Defective Strand-Displacement DNA Synthesis Due to Accumulation of Thymidine Analogue Resistance Mutations in HIV-2 Reverse Transcriptase

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ABSTRACT: Retroviral reverse transcriptases (RTs) have the ability to carry out strand displacement DNA synthesis in the absence of accessory proteins. Although studies with RTs and other DNA polymerases suggest that fingers subdomain residues participate in strand displacement, molecular determinants of this activity are still unknown. A mutant human immunodeficiency virus type 2 (HIV-2) RT (M41L/D67N/K70R/S215Y) with low strand displacement activity was identified after screening a panel of purified enzymes, including several antiretroviral drug-resistant HIV-1 and HIV-2 RTs. In HIV-1, resistance to zidovudine and other thymidine analogues is conferred by different combinations of M41L, D67N, K70R, L210W, T215F/Y, and K219E/Q (designated as thymidine analogue resistance-associated mutations (TAMs)). However, those changes are rarely selected in HIV-2. We show that the strand displacement activity of HIV-2_{ROD} mutants M41L/S215Y and D67N/K70R was only slightly reduced compared to the wild-type RT. In contrast, mutants D67N/K70R/S215Y and M41L/D67N/K70R/S215Y were the most defective RTs in reactions carried out with nicked and gapped substrates. Moreover, these enzymes showed the lowest nucleotide incorporation rates in assays carried out with strand displacement substrates. Unlike in HIV-2, substitutions M41L/T215Y and D67N/K70R/T215Y/K219Q had no effect on the strand displacement activity of HIV-1_{BH10} RT. The strand displacement efficiencies of HIV-2_{ROD} RTs were consistent with the lower replication capacity of HIV-2 strains bearing the four major TAMs in their RT. Our results highlight the role of the fingers subdomain in strand displacement. These findings might be important for the development of strand-displacement defective RTs.

KEYWORDS: strand displacement, HIV, reverse transcriptase, antiretroviral drugs, DNA polymerases

Retroviral reverse transcriptases (RTs) are responsible for the conversion of the single-stranded viral genomic RNA into an integration-competent double-stranded DNA.¹ RTs are multifunctional enzymes with RNA- and DNA-dependent DNA polymerase and endonuclease (RNase H) activities. Reverse transcription is initiated from a cellular tRNA primer that binds near the 5' end of the viral RNA at the primer binding site (PBS). Minus-strand DNA synthesis proceeds while the RNase H activity of the RT degrades the genomic RNA and facilitates the template switch by which DNA synthesis can continue from the 3' end of the viral RNA. The transferred strand continues its elongation until completion. RNA polypurine tracts (PPTs) of around 15 nucleotides are resilient to cleavage by the RT's RNase H activity and serve as primers for plus-strand DNA synthesis. PPT elongation continues until the RT copies a part of the tRNA molecule. Then, complementary copies of the PBS sequences in plus- and minus-strand DNA anneal, and after a second strand transfer event, DNA synthesis of both strands resumes to

generate a double-stranded proviral DNA with long terminal repeats (LTRs).^{1,2}

Retroviral RTs have the ability to displace a nontemplate strand while synthesizing the proviral DNA. This property known as strand displacement is essential to complete the second strand transfer reaction.^{3–5} Human immunodeficiency virus type 1 (HIV-1) RT catalyzes strand displacement activity in the absence of accessory factors.^{6,7} However, strand displacement DNA synthesis can be enhanced by the addition of *Escherichia coli* single-stranded binding protein or human replication protein A.^{5,8} In contrast, retroviral nucleocapsid (NC) proteins have a relatively minor impact on the rates of

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Figure 1. Strand displacement DNA synthesis by WT and mutant HIV-2_{ROD} RTs using a nicked substrate. A schematic representation of the M54/3TRP/4TRP complex used in strand displacement (SD) assays is shown above. Nucleotide sequences of relevant oligonucleotides are shown below. Control experiments were carried out with the 54/22-mer M54/3TRP. The concentration of template-primer or strand displacement complexes in the assays was 30 nM. Reactions contain active RT concentrations of 180 nM, and each dNTP was supplied at 100 μ M (final concentration). Aliquots were removed after incubating the samples for 0.5, 1, 5, and 10 min in control (C) assays using M54/3TRP, and at 0.5, 1, 2, 5, and 10 min, in assays carried out with the strand-displacement substrate (SD). Figure shows representative gels from at least four independent experiments.

RNA and DNA displacement synthesis.^{5,8–10} Biophysical studies suggest that effects of the NC protein could be due to a reduction in the time required for the RT to form a new initiation complex after dissociating from the template-primer,¹¹ an observation that is consistent with the dominant effect of pausing and reinitiation events on strand displacement.

During HIV-1 reverse transcription, strand displacement is required to eliminate any RNA fragments not cleaved by the RNase H activity of the RT. After the second strand transfer event, the viral RT displaces the tRNA bound to the PBS region at the 3' end of the minus-strand DNA, and synthesizes the LTR sequence of approximately 600 bp forming the minus-strand 5'-LTR, as well as a central DNA flap that requires a 100 nt-displacement near the center of the proviral DNA. This DNA flap results from the elongation of a central PPT and could participate in the nuclear import of the HIV-1 genome prior to integration.^{10,12,13} Reverse transcription is a major target for treatment of the acquired immune deficiency syndrome (AIDS) and HIV RT inhibitors constitute the backbone of current antiretroviral therapies. However, despite its relevance in retrovirus replication, the molecular determinants of strand displacement synthesis are still poorly understood.

