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Biochemical Characterization of HIV-1 Reverse Transcriptases Encoding Mutations at Amino Acid Residues 161 and 208 Involved in Resistance to Phosphonoformate

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ABSTRACT. Mutations at amino acid residues 161 (Q161L) and 208 (H208Y) of the reverse transcriptase (RT) have been identified in HIV-1 variants which are resistant to phosphonoformate (PFA). In the present study, we report on the biochemical properties of recombinant RTs (rRTs) carrying either one or both of the above mutations. We also report on their susceptibility to PFA and to nucleoside (NRTI) and non-nucleoside (NNRTI) RT inhibitors. Like the wild-type (wt) enzyme, mutant rRTs H208Y and Q161L/H208Y showed a preference for Mg^{2+} over Mn^{2+}, whereas the Q161L rRT preferred Mn^{2+}. The three mutant rRTs showed degrees of PFA resistance which differed according to the template-primer used, and steady-state kinetic studies revealed an inverse correlation between their degree of PFA resistance, affinity for deoxynucleoside triphosphates (dNTPs) and catalytic efficiency (k_{cat}/K_m ratio). These results indicated that HIV-1 rRTs bearing mutations at codons 161 and/or 208 had altered dNTP binding sites which led to a PFA-resistant phenotype. However, unlike the corresponding mutant viruses, which are hypersensitive to 3′-azido-3′-deoxythymidine (AZT), 11-cyclopropyl-5,-11-dihydro-4-methyl-6H-dipyridol[3,2-b:2′,3′,-e]diazepin-6-one (Nevirapine) and (+)-(5S)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)-imidazo[4,5,1-jk][1,4]benzodiazepin-2(1H)-thione. (TIBO R82150), the mutant RTs Q161L and Q161L/H208Y were resistant to 3′-azido-3′-deoxythymidine triphosphate (AZTTP) and as susceptible as the wt enzyme to Nevirapine and TIBO R82150. Overall, these results suggest that codons 161 and 208 of the HIV-1 RT gene are involved in substrate binding as well as in NRTI recognition, and provide more insights into the mechanism by which HIV-1 becomes resistant to PFA. BIOCHEM PHARMACOL **56**;12:1583–1589, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. HIV-1; reverse transcriptase; PFA; foscarnet; resistance; chemotherapy

PFA§ is a PP_i analog that targets the DNA polymerase of herpesviruses as well as the RT of retroviruses [1]. Inhibition of viral polymerases is noncompetitive with respect to dNTPs, consistent with the fact that PFA binds near the catalytic site and, by forming dead-end complexes, prevents enzyme translocation/release from template [1].

In the clinic, PFA is most commonly used for the treatment of infections caused by 9-[(2-hydroxyethoxy) methyl]-guanine-resistant HSVs [2] and the therapy of cytomegalovirus retinitis whose occurrence is increased in AIDS patients [3]. A direct effect of the drug against HIV-1 has also been observed in clinical trials and, despite its poor solubility and systemic toxicity, its use in combination with other antiretroviral therapies has been encouraged [4–6].

PFA-resistant herpesviruses and HIV-1 have been isolated both *in vitro* and *in vivo*, and their emergence has been associated with mutations occurring in the *pol* genes [7–12]. Drug-resistant HIV-1 variants have been shown to carry mutations in the RT gene at several positions, including codons 161 (Q161L) and 208 (H208Y). Crystallographic studies have revealed that the amino acid residue 161 lies just beneath the putative dNTP binding site in the αE helix of the p66 subunit, whereas residue 208 is located in the αF

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§ *Abbreviations:* activated DNA, DNase I-activated calf thymus DNA; AZT, 3′-azido-3′-deoxythymidine; AZTTP, 3′-azido-3′-deoxythymidine triphosphate; D4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; D4TTP, 2′,3′-didehydro-2′,3′-dideoxythymidine triphosphate; dNTP, deoxynucleotide triphosphate; HSV-1, herpes simplex virus type 1; Nevirapine, (11-cyclopropyl-5,-11-dihydro-4-methyl-6H-dipyridol[3,2-b:2′,3′,-e]diazepin-6-one); NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PFA, phosphonoformate, foscarnet; PP_i, pyrophosphate; rRT, recombinant reverse transcriptase; TIBO R82150, (+)-(5S)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)-imidazo[4,5,1-jk][1,4]benzodiazepin-2(1H)-thione.

