-cycle-related kinases, such as CDK1, 2 and 4, and also CDKs involved in transcriptional regulation such as CDK8, 9, 12 and 13 (see for details [28]). We previously showed that increased phosphorylation of CDK9 Ser-175 residue leads to a decreased

cellular activity of CDK9/cyclin T1 and reduces RNA polymerase II phosphorylation *in vivo* [29]. Reduced CDK9 Ser-175 phosphorylation may enhance HIV-1 transcription by preventing CDK9/cyclin T1 interaction with Brd4 thereby making CDK9/cyclin T1 available for recruitment by HIV-1 Tat protein [30]. Jonathan Karn and colleagues recently showed that phosphorylation of CDK9 Ser-175 occurs during the induction of latent HIV-1 provirus and that Tat Lys-12 forms a hydrogen bond with CDK9's phospho-Ser175 residue [30]. Thus, interaction between Lys-12 of Tat and phosphorylated CDK9's Ser-175 might facilitate reactivation of latent HIV-1 provirus by enhancing the binding of Tat to P-TEFb [30]. We have also recently demonstrated that phosphorylation of CDK9 Ser-90 by CDK2 alters CDK9 association with 7SK snRNP and upregulates HIV-1 transcription [31]. Taken together, recent studies identified CDK9 phosphorylation on multiple residues as an important mediator of HIV-1 transcription and suggest potential therapeutic targeting of these phosphorylation events for HIV-1 transcription inhibition or for the reactivation of latent HIV-1 provirus.

3. REGULATION OF HIV-1 TRANSCRIPTION BY PROTEIN PHOSPHATASE-1

We and others have previously shown that protein phosphatase-1 (PP1) dephosphorylates CDK9's Thr-186 [29, 32]. We also showed that PP1 dephosphorylates CDK9 Ser-175 [29]. PP1 holoenzyme consists of a constant catalytic subunit (PP1c) and a variable PP1 interacting subunit such as nuclear inhibitor of PP1 (NIPP1), PP1 nuclear targeting subunit (PNUTS), Sds22 and others [33]. PP1c interacts with its various regulatory subunits through the combination of short distinct binding motifs that define the cellular localization, catalytic activity and substrate-specificity of the PP1 holoenzyme [34]. Recent genome-wide promoter binding analysis showed that PP1 holoenzyme targets hundreds of cellular promoters by its major nuclear regulatory subunits, PNUTS, NIPP1 and RepoMan, suggesting PP1's potential to regulate the expression of many cellular transcripts [35]. While a PP1 regulatory subunit that recruits PP1 to P-TEFb is yet to be identified, a study by Andrew Rice and colleagues showed that PNUTS associates with CDK9/cyclin T1, and siRNA-mediated knock-down of PNUTS upregulates HIV-1 transcription [36]. Our previous study showed that PP1 can participate in host cell transcription by dephosphorylating RNAPII CTD [37]. In addition, we showed that NIPP1/PP1 holoenzyme associates with RNAPII [38]. Thus, RNAPII-associated PP1 can potentially regulate host cell transcription by controlling phosphorylation of RNAPII or CDK9. NIPP1 can silence transcription as it constitutes part of a protein silencing complex that includes the transcriptional repressor EED and histone deacetylase HDAC2 [39, 40]. NIPP1 can also activate transcription as it induces expression of mesenchymal genes in HeLa cells [41]. We recently reported that NIPP1 represses expression of neuregulin-1, a heparin-binding neurotrophic factor [42]. Thus, deregulation of PP1 can enhance transcription of normally repressed genes, including integrated HIV-1 provirus.

