Novel strategy to inhibit HIV by targeting integrase/INI1 interaction based on the TAR RNA mimicry

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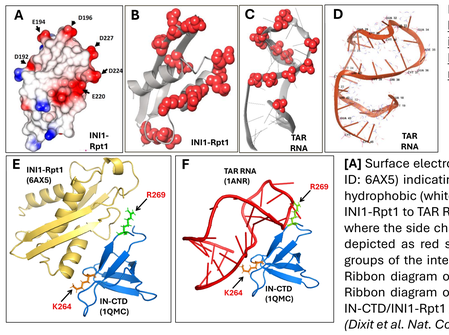
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Background

Targeting host-virus interactions is an attractive strategy to overcome the problem of HIV-1 drug resistance. INI1/SMARCB1 is an HIV-1 integrase (IN)-interacting host factor required for assembly and particle production. Studies from our laboratory has indicated that IN binding Rpt1 domain of INI1 (INI1-Rpt1) and viral TAR RNA structurally mimic each other **(Fig 1A)** and bind to the same surface on IN **(Fig 1B)** (***Dixit et al. Nat. Comm. 2021***). IN mutants defective for binding to INI1 are impaired for virion morphogenesis and infection. Because of the INI1-Rpt1 mimicry to TAR, we hypothesize that drugs that target IN-INI1 interaction will be "dual acting", disrupting both IN-INI1 and IN-TAR interactions, and induce particle morphogenesis defects to inhibit HIV-1 replication.



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Figure 1: TAR RNA mimicry of INI1-Rpt1 domain and molecular docking to compare INI1-Rpt1$_{183-304}$ and TAR RNA binding to IN-CTD

[A] Surface electrostatic computation of INI1$_{183-245}$ NMR structure (PDB ID: 6AX5) indicating negatively (red) and positively (blue) charged, and hydrophobic (white) residues. **[B, C]** Cartoon illustrating the similarity of INI1-Rpt1 to TAR RNA. Ribbon diagram of NMR structure of INI1 Rpt1 (B) where the side chains of all 11 negatively charged surface residues are depicted as red spheres, and that of TAR RNA (C) where phosphate groups of the interacting nucleotides are depicted as red spheres. **[D]** Ribbon diagram of NMR structure of TAR RNA (PDB ID: 1ANR). **[E, F]** Ribbon diagram of modeled complexes showing interface residues of IN-CTD/INI1-Rpt1 (E) and IN-CTD/TAR RNA (F) complexes. *(Dixit et al. Nat. Comm. 2021)*

Designing of the Stapled Peptide from INI1-Rpt1 a-Helix 1

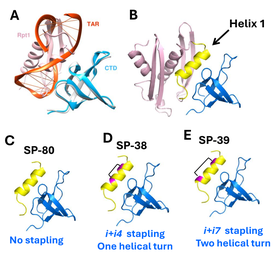
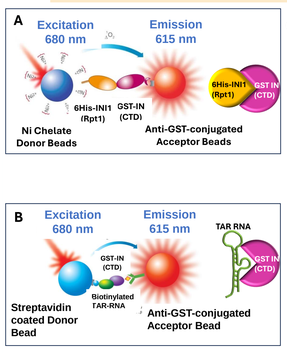


Figure 2: Designing of peptides based on structural mimicry between INI1-Rpt1 domain and TAR RNA

[A] Superimposition of the IN-CTD (blue) /INI1-Rpt1(pink) and IN-CTD (white)/TAR RNA (orange) complexes shows the identical orientation of IN-CTD and overlap of INI1-Rpt1 and TAR RNA regions in the three-dimensional space. **[B]** IN-CTD (in blue) complexed with INI1-Rpt1 (in pink). Helix 1 of INI1-Rpt1 at the interface of IN-CTD/INI1-Rpt1 complex overlaps with the IN-binding region of TAR RNA. The interface helix of INI1-Rpt1 domain is highlighted in yellow. **[C-E]** Ribbon diagrams of linear and stapled peptides designed from the α-helix 1 of INI1 Rpt1 region. Pair of stapled residues on SP38 and SP39 are respectively indicated as 'i and i+4' and 'i and i+7'.

