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Targeting HIV Transcription: The Quest for a Functional Cure

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Abstract

Antiretroviral therapy (ART) potently suppresses HIV-1 replication, but the virus persists in quiescent infected $CD4^+$T cells as a latent integrated provirus, and patients must indefinitely remain on therapy. If ART is terminated, these integrated proviruses can reactivate, driving new rounds of infection. A functional cure for HIV requires eliminating low-level ongoing viral replication that persists in certain tissue sanctuaries and preventing viral reactivation. The HIV Tat protein plays an essential role in HIV transcription by recruiting the kinase activity of the P-TEFb complex to the viral mRNA's stem–bulge–loop structure, TAR, activating transcriptional elongation. Because the Tat-mediated transactivation cascade is critical for robust HIV replication, the Tat/TAR/P-TEFb complex is one of the most attractive targets for drug development. Importantly, compounds that interfere with transcription could impair viral reactivation, low-level ongoing replication, and replenishment of the latent reservoir, thereby reducing the size of the latent reservoir pool. Here, we discuss the potential importance of transcriptional inhibitors in the treatment of latent HIV-1 disease and review recent findings on targeting Tat, TAR, and P-TEFb individually or as part of a complex. Finally, we discuss the impact of extracellular Tat in HIV-associated neurocognitive disorders and cancers.

1 Introduction

Antiretroviral therapy (ART) potently suppresses replication of human immunodeficiency virus (HIV) driving viral loads to undetectable levels (<50 copies/ml), but fails to permanently eradicate the virus (Chun et al. 1997; Finzi et al. 1997; Wong et al. 1997). Unfortunately, HIV still persists mostly in latently infected memory $CD4^+$T cells in individuals on suppressive ART, and these cells represent a long-lasting source of resurgent virus upon the interruption of ART (Finzi et al. 1999). The long half-life of infected memory $CD4^+$T cells is partly responsible for the lifelong persistence of HIV (Finzi et al. 1999; Siliciano et al. 2003). In addition to latently infected cells, persistence can also be attributed to ongoing low levels of viral replication in infected subjects on ART (Fletcher et al. 2014; Palmer et al. 2008). Cell-associated viral RNA can be detected in gut and lymph nodes, suggesting continuous viral production in these compartments during ART and these anatomical reservoirs may constitute viral sanctuaries (Yukl et al. 2010).

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As current anti-HIV drugs do not inhibit transcription from integrated viral genomes and do not prevent viral particle release from stable cellular reservoirs, novel classes of antiretrovirals (ARVs) are needed to inhibit these processes. An ideal drug candidate should be able to inhibit viral production from integrated viral genomes and permanently silence HIV transcription.

In newly infected cells, cellular transcription factors such as NF-κB initiate HIV basal transcription at the 5′ long-terminal repeat (LTR) but result in short, abortive viral transcripts due to RNA polymerase II (RNAPII) pausing shortly after promoter clearance (Toohey and Jones 1989). An RNA stem–loop structure called transactivation response element (TAR) spontaneously forms within the first 59 nucleotides of each viral transcript. The viral protein Tat, a 101 amino acid protein, is initially expressed from rare full-length transcripts that are multiply spliced. After acetylation of Tat at lysine 28 by the p300/CBP-associated factor (PCAF), Tat recruits the positive transcription elongation factor b (P-TEFb) [composed of cyclin T1 and cyclin-dependent kinase 9 (CDK9)] from a large inactive complex composed of 7SK snRNA, the methylphosphate capping enzyme, MePCE, the La-related protein, LARP7, and HEXIM1 proteins (Fig. 1) (Barboric et al. 2007; Krueger et al. 2008; Sedore et al. 2007). Tat binds to P-TEFb, and the complex binds the TAR RNA (D'Orso and Frankel 2010). Tat binds to TAR by a specific arginine-rich basic domain between residues 49 and 57. Once in close proximity to the pre-initiation complex, autophosphorylated CDK9 (Garber et al. 2000) phosphorylates negative elongation factors DSIF and NELF, converting DSIF into a positive elongation factor and causing NELF to release from the complex. In addition, CDK9 phosphorylates serine 2 of the RNAPII C-terminal domain (CTD) heptapeptide repeat, allowing the interaction of RNAPII with additional factors involved in productive transcription elongation (Fig. 1) [Reviewed in (Ott et al. 2011)]. Tat is released from TAR and P-TEFb after being acetylated at lysine 50 by p300/CBP and hGCN5. Freed Tat can then recruit factors such as PCAF and SWI/SNF leading to further chromatin remodeling enhancing HIV transcription elongation. Studies based on chromatin immunoprecipitation and fluorescence recovery after photobleaching suggested that Tat and P-TEFb could stay on the elongating RNAPII throughout the transcription of the entire HIV gene and could undergo several cycles of association/dissociation during the elongation process (Bres et al. 2005; Molle et al. 2007). The elongation complex is then converted into a highly processive unit and promotes the synthesis of full-length viral transcripts by more than 100-fold (Cullen 1986).

During ART, HIV replication in a subset of memory $CD4^+$T cells is progressively silenced and the HIV-1 provirus is maintained in a latent transcriptional state by a multitude of molecular mechanisms. These include low levels of Tat or P-TEFb, absence of cellular transcription factors NF-κB and NFAT, presence of repressors such as CBF-1 and YY1 as well as transcriptional interference [reviewed in (Margolis 2010; Mbonye and Karn 2011)]. Entry into latency is also regulated by specific epigenetic chromatin modifications of nucleosomes present at the HIV promoter, notably, deacetylation and methylation of histone N-terminal tails by specific enzymes such as histone deacetylase 1 (HDAC1) and Suv39H1 (Margolis 2010; Mbonye and Karn 2011). During transcriptional reactivation, Tat recruits chromatin remodeling factors, such as SWI/SNF, that are responsible for changes in the local chromatin structure (Easley et al. 2010; Mahmoudi 2012) and histone acetyl

transferases (HAT) such as p300/CBP, which can reverse the effects of histone deacetylation (Fig. 1) (Benkirane et al. 1998; Lusic et al. 2003).