4. SMALL MOLECULE COMPOUNDS THAT TARGET TAT-MEDIATED TRANSCRIPTION

Many small molecules that inhibit HIV-1 transcription were described in details [43]; thus we provide only a brief update. Didehydrocortistatin A (dCA) is a recently identified Tat inhibitor (see Fig. 1 for mechanisms of inhibition, and Fig. 2 for structure) that potently inhibits HIV-1 transcription and reduces residual levels of viral transcription in several cell culture models of HIV latency including *ex vivo* treated primary CD4⁺T cells isolated from virally suppressed patients [44]. The dCA establishes a nearly permanent state of latency in infected primary cells in culture [44]. Triptolide, a diterpenoid epoxide isolated from *Tripterygium wilfordii Hook F*, a Chinese medicinal herb, possesses a broad range of bioactivities, including anti-inflammatory, immunosuppressive, anti-tumor properties and inhibits HIV-1 transcription and replication by promoting proteasome degradation of Tat protein [45]. This compound is currently in phase 3 clinical trial in China. A derivative of 6-aminoquinolone, WM5 binds to TAR RNA with micromole IC₅₀ and inhibits HIV-1 replication [46]. Tabarrini and colleagues have modified this compound and successfully tested it against human cytomegalovirus (CMV) and hepatitis C viruses [47, 48]. Previously reported CDK9 inhibitors, DRB [49] and flavopiridol [50, 51] were toxic for the cells in the tested range. They target the active center of CDK9 and inhibit all cellular transcripts. DRB [49], flavopiridol [50, 51] and also R-roscovitin [52] were shown not to be exclusively selective for CDK9. A new CDK9 inhibitor with improved selectivity (LDC000067, abbreviated as LDC067) is based on a 2, 4-aminopyrimidine scaffold. It was identified in a small molecule screen of a chemical library designed to bind to the ATP binding pockets in kinases [53]. LDC067 inhibits CDK9 *in vitro* with an IC₅₀ of 44 ± 10 nM with the selectivity for CDK9 over other CDKs in the range of 55-fold (vs. CDK2) to over 230-fold (vs. CDK6 and CDK7) [53]. Okamoto and colleagues recently reported another novel CDK9 inhibitor, FIT-039, that inhibits HIV-1 replication [54]. The compound previously showed broad spectrum antiviral activities against herpes simplex virus (HSV)-1 and HSV-2, adenovirus and CMV [54]. In latently infected OM-10.1 cells, FIT-039 reduces NF-κB translocation into the nucleus and increases IκB expression in the cytoplasm thus targeting both CDK9 and NF-κB-dependent transcription activation [54].

Availability of CDK9/cyclin T1/Tat structure [55, 56] opened new possibilities for the design of small molecules aimed at disrupting the interaction of Tat with CDK9/cyclin T1. A high-throughput docking of small molecules mimicking a Tat-derived HIV-1 inhibitory peptide onto the binding pocket of CDK2 identified small molecules disrupting the cyclin/CDK interaction [57]. The discovered compound F07#13 inhibited HIV-1 transcription because of the similarity of cyclin/CDK interaction pocket between CDK2 and CDK9. Compound C3 was recently identified by *in silico* screening of Tat/TAR recognition motif in cyclin T1 [58] (Fig. 2). C3 compound inhibited phosphorylation of RNAPII CTD Ser-2 and prevented HIV-1 reactivation at 160–600 nanomolar range of concentrations in TNF-α treated OM-10.1 and U1 cells.

5. TARGETING PP1 FOR INHIBITION OF HIV-1 TRANSCRIPTION

Our previous studies showed that HIV-1 Tat interacts with PP1 and translocates it to the nucleus [59]. The highly conserved Q³⁵VCF³⁸ sequence of Tat interacts with the “RVxF”-accommodating cavity of PP1 helping to shuttle PP1 to the nucleus [59]. While Tat interaction with PP1 is weak, in the range of 1–10 μ M, the introduction of an “ideal” RVTF sequence into Tat led to the inhibition of HIV-1 transcription [59]. Tat RVTF bound stronger to PP1 and, as a result, was not able to bind P-TEFb [59]. Thus, we hypothesized that the intrinsically weak Tat-PP1 interaction can be targeted by a therapeutic small molecule as it will not affect the stronger nanomolar range interactions of PP1 with host cell regulatory subunits. As a proof of principle, we showed that expression of nuclear inhibitor of PP1 (cdNIPP1), 98 amino acid long peptide that localizes to nucleus and sequesters PP1, as part of HIV-1 genome efficiently inhibited replication of the modified HIV-1 [60] (Fig. 1). We also showed that 293T cells stably expressing cdNIPP1 were viable and that HIV-1 transcription was suppressed in these cells [60]. In the cdNIPP1 expressing cells, CDK9 Thr-186 phosphorylation was increased and P-TEFb was associated with large molecular weight complex [60]. Dephosphorylation of CDK9 Thr-186 releases CDK9 from the large P-TEFb [61]. However, CDK9 Thr-186 dephosphorylation by PPM1A and PPM1B phosphatases blocked CDK9 activity leading to CDK9 proteasome degradation [62]. Thus, sequestration of PP1 or targeting it with small molecules to dissociate Tat appears to be a feasible approach to modulate HIV-1 transcription.