HIV-1 RTs are p66/p51 heterodimers, with amino acid residues Trp24, Pro25, Phe61, Ile63, Leu74, and Asp76 (in the 66-kDa subunit), interacting with the template 5'-overhang in the vicinity of the nucleotide binding site.¹⁴ In agreement with these observations, nonconservative substitutions at positions 24 and 61 in HIV-1 RT (i.e., W24G and F61G) render unstable p66/p51 heterodimers and impair RT-template/primer binding.¹⁵

Phe61 contacts the template strand immediately ahead of the nucleotide binding site. Substitutions of this residue, located next to the β 3- β 4 hairpin loop of the fingers subdomain of p66, produced remarkable alterations in the strand displacement activity of HIV-1 RT.¹⁶ Thus, F61W showed reduced DNA displacement synthesis compared to wild-type (WT) RT, while F61A, F61L, and F61Y were at least as efficient as the WT enzyme in displacing a nontemplate strand during DNA synthesis.¹⁶ Although F61W showed significant polymerase activity,¹⁷ mutant viruses carrying any of those mutations were found to be defective in phenotypic assays.¹⁸ Further characterization of those mutants suggested an unexpected defect in RNase H function that could affect PPT removal during plus-strand DNA synthesis. In contrast, amino acid substitutions at the equivalent position in the murine leukemia virus (MLV) RT (i.e., L99A, L99F, L99Y, and L99W) had no effect on strand displacement and showed

polymerization activities similar to those of the WT enzyme.¹⁹ In contrast, substituting Ala for Tyr64 in MLV RT (equivalent to Trp24 in HIV-1 RT) rendered a polymerase with impaired capacity to carry out DNA and RNA displacement synthesis without affecting polymerase processivity or RNase H activity. However, the mutant virus was not viable due to an extensive defect in reverse transcription.¹⁹

Amino acid substitutions emerging in HIV-1 RTs from virus selected in the presence of antiretroviral drugs locate at the nucleotide binding site or its vicinity, and very often involve residues of the RT fingers subdomain.²⁰ However, despite extensive characterization of HIV RT mutants associated with resistance to nucleoside and nonnucleoside RT inhibitors (NRTIs and NNRTIs, respectively),²⁰ there are no reports describing drug resistance-associated mutant RTs with normal DNA polymerase activity but showing impaired strand displacement activity.

In this study, we describe HIV-2_{ROD} RT variants containing different combinations of frequent thymidine analogue resistance-associated mutations (TAMs) (M41L, D67N, K70R, and S215Y) which are defective in strand displacement activity. These enzymes showed good catalytic efficiency in RNA- and DNA-dependent DNA synthesis reactions. However, using nicked duplex DNA substrates, we demonstrate that the accumulation of TAMs renders HIV-2 RTs with reduced nucleotide incorporation efficiency at the 3' end of the primer. Our results underline the contribution of all TAMs to the observed phenotype although the addition of M41L and/or S215Y to the double mutant D67N/K70R is necessary to detect defects in DNA and RNA displacement synthesis using nick or gapped substrates. Interestingly, despite the sequence similarity between HIV-1 and HIV-2 RTs, TAMs are rarely found in nucleoside analogue-resistant HIV-2. Unlike in the case of HIV-1, the presence of the four classical TAMs (M41L/D67N/K70R/S215Y) in HIV-2 did not produce an AZT-resistant virus.²¹

RESULTS

Screening and Identification of RTs with Defective Strand Displacement Activity. Strand displacement was measured by using a single-stranded DNA of 54 nucleotides (M54), annealed to oligonucleotides of 22 and 32 bases in length (Figure 1). The 22-mer (3TRP) was used as primer and labeled at its 5' end, whereas the 32-mer (4TRP) was the potential obstacle for DNA synthesis and the displaced oligonucleotide when 3TRP was extended in the presence of dNTPs. M54 was selected because it mimics a *pol* sequence found in the HIV-1_{HXB2} genome (nucleotides 3723–3775 of GenBank accession no. K03455), identified as part of a recombination hot spot found in the HIV-1 genome.²²

Defects in strand displacement were assessed after comparing primer elongation efficiencies obtained with M54/3TRP/4TRP with those obtained with template-primer M54/3TRP. Initially, we used these assays to identify RTs with defective strand displacement activity using a panel including >30 previously characterized mutant and wild-type (WT) enzymes of HIV-1 (group M-subtype B strains BH10 and NL4–3 and group O strain ESP49) and HIV-2 (ROD strain) (Table 1). The largest reductions in strand displacement activity were observed with HIV-2 RTs containing four TAMs. As shown in Figure 1, primer extensions using the control template-primer M54/3TRP were similar for WT HIV-2_{ROD} RT and the quadruple mutant M41L/D67N/K70R/S215Y.

Table 1. Strand Displacement Efficiencies of HIV-1 and HIV-2 RTs^a

RTs		strand displacement efficiency
virus	amino acid substitutions	
HIV-1 _{BH10}	WT	+++
	M41L/T215Y	+++
	D67N/K70R	+++
	D67N/K70R/T215Y/K219Q	+++
	V75A	+++
	V75F	+++
	V75I	+++
	L92P	++
	E138K	+++
	E478Q	+++
HIV-1 _{NL4–3}	Δ67	+++
	Δ67/T69G/K70R	+++
	Δ67/T69G/K70R/K219E	+++
HIV-1 _{ESP49}	WT	+++
	F61A/K358R/A359G/S360A	++
	K65R/L92P/K358R/A359G/S360A/E478Q	+++
	V75I	+++
	V75I/D443N	++
	L92P/K358R/A359G/S360A	++
	L92P/K358R/A359G/S360A/D443N	++
	L92P/K358R/A359G/S360A/E478Q	++
	V148I/K358R/A359G/S360A	+++
	T355A/Q357M/K358R/A359G/S360A	+++
	K358R/A359G/S360A	+++
	K358R/A359G/S360A/D443N	+++
	K358R/A359G/S360A/E478Q	+++
	D443N	++
	D67N/K70R	++
HIV-2 _{ROD}	WT	+++
	M41L/D67N/K70R/S215Y	+
	M41L/S215Y	++
	T60I/K65R/M73K	+++
	K65R	+++
	D67N/K70R	++

^aHIV-1_{BH10} and HIV-1_{NL4–3} RTs derived from HIV-1 strains of group M, subtype B. HIV-1_{ESP49} RT is a well-characterized enzyme from an HIV-1 group O strain. HIV-2_{ROD} RT derives from a group A HIV-2 strain. Experiments were carried out with template-primer M54/3TRP and strand displacement complex M54/3TRP/4TRP. +++, indicates that the elongation patterns were similar for both complexes, and showed substantial amounts of fully elongated product in reactions carried out for 1 min with M54/3TRP/4TRP. ++, indicates delayed elongation with M54/3TRP/4TRP relative to M54/3TRP. The amounts of fully elongated primers after 2 min was very small (<2%) with M54/3TRP/4TRP. +, indicates a clear defect in elongation reactions with M54/3TRP/4TRP. Using this DNA complex, the amounts of fully elongated primers remain very small (<2%), up to 5 min-incubation. Representative examples are given in Figure 1.

