A New Generation of Peptide-based Inhibitors Targeting HIV-1 Reverse Transcriptase Conformational Flexibility*

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The biologically active form of human immunodeficiency virus (HIV) type 1 reverse transcriptase (RT) is a heterodimer. The formation of RT is a two-step mechanism, including a rapid protein-protein interaction “the dimerization step,” followed by conformational changes “the maturation step,” yielding the biologically active form of the enzyme. We have previously proposed that the heterodimeric organization of RT constitutes an interesting target for the design of new inhibitors. Here, we propose a new class of RT inhibitors that targets protein-protein interactions and conformational changes involved in the maturation of heterodimeric reverse transcriptase. Based on a screen of peptides derived from the thumb domain of this enzyme, we have identified a short peptide PAW that inhibits the maturation step and blocks viral replication at subnanomolar concentrations. PAW only binds dimeric RT and stabilizes it in an inactive/non-processive conformation. From a mechanistic point of view, PAW prevents proper binding of primer/template by affecting the structural dynamics of the thumb/fingers of p66 subunit. Taken together, these results demonstrate that HIV-1 RT maturation constitutes an attractive target for AIDS chemotherapeutics.

Human immunodeficiency virus type I (HIV-1) is the primary cause of AIDS, a slow progressive and degenerative disease of the human immune system. Despite recent therapeutic developments and the introduction of highly active antiretroviral therapy, the rapid emergence of drug-resistant viruses against all approved drugs together with inaccessible latent virus reservoirs and side effects of currently used compounds have limited the efficacy of existing anti-HIV-1 therapeutics (1). Therefore, there is still an urgent need for new and safer drugs, active against resistant viral strains or directed toward novel targets in the replicative cycle, which will be useful for multiple drug combination.

HIV-1 reverse transcriptase (RT) plays an essential multifunctional role in the replication of the virus, by catalyzing the synthesis of double-stranded DNA from the single strand retroviral RNA genome (2, 3). The majority of the chemotherapeutic agents used in AIDS treatment target the polymerase activity of HIV-1 RT, such as nucleoside reverse transcriptase inhibitors (NRTI) or non-nucleoside inhibitors (NNRTI) (4). The biologically active form of RT is an asymmetric heterodimer that consists of two subunits, p66 and p51, derived from p66 by proteolytic cleavage of the C-terminal RNase H domain (2, 3, 5).

The polymerase domain of both p66 and p51 subunit can be subdivided into four common subdomains: fingers, palm, thumb, and connection (6–10). Determination of the three-dimensional structures of RTs has revealed that, although the folding of individual subdomains is similar in p66 and p51, their spatial arrangement differs markedly (11). The p66 subunit contains both polymerase and RNase H active sites. The p66-polymerase domain folds into an “open,” extended structure, forming a large active site cleft with the three catalytic residues (Asp110, Asp183, and Asp186) within the palm subdomain exposed in the nucleic acid binding site. The primer grip is responsible for the appropriate placement of the primer terminus at the polymerase active site and is involved in translocation of the primer-template (p/t) following nucleotide incorporation (12–14). In contrast, p51 predominantly plays a structural role in the RT heterodimer, by stabilizing the dimer interface thereby favoring loading of the p66 onto the p/t and maintaining the appropriate enzyme conformation during initiation of reverse transcription (15).

To propose new classes of HIV inhibitors, extensive efforts have been made in the design of molecules that target protein-protein interfaces required for viral entry, replication, and maturation (16–19). We (20, 22) and others (5, 24) have proposed that the heterodimeric organization of RT constitutes an interesting target for the design of new inhibitors. The formation of the active heterodimeric HIV-1 RT occurs in a two-step process. First a rapid association of the two subunits (dimerization step) via their connection sub-domains thereby yielding an...
inactive intermediate RT, followed by a slow conformational change of this intermediate (maturation step), generating the biologically active form of this enzyme. The maturation step involves contacts between the thumb of p51 and the RNase H of p66 as well as between the fingers of p51 with the palm of p66 (20–23). NNRTIs have been reported to interfere with RT dimerization and to modulate the overall stability of the heterodimeric RT depending on their binding site on RT (24–29). NNRTIs, including Efavirenz and Nevirapine, have been shown to promote HIV-1 RT maturation at the level of the Gag-Pol protein and to affect viral protease activation, resulting in the suppression of viral release from infected cells (30, 31). Conversely, NNRTIs such as TSAO and BBNH derivatives act as destabilizers of RT subunit interaction (27).

We have demonstrated that preventing or controlling RT dimerization constitutes an alternative strategy to block HIV proliferation and has a major impact on the viral cycle (32). In protein-protein interactions the binding energy is not evenly distributed across the dimer interface but involves specific residues “hot spots” that stabilize protein complexes. We have shown that the use of small peptides targeting hot spot residues required for RT dimerization constitutes a new strategy to inhibit HIV-1 RT (16, 17) and have described a decapeptide “Pep-7”-mimicking p66/p51 interface that prevents RT dimerization by destabilizing RT subunit interactions and that blocks viral replication (23, 32).

The thumb domain plays an important role in the catalysis and integrity of the dimeric form of RT, thereby constituting a potential target for the design of novel antiviral compounds (7, 8, 22). The p66-thumb domain is involved in p/t binding and polymerase activity of RT (7, 8, 13), and p51-thumb domain is required for the conformational changes associated with RT dimer maturation (22). We have designed a peptide, Pep-A, derived from a structural motif located between residues 284 and 300, corresponding to the end of helix 8l, the loop connecting helices 8l and 9l and a part of helix 9l. This peptide is a potent inhibitor of RT interfering with the conformational change associated with full activation of the enzyme. However, although it significantly blocks RT maturation in vitro, it lacks antiviral activity (22). In the present work, we have designed and evaluated a series of peptides derived from the thumb subdomain of RT using Pep-A as a template. We have identified a 17-residue peptide P_{AW}, which constitutes a potent inhibitor of RT-polymerase activity of HIV-1 RT in vitro. We have demonstrated that P_{AW} inhibits RT maturation and abolishes viral replication without any toxic side-effects. The characterization of the mechanism through which P_{AW} inhibits RT, combining steady-state and pre-steady-state methods, together with size-exclusion chromatography has revealed that P_{AW} only binds dimeric RT and stabilizes it in an inactive/non processive dimeric conformation that prevents the proper binding of p/t. Taken together, these results demonstrate that conformational flexibility of HIV-1 RT during maturation constitutes an attractive target for AIDS chemotherapeutics.

