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Molecular basis of the association of H208Y and thymidine analogue resistance mutations M41L, L210W and T215Y in the HIV-1 reverse transcriptase of treated patients

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ABSTRACT

Thymidine analogue resistance mutations (TAMs) in HIV-1 reverse transcriptase (RT) associate in two clusters: (i) TAM1 (M41L, L210W and T215Y) and TAM2 (D67N, K70R, K219E/Q, and sometimes T215F). The amino acid substitution H208Y shows increased prevalence in patients treated with nucleoside analogues and is frequently associated with TAM1 mutations. We studied the molecular mechanism favoring the selection of H208Y in the presence of zidovudine, tenofovir and other nucleoside RT inhibitors (NRTIs). NRTI susceptibility was not affected by the addition of H208Y in phenotypic assays carried out in MT-4 cells using recombinant HIV-1 containing wild-type (subtype B, BH10), H208Y, M41L/L210W/T215Y or M41L/H208Y/L210W/T215Y RTs. However, enzymatic studies carried out with purified RTs revealed that in the presence of M41L/L210W/T215Y, H208Y increases the RT's ability to unblock and extend primers terminated with zidovudine, tenofovir and in a lesser extent, stavudine. These effects were observed with DNA/DNA complexes (but not with RNA/DNA) and resulted from the higher ATP-dependent excision activity of the M41L/H208Y/L210W/T215Y RT compared with the M41L/L210W/T215Y mutant. The increased rescue efficiency of the M41L/H208Y/L210W/T215Y RT was observed in the presence of ATP but not with GTP or ITP. Molecular dynamics studies predict an alteration of the stacking interactions between Tyr^{215} and the adenine ring of ATP due to long-distance effects caused by tighter packaging of Tyr^{208} and Trp^{212}. These studies provide a mechanistic explanation for the association of TAM-1 and H208Y mutations in viral isolates from patients treated with NRTIs.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS). To date, thirteen different compounds inhibiting the viral reverse transcriptase (RT) have been approved for treatment of HIV infection. Eight of those drugs are nucleos(t)ide reverse transcriptase inhibitors (NRTIs). NRTIs act as chain-terminators due to the lack of a 3′-OH group on the ribose ring (reviewed in Menéndez-Arias, 2008). Retroviral RTs synthesize a double-stranded proviral DNA from the genomic single-stranded viral RNA. The HIV-1 RT is a heterodimer composed of two subunits of 66 and 51 kDa, designated as p66 and p51, respectively. The p66 subunit contains a DNA polymerase domain (composed of fingers, palm and thumb subdomains) and an RNase H domain. Both domains are joined by a connection subdomain. The smaller subunit (p51) has the same amino acid sequence as p66 but lacks the RNase H domain that extends from residues 441 to 560 (Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993; Huang et al., 1998).

HIV-1 resistance to NRTIs can be conferred by mutations acting through a discrimination mechanism by which the RT facilitates the incorporation of the natural dNTP over the nucleoside analogue in its triphosphate form (Sarafianos et al., 1999; Ray et al., 2002; Deval et al., 2004), or through an excision mechanism by which primer-terminating NRTIs can be removed from the 3′ end of the DNA in the presence of pyrophosphate (PPi), ATP or an alternative PPi donor. The excision reaction renders the free 3′ end of the primer and a tri- or tetra-phosphorylated product (Arion et al., 1998; Meyer et al., 1998). The likely physiological PPi donor is ATP

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(Meyer et al., 1999; Smith et al., 2005). Resistance to cytidine analogues [e.g. lamivudine (β-L-(−)-2′,3′-dideoxy-3′-thiacytidine; 3TC) or emtricitabine (β-L-(−)-2′,3′-dideoxy-5-fluoro-3′-thiacytidine; FTC)] is usually conferred by mutations M184I or M184V that exert their effects through a nucleotide discrimination mechanism (Sarafianos et al., 1999). In contrast, thymidine analogue resistance mutations (TAMs) such as M41L, D67N, K70R, L210W, T215F/Y and K219E/Q enhance NRTI excision on primers preferentially terminated with zidovudine (3′-azido-3′-deoxythymidine; AZT), stavudine (2′,3′-didehydro-2′,3′-dideoxythymidine; d4T) or tenofovir (reviewed in Menéndez-Arias, 2013). TAMs facilitate excision by forming a binding site for the adenine moiety of ATP and a network of hydrogen bonds that place the phosphate groups in a proper orientation for the pyrophosphorolytic reaction (Tu et al., 2010).

Clinical studies have shown that TAMs associate in two different clusters: TAM1 including mutations M41L, L210W and T215Y, and TAM2 that is formed by the association of D67N, K70R, K219E or Q, and sometimes T215F (Yahi et al., 1999; Cozzi-Lepri et al., 2005). A number of accessory mutations associated with any of both clusters have been identified after analyzing HIV-1 genotypes obtained from patients receiving antiretroviral therapy (reviewed in Menéndez-Arias, 2013). The mechanisms by which accessory mutations contribute to drug resistance have been determined only in a few cases. For example, among those associated with the TAM1 cluster, E44D enhances nucleotide analogue excision (Girouard et al., 2003), R284K acts by increasing nucleotide incorporation after unblocking the inhibitor-terminated primer (Betancor et al., 2012), and N348I decreases the RNase H activity, while promoting primer unblocking in RNA/DNA complexes (Yap et al., 2007).

Previous studies have shown an increased prevalence of H208Y in patients treated with nucleoside analogues (Gonzales et al., 2003; Stürmer et al., 2003; Rhee et al., 2005; Saracino et al., 2006; Svicher et al., 2006; Nebbia et al., 2007; Shahriar et al., 2009; von Wyl et al., 2010; Betancor et al., 2012). In addition, the low prevalence of H208Y in untreated patients (<1%) has been found to increase by more than 10-fold in patients treated with NRTIs (Rhee et al., 2005; Nebbia et al., 2007; von Wyl et al., 2010; Betancor et al., 2012). H208Y frequently associates with mutations of the TAM1 cluster (Gonzales et al., 2003; Stürmer et al., 2003; Saracino et al., 2006; Svicher et al., 2006; Nebbia et al., 2007; von Wyl et al., 2010; Betancor et al., 2012). It has been proposed that the emergence of M41L and T215Y precedes the development of H208Y (Stürmer et al., 2003). In this work, we have studied the influence of H208Y in HIV-1 NRTI susceptibility in the presence and in the absence of TAM1 mutations. Enzymatic studies demonstrate that H208Y enhances ATP-mediated excision of NRTIs in DNA-DNA primer-template complexes, when accompanied by substitutions M41L, L210W and T215Y.

2. Materials and methods

2.1. Mutagenesis

Site-directed mutagenesis was carried out with the QuikChange Site-Directed Mutagenesis kit (Stratagene) by following the manufacturer's instructions. As templates we used p66RTB, a plasmid containing the nucleotide sequence that encodes for the 66-kDa subunit of the HIV-1 group M subtype B (BH10 strain) RT (Matamoros et al., 2005), and previously described derivatives carrying RT mutations M41L/T215Y and M41L/L210W/T215Y (Betancor et al., 2010, 2012). Mutagenic primers 5′-GGAGCTGAGACAATATCTGTGGAGGTGGGG-3′ and 5′-CCCCACCTCCACAGATATTG TCTCAGCTCC-3′ were used to introduce the H208Y mutation in the plasmid containing the M41L/L210W/T215Y complex. All introduced mutations were confirmed by DNA sequencing.