However, this mutant was defective in strand displacement activity, as demonstrated by the lower elongation rates obtained with the M54/3TRP/4TRP complex. In these assays, fully extended primer was observed only after 10 min incubation with M41L/D67N/K70R/S215Y HIV-2 RT. In contrast, experiments run in parallel showed that the double

Figure 2. Strand displacement DNA synthesis by WT and mutant HIV-2_{ROD} RTs using gapped substrates. (A) Schematic representation of the substrates used in the strand displacement (SD) assays. DNA primers used in the assays are indicated above each gel. Panels show data obtained with strand displacement complexes containing gaps of one (B), two (C), or five (D) nucleotides. Control experiments (C) were carried out without the displaced oligonucleotide 4TRP (i.e., template-primers M54/3TRP-17, M54/3TRP-20 and M54/3TRP-21). The concentration of template-primer or strand displacement complexes in the assays was 30 nM. Reactions contain active RT concentrations of 180 nM, and each dNTP was supplied at 100 μ M (final concentration). Aliquots were removed after incubating the samples for 0.5, 1, 5, and 10 min in control (C) assays using M54/3TRP-17, M54/3TRP-20, and M54/3TRP-21, and at 0.5, 1, 2, 5, and 10 min, in assays carried out with the strand-displacement substrate (SD). Figure shows representative gels from at least three independent experiments.

mutant D67N/K70R was not defective in strand displacement activity, although the amount of full-length product obtained with the M54/3TRP/4TRP complex after incubating the sample for 30 s was slightly reduced in comparison with the WT enzyme (see first time point in the SD panels of WT_{ROD} and D67N/K70R_{ROD} in Figure 1).

Analysis of the Strand Displacement Efficiency Using Gapped Substrates. The effects of the composition of strand displacement substrates in primer extension efficiency were determined for the WT HIV-2_{ROD} RT, as well as for mutants D67N/K70R and M41L/D67N/K70R/S215Y. The influence of the gap between the 3' end of the primer and the 5' end of the displaced oligonucleotide was tested with complexes M54/3TRP-21/4TRP, M54/3TRP-20/4TRP, and M54/3TRP-17/4TRP, containing gaps of one, two, and five nucleotides,

respectively. Results were compared with those obtained with the reference substrate (M54/3TRP/4TRP) that has no gaps but a nick between oligonucleotides 3TRP and 4TRP. As shown in Figure 2B, a single-nucleotide gap had little impact on strand displacement with all three tested RTs, although the amount of full-length products was reduced in the presence of displaced DNA, in reactions catalyzed by the quadruple mutant. The differences between WT and mutant M41L/D67N/K70R/S215Y HIV-2 RTs were more pronounced in assays carried out with substrates containing gaps of two or five nucleotides. Increased levels of shorter products and sharply decreased synthesis of full-length products were observed at shorter incubation times with M41L/D67N/K70R/S215Y_{ROD} RT, using M54/3TRP-20/4TRP (Figure 2C) and M54/3TRP-17/4TRP (Figure 2D) as substrates. In

Figure 3. Patterns of strand displacement DNA synthesis by HIV RTs with gapped DNA under pre-steady-state conditions. Assays were carried out with template-primer M54/3TRP-17 (C, control) and complex M54/3TRP-17/4TRP (SD, strand displacement conditions). Complex and active RT concentrations in these assays were 50 and 75 nM, respectively. Reactions were carried out in the presence of 100 μ M of each dNTP. In all assays, aliquots were collected after 10, 25, 50, 75, 100, 200, 400, 600, 1000, 2000, 4000, and 6000 ms. Black triangles and dots below the gels are used to indicate the position of samples taken after 200 and 2000 ms, respectively. The unextended primer (3TRP-17) is shown in lane C. Representative gels from at least two independent experiments are shown.

contrast, elongation patterns obtained with WT_ROD and D67N/K70R_ROD RTs were similar for all tested DNAs, and differences between both enzymes were smaller.

Similar results were obtained when the displaced template (i.e., 4TRP) was substituted by an oligonucleotide containing the same complementary sequence, but a 10-nucleotide unpaired overhang (4TRP-CT) (Figure S1). Elongation patterns were similar for complexes containing either 4TRP or 4TRP-CT, suggesting that the flap had little impact on the strand displacement activity of the tested RTs. In any case, the strand displacement defect of M41L/D67N/K70R/S215Y_ROD RT was clearly detected with 4TRP-CT, using both nicked and gapped substrates (Figure S1). These results highlight the defect of the M41L/D67N/K70R/S215Y mutant in different sequence contexts.

Strand Displacement Synthesis under Pre-Steady-State Conditions. Kinetics of DNA synthesis were also measured under single turnover reaction conditions using the

M54/3TRP-17/4TRP complex. As shown in Figure 3, all tested RTs synthesized full-length products after long incubation times, when the standard template-primer M54/3TRP-17 was used (see the last two lanes in control experiments, corresponding to aliquots taken at 4 and 6 s). However, adding the 4TRP oligonucleotide to the M54/3TRP-17 template-primer impaired their elongation capacity. D67N/K70R/S215Y_ROD and M41L/D67N/K70R/S215Y_ROD RTs were defective in strand displacement activity under pre-steady-state conditions, and showed limited extension of the primer even at the longest incubation times. On the contrary, WT_BH10, and ROD RT mutants M41L/S215Y and D67N/K70R were able to synthesize products of 33 nucleotides after relatively short incubation times (200 ms), with average lengths of extended primers in the range of 22.3 to 23.7 nucleotides (Figure S2). At this time point, D67N/K70R/S215Y_ROD and M41L/D67N/K70R/S215Y_ROD RTs were less efficient, with average lengths below 21

Figure 4. Nucleotide incorporation activity of mutant HIV-2_{ROD} RTs. Assays were carried out with template-primer M54/3TRP (C, control) and M54/3TRP/4TRP (SD, strand displacement conditions). Nucleotide incorporation assays were carried out in the presence of 25 μ M dCTP. DNA complexes and active RT concentrations were 30 nM and 4.5 or 6 nM, respectively. Aliquots were taken after incubating the samples for 10, 20, 30, and 40 s in control experiments (C) and 1, 2, 5, 10, 20, 30, 45, and 60 min, in assays carried out with the M54/3TRP/4TRP complex. Representative gels from at least two independent experiments are shown.

