Peptides Mimicking the β7/β8 loop of HIV-1 Reverse Transcriptase p51 as "Hotspot-Targeted" Dimerization Inhibitors

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KEYWORDS: constrained peptides, dimerization hotspot, dimerization inhibitors, HIV reverse transcriptase.

ABSTRACT: A conformationally constrained short peptide designed to target a protein-protein interaction hotspot in HIV-1 reverse transcriptase (RT) disrupts p66-p51 interactions and paves the way to the development of novel RT dimerization inhibitors.

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) plays a crucial role in the life cycle of HIV-1 and is a key target for antiretroviral therapy.[1] The majority of currently approved anti-HIV-1 RT drugs interfere with the DNA polymerase activity of the enzyme. However, drug resistance and other undesired side effects highlight the need to identify novel classes of inhibitors that interact with this enzyme by alternative mechanisms.[1-3]

The mature HIV-1 RT is a heterodimer composed of 66- and 51-kDa subunits, designated as p66 and p51, respectively (Figure 1).[4,5] Although both subunits bind to the nucleic acid substrate, the p66 subunit possesses both DNA polymerase and RNase H activities whereas the p51 subunit provides structural support to p66 by stabilizing the dimer interface and promoting its loading onto the template primer.[4-9]

Since HIV-1 RT is an obligate dimer,[9,10] inhibition of the dimerization process constitutes an alternative approach to conventional RT inhibitors that compete with the incoming nucleotide substrate, prevent DNA chain elongation, or induce a detrimental conformational change in the enzyme active site. Disrupting the interactions that stabilize the p66/p51 dimer is very challenging and although several RT dimerization inhibitors have been reported[9,11,12] this type of inhibitors has not reached the clinic yet. Representative examples include short peptides mimicking the major contact areas of different interfacial regions of the p66/p51 heterodimer such as the 19-mer of sequence FKLPIQKETWETWWTEYWQ and its truncated 10-mer variant of sequence KETWETWWTE. These synthetic tryptophan-rich peptides derived from the tryptophan repeat motif in the connection subdomain of p66 between positions 384-407 of p66 were found to be HIV-1 RT inhibitors.[11,13] Although the 19-mer was shown to inhibit the association of RT subunits *in vitro*, it was unable to promote dissociation of the heterodimer.[11] The 10-mer proved to be more effective as an RT dimerization inhibitor[13] and it was proposed that it interacts in a cleft between the fingers and the connection subdomains of p51 thereby destabilizing the dimer conformation and triggering dissociation.[12] Prof. Botta's team reported some small molecules that inhibited, in a dose-dependent manner, the HIV-1 RT dimerization process by reducing the association of p66 and p51 subunits.[14,15] These molecules, of which **MAS0** (Figure 1) is considered the prototype, were obtained by a virtual screening approach in the p66 binding pocket defined by Trp402 and Trp410, followed by optimization.[14,15] **MAS0** and analogues inhibited the RNase H and polymerase activities of the enzyme.

The RNase H domain of p66 and the p51 thumb subdomain make up a second dimerization interface that plays an important role in the maturation of the enzyme. A peptide containing the thumb subdomain residues 284-300 (RGTKALTEVIPLTEEAE) was found to inhibit the RT's DNA polymerase activity while preventing RT maturation and blocking HIV replication in cell culture.[16] A third important p66/p51 interacting region involves a β-hairpin loop in the fingers subdomain of p51 that is flanked by Pro133 and Pro140.[17,18] This so-called β7-β8 loop interacts with the palm domain of p66 and contributes to the stability of p51/p66 heterodimer and to the catalytic function of p66.[19]