2.2. HIV drug susceptibility, replication and growth competition assays

Assays were carried out with recombinant HIV-1 variants generated after co-transfecting MT-4 cells with an RT-deleted HXB2-D clone previously linearized with BstEII and a PCR product containing the nucleotide sequence encoding for the 66-kDa subunit of the RT (Kellam and Larder, 1994). The RT-coding region of recovered viruses was fully sequenced in order to check for reversions or undesired mutations. MT-4 cells and the deleted HXB2-D clone were obtained from the AIDS Reagent Program (Medical Research Council). MT-4 cells were used to propagate the recombinant virus. Its susceptibility to NRTIs was determined as previously described, using a multiplicity of infection of 0.003 (Betancor et al., 2010). Viral replication kinetics were assayed by infecting peripheral blood mononuclear cells (PBMCs) (mixed from three healthy donors), previously stimulated with phytohemagglutinin and interleukin 2 (Puertas et al., 2009). In these assays, viral replication rates were determined from the amounts of HIV-1 p24 antigen produced during the exponential phase of viral growth, as described (Puertas et al., 2009). Growth competition experiments in MT-4 cells were carried out in the absence of drug (Prado et al., 2004). A multiplicity of infection of 0.001 was used and competing viruses were mixed at ratios of 80:20, 50:50 and 20:80. RT inhibitors were obtained from the NIH AIDS Research and Reference Reagent Program.

2.3. RT purification

Recombinant RTs were expressed and purified as previously described (Boretto et al., 2001; Matamoros et al., 2005). RT p66 subunits containing His_6 tags at their C-terminal end were co-expressed with the HIV-1 protease in *Escherichia coli* XL1 Blue to obtain p66/p51 heterodimers. These heterodimers were then purified by ionic exchange chromatography, followed by immobilized metal affinity chromatography on Ni^{2+}-nitriloacetic acid-agarose (Matamoros et al., 2005). Purity of the enzymes was checked by SDS–polyacrylamide gel electrophoresis and RT concentrations were determined by active site titration as described (Kati et al., 1992; Menéndez-Arias, 1998).

2.4. Nucleotides, templates and primers

Stock solutions (100 mM) of dATP, dCTP, dGTP, dTTP, ATP, CTP and GTP were obtained from GE Healthcare. ITP was obtained from Sigma–Aldrich. AZTTP and carbovir-triphosphate (CBVTP) were purchased from Moravek Biochemicals (Brea, CA), while d4TTP and tenofovir diphosphate were obtained from TriLink Biotechnologies (San Diego, CA) and Sierra Bioresearch (Tucson, AZ), respectively. Prior to use, nucleoside triphosphate solutions were treated with inorganic pyrophosphatase (Roche), as previously described (Mas et al., 2002). Synthetic oligonucleotides were obtained from Life Technologies. Primers 25PGA, 31Trna and D38rna were labeled at their 5′ termini with [γ-^{32}P]ATP (Perkin Elmer) and T4 polynucleotide kinase (Promega) before annealing them to their complementary strand.

2.5. Primer unblocking and extension assays

RT-catalyzed DNA rescue reactions were carried out with DNA/DNA and RNA/DNA duplexes D38/25PGA, D38C/25PGA, D38T/25PGA, D38rna/25PGA and D38Trna/25PGA. After the phosphorylation of the primer and its annealing to the template, the template-primer complex (75 nM) and the RT (60 nM) were preincubated for 10 min at 37 °C in 50 mM Hepes pH 7.0 buffer, containing 15 mM NaCl, 15 mM magnesium acetate, 130 mM potassium acetate, 1 mM dithiothreitol and 5% (w/v) polyethylene glycol (Matamoros et al., 2004; Betancor et al., 2012). Reactions

Table 1
Susceptibility of HIV-1 constructs to RT inhibitors.

RTs	IC_{50} (nM)[a]				
	AZT	d4T	Tenofovir [b]	Abacavir	Emtricitabine
WT	3.1 ± 1.3	297.8 ± 72.0	9.1 ± 3.8	1724 ± 406	405 ± 58
H208Y	4.0 ± 2.2 (1.3)	195.0 ± 65.1 (0.7)	10.0 ± 3.8 (1.1)	1704 ± 196 (1.0)	718 ± 72 (1.8)
M41L/T215Y	19.7 ± 8.8 (6.4)	395.0 ± 117.6 (1.3)	18.5 ± 5.7 (2.0)	2735 ± 164 (1.6)	1081 ± 261 (2.7)
M41L/L210W/T215Y	16.3 ± 5.9 (5.3)	320.0 ± 125.2 (1.1)	18.0 ± 8.3 (2.0)	1990 ± 260 (1.2)	686 ± 119 (1.7)
M41L/H208Y/L210W/T215Y	19.0 ± 5.7 (6.1)	338.8 ± 60.7 (1.1)	27.9 ± 9.4 (3.1)	1600 ± 198 (0.9)	737 ± 60 (1.8)

[a] The IC_{50} values shown are the averages ± standard deviations of at least three tests, with each one performed six times. The fold increase in IC_{50} relative to the wild-type HXB2 virus control carrying the RT sequence of BH10 is shown between parentheses.
[b] Experiments were carried out with the water soluble diester prodrug tenofovir disoproxil fumarate.

were initiated by adding AZTTP, d4TTP, CBVTP or tenofovir-diphosphate at a final concentration of 25 μM. After incubating the sample for 10 min at 37 °C, rescue reactions were initiated by adding a mixture of all four dNTPs at a final concentration of 100 μM each, together with the PPi donor (e.g. ATP, CTP, GTP or ITP). To avoid the formation of dead-end complexes (Meyer et al., 1998, 2000), the next complementary dNTP (dATP or dTTP, under our assay conditions) was supplied at a final concentration of 1 μM. Rescue reaction catalytic rates (k_{res}) were obtained in the same conditions described above, using different rNTP concentrations.

In the case of the RNA/DNA rescue reactions, the procedure was similar to that described above but the final concentrations of NRTIs and dNTPs were 50 μM and 200 μM, respectively. Aliquots were removed between 0 and 60 min of incubation at 37 °C, and the reactions were stopped by adding an equal amount of sample loading buffer (10 mM EDTA in 90% formamide containing 3 mg/ml xylene cyanol FF and 3 mg/ml bromophenol blue). Products were analyzed by sequencing gel electrophoresis (20% (w/v) polyacrylamide/8 M urea in Tris borate-EDTA buffer) and quantified by using a BAS1500 scanner (Fuji) and the software TINA 2.09 (Raytest Isotopenmessgerate GmbH, Staubenhardt, Germany).

2.6. Pre-steady-state kinetics of the ATP-dependent excision reaction

The primer 25PGA was blocked at its 3′ end with d4TTP using terminal deoxynucleotidyltransferase (Roche) (Mas et al., 2002), and free nucleotides were eliminated after at least three passages through a mini Quick Spin column (Roche). The blocked primer ($25PGA^{d4T}$) was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and then annealed to the DNA template D38 to obtain the $D38/25PGA^{d4T}$ complex (Matamoros et al., 2009). The primer 25PGA (previously annealed to D38 or D38rna) was blocked with AZT in the presence of wild-type HIV-1_{BH10} RT or the RNase H-deficient mutant HIV-1 group O RT V75I/E478Q (Álvarez et al., 2009), respectively, by following a previously described procedure (Betancor et al., 2012). Excision rates (k_{obs}) for d4T monophosphate (d4TMP) and AZT monophosphate (AZTMP) were determined under single turnover conditions (Betancor et al., 2010). The formation of product over time was fitted to a single exponential decay: $[P] = A \times e^{-k_{obs} \times t}$, where [P] is the concentration of product.