2 HIV Transcription Inhibitors and Deep-Latency

Tat is an attractive target for therapeutic intervention because it has no cellular homologs and it is expressed early in the viral life cycle. Direct inhibition of Tat blocks the feedback loop that drives exponential production of viral progeny. An ideal anti-HIV small-molecule candidate should have a drug-like structure, be soluble in physiologic conditions, inexpensive, used at low posology and able to penetrate sanctuary sites such as gut, lymph nodes and the brain. In the specific case of a transcription inhibitor, it is essential that the compound inhibits only Tat-dependent transcription without affecting cellular transcription. This can be accomplished by directly targeting Tat/TAR interaction or the recruitment of P-TEFb by Tat to the transcriptionally paused RNAPII. A specific Tat-dependent transcription inhibitor should not disrupt HIV basal transcription, which is promoted by cellular transcription factors shared with cellular genes (e.g., NF-κB, Sp1, NFAT, etc) (Cullen 1991; Jeang et al. 1993; Pessler and Cron 2004), and this explains why anti-Tat molecules are unable to fully inhibit acute HIV replication, unlike ARVs targeting other viral proteins (Mousseau et al. 2012). However, in contrast with ARVs that have no activity on viral expression from cells containing an integrated provirus and can only block de novo infection, an HIV transcription inhibitor mediates complete transcriptional inhibition in these cells (Mousseau et al. 2012).

Several strategies have been employed for eradicating the latent HIV reservoir including ART regimen intensification, vaccines, HDAC inhibitors, gene therapy, and stem cell transplantation (Deeks et al. 2012). The most commonly explored strategy focuses on purging the reservoirs using anti-latency agents such as HDAC inhibitors, while preventing novel infections by maintaining ART (Deeks 2012). This approach is based on the premise that reactivation of HIV from latency will kill the infected cells or allow the immune system to eliminate them. However, a clinical trial using the HDAC inhibitor Vorinostat demonstrated that the reactivation of latently infected $CD4^+$T cells did not result in a measurable decrease in the size of the latent reservoir (Archin et al. 2012). Moreover, Siliciano and colleagues showed that the current methods to measure the latent reservoir (viral outgrowth assay) could have underestimated its size by up to 60-fold, and multiple reactivation events would be necessary for the required viral reactivation of every single latently infected cell (Ho et al. 2013). Each eradication approach has its own inherent challenges and none have demonstrated definitive success. The countless failures in obtaining total HIV eradication have brought forth the concept of a “functional cure”—defined by the persistence of HIV genetic material in the body without detectable viral replication in the absence of ART, without loss of $CD4^+$T cells, no clinical progression, and lack of HIV transmission.

An alternative approach that represents a significant departure from established paradigms of eradicating latent reservoirs involves the use of a therapeutic agents targeting HIV transcription. These could potentially suppress residual levels of viral transcription in latently infected cells, thereby establishing a state of deep-latency refractory to viral

reactivation. Specifically, rather than activating the endogenous latent reservoir, one would drive the residual transcription that occurs during ART into long-term deep-latency. Despite the persistence of the HIV genetic material in the body, a transcriptional inhibitor treatment combined with ART would be aimed at reducing the size of the latent reservoir pool by potentially blocking ongoing viral replication, as well as reactivation events or “blips” (Jones and Perelson 2007; Sklar et al. 2002) that replenish the viral latent reservoir, a key limitation of current ART (Fig. 2).

Transcriptional inhibitors could also reduce morbidities associated with persistent levels of immune activation caused by low-level ongoing replication in subjects on suppressive ART (Hunt 2010). Moreover, by suppressing viral transcription to a state of deep-latency refractory to reactivation, ART could potentially be interrupted without viral rebound to alleviate patient’s side effects.

3 Extracellular Tat

ART curtails HIV replication; however, there is a paradoxical increase in the prevalence of HIV-associated neurocognitive disorders (HAND) (Bagashev and Sawaya 2013), which include a group of syndromes that range from undetectable neurocognitive impairments to severe forms of encephalitis/dementia (Gannon et al. 2011). A commonly accepted explanation for HAND is the inability of various ART regimens to pass through the blood–brain barrier (BBB), thus rendering the brain a viral sanctuary (Bagashev and Sawaya 2013). HIV-1 does not infect neurons; therefore while still debated, neurotoxicity has been postulated to be mediated by extracellular Tat upon its direct passage through the BBB or after secretion from infected glia cells (Li et al. 2009). Tat uptake by glial cells (King et al. 2006; Ma and Nath 1997) or interaction with certain cell receptors on a variety of cell types has been widely reported (Albini et al. 1996, 1998; Debaisieux et al. 2012; Ensoli et al. 1993; Fawell et al. 1994; Rusnati and Presta 2002). A high level of Tat mRNA has been found in the brain of HAND patients (Del Valle et al. 2000; Hofman et al. 1994; Hudson et al. 2000; Li et al. 2009; Wesselingh et al. 1993; Wiley et al. 1996), and anti-Tat antibodies were detected in their serum (Aldovini et al. 1986) or their cerebrospinal fluid (Bachani et al. 2013). Moreover, injection of Tat or stable expression of Tat in brain showed neurological symptoms similar to those associated with HAND (Gorantla et al. 2012; Kim et al. 2003; Li et al. 2009; Weeks et al. 1995; Zucchini et al. 2013).