(Dixit et al. Nat. Comm. 2021)

INI1-Rpt1 derived stapled peptides inhibit both IN-INI1 and IN-RNA interactions



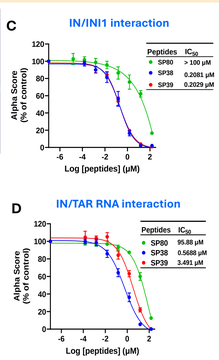


Figure 3: Alpha assay to determine the effect of Stapled peptides to inhibit IN/INI1 and IN/RNA interactions

[A, B] Schematic representations of the working principle of alpha assay for detecting protein-protein (A) and protein-RNA (B) interactions. **[C, D]** Graphs represent non-linear regression curve fitting of the dose-dependent effects of different peptides (SP38, SP39, and SP80) on IN-CTD/INI1$_{183-304}$ (C) and IN-CTD/TAR-RNA (D) interactions, as determined through Alpha assays. The respective IC$_{50}$ values are indicated. Stapling dramatically reduced the IC$_{50}$ value of the peptide inhibitor by several folds for IN-CTD/INI1 and IN-CTD/TAR-RNA interactions. However, the stapled peptide SP39 inhibited this interaction less efficiently than SP38 SP38 inhibited IN-CTD/INI1 and IN-CTD/TAR-RNA interactions with similar IC$_{50}$ values.

INI1-derived stapled peptide inhibits HIV-1 replication in a multiday infection assay to 5 logs

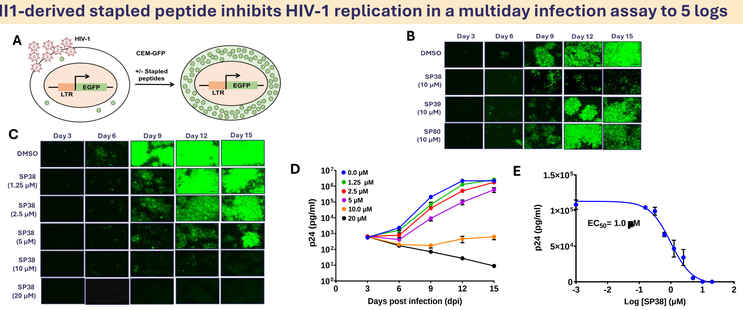


Figure 4: INI1-derived stapled peptide inhibits HIV-1 replication in a multiday infection assay

[A] Schematic representation to determine the effect of SP38 using CEM-GFP cells as reporter. CEM-GFP cells have an integrated copy of EGFP gene downstream of LTR. Upon HIV-1 infection, EGFP is expressed which is detectable by fluorescence microscopy. **[B]** Representative fluorescent images from vehicle (DMSO) and different peptide (SP38, SP39 and SP80) treated CEM-GFP cells, captured at different time-points (days 3, 6, 9, 12 and 15) to indicate the effect of peptides on multiday HIV-1$_{NL4-3}$ replication. **[C, D]** Fluorescent images of CEM-GFP cells infected with HIV-1$_{NL4-3}$ (C) and p24 levels in the culture supernatant of these cells (D) over a span of 15 days demonstrating the dose-dependent inhibition of HIV-1 replication by SP38. **[E]** Non-linear regression curve fitting analysis to determine the EC$_{50}$ of SP38 for effectively reducing HIV-1 replication to half in CEM-GFP cells. **Note that the EC$_{50}$ is 1.0 µM, which is very close to the IC$_{50}$ obtained in the biochemical assays for IN-INI1 and IN-RNA interactions.**

Method

Using structural information on INI1-Rpt1/IN-(C-terminal domain) CTD complex, hydrocarbon stapled peptides derived from interface α1 helix of INI1-Rpt1 were designed and commercially synthesized

These peptides were tested for their effects on IN-INI1, INI-RNA interactions using Alpha Assay and on HIV-1 replication using multiday infection assay in T-cell lines, PBMCs and primary macrophages.

Cell penetrability and cytotoxicity of the functional peptide were assessed through fluorescence microscopy and MTS asay, respectively.