2.7. DNA binding affinity

Dissociation equilibrium constants (K_d) for wild-type and mutant RTs and DNA duplexes were obtained with the D38/25PGA template-primer by following a previously described procedure (Betancor et al., 2010).

2.8. RNase H assays

RNase H activity of RTs was determined with the template-primer 31Trna/21P in the previously described conditions (Álvarez et al., 2009; Betancor et al., 2012).

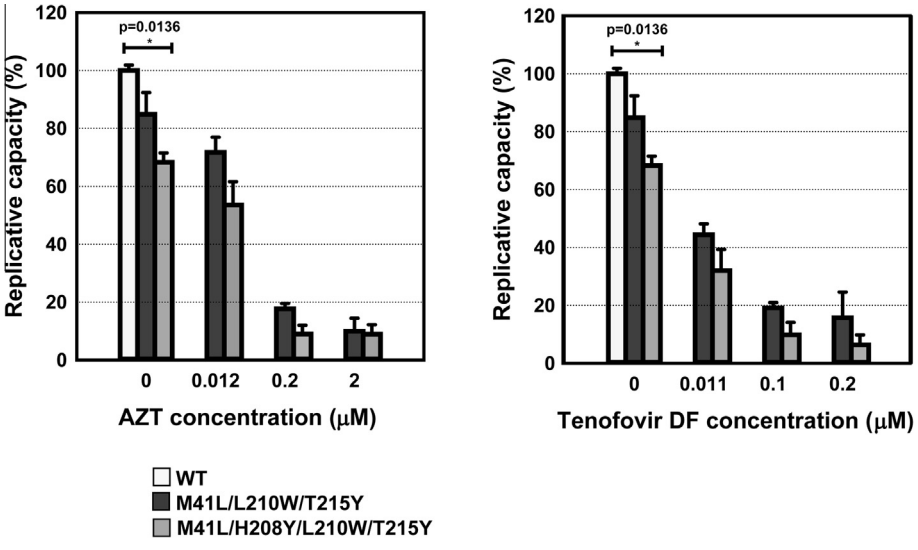


Fig. 1. Replication kinetics of HIV-1 clones bearing WT and mutant RTs in absence and presence of AZT (left) and tenofovir disoproxil fumarate (tenofovir DF) (right). In each case, histograms show the relative replication capacity (%), compared to the WT virus in the absence of drug, based on the slopes of p24 antigen production of each recombinant virus after infection of stimulated PBMCs. Statistical analyses were performed by using the Student *t* test. In all cases, differences between mutants M41L/L210W/T215Y and M41L/H208Y/L210W/T215Y were not significant.

2.9. Homology modeling and molecular dynamics

Structural models of mutant RTs M41L/L210W/T215Y and M41L/H208Y/L210W/T215Y in complex with double-stranded DNA and AZT adenosine dinucleoside tetraphosphate (AZTppppA) were constructed by standard homology modeling techniques (Kisic et al., 2008). The coordinates of the mutant M41L/D67N/K70R/T215Y/K219Q RT in the ternary complex of HIV-1 RT/double-stranded DNA/AZTppppA (Protein Data Bank file 3KLE) (Tu et al., 2010) were used as template. Molecular dynamics simulations based on the models and the crystal structure were performed as previously described for wild-type HIV-1 RT (Mendieta et al., 2008). The system included the DNA polymerase domain (residues 1–389) of p66, a 15/12-mer DNA/DNA template-primer and residues 3–82 of p51. Two Mg^{2+} ions were included in the system. The total simulation lengths were longer than 10 ns, and the analysis of trajectories was performed as previously described (Mendieta et al., 2008).

3. Results

3.1. Phenotypic assays with recombinant HIV-1

Previous analysis of HIV-1 *pol* sequences obtained from patients treated with NRTIs showed the association of H208Y and TAMs, particularly with those classified as belonging to the TAM1 cluster (i.e. M41L, L210W and T215Y) (Gonzales et al., 2003; Stürmer et al., 2003; Saracino et al., 2006; Svicher et al., 2006; Nebbia et al., 2007; von Wyl et al., 2010; Betancor et al., 2012). However, our assays carried out with recombinant HIV-1 showed that the influence of H208Y on NRTI susceptibility either alone or in combination with M41L, L210W and T215Y was not significant, although a relatively