**EXPERIMENTAL PROCEDURES**

**Materials**—Poly(rA)-oligo(dT) and [3H]dTTP (1 μCi/μl) were purchased from Amersham Biosciences. dTTP was from Roche Molecular Biochemicals, Roche Diagnostics (Meylan, France). MF membrane (25 mm, 0.45 μm) filters for RT assay were purchased from Millipore (Molsheim, France). Primer and template oligonucleotides were from MWG Biotech AG (Ebersberg, Germany). A 19/36-mer DNA/DNA primer/template was used for steady-state fluorescence titration and stopped-flow experiments, with 5’-TCCCTGTCGCGCGCCACT-3’ for the primer strand and 5’-TGGAAAATCTTCATG-CAGTGGCCGCCGAACAGGGA-3’ for the template strand. The sequence of the template strand corresponds to the sequence of the natural primer binding site (PBS) of HIV-1 (33). The primer was labeled at the 3’-end with 6-carboxyfluorescein on thymine base. Primer and template oligodeoxynucleotides were separately resuspended in water and diluted to 100 μM in annealing buffer (25 mM Tris, pH 7.5, and 50 mM NaCl). Oligonucleotides were mixed together and heated at 95 °C for 3 min, and then cooled to room temperature for 1 h.

**Expression and Purification of HIV-1 RT Proteins**—His-tagged RTs were expressed and purified as previously described (23, 34). Briefly, M15 bacteria (Qiagen) were separately transformed with all the constructs of p51 and p66 subunits. Cells were grown at 37 °C up to ~0.3 A_{595}, then cultures were cooled to 20 °C and induced overnight with 0.5 mM isopropyl-1-thio-β-d-galactopyranoside. Bacterial cultures expressing His-tagged p66 subunit were mixed with cultures expressing the His-tagged p51 subunit to enable dimerization during sonication. For protein isolation and initial purification, the filtered supernatant was applied onto a Hi-Trap chelating column equilibrated with 50 mM sodium phosphate buffer, pH 7.8, containing 150 mM NaCl supplemented with 50 mM imidazole. The heterodimeric p66/p51 RT was eluted with an imidazole gradient and finally purified by size-exclusion chromatography on a HiLoad 16/60 Superdex 75 column equilibrated with a 50 mM Tris, pH 7.0, buffer containing 1 mM EDTA and 50 mM NaCl. Recombinant untagged HIV-1 BH42 RT was expressed in *Escherichia coli* and purified as previously described (35). Highly homogeneous preparations from co-expression of the p66 and p51 subunits were stored in −80 °C in buffer supplemented with 50% glycerol. Protein concentrations were determined at 280 nm using a molar extinction coefficient of 260 450 M⁻¹·cm⁻¹.

**Peptide Synthesis**—Pep-A-derived peptides were purchased from Gl Biochem, (Shanghai, China) and Genepep, SA (Prades le Lez, France). Pep-1 and P_{AW} were synthesized using an (fluorenylmethoxy)carbonyl (Fmoc) continuous (Pionnier, Applied Biosystems, Foster City, CA) starting from Fmoc-polyamide linker-poly(ethylene glycol)-polystyrene resin at a 0.05-mmol scale. Peptides were purified by semi-preparative reversed-phase high performance liquid chromatography (HPLC) (C18 column Interchrom UP5 WOD/25 M Uptisphere 300 5 ODB, 250 mm × 21.2 mm) and identified by electrospray mass spectrometry. P_{AW} (1 mM) was coupled to FITC using maleimide-FITC (Molecular Probes, Inc., 5 mM) through overnight incubation at 4 °C in PBS (Amersham Biosciences). Fluorescently labeled peptide was further purified by reversed-phase HPLC using a C18 reverse-phase HPLC column (Interchrom UP5 HDO/25 M Modulo-cart Uptisphere, 250 mm × 10 mm) then identified by electrospray mass spectrometry.
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RT-Polymerase Assay—RNA-dependent-DNA RT-polymerase activity was measured in a standard reaction assay using poly(rA)-(dT)₁₅ as p/t as previously described (5). Briefly, 10 μl of RT at 20 nM was incubated at 37 °C for 30 min with 20 μl of reaction buffer (50 mM Tris, pH 8.0, 80 mM KCl, 6 mM MgCl₂, 5 mM DTT, 0.15 μM poly(rA-dT), 15 μM dTTP, 0.3 μCi of [³H]dTTP). For peptide evaluation, HIV-1 RT was incubated with increasing concentrations of peptide inhibitors for 23 h, and polymerase reaction was initiated by adding reaction buffer. Reactions were stopped by precipitation of nucleic acids with 5 ml of 20% trichloroacetic acid solution for 2 h on ice, then filtered using a multiwell-sample collector (Millipore), and washed with 5% trichloroacetic acid solution. Filters were dried at 55 °C for 30 min, and radionucleotide incorporation was determined by liquid scintillation spectrometry. Data were fitted using a Dixon plot reporting the reciprocal of the velocity (1/v) as a function of inhibitor concentrations. Kᵣ values for the different peptides were estimated from the intercept on the concentration axis (36).

Steady-state Fluorescence Experiments—Fluorescence experiments were performed in buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM MgCl₂, and 1 mM DTT, at 25 °C, using a SPEX-PAT spectrofluorometer in a 1-cm path-length quartz cuvette, with a band-pass of 2 nm for excitation and emission, respectively. Excitation was performed at 492 nm, and emission spectra were recorded from 500 to 600 nm. According to fluorescence experiments, a fixed concentration of FAM-labeled (19/36) p/t (50 nM) or of FITC-Pₐₚ (200 nM) was titrated with increasing protein concentrations from 5 nM to 1 μM. Data were fitted as previously described (34, 37), using a quadratic equation (GraFit, Erithacus Software).