Tat has been proposed to mediate neurotoxicity by several mechanisms: induction of oxidative stress (Romani et al. 2010), BBB damage (Bagashev and Sawaya 2013; Li et al. 2009; Strazza et al. 2011), up-regulation of genes such as monocyte chemoattractant protein-1 (MCP-1), TNF-α, and metalloprotease 9 (Li et al. 2009; Romani et al. 2010), its chemotaxic function (Li et al. 2009), inhibition of autophagy in macrophages (Van Grol et al. 2010), and activation of N-methyl-D-aspartate receptors (Campbell and Loret 2009; Li et al. 2009). Tat’s basic domain and cysteine-rich region could play an important role in these neurotoxic effects (Li et al. 2009), and patients infected with HIV-1 subtype C, containing a mutation in Tat’s cysteine region, tend to have less prevalence of HAND (Li et al. 2009). Extracellular Tat has also been associated with acquired immune deficiency syndrome (AIDS)-associated cancers such as Kaposi’s sarcoma, often developing more aggressively in

HIV-infected patients (Johri et al. 2011; Nunnari et al. 2008; Romani et al. 2010). Tat's basic domain, by competing with basic fibroblast growth factor for heparin sulfate proteoglycan, may induce spindle cell growth in Kaposi's sarcoma (Campbell and Loret 2009). Compounds interacting with the TAR-binding domain of Tat [such as dCA, see Sect. 4.2 (Mousseau et al. 2012)] may have the ability to counteract the neurotoxic and cancer-promoting properties of Tat by either reducing its production at the transcription level and by directly inhibiting its pathogenic activity (Mediouni et al. 2015).

Here, we review a selection of new small-molecule inhibitors of the Tat/TAR and the Tat/cyclin T1/CDK9 complexes. For a more exhaustive collection of HIV transcription inhibitors, we suggest the following publications (Baba 2006; Massari et al. 2013; Mousseau and Valente 2012; Richter and Palu 2006; Stevens et al. 2006). When applicable, we will direct the reader to the most recent and appropriate review.

4 Tat/TAR Interaction

Tat recruits P-TEFb complex to the nascent TAR element of the viral RNA to promote transcriptional elongation, greatly enhancing HIV mRNA synthesis. As expected, disrupting Tat/TAR interaction dramatically reduces viral production and hence has been the focus for the development of numerous antiviral compounds (Massari et al. 2013; Mousseau and Valente 2012). However, none has yet reached the clinic, and only one has recently entered the clinical trial pipeline (NCT02219672). The discovery of a safe Tat-dependent transcriptional inhibitor would be of tremendous value, as it may reduce residual viremia from latently infected $CD4^+$T cells and repress HIV reservoir replenishment, the major hurdle in the ongoing race for an HIV cure.

Here, we will focus on recent and specific small-molecule inhibitors targeting TAR or Tat. Other strategies including vaccine, ribozyme, aptamer, oligonucleotide-based antisense and non-small inhibitors such as aminoglycoside and peptide-based structure are reviewed elsewhere (Burnett and Rossi 2012; Campbell and Loret 2009; Eekels and Berkhout 2011; Massari et al. 2013; Mulhbacher et al. 2010; Richter and Palu 2006; Turner et al. 2006; Zeller and Kumar 2011).

4.1 TAR

TAR is a small non-coding hairpin RNA that folds into a well-defined three-dimensional structure. TAR is extremely well conserved among HIV clades and is a very attractive target for drug development. However, specifically targeting TAR with small molecules has proven challenging given the properties common to all RNAs such as negative charge, large flexibility, and reduced diversity of chemical composition. There is no crystal structure of the Tat/TAR complex, only structures of small-molecule ligands or Tat-derived peptides interacting with TAR exist to help guide the design of small-molecule inhibitors (Aboul-ela et al. 1995; Puglisi et al. 1992).

One of the most interesting TAR inhibitors reported to date is **WM5** (Fig. 3), a 6-aminoquinolone, able to specifically block Tat/TAR interaction by binding to TAR at the micromolar range (Cecchetti et al. 2000; Parolin et al. 2003; Richter et al. 2004). This

compound inhibits Tat-dependent LTR activity and inhibits acute viral replication in peripheral blood mononuclear cells (PBMCs), but with a low therapeutic index (TI) (Tabarrini et al. 2004). Successive structure-activity relationship studies resulted in several WM5 derivatives with anti-HIV properties (Tabarrini et al. 2010). **HM13N** and **NM13** are the best quinolone-based inhibitors of Tat-dependent transcription described so far (Fig. 3) (Massari et al. 2010; Tabarrini et al. 2011). HM13N inhibits both HIV-1 and HIV-2 in MT-4 cells and displays a reasonably good TI in chronically and latently infected cell lines. NM13, is selective only to HIV-1 with an half-maximal inhibitory concentration (IC_{50}) = 80 nM and a TI ≥ 3,707 in MT-4 cells, but has poor solubility (Tabarrini et al. 2011). Contrary to WM5, none of these two compounds bind specifically to the Tat/TAR complex, acting by an unknown mechanism. Two related 6-desfluoroquinolones, HM12 and HM13, inhibit in vivo TNF-α reactivation from latently infected OM-10.1 cells, a promyelocytic cell line, when engrafted in hu-SCID mice (Stevens et al. 2007; Tabarrini et al. 2008). These compounds are currently in development to improve their solubility and reduce cytotoxicity.

Recently, Stelzer et al. (2011) has developed a molecular dynamic software to screen novel TAR-binding small molecules. A library of 51,000 compounds were virtually screened onto a RNA dynamic ensemble of 20 conformers of TAR to help account for large degrees of RNA conformational adaptation during docking. A fluorescence-based binding assay confirmed several of the hits bound to TAR and inhibited binding to Tat. Netilmicin, an aminoglycoside antibiotic, bound TAR with the highest specificity and blocked viral replication in an HIV-1 indicator cell line with an IC_{50} of 23 μM. Netilmicin might, however, not be specific against HIV, just as previously observed for other aminoglycoside derivatives (Lapidot et al. 2008). Nevertheless, this study demonstrated the usefulness of high-throughput in silico screening to develop new TAR inhibitors.