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helix and is related neither to the dNTP nor to the template-primer binding sites [11, 13].

In vitro studies with HIV-1 variants have shown that, when present alone, the Q161L and H208Y mutations confer 5- and 2-fold resistance to PFA, respectively, whereas, when present together, they result in a higher degree of resistance (ninefold) [11]. In addition, they also confer hypersusceptibility to AZT (up to 90-fold), Nevirapine (up to 30-fold), and TIBO R82150 (up to 20-fold) [11].

The identification of PFA-resistant mutants has made it possible to evaluate the effects of amino acid changes within sites involved in substrate recognition and/or catalytic processing, but no data are available on the biochemical properties of mutant RTs. Therefore, we investigated the biochemical basis of PFA resistance and the functional role of amino acid residues 161 and 208 by evaluating the impact of the Q161L and H208Y mutations, singly or together, on the rRT optimal cation requirements, steady-state kinetic constants and susceptibility to PFA, PP_i, AZT, Nevirapine, and TIBO R82150.

MATERIALS AND METHODS

rRT Cloning and Purification

Mutagenized HIV-1 RT genes carrying either one or both mutations at codons 161 and 208 were obtained as described previously [11]. The RT coding region, cloned in the pALTER mutagenesis vector (Stratagene), was digested with endonucleases *Nco*-I and *Hind*-III and transferred into the p*Trc*99A expression vector (Pharmacia). Plasmids were then electroporated into *Escherichia coli* JM109. Protein expression and purification were performed as described previously [14]. Briefly, crude bacterial extracts were dialyzed for 6 hr in 50 mM of Tris-HCl pH 7.5, 5 mM of dithiothreitol (DTT), 2 mM of EDTA, 1 mM of phenylmethylsulfonyl fluoride, 10% (v/v) glycerol (solution A), and then sequentially loaded onto DE52, DEAE-cellulose (Whatman) and P11 cellulose phosphate (Whatman) columns. P11 columns were eluted with a linear gradient of 1 M of NaCl dissolved in solution A. Fractions were collected and assayed for peak rRT activity. Purity of wt and mutant p66/p66 homodimers was checked by SDS-PAGE (data not shown).

rRT Assays

Template-primers were purchased from Pharmacia, dNTPs from Sigma and [^{3}H]dNTP from Amersham. Standard rRT assays were performed at 37° for 30 min in a 50 μL reaction mixture containing 50 mM of Tris-HCl, pH 7.8, 1 mM of DTT, 80 mM of KCl, 6 mM of $MgCl_2$, 0.1 mg/mL of BSA and 10 μM of [^{3}H]dGTP or [^{3}H]TTP (1 Ci/mmol), according to the template-primer used. When the template-primer was poly(rC)-$(dG)_{12-18}$, poly(rA)-$(dT)_{10}$ or poly(dC)-$(dG)_{12-18}$ (0.5 OD_{260} unit/mL), 4×10^{-3}, 1.6×10^{-3} or 4×10^{-3} enzyme units were used, respectively [15]. One rRT unit is defined as the amount of enzyme necessary to incorporate 1 nmol of [^{3}H]TMP into the poly(rA)-$(dT)_{10}$ template in 1.0 min at 37°. When activated DNA (0.2 mg/mL) was used as template-primer, the reaction mixture contained 10 μM of [^{3}H]dGTP (1 Ci/mmol), 20 μM each of dATP, dCTP and TTP, and 6×10^{-3} enzyme units. At the end of incubation, reaction mixtures (40 μL) were spotted on glass filters (Whatman GF/A) and the trichloroacetic acid-insoluble radioactivity was determined [15]

Kinetic Studies

Michaelis–Menten kinetics were performed using Lineweaver–Burk plots; v was expressed as pmol/min and [S] as μM. K_I values were calculated by replotting the intercept of the slope versus the drug concentration; k_{cat} values were expressed as V_{max}/[E] ratio.