HPLC Size-exclusion Chromatography—Chromatography was performed using one (Phenomenex S3000) or two HPLC columns in series (Phenomenex S3000 followed by Phenomenex S2000, both 7.5 mm × 300 mm). Samples containing 3–10 μM of RT or p51 were applied onto one or two HPLC columns and eluted with 200 mM potassium phosphate (pH 7.0) at a flow rate of 0.5 ml/min⁻¹ (5).

Rapid Kinetic Experiments—Binding kinetics of p/t onto HIV-1 RT were performed with a FAM-labeled p/t in buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM MgCl₂, and 1 mM DTT, using a stopped-flow apparatus (Hi-Tech Scientific, Salisbury, UK) at 25 °C. A fixed concentration of FAM-labeled p/t (20 nM) was rapidly mixed with increasing concentrations of RT or RT-Pₐₚ complex formed at a 1/20 molar ratio (25–400 nM). 6-Carboxyfluorescein fluorescence was excited at 492 nm, and emission was detected through a filter with a cut-off at pH 7.0. Data acquisition and analysis were performed according to a single-exponential equation.

Dissociation kinetics of HIV-1 RT were monitored by using bis-ANS as extrinsic probe. Changes in bis-ANS (bis-ANS) fluorescence provide a good signal-to-probe variation in the exposure of the hydrophobic regions associated to RT dissociation in a time-dependent manner. 0.5 μM RT was dissociated in the presence of 0.8 μM bis-ANS, by adding 10% acetonitrile in the absence or in the presence of 10 μM Pₐₚ. Kinetics of dissociation were monitored by following fluorescence resonance energy transfer between tryptophan residues of RT and bis-ANS. Excitation of RT-Trp residues was performed at 290 nm, and the increase of bis-ANS fluorescence emission at 490 nm was detected through a 420 nm cut-off filter. Data acquisition and analysis were performed using KinetAsyst 3 software (Hi-Tech Scientific), and traces were fitted according to a single-exponential equation.

Cell Culture, Transfection, and Indirect Immunofluorescence Microscopy—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were grown on glass coverslips to 75% confluency, then transfected with pcDNA3-p66RT plasmid using Lipofectamine 2000 reagent according to the manufacturer’s instructions (Invitrogen). For colocalization experiments, cells were subsequently cultured for 32 h, before incubation with FITC-Pₐₚ or FITC-Pₐₚ/Pep-1 (complex obtained at a molar ratio 1/10) for 1 h. Coverslips were extensively rinsed with PBS, and cells were fixed in 4% paraformaldehyde for 10 min and permeabilized in 0.2% Triton. After saturation in PBS supplemented with bovine serum albumin 1% for 1 h, cells were incubated overnight with monoclonal 8C4 anti-HIV-1 RT antibody (AIDS Research Reference Reagent Program, National Institutes of Health, diluted 1:100 in PBS-bovine serum albumin 1%), followed by Alexa-555 anti-mouse (Molecular Probes). Immunofluorescence detection of HIV-1 RT and FITC-Pₐₚ was performed by epifluorescence microscopy using a PL APO 1.4 oil PH3 objective on a Leica DMRA 1999 microscope. Three-dimensional reconstitution of the 20 frames (interval, 0.3 μm) realized from z stacking was performed using Imaris 6.0 software.

ATCC H9 cells, stably transfected with pNL4.3 V-R+ plasmid and constitutively expressing Gag-Pol HIV-1 proteins (obtained from Dr. R. Marquet, Institut de Biologie Moléculaire et Cellulaire, France) were used for RT pulldown experiments. H9 cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 10% (v/v) fetal calf serum, 1% antibiotics (streptomycin 10,000 mg/ml, penicillin, 10,000 IU/ml) and G418 (1 mg/ml). Pₐₚ was incubated with 500 μl of activated CNBr-activated Sepharose 4B beads at 4 °C overnight. After centrifugation, supernatants were removed, and the beads were incubated with glycine, pH 8.0, for 2 h at 4 °C with gentle stirring. The beads were then washed with 0.1 M sodium acetate buffer (pH 4.0), then 0.5 M bicarbonate buffer, and finally in PBS, three times each. The peptide bound to the beads were then saturated for 30 min in PBS/bovine serum albumin 0.1% and then incubated for 1 h at 4 °C with equal amounts of H9 cells lysed for 30 min on ice in lysis buffer (Tris, 20 mM, pH 7.2, NaCl, 400 mM, EDTA, 1 mM, DTT, 1 mM, and Protease inhibitors, EDTA free) and sonicated 2 × 5 s at 20%. Beads were washed with lysis buffer then twice with PBS, and the bound proteins were finally separated on
15% SDS-PAGE gel and analyzed by Western blotting using monoclonal 8C4 anti-HIV-1 RT antibody.

**Antiviral Assay**—The anti-HIV activities of the whole series of peptides were assayed according to previously described methods (38). Phytohemagglutinin-P (PHA-P)-activated peripheral blood mononuclear cells (PBMCs) were infected with the reference lymphotropic HIV-1-LAI strain (39). Virus was amplified in vitro on PHA-P-activated PBMCs. Viral stock was titrated using PHA-P-activated PBMCs, and 50% tissue culture infectious doses (TCID_{50}) were calculated using Kärber’s formula (40). PBMCs were pretreated for 1 h with increasing concentrations of peptide (from 100 to 0.1 nM), then infected with 100 TCID_{50} of the HIV-1-LAI strain. Peptides were maintained throughout the culture, and cell supernatants were collected at day 7 post-infection and stored at −20 °C. Viral replication was measured by quantifying RT activity in cell culture supernatants. In parallel, cytotoxicity of the compounds was evaluated in uninfected PHA-P-activated PBMCs by colorimetric 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide assay on day 7 (41). Experiments were performed in triplicate and repeated with another blood donor. Data analyses were performed using SoftMax®Pro 4.6 micro-computer software: percentages of inhibition of RT activity or of cell viability were plotted versus concentration and fitted with quadratic curves; 50% effective doses (ED_{50}) and cytotoxic doses (CD_{50}) were calculated.