Table 2. Steady-State Kinetic Parameters for the Incorporation of dCTP at the 3' End of 3TRP in M54/3TRP Template-Primers with or without the Oligonucleotide 4TRP^a

RTs	control			strand displacement conditions			preference ^b
	k_{cat} (min ⁻¹)	K_{m} (nM)	$k_{\text{cat}}/K_{\text{m}}$ (nM/min)	k_{cat} (min ⁻¹)	K_{m} (nM)	$k_{\text{cat}}/K_{\text{m}}$ (nM/min)	
WT_ROD	2.8 $\pm$ 0.3	473.9 $\pm$ 194.8	(5.9 $\pm$ 2.5) $\times 10^{-3}$	0.077 $\pm$ 0.007	1435 $\pm$ 575	(5.4 $\pm$ 2.2) $\times 10^{-5}$	109.3 $\pm$ 64.1
M41L/S215Y_ROD	5.3 $\pm$ 0.4	591.0 $\pm$ 125.7	(8.9 $\pm$ 2.0) $\times 10^{-3}$	0.12 $\pm$ 0.01	594.9 $\pm$ 158.8	(2.0 $\pm$ 0.1) $\times 10^{-4}$	44.6 $\pm$ 16.8
D67N/K70R_ROD	13.9 $\pm$ 1.3	184.6 $\pm$ 60.9	0.0754 $\pm$ 0.0258	0.27 $\pm$ 0.01	238.5 $\pm$ 38.6	(1.13 $\pm$ 0.19) $\times 10^{-3}$	66.7 $\pm$ 25.5
M41L/D67N/K70R_ROD	5.9 $\pm$ 0.5	461.1 $\pm$ 129.5	0.0129 $\pm$ 0.0038	0.094 $\pm$ 0.008	249.3 $\pm$ 106.5	(3.8 $\pm$ 1.7) $\times 10^{-4}$	33.8 $\pm$ 18.1
D67N/K70R/S215Y_ROD	4.5 $\pm$ 0.3	347.5 $\pm$ 85.7	0.0130 $\pm$ 0.0033	0.055 $\pm$ 0.004	117.9 $\pm$ 54.0	(4.7 $\pm$ 2.2) $\times 10^{-4}$	27.9 $\pm$ 14.8
M41L/D67N/K70R/S215Y_ROD	3.5 $\pm$ 0.3	827.2 $\pm$ 227.1	(4.2 $\pm$ 1.2) $\times 10^{-3}$	0.052 $\pm$ 0.004	325.7 $\pm$ 107.9	(1.6 $\pm$ 0.5) $\times 10^{-4}$	26.1 $\pm$ 11.5
WT_BH10	4.0 $\pm$ 0.3	105.6 $\pm$ 32.5	0.0382 $\pm$ 0.0121	0.190 $\pm$ 0.007	56.3 $\pm$ 11.5	(3.4 $\pm$ 0.7) $\times 10^{-3}$	11.3 $\pm$ 4.3

^aStrand displacement conditions and control experiments, respectively. Data shown are the mean values $\pm$ standard deviation. Each of the assays was performed independently at least three times. ^bPreference = [$k_{\text{cat}}/K_{\text{m}}$ (control)]/ $k_{\text{cat}}/K_{\text{m}}$ (strand displacement conditions)].

nucleotides and bands longer than 26 nucleotides almost undetectable. The longest primer extensions (33 nucleotides) obtained with the quadruple mutant under strand displacement conditions were observed after incubating the sample for 1–2 s (Figure 3).

Nucleotide Incorporation Kinetics Using Nicked Substrates. Although primer extension estimates consistently showed the strand displacement defect of M41L/D67N/K70R/S215Y_ROD RT, a quantitative assessment of the data was difficult because observed products result from successive additions of individual nucleotides. Data obtained with

complex M54/3TRP-17/4TRP suggest that a limiting step in the strand displacement process occurs after the gap is filled. This stage of the reaction can be mimicked with complex M54/3TRP/4TRP. Using this DNA that represents a nicked strand displacement substrate, we measured nucleotide incorporation at the 3' end of 3TRP in the absence and in the presence of the displaced oligonucleotide (4TRP) for HIV-2 RTs containing different combinations of TAMs. As shown in Figure 4, dCTP incorporation rates for mutant RTs D67N/K70R/S215Y_ROD and M41L/D67N/K70R/S215Y_ROD using the M54/3TRP/4TRP complex were low or almost

Figure 5. Strand displacement RNA-dependent DNA synthesis by HIV RTs bearing different combinations of TAMs. Assays were carried out with template-primer M54 RNA/3TRP-17 (C, control) and complex M54 RNA/3TRP-17/4TRP (SD, strand displacement conditions), in the presence of 25 μ M thujaplicinol. Sequence below the schematic representation of the SD complex corresponds to the M54 RNA template. The RNA secondary structure of M54 RNA/3TRP-17 was predicted online with software developed at the University of Rochester Medical Center (<https://rna.urmc.rochester.edu/RNAstructureWeb/>).²³ Red arrow indicates template position +15, corresponding to a pause site in the elongation reaction. Primer extension reactions carried out in the presence of 30 nM template-primer (or strand displacement complex), 20 nM active RT, and 100 μ M of each dNTP. Aliquots were taken at 0.5, 1, 2, 5, and 10 min in both control and strand displacement assays. Representative gels from at least two independent experiments are shown.

negligible, while substantial amounts of nucleotide were incorporated under nondisplacement conditions. These findings suggested that nucleotide incorporation per se could be a major contributor to the strand displacement defect.

Steady-state kinetic parameters revealed that tested RTs showed important differences in catalytic turnover in the presence of control template-primer substrates (i.e., M54/3TRP) (Table 2). Although nucleotide turnover rates (k_{cat}) were similar for all tested RTs (2.8–5.9 min^{-1}), higher values were observed for the double mutant D67N/K70R_ROD RT (13.9 min^{-1}). In these control experiments, WT_ROD and WT_BH10 RTs showed similar nucleotide incorporation rates (less than 2-fold differences in k_{cat} values), although the WT HIV-2 enzyme showed a 6-fold lower catalytic efficiency (k_{cat}/K_m). Interestingly, in the presence of displaced oligonucleotide (i.e., 4TRP), the highest k_{cat} values were obtained for WT_BH10 RT and mutants M41L/S215Y_ROD and D67N/K70R_ROD (>0.12 min^{-1}). In contrast, mutant RTs D67N/K70R/S215Y_ROD and M41L/D67N/K70R/S215Y_ROD showed very low catalytic rates in the presence of displaced oligonucleotide ($k_{\text{cat}} < 0.06 \text{ min}^{-1}$), with the quadruple mutant showing the lowest catalytic efficiency under these conditions.