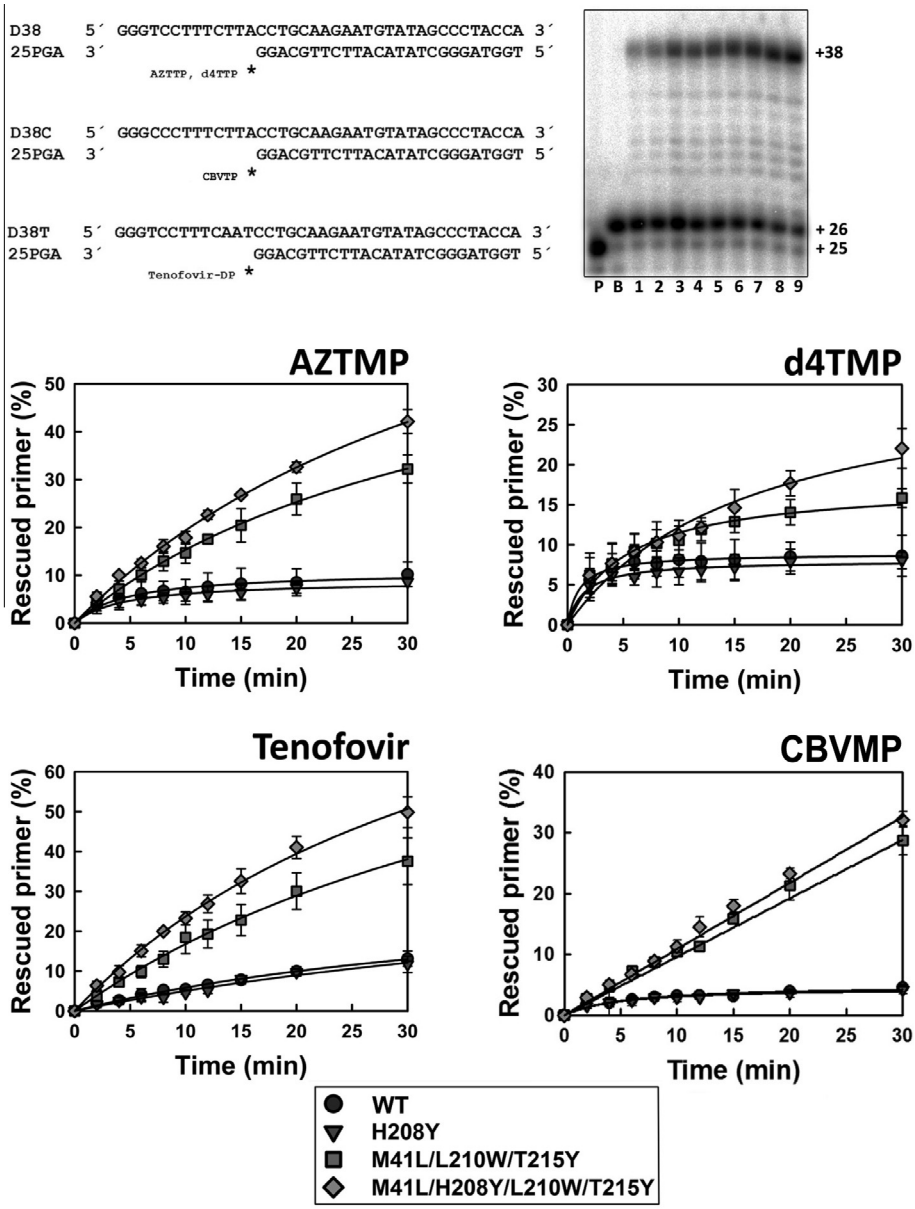


Fig. 2. DNA rescue activity of WT and mutant RTs from AZTMP-, d4TMP-, carbovir monophosphate (CBVMP)- and tenofovir-terminated template-primers. Reactions were carried out using the heteropolymeric DNA/DNA complexes shown in the top panel. Primers (lane P) were blocked with a nucleotide analogue to generate a 26-nucleotide blocked primer (lane B). Extension of the primer to the final product of 38 nucleotides requires the previous excision of the inhibitor. Lanes 1–9 represent aliquots taken after 2, 4, 6, 8, 10, 12, 15, 20 and 30 min after adding a mixture containing all four dNTPs and 3.2 mM ATP. Time course experiments of primer rescue reactions initiated from template-primers blocked with the indicated NRTI are shown below. Template-primer and active RT concentrations in these assays were 30 and 24 nM, respectively. The values given (average ± standard deviation [error bars]) were obtained from three independent experiments.

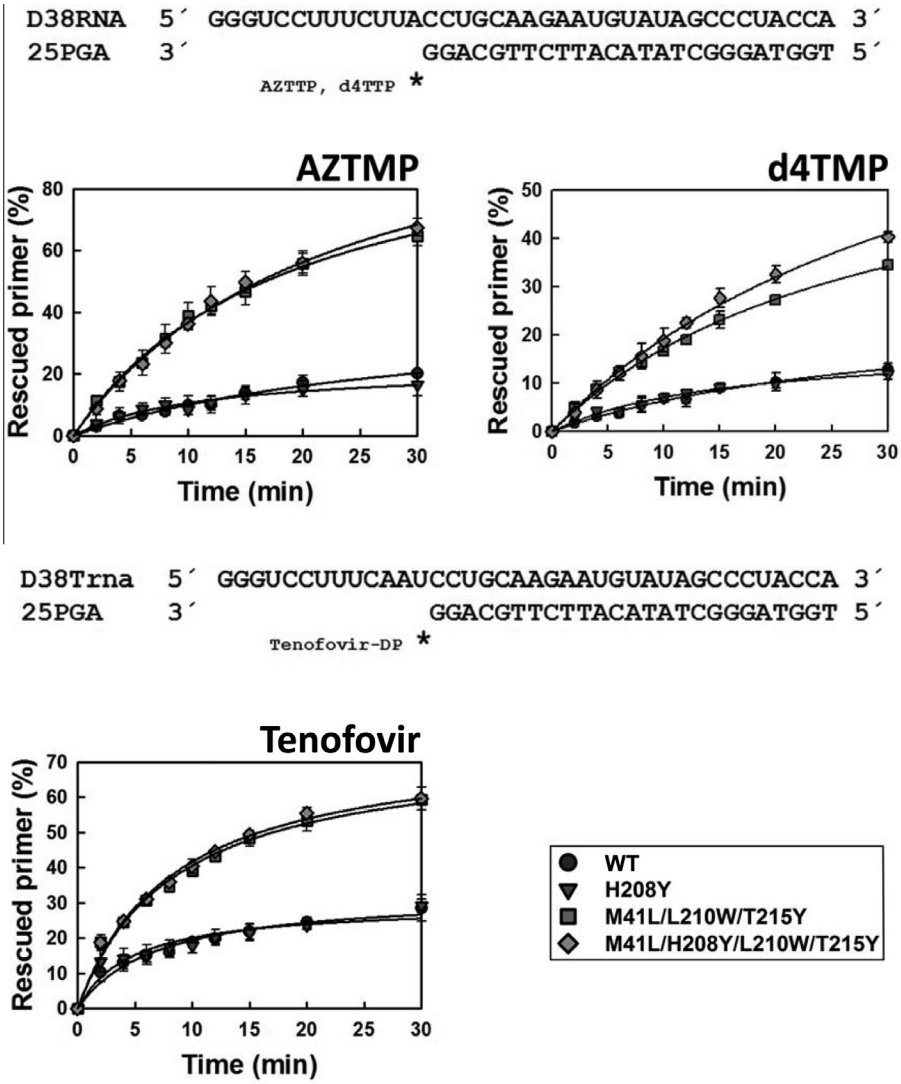


Fig. 3. RNA-dependent rescue activity of primers blocked with AZTMP, d4TMP and tenofovir. Time course experiments of the excision reaction were carried out in the presence of 3.2 mM ATP. Template-primers used are indicated above their corresponding graphs. All dNTPs in the assay were supplied at 200 μM, except dATP or dTTP (depending on the assay) whose concentration was 2 μM. Template-primer and active RT concentrations in these assays were 30 and 24 nM, respectively. The values (average ± standard deviations [error bars]) were obtained from three independent experiments.

small increase in tenofovir resistance was observed for HIV-1 strains bearing RT mutations M41L/H208Y/L210W/T215Y, in comparison with those having the combination M41L/L210W/T215Y (Table 1). This effect on tenofovir resistance has been previously reported for viruses having more complex TAM combinations (e.g. HIV-1 group M subtype B virus bearing mutations M41L, D67N, V75M, A98G, V118I, G190Q, L210W, T215Y and K219Q) (Malik et al., 2009). As expected, significant differences in NRTI susceptibility between TAM-containing HIV-1 and the reference wild-type strain were observed with M41L/T215Y, M41L/L210W/T215Y and M41L/H208Y/L210W/T215Y and AZT resistance, as well as the mutant M41L/H208Y/L210W/T215Y and tenofovir resistance ($p < 0.01$, unpaired Student's t test).

Competition experiments carried out in MT-4 cells in the absence of drugs showed that the recombinant HIV-1 bearing the M41L/H208Y/L210W/T215Y RT was outgrown by the mutant M41L/L210W/T215Y (Supplementary Fig. 1). These results were consistent with viral replication efficiencies determined in PBMCs (Fig. 1). At lower AZT or tenofovir concentrations the mutant M41L/L210W/T215Y showed higher replicative capacity although the differences with the recombinant virus having the M41L/H208Y/L210W/T215Y RT were not statistically significant. In contrast, at higher AZT concentrations the differences between both viruses were almost undetectable, thereby suggesting that the

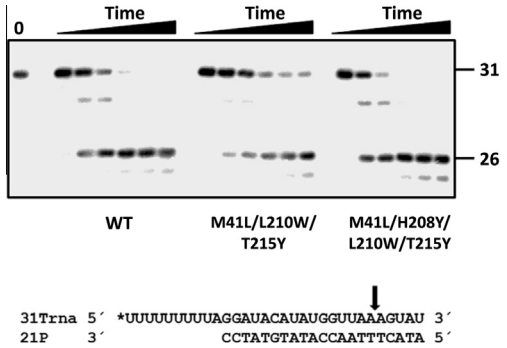


Fig. 4. RNase H activity of wild-type and mutant RTs. Cleavage of the [^{32}P]31Trna/21P substrate (50 nM) was carried out in the presence of the corresponding RT at 50 nM (active enzyme concentration). Time points were taken after 20 s, 40 s, 1, 2, 3 and 4 min. The arrow in the RNA template sequence is used to indicate the cleavage site.

selection of H208Y-containing HIV-1 variants could be favored only at high drug concentrations.