**RESULTS**

**Design and Evaluation of Pep-A-derived Peptides**—We have previously demonstrated that the thumb domain of p51 subunit is involved in activation of heterodimeric RT and that a 17-residue peptide, Pep-A, corresponding to an extremely well conserved structural motif located between amino acids “284 and 300,” can affect the maturation of HIV-1 RT (22). To optimize and identify major residues in Pep-A required for RT inhibition, a series of new peptides was derived from Pep-A sequence (RGTKALTEVIPLTEEAEC). First, the N-terminal arginine of Pep-A was removed to improve the solubility and facilitate the synthesis of Pep-A-derived peptides, and then additional peptides were then generated by performing an alanine scan on P1. Pep-A-derived peptides were evaluated using a standard polymerase RT assay, and $K_i$ values extrapolated using Dixon plot analysis (36) are reported in Table 1.

All peptides affected the polymerase activity of RT in a dose-dependent manner, and four peptides, P1 ($K_i$: 7.5 μM), P6 ($K_i$: 5.7 μM), P10 ($K_i$: 7.3 μM), and P11 ($K_i$: 7.0 μM), possess an inhibition constant <10 μM (Fig. 1). As a reference, we show that Pep-A inhibits RT-polymerase activity with an inhibition constant value of 35 μM. Peptide analysis reveals that removing the Arg^1 residue in Pep-A increases the potency of the peptide (P1) 5-fold. In comparison to P1, mutation of residues Gly^7, Ala^4, Glu^2, and Leu^11 into alanine significantly affects the potency of the peptide suggesting that the side chains of these residues are required for the interaction with RT. The nature of the side chain of Glu^7 seems to be a major requirement for the interaction with RT, because its substitution by alanine (P8), reduces the efficiency of the peptide 8-fold. In contrast, Lys^2, Thr^6, Val^8, and Glu^14 residues have a minor impact because their mutation into alanine only reduces their potency by a factor of 2. Interestingly, the hydrophobic character of Ala^4 and Val^8 side chains plays a role in the binding of the peptide to RT, and reducing their length affects the potency of the corresponding peptides to inhibit RT 2.7- and 2-fold, respectively. Taking into account that Trp residues are generally involved in stabilization of protein-protein interfaces, the two residues Ala^4 and Val^8 were mutated into Trp, to favor the binding of the peptide to RT. As shown in Fig. 1, the corresponding peptide P_{AW} significantly inhibits RT polymerase activity with an inhibition constant ($K_i$) of 0.7 μM, revealing that mutation of these two residues into Trp improves peptide efficiency 50-fold over Pep-A and 10-fold in comparison to the best lead peptide from the Ala scan (P6) (Fig. 1 and Table 1).

**Antiviral Potency of Pep-A-derived Peptides**—Antiviral activity of the five peptide leads (P1, P6, P10, P11, and P_{AW}) was

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**Table 1**

| Peptides | Sequences | $K_i$ (μM) |
|----------|-----------|------------|
| PepA     | RGTKALTEVIPLTEEAEC | 35 ± 5 |
| P1       | GTKALTEVIPLTEEAEC   | 7.5 ± 2.3 |
| P2       | ATKALTEVIPLTEEAEC   | 28 ± 11 |
| P3       | GAKALTEVIPLTEEAEC   | 10.3 ± 2.1 |
| P4       | GTKALTEVIPLTEEAEC   | 15 ± 2.9 |
| P5       | GTKALTEVIPLTEEAEC   | 20 ± 3.7 |
| P6       | GTKAAATEVIPLTEEAEC  | 5.7 ± 2.3 |
| P7       | GTKALAEVIPLTEEAEC   | 13.5 ± 2.1 |
| P8       | GTKALFAVIPLTEEAEC   | 57 ± 19 |
| P9       | GTKALPAVIPLTEEAEC   | 15 ± 7.3 |
| P10      | GTKALTEVIPLTEEAEC   | 7.3 ± 2.9 |
| P11      | GTKALTEVIPLTEEAEC   | 7 ± 1.4 |
| P12      | GTKALTEVIPLTEEAEC   | 22 ± 3 |
| P13      | GTKALTEVIPLTEEAEC   | 10.2 ± 2.5 |
| P14      | GTKALTEVIPLTEEAEC   | 14 ± 3 |
| P15      | GTKALTEVIPLTEEAEC   | 14.2 ± 2.2 |
| P_{scramble} | GAKTEALTEVIPLTEEAEC | 61 ± 12 |
| P_{AW}   | GTKWLTEWIPLTAEAEC   | 0.7 ± 0.2 |
| P_{AW-FITC} | GTKWLTEVIPLTAEAEC-FITC | 2.7 ± 0.7 |

$^a$ RT polymerase activity was measured as described under “Experimental Procedures.” The inhibition constants $K_i$ were calculated from Dixon plots, and reported data correspond to the mean of three separate experiments.
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evaluated on PHA-P-activated PBMCs infected with HIV-1-LAI. Results were reported as 50% efficient concentration (EC\textsubscript{50}) and selectivity index corresponding to the ratio between EC\textsubscript{50} and the cytotoxic concentration (CC\textsubscript{50}) inducing 50% death of uninfected PBMCs and relative to Pep-A and P8 (Table 2). To avoid any limitation due to the poor ability of peptides to cross cellular membranes, they were associated to the peptide-based nanoparticle delivery system Pep-1, at a 1/10 molar ratio.

TABLE 2
Antiviral activity of Pep-A and PAW\textsubscript{derived} peptides
\begin{tabular}{lll}
Peptides & EC\textsubscript{50}\textsuperscript{a} & Selectivity index\textsuperscript{b} \\
--------- & ------ & ----- \\
P1/Pep-1 & 78.2 & ND\textsuperscript{c} \\
P6/Pep-1 & 170 & >3 \\
P8/Pep-1 & 290 & >3 \\
P10/Pep-1 & 140 & ND \\
P\textsubscript{A} & >1000 & ND \\
P\textsubscript{A}/Pep-1 & 1.8 & >550 \\
P18/Pep-1 & >1000 & ND \\
P24/Pep-1 & 2.3 & >1200 \\
P26/Pep-1 & >1000 & ND \\
P27 & >1000 & ND \\
P27/Pep-1 & <0.32 & >3100 \\
\end{tabular}

\textsuperscript{a} Anti-HIV activity was evaluated on PHA-P-activated PBMCs infected with HIV-1-LAI strain. EC\textsubscript{50} values correspond to the 50% effective peptide dose.

\textsuperscript{b} The selectivity index corresponds to the ratio between EC\textsubscript{50} and the cytotoxic concentration (CC\textsubscript{50}) inducing 50% death of uninfected PBMCs.