TAM-containing RTs were also compared using primer extension assays carried out in the absence or presence of 4TRP (Figure S3), but normalizing the RT amounts, according to the catalytic turnover obtained with substrate M54/3TRP. Time points showing the accumulation of full-length products in control experiments (C lanes in Figure S3) revealed similar

patterns for all five enzymes. However, the corresponding side-by-side comparisons carried out with the strand displacement substrate (M54/3TRP-17/4TRP) showed reduced extension efficiencies for all TAM-containing RTs as compared with a WT enzyme. Nevertheless, in agreement with results obtained under pre-steady-state conditions, extensions obtained with D67N/K70R/S215Y_ROD and M41L/D67N/K70R/S215Y_ROD RTs were inefficient, with almost undetectable elongation at short incubation times and reduced amounts of full-length products in the last time point of the reaction. Complementary assays carried out with TAM-containing HIV-2_{ROD} RTs bearing additional mutations at positions 75, 181, or 210 revealed similar extension patterns under control and strand displacement conditions (Figure S4), suggesting the dominant effect of TAMs D67N, K70R and S215Y.

In contrast, those mutations had no effect on strand displacement activity when present in the HIV-1_{BH10} RT. As shown in Figure S5, little differences were detected between the strand displacement activities of WT_BH10 RT and mutant M41L/T215Y_BH10 and D67N/K70R/T215Y/K219Q_BH10 RTs, when using the M54/3TRP-17/4TRP complex, thereby confirming our initial assessments obtained with the nicked substrate (M54/3TRP/4TRP) (Table 1).

Strand Displacement Assays Using Template RNA. Further evidence of the strand displacement defect of the M41L/D67N/K70R/S215Y_ROD RT was obtained from assays carried out with the complex M54 RNA/3TRP-17/4TRP, where the template M54 was an RNA oligonucleotide of 54 bases. As shown in Figure 5, WT_BH10 and mutant

Figure 6. Effects of TAMs on the replication kinetics of recombinant HIV-2 clones bearing mutant or WT RTs. The production of antigen HIV-2 p24 in culture supernatants was determined at days 1, 2, 3, 4, 5, and 6. For each virus, the slope of the plot provides an estimate of the viral replication capacity. The histogram on the right compares the slopes obtained for each mutant. Values represent mean values $\pm$ standard deviations obtained from at least three independent experiments.

Figure 7. Crystal structure of HIV-1 RT showing the location of residues 41, 61, 67, 70 and 215. View of the ternary complex of HIV-1_{HXB2} RT, bound to double-stranded DNA and dTTP (PDB coordinates from file 1RTD). The RT subunits are represented by cyan (p66) and green (p51) ribbons. The template and primer strands are shown with red and orange sticks (left panel) or cartoons (right panel). In both panels, sphere representations indicate the location of the incoming dTTP (pink), and residues 61 (purple), 67 and 70 (green), and 41 and 215 (yellow) of the 66-KDa subunit of the RT.

D67N/K70R_{ROD} had similar primer extension patterns, while in the presence of 4TRP, the WT_{ROD} RT was able to synthesize full-length products although in limited amounts and after relatively long incubation times. In contrast, assays carried out with M54 RNA/3TRP-17/4TRP and mutant M41L/D67N/K70R/S215Y_{ROD} RT (SD samples) showed low primer extension efficiencies, consistent with its defective phenotype. All RTs showed major pauses at positions +5 and +6 in strand displacement assays. However, bands at positions +15 or +19 representing longer elongation products were not detected in assays carried out with mutant M41L/D67N/K70R/S215Y_{ROD} RT. All assays were carried out in the presence of the ribonuclease H inhibitor β -thujaplicinol²⁴ to

avoid any potential interference with the DNA synthesis reaction of the RT-associated ribonuclease H activity found in tested RTs.

Effects of TAM Combinations on the HIV-2 Replication Capacity. Viral replication kinetics assays carried out in MT-4 cells showed that the accumulation of TAMs in HIV-2 had a negative effect on viral fitness (Figure 6). Growth kinetics curves were analyzed by fitting a linear model to the log-transformed HIV-2 p24 data during the exponential growth phase. The slope of p24 antigen production provides a reliable estimate of the viral replication capacity.²⁵ As shown in Figure 6, the presence of the four major TAMs (M41L, D67N, K70R, and S215Y) produced a significant decrease in viral replication

capacity. Viral titers measured three or 4 days postinfection were 2 orders of magnitude lower for the mutant virus containing M41L/D67N/K70R/S215Y_{ROD} RT than for the WT HIV-2. This effect was observed with the two viruses containing the four TAMs. However, differences between the WT and the double mutant D67N/K70R were not significant. These findings are consistent with the partial loss of function shown by the RTs defective in strand displacement activity described in this work.

DISCUSSION

Strand displacement reactions play relevant roles in many biological processes, including DNA replication and recombination, cotranscriptional RNA folding, spliceosome assembly, and in the regulation of bacterial mRNA levels through riboswitches (for reviews, see refs 26–28). Strand displacement activity of replicative polymerases is usually enhanced by accessory factors that cover the DNA substrates. Examples are PCNA, β -clamp, thioredoxin, UL44, and the β subunit of the DNA polymerase γ .²⁹ In contrast, the presence of subdomains (i.e., terminal protein regions) that encircle the synthesized DNA facilitate the strong strand displacement activity of bacteriophage Φ 29 DNA polymerase, as well as related enzymes of the B2 family.³⁰

Strand displacement is required for the replication of retroviruses and retroelements (e.g., LTR- and non-LTR retrotransposons), and RTs encoded within their genomes are able to complete reverse transcription in the absence of accessory factors^{6,7,9,31} (reviews in refs 1, 2). However, those enzymes lack structural motifs or subdomains channeling the DNA during polymerization. HIV-1 and HIV-2 RTs are heterodimeric enzymes with an active DNA polymerase catalytic site in the large subunit. Catalytic residues (Asp110, Asp185, and Asp186) coordinate with two divalent cations (Mg^{2+}) that are required for nucleotide incorporation. Residues in the vicinity, such as Lys65, Arg72, Asp113, Ala114, Tyr115, and Gln151 are involved in interactions with the incoming dNTP.¹⁴ The β 3- β 4 hairpin loop in the fingers subdomain (residues 64–72) also plays an important role in dNTP binding and resistance to nucleoside analogues. Phe61 and Ile63 at the base of the hairpin contact nucleotide +1 at the template strand (Figure 7). The involvement of these residues in strand displacement activity was suggested based on a potential effect on base pairing between the template overhang and the 5' end of the displaced oligonucleotide. The large reduction in strand displacement activity shown by mutant F61W was consistent with the proposed model.¹⁶ However, amino acid substitutions of the equivalent residues in MLV RT (i.e., L99W, L99A, etc.) had negligible effects on strand displacement activity.¹⁹

