MECHANISMS OF NUCLEOSIDE ANALOGUE RESISTANCE ASSOCIATED WITH THE SELECTION OF HIV-1 REVERSE TRANSCRIPTASE THUMB SUBDOMAIN POLYMORPHISMS

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BACKGROUND: A cross-sectional study involving >1800 *pol* sequences from HIV-infected patients under treatment in Spanish hospitals revealed the increased prevalence of reverse transcriptase (RT) thumb subdomain polymorphisms such as Pro272, Arg277 and Thr286 in patients failing therapy with different nucleoside analogue combinations. The RT thumb subdomain plays a role in DNA polymerization and contributes to stabilizing the p66/p51 heterodimer. Amino acid changes in the thumb subdomain could affect the balance between excision and template RNA degradation, as reported for several connection subdomain and RNase H domain mutations.

METHODS: Recombinant HIV-1 containing the wild-type RT (subtype B, BH10) and mutant derivatives bearing the substitutions M41L/T215Y, M41L/T215Y/P272A/R277K/T286A and P272A/R277K/T286A were obtained. *In vitro* drug susceptibility was assayed on MT-4 cells and replication kinetics with or without inhibitor was determined by quantification of $p24^{Gag}$ antigen production in PBMCs. RT variants with the mutations indicated above, and the excision-proficient mutant M41L/A62V/T69SSS/K70R/T215Y were purified. Chain terminator excision activity, ability to form stable ternary complexes in the presence of the next complementary dNTP, equilibrium dissociation constants with different template-primers, and RNase H activity were determined for all RTs.

RESULTS: Amino acid substitutions P272A/R277K/T286A had a minor impact on phenotypic RT inhibitor susceptibility (Table 1). However, those mutations produced a significant reduction of the viral replication capacity in PBMCs, in the presence of zidovudine, both in a wild-type sequence context, and in the presence of M41L/T215Y (Figure 1).

In studies carried out with recombinant enzymes, we demonstrate that RT thumb subdomain mutations decrease primer-unblocking activity on RNA/DNA complexes, but not on DNA/DNA template-primers (Figures 2 and 3). These effects were shown with primers terminated with thymidine analogues (*i.e.*, zidovudine and stavudine) as well as with carbovir (the relevant derivative of abacavir), and were more pronounced when mutations were introduced in a wild-type sequence context. The excision rate of stavudine-monophosphate in the presence of ATP 3.2 mM was 0.0032 min^{-1} for wild-type BH10 RT and about 2.5 times lower for the mutant P272A/R277K/T286A (Figure 4). Differences between M41L/T215Y and M41L/T215Y/P272A/R277K/T286A were not significant in these assays.

RT thumb subdomain mutations increased by two-fold the apparent K_d for RNA/DNA without affecting the K_d for DNA/DNA substrates (Table 2). RNase H assays carried out with blocked and unblocked RNA/DNA complexes did not reveal significant differences in the kinetics of the reaction, or in the primary and secondary cleavage patterns (Figure 5).

TABLE 1
Susceptibility of HIV-1 constructs to RT inhibitors

	IC_{50} (nM)						
RTs	AZT	d4T	Abacavir	ddI	Tenofovir	Lamivudine	Nevirapine
WT	5.1 ± 2.6	315.6 ± 44.9	1280.8 ± 242.4	2210.4 ± 363.6	181.0 ± 38.6	1014.1 ± 39.3	44.0 ± 15.7
P272A/R277K/T286A	6.1 ± 2.3 (1.2)	289.3 ± 50.9 (0.9)	1169.8 ± 112.1 (0.9)	2499.8 ± 894.9 (1.1)	221.1 ± 28.5 (1.2)	1519.2 ± 486.0 (1.5)	23.1 ± 4.1 (0.5)
M41L/T215Y	14.5 ± 0.8 (2.8)	399.2 ± 54.3 (1.3)	1526.1 ± 236.7 (1.2)	1888.8 ± 604.0 (0.9)	264.4 ± 38.5 (1.5)	1988.7 ± 628.1 (1.9)	22.8 ± 14.4 (0.5)
M41L/T215Y/P272A/R277K/T286A	10.7 ± 6.5 (2.1)	378.6 ± 90.3 (1.2)	1768.2 ± 188.1 (1.4)	2562.4 ± 710.3 (1.2)	261.4 ± 78.3 (1.4)	1558.8 ± 854.2 (1.5)	29.1 ± 24.2 (0.7)

The IC_{50} values represent the mean ± standard deviation 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 in parenthesis.