6. TARGETING PP1/TAT INTERACTION WITH SMALL MOLECULES

Based on the crystal structure of PP1 in complex with RFTV peptides, we designed a library of small molecule compounds [31]. About 300, 000 compounds from the Enamine (Kiev, Ukraine) stock collection were screened *in silico*. We selected 262 compounds with best binding scores and a geometry close to the RVxF binding motif of PP1 were further evaluated for inhibition of HIV-1 replication *in vitro* [31]. Compound 1H4 (see Figs. 1 and 3) inhibited viral replication in MT4 cells infected with pNL4-3 HIV-1 with IC₅₀=25 μ M [31]. Further modifications of 1H4 compound yielded a derivative compound, 1E7-03 [63] (Fig. 3) which inhibited HIV-1 transcription and replication *in vitro* in cultured and primary cells with low micromole IC₅₀ and without toxicity. 1E7-03 competed with Tat-derived QVCF sequence containing peptide for binding with PP1 *in vitro* in a dose-dependent manner and also reduced nuclear targeting of PP1 by Tat in cultured cells [63].

Thus, targeting Tat binding to PP1 with small molecules disrupts Tat-mediated transactivation of HIV-1 transcription and can potentially silence low-level HIV-1 transcription as well as inhibit viral replication in stable reservoirs.

7. PP1-TARGETING SMALL MOLECULE ACTIVATORS OF HIV-1 TRANSCRIPTION

While screening for HIV-1 inhibitory small molecules, we identified several compounds that induced HIV-1 transcription [63, 64]. These compounds included both 1, 2, 3, 4-tetrahydraacridine-based compounds (1H4 derivatives) and additional compounds that

contained sulfonamide linker and were structurally distinct from 1, 2, 3, 4-tetrahydracridine series [64]. We obtained a library of homologues from the Enamine stock collection and screened it using one round HIV-1 infection with pseudotyped VSVG HIV-1-LacZ. This replication deficient virus is capable of infecting T cells, integrating and expressing luciferase from the integrated provirus, but cannot produce envelope because of the point mutations. Thus only single replication cycle is achieved which allows more accurate analysis of HIV-1 transcription. We identified compound 3, which was renamed small molecule activator of PP1 (SMAPP1) (Figs. 1 and 3). SMAPP1 induced HIV-1 replication in latently infected Jurkat T cells, THP-1 cells and primary CD4⁺ T cells and also in latently infected ACH-2 and OM 10.1 cells [64]. *In vitro* analysis showed that SMAPP1 bound PP1 in surface plasmon resonance-based Bia-core assay and enhanced dephosphorylation of recombinant PP1 substrate by purified PP1. In SMAPP1-treated cells, phosphorylation of CDK9 Ser-90 and Thr-186 was increased. Proteomic analysis showed that SMAPP1 increased expression of P-TEFb and PP1-related proteins including a PP1-regulatory subunit, Sds22, which was confirmed by immunoblotting analysis. *In silico* molecular docking of SMAPP1 showed its preferential interaction with PP1 via the binding site that is located in the C-terminal groove of PP1. Interestingly, analysis of the cellular proteome of CEM T cells treated with SMAPP1 [64] showed upregulation of NRG-1 among proteins whose expression was increased by SMAPP1 treatment. Thus our study identified a novel class of PP1-targeted compounds that activate latent HIV-1 provirus and that may be useful for future anti-HIV-1 therapeutics targeting HIV-1 eradication. While SMAPP1 was stable in tissue culture media, incubation in murine serum led to its rapid degradation (in less than 15 mins) [65], making this compound not suitable for use in HIV-1 infected humanized mice. To improve stability and facilitate the delivery of SMAPP1, we packaged the compound in polymeric polyethylene glycol polymethyl methacrylate (PEG-PMMA) nanoparticles. SMAPP1 was released from PEG-PMMA nanoparticles during the first 24 hrs and the release continued for up to 120 hrs [65]. Treatment of the HIV-1-infected cells with the SMAPP1-loaded nanoparticles induced HIV-1 transcription by about 50% compared to the untreated control, while blank PEG-PMMA nanoparticles showed no activation of HIV-1 transcription [65]. Thus, nanoparticle formulations can aid in improving stability of HIV-1 transcription targeting compounds.