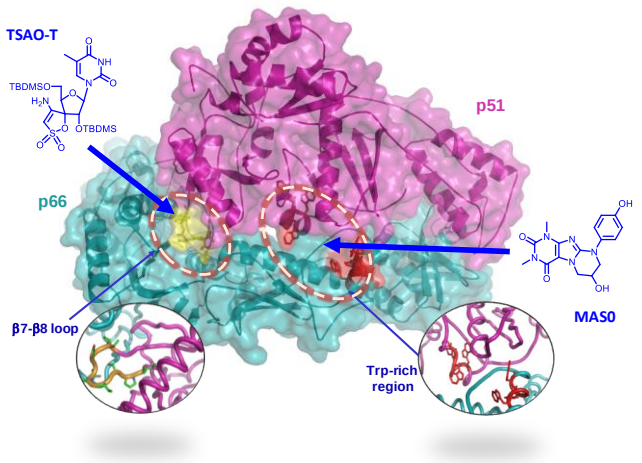

Figure 1. Three-dimensional crystal structure of HIV-1 RT heterodimer and spatial location of the β7-β8 loop of the p51 subunit in the fingers subdomain and of the Trp repeat motif in the connection subdomain of the p66 subunit. Interaction sites for **TSAO-T** and **MAS0** small-molecule dimerization inhibitors.

The loop is also part of the "floor" of the non-nucleoside RT inhibitors (NNRTIs) binding pocket.[8,10]

A few years ago we reported on some small molecules that interact at the p51 subunit and destabilize the HIV-1 RT p66/p51 interaction.[17,20,21] In particular, TSAO-T (the prototype of a unique family of allosteric NNRTIs)[17,20,21] was shown to destabilize the heterodimeric HIV-1 RT and decrease its ability to bind DNA (Figure 1).[22] Therefore, TSAO-T was the first example of a small non-peptide molecule that inhibits the dimerization process of the enzyme upon transient binding to the β7-β8 of the p51 subunit.[23-26] On the basis of results from computational studies it was proposed that TSAO molecules bind at the p66/p51 dimer interface, making extensive use of the β7-β8 loop of the p51 subunit.[26] In fact, a key interaction for TSAO molecules binding to HIV-1 RT involves Glu138 in the β7-β8 loop[27] and TSAO derivatives consistently select for a E138K resistance mutation in HIV-1 RT, hence this loop was identified as a "hot spot" for enzyme dimerization.[26] In addition, the crystal structure of the HIV-1 RT/TSAO-T complex showed a hyperexpansion of the NNRTI-binding pocket (NNIBP) and a significant rearrangement of RT subdomains.[28] Consequently, taking together the crystal structure, molecular modeling, resistance data and biochemical results, it seems reasonable to assume that Glu138 and surrounding residues in the β7-β8 loop of p51 may form a transient site for binding of TSAO inhibitors prior to their final docking inside the NNIBP.

A segment of the β7-β8 loop has a sequence (SINNETPG) that is unique for HIV-1 RT. Amino acids of this loop in p51 (Ile135, Asn136, Asn137, Glu138 and Pro140) make contact with the p66 subunit (including P95) and contribute significantly to the dimerization binding energy[13,15,17] (Figure 2). Four of these residues (Asn136, Asn137, Glu138, and Thr139) establish several hydrogen bonding interactions with amino acids in the p66 subunit[19] while Asn136 plays a key role in heterodimer stability.[29] Due to the dual donor/acceptor character for hydrogen bonding of the carboxamide side chain of Asn136 this amino acid can make two hydrogen bonds to p66 backbone CO and NH groups of His96. Moreover, the backbone of p51 Asn136 also interacts through hydrogen bonds with Thr181 and Val381 of the p66 subunit. On the other hand, Glu138 (key residue for TSAO molecules) interacts with the side chains of Tyr181 and Lys103 of the p66 subunit, thus contributing to the heterodimer's stability.[21,26] Nonconservative substitutions of those amino acids[29-32] must have a large impact on the RT heterodimer interface because they often render catalytically inactive enzymes.[27-29] Alanine-scanning mutagenesis of Ser134, Ile135, Asn136, Asn137, Thr139 and Pro149 residues[31] corroborated the pivotal role of Asn136 in HIV-1 RT heterodimer stability and function.[30-32]