\textsuperscript{c} ND, value not determined.

Pep-1 has been successfully used for the delivery of peptides and proteins into numerous cell lines as well as in vivo (42, 43). The inability of free peptides to block viral replication is directly associated to their poor cellular uptake as reported in Fig. 2A for fluorescently labeled peptide (FITC-PAW). In contrast, when complexed at a molar 1/10 ratio with the Pep-1 delivery system, FITC-PAW rapidly (in <1 h) enters cells (Fig. 2B). FITC-PAW localization and RT-PAW interaction were characterized using three-dimensional reconstitution of frames from z stacks. Three-dimensional image analysis reveals that PAW does not enter the nucleus and partially localizes with RT at the periphery of the nucleus (Fig. 2, E and F).

When associated with Pep-1, peptides P1, P6, P10, and P11 block viral proliferation with IC\textsubscript{50} values in the low micromolar range, which correlates with their ability to inhibit HIV-1 RT \textit{in vitro} (Table 2). In contrast, in agreement with previous findings no antiviral activity was observed with Pep-A when associated to Pep-1 (22). When complexed with Pep-1, PAW exhibits a marked antiviral activity with an EC\textsubscript{50} of 1.8 nm and a therapeutic/selectivity index of ~550. The 44- and 161-fold greater potency of PAW over peptides harboring mutations at Glu7 (P8) or lacking Trp residues (P1) confirms the requirement of these residues for targeting RT both \textit{in vitro} and \textit{in cellulo}. PAW constitutes a powerful inhibitor of polymerase activity and possesses a very potent antiviral activity without any toxic effect. We therefore, further investigated its mechanism of action on RT.

PAW Peptide Interacts with HIV-1 RT \textit{in a Cellular Context}—To confirm that PAW targets HIV-1 RT in a cellular context, we further investigated its ability to form stable complexes with HIV-1 RT expressed in cells by using pulldown experiments. The peptides PAW and P8, covalently associated with CNBr-Sepharose beads, were incubated in the presence of cell lysates of H9 cells expressing Gag-Pol gene products of HIV-1. Analysis of the presence of RT by Western blotting revealed that only PAW was able to form a stable complex with RT in a cellular context and to retain RT on beads (Fig. 2G). In contrast, no RT was associated to free or P8 beads.

Binding of PAW Peptide to the Dimeric Form of HIV-1 RT—To further understand the mechanism through which PAW inhibits RT, we investigated its potency to interact with the dimeric form of HIV-1 RT \textit{in the absence or presence of DNA/DNA p/t}. The binding of PAW to RT was monitored using a fluorescently labeled peptide (FITC-PAW). We first evaluated the impact of PAW labeling on the C-terminal cysteine

FIGURE 2. HIV-1 RT interacts with PAW\textsubscript{in cultured cells}. A–F, Cellular localization of PAW and its interaction with HIV-1 RT \textit{in cellulo} was monitored using HeLa cells expressing RT transfected with FITC-PAW\textsubscript{Pep-1} complex formed at a 1/10 molar ratio. HIV-1 RT (Alexa 555 secondary antibody) and FITC-PAW were visualized, respectively, through a Cy3 and a GFP filter. HeLa cells transfected with free FITC-PAW\textsubscript{A} or complexed with Pep-1 (B). Cultured HeLa cells were transfected (D) with pcDNA3-p66RT. HeLa cells co-transfected with both pcDNA-p66RT and FITC-PAW\textsubscript{Pep-1} at a 1/10 molar ratio. The RT-PAW co-localization was analyzed by the three-dimensional image reconstitution with Imaris 6.0 software of 20 frames from z stacks (E and F). RT, PAW, and nuclear staining with Hoechst are reported in red, green, and blue, respectively. Global view (D), three-dimensional image analysis of a selected cell (red arrow) reveals that PAW and RT localize in the cytoplasm at the periphery of the nucleus. F, zoom of the box reported in panel E. G, interaction between PAW and HIV-1 RT detected in a CNBr pulldown assay. Experiments were performed as described under “Experimental Procedures.” 30 \mu g (total protein) per lane were separated on 15% SDS-PAGE and subjected to Western blotting using rabbit anti-RT antibody. Lanes correspond to control free beads, P8 and PAW beads, and total proteins loaded on the gel, respectively.
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The binding of PAW to RT was monitored by following the quenching of extrinsic FITC fluorescence associated with its binding was of 57%, and the affinity of FITC-PAW for RT increased 5-fold ($K_d = 149 \pm 38 \text{ nm}$) for p66DM/p51wt (Fig. 3A).

Effect of PAW Peptide on Primer/Template Binding to HIV-1 RT—The impact of PAW peptide on the ability of HIV-1 RT to bind p/t was then investigated at both steady-state and pre-steady-state levels using a 19/36-mer p/t labeled at the 3’-end of the primer with FAM-derivative as previously described (34, 37). As reported in Fig. 4A, the presence of a saturating concentration of PAW (10 $\mu$m) decreases the affinity of fluorescently labeled p/t for RT 4.5-fold with a $K_d$ value of 99 ± 40 nm in comparison to 22 ± 5 nm obtained in the absence of PAW. The binding of unlabeled p/t induces a 42% change in the fluorescence of PAW-FITC pre-bound to RT and leads to a similar $K_d$ value of 66.5 ± 19 nm (Fig. 4A). These results suggest that p/t interacts close to PAW binding site on RT, inducing a change in the orientation of FITC linked to PAW, but does not share the same binding site.

RT-p/t pre-steady-state binding kinetics follow a three-step mechanism in the presence or in the absence of PAW, including a rapid diffusion controlled second order step leading to the formation of the RT-p/t collision complex, followed by two slow, concentration-independent, conformational changes (34). The plot of the pseudo-first order rate constant for the initial association of the p/t with RT against RT concentration is linear. In the absence of PAW, $k_{+1}$ and $k_{-1}$ rate constant values of $4.23 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ and 29.9 $\text{s}^{-1}$ were calculated from the slope and the intercept with the $y$ axis of the graph (Fig. 4, B and C). Analysis of the second and third slow phases yielded rate constants of $k_2 = 5.8 \text{ s}^{-1}$ and $k_3 = 0.76 \text{ s}^{-1}$ for RT. The presence of PAW did not alter the overall $K_d$ for the initial formation of the RT-p/t complex as both the “on” ($k_{+1} = 1.05 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$) and the “off” ($k_{-1} = 7.9 \text{ s}^{-1}$) rates of the first step are decreased by ~4-fold. In contrast, the presence of PAW on RT significantly reduced the rate constants of the slow conformational steps ($k_2 = 1.99 \text{ s}^{-1}$ and $k_3 = 0.22 \text{ s}^{-1}$), affecting the proper binding of the p/t (Fig. 4, B and C).