3.2. Effect of H208Y substitution on the ATP-dependent rescue efficiency of primers blocked with NRTIs

Unblocking and extension of AZTMP-, d4TMP-, carbovir monophosphate (CBVMP)- and tenofovir-terminated DNA primers by wild-type and mutant RTs was first analyzed by using DNA/DNA complexes (Fig. 2). In the presence of 3.2 mM ATP, wild-type and mutant H208Y RT showed very low or negligible rescue activity. In contrast, the rescue activity of RTs bearing TAM1 mutations was quite significant. Addition of H208Y produced a further increase in the rescue efficiency with all four NRTIs, particularly with AZTMP- and tenofovir-terminated primers (Fig. 2). In contrast, similar assays carried out with RNA/DNA template-primers failed to show differences in rescue efficiency between M41L/L210W/T215Y and M41L/H208Y/L210W/T215Y RTs (Fig. 3). Interestingly, under pre-steady-state conditions the RNase H cleavage rate obtained with the wild-type enzyme was $0.68 \pm 0.05\ min^{-1}$. However, the k_{obs} obtained for mutant M41L/H208Y/L210W/T215Y RT was $0.93 \pm 0.13\ min^{-1}$, more than twofold higher than the rate obtained with the M41L/L210W/T215Y RT (k_{obs} $0.41 \pm 0.08\ min^{-1}$) (Fig. 4). These results suggest that the effects of H208Y on rescue efficiency using DNA/DNA template-primers would be mitigated in RNA/DNA complexes, due to the higher RNase H activity of the M41L/H208Y/L210W/T215Y RT relative to the M41L/L210W/T215Y mutant. This higher endonuclease activity is expected to decrease rescue efficiency as a result of the lower stability of the template-primer. Excision would be reduced because RNA/DNA complexes containing shorter RNA templates would have higher dissociation rates and therefore, the amount of NRTI available for excision would be reduced.

3.3. ATP-dependent excision activity of M41L/L210W/T215Y and M41L/H208Y/L210W/T215Y RTs

Rescue reactions described above involve an unblocking step (ribonucleotide-dependent excision) followed by extension of the primer. Experiments carried out with a DNA/DNA complex containing an AZT-terminated primer showed that the M41L/H208Y/L210W/T215Y RT had increased excision activity in comparison with mutant M41L/L210W/T215Y (Fig. 5). These effects were observed over a wide range of RT concentrations, suggesting that the observed differences between both enzymes would be independent of the ratio of enzyme to NRTI-terminated primer-template. These results were further supported by the similar DNA/DNA binding affinities obtained for both mutant RTs. Thus, double-stranded DNA dissociation constants (K_d) obtained for M41L/L210W/T215Y and M41L/H208Y/L210W/T215Y RTs were 2.37 ± 0.65 nM and 2.65 ± 0.39 nM, respectively. In reactions carried out with complexes containing d4T-terminated primers, the M41L/H208Y/L210W/T215Y RT showed slightly higher excision rates, although differences were not significant (data not shown). Unfortunately, tenofovir excision could not be measured because after removal of this NRTI (an adenosine derivative), the RT incorporates the PPi donor (i.e. ATP) that is supplied at millimolar concentration rendering a primer with the same length as the one terminated with tenofovir. Similar observations have been previously reported in other studies (Iyidogan and Anderson, 2012). Finally, consistent with results obtained in ATP-dependent rescue assays of AZTMP-blocked primers annealed to RNA templates, excision of AZTMP from D38rna/25PGA^{AZTMP} complexes showed similar rates for M41L/L210W/T215Y and M41L/H208Y/L210W/T215Y RTs ($0.065 \pm 0.008\ min^{-1}$ and $0.068 \pm 0.007\ min^{-1}$, respectively).

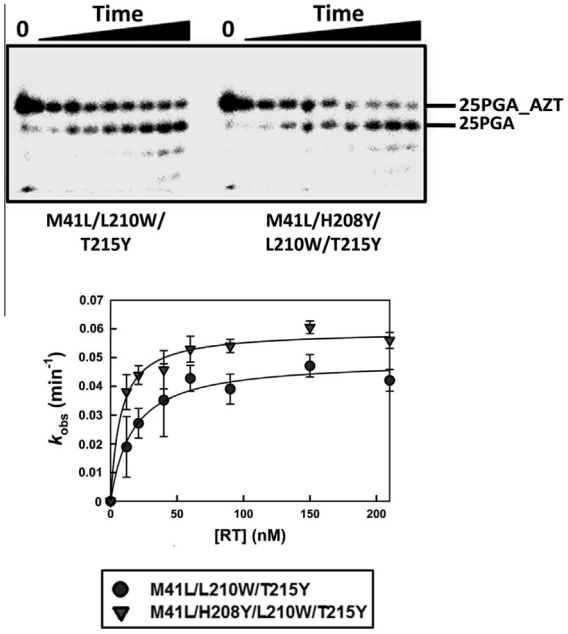


Fig. 5. Kinetics of ATP-dependent excision of AZTMP from a blocked DNA/DNA template-primer. Template D38 was annealed to a 26-nt primer obtained after addition of AZTMP to the 3′-OH of 25PGA. Assays were carried out in the presence of 3.2 mM ATP with M41L/L210W/T215Y and M41L/H208Y/L210W/T215Y RTs. The template-primer concentration in these assays was 30 nM, and the active RT concentrations used ranged from 12 to 210 nM. Results in the gel shown above were obtained by using a 90 nM concentration of active RT. Aliquots were removed after 2, 5, 10, 15, 20, 30, 45, 60 and 80 min of incubation at 37 °C. The dependence of the excision rate (k_{obs}) on the corresponding RT concentration is represented below for both mutant RTs.