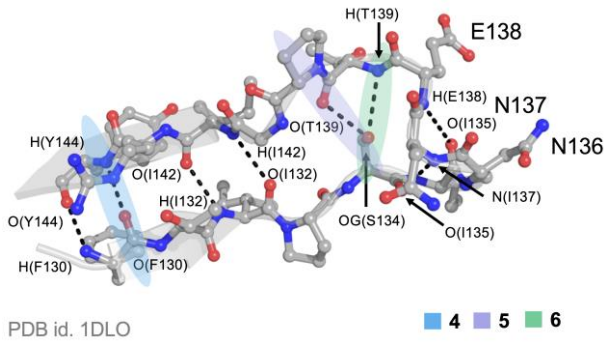

Figure 2. Relevant intramolecular hydrogen bonds that help to maintain the secondary structure of the p51 β7/β8 loop in the p66-p51 heterodimer. The H-bonds surrogated by covalent bonds in the designed peptides **4**, **5** and **6** are highlighted.

Collectively, the published data summarized above strongly suggest that the β7-β8 loop of the p51 subunit of HIV-1 RT plays a key role in enzyme dimerization and could be a novel and attractive pharmacological target for RT inhibition. Our previous models and site-directed mutagenesis results[19-22] inspired the design of several novel constrained "minimalistic" peptides containing part of the β7-β8 loop sequence (Chart 1). We herein report their synthesis, NMR and molecular dynamics conformational studies, and biological evaluation. Our results provide proof of concept that this largely unexplored region of the HIV-1 RT interface is a druggable target thereby supporting further studies that can lead to novel classes of RT dimerization inhibitors.

The rational design of synthetic peptides derived from the p51 β7/β8 loop of the native protein was based on two general considerations: (1) the conformational features of the β-turn region and the residues important for dimerization should be conserved and (2) the backbone flexibility of the peptides should be minimized to maintain and stabilize the bioactive conformation. To this end, the three-dimensional structure of HIV-1 RT[19] provided important information for the design of peptides interfering with the dimerization process of this enzyme. A detailed analysis of many crystallographic structures revealed the presence of two consecutive β-turns in the central INNE sequence of the β-hairpin and up to eight intramolecular hydrogen bonds within the 130-144 stretch (Figure 2). The two consecutive β-turns appear structured by virtue of hydrogen bonds between the hydroxyl group of the side-chain of Ser134 and the backbone oxygen of Thr139 and also between the backbone carbonyl oxygen of Ser134 and the backbone NH group of Asn137.

Since the double β-turn should be preserved in the novel designed peptides, we focused on the synthesis of restrained peptides based on the linear peptide **1** (Chart 1). Linear peptides are known to be flexible and proteolytically unstable. In contrast, conformationally restrained free peptides usually overcome these limitations in solution.[33,34] Two strategies were followed to introduce those restraints in their conformation. First, the turn-forming ^{D}Pro-^{L}Pro template[35] was introduced at the sequence termini to link Phe131 and Tyr146 and produce the cyclic peptide **2** (Chart 1). The 17-mer **2** was synthesized by solid phase peptide chemistry, followed by macrocyclization, deprotection and cleavage (see Supporting Information). The second strategy involved the introduction of disulfide linkages as surrogates of the three hydrogen bonds highlighted in Figure 2. Thus, the disulfide-containing cyclic 13-mer **4** was prepared by introducing cysteine residues at the two ends of the linear peptide (**3**, IPSINNETPGI) corresponding to residues 130-144 (Chart 1). The disulfide bond in **4** thus acts as a surrogate of the hydrogen bond formed between the carbonyl oxygen of Phe130 and the amide nitrogen of Tyr144 (O=C(F130)− HN(Y144), Figure 2).

Chart 1. Sequences of the designed peptides derived from the β7-β8 loop of the p51 subunit of HIV-1 RT.