The mechanism of action of the functional peptide was tested by assessing their effect on viral protein expression using Western blot analysis, particle production by p24 AlphLisa, effect on late events using single cycle infection assays, infectivity and particle morphogenesis, using virology and transmission electron microscopy (TEM) analyses.

Key background information:

- INI1-Rpt1 and TAR-RNA structurally mimic each other and bind to the same surface of IN.
- Both IN/INI1 and IN/TAR-RNA interactions are crucial for HIV-1 particle morphogenesis

Hypothesis:

- Inhibitors that target IN/INI1 interaction can be 'dual acting' and they can inhibit both IN/INI1 and IN/TAR-RNA interactions.
- Such inhibitors can impair particle morphogenesis and inhibit HIV-1 replication.

Main Finding:

- INI1-Rpt1 interface alpha Helix 1-derived stapled peptides inhibit both IN/INI1 and IN/TAR interactions.
- INI1-derived stapled peptides inhibit HIV-1 replication without cytotoxicity.
- The Stapled peptides selectively impair particle morphogenesis without inhibiting viral protein expression, assembly, budding and processing.
- INI1-derived Stapled peptide represent a "new-in-class" inhibitor that dually target IN/INI1 and IN/TAR RNA interactions.

INI1-derived stapled peptide SP38 penetrate the cells and has no cytotoxicity

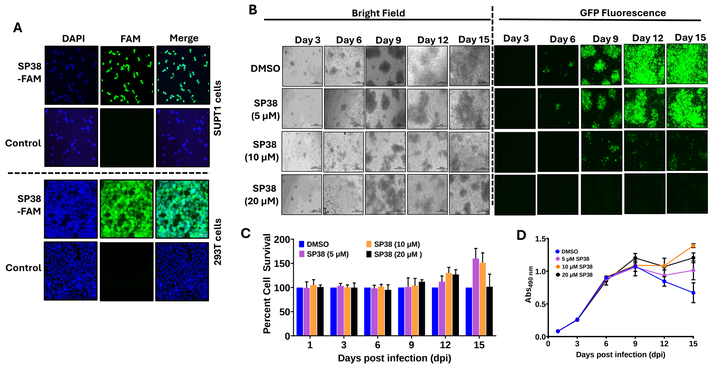


Figure 5: INI1-derived stapled peptide SP38 exhibits efficient cell penetration without causing cytotoxicity

[A] Representative images of SUPT1 and 293T cells showing cellular uptake of fluorescein amidite (FAM) conjugated SP38 (SP38-FAM) as green fluorescence. DAPI indicates the nuclear staining, and the number of cells present. **[B-D]** Compilation of representative brightfield and fluorescent images of HIV-1$_{NL4-3}$ infected CEM-GFP cells (B), and percent cell survival (C) and cell proliferation (D), determined through MTS assay, in the presence and absence of inhibitory concentrations of SP38. **These results confirm the absence of cytotoxicity by SP38 treatment at concentrations required for complete inhibition of HIV-1 replication**.

Stapled peptide inhibits HIV-1 replication in PBMCs and primary macrophages

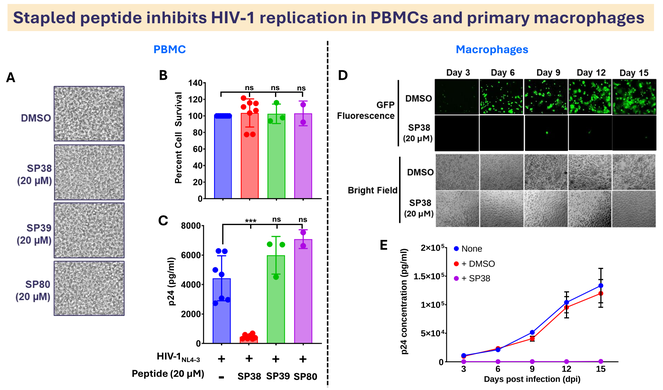


Figure 6. INI1-derived stapled peptide SP38 inhibits HIV-1 replication in PBMCs and primary macrophages without cytotoxicity