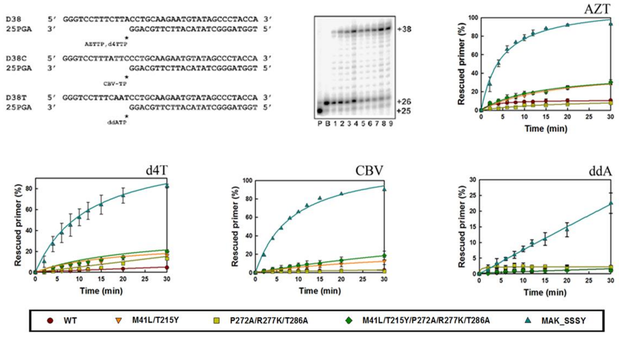

Figure 2. ATP-mediated excision of AZTMP, d4TMP, CBVMP and ddAMP from DNA/DNA template-primers by WT and mutant RTs. Reactions were carried out with 38/25-mer DNA/DNA heteropolymeric complexes (sequences shown above). First, the inhibitor was incorporated at position +1 (indicated with an asterisk) of the 25-nucleotide primer (*lane P*) to generate a 26-nucleotide product (lane B). Excision of inhibitor and further primer extension in the presence of 3.2 mM and a mixture of dNTPs lead to the formation of a fully extended 38-nucleotide product. A representative time course of a primer rescue reaction is shown in *lanes 1-9* that correspond to aliquots removed 2, 4, 6, 8, 10, 12, 15, 20 and 30 min after the addition of 3.2 mM ATP (upper right panel). Time courses of primer rescue reactions initiated from inhibitor-terminated primers are given below. All dNTPs in the assays were supplied at 100 μM, except for dATP whose concentration was 1 μM. Template-primer and active RT concentrations in these assays were 30 and 24 nM, respectively. Represented values were obtained from three independent experiments. The MAK_SSSY RT is an excision-proficient enzyme that contains a Ser-Ser insertion between codons 69 and 70, and mutations M41L, A62V, T69S, K70R and T215Y.

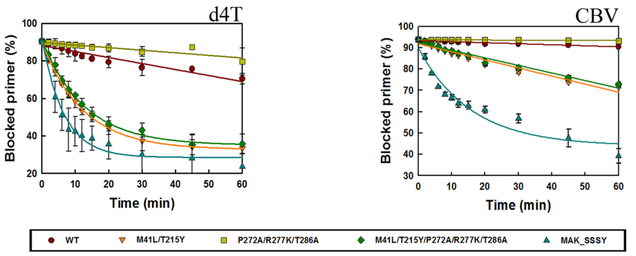

Figure 4. Kinetics of the ATP-dependent excision of d4TMP and CBVMP from RNA/DNA template-primers. Time courses for the excision reaction catalyzed by WT and mutant RTs (250 nM), of d4TMP- or CBVMP-terminated primers (26-mers) annealed to their corresponding 38-nucleotide DNA templates (30 nM) were determined in the presence of 3.2 mM ATP. The calculated k_{obs} values for the d4TMP excision reaction were 0.0032 ± 0.0003 min^{-1} for WT RT, 0.0014 ± 0.0002 min^{-1} for mutant P272A/R277K/T286A, 0.0810 ± 0.0025 min^{-1} for mutant M41L/T215Y, 0.0782 ± 0.0044 min^{-1} for mutant M41L/T215Y/P272A/R277K/T286A, and 0.153 ± 0.018 min^{-1} for the MAK_SSSY RT. For the excision of CBVMP, the k_{obs} values for mutants M41L/T215Y, M41L/T215Y/P272A/R277K/T286A and MAK_SSSY were 0.0038 ± 0.0003 min^{-1}, 0.0035 ± 0.0002 min^{-1} and 0.0629 ± 0.0138 min^{-1}, respectively.