8. REGULATION OF HIV-1 TRANSCRIPTION BY CDK2

Our early study and a parallel study from John Brady's group showed that HIV-1 transcription is activated by Tat in the G1 phase, but not in the G2 phase [66, 67]. This observation led to the identification, by us, and later by Kashanchi's group, of CDK2/cyclin E as a key player in HIV-1 transcription [68, 69]. We showed that HIV-1 was inhibited in CDK2 knock-down cells [70] and also in macrophages, differentiated from induced pluripotent stem cells. These cells were engineered from CDK2 knock-down epithelial cells [71]. The findings suggested that CDK2 was critical for HIV-1 transcription and replication. A functional link between CDK9 and CDK2 was found when we analyzed phosphorylation of CDK9 by CDK2 *in vitro*. CDK9 was phosphorylated on Ser-90 located within (⁹⁰SPYNR⁹⁴) consensus CDK2 phosphorylation site [31]. The level of phosphorylation was reduced after knockdown of CDK2. Alanine mutation of CDK9 Ser-90 reduced CDK9

association with the large P-TEFb complex and inhibited HIV-1 transcription. In contrast, CDK9 S90D mutant remained associated with the large P-TEFb complex whose expression induced Tat-dependent HIV-1 transcription [31]. Molecular modeling showed that CDK9 Ser-90 was solvent exposed and located on a flexible loop. These findings identified a novel regulatory phosphorylation site on CDK9 that may be targeted for activation or inhibition of HIV-1 transcription.

Recent studies identified a new CDK2 target, SAM domain and HD domain-containing protein 1 (SAMHD1) that controls the cellular deoxyribonucleoside triphosphate (dNTP) pool sizes through dNTP hydrolysis and inhibits HIV-1 reverse transcription [72–74]. SAMHD1 activity was shown to be controlled by phosphorylation on Thr-592 by CDK1/cyclin A [75]. Also CDK6-cyclin D3 was implicated in SAMHD1 phosphorylation control [76], and CDK2 was shown to phosphorylate SAMHD1 [77]. Increased SAMHD1 phosphorylation blocks its activity and lifts restriction of HIV-1 reverse transcription (Fig. 4).

9. INHIBITION OF HIV-1 TRANSCRIPTION WITH IRON CHELATORS

HIV-1 replication is dependent on host cell enzymes some of which require intracellular iron for their activity. HIV-1 reverse transcription is dependent on host cell ribonucleotide reductase (RNR) which contains non-heme iron [78]. Enzymes involved in HIV-1 transcription, viral mRNA translation and viral assembly also require iron-containing enzymes [79]. HIV-1 transcription is regulated by CDK2, which is deregulated by intracellular iron depletion ([80, 81] and described above). I κ B kinase activation is dependent on iron efflux [82–84], induces NF- κ B and contributes to the activation of HIV-1 promoter [85]. We previously showed that expression of the iron export protein, ferroportin, inhibited HIV-1 transcription [86] (see Fig. 1). Treatment of HIV-1 infected CD4+ T cells and macrophages with iron and hepcidin, a hormone peptide that promotes ferroportin degradation (see Fig. 1), induced HIV-1 replication [86] suggesting that ferroportin restricted HIV-1 expression. We recently investigated the mechanism of reduced HIV-1 *ex vivo* infection in PBMC obtained from patients with sickle cell disease [87]. We observed the expression of ferroportin, reduction of intracellular iron, decreased CDK2 activity and reduced SAMHD1 phosphorylation (Fig. 4), and increased expression I κ B α (Fig. 1), cumulatively leading to the inhibition of HIV-1 transcription and reverse transcription [87]. Cellular iron levels can also be increased by Nef, an HIV-1 accessory protein, that down regulates the expression of human hemochromatosis protein (HFE). HFE controls iron uptake by macrophages and is mutated in hereditary hemochromatosis [88]. Deregulation of HFE by Nef increases iron levels that coincides with increased HIV-1 gag expression suggesting a beneficial effect of increased iron on the production of HIV-1 virions and viral replication [88].