Davidson et al. (2011) discovered novel non-charged drug-like small molecules targeting TAR using nuclear magnetic resonance (NMR) ligand-based screening. In this study, an arginine derivative was used as a probe to bind to the Tat binding region of TAR (similar to how arginine of the Tat basic domain would bind to TAR), to lock TAR into a specific conformation more favorable to the binding of drug-like compounds. Reorganization of the RNA structure led to the formation of a pocket in the major groove allowing the binding of six compounds. It will be important to validate these hits in HIV cellular models. This study was proof of concept for the use of ligand-based NMR screening to identify new small molecules to target TAR.

4.2 Tat

Given Tat's crucial requirement for viral gene expression and its role in maintenance or reactivation of latency, the search for Tat inhibitors has been an intense area of research over the years. However, small molecules that inhibit Tat, either by direct interaction, by its degradation or by structurally modifying it, are rare (Kalantari et al. 2009; Mousseau et al. 2012; Narayan et al. 2011; Wan and Chen 2014). This lack of true Tat inhibitors in the literature might be a reflection of the employed screening strategies. For example, screening for Tat-dependent transcription inhibitors using the widely accepted cellular HIV LTR-

reporter system may result in the identification of off-target inhibitors, affecting additional host cellular factors important to HIV transcription.

Nevertheless, in two separate studies, Prabhu and co-workers identified two small molecules inhibiting Tat-mediated activation of HIV-1 transcription using an HIV LTR-Luc reporter assay activated by Tat transfection (Kalantari et al. 2009; Narayan et al. 2011). The first compound, the **cyclopentenone prostaglandin 15d-PGJ$_2$** was previously reported to inhibit HIV replication (Fig. 3) (Rozera et al. 1996). This compound is an arachidonic acid-derived endogenous Michael acceptor electrophiles (MAEs) presenting an α,β-unsaturated carbonyl functionality (enone) and is capable of forming covalent Michael adducts by interacting with the cysteine sulfhydryls of certain proteins. At 3.1 μM, the compound 15d-PGJ$_2$ reduces Tat-dependent transcription by 80 % and HIV replication by 75 % in acutely HIV-1 infected U937 cells. The second inhibitor discovered, **celastrol**, a tripernoid MAE, inhibits Tat-mediated transactivation by 80 % using a non-toxic concentration of 150 nM (Fig. 3). But, HIV replication inhibition measured by p24 enzyme-linked immunosorbent assay and quantitative reverse transcription polymerase chain reaction in acutely infected U937 was only 50 %. Celastrol is very toxic, since viability is reduced by 50 % in U937 cells at 250 nM. The relevance of these findings lies in the mechanism by which celastrol and 15d-PGJ$_2$ interact with Tat, forming a covalent bond with the Cys thiols of the viral protein. In the case of celastrol, the secondary structure of Tat was altered even though it did not impair the binding to TAR, suggesting a probable deficient role of Tat in P-TEFb recruitment. Both of these two compounds need to be further optimized to increase their TI.

A bis-triazoloacridone compound, **temacrazine**, was found to inhibit HIV replication in acute, chronic, and latent cells in the nanomolar range (Fig. 3) (Turpin et al. 1998). The compound was first identified based on its antitranscriptional activity in cancer and appears to be a selective inhibitor of HIV transcription functioning via an unknown mechanism. It was suggested it affects a still unidentified highly specific viral target required for HIV-1 transcription. No additional follow-up on temacrazine has been reported since, suggesting that some undesirable properties prevented its development as an anti-HIV drug.

More recently, a group reported that **triptolide**, a diterpenoid epoxide isolated from *Tripterygium wilfordii*, was able to inhibit HIV-1 transcription by accelerating Tat degradation in a specific manner (Fig. 3) (Wan and Chen 2014). While not tested for direct interaction with Tat, the N-terminus of Tat (1–57) and the nuclear localization of Tat seemed to be required for Tat degradation. The reported IC_{50} in PBMCs averaged 1.3 nM, but the compound was quite toxic with a half-maximal cytotoxic concentration (CC_{50}) around 13 nM limiting its use in its current form. Nonetheless, Triptolide has entered several clinical trials for different targets such as gastrointestinal diseases, rheumatoid arthritis, and HIV. The HIV clinical trial is currently in phase III (NCT02219672) and is performed in combination with a cocktail of ARVs to study its impact on the size of the HIV-1 reservoir, in naïve-ART Chinese patients currently in the acute phase of HIV-1 infection.

Finally, our laboratory has reported that **didehydro-cortistatin A** (**dCA**), an analogue of a natural steroidal alkaloid isolated from a marine sponge, inhibits Tat-mediated transactivation of the HIV-integrated provirus by binding specifically to the TAR-binding

domain of Tat (Fig. 3) (Mousseau et al. 2012). We demonstrated that dCA inhibits transcription initiation and elongation from the viral promoter in chronically infected cells at subnanomolar concentrations without cell-associated toxicity (IC_{50} ranges from 1 pM to 2 nM; CC_{50} = 20 μM). Moreover, long-term treatment of chronically infected cells reduced viral mRNA to undetectable levels and dCA discontinuation does not result in viral rebound suggesting that dCA promotes rapid and prolonged silencing of the HIV promoter. Most importantly, we demonstrated that dCA could abrogate spontaneous as well as antigenic viral particle release from $CD4^+$T cells explanted from virally suppressed subjects on ART.

Hence, by blocking Tat-dependent transcription, a Tat inhibitor such as dCA may reduce reactivation of latently infected cells and block low-ongoing replication, thereby controlling replenishment of the viral reservoir, which upon death of the long-lived latently infected memory $CD4^+$T cells could result in a reduction of the reservoir pool over time.

