

Protein Phosphatase-1 as a New Target for Anti-HIV-1 Therapeutics



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ABSTRACT

Background:

- Our recent studies indicate that CDK2 and protein phosphatase-1 (PP1) regulate HIV-1 transcription
- Inhibition of CDK2 prevents association of CDK9 with cyclin T1
- HIV-1 Tat binds to PP1 through the Tat's Q²⁵VCF³⁸ sequence and translocates it to the nucleus.
- Disruption of Tat-PP1 interaction inhibits HIV-1 transcription
- RVxF-accommodating cleft of PP1 was selected as targeted binding site
- We analyzed the molecular mechanisms of regulation of HIV-1 transcription by PP1 and CDK2
- Developed as a proof-of-principle small molecular inhibitors of PP1

Methods:

- Phosphorylation and dephosphorylation of CDK9 by CDK2 and PP1 was analyzed
- PP1 inhibitory peptide was expressed in place of nef and HIV-1 replication was analyzed
- Virtual screening of PP1 inhibitors was performed against PP1 crystal structure using QXP docking program and library of Enamine compounds
- A library of 282 compounds was assayed for the inhibition of HIV-1 transcription and viral replication

Results:

- CDK2 directly phosphorylates CDK9 *in vitro* and PP1 dephosphorylates the CDK2-phosphorylated CDK9
- Inhibition of PP1 reduces CDK9 activity and increases its association with 7SK RNA
- Expression of PP1 inhibitor as part of HIV-1 genome inhibited HIV-1 replication
- Screen of targeted small molecule inhibitors of PP1 resulted in identification of 1H4 compound that inhibited HIV-1 transcription and replication

Conclusions:

- Phosphorylation of CDK9 by PP1 allows dissociation of inhibitory 7SK RNA and HEXIM1 protein; then phosphorylation by CDK2 might help CDK9 to associate with cyclin T1 and regain the kinase activity
- Developed, as a proof of concept, a small molecule inhibitor of PP1 that inhibit HIV-1 transcription and replication

RESULTS

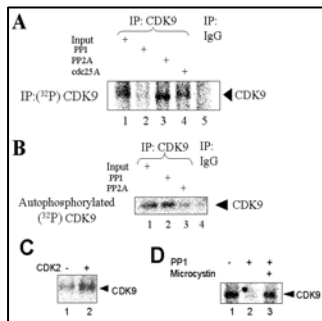


Fig. 1. CDK2 and PP1 phosphorylate and dephosphorylate CDK9. A. PP1 dephosphorylates CDK9. CDK9 was phosphorylated in 293T cells pulsed with ³²P orthophosphate in the presence of okadaic acid (8). CDK9 was immunoprecipitated from cellular lysates and subjected to dephosphorylation with PP1, PP2A or cdc25A. B. Autophosphorylated CDK9 was immunoprecipitated from 293T cells, supplemented with ³²P-ATP and allowed to autophosphorylate itself. The kinase reaction was blocked by the addition of EDTA and CDK9 was subjected to dephosphorylation by PP1 or PP2A. C. CDK2 phosphorylates CDK9. Recombinant CDK9/cyclin T1 was incubated in the presence of ³²P-ATP without (lane 1) or with recombinant CDK2/cyclin E (lanes 2). D. PP1 dephosphorylates CDK9 that was phosphorylated by CDK2. Recombinant CDK9/cyclin T1 was phosphorylated by recombinant CDK2/cyclin E with

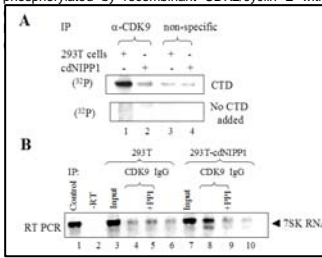


Fig. 2. A, CDK9 phosphorylation is impaired in the cells expressing cdiNPP1. CDK9 was immunoprecipitated from 293T cells or 293T cells expressing cdiNPP1 and subjected to CTD phosphorylation. B. Expression of cdiNPP1 increases CDK9 association with 7SK RNA, which can be removed by treatment with PP1. RT-PCR of 7SK associated with CDK9 precipitated from 293T (lanes 3-5) or 293T-cdiNPP1 cells (lanes 7-9). Treatment with PP1 reduces CDK9-associated 7SK RNA (lane 9). As 7SK RNA association requires T186 phosphorylation - PP1 might dephosphorylate T186 and regulate binding of 7SK RNA.