Iron chelators, deferoxamine (DFO) and deferiprone inhibited HIV-1 replication in human PBMCs and macrophages but the inhibition was attributed to a decrease in cell proliferation [89]. Similar to DFO and deferiprone, orally active bidentate chelators, CP502 and CP511 inhibited HIV-replication by reducing cellular proliferation [90] (see structures in Fig. 5). In

cultured T-cells, iron treatment increased HIV-1 viral replication, whereas iron chelation with DFO reduced viral replication [91].

Des Richardson's group showed that CDK2 was inhibited by iron chelators [13, 59, 60]. This observation prompted us to test the effect of iron chelators on HIV-1 transcription. We found that HIV-1 transcription was inhibited in T-cells treated with iron chelator 311 (developed by Des Richardson) and ICL670 [80] (Fig. 5). ICL670 (also known as deferasirox or Exjade) is approved for clinical use in USA [92] but required higher concentrations (~ 100 μ M) for HIV-1 inhibition in cultured cells compared to HIV-1 inhibition by 311 (~ 3 μ M) [80]. Both 311 and ICL670, but not DFO inhibited cellular activities of CDK2 and CDK9 [80]. ICL670 and 311 prevented the association of CDK9 with cyclin T1 and reduced phosphorylation of RNAPII CTD Ser-2 residues [80], further supporting the idea that iron chelators can target HIV-1 transcription. Another potential target of iron chelators is ribonucleotide reductase 2 (RNR2), which contains non-heme iron and whose enzymatic activity is inhibited by DFO and 311 [93]. Inhibition of RNR2 by resveratrol and its analogs prevents HIV-1 infection in synergy with cART inhibitors [94]. Di-2-pyridylketone thiosemicarbazone (DpT) and 2-benzoylpyridine thiosemicarbazone (BpT)-based tridentate iron chelators markedly inhibit HIV-1 transcription and virus replication at much lower concentrations than 311 or ICL670 [81] (Fig. 5). Bp4aT and Bp4eT iron chelators inhibit CDK2 activity and also CDK9 activity [81]. Phenyl-1-pyridin-2yl-ethanone (PPY)-based iron chelators, PPYeT and PPYaT inhibit HIV-1 transcription by decreasing CDK2 and CDK9 activities, and also upregulating IKB α expression and downregulating nuclear NF- κ B [95] (Fig. 5). Recently, the topical fungicide ciclopirox, and the iron chelator deferiprone, were shown to inhibit HIV-1 gene expression at the level of transcription initiation by interfering with the hypusine modification of eIF5a [96] (Fig. 4). Treatment with deferiprone induced apoptosis in HIV-1-infected cells through mitochondrial membrane depolarization leading to permanent elimination of HIV-1 infected cells in culture [97]. In a follow-up exploratory trial, patients were treated with deferiprone [98]. Those patients who achieved 150 μ M deferiprone supra-threshold level in serum and cumulatively received 17 oral doses demonstrated strong HIV-1 RNA decline while on-drug and also for up to 8 weeks off-drug without viral rebound [98]. The authors proposed that deficiency of hypusine-containing eIF5A impedes the translation of mRNAs that encode proline clusters that is present in Gag/p24, and induces apoptosis in HIV-infected cells. Taken together, our studies and studies from other groups point to iron chelators and hypusination inhibitor as a novel therapeutic avenues for HIV-1 transcription and translation inhibition.