Effect of PAW on the Stability and Dimerization of HIV-1 RT—The impact of PAW on the stability and formation of heterodimeric RT was investigated in detail by size-exclusion chromatography as previously described (17). As reported in Fig. 5A, heterodimeric RT incubated or not in the presence of an excess of PAW (100 $\mu$m) for 1 h and 30 min at room temperature, is fully dimeric and eluted as a single peak at 16.7 min. The interaction of PAW with RT was monitored by size-exclusion chromatography using HIV-1 RT preincubated with FITC-PAW. Chromatography analysis reveals that FITC-PAW co-elutes with heterodimeric RT in a single peak at 16.7 min (Fig. 5A), demonstrating that PAW binds heterodimeric RT and does not induce RT dissociation. We
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FIGURE 4. Impact of PAW peptide on the binding of primer/template to HIV-1 RT. A, titration of fluorescently labeled p/t binding to RT (■) or RT-PAW (○). B, titration of FITC-PAW/RT binding to p/t (▲). A fixed 50 nM concentration of fluorescently labeled p/t was titrated with increasing concentrations of RT or RT-PAW. The binding of p/t to RT was monitored by following the quenching of p/t extrinsic fluorescence at 512 nm, upon excitation at 492 nm. A fixed 100 nM concentration of FITC-PAW/RT complex was titrated by increasing concentrations of p/t (18/36). The binding of FITC-PAW/RT to p/t was monitored by following the quenching of FITC-PAW extrinsic fluorescence at 512 nm, upon excitation at 492 nm. Kd values were calculated using a quadratic equation as previously described (20) and correspond to the mean of at least three separate experiments. Kinetics of binding was calculated using a three-exponential equation. D, secondary plot of the dependence of the fitted pseudo-first order rate constants for the first phase on RT (■) or RT-PAW (○) concentration.

FIGURE 5. Binding of PAW to heterodimeric RT as monitored by size-exclusion chromatography. A, heterodimeric RT (2.3 μM) was incubated in the presence of PAW (10 μM) for 1 h and 30 at room temperature, then applied onto a gel filtration column and eluted with 200 mM potassium phosphate buffer, pH 7.0. B, heterodimeric RT (10 μM) was incubated in the presence of FITC-PAW (150 μM) for 2 h at room temperature then partially dissociated by 10% acetonitrile for 30 min and analyzed by gel filtration. Proteins were monitored at 280 nm (solid line) and fluorescein-labeled peptide at 492 nm (dashed line).

Then evaluated the ability of PAW to interact with p66 or p51 monomeric forms. Experiments performed with a partially dissociated RT-PAW (50%) complex by 10% acetonitrile, showed that PAW remains associated only with the dimeric fraction of RT and does not bind monomeric p66 or p51 subunits, which are eluted at 17.5 min and 18.2 min, respectively (Fig. 5B).

We then investigated the ability of PAW to prevent HIV-1 RT dimerization. Dissociation of RT was achieved at room temperature with 17% acetonitrile, and then association of the subunits was induced by a 10-fold dilution of the sample in an acetonitrile-free buffer in the absence or presence of 100 μM of PAW. At this concentration (1.7%) of acetonitrile no dissociation of RT could be detected. As shown in Fig. 6A, heterodimeric RT was fully re-associated 5 h after dilution in an acetonitrile-free buffer, both in the absence or in the presence of PAW (100 μM), indicating that PAW does not block RT dimerization (Fig. 6A).

The impact of PAW was further investigated on the kinetics of RT dimerization. The level of dimeric RT was evaluated 30 min and 2 h, respectively, after dilution in free acetonitrile buffer by size-exclusion chromatography (Fig. 6, B and C). In the presence of PAW 21 and 59% of dimeric RT was quantified after 30 min and 2 h, respectively (Fig. 6B). In comparison only 16% (30 min) and 29% (2 h) of dimeric RT were detected in the absence of peptide (Fig. 6C), suggesting that the presence of PAW favors the kinetics of RT dimerization.

PAW Peptide Favors Dimerization of the Small p51 Subunit—p51 subunits are mainly monomeric, and dissociation constants for p51/p51 homodimer have been reported to be either in the micromolar (25) or millimolar (5) range depending on the technology used to quantify the interactions. We have investigated the ability of PAW to favor p51/p51 dimerization by size exclusion chromatography, using two HPLC columns in series. Experiments were performed at a p51 concentration of 3.5 μM at which it is entirely monomeric and elutes as a single peak at 32.7 min (Fig. 7). Monomeric p51 (3.5 μM) was incubated in the presence of FITC-labeled PAW (20 μM) for 1 h at room temperature then analyzed by size exclusion chromatography. As reported in Fig. 7, in the presence of fluorescently labeled PAW 4.6% of p51 are dimeric and associated to PAW, suggesting that
PAW promotes p51/p51 homodimer and only p51/p51 homodimer. PAW Peptide Prevents HIV-1 RT Dissociation—Finally, the impact of PAW on HIV-1 RT stability and dissociation were investigated at the steady state level by size exclusion chromatography and at the pre-steady-state level by stopped-flow rapid kinetics. HIV-1 RT was preincubated in the presence of 100 μM PAW, for 2 h, prior dissociation with 17% or 10% of acetonitrile, and the level of dimeric form was then assessed by size exclusion chromatography and the rate of dissociation by pre-steady-state kinetics. As reported in Fig. 8A, the presence of PAW protected RT from the acetonitrile dissociation, because 17% remained dimeric whereas “free” RT was completely dissociated with 17% acetonitrile.