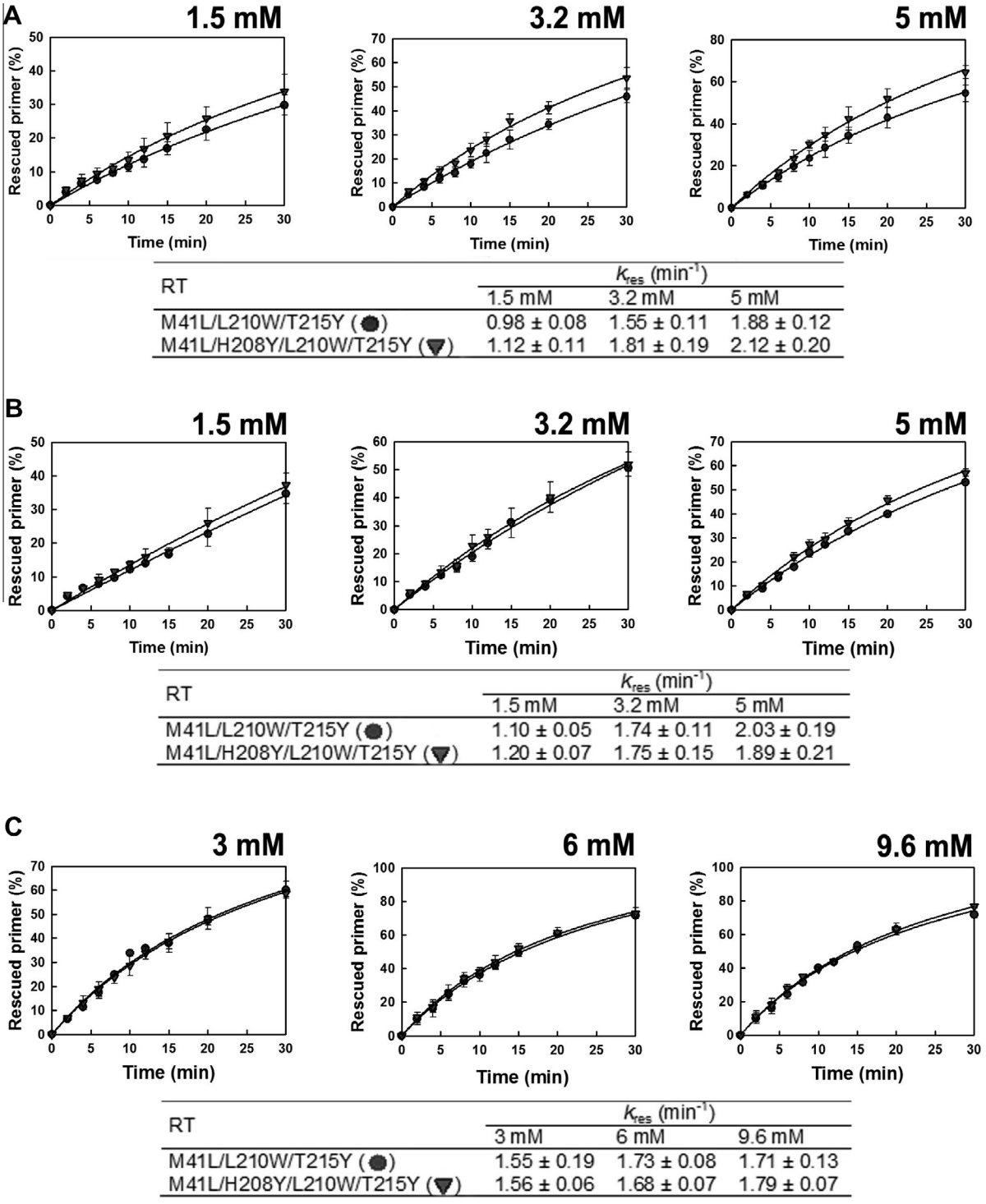
3.4. The increased rescue efficiency of the M41L/H208Y/L210W/T215Y RT can be modulated by the ribonucleoside triphosphate used as PPi donor

Assays carried out in the presence of millimolar concentrations of ATP, ITP or GTP showed that the increased rescue efficiency of the M41L/H208Y/L210W/T215Y RT relative to the M41L/L210W/T215Y mutant was observed only when ATP was used as the PPi donor (Fig. 6). Assuming that rescue efficiencies are dominated by the catalytic rate of the ribonucleotide-dependent excision reaction, we determined the catalytic rates of the rescue reactions (k_{res}) in the presence of different ribonucleotides. In agreement with the results obtained in rescue assays, when ATP was used as PPi donor, the k_{res} value was significantly higher for the M41L/H208Y/L210W/T215Y RT than for mutant M41L/L210W/T215Y (i.e. at 3.2 mM concentration, the obtained k_{res} values were $1.81 \pm 0.19\ min^{-1}$ and $1.55 \pm 0.11\ min^{-1}$, respectively; $p < 0.05$, unpaired Student's t test). However these differences were not significant for GTP or ITP at concentrations in the range 1.5–9.6 mM (Fig. 6). These results suggest that differences obtained in ATP-dependent rescue reactions could be related to the presence of an amino group at position 6 of the purine ring, instead of an oxygen atom (as occurs in GTP or ITP).

Interestingly, in the presence of TAMs M41L/T215Y, the addition of either H208Y or L210W had no effect on the rescue rates of primers terminated with AZT when using 3.2 mM ATP or 9 mM GTP as PPi donors (Fig. 7). At these high ATP or GTP concentrations rescue rates were close to the maximum rate of the reaction.

3.5. Structural models of mutant RTs complexed with double-stranded DNA and AZTppppA

The interactions between residues 208, 210 and 215 with the incoming ATP were analyzed using molecular dynamics



RT	k_{res} (min^{-1})		
	1.5 mM	3.2 mM	5 mM
M41L/L210W/T215Y (●)	0.98 ± 0.08	1.55 ± 0.11	1.88 ± 0.12
M41L/H208Y/L210W/T215Y (▼)	1.12 ± 0.11	1.81 ± 0.19	2.12 ± 0.20

RT	k_{res} (min^{-1})		
	1.5 mM	3.2 mM	5 mM
M41L/L210W/T215Y (●)	1.10 ± 0.05	1.74 ± 0.11	2.03 ± 0.19
M41L/H208Y/L210W/T215Y (▼)	1.20 ± 0.07	1.75 ± 0.15	1.89 ± 0.21

RT	k_{res} (min^{-1})		
	3 mM	6 mM	9.6 mM
M41L/L210W/T215Y (●)	1.55 ± 0.19	1.73 ± 0.08	1.71 ± 0.13
M41L/H208Y/L210W/T215Y (▼)	1.56 ± 0.06	1.68 ± 0.07	1.79 ± 0.07

Fig. 6. Effect of the ribonucleotide used as PPi donor on the rescue activity of primers terminated with AZTMP in reactions catalyzed by M41L/L210W/T215Y and M41L/H208Y/L210W/T215Y mutant RTs. Rescue assays with D38/25PGA^{AZTMP} template/primers were carried out in the presence of (A) 1.5, 3.2 and 5 mM ATP, (B) 1.5, 3.2 and 5 mM ITP, and (C) 3, 6 and 9.6 mM GTP. All assays were carried out in the presence of 30 nM template-primer, 24 nM RT and 100 μM of each dNTP, except for dATP, whose concentration was 1 μM. The values (average ± standard deviation [error bars]) were obtained from three independent experiments.

simulations based on molecular models constructed by using as template the crystal structure of the ternary complex of M41L/D67N/K70R/T215Y/K219Q RT/double-stranded DNA/AZTppppA (PDB file 3KLE). Although the crystal structure contains only one Mg^{2+} ion, our molecular dynamics simulations demonstrated that in the presence of two Mg^{2+} ions, catalytic aspartic acid residues (Asp^{110}, Asp^{185} and Asp^{186}) showed a better geometry, consistent with their participation in the catalytic mechanism of DNA polymerization.

Molecular models of M41L/H208Y/L210W/T215Y and M41L/L210W/T215Y RTs based on the refined crystal structure containing two Mg^{2+} ions showed similar interactions at the catalytic site, but there were significant differences that affected the conformation of the adenine moiety in the AZTppppA molecule and the