RESULTS

Definition of Optimal Assay Conditions and Ionic Requirements

We first determined the optimal reaction conditions of the RNA-dependent DNA polymerase activity of wt and mutant rRTs (data not shown). The four enzymes showed maximum incorporation rates under the assay conditions reported in Materials and Methods.

Previous work [16, 17] has shown that mutations in the HIV-1 RT gene at positions 89 and 90 confer both PFA resistance and altered preference for divalent cations. Thus, in order to assay the behavior of the mutant enzymes under optimal conditions, we evaluated whether this was also true for enzymes bearing amino acid substitutions at positions 161 and/or 208. Unlike Mg^{2+} requirements, which resulted to be 6 mM for all rRTs, the optimal Mn^{2+} concentrations were found to differ among test rRTs as follows: 0.15 mM for the wt enzyme, 0.40 mM for the Q161L rRT, and 0.10 mM for both H208Y and Q161L/H208Y rRTs (data not shown).

When we measured the RNA-dependent DNA polymerase activities of wt and mutant enzymes in the presence of optimal Mg^{2+} or Mn^{2+} concentrations, we found that the wt, H208Y and Q161L/H208Y rRTs had higher incorporation rates in the presence of Mg^{2+} than Mn^{2+} (5-, 2.2-, and 2.5-fold, respectively), whereas the Q161L rRT was 2.5-fold more active in the presence of Mn^{2+} (Fig. 1).

PFA Susceptibility of Wt and Mutant rRTs

We then evaluated the PFA susceptibility of the RNA- and DNA-dependent DNA polymerase activities of wt and mutant enzymes using various template-primers (Table 1). With poly(rA)-$(dT)_{10}$, the Q161L and Q161L/H208Y rRTs were less susceptible to PFA than the wt enzyme (11- and 4-fold, respectively), whereas H208Y showed a susceptibility comparable to that of the wt RT (Fig. 2).

When poly(rC)-$(dG)_{12-18}$ was used, higher IC_{50} values

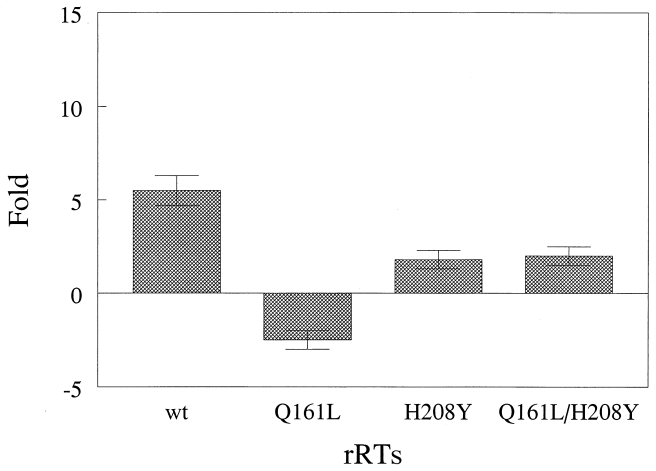

FIG. 1. Mg^{2+}/Mn^{2+} preference of wt and mutant rRTs. Assays were performed as described under Materials and Methods using poly(rA)-$(dT)_{10}$ and [^{3}H]-TTP as substrates. When present, Mg^{2+} was always 6 mM, whereas the Mn^{2+} concentration was 0.15 mM for the wt enzyme, 0.4 mM for Q161L rRT and 0.1 mM for both H208Y and Q161L/H208Y rRTs. Columns represent the rRT preference for Mg^{2+} or Mn^{2+} expressed as the ratio of the pmol of [^{3}H]-TMP incorporated in the presence of Mg^{2+} to those incorporated in the presence of Mn^{2+}. Data shown are mean ± SD from three independent determinations.