Despite those contradictory findings, it has been shown that in *E. coli* DNA polymerase I nonconservative substitutions S769A/F771A and R841A in the fingers subdomain of the enzyme are deleterious for strand displacement activity.³² Arg841 interacts with template nucleotide +1 while Ser769 and Phe771 contact the 5' end of the displaced oligonucleotide as predicted by modeling studies of the Klenow fragment of *Bacillus stearothermophilus* DNA polymerase I and double-stranded DNA.³²

Our data show that the strand displacement activity of HIV RTs bearing β 3- β 4 hairpin loop substitutions D67N/K70R is similar to that shown by WT enzymes, but in HIV-2 RT, the addition of M41L or S215Y produces defective enzymes with

reduced strand displacement activity. This effect is even more pronounced with the quadruple mutant (i.e., M41L/D67N/K70R/S215Y). In agreement with these findings, the replication capacity of HIV-2_{ROD} bearing mutations D67N/K70R was similar to that shown by the WT virus, but higher than the one shown by mutant HIV-2 bearing mutations M41L/D67N/K70R/S215Y or M41L/D67N/K70R/I75V/S215Y in the viral RT. Met41 locates at the fingers subdomain while Ser215 is part of the palm subdomain (Figure 7). However, both residues are away from the template-primer and they are not expected to participate in direct interactions with the nucleic acid substrate of the RT. M41L together with S215Y had a small impact on the strand displacement activity of the HIV-2 RT.

The mutant M41L/D67N/K70R/S215Y_{ROD} RT shows defective strand displacement activity with all tested substrates, including nicked and gapped oligonucleotide complexes (Figure 2). These defects were also observed when flaps were introduced at the 5' end of the displaced DNA. Nucleotide incorporation kinetics experiments carried out with a nicked complex reveal that the reduced strand displacement activity of mutants D67N/K70R/S215Y and M41L/D67N/K70R/S215Y could be related to their lower turnover rates in the presence of displaced oligonucleotide. Thus, their catalytic constants (k_{cat}) for nucleotide incorporation in these conditions are particularly low for mutant D67N/K70R/S215Y_{ROD} and M41L/D67N/K70R/S215Y_{ROD} RTs ($k_{cat} < 0.06 \text{ min}^{-1}$), although their catalytic turnover rates were similar to those obtained with other RTs using control M54/3TRP template-primers. In agreement with those findings, DNA polymerization reactions carried out under pre-steady-state conditions show that the accumulation of TAMs renders RTs with lower turnover nucleotide incorporation rates and poorly extended primers when using gapped substrates (Figure 3). In the absence of displaced oligonucleotide, all RTs showed relatively high polymerization activity, although the double mutant M41L/S215Y showed a lower turnover rate. This finding is consistent with previous reports showing that WT and mutant S215Y HIV-2 RT had low DNA polymerization activity in assays carried out with homopolymeric template-primers.³³ In mutants D67N/K70R/S215Y and M41L/D67N/K70R/S215Y, the defect appears to be compensated by the presence of D67N/K70R.

HIV-2 is intrinsically resistant to non-nucleoside reverse transcriptase inhibitors, to several protease inhibitors, and to enfuvirtide (a fusion inhibitor).³⁴ Integrase strand transfer inhibitor-based regimens or protease inhibitor-based therapies are the recommended treatment options for HIV-2-infected persons.³⁵ The NRTIs tenofovir and emtricitabine constitute the backbone of the most effective antiretroviral therapies against HIV-1 and HIV-2, and are preferred to other combinations such as zidovudine and lamivudine.^{34,35} However, thymidine analogues such as zidovudine and stavudine are still employed as first line combination antiretroviral therapies in low- to middle-income countries,³⁶ and TAMs are frequently observed in transmitted HIV variants.^{37,38} In HIV-2, the combination of amino acid substitutions K65R, Q151M, and M184V at the nucleotide binding site of the RT confers class-wide NRTI resistance.^{34,39} Unlike in HIV-1, TAMs are rarely selected in HIV-2^{39–41} where their accumulation does not confer resistance to zidovudine and other nucleoside analogues.^{21,33} Comprehensive analysis of sequences deposited in GenBank and other

resources revealed only one reported case of an HIV-2 RT containing three or more TAMs. Specifically, the combination D67N/K70R/S215Y was found in the RT of a virus isolate obtained from an untreated patient infected in Equatorial Guinea.⁴² The partial sequence of the *pol* gene allowed its classification as an HIV-2 group B variant, but unfortunately, the virus was poorly characterized and clinical information on the evolution of the patient has not been reported.

Biochemical assays described above reveal that D67N/K70R/S215Y_{ROD} RT is also defective in strand displacement activity, showing elongation patterns that were similar to those obtained with the quadruple mutant (Figures 3 and S3). In contrast, the strand displacement activities of WT and mutant D67N/K70R/T215Y/K219Q HIV-1_{BH10} RTs were very similar. We have previously shown that the presence of Met73 and Ile75 in the fingers subdomain of the HIV-2 RT restricts the development of resistance to nucleoside analogues through the excision pathway.²¹ Modeling studies predicted that different conformations for the β 3- β 4 hairpin loops of HIV-1 and HIV-2 RTs could affect hydrogen bond interactions at position 70, necessary for nucleoside analogue excision.²¹ In addition, we have now shown that the accumulation of TAMs in HIV-2 renders RTs with reduced strand displacement activity, while the expected loss of viral fitness has been confirmed by the lower replication capacity of mutant HIV-2 bearing RTs containing 4 TAMs.