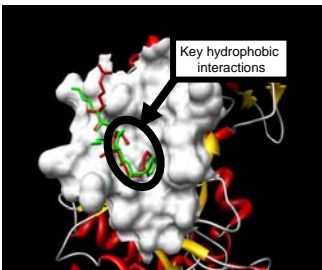


Fig. 3. Superposition of (KR)VxP peptides from Gm and MYPT1 bound to PP1. Gm's peptide (RVSF) is in red and MYPT1's peptide (KVVF) in green. Val and Phe sidechains occupies similar positions.

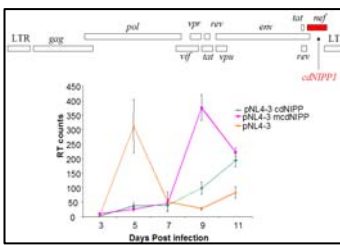


Fig. 4. Inhibition of PP1 prevents HIV-1 replication. Central domain of NIP1, wt and mutant were cloned in place of Nef in pNL4-3 vector. Recombinant HIV-1 viruses were collected from supernatant of HeLa cells transfected with corresponding pNL4-3 vectors. Equal amount of viruses (as measured by RT) were used to infect cultured T cells. Viral RT was analyzed in the supernatants at the indicated time points. Expression of WT cdiNPP1 practically blocked HIV-1 replication whereas expression of cdiNPP1 shifted viral replication. This experiment shows that inhibition of PP1 prevents HIV-1 replication.

LIBRARY DESIGN

Compound database preparation

Enamine's stock was processed according to drug-like rules with two exceptions

- 280 < MW < 550
- Rotatable bonds < 10
- Over 300 000 compounds selected

Target molecule analysis

- Tat binds to the PP1's hydrophobic groove normally occupied by an RVxF containing PP1 regulatory subunit such as NIP1-derived KNSRVTFSE peptide. The Kd difference in the binding of Tat and KNSRVTFSE peptide are at three orders of magnitude. Therefore inhibitor should fit into this Kd window
- Due to absence of actual binding pocket no use of scoring function
- Focusing on hydrophobic interactions of Phe and Val side chains of known peptides

Docking studies were performed in QXP program

| Plate | ID | IC50 in CEM-GFP cells | IC50 in 293T | Toxicity (PI) |
|----------|-----|-----------------------|--------------|---------------|
| Plate 01 | G03 | < 7 μM | > 40 μM | - |
| Plate 01 | H04 | < 10 μM | < 5 μM | - |
| Plate 01 | B07 | < 25 μM | - | - |
| Plate 01 | C07 | < 10 μM | > 10 μM | - |
| Plate 01 | G10 | < 20 μM | - | - |
| Plate 01 | D12 | < 20 μM | - | - |
| Plate 02 | D02 | < 25 μM | - | - |
| Plate 02 | C03 | < 25 μM | > 10 μM | - |
| Plate 02 | D03 | < 10 μM, but only 50% | - | - |
| Plate 02 | C03 | < 10 μM | - | - |
| Plate 02 | C04 | > 10 μM | - | - |
| Plate 02 | C05 | > 10 μM | > 10 μM | - |
| Plate 02 | B07 | < 6 μM | - | - |
| Plate 02 | E09 | < 25 μM | > 10 μM | - |
| Plate 02 | G10 | < 25 μM | - | - |
| Plate 02 | D12 | < 6 μM | - | - |
| Plate 03 | E01 | < 25 μM | - | - |
| Plate 03 | G01 | < 25 μM | - | - |
| Plate 03 | C02 | > 10 μM | - | - |
| Plate 03 | F03 | > 10 μM | - | - |
| Plate 03 | A06 | > 10 μM | > 10 μM | - |
| Plate 03 | A08 | > 10 μM | > 10 μM | - |
| Plate 03 | C08 | > 10 μM | > 10 μM | - |

Fig. 5. Screening of a targeted library. A targeted library of small molecular weight compounds (MW=500 Da) was generated from a virtual screen of Enamine's stock collection as described above. The compounds were diluted to 5 nM concentration in DMSO and added to CEM-GFP T-cells containing integrated HIV-1 LTR-GFP reporter that were infected with adenovirus expressing Tat, Ad-Tat. The initial screen was performed at 25 μM concentration of compounds in 96-well plates. HIV-1 transcription was measured as gain of GFP fluorescence. The inhibition is shown in the Figure as percent of untreated control. Toxicity was analyzed by trypan blue exclusion assay. After the initial screening, we identified about 60 compounds that at 25 μM concentration inhibited HIV-1 transcription at least by 75%. Based on this first screen, we chose 60 compounds for the second screen. In the second screen we determined IC50s and also determined cytotoxicity by the uptake of propidium iodide. The following Table shows the compounds that were inhibitory.