1 (linear) FTIPSINNETPGIRY

2 (cyclic) ^{L}PFTIPSIN
N
^{D}PYRIGPTE

3 (linear) IPSINNETPGI

4 (cyclic) CIPSINNETPGIC

5 (cyclic) CSINNETPC

6 (cyclic) CINNEC

Preliminary testing revealed that only the disulfide-containing cyclic peptide **4** showed some inhibition of HIV-1 RT polymerase activity whereas the ^{D}Pro-^{L}Pro-containing peptide **2** and the linear peptides **1** and **3** were inactive. In light of these findings, and with the aim of determining the minimal sequence required for biological activity, we also designed and studied some truncated disulfide-containing cyclic peptides. Our strategy for further optimization involved the replacement of the hydrogen bond established between the side chain of Ser134 with either the carbonyl oxygen or the amide proton of Thr139 (Oγ(S134)-O=C(T139) or Oγ(S134)-HN(T139) respectively, Figure 2). Thus, the 13-mer was truncated to yield the cyclic 9-mer **5** (CSINNETPC) and the cyclic 6-mer **6** (CINNEC) (Chart 1) which could better mimic the bioactive conformation observed for the β7/β8 loop in the crystallographic studies.

It has been reported that by using differential scanning fluorimetry (DSF) it is possible to determine whether an RT inhibitor is a destabilizer or stabilizer of the p51/p66 heterodimer by the decrease or increase, respectively, caused in the melting temperature (T_m).[36] Compounds binding close to the p51/p66 heterodimer interface have been reported to reduce the T_m of RT.[37] Thus, to determine if **1-6** were able to stabilize or destabilize the p51/p66 RT heterodimer, we studied the effects of these peptides on the thermal stability of HIV-1 RT p51/p66 heterodimer by performing DSF experiments. The results are shown in Table 1 in which **MAS4** (an analogue of the HIV-1 RT dimerization inhibitor **MAS0**)[14,15] and **TSAO-T** are included as reference compounds. As shown in Table 1, an important T_m decrease (ΔT_m = 13.6─15.8 °C) was observed in the presence of **1-6** that was much larger than that obtained with the reference compounds **MAS4** and **TSAO-T** (ΔT_m = 2.6 and 1.4 °C, respectively). Thus, at 100 μM, **1-6** destabilize the RT heterodimer in a way that is consistent with their putative binding to the heterodimer interface.

Table 1. T_m detected for the signal of ligand-bound RT and shift (ΔT_m) with respect to the apo form of the enzyme.

Ligand	T_m (°C)[a]	ΔT_m (°C)[b]
RT WT	54.6 ± 0.2	
TSAO	53.2 ± 0.3	- 1.4
MAS4	52.0 ± 0.3	- 2.6
1	40.1 ± 0.2	- 15.6
2	39.6 ± 0.3	- 15.0
3	41.0 ± 0.2	- 13.6
4	41.0 ± 0.7	- 14.5
5	38.8 ± 1.1	- 15.8
6	38.8 ± 0.5	- 15.8

[a]*Tm* (-dI/dT max). [b]Shift in HIV-1 RT melting temperature measured in the presence of ligand. *Tm* values are the average of three independent measurements.

Inhibition of HIV-1 RT dimerization was assessed by using a previously described high-throughput (HTS) screening assay based on the interaction of p66 and p51 containing a FLAG epitope and a poly-histidine tail, respectively.[25] Briefly, after immobilization of the His-p51 subunit on nickel-coated plates (HIS-Select®, Sigma) and addition of purified FLAG-p66, the extent of dimerization is estimated in an ELISA using specific primary antibodies that recognize the FLAG epitope located at the N-terminus of p66. When these assays were carried out in the presence of **3-6**, the linear peptide **3** did not inhibit RT dimerization whereas cyclic peptides **4-6** were active in the micromolar range (Table 2). The best inhibitor was the cyclic hexapeptide **6** with an IC_{50} of 37 ± 14 μM.

Table 2. Inhibition of HIV-1 RT dimerization and DNA polymerase activity by peptides derived from the β7/β8 loop.