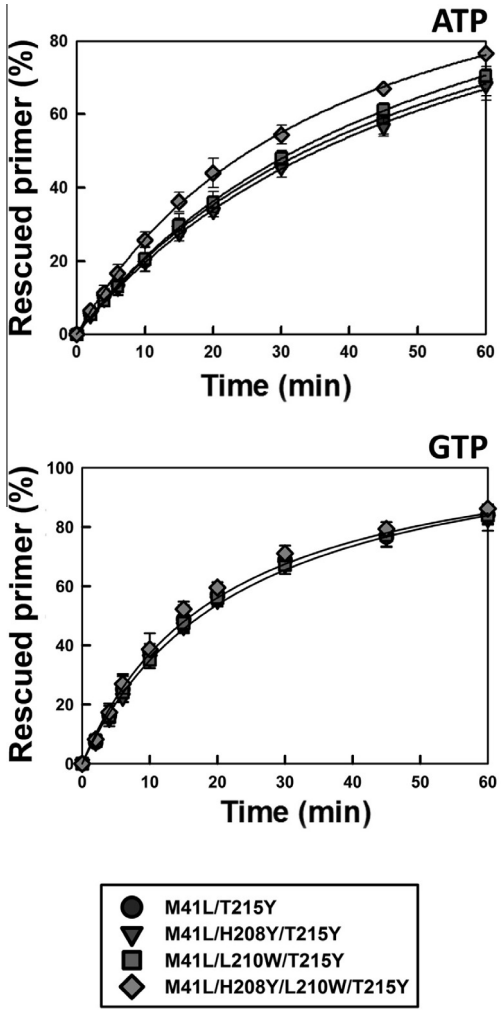


Fig. 7. Effect of L210W on the DNA-dependent AZTMP-terminated primer rescue activity of RTs bearing mutations M41L and T215Y (with or without H208Y). Time course experiments with mutant RTs (M41L/T215Y, M41L/H208Y/T215Y, M41L/L210W/T215Y and M41L/H208Y/L210W/T215Y) were carried out in the presence of 3.2 mM ATP or 9 mM GTP. The template-primer concentration was 30 nM and RTs were supplied at 24 nM (active site concentration). All dNTPs in the assay were supplied at 100 μM, except for dATP whose concentration was 1 μM. The values (average ± standard deviation [error bars]) were obtained from three independent experiments.

side-chains of neighboring residues. As shown in Fig. 8a, the conformation of AZTppppA in the complex containing M41L/H208Y/L210W/T215Y RT was very similar to that found in the crystal structure obtained with the excision-proficient M41L/D67N/K70R/T215Y/K219Q mutant, with Tyr^{215} making stacking interactions with the adenine ring of AZTppppA. In contrast, in complexes containing mutant M41L/L210W/T215Y RT, the adenine moiety of AZTppppA is located in a rather different position, where stacking interactions with the side-chain of Tyr^{215} have been lost (Fig. 8b). However, phosphates in AZTppppA remain in a conformation compatible with excision.

Differences between M41L/H208Y/L210W/T215Y and M41L/L210W/T215Y RTs also affect the position of the side-chain of Trp^{210} that in the model of M41L/L210W/T215Y RT pushes the side-chain of Tyr^{215} towards the adenine ring of AZTppppA, causing a steric clash that affects the conformation of the adenine moiety. A close examination of the interactions found at positions 208–215 reveal a role for residues 208–212 that form the C-terminal end of α-helix F. Interestingly, in the M41L/H208Y/L210W/T215Y RT structure, the side-chains of Tyr^{208} and Trp^{212} form a stacking interaction with the hydroxyl group of Tyr^{208} located only 2.99 Å away from the –NH– of the Trp^{212} indole. This interaction affects the conformation of Arg^{211} and indirectly the positioning of the side-chain of Trp^{210}, that shows a downwards location relative to the Tyr^{215} ring. In contrast, in the M41L/L210W/T215Y RT structure, the presence of His^{208} reduces its potential interaction with Trp^{212}, resulting in an upwards conformation of Trp^{210} that moves the side-chain of Tyr^{215}, altering its interaction with the adenine moiety of AZTppppA, and thereby modifying the ATP binding pocket.

4. Discussion

The H208Y substitution is a rare mutation in viruses isolated from naïve patients. According to the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu/; accessed on March 3rd, 2014), the prevalence of H208Y in HIV-1 group M subtype B RTs found in untreated individuals has been estimated around 0.2%. Clinical studies showed an increased prevalence of H208Y associated with the accumulation of amino acid substitutions of the TAM1 mutational pathway (i.e. M41L, L210W and T215Y). However, there is a lack of evidence supporting the association between H208Y and TAM1 mutations from a molecular perspective. Previous studies have shown that H208Y alone or in combination with Q161L confers low-level resistance to foscarnet (Mellors et al., 1995; Tramontano et al., 1998), while the combination Q161L/H208Y suppresses AZT resistance mediated by TAM2 mutations (i.e. D67N/K70R/T215F/K219E) (Meyer et al., 2003).

The simultaneous presence of accessory mutations H208Y/R211K/L214F in HIV-1 variants containing several TAMs (e.g. M41L, D67N, K70R, L210W, T215Y and K219E) has been observed in patients failing treatment with zidovudine and lamivudine (Stoeckli et al., 2002; Stürmer et al., 2003). In phenotypic drug susceptibility assays, the combination of those three accessory mutations increased AZT resistance when added to an RT background containing six TAMs (Stürmer et al., 2003). We have previously shown that in the presence of zidovudine, L214F enhanced the viral replication capacity of HIV variants containing the TAM1 mutational triad (M41L/L210W/T215Y), without having a significant impact on AZT resistance (Puertas et al., 2009). On the other hand, we found only small differences in the IC_{50}s obtained for AZT and other NRTIs with recombinant HIV-1 bearing RT mutations M41L/L210W/T215Y and M41L/H208Y/L210W/T215Y, although viruses in our study contained Leu instead of Phe at position 214 of the RT. Growth competition assays carried out in MT-4 cells in the absence of drugs showed the lower fitness of virus containing the M41L/H208Y/L210W/T215Y RT in comparison with those having the combination M41L/L210W/T215Y. This detrimental effect of H208Y in viral fitness has also been previously reported for virus having other combinations of TAMs such as M41L, D67N, L210W, T215Y and K219N (Malik et al., 2010). In PBMCs, the impact of H208Y in the replication capacity of recombinant viruses having TAM1 mutations was not significant, although a slightly deleterious effect was observed at different AZT or tenofovir concentrations and in the absence of drugs. Interestingly, at the highest AZT concentration tested the differences in replication capacity between mutants M41L/L210W/T215Y and M41L/H208Y/L210W/T215Y were attenuated. Taken together, these results suggest that the selection of H208Y may be determined by the sequence background of the RT, where other secondary mutations could have a subtle but significant effect in the viral fitness of H208Y-containing variants.

Biochemical assays described in this paper revealed that in the presence of TAM1 mutations, H208Y increases the RT's ability to unblock and extend primers terminated with AZT, d4T or tenofovir.