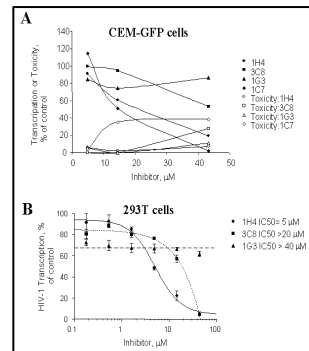


Fig. 6. Inhibition of HIV-1 transcription by small molecule mimetics of QVCF peptide. We further validated the identified compounds by analyzing them side-by-side for the inhibition of HIV-1 transcription in CEM-T cells and 293T cells. The only compounds that significantly inhibited HIV-1 transcription in transiently transfected 293T cells was 1H4. Panel A, an inhibition of HIV-1 transcription in CEM-T cells and measurements of cytotoxicity by the compounds 1H4, 3C8, 1G3 and 1C7. The compounds 1H4 and 3C8 were not toxic and inhibited HIV-1 transcription. In contrast, 1C7 compound potently inhibited HIV-1 transcription but also demonstrated a high cytotoxicity. The 1G3 compounds was neither inhibitory nor toxic. Panel B, the effect of compounds 1H4, 3C8, and 1G3 on HIV-1 transcription in 293T cells transfected with Tat-expressing vector, HIV-1 LTR-LacZ reporter and CMV-EGFP reporter.

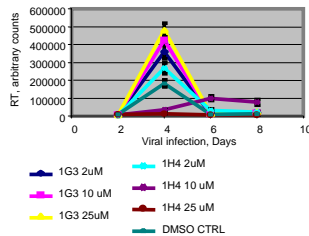


Fig. 7. Inhibition of HIV-1 replication by 1H4 compound. MT4 cells were infected with recombinant pNL4-3 HIV-1 and treated with different concentrations of 1H4 compound. The cells were also treated with 1G3 compound as a control. The 1H4 compound inhibited HIV-1 replication at 10 μM or 25 μM concentrations. The 1G3 compound did not inhibit HIV-1 replication but in contrast induced HIV-1 replication by about 2-fold. Thus, HIV-1 replication is inhibited by the 1H4 compound.

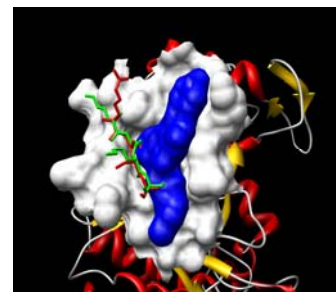


Fig. 8. Model of interaction of 1H4 and PP1. Inhibitor occupies hydrophobic sites but positions differently from RVxF peptides.

CONCLUSIONS

- We have shown that HIV-1 transcription is inhibited when interaction of HIV-1 Tat with PP1 is disrupted by a small molecule compound
- Discovered and validated compound 1H4 to be inhibitory for HIV-1

ACKNOWLEDGEMENTS

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INTRODUCTION

Our recent studies showed that host cell protein phosphatase-1 (PP1) is required for HIV-1 transcription (1). Inhibition of PP1 in cultured cells inhibited Tat-induced HIV-1 transcription and viral replication. Tat targets PP1 to the nucleus through the binding of HIV-1 Tat's Q²⁵VCF³⁸ sequence to the RVxF-accommodating cleft of PP1. We also recently showed that CDK2 phosphorylates Tat during viral replication (2) and that inhibition of CDK2 activity by iron chelators inhibited CDK9 activity and HIV-1 transcription (3). Here we show that CDK2 and PP1 phosphorylate and dephosphorylate CDK9 and designed small molecular inhibitors to disrupt the

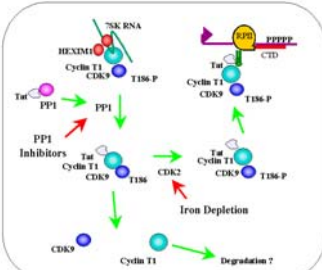


Fig. 1. Model of PP1-mediated regulation of HIV-1 transcription. Dynamic association of Tat's Q²⁵VCF³⁸ sequence with PP1's RVxF-accommodating cleft helps Tat to bind PP1 and translocates it to the nucleus. Nuclear PP1 dephosphorylates CDK9 on Thr186 and allows dissociates from 7SK RNA and HEXIM1 protein. Then CDK9 undergoes phosphorylation by CDK2 to regain the enzymatic activity. Tat recruits reactivated CDK9/cyclin T1 to HIV-1 TAR RNA where CDK9 phosphorylates RNA polymerase II C-terminal domain (CTD) and induces HIV-1 transcription.

References

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