Peptide	IC_{50} (μM)		
	Dimerization[a]	Nucleotide incorporation activity[b]	DNA polymerization (reassociation assay)[c]
1	ND	>145	ND
2	ND	>145	ND
3	>150	>145	ND
4	137 ± 34	>145	>250 (<5)
5	61 ± 15	>145	>250 (43)
6	37 ± 14	109.4 ± 30.8	203 ± 35

[a]Values were determined with the FLAG-p66/His-p51 dimerization assay.[25] [b] Single nucleotide incorporation assays were carried out with D38/25PGA, a 38/25mer DNA/DNA template primer, and wild-type HIV-1 RT. Reported values are the average ± S.D. of three independent experiments. Nevirapine was used as control in these assays and showed an IC_{50} of 0.57±0.14 μM. [c]The amount of polymerized DNA was determined after addition of [^{3}H]dTTP, using poly(rA)/oligo(dT)$_{16}$ as template-primer. The RT was pre-incubated with the corresponding inhibitor, in the presence or absence of 14% acetonitrile for 15 h at 37 ºC. Before adding the nucleotide, acetonitrile concentration was reduced to <1% to measure the amount of reassociated heterodimer through its enzymatic activity. Reported values are the average ± S.D. of three independent

experiments. Numbers within parentheses represent the percentage of inhibition at 200 μM. ND, not determined.

Although the peptides mimicking the β7/β8 loop were able to inhibit HIV-1 RT dimerization, they had little impact on the RT-associated enzymatic activities. As expected, the RNase H activity was not affected by the presence of **1**-**6** at concentrations as high as 100 μM (the highest concentration tested in these assays). However, single nucleotide incorporation assays carried out with a 38/25-mer DNA/DNA template-primer showed that **6** was able to inhibit the reaction although at relatively high concentrations (IC_{50} = 109.4 ± 30.8 μM) (Table 2). Other tested oligopeptides showed less than 5% inhibition at concentrations above 145 μM in these assays. We also found that **6** interferes with the recovery of the DNA polymerase activity after re-association of p66 and p51 in assays carried out with poly(rA)/oligo(dT)$_{16}$. Full dissociation of the HIV-1 RT heterodimer to folded monomers can be achieved in the presence of acetonitrile at concentrations in the range of 12-20%.[10] However, this process is reversible and if the concentration of acetonitrile is reduced below 1%, a significant amount of DNA polymerase activity can be recovered.

We carried out assays in which HIV-1 RT subunits were dissociated in the presence of 14% acetonitrile. After 15 h of incubation in the presence or absence of inhibitor (**4**-**6** in these assays), acetonitrile concentrations were quickly reduced to <1% and primer elongation activity was measured using [^{3}H]dTTP. Control assays were carried out in the absence of acetonitrile. While **4** had no effect on the recovery of DNA polymerase activity at concentrations as high as 250 μM, **6** showed an IC_{50} of 203 ± 35 μM (Table 2). These results are consistent with those obtained from single-nucleotide incorporation and FLAG-p66/His-p51 dimerization inhibition experiments, and therefore provide further support to our contention that the pocket in p66 harboring the β7/β8 loop of p51 is a target for RT dimerization inhibitors.

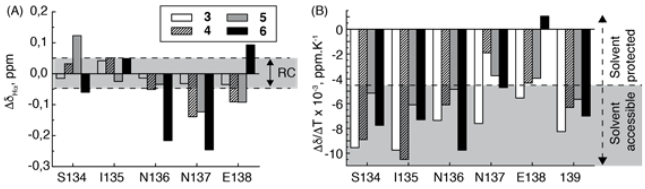

Figure 3. (A) Bar plot showing the $\Delta\delta_{H\alpha}$ ($\Delta\delta_{H\alpha}$ = $\delta_{H\alpha}^{observed}$ - $\delta_{H\alpha}^{RC}$, ppm) values for residues 134-138 for peptides **3**-**6** in aqueous solution at pH 5.5 and 5 ºC. The random coil (RC) range[38] is indicated in a grey background. (B) Bar plot showing the temperature coefficients for the amide protons of residues 134-139. Values more negative than -4.5×10^{-3} ppm·K^{-1} (grey background) indicate solvent-accessible HN protons and those less negative are indicative of solvent-protected ones. The inset indicates the bar pattern corresponding to each peptide in both panels.