[A, B] Bright field images (A) and percent cell survival (B) of activated PBMCs infected with HIV-1$_{NL4-3}$ in the presence and absence of different peptides (SP38, SP39 and SP80) showing no cytotoxicity. **[C]** Graph representing the effect of different peptides (SP38, SP39 and SP80) on HIV-1 infection of activated PBMCs determined through p24 levels in culture supernatants of infected and non-infected cells. SP38 treatment significantly reduced HIV-1$_{NL4-3}$ replication in these PBMCs 14 days post-infection. **[D]** Fluorescent and bright field images of primary macrophages 3, 6, 9, 12 and 15 days after infection with HIV-1AD8-GFP virus in the presence and absence of SP38. Cells were either pre-treated with SP38 for 2 h before infection or co-treated during infection. **[E]** Effect of SP38 on HIV-1 infection of primary macrophages represented graphically as p24 levels in culture supernatants of infected and non-infected cells. Representative images are from one of three independent experiments. Cytotoxicity and p24 data are mean ± SE of these experiments.

Reference:

Dixit, U., Bhutoria, S., Wu, X. *et al.* INI1/SMARCB1 Rpt1 domain mimics TAR RNA in binding to integrase to facilitate HIV-1 replication. *Nat Commun* 12, 2743 (2021). https://doi.org/10.1038/s41467-021-22733-9

Funding source: NIH R21 AI156932 and R01 AI170206-01-A1 to Ganjam V. Kalpana

INI1-derived stapled peptide does not affect viral protein expression, processing and particle production

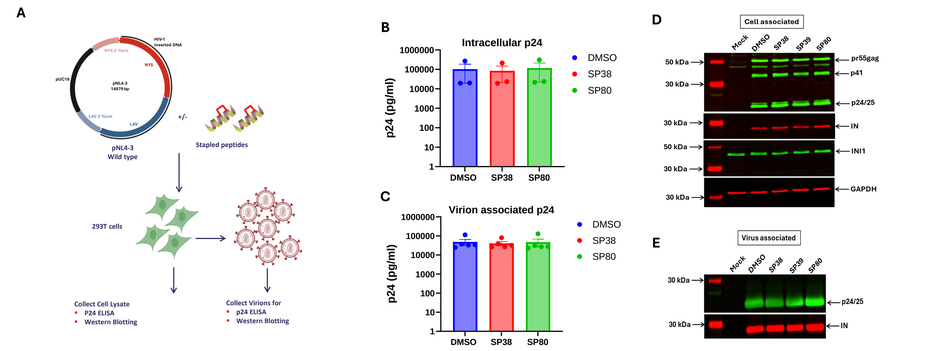


Figure 7. INI1-derived stapled peptide SP38 does not affect particle production, viral protein production, and processing

[A] Schematic representation of the strategy to investigate the effect of SP38 during late events of HIV-1 replication. Producer 293T cells were transfected with the plasmid pNL4-3, in the presence or absence of peptides (SP38 and SP80), and virions and cell lysates were collected after 24 h. Production of viral protein and release of virions were assessed through determining p24 using AlphaLISA assay of cell lysates and supernatants. Infectivity of these particles was tested through multiday infection assay in CEM-GFP cells. Effect of the peptides on particle morphogenesis was checked through transmission electron microscopy (TEM) and ability of the treated virions to undergo early events by qRT-PCR. **[B, C]** Graphs showing intracellular (lysate) (B) and virion-associated (supernatant) (C) p24 levels confirm that SP38 affected neither the viral protein expression nor particle production. **[D]** Western blots depicting the proteolytic processing of Gag polyprotein, and levels of IN and INI1 in host cells show no effect of the stapled peptide SP38 on viral protein production and processing in the host cells. The GAPDH blot confirms that equal amounts of protein in each lysate have been subjected to Western blot analysis.Graphs showing virion associated and intracellular p24 levels confirmed that the peptides affected neither the viral protein translation nor particle production.**[E]** Images of scanned western blots of p24 and IN from virus particles produced in the presence/absence of different peptides (SP38, SP39, and SP80) showed no effect on viral protein production.