5 Inhibition of P-TEFb, an Essential Cellular Complex for HIV-1-Activated Transcription

Several host cell factors have essential roles in HIV-1 transcriptional activation and may serve as potential targets for antiviral chemotherapy, as long as cell survival is not impaired [reviewed in (Coley et al. 2009; Massari et al. 2013; Mousseau and Valente 2012; Stevens et al. 2006)]. One advantage to this approach is that unlike current ARVs acting on viral proteins, targeting cellular factors would unlikely lead to viral resistance. In this section, we will focus on recent small-molecule inhibitors of the P-TEFb complex and its kinase activity.

5.1 CDK9

Finding a highly selective and non-cytotoxic CDK9 inhibitor is a difficult task due to its dual role in cellular and HIV transcription (Klebl and Choidas 2006; Nemeth et al. 2011; Wang and Fischer 2008). Nevertheless, major efforts have been made to find small-molecule inhibitors targeting specifically the CDK9 activity and the function of P-TEFb complex in HIV replication. The crystal structure of CDK9/cyclin T1/Tat complex has recently been resolved, revealing a main interaction between Tat and cyclin T1, but also with the T-loop of CDK9 (Gu et al. 2014; Tahirov et al. 2010). These studies suggested a conformational change in P-TEFb upon Tat binding, which opened the possibility to design inhibitors targeting specifically the interface of this viral protein/cellular host complex, without affecting Tat-free P-TEFb complexes, thereby avoiding toxicity (Narayanan et al. 2012; Ramakrishnan et al. 2012; Sedore et al. 2007).

Among the first, CDK9 inhibitors reported to inhibit HIV were the nucleotide analogue DRB (Biglione et al. 2007; Marciniak and Sharp 1991), flavopiridol (Chao et al. 2000), and R-roscovitine (Wang et al. 2001). DRB and flavopiridol have a fair selectivity for CDK9 over other CDKs, but displayed a small TI. Flavopiridol potently inhibits both, CDK9 activity with an IC_{50} of 6 nM, and HIV replication at 10 nM, but chemical derivatives only improved selectivity and survival to a small degree (Ali et al. 2009). Based on the reported P-TEFb structure in complex with flavopiridol (Baumli et al. 2008), a recent computer-aided

design study identified the small-molecule 2-phenylquinazolinone derivative #37, as an inhibitor of both CDK9 and CDK2 activity. This molecule inhibits phorbol myristate acetate —activated chronically infected OM-10.1 cells, with an IC_{50} of 4 μM and a CC_{50} of 345 μM (Sancineto et al. 2013).

A derivative of a Chinese antileukemia drug, indirubin-3′-monoxime (IM), was reported to be a specific CDK9 inhibitor, reducing Tat-induced viral expression in both PBMCs and macrophages, in the single-digit micromolar range with no associated toxicity (Heredia et al. 2005; Toossi et al. 2012). More recently, IM was shown to reduce viral replication of two multidrug-resistant HIV reverse transcriptase (RT) molecular clones in humanized mice (Heredia et al. 2014).

The third generation of R-roscovitine, an ATP analogue named CR8#13 (Carpio et al. 2010), inhibits Tat-activated transcription by targeting specifically CDK9 (Narayanan et al. 2012). CR8#13 displays an IC_{50} of 10 nM in TNF-α activated chronically infected OM-10.1 cells and inhibits RT activity by 90 % in PBMCs at 100 nM without displaying major toxicity.

Recently, a high-throughput docking of small molecules mimicking a Tat-peptide onto the binding pocket of CDK2 was used to find small molecules disrupting the cyclin/CDK interaction (Van Duyne et al. 2013). Upon optimization, the second-generation compound named **F07#13** inhibited viral replication by 90 % in Tat-transfected HLM-1 cells (HLM-1 carries a provirus with a mutation in Tat) with an IC_{50} of 0.12 μM, without affecting basal transcription (Fig. 3). Given the close homology of the interface of cyclin/CDK interaction sites between CDK2 and CDK9, Van Duyne et al. tested this compound on the CDK9/cyclin T1/Tat interface pocket. F07#13 specifically inhibits HIV-1-activated transcription with preference toward targeting the complex when associated with Tat. Furthermore, HIV-1 infection in humanized mice was partially inhibited during a two-week treatment period without apparent toxicity (Van Duyne et al. 2013).

Of note, the phosphinic acid #93 was shown to be the most specific ATP-competitive inhibitor of CDK9/cyclin T1 kinase activity with an IC_{50} of 142 nM (Nemeth et al. 2014). This compound appeared to have some antiviral activity in one HIV-1 proliferation assay in MT4 cells even though further validation tests should be performed to ascertain efficacy against HIV in more relevant HIV-infected models.

The ability of compounds to distinguish between the different P-TEFb complexes (Narayanan et al. 2012; Ramakrishnan et al. 2012; Sedore et al. 2007) would favor specificity of HIV inhibition and limit off-target effects on cellular gene expression.

5.2 Cyclin T1

Cyclin T1 and cyclin T1/Tat interaction have also been sought after as potential targets to inhibit HIV transcription. Cyclin T1 interacts with the loop of TAR and with Tat through a critically conserved cysteine (Bieniasz et al. 1998; Garber et al. 1998).

Several non-small-molecule inhibitors have been explored such as cyclin T1 intrabodies (Bai et al. 2003), microRNA-198 (Sung and Rice 2009), cyclin T1-dominant negative mutants (Jadlowsky et al. 2008a, b), and the overexpression of HEXIM1/2 (Fraldi et al. 2005). Recently, an in silico screening targeting the Tat/TAR RNA recognition motif of cyclin T1 identified the compound **C3**, which inhibits association of cyclin T1/Tat, as well as Tat-mediated LTR transcription in a reporter assay (Fig. 3) (Hamasaki et al. 2013). C3 also inhibits HIV reactivation from TNF-α activated OM-10.1 and U1 cells at high nanomolar IC_{50} and with a $CC_{50} > 10$ μM.