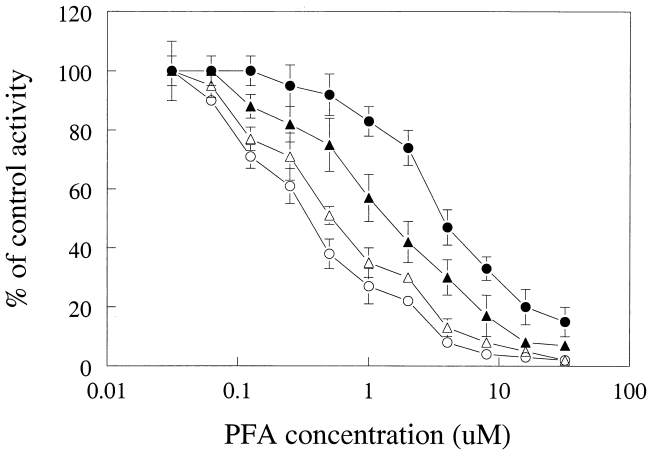

FIG. 2. PFA susceptibility of wt and mutant rRTs. Assays were performed as described under Materials and Methods using poly(rA)-$(dT)_{10}$ and [^{3}H]-TTP as substrates. Data shown are mean ± SD from three independent determinations. wt (○), Q161L (●), H208Y (△), and Q161L/H208Y (▲).

(Table 1) were obtained, but this was not surprising since the inhibitory potency of PFA is known to vary according to the template-primer used [1]. Unexpectedly, however, the degree of PFA resistance of mutant rRTs also showed a template-primer dependency. In fact, when compared to the wt enzyme, Q161L and Q161L/H208Y rRTs resulted 22- and 12-fold less susceptible to PFA, respectively, whereas H208Y was fully susceptible.

When the PFA susceptibility of the DNA-dependent DNA polymerase activity was evaluated, a different pattern was obtained. With poly(dC)-$(dG)_{12-18}$, the Q161L/H208Y rRT was the least susceptible (fourfold) to PFA, followed by the Q161L rRT (2.5-fold) and the H208Y rRT, which was fully susceptible. Similar results were obtained with activated DNA as template-primer.

Characterization of the Kinetic Constants of Wt and Mutant rRTs

Since PFA is a PP_i analog, mutations at codons 161 and/or 208 could be expected to affect the function of the HIV-1 RT active site. Therefore, we evaluated whether the rRTs carrying the above mutations showed altered affinity for dNTPs (Table 2).

When the RNA-dependent DNA polymerase activity was evaluated, Q161L, Q161L/H208Y and H208Y rRTs showed K_m values for TTP higher than the wt enzyme (6.2-, 3.2-, and 2.4-fold, respectively). As far as the K_m of dGTP was concerned, the same was true for both Q161L and Q161L/H208Y rRTs, whereas the H208Y rRT showed the same K_m as the wt enzyme.

When the DNA-dependent DNA polymerase activity was evaluated, K_m values for dGTP significantly higher than those for the wt enzyme were shown by Q161L/H208Y and Q161L rRT using poly(dC)-$(dG)_{12-18}$, and by Q161L/H208Y and Q161L rRTs using activated DNA. On the other hand, the affinity for dGTP shown by the H208Y rRT with poly(rC)-$(dG)_{12-18}$, poly(dC)-$(dG)_{12-18}$ and activated DNA was close to that of the wt enzyme. These results indicated that no matter which DNA polymerase function of the HIV-1 rRTs was considered, the degree of PFA resistance and the affinity for dNTPs were inversely related.