INI1-derived stapled peptide make the virus particles non-infectious, malformed and defective for early events

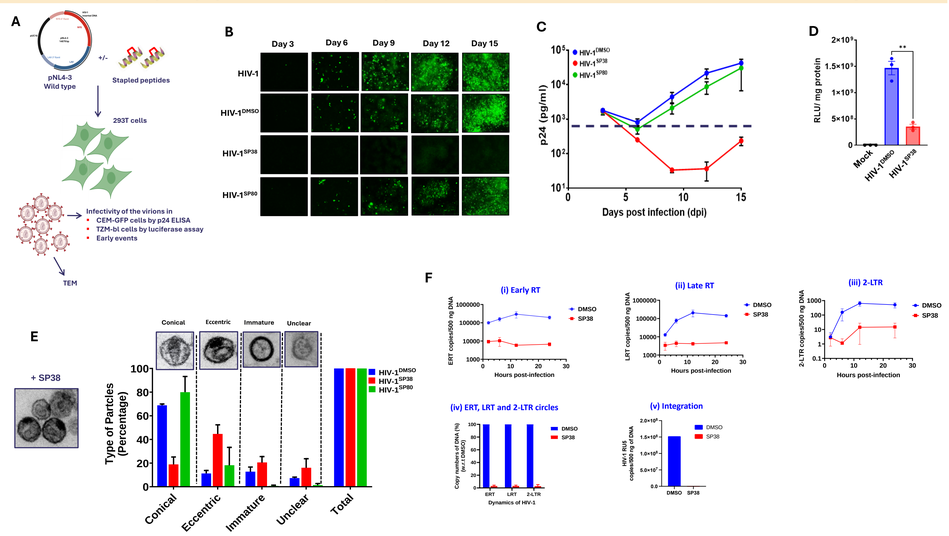


Figure 8. Virus particles produced in the presence of INI1-derived stapled peptide are defective for infection, morphology, and early events

[A] Schematic representation of the strategy to investigate the effect of SP38 during late events of HIV-1 replication. Producer 293T cells were transfected with the plasmid pNL4-3, in the presence or absence of peptides (SP38 and SP80), culture supernatants containing the produced virions were collected after 24 h, tested for infectivity and early events, and checked through TEM for morphological anomaly(ies). **[B]** Fluorescent images of CEM-GFP cells after 3, 6, 9, 12 and 15 days of infection with virions produced in the presence and absence of stapled (HIV-1^{SP38}) and linear (HIV-1^{SP80}) peptides showing significantly reduced infectivity of HIV-1^{SP38} particles. **[C]** Analysis of the p24 levels in the supernatant of CEM-GFP cells infected with HIV-1^{SP38} and HIV-1^{SP80} virions over 15 days shows decreased p24 levels corroborating with reduced infectivity of HIV-1^{SP38} particles. **[D]** Reduced infectivity of HIV-1^{SP38} particles is further confirmed through luciferase assay using the TZM-bl reporter cells. Representative images are from one of three independent experiments. p24 and luciferase data are mean ± SE of three independent experiments. **[E]** TEM analysis of HIV-1^{SP38} virions showed the particles to be predominantly eccentric. **[F]** Graphical representation of different particle morphologies in the virus pools produced in the presence of DMSO (HIV-1^{DMSO}), stapled peptide SP38 (HIV-1^{SP38}) and linear peptide SP80 (HIV-1^{SP80}). The data represented is mean ± SE of at least 200 particles counted from each population and confirms the predominance of eccentric particles in the HIV-1^{SP38} pool. **[F]** SP38 treatment significantly reduced the copies of early reverse transcripts (ERTs) (i), late reverse transcripts (LRTs) (ii), and 2-LTRs (iii), particularly, by 12 h post-infection (iv) and also inhibited integration, as determined by Alu-Gag PCR (v).

Conclusions

- SP38, a stapled peptide derived from INI1 α1 helix inhibits both IN/INI1 and IN/RNA interactions *in vitro* and in cells.
- SP38 inhibits HIV-1 replication in T-cells, PBMCs and primary macrophages.
- SP38 does not inhibit HIV-1 assembly, budding and particle production.
- SP38 selectively inhibits particle morphogenesis, consistent with our hypothesis.
- SP38 is a prototype for a novel class of potential anti-HIV-1 drug candidates.
- SP38 also holds promise in "shock and kill" therapy.