The protection by PAW of acetonitrile-associated RT dissociation was further investigated by monitoring pre-steady-state dissociation kinetics of HIV-1 RT, using bis-ANS as an extrinsic probe (46). Binding of bis-ANS to dissociated RT resulted in a large increase in the fluorescence of the probe due to noncovalent interactions of bis-ANS to exposed hydrophobic surfaces on RT subunits, therefore providing a good signal for following RT dissociation in a time-dependent manner. Experiments were performed by adding bis-ANS to HIV-1 RT prior dissociation of the enzyme by 10% acetonitrile and monitoring FRET between exposed Trp of RT and Bis-ANS. As reported in Fig. 8B, the kinetics of increased ANS fluorescence upon dissociation of RT in the absence of PAW followed a single-exponential reaction, with a dissociation rate constant $k_{\text{dis}}$ of $5.30 \pm 0.01 \text{ s}^{-1}$, which was reduced 3.8-fold ($k_{\text{dis}} = 1.42 \pm 0.007 \text{ s}^{-1}$), when RT was incubated with PAW.

Optimization of PAW and Selection of a Minimal Inhibitory Peptide Motif—Taken together our results demonstrate that PAW constitutes a potent conformational inhibitor of RT and exhibits a potent antiviral activity. To define the minimal peptidic sequence for RT inhibition, new peptides derived from PAW were designed and evaluated (Table 3). As the interaction between PAW and RT seems to involve both the N-terminal part and Trp residues of the peptide, the peptidic sequence was shortened at the N- and/or C-terminal extremities, and the positional effect of the Trp was evaluated. All peptides were tested in standard RT assays (Table 3) and were evaluated on PBMCs infected by HIV-1-LAI (Table 2). Reducing PAW sequence by two residues at the N terminus reduced efficiency 2.5-fold ($P_{26}: K_i = 1.8 \pm 0.7 \mu M$). In contrast, the five last residues at the C terminus of PAW can be removed without affecting its potency to inhibit RT polymerase activity or to block viral replication ($P_{24}: K_i = 0.7 \pm 0.05 \mu M$ and EC$_{50} = 2.3 \text{ nM}$). That P18 does not inhibit RT polymerase activity confirms that the Trp residues form the major interface with RT.
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FIGURE 8. PAW peptide prevents HIV-1 RT dissociation. A, PAW-associated protection of RT from the acetonitrile dissociation as monitored by size exclusion chromatography. First, HIV-1 RT (8.7 μM) was incubated in the presence (dashed line) or absence (solid line) of fluorescently labeled PAW (100 μM) then dissociated by 17% acetonitrile for 30 min at room temperature and applied onto a size exclusion chromatography. B, kinetics of RT dissociation induced by acetonitrile. RT (0.5 μM) was dissociated in the presence of 0.8 μM bis-ANS, by adding 10% acetonitrile in the absence (black line) or presence of 5 μM PAW (gray line). The kinetics of dissociation was monitored by following the fluorescence resonance energy transfer between trypthophan of RT and bis-ANS. Tryptophan excitation was performed at 290 nm, and the increase of bis-ANS fluorescence emission at 490 nm was detected through a 420 nm cut-off filter. Data acquisition and analysis were performed using KinetAsyst 3 software (Hi-Tech Scientific), and traces were fitted according to a single exponential equation.

TABLE 3

| Peptides | Sequences          | $K_i$ μM |
|----------|--------------------|----------|
| PAW      | GTKWLTEWLP/LTEAEAC | 0.7 ± 0.2 |
| P16      | GTKW/LTEWLP/LTEAEAC| 14 ± 4   |
| P17      | GTKW/LTEWLP/LTEAEAC| 35 ± 11  |
| P18      | GTKW/LTEWLP/LTEAEAC| 53 ± 12  |
| P19      | GTKW/LTEWLP/LTEAEAC| 49 ± 9   |
| P24      | GTKW/LTEWLP/LTEAEAC| 0.7 ± 0.05 |
| P26      | KWL/LTEWLP/LTEAEAC | 1.8 ± 0.7 |
| P27      | KWL/LTEWLP/LTEAEAC | 0.05 ± 0.01 |
| P28      | KWL/LTEWLP/LTEAEAC | 2 ± 0.6  |
| P29      | KWL/LTEWLP/LTEAEAC | 1 ± 0.4  |

$^a$ RT polymerase activity was measured as described under “Materials and Methods.” The inhibition constants $K_i$ were calculated from Dixon plots and reported data correspond to the mean of three separate experiments.

Trp$^6$ to position 9 (P16) and both Trp$^4$ and Trp$^8$ to positions 5 and 9 (P17) reduced the efficiency of the corresponding peptides 20-fold ($K_i$: 14 ± 4 μM) and 50-fold ($K_i$: 35 ± 11 μM), respectively. Interestingly, removing the last two residues of PAW increases its efficiency 14-fold (P27: $K_i$: 50 ± 0.01 nM) and is also associated with an increase in its antiviral activity with an IC$_{50} < 0.32$ nm and a therapeutic/selectivity index $> 3100$.

DISCUSSION

Targeting the conformational flexibility of heterodimeric RT has provided new concepts for the design of drugs active on viruses resistant to currently used RT inhibitors (5, 17, 26, 28, 47). RT activation involves a two-step dimerization process initiated by a rapid monomer/monomer association generating an inactive intermediate heterodimer, followed by a slow isomerization yielding the biologically active enzyme (5, 20). Considering that HIV-1 RT is extremely stable (25, 46, 48), selection of compounds that are able to dissociate the complex remains challenging. In contrast, because maturation of RT requires less energy than dimerization, targeting conformational changes involved is a very attractive approach for the design of novel antiviral compounds. Maturation of the inactive intermediate heterodimer corresponds to conformational changes involving interactions between the thumb of p51 and the RNase H of p66 and between the fingers of p51 and the palm of p66 (20). We previously demonstrated that the thumb domain of p51 plays a major role in RT maturation and that a synthetic peptide (Pep-A) derived from this domain selectively inhibited activation of HIV-1 RT (22). In the present work, we report the design of a new generation of peptide inhibitors derived from the thumb domain and have identified a lead peptide PAW that efficiently blocks both maturation of RT and viral replication.