In addition to their importance as a target for antiretroviral therapy, RTs are also valuable biotechnological tools for their ability to synthesize DNA using RNA templates. WT and engineered RTs of avian myeloblastosis virus, MLV, HIV-1, and *Geobacillus stearothermophilus* group II introns have been developed into more efficient tools to study gene expression by increasing catalytic efficiency, processivity, thermostability, and fidelity of DNA synthesis.^{43,44} In addition, the recent development of novel technologies such as cDNA microarrays and deep RNA sequencing (RNA-seq) has opened new opportunities to exploit other intrinsic properties of retroviral RTs.^{45,46} In transcriptomics, the preparation of RNA libraries is important to obtain valuable data particularly for lowly expressed RNA species.⁴⁷ The strong strand displacement activity shown by retroviral RTs contributes to an uneven transcript coverage where 3' terminal sequences of genes are overrepresented.⁴⁸ Several methods including the use of specific linkers that hybridize to the mRNA have been used to circumvent these problems and generate more uniform read coverages. In this context, RTs defective in strand displacement activity could be useful for future developments in transcriptomics technologies.

METHODS

RT Expression and Purification. Heterodimeric (p66/p51) WT and mutant HIV-1 RTs were expressed and purified as described previously.^{21,49–57} Histidine-tagged WT HIV-2_{ROD} RT and mutants M41L/D67N/K70R/I75V/I181Y/S215Y, M41L/D67N/K70R/I75V/N210L/S215Y, M41L/D67N/K70R/I75V/S215Y, M41L/D67N/K70R/S215Y, and D67N/K70R were obtained as p68/p54 heterodimers after coexpression of the large subunit of the HIV-2_{ROD} RT with the HIV-2 protease.^{21,58} Enzymes were kept in 50 mM Tris-HCl, pH 7.0, containing 25 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 10% glycerol, and quantified by active site titration before biochemical studies.^{59,60} RT concentrations used in enzymatic assays were those obtained

from active site titrations, except for strand displacement assays shown in Figures S3 and S4 where enzyme concentrations were normalized to the activity shown by the WT BH10 RT.

Mutagenesis. Site-directed mutagenesis was carried out by using the standard QuikChange (Stratagene) protocol. HIV-2 RT mutants (M41L/S215Y, M41L/D67N/K70R, and D67N/K70R/S215Y) were obtained by using as template the pT5m expression plasmid encoding the p68 subunit of the WT HIV-2_{ROD} enzyme,⁶¹ or the RT bearing mutations D67N and K70R.²¹ Complementary mutagenic primers (5' GAAATCTGTGAAAACTGGAAAAAGAAG 3' and 5' CTTCTTTTCCAGTTTTTCACAGATTTC 3', for M41L, and 5' TGGCCTAGGATTTTATACCCCAGATGA 3' and 5' TCATCTGGGGTATAAAATCCTAGGCCA 3', for S215Y) were used to amplify the entire pT5m plasmid (containing the nucleotide sequence encoding WT or D67N/K70R HIV-2_{ROD} RT), in thermocycling reactions carried out with high-fidelity Pfu DNA polymerase. After mutagenesis, the entire RT-coding regions were sequenced and, if correct, used for RT expression and purification.

Strand Displacement Assays. Displacement and non-displacement substrates were obtained with DNA or RNA templates of 54 nucleotides (i.e., M54 DNA and M54 RNA), and DNA primers of 17, 20, 21, and 22 bases depending on the assay (designated as 3TRP-17, 3TRP-20, 3TRP-21, and 3TRP, respectively). Nontemplate DNA strands of 32 nucleotides (4TRP) and 44 nucleotides (4TRP-CT) were used to prepare displacement substrates.

DNA Primers (20 pmol) were labeled at their 5' termini with 10 μ Ci of [γ -³²P] ATP (PerkinElmer) and T4 polynucleotide kinase (New England BioLabs) before annealing to M54 templates.⁶² M54/3TRP and related nondisplacement complexes containing primers 3TRP-17, 3TRP-20, or 3TRP-21, were obtained by adding 30 pmol of the corresponding primer (i.e., 3TRP, 3TRP-17, 3TRP-20, or 3TRP-21) to 60 pmol of M54 and heating the sample at 100 °C for 4 min in 50 μ L of 50 mM Tris-HCl pH 8.0 buffer, with 50 mM KCl and 6 mM MgCl₂, followed by slowly cooling at room temperature. M54/3TRP/4TRP (and related complexes containing primers 3TRP-17, 3TRP-20, or 3TRP-21) were obtained by the same procedure, but adding 90 pmol of 4TRP or 4TRP-CT to the mixture of 3TRP and M54. Displacement complexes (e.g., M54/3TRP/4TRP and complexes with 3TRP-related primers), were incubated at 100 °C for 4 min in 50 μ L of 50 mM Tris-HCl pH 8.0 buffer, containing 50 mM KCl and 6 mM MgCl₂, and kept at 70 °C for 30 min. Then, complexes were maintained at 33 °C for 30 min and diluted to 300 nM final concentration in 50 μ L of 50 mM Tris-HCl pH 8.0 buffer, containing 50 mM KCl, 6 mM MgCl₂, and 5 mM DTT. The same procedures were followed to obtain complexes M54 RNA/3TRP-17 and M54 RNA/3TRP-17/4TRP used in strand displacement reactions carried out with RNA templates.

Strand displacement reactions were carried out with displacement and nondisplacement substrates (e.g., M54/3TRP/4TRP and M54/3TRP complexes, respectively). RTs (10–180 nM) were preincubated 10 min at 37 °C with the corresponding template-primer (with or without the displaced oligonucleotide) at 60 nM concentration, in 20 μ L of 50 mM Tris-HCl pH 8.0 buffer, containing 50 mM KCl, 6 mM MgCl₂, and 5 mM DTT. Then, DNA polymerization reactions were initiated by adding 20 μ L of 50 mM Tris-HCl pH 8.0 buffer, containing 50 mM KCl, 6 mM MgCl₂, 5 mM DTT and a mixture of all dNTPs at a final concentration of 100 μ M each.

Aliquots of 4 μ L were removed at different times and mixed with 4 μ L of stop solution (10 mM EDTA in 90% formamide, containing 3 mg/mL xylene cyanol and 3 mg/mL bromophenol blue). Reactions with M54 RNA templates were carried out under the same conditions, although the preincubation step was reduced to 2 min, and the concentration of the RNase H inhibitor thujaplicinol was maintained at 25 μ M throughout the assay. In all cases, reaction products were visualized on denaturing 20% polyacrylamide-8 M urea gels, after exposure to phosphorimaging plates. A BAS1500 scanner (Fuji) and the program Tina version 2.09 (Raytest Isotopenmessgerate GmbH, Staubenhardt, Germany) were used for assay documentation and determination of elongation yields.