The compounds described above such as dCA, triptolide, the WM5 derivatives, F07#13 or C3 targeting either Tat, TAR, P-TEFb represent great steps forward in the quest for efficacious HIV transcription inhibitors of integrated provirus in latently infected cells.

6 Inhibition of Extracellular Tat

Small molecules that inhibit extracellular Tat are scarce. Of note, the small-molecule inhibitor of Tat-dependent transcription 15d-PGJ_2 (see Sect. 4.2 and Fig. 3), was shown to inhibit the inflammatory response by blocking Tat-induced MCP-1 production in the hippocampus (Kim et al. 2012). Several non-small molecules were reported to bind extracellular Tat and inhibit its related functions in HAND. Sulfated polymannuroguluronate (SPMG), a sulfated polysaccharide was shown to bind the Tat basic domain (Hui et al. 2006), a triad of basic amino-acids in Tat (K12, K41, R78) (Wu et al. 2011) as well as the CD4 receptor (Miao et al. 2004). This molecule was reported to have several positive effects on HAND. It inhibited T-lymphocyte apoptosis by combating oxidative damage of mitochondria (Miao et al. 2005) and blocked Tat-induced neuronal cell death implicated in HIV-associated dementia (Hui et al. 2008). SPMG also inhibits HIV-1 Tat-induced angiogenesis in Kaposi's sarcoma (Lu et al. 2007).

It is known that heparin sulfate proteoglycan acts as a receptor of extracellular Tat to help internalization. Rusnati et al. (1998, 2001) have shown that polyanionic heparin-like compounds are able to block extracellular Tat uptake by interacting with its basic domain. For instance, polysulfonated distamycin A derivatives (PNU145156E and PNU153429) were found to interact and sequester extracellular Tat in the extracellular space as well as to inhibit intracellular Tat when these compounds were introduced by lipofection into the cells (Corallini et al. 1998). These compounds blocked Tat-induced neoangiogenesis in T53c14 cells (Corallini et al. 1998) and delay Tat-induced tumor growth and neovascularization in Kaposi's sarcoma-like tumor model (Possati et al. 1999).

Recently, our laboratory has shown that the Tat inhibitor dCA was able to block extracellular Tat uptake in microglia-like and astrocyte cell line models by 40 and 45 %, respectively (Mediouni et al. 2015). In addition, in the astrocytic cell line U87MG, the Tat-mediated release of the key inflammatory signaling proteins IL-1β, TNF-α, and MCP-1 was reverted by dCA treatment. Finally, using a mouse model that specifically expresses Tat in astrocytes, we demonstrated that dCA reverses the potentiation by Tat of cocaine-mediated reward using conditioned place preference experiments (Mediouni et al. 2015).

Extracellular Tat can bind via its Arg78-Gly79-Asp80 (RGD) domain to $\alpha_v\beta_3$ integrin present in endothelial cells and induce cell proliferation, motility, and neovascularization, all processes required in cancer (Urbinati et al. 2005). SCH221153, an RGD-peptidomimetic compound, binds to Tat and prevents the interaction with $\alpha_v\beta_3$ integrin, thereby inhibiting angiogenic responses triggered by Tat in chick-embryo membrane (Urbinati et al. 2005). However, RGD domain is not present in all Tat variants limiting its wide spread use.

Given the effects of Tat not only in transcription but most likely in AIDS associated pathologies, a Tat inhibitor would be of extreme benefit and hence more efforts should be put into advancing into the clinic such a long-awaited compound.

7 Conclusions

Despite two decades of research and the discovery of numerous compounds targeting Tat, TAR, P-TEFb, or their respective interactions, no small-molecule inhibitor has yet reached the clinic. The reason for this discrepancy may come from the methods employed to screen for Tat-dependent transcription inhibitors. Historically, the LTR-reporter assay with co-expression of Tat was widely used, but this type of assay leaves room for the identification of non-Tat specific inhibitors, eventually leading to cellular toxicity. So far, only two compounds have entered clinical trials: a benzodiazepine derivative, Ro 24-7429, developed by Roche in the 90s, and triptolide, a natural compound extracted from a vine used in traditional Chinese medicine. Ro 24-7429 was first evaluated, but side effects in the nervous system and absence of activity in patients cut these efforts short (Haubrich et al. 1995; Hsu et al. 1993). Currently, triptolide is in phase III and is being tested alongside ARVs to measure its impact in the HIV reservoir of naïve-ART patients in acute phase (NCT02219672).

Nevertheless, given the crucial role of Tat, TAR, and P-TEFb in viral transcription, these remain outstanding targets for the discovery of new small-molecule inhibitors of HIV replication and more so in the current context of treating latent HIV disease. A transcription inhibitor would be an exceptional addition to the current arsenal of ARVs as it would block viral reactivation from latently infected $CD4^+$T cells, reduce low-ongoing viral replication from viral sanctuaries, and prevent reservoir replenishment.

A transcription inhibitor could potentially control HIV reactivation from latency even in the absence of ART by establishing a state of deep-latency, which would be refractory to viral reactivation when ART is discontinued (Fig. 4). One could also speculate that such inhibitors would accelerate the rate of clearance of latently infected cells by reducing replication and replenishment of the latent reservoir. Thus, the latent pool of cells in an infected individual would be stabilized and death of the long-lived infected memory T cell would result in a continuous decay of this pool over time, possibly culminating in the long-awaited sterilizing cure.

Among the recently identified HIV transcriptional inhibitors, dCA, an analogue of a natural compound isolated from a marine sponge, is one of the most promising. dCA binds directly to Tat and inhibits HIV transcription in a Tat-dependent manner in both acutely and chronically infected cells in the nanomolar range with no associated toxicity. Treatment of

chronically infected cells with dCA resulted in a 2-log reduction in mRNA levels. More importantly, arrest of the treatment did not result in viral rebound upon drug removal for the duration of the experiment (one month) (Mousseau et al. 2012). In addition, dCA inhibits spontaneous viral release from latently infected $CD4^+$T cells isolated from aviremic patients undergoing ART. As such, dCA might correspond to the type of the transcription inhibitor that would control reactivation and reservoir replenishment with the elusive goal of a cure.