All oligopeptides including **6** were also tested for their anti-HIV activity in cell culture and they were shown to be inactive at subtoxic concentrations (data not shown), most likely due to poor cellular uptake.

To explain the differences in activity found between linear and cyclic peptides, comparative conformational studies in aqueous solution using NMR and molecular dynamics (MD) simulations were carried out. Once the ^{1}H and ^{13}C chemical shifts (Figure S1–S4 and Tables ST1–ST4) of **3**-**6** in aqueous solution were assigned, NOE connectivities, ^{1}H and ^{13}C chemical shifts, and amide temperature coefficients were examined to determine the structures adopted by the peptides.[38] Since the linear peptide **3** (residues 132-142) displays ^{1}Hα, ^{13}Cα and ^{13}Cβ chemical shifts deviations (Figures 3A and S5), amide temperature coefficients (Figure 3B and Table ST5) within the random coil range ($|\Delta\delta_{H\alpha}| \leq 0.05$ ppm, ($|\Delta\delta_{C\alpha}| \leq 0.04$ ppm, $|\Delta\delta_{C\beta}| \leq 0.05$ ppm, $|\Delta\delta/\Delta T| \geq 4.5 \times 10^{-3}$ ppm·K^{-1}) and no detectable non-sequential NOE, we conclude that it is mostly a random coil in solution. In contrast, the NMR data for cyclic **4**-**6** clearly indicates that they exhibit some preferred ordered conformations. The Hα, ^{13}Cα and ^{13}Cβ chemical shift deviations for some residues of the cyclic peptides are large (Figures 3A and S5), particularly in the case of the shortest peptide **6**. The amide temperature coefficients of residues N137 and E138 in the cyclic peptides exhibit values that are below -4.5 ppm·K^{-1} (Figure 3B and Table ST5) and hence are strongly suggestive of low solvent accessibility and involvement in intramolecular hydrogen bonding, as observed in many X-ray crystal structures (e.g. PDB id. 1DLO). Furthermore, the cyclic peptides display non-sequential NOEs and dihedral angles derived from the ^{1}Hα, ^{13}Cα and ^{13}Cβ chemical shifts (Tables ST1, ST6, and ST7) that support well-defined 3D structures comparable to that of β7/β8 loop in the p51 subunit of HIV-1 RT, particularly in the case of the minimalistic cyclic peptides **4** and **6** (Figures 4 and S6).

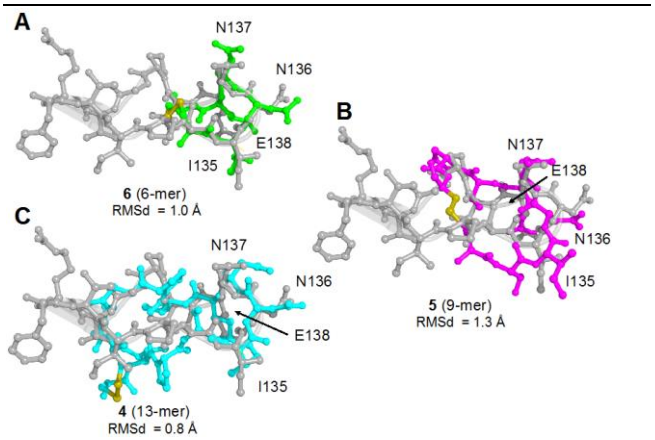

Figure 4. Superposition of the β7/β8 loop in p51 as found in a representative X-ray crystal structure of HIV-1 RT (in grey, PDB id. 1DLO) and the NMR-derived average structure of cyclic peptides (A) **6** (green), (B) **5** (magenta), and (C) **4** (cyan). Only the backbone atoms of residues 134-139 were considered. Residues I135, N136, N137, and E138 are labeled. In the cyclic peptides, the Cys disulfide linkages are shown in yellow. RMSd = root-mean-square deviation.