Pre-Steady-State Kinetics. DNA primers (95 pmol) were labeled at their 5' termini with 30 μ Ci of [γ - 32 P] ATP (PerkinElmer) and T4 polynucleotide kinase (New England BioLabs) before annealing to the M54 template.⁶² M54/3TRP-17 was obtained by adding 240 pmol of the 3TRP-17 primer to 480 pmol of M54, after heating the sample at 100 °C for 4 min followed by slowly cooling at room temperature. M54/3TRP-17/4TRP was obtained by the same procedure, but adding 720 pmol of 4TRP to the mixture of 3TRP-17 and M54. The strand displacement complex (M54/3TRP-17/4TRP) was incubated at 100 °C for 4 min. This complex was then maintained at 33 °C for 30 min. M54/3TRP-17 and M54/3TRP-17/4TRP were prepared at a concentration of 2 μ M.

DNA primer elongation was determined under pre-steady-state conditions using different RTs and complexes M54/3TRP-17 or M54/3TRP-17/4TRP. Assays were carried out with a rapid quench-flow instrument (model QFM-400, Bio-Logic Science Instruments, Claix, France), upgraded with a mixer cross and a special mixer (Bio-Logic) that allowed more accurate monitoring of enzymes with turnovers higher than 20 s⁻¹.^{51,56,63} Reactions were initiated by adding 12 μ L of a mixture of all dNTPs at 200 μ M each, diluted in a 50 mM Tris-HCl pH 8.0 buffer, containing 50 mM KCl and 24 mM MgCl₂, to 12 μ L of a solution of 50 mM Tris-HCl pH 8.0 buffer, containing 50 mM KCl, 100 nM M54/3TRP-17 or 100 nM M54/3TRP-17/4TRP complexes and 150 nM RT. At appropriate times (in the range of 10–6000 ms), reactions were stopped with EDTA (0.3 M final concentration). Reaction products were separated by electrophoresis on a denaturing 20% (w/v) polyacrylamide/8 M urea gel and analyzed by phosphorimaging with a BAS1500 scanner (Fuji).

Nucleotide Incorporation Assays. Kinetic parameters for single-nucleotide incorporation under steady-state conditions were obtained as previously described.⁵¹ Assays were carried out in 40 μ L of 50 mM Tris-HCl pH 8.0 buffer, containing 50 mM KCl, 6 mM MgCl₂ and 5 mM DTT. Active RT concentrations were in the range of 10–15 nM, and DNA/DNA complexes M54/3TRP and M54/3TRP/4TRP were added to 30 nM (final concentration). After incubating the RT with the corresponding DNA complex for 10 min at 37 °C, reactions were initiated by adding dCTP at appropriate concentrations, in the range of 0.0024 μ M to 75 μ M. Aliquots of 4 μ L were withdrawn at appropriate times and mixed with equal amounts of the EDTA/formamide solution described above to obtain the corresponding product against time plots, at each substrate concentration. Samples were resolved by electrophoresis on a denaturing 20% polyacrylamide-urea gel and primer extension was measured using a BAS1500 scanner. The Michaelis–Menten constants (K_m) and catalytic rates of

nucleotide incorporation (k_{cat}) were obtained after fitting the nucleotide incorporation data to the Michaelis–Menten equation using the GraphPad Prism version 6.00 software (GraphPad Software, La Jolla, CA, USA).

Replication Capacity Assays. Infectious viral stocks from recombinant proviral clones obtained after cotransfection with three DNA fragments covering the full-length HIV-2 proviral DNA²¹ were obtained by electroporation of MT-4 cells (NIH AIDS Research and Reference Reagent Program). Seven days after transfection, supernatants were collected and p24Gag concentration was determined with an enzyme-linked immunosorbent assay (INNOTEST HIV Antigen mAb). Chemiluminescence titration assays in HeLa-derived TZM-bl cells (NIH AIDS Research and Reference Reagent Program) were used to determine the mean 50% tissue culture infective dose(s) (TCID₅₀) of each viral stock, as previously described.⁶⁴

Viral replication kinetics of mutant and WT viruses were assayed by infecting 1×10^6 MT-4 cells with 1000 TCID₅₀ of each viral stock (multiplicity of infection of 0.001).²⁵ After incubation for 4 h at 37 °C in the presence of 5% CO₂, cells were washed twice with phosphate-buffered saline and resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum. Viral replication was quantified by measuring HIV-2 p24 capsid protein production in the culture supernatant for 6 days. Growth kinetics were analyzed by fitting a linear model to the log-transformed p24 data during the exponential growth phase by maximum likelihood methods using GraphPad Prism software.²⁵ The slopes of the plots were determined to estimate viral replication capacities, and the significance of differences between slopes were calculated using an unpaired *t* test with Welch's correction.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsinfectdis.9b00512>.

Figure S1, analysis of the effects of 5' overhangs in the displaced oligonucleotide in the strand displacement activity of WT and mutant HIV-2_{ROD} RTs; Figure S2, quantitative analysis of primer extensions obtained under pre-steady-state conditions; Figure S3, results of strand displacement DNA synthesis assays carried out with HIV RTs bearing different combinations of TAMs (WT_BH10, D67N/K70R_ROD, M41/D67N/K70R_ROD, D67N/K70R/S215Y_ROD, M41L/D67N/K70R/S215Y_ROD, M41L/S215Y_ROD, and WT_ROD); Figure S4, results of strand displacement DNA synthesis assays carried out with HIV-2 RTs bearing the four TAMs together with additional amino acid substitutions such as I75V, I181Y, and N210L; Figure S5, results of strand displacement DNA synthesis assays carried out with WT and mutant HIV-1 RTs M41L/T215Y and D67N/K70R/T215Y/K219Q (PDF)

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Author Contributions

M.A.M. and L.M.-A. designed research; S.M.-A., M.A., and M.N. carried out experimental work; S.M.-A., M.A., M.N., M.A.M., and L.M.-A. analyzed data; S.M.-A., M.A.M., and L.M.-A. wrote the paper.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

HIV, human immunodeficiency virus; MLV, murine leukemia virus; NC, nucleocapsid; PPT, polypurine tract; LTR, long terminal repeat; NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PBS, primer binding site; RT, reverse transcriptase; TAM, thymidine analogue resistance mutation; WT, wild-type.

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