Spatial and functional relationships among residues involved in substrate binding and their modulation of catalytic processes are not fully understood. Therefore, it was interesting to evaluate whether mutations affecting the

TABLE 1. Template-primer-dependent susceptibility of wt and mutant HIV-1 rRTs to inhibition by PFA

	IC_{50} (μM)			
rRT	**poly(rA)-$(dT)_{10}$**	**poly(rC)-$(dG)_{12-18}$**	**poly(dC)-$(dG)_{12-18}$**	**Activated DNA**
Wt	0.37 ± 0.04	2.7 ± 0.1	2.4 ± 0.7	1.0 ± 0.2
Q161L	4.0 ± 0.8 (10.8)*	61.0 ± 3.0 (22.6)	6.0 ± 0.8 (2.5)	1.7 ± 0.3 (1.7)
H208Y	0.52 ± 0.03 (1.4)	2.7 ± 0.2 (1.0)	3.3 ± 1.0 (1.4)	1.2 ± 0.2 (1.2)
Q161L/H208Y	1.45 ± 0.3 (3.9)	34.0 ± 3.0 (12.6)	9.7 ± 2.0 (4.0)	3.0 ± 0.2 (3.0)

Data represent the average ± SD from three independent determinations performed as described under "Materials and Methods."

*Numbers in brackets represent the fold of increase of the IC_{50} values of mutant rRTs, as compared with the wt rRT.

TABLE 2. Kinetic constants of wt and mutant HIV-1 rRT on four different template-primers

rRT	Poly(rA)-$(dT)_{10}$			Poly(rC)-$(dG)_{12-18}$			Poly(dC)-$(dG)_{12-18}$			Activated DNA		
	K_m TTP*	k_{cat}	k_{cat}/K_m	K_m dGTP	k_{cat}	k_{cat}/K_m	K_m dGTP	k_{cat}	k_{cat}/K_m	K_m dGTP	k_{cat}	k_{cat}/K_m
Wt	5.0 ± 1	18 ± 8	3.6	6.0 ± 2	12 ± 5	2.0	3.0 ± 0.5	15 ± 3	5.0	2.0 ± 0.6	2.5 ± 0.7	1.2
Q161L	31 ± 5 (6.2)†	45 ± 3 (2.5)	1.5 (2.4)‡	77 ± 9 (12.8)	65 ± 10 (5.4)	0.8 (2.5)	7.0 ± 0.3 (2.3)	39 ± 2 (2.6)	5.6 (0.9)	4.1 ± 0.1 (2.1)	25.0 ± 10 (10)	6.1 (0.2)
H208Y	12 ± 2 (2.4)	9.0 ± 1 (0.5)	0.7 (5.1)	5.0 ± 1 (0.8)	5.0 ± 2 (0.4)	1.0 (2.0)	4.0 ± 0.1 (1.3)	4.0 ± 2 (0.3)	1.0 (5.0)	3.0 ± 1.0 (1.5)	0.7 ± 0.3 (0.3)	0.2 (5.2)
Q161L/ H208Y	16 ± 3 (3.2)	19 ± 2 (1.1)	1.2 (3.0)	59 ± 2 (9.8)	21 ± 4 (1.7)	0.4 (5.0)	16 ± 5.0 (5.3)	14 ± 3 (0.9)	0.9 (5.5)	3.6 ± 1.0 (1.8)	2.1 ± 1.0 (0.8)	0.6 (2.0)

Data represent the average ± SD from three independent determinations performed as described under "Materials and Methods."
*Kinetic constants were determined by Lineweaver-Burk plots, K_m values are expressed as μM concentration, k_{cat} values as min^{-1}.
†Numbers in brackets represent the fold increase of K_m or K_{cat} ratio of mutant rRTs, as compared with wt rRT.
‡Numbers in brackets represent the fold decrease of the k_{cat}/K_m ratio of mutant rRTs, as compared with wt rRT.

affinity for dNTPs could also affect the catalytic function of the PFA-resistant rRTs. When compared to the wt rRT (Table 2), the Q161L rRT showed turnover rates 2.5- to 10-fold higher, depending on the template-primer used; the Q161L/H208Y showed no significant changes, whereas the H208Y rRT showed a 2- to 3-fold decrease. However, when k_{cat}/K_m ratios were considered, mutant rRTs showed a 2- to 5-fold decrease in catalytic efficiency with respect to the wt enzyme, regardless of the template-primer used (Table 2). The sole exception was the Q161L rRT which showed the same k_{cat}/K_m ratios of the wt rRT with poly(dC)-$(dG)_{12-18}$, and a fivefold higher ratio when assayed with activated DNA.