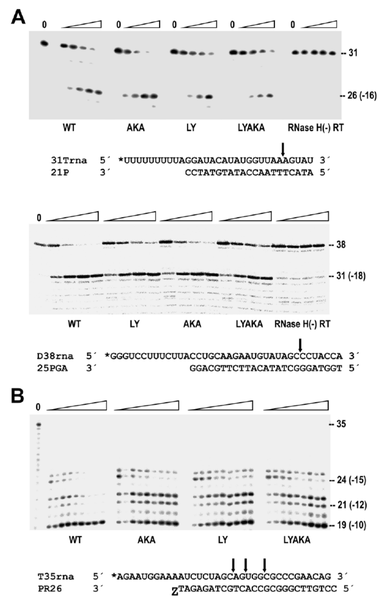

Figure 5. RNase H activity of WT and mutant RTs. (A) [^{32}P]RNA/DNA substrates (50 nM) were cleaved at 37ºC in the presence of the corresponding RT at 100 nM (active enzyme concentration). The template-primer sequences are shown below. Time points were obtained after incubating the samples for 1, 2, 4 and 8 min in the case of 31Trna/21P, and 10, 20, 30 and 40 s, in the case of D38rna/25PGA. (B) Representative autoradiogram of the RNase H cleavage activity of WT and mutant RTs during ATP-mediated AZTMP excision. Assays were performed with the template-primer shown below (at 20 nM) in the presence of 200 nM RT. Time points in the experiments were 10, 20, 30, 45, 60, 90, 120, 150 and 180 min, respectively. Arrows are used to indicate cleavage sites. The labeled 5′ ends of the templates are marked with asterisks. Mutant RTs: AKA, P272A/R277K/T286A; LY, M41L/T215Y; LYAKA, M41L/T215Y/P272A/R277K/T286A. An RNase H-deficient RT (HIV-1 group O RT with mutations V75I/E478Q) was included as a control.

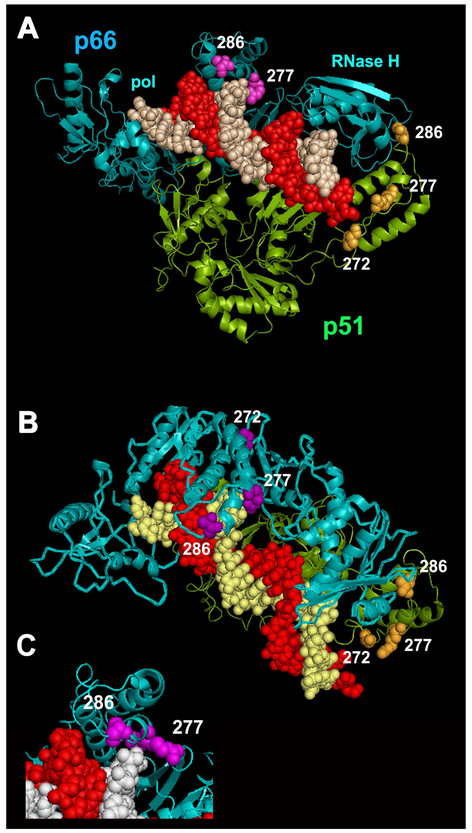

Figure 6. Structure of HIV-1 RT in complex with RNA/DNA. The RT subunits are represented as ribbon diagrams (cyan for p66 and green for p51). The DNA primer is shown in red, while the RNA template is shown in white (panel A) or yellow (in panel B). The side chains of relevant residues of the thumb subdomains of p66 and p51 are shown in magenta and orange, respectively, using a Corey-Pauling-Koltun (CPK) model. Structures were drawn with the PyMOL molecular viewer. Atom coordinates were taken from PDB file 1HYS. Side and top views are shown in (A) and (B), respectively. (C) shows the location of positions 277 and 286 in the p66 subunit in an HIV-1 RT complexed with a DNA/DNA template/primer.