Three independent unrestrained MD simulations for **4–6** in explicit water, each lasting 200 ns (*i.e.* 0.6 μs per peptide), were also run in explicit aqueous solution starting from conformations derived from the β7-β8 loop of HIV-1 RT.[4] The evolution of the root-mean-squared deviation (RMSd, Å) of the central INNE amino acid stretch provided a measure of the structural stability, which showed values <0.5 Å for most of the time (Figure S7). In other words, the cyclic peptides maintain an overall conformation close to that found in the β7-β8 loop of HIV-1 RT, even in the absence of the enzyme. In all of these peptides, the S134–I135 and N137–E138 intramolecular NH–O=C hydrogen bonds are kept for almost the whole

length of the MD simulations (Figure S8), in excellent agreement with the NMR data showing strong protection of the NH(E138)–O=C(I135) from the solvent in **4** and **6**. These two peptides display ψ and ϕ angles very close to those found in the β7-β8 loop of HIV-RT p51 subunit although some residues can also temporarily visit other regions of the Ramachandran plot throughout the MD simulation time (*e.g.* E138 in **4** and **6**, or N136 in **5** (Figure S9). This is because the absence of any restraints in our MD simulations allowed the identification of other minor conformations that are only transiently visited (Figure S10). In any case, both the NMR experiments and the theoretical studies show that the central region of the three peptides can adopt a three-dimensional structure that resembles that of the p66-bound β7-β8 loop of the p51 subunit of HIV-1 RT.

Finally, by measuring some extra intramolecular hydrogen bonds beyond the INNE motif in the larger cyclic peptides **4** and **5** (Figure S11), we identified a major cluster of conformers of **5** displaying two additional intramolecular hydrogen bonds, one between Oγ(S134) and HN(T139) and another between O(T139) and HN(C133). The former is present in the native protein but the latter is novel because in the p51 subunit that amide proton is absent because Pro133 occupies that position. Interestingly, the latter hydrogen bond occurs between Oγ(S134) and HN(T139) in **4**.

Taken together, our results show that, amongst the peptides synthesized to mimic the β7-β8 loop of the p51 subunit of HIV-1 RT with the aim of inhibiting enzyme dimerization, the cyclic peptides **4**–**6** adopt structures in aqueous solution that are highly similar to that observed in the native protein and preserve some of the intramolecular hydrogen bonds present in the loop. In contrast, linear peptides **1** and **3** show random conformations in solution. *In vitro* evaluation of these peptides revealed that **6** can actually destabilize the HIV-1 RT dimer and also moderately inhibit the DNA polymerase activity of this enzyme. These results provide proof of concept that the identified hotspot at the p66-p51 interface is druggable and pave the way to the development of novel RT dimerization inhibitors.

ASSOCIATED CONTENT

Supporting Information

Synthetic and biological procedures, NMR data and molecular modeling methods. The Supporting Information is available free of charge on the ACS Publications website. (PDF).

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The manuscript was written through contributions of all authors.

ACKNOWLEDGMENT

We are immensely grateful to Prof. Emeritus Jan Balzarini (KU Leuven) for his continuous example, support and encouragement over the years. We are thankful to Prof. Antonio Jiménez's lab members at UAH for collaboration and many helpful discussions. We thank the Spanish MINECO/MEC (project SAF20015-64629-C2 to M.J.C. and F.G.) and BIO2016-76716-R (to L.M.A.) for financial support. A Fundación Ramón Areces institutional grant to CBMSO is gratefully acknowledged. P.A.S.M. thanks the University of Vienna for allowing the use of its computational facilities.

ABBREVIATION

TSAO, 2',5'-bis-*O*-(*tert*-butyldimethylsilyl)-3'-spiro-5"-(4"-amino-1",2"-oxathiole-2",2-dioxide) pyrimidine.

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Peptides Mimicking the β7/β8 loop of HIV-1 Reverse Transcriptase p51 as “Hotspot-Targeted” Dimerization Inhibitors

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Table of Content Graphic.

Conformationally constrained short peptides were designed to target a protein-protein interaction hotspot in HIV-1 reverse transcriptase (RT) to disrupt p66-p51 interactions.