Susceptibility of Wt and Mutant rRTs to NRTIs and NNRTIs

Substitution at amino acid residue 161, with or without substitution at residue 208, confers increased susceptibility to AZT, Nevirapine and TIBO R82150 on PFA-resistant variants [11]. This observation, coupled with the above reported findings that PFA-resistant rRTs have altered affinity for dNTPs, led us to evaluate the susceptibility of mutant enzymes to some nucleoside analogs and NNRTIs.

When poly(rA)-$(dT)_{10}$ and TTP were used as substrates, AZTTP competitively inhibited all the enzymes assayed (data not shown). Surprisingly, the K_I values for the Q161L and Q161L/H208Y rRTs were higher (6.1- and 5.2-fold, respectively) than that for the wt rRT, whereas the K_I for the H208Y rRT was similar (Table 3), suggesting that Q161L mutation induces an AZTTP-resistant phenotype. Competitive inhibition was also observed with D4TTP (data not shown). In this case, K_I values for the Q161L and Q161L/H208Y rRTs were still higher (32- and 27-fold, respectively) than that for the wt rRT (Table 3), whereas that for the H208Y was closer (threefold higher) to that for the wt enzyme. This suggest that in contrast to what was observed with infectious viruses, mutations at codons 161 and/or 208 induce PFA-resistant rRTs which also resist to AZT and D4T metabolites.

The susceptibility of mutant rRTs to Nevirapine and TIBO R82150 was determined with poly(rC)-$(dG)_{12-18}$, due to the fact that, on the one hand, mutant rRTs showed the highest alteration in both affinity for dNTPs and PFA susceptibility with this template-primer (Table 1); on the other hand, Nevirapine and TIBO R82150 have shown the most potent inhibition of the wt HIV-1 rRT with poly(rC)-$(dG)_{12-18}$ [15]. In this case, none of the mutant enzyme differed significantly from the wt rRT in sensitivity to Nevirapine or TIBO R82150 (Table 3).

PP_i Susceptibility of Wt and Mutant rRTs

Previous studies have shown that although the PFA-resistant HSV-1 DNA polymerase is 10- to 20-fold less susceptible to PFA, it is only 2- to 3-fold less susceptible to PP_i, indicating that PP_i and PFA possess different binding

TABLE 3. Inhibition constants of wt and mutant HIV-1 rRTs for NRTI and NNRTI

	K_I*		IC_{50}†		
rRT	AZTTP (nM)	D4TTP (nM)	Nevirapine (μM)	TIBO R82150 (μM)	PP_i (mM)
Wt	22 ± 5	1.4 ± 0.1	0.75 ± 0.2	0.87 ± 0.1	1.2 ± 0.2
Q161L	134 ± 20 (6.1)	46 ± 4 (32)	0.33 ± 0.1 (2.3)‡	0.89 ± 0.1 (1.0)	2.5 ± 0.5 (2.1)
H208Y	15 ± 2 (0.7)	4 ± 1 (2.8)	0.37 ± 0.1 (2.0)	0.77 ± 0.1 (1.1)	2.7 ± 0.7 (2.2)
Q161L/H208Y	114 ± 6 (5.2)	39 ± 7 (27)	0.67 ± 0.1 (1.1)	0.46 ± 0.1 (1.9)	3.5 ± 0.5 (2.9)

Data represent the average ± SD from three independent determinations performed as described under "Materials and Methods."

*K_I values were calculated by replotting the slopes of Lineweaver–Burk plots versus drug concentrations. Reactions were performed using poly(rA)-$(dT)_{10}$ (fixed) and TTP (variable) as substrates.