TABLE 2
Dissociation equilibrium constants for WT and mutant HIV-1 RTs and DNA/DNA and RNA/DNA template-primers

	Apparent K_d (nM)	
RTs	DNA/DNA	RNA/DNA
WT	2.19 ± 0.34	1.23 ± 0.33
P272A/R277K/T286A	1.75 ± 0.44	2.76 ± 0.45
M41L/T215Y	3.13 ± 0.04	1.14 ± 0.30
M41L/T215Y/P272A/R277K/T286A	2.77 ± 0.31	2.09 ± 0.46

The K_d values for DNA/DNA binding were obtained with the template-primer D38/25PGA. The RNA/DNA template-primer used in these experiments was D31rna/25PGA (sequence given below).

D31rna 5′ GGGUCCUUUCUUACCUGCAAGAAUGUAUAGC 3′
25PGA 3′ GGACGTTCTTACATATCGGGATGGT 5′

Reported values are the averages ± standard deviations, obtained from three independent experiments.

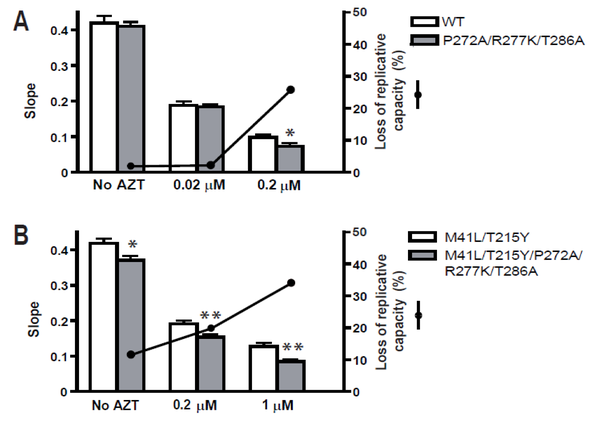

Figure 1. Replication kinetics assay in the absence and in the presence of AZT. The slope of p24 antigen production of each virus after infection of PBMCs (mixed from two donors) is shown in bars. Comparisons of WT *versus* mutant P272A/R277K/T286A and mutant M41L/T215Y *versus* M41L/T215Y/P272A/R277K/T286A are shown in panels A and B, respectively. The significance of the difference between slopes was calculated using the GraphPrism v. 4 software (*, $p<0.05$; **, $p<0.01$). Filled circles (•) represent the percentage loss of replication capacity of the recombinant HIV-1 variants containing mutations P272A, R277K and T286A in the absence (A) or in the presence (B) of M41L/T215Y, under different assay conditions.

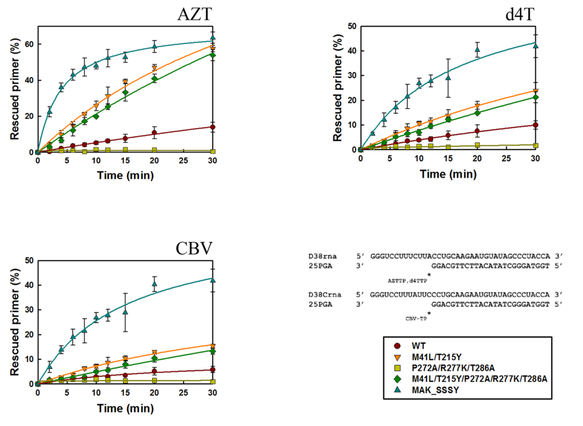

Figure 3. ATP-mediated excision of AZTMP, d4TMP and CBVMP from RNA/DNA template-primers by WT and mutant RTs. Time courses of excision reactions were carried out in the presence of 3.2 mM ATP. Template-primers used are indicated below. All dNTPs in the assays were supplied at 200 μM, except for dATP whose concentration was 2 μM. Template-primer and active RT concentrations in these assays were 10 and 30 nM, respectively. Represented values were obtained from three independent experiments.

CONCLUSIONS: Thumb subdomain polymorphisms P272A, R277K and T286A have a negative effect on viral fitness in the presence of drugs. Our results show that those mutations decrease the excision activity on RNA/DNA template-primers through an RNase H-independent mechanism. The interaction of Arg277 with the phosphate backbone of the RNA template in HIV-1 RT bound to RNA/DNA, and the location of Thr286 close to the RNA strand (Figure 6), are consistent with a role of thumb polymorphisms in decreasing the nucleoside RT inhibitor excision activity on RNA/DNA template-primers, by affecting interactions with the template-primer duplex without involvement of the RNase H activity of the enzyme.

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