10. ANTI-HIV-1 ACTIVITY OF CURCUMIN AND ITS ANALOGUES

Curcumin (CUR), a multi-target natural compound from turmeric, has been reported to affect multiple steps of HIV-1 infection and multiplication including HIV-1 reverse transcription, integration, transcription and maturation (Fig. 4). To date, many of the curcumin analogues have been developed to improve bioavailability and metabolic stability, and some of these analogues inhibit HIV-1. Curcumin and its derivatives, including reduced curcumin, allylcurcumin (ACUR), and tocopherylcurcumin (TCUR), were previously shown to inhibit Tat induced transcription of HIV-1 LTR driven reporter in HeLa cells [99].

Curcumin was also shown to inhibit a chloramphenicol acetyl transferase reporter expression from HIV-1 LTR activated by UV-irradiation that was attributed to the reduction of NF- κ B activation [100]. Besides NF- κ B, AMPK and HDAC1 pathways were shown to be targeted by curcumin through the reduction of HIV-Tat protein acetylation by p300 [101]. Recently, curcumin was also shown to promote proteosomal degradation of Tat protein leading to the inhibition of Tat-induced HIV-1 transcription and reduction of virion production [102]. In our recent study, curcumin A, a pentanone analogue of curcumin (Fig. 6), inhibited HIV-1 with higher potency ($IC_{50}=2\text{ }\mu\text{M}$) than the parental curcumin ($IC_{50}=12\text{ }\mu\text{M}$) in HIV-1 infected PBMCs [103]. Analysis of specific steps of HIV-1 replication showed that curcumin A inhibited HIV-1 RT, but not Tat induced or basal HIV-1 transcription [103]. It is not fully clear why we did not detect HIV-1 transcription inhibition. One explanation is that we employed much lower concentrations of curcumin than previously reported for HIV-1 inhibition. Additionally, we observed 50% reduction in the viability of CEM T cells and PBMCs treated with $1.3\text{ }\mu\text{M}$ and $35\text{ }\mu\text{M}$ curcumin, correspondingly [103]. The recently reported proteosomal degradation of Tat required at least $40\text{ }\mu\text{M}$ degradation of Tat required at least $40\text{ }\mu\text{M}$ concentration of curcumin [102], therefore this effect could be due to the induction of apoptosis. Importantly, curcumin A inhibited HIV-1 more potently compared to curcumin in HIV-1 infected PBMCs [103]. Curcumin A also inhibited the expression of proinflammatory cytokines such as TNF- α and IL-6, which favor HIV-1 replication [104].

In vitro studies demonstrated that curcumin might also inhibit HIV-1 integrase (IN) ($IC_{50}=40\text{ }\mu\text{M}$) and that it interacts with catalytic IN core within amino acids 50–212 [105]. *In silico* docking analysis revealed a configuration of the proposed curcumin-binding site in IN [106] which still needs to be backed by the structural data and *in vivo* proof of the IN inhibition. Additional curcumin analogues were developed and showed to inhibit HIV-1 integrase *in vitro* including rosmarinic acid and dicaffeoylmethane ($IC_{50} < 10\text{ }\mu\text{M}$) [107] (Fig. 6). Curcumin was found to be also an inhibitor of HIV-1 protease *in vitro* ($IC_{50} = 100\text{ }\mu\text{M}$), and the inhibition significantly improved by a complexation of curcumin with boron ($IC_{50}=6\text{ }\mu\text{M}$) [108]. Docking of curcumin to the HIV-1 protease active site revealed a very good fit with extensive hydrogen bonding [106]. Curcumin analog, (E)-2-(3, 4-dimethoxybenzylidene)-6-((E)-3-(3, 4-dimethoxyphenyl)-acryloyl) cyclohexanone (2e) (Fig. 6) was the strongest HIV-1 protease inhibitor in vitro among the tested curcumin analogs [109].

Curcumin consists of two methoxyphenol rings connected by a conjugated heptadienedione. The presence of the active methylene group and α , β -unsaturated diketone moiety is likely to cause instability of curcumin under physiological conditions, poor absorption, and fast metabolism [110]. Previous SAR studies suggested that substituting a shorter linker for the unstable α , β -unsaturated diketone linker would improve the stability of curcumin without compromising its activity. We described mono-carbonyl curcumin analogues as well as cyclopentanone and cyclohexanone analogues that were designed to eliminate the methylene group and one carbonyl group in order to reduce the instability of curcumin and improved its anti-HIV activity [103]. The most potent analog, curcumin A, showed improved stability in complete media supplemented with bovine serum, in serum-free media and in phosphate-buffered saline (PBS). Curcumin A was also stable in murine serum for up to 24 hrs, whereas the parental curcumin had a half-life of 10 hrs.