†Compound concentration required to inhibit rRT activity by 50%. Reactions were performed using as substrates poly(rC)-$(dG)_{12–18}$ and dGTP (for assays with Nevirapine and TIBO R82150) or poly(rA)-$(dT)_{10}$ and TTP (for assays with PP_i).

‡Numbers in brackets represent the fold increase (AZTTP, D4TTP and PP_i) or decrease (Nevirapine and TIBO R82150) of the K_i values or IC_{50} values of mutant rRTs, as compared with the wt rRT.

domains within the active site of the enzyme [18]. Therefore, the PP_i susceptibility of wt and mutant rRTs was determined using poly(rA)-$(dT)_{10}$ as template-primer. As shown in Table 3, the IC_{50} values of PP_i against mutant rRTs were 2- to threefold higher than those against the wt rRT, indicating the existence of different binding domains for PP_i and its analog PFA in the HIV-1 rRT as well.

DISCUSSION

PFA-resistant HIV-1 isolated *in vitro* and *in vivo* encode mutations at codons 161 and/or 208 of the RT gene. In this study, we performed a biochemical characterization of the HIV-1 rRTs carrying either one or both of the above mutations.

The changes in preference for Mg^{2+} over Mn^{2+} shown by rRTs mutated at codons 161 and 208 (particularly impressive is that shown by the Q161L rRT) suggest that amino acid residues involved in PFA resistance may also be responsible for cofactor recognition and binding. On the one hand, this notion is reinforced by the fact that an analogous phenomenon has already been shown for PFA-resistant HSV-1 DNA polymerase [18]. On the other hand, the fact that HIV-1 rRTs carrying mutations at amino acids 89 [16] and 90 [17] show a PFA-resistant phenotype and an alteration of the preference for divalent cations suggests that other amino acid residues may be functionally involved in cofactor recognition. The mechanism of such involvement is complicated by the fact that binding of divalent cations occurs at two sites in the RT, and that divalent cations also bind to nucleic acids and dNTPs, thus leading to different template and substrate complexes depending on the cation used and its concentration [19]. Therefore, it cannot be excluded that PFA-resistant rRTs may be affected in the formation of such complexes.

We showed that PFA resistance involves both the RNA- and DNA-dependent DNA polymerase functions of the RT and that different mutations confer different levels of resistance that depend on the template-primer used. This dependence is probably due to the fact that the binding of template-primers to both Q161L and Q161L/H208Y rRTs induces conformational changes that impinge on the PFA site to an extent that depends on the template-primer. This in turn may differently affect the ability of PFA to interact with its site and, thus, to inhibit the RT function.

Steady-state kinetic analysis revealed that, when compared to the wt enzyme, mutated rRTs show higher K_m values for dNTPs but unaltered K_m values for the four template-primers used (data not shown). Interestingly, the decrease in affinity for dNTPs of the mutant enzymes is not compensated by an increase in the turnover number. In fact, the catalytic efficiency of the mutant enzymes is lower than that of the wt rRT, suggesting that, overall, the binding of the natural substrates to the mutant rRTs may be somewhat weaker than the binding to the wt enzyme. The observation that variants bearing the Q161L and/or the H208Y substitution replicate as efficiently as wt viruses [11] may suggest that such a decrease in catalytic efficiency is not sufficient per se to alter viral growth kinetics. This is supported by the observation that the M184A variant that shows a slow replication kinetics [20] also shows a 10- to 50-fold decrease in the catalytic efficiency of its rRT [21].