Adding to the benefits, a Tat inhibitor targeting either the Tat basic domain or the cys-rich domain may directly impact the appearance of neurotoxicity and development of HAND as well as reduce Tat cancer-causing properties (Fig. 4). The addition of a transcription inhibitor to ART could be of immense value to control viral reactivation and decrease the viral reservoir, a key limitation in the current multi-therapy treatment.

Abbreviations

ART	Antiretroviral therapy
HIV	Human immunodeficiency virus
ARVs	Antiretrovirals
LTR	5′ long-terminal repeat
RNAPII	RNA polymerase II
TAR	Transactivation response element
PCAF	p300/CBP-associated factor
P-TEFb	Positive transcription elongation factor b
CDK9	Cyclin-dependent kinase 9
CTD	C-terminal domain
HDAC	Histone deacetylase
HAT	Histone acetyl transferase
HAND	HIV-associated neurocognitive disorders
BBB	Blood–brain barrier
MCP-1	Chemoattractant protein-1
PBMC	Peripheral blood mononuclear cell
TI	Therapeutic index
IC_{50}	Half-maximal inhibitory concentration
NMR	Nuclear magnetic resonance
MAE	Michael acceptor electrophile
CC_{50}	Half-maximal cytotoxic concentration
dCA	didehydro-cortistatin A

RT	Reverse transcriptase
SPMG	Sulfated polymannuroguluronate
AIDS	Acquired immune deficiency syndrome

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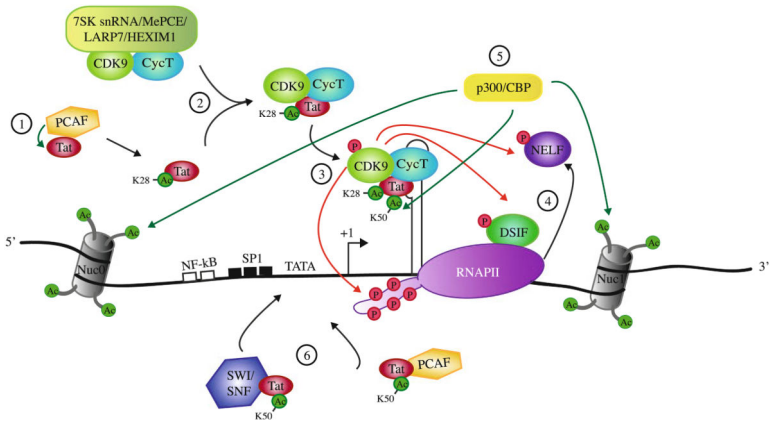

Fig. 1.

HIV-1 transcription elongation. *1* Upon Tat acetylation on Lys28 by PCAF, *2* Tat recruits P-TEFb (CDK9/cyclin T1) from a large inactive complex with 7SK snRNA/MePCE/LARP7/HEXIM1. *3* Tat/P-TEFb complex binds to TAR. CDK9 phosphorylates Ser2 of the RNAPII CTD, stalled shortly after transcription initiation. *4* CDK9 phosphorylates the negative elongation factor NELF, which is released from RNAPII, and DSIF that becomes a positive transcription elongation factor. *5* Tat is acetylated at Lys50 by p300/CBP, resulting in the release of the Tat/P-TEFb complex from TAR. p300/CBP acetylates Nuc0 and Nuc1 allowing for chromatin remodeling. *6* Tat recruits to the initiation start site SWI/SNF, PCAF, and additional factors not depicted here to further promote transcription. *Green arrow* acetylation; *red arrow* phosphorylation

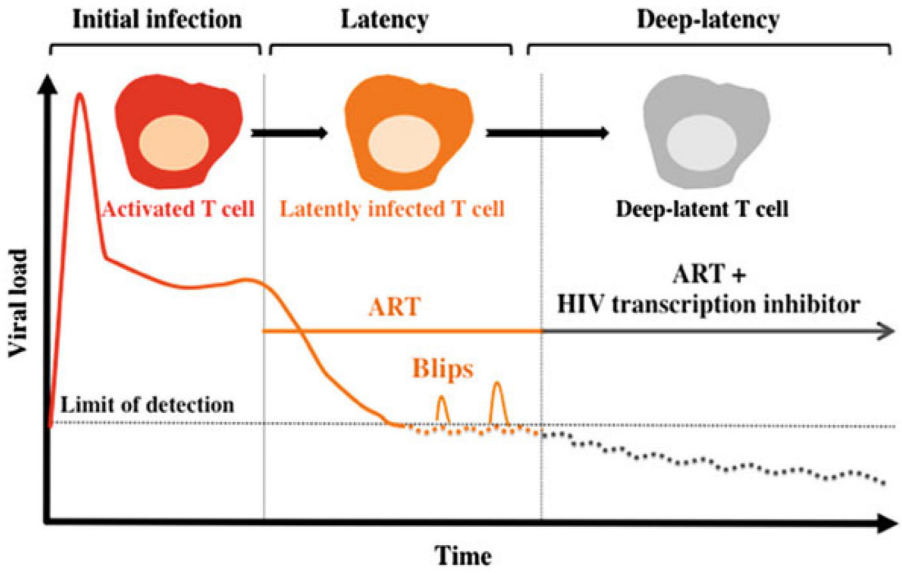

Fig. 2.