Taken together, curcumin and its analogues can potentially inhibit different replication steps of HIV-1 life cycle. Because, most of the research on curcumin was conducted *in vitro* and at high curcumin concentrations, its relevance to the curcumin activity *in vivo* remains to be determined. Further detailed and structural modification of curcumin is needed to further clarify its mechanism of action and its suitability for HIV-1 inhibition.

CONCLUSION

cART is efficient in eradicating circulating virus in plasma by inhibiting productive HIV-1 infection. However, current drugs are not able to completely eliminate virus [111], since HIV-1 is capable of maintaining latent infection in stable reservoirs such as resting CD4⁺ T cells, naive T cells and CD34⁺ multipotent hematopoietic stem cells [112, 113]. Furthermore, interruption of therapy leads to the rapid increase of viral population [4, 114], whereas a long combinational cART treatment has negative side effects including fatigue, diarrhea [115], neurocognitive abnormalities and cardiovascular complications [116–118]. Our review describes HIV-1 inhibitory small molecules including those that interfere with PP1 and intracellular iron. Latent HIV-1 provirus can be silenced with PP1-targeting HIV-1 inhibitors such as 1E7-03 [63] or iron chelators which can be used alone or in combination with cART. Altogether, HIV-1 inhibitory compounds can be developed into a supplemental therapy for inhibition of HIV-1 transcription that can augment the existing cART treatment. In addition, HIV-1 activating compounds can be used in “kick and kill” therapy in which HDAC inhibitors and other HIV-1 activating drugs are used to activate the virus followed by cART. Reversing of HIV-1 latency can be achieved with SMAPP1 analogs which will have to be used with cART to prevent virus rebound. Taken together, the studies described in our review point to PP1 as a new drug target, intracellular iron as subject for iron chelation and novel curcumin analogs that can be further developed for novel HIV-1 transcription-targeting therapeutics.

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ABBREVIATIONS

311	<i>2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone</i> iron chelator
ACUR	Allyl -curcumin
AFF	AF4 family of transcription factors involved in leukemia
AF9 (MLL-AF9)	mixed lineage leukemia (MLL)-AF9 fusion protein resulted from the most common translocation in acute myeloid leukemia (AML), t(9; 11)
BpT	2-benzoylpyridine thiosemicarbazone-based iron chelator

cART	combination antiretroviral therapy
CDK	cell cycle dependent kinase
cdNIPP1	an 82-residue PP1-binding fragment of the central domain of NIPP1
CMV	cytomegalovirus
COUP-TF	Chicken Ovalbumin Upstream Promoter transcription factor
CTD	C-terminal domain of RNA polymerase II
dCA	didehydro-cortistatin A
dNTP	deoxyribonucleoside triphosphates
DFO	deferoxamine
DpT	Di-2-pyridylketone thiosemicarbazone
DRB	<i>5, 6-Dichloro-1-β-D-ribofuranosylben-zimidazole</i>
DSIF/Spt4/Spt5/	DRB-sensitivity inducing complex composed of Spt4 and Spt5 homologs
EED	<i>Embryonic Ectoderm Development protein</i> , transcriptional repressor and a member of the Polycomb-group (PcG) family
ELL	Eleven-Nineteen Lysine-rich Leukemia, RNA polymerase II elongation factor
ENL4	echinoderm microtubule associated protein like 4
FPN	ferroportin, <i>solute carrier family 40 member 1 (SLC40A1) iron export protein</i>
HATs	histone acetyl transferases
HDAC	histone deacetylase
HEXIM	<i>Hexamethylene Bisacetamide Inducible</i> protein
HFE	human hemochromatosis protein
HMGA1	by high mobility group AT-hook 1 protein
HSV	herpes simplex virus
IKB α	NF-κB inhibitor α
IL6	interleukin-6