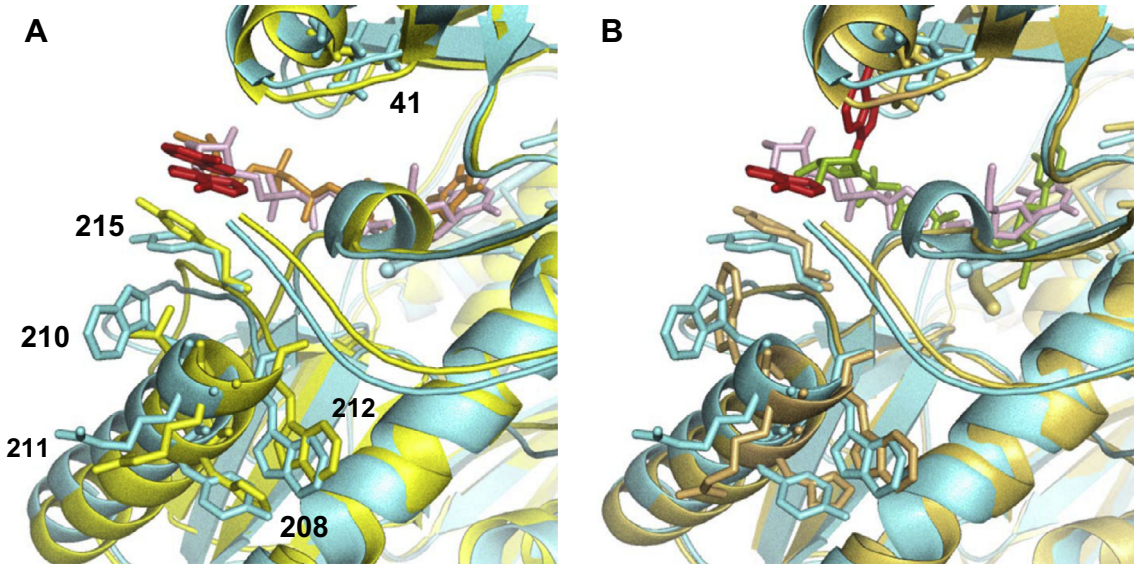


Fig. 8. Comparison of the structural models of mutant M41L/L210W/T215Y and M41L/H208Y/L210W/T215Y RTs in complex with double-stranded DNA and AZTppppA. (A) Superposition of the refined crystal structure of M41L/D67N/K70R/T215Y/K219Q RT/double-stranded DNA/AZTppppA and the equivalent molecular model of the complex containing M41L/H208Y/L210W/T215Y RT. Cartoon representations of the polypeptide backbone of the M41L/D67N/K70R/T215Y/K219Q and M41L/H208Y/L210W/T215Y RTs are shown in yellow and cyan, respectively. Stick representations are used to indicate the location of relevant residues at positions 41, 208–212 and 215. The AZTppppA molecule in the refined crystal structure is shown in orange, and in the molecular model containing the M41L/H208Y/L210W/T215Y RT is shown in purple. Adenine rings are shown in red. (B) Superposition of the refined molecular models of the ternary complexes containing M41L/H208Y/L210W/T215Y and M41L/L210W/T215Y RTs (cyan and wheat, respectively). Purple and green stick representations are used to show the AZTppppA molecules in complexes containing M41L/H208Y/L210W/T215Y and M41L/L210W/T215Y RTs, respectively. In both cases, adenine moieties are shown in red. Structures were drawn using the PyMol molecular software (http://www.pymol.org). Atom coordinates were taken from PDB file 3KLE (Tu et al., 2010). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The increased rescue efficiency of NRTI-terminated primers by the M41L/H208Y/L210W/T215Y RT can be attributed to its higher ATP-dependent excision activity. This effect was observed over a wide range of RT concentrations suggesting that this mechanism of resistance could operate at different multiplicities of infection. Previous studies on the role of accessory mutations in the acquisition of NRTI resistance revealed significant differences in rescue efficiency when terminated-primers were annealed to RNA or DNA templates (Betancor et al., 2010, 2012). We did not find significant differences in rescue efficiencies between M41L/H208Y/L210W/T215Y and M41L/L210W/TY RTs in assays carried out with NRTI-terminated primers annealed to RNA. This could be explained by the higher RNase H activity of the M41L/H208Y/L210W/T215Y mutant that could decrease the stability of the RNA template during the rescue reaction. A similar phenotype was previously described for R284K, an amino acid substitution that increased the DNA polymerase activity of the HIV-1 RT in the presence of TAM1 mutations (Betancor et al., 2012).

Structural studies have shown that TAMs facilitate the ATP-dependent phosphorolytic activity by creating a network of hydrogen bonds between the PPi moiety of the donor molecule and RT residues at positions 67, 70 and 219, as well as hydrophobic residues that facilitate ATP binding through stacking interactions with Tyr^{215} (Tu et al., 2010). In our modeling studies, based on the crystal structure of RT bound to double-stranded DNA and a product of the excision reaction (i.e. AZTppppA), we assumed that the ATP moiety of AZTppppA adopts a conformation similar to that predicted for the PPi donor.

Interestingly, in the complex containing M41L/H208Y/L210W/T215Y RT, the adenine moiety occupies a similar location as in the crystal structure containing the excision-proficient M41L/D67N/K70R/T215Y/K219Q RT. In the M41L/H208Y/L210W/T215Y RT model, the amino group of the adenine points towards the side-chain of Tyr^{215}. This observation is consistent with the differential effects observed in ATP- *versus* GTP- or ITP-mediated rescue reactions. In contrast, the adenine moiety is stabilized in a rather different conformation in the model containing the M41L/L210W/T215Y RT. Although, this enzyme has excision activity, the differences in the conformation of the ATP moiety of AZTppppA could explain why the excision rate is reduced compared with that shown by the M41L/H208Y/L210W/T215Y RT. It should be noted, however, that the conformation of AZTppppA in the complex containing M41L/L210W/T215Y RT retains an adequate orientation of the phosphate groups in the ATP moiety, unlike in the case of the previously described ternary complexes containing the wild-type RT (Tu et al., 2010) that is devoid of ATP-dependent phosphorolytic activity.

Our modeling studies reveal that a key factor explaining the differences between the excision rates of M41L/H208Y/L210W/T215Y and M41L/L210W/T215Y RTs is the proper packaging of residues at the C-terminal end of α-helix F. Stacking interactions between the adenine ring of ATP and the side-chain of Tyr^{215} are important for excision. The proper positioning of Tyr^{215} can be affected by stacking interactions with Trp^{210} and the relative orientation of those residues influences the conformation of the ATP binding pocket. Tyr^{208} in the M41L/H208Y/L210W/T215Y RT interacts with the conserved Trp^{212}, while affecting the conformation of the side-chain of Trp^{210}. Despite these conformational changes, stacking interactions between Tyr^{215} and the adenine ring are maintained in this complex (Fig. 8). In contrast, in the absence of H208Y, the interactions at the C-terminal end of α-helix F appear to be weaker and the side-chain of Trp^{210} moves towards Tyr^{215}, destabilizing its interaction with the ATP donor.

These observations are consistent with the results of primer unblocking and extension assays that showed that the enhancing effect of H208Y on the ATP-dependent excision activity could be detected only in the presence of L210W. In agreement with previous reports (Girouard et al., 2003), the addition of L210W had no

effect on the ATP-dependent phosphorolytic activity of the M41L/T215Y RT (Fig. 7). Further support of the coordinated action of H208Y and L210W was obtained from a recent search in the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu/; accessed on March 3rd, 2014) that rendered 1887 RT sequences that contained mutations M41L, H208Y and T215Y. However, only 122 of them (less than 6.5%) had Leu at position 210.

In addition to H208Y and L210W, a number of amino acid substitutions at the C-terminal end of α-helix F or its vicinity have been identified as secondary mutations emerging under therapy with AZT and other NRTIs. Examples are Q207D that decreases AZT susceptibility by increasing the relative fitness of AZT-resistant HIV-1 (Lu et al., 2005), L214F that enhances the viral replication capacity of HIV-1 variants containing Tyr^{215} in the presence of AZT (Puertas et al., 2009), and R211K that combined with H208Y and L214F was found to be more prevalent in AZT-resistant clinical isolates (Stürmer et al., 2003). Results reported in this paper suggest that subtle effects on the packaging of α-helix F could have a significant impact on the stacking interaction between Tyr^{215} and the nitrogen base of the ribonucleotide PPi donor, and therefore on ATP-mediated excision. A complex array of interactions involving residues at positions 208, 210, 212, and possibly 214 may have a direct impact on the stacking interaction between the side-chain of Tyr^{215} and the adenine ring of ATP which plays a key role in excision and NRTI resistance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.03.004.

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