Viruses bearing mutations Q161L and Q161L/H208Y have been reported to be resistant to PFA and hypersusceptible to AZT [11], whereas their rRTs proved to be resistant to both PFA and AZTTP. However, since such amino acid substitutions also impact dNTP binding, it is more appropriate to compare the differences in the K_m/K_I ratios of wt and mutant rRTs to determine whether the utilization of the nucleoside substrate or analog has been more significantly compromised (Table 4). When these

TABLE 4. Comparative evaluation of the K_m/K_I ratios of wt and mutant HIV-1 rRTs with different drugs

	K_m/K_I ratio				
rRT	PFA*	PFA†	AZTTP*	D4TTP*	PP_i*
Wt	13	2.2	227	3571	0.0042
Q161L	8	1.3	231	674	0.0124
H208Y	23	1.9	800	3000	0.0044
Q161L/H208Y	11	1.6	140	410	0.0046

Data are taken from Tables 1, 2, and 3. For PFA and PP_i the IC_{50} values were used since they are non-competitive inhibitors of HIV-1 RT and their IC_{50} and K_i values coincide [22].

values are analyzed for Q161L and Q161L/H208Y rRTs in comparison to wt rRT, no significant differences are seen for AZTTP, whereas a 5- to 8-fold difference is seen for D4TTP. These data indicate that TTP and AZTTP have been affected to the same extent by these mutations, whereas D4TTP has been affected to a greater extent. In contrast, H280Y rRTs show a K_m/K_I ratio for AZTTP threefold higher than that of the wt enzyme, whereas the ratio for D4TTP is unchanged.

The reason for the striking difference between the behavior of the viruses and their rRTs remains unexplained. However, mutant viruses in cell culture and the corresponding RTs in enzyme assays often show different susceptibilities to selecting drugs. For instance, HIV-1 carrying the amino acid substitutions D67N, K70R, T215Y, and K219Q shows a 120-fold resistance to AZT in cell culture [23], whereas its rRT shows an unchanged K_I value for AZTTP and a decreased K_m value for TTP [24].

Even though PFA is a noncompetitive inhibitor of the HIV-1 RT [1], the analysis of the variation of the K_m/K_I ratio for this drug between mutant and wt enzymes may provide some insights into the mechanism by which the virus becomes resistant to PFA. The lack of differences in the K_m/K_I ratios for PFA between mutant and wt rRTs suggests that the virus becomes resistant to PFA by selecting mutations that alter the nucleotide substrate binding site; these mutations do not impair viral replication but lead to the PFA-resistant phenotype.

It is worth noting that this hypothesis is consistent with the observation that in the case of amino acid substitution A90V, the reduction in PFA susceptibility of the HIV-1 rRT was also proportional to the reduction in the affinity for dNTPs [17].

The NNRTIs strongly decrease the catalytic rate of the HIV-1 RT [25]; therefore, their binding site is functionally related to the active site of the enzyme. Amino acid substitutions at residues 103, 181 and 188 of RT, involved in HIV-1 resistance to NNRTI [26], have been reported to alter the susceptibility of the rRT to PFA [27]. For instance, when compared to the wt enzyme, rRTs bearing mutations K103N, Y181C and Y188H rRTs were more susceptible to PFA by 16-, 2- and 25-fold, respectively; Y188C rRT was as susceptible as wt rRT and Y181W rRT was 26-fold less susceptible to PFA than the wt enzyme. On the other hand, viruses carrying the Q161L and Q161L/H208Y amino acid substitutions have been reported to be hypersusceptible to Nevirapine and TIBO R82150 [11]. We have observed that Q161L and Q161L/H208Y rRTs were not significantly more susceptible to the latter NNRTIs, indicating, also in this case, a lack of correlation between the susceptibility of the viruses and their recombinant enzymes.

Finally, the decrease in susceptibility to PFA of the mutant rRTs does not correlate with their decrease in susceptibility to PP_i. This is particularly true for the Q161L rRT and suggests that the PFA binding site does not completely overlap the PP_i binding site. It is possible that 1) PFA binds to determinants involved in the recognition of other portions of the dNTP molecule in addition to those involved in the recognition of phosphates β and γ; or that 2) after the catalysis of the phosphate bond, the conformation of determinants involved in the binding of phosphates β and γ is modified to allow PP_i release. Thus, the binding of PFA and PP_i to RT, though involving the same site and the same determinants, differs because it takes place before and after, respectively, the above-mentioned conformational change.

Studies of the effect of the introduction of different amino acids at these positions could provide further understanding of the HIV-1 RT function.

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