IN	HIV-1 integrase
Lac Z	a gene present in E.coli that encodes β -galactosidase protein
LARP7	<i>La Ribonucleoprotein Domain Family Member 7</i> protein
LTR	HIV-1 long terminal repeat
MePCE	methylphosphate capping enzyme
NELF	the negative elongation factor
NF-κB	nuclear factor κ B
NIPP1	nuclear inhibitor of PP1
PNUTS	PP1 nuclear targeting subunit
PP1	protein phosphatase-1
PP1c	catalytic subunit of protein phosphatase-1
PPY	phenyl-1pyridin-2yl-ethanon
P-TEFb	positive transcription elongation factor b
RCUR	Reduced curcumin
RepoMan	PP1 regulatory subunit
RNAPII	RNA polymerase II
RNR	ribonucleotide reductase
RVxF	PP1-binding motif
7SK RNA	small nuclear RNA
SAMHD1	SAM domain and HD domain-containing protein 1
SAR	structure-activity relationship
Sds22	PP1 regulatory subunit
SEC	super-elongation complex
SMAPP1	small molecule activator of PP1
snRNP	small nuclear ribonucleotide protein complex
STIP2	COUP-TF interacting protein 2
SWI/SNF	<i>SWItch/Sucrose Non-Fermentable, a nucleosome remodeling complex</i>
TAR RNA	HIV transactivation response RNA

Tat	HIV-1 <i>trans-activating regulatory protein</i>
TNF-α	<i>tumor necrosis factor alpha</i>
TCUR	Tocopheryl-curcumin
VSVG	vesicle stomatitis virus g glycoprotein

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Fig. 1. Schematic representation of the HIV-1 transcription regulation

The figure shows a network of host cell factors that regulate Tat-activated HIV-1 transcription. Cellular iron (Fe^{2+}), the iron export protein ferroportin (FPN) and the FPN-inhibitory hormone hepcidin are shown. Reduction of cellular iron leads to CDK2/cyclin E inhibition and IKB α upregulation. Recruitment of CDK9/cyclin T1 by Tat from 7SK snRNP to TAR RNA leads to dissociation of NELF and recruitment of super elongation complex factors, AFF4, ELL2 and ENL4/AF9. Boxes indicate various HIV-1 transcriptional inhibitors and activators described in the review.

Fig. 2. Small molecules targeting Tat-mediated transcription

The following small molecules are shown: Tat-targeting HIV-1 transcription inhibitor (dCA); TAR RNA binding inhibitor (WM5); Tat proteasomal degradation inhibitor (Triptolide); CDK9 inhibitors (DRB, Flavopiridol, R-roscovitin, LDC067, and FIT-039); CDK2/CDK9 inhibitor identified by structure-based drug design (F07#13); and Tat/CycT1 complex inhibitor (C3).

Fig. 3. Small molecules targeting PP1

Shown are the structures of PP1-targeting HIV-1 transcription inhibitors, 1H4 and 1E7-03 and a PP1-targeting activator of HIV-1 transcription, SMAPP1.

Fig. 4. Steps of HIV-1 replication cycle targeted by small molecules

HIV-1 replication cycle is shown. Indicated are the mechanism of HIV-1 inhibition by SAMHD1 and the effect of iron chelators that inhibit CDK2/cyclin E; curcumin analogs and hypusine modification inhibitors (cicliopirox and deferiprone).

Fig. 5. Iron chelators that were shown to inhibit HIV-1

The following iron chelators are shown: inhibitors of HIV-1 replication (Deferiprone, CP502 and CP511); inhibitors of HIV-1 transcription that disrupt the interaction of CDK9 with Cyclin T1 (311 and ICL670); inhibitors of HIV-1 transcription and replication (DpT, BpT, Bp4aT and Bp4eT); and inhibitors of HIV-1 transcription that inhibit CDK2 and CDK9 activities (PPYaT and PPYeT).

Fig. 6. Curcumin and it analogues with HIV-1 inhibition

The following curcumin analogs are shown: curcumin A, an inhibitor of HIV-1 reverse transcription; RA and DCM, inhibitors of HIV-1 integrase; RCUR, ACUR and TCUR, inhibitors of HIV-1 Tat-regulated transcription; and 2e, inhibitor of HIV-1 protease.