Hypothetically, a transcription inhibitor could promote deep-latency and block reactivation. High levels of circulating virus are observed in the blood of infected individuals upon HIV infection. Upon ART initiation, viral mRNA is reduced to below 50 copies/ml and memory $CD4^+$T cells remain latently infected. If ART is halted, there is an immediate rebound of virus production that correlates with $CD4^+$T-cell reactivation. Combining a transcription inhibitor to ART could induce a state of deep-latency that would lead to the reduction in size of the viral reservoir

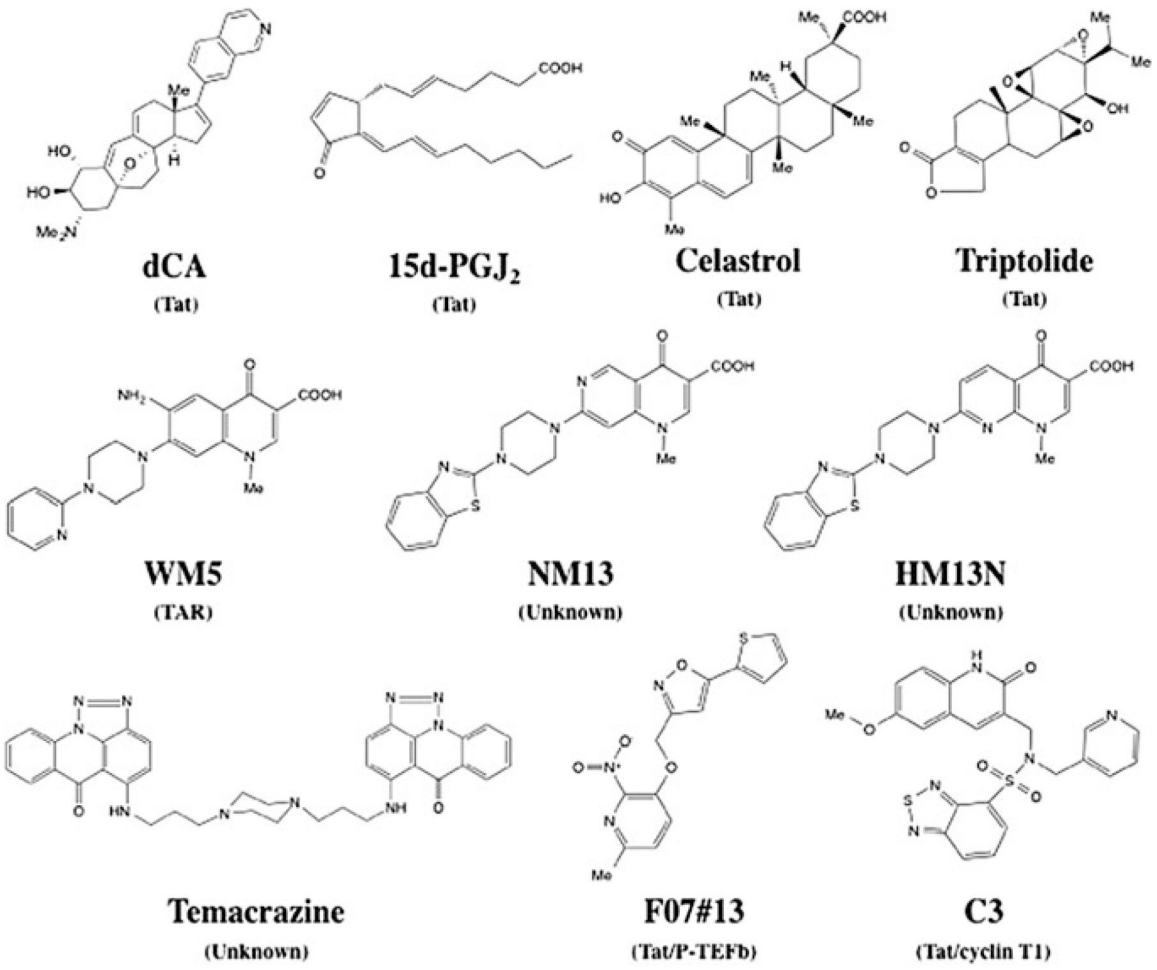

Fig. 3.

Structure of selected HIV transcription inhibitors. *Brackets* target of the compound

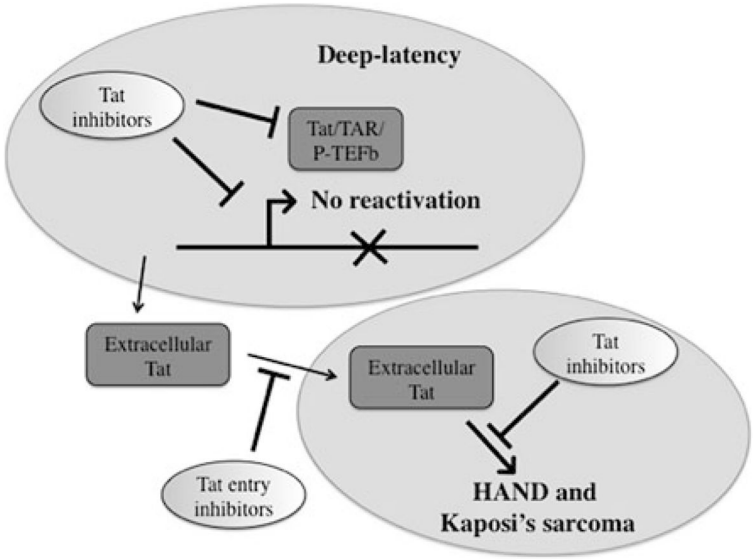

Fig. 4.

Multiple targets of a Tat inhibitor. The primary role of a Tat inhibitor is to block HIV transcription to promote a state of deep-latency and inhibit HIV reactivation. A Tat inhibitor might also reduce uptake of extracellular Tat by blocking its interaction with cellular receptors. Finally, a Tat inhibitor might impact the effect of extracellular Tat in the induction of pathways resulting in HAND or Kaposi's sarcoma