PAW Peptide Preferentially Binds Dimeric RT in the “Open” Conformation—From size exclusion chromatography, we clearly demonstrated that PAW only binds dimeric forms of RT (p66/p51 and p51/p51). Moreover, as already reported for Pep-A (22), PAW does not induce heterodimer dissociation nor prevent the monomer/monomer association, and instead significantly increases stability of the heterodimer and favors dimerization. According to the two-step process mechanism, we propose that PAW blocks RT maturation by stabilizing the inactive intermediate of RT in a non-progressive conformation.

Determination of crystal structures of the HIV-1 RT associated or not with a p/t has revealed that the binding of p/t to RT triggers major conformational changes in the overall structure of the enzyme, including the increase in the compactness together with conversion of RT from a “closed” to an open conformation (7–9). The structure RT adopts two conformational states: a closed conformation stabilized by interactions between fingers and thumb domains of p66 and an open conformation associated with a change in the orientation of the thumb domain and a shift of the fingers domain, which are induced by p/t binding (8, 9). We demonstrate that PAW tightly binds RT preferentially in the open conformation, because its affinity is increased 5-fold in the presence of p/t. The dynamics of the thumb and fingers domains of p66 and the conformational changes of RT associated with p/t binding exposes the binding site of PAW, was strengthened by the fact that mutation of Phe$^{61}$ into glycine on the fingers domain of p66 subunit altered binding of PAW to RT (6-fold). Phe$^{61}$ is located in the fingers domain and together with Trp$^7$ and Arg$^{28}$ is involved in the stabilization of the closed conformation of RT, by contacting the loop between helices αl and αJ of the thumb domain. Given that mutation of Phe$^{61}$ favors the open conformation of RT (34), but dramatically reduces the affinity of PAW for RT, suggests that this residue is directly involved in the binding of PAW. In addition, the PAW binding site is located close to this residue on the thumb or the fingers domain of p66.
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$P_{AW}$ Peptide Blocks RT in an Inactive Conformation Altering Proper Binding of Primer/Template—The initial monomonomer interaction yields an intermediate dimer lacking of polymerase and RNase H activities, then corrected organization of the catalytic and the p/t binding sites occurs during a slow maturation step (20). We propose that $P_{AW}$ interacts directly with the intermediate inactive form, which stabilizes in a non-processive conformation. From a mechanistic point of view, $P_{AW}$ acts as non-competitive inhibitor affecting conformational changes required for proper folding of the p/t binding site. $P_{AW}$ does not displace the RT-p/t complex but reduces both “off” and “on” rates of the collisional binding of p/t to RT; it did not affect the overall dissociation constants of the collisional step, with $k_d$ values of 70.4 nM and 70.6 nM obtained in the presence or the absence of $P_{AW}$, respectively. In contrast, $P_{AW}$ dramatically affects the proper binding and conformational changes that place correctly the p/t for catalysis. The corresponding rate constants are reduced by 3-fold ($k_a$) and 3.4-fold ($k_d$), respectively. Taken together that $P_{AW}$ binds heterodimeric and homodimeric RTs (p66/p51 and p51/p51), preferentially in the open conformation, and that it specifically inhibits polymerase activity and not RNase H, demonstrates that $P_{AW}$ interacts with the inactive intermediate form of RT and prevents conformational changes required for the proper folding of the p/t binding site.

Similarly, NNRTIs are non-competitive inhibitors that block RT in a non-processive conformation (24–28). NNRTIs bind near the RT–polymerase catalytic site and affect the dynamics of thumb–finger domain interaction on p66 subunit and maintain RT in an open conformation (6, 8, 10). Although the molecular mechanism by which NNRTIs inhibit RT is not entirely clear, evidence reports that binding of NNRTIs restricts the mobility of the thumb domain, slowing or preventing p/t translocation and thereby inhibiting elongation of nascent DNA. NNRTIs such as Efavirenz favor RT dimerization in vitro and in cultured cells (24), but in contrast to $P_{AW}$, they improve the binding of p/t (35, 49), which excludes that $P_{AW}$ and NNRTI binding sites overlap and favors a $P_{AW}$ binding site close to the interface between the thumb and fingers domain of p66.

$P_{AW}$ is a Potent Antiviral Compound—When associated with Pep-1-based nanoparticles, $P_{AW}$ is a potent non-toxic inhibitor of viral replication ($EC_{50}$, 1.8 nM). This peptide constitutes a major improvement in comparison to Pep-A, which is 50-fold less potent in vitro on RT activity and does not exhibit any antiviral activity in the same delivery conditions. We demonstrate that, when delivered into cells using Pep-1, $P_{AW}$ interacts with RT in both cells expressing only p66 and in the context of the full Pol-polyprotein. Taken together, this result, combined with the fact that $P_{AW}$ $EC_{50}$ values on HIV-LAI are similar to its dissociation constant for RT, confirms that $P_{AW}$ inhibition occurs via conformational changes on RT and not through direct inhibition of polymerase activity. The analysis of $P_{AW}$ sequence has revealed that essential residues involved in RT binding and antiviral activity are located in the N terminus of the peptide. In particular, Glu, Leu, Trp, and Trp are required for binding of $P_{AW}$ to RT. The hydrophobic character of the side chain of Trp residues at positions 4 and 8 plays a major role in stabilizing the $P_{AW}$-RT complex. We show that $P_{AW}$ can be reduced to 12 residues (P24) without affecting either its in vitro or in cellulo potency. Interestingly, removing the last two residues of $P_{AW}$ (P27) increases in vitro and antiviral efficiency 12-fold and 10-fold, respectively. These results suggest that, although the Trp residues are key residues for the potency of the peptide, residues 12–14 in the sequence of $P_{AW}$ are also required to stabilize $P_{AW}$ in its binding site.

CONCLUSIONS

In the present work, we have demonstrated that the dynamics of the thumb/thumb domains of p66 play an essential role in the stabilization and maturation of heterodimeric HIV-1 RT. As such, we have established a proof of concept that targeting conformational changes required for RT flexibility can lead to highly potent antiviral molecules. We have identified a new RT inhibitor, $P_{AW}$, that alters finger/thumb dynamics and maintains RT in a non-processive conformation, by altering the proper binding of p/t to RT. Development of this type of inhibitor together with a better knowledge of its mechanism at the viral level will provide new perspectives for designing specific inhibitors of the “niche” of highly resistant strains.

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