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The N137 and P140 amino acids in the p51 and the P95 amino acid in the p66 subunit of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase are instrumental to maintain catalytic activity and to design new classes of anti-HIV-1 drugs

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Abstract Amino acids N137 and P140 in the p51 subunit of HIV-1 reverse transcriptase (RT) are part of the β7–β8-loop that contributes to the formation of the base of the non-nucleoside RT inhibitor (NNRTI)-binding pocket and makes up a substantial part of the dimerization interface. Amino acid P95 in p66 also markedly contributes to the dimerization binding energy. Nine RT mutants at amino acid 137 were constructed bearing the mutations Y, K, T, D, A, Q, S, H or E. The prolines at amino acid positions 95 and 140 were replaced by alanine in separate enzymes. We found that all mutant RT enzymes showed a dramatically decreased RNA-dependent DNA polymerase activity. None of the mutant RT enzymes showed marked resistance against any of the clinically used NNRTIs but they surprisingly lost significant sensitivity for NRTIs such as ddGTP. The denaturation analyses of the mutant RTs by urea are suggestive for a relevant role of N137 in the stability of the RT heterodimer and support the view that the β7–β8 loop in p51 is a hot spot for RT dimerization and instrumental for efficient polymerase catalytic activity. Consequently, N137 and P140 in p51 and P95 in p66 should be attractive targets in the design of new structural classes of RT inhibitors aimed at compromising the optimal interaction of the β7–β8 loop in p51 at the p66/p51 dimerization interface.

Keywords: Anti-HIV-1 drugs; HIV-1; p51; p66

1. Introduction

In the treatment of human immunodeficiency virus type 1 (HIV-1) infections an important number of clinically used drugs interfere with the viral reverse transcriptase (RT). To date, three major categories of RT inhibitors can be distinguished: (i) 2′,3′-dideoxynucleoside analogs designated nucleoside RT inhibitors (NRTIs), (ii) acyclic nucleoside phosphonate analogs designated nucleotide RT inhibitors (NtRTIs), and (iii) non-nucleoside RT inhibitors (NNRTIs) [1,2]. Despite the diversity of available compounds, HIV variants resistant to these classes of RT-directed drugs have become increasingly prevalent in drug-experienced patients. Resistance towards NNRTIs is primarily associated with mutations of the amino acids lining the lipophilic NNRTI binding pocket [3,4].

Residues of major importance in the RT structure are often very highly conserved. Previous studies have revealed that mutations of conserved amino acids such as W229 [5], Y318 [6] or W401 [7] of HIV-1 RT result in a severe decrease of the catalytic activity of the enzyme. Interestingly, the W229 and Y318 mutations did not result in a markedly altered sensitivity of the RT to most NNRTIs. Therefore, it would be unlikely that treatment of HIV-1 infected cells with NNRTIs targeting these conserved amino acids would result in selection of resistance mutations at these amino acid positions [5,6].

The catalytic activity of the RT is solely observed with the (hetero)dimeric form of the enzyme [8]. Thus, compounds that interfere with the p66/p51 interface may be potentially selective dimerization inhibitors of RT [9]. An important region at the p66/p51 interface is the β7–β8-loop in p51 comprising amino acids from P133 to P140 (PSINNETP) that fits into a groove-like structure made up by part of the template/primer binding region in the p66 subunit [10,11]. The tip of this loop also contributes to the formation of the 'floor' of the NNRTI binding pocket. The amino acids of this loop in p51 that make contact with the p66 subunit (including P95) are I135, N136, N137, E138 and P140 [12]. The importance of this loop for structural support and polymerase function of the heterodimeric RT was studied by either deletion, or by alanine substitution of amino acids N136, N137, E138 and T139 [10]. In our study, we introduced nine different amino acids (Y, K, T, D, A, Q, S, H and E) at the highly conserved amino acid position N137 by site-directed mutagenesis. In addition, we replaced two prolines by an alanine (P95 in p66 and P140 in p51) in two separate RT enzymes. These amino acids (i) are highly conserved among all lentiviruses, (ii) make up an important part of the dimerization interface [10–12], (iii) contribute to the formation of the bottom of the NNRTI pocket [11,12], and (iv) have not yet been reported as susceptible to mutation

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under the selective pressure of NNRTIs (or any other drugs) in cell culture. Given these important characteristics, we investigated whether these amino acids may act as potential targets for the design of novel NNRTI or p66/p51 dimerization inhibitors.

We found that all the mutant enzymes studied markedly lost catalytic activity but kept high sensitivity to the inhibitory effect of NNRTIs. Therefore, we conclude that amino acid N137, together with P95 and P140, should be considered as target amino acids in the design of novel NNRTIs or dimerization inhibitors with the aim to obtain a more favorable drug resistance profile than that of existing HIV-1 RT inhibitors.

2. Materials and methods

2.1. Compounds

[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″- (4″-amino-1″,2″-oxathiole-2″, 2″-dioxide) derivatives of N^3-methylthymine (TSAO-m^{3}T) and thymine (TSAO-T) were synthesized as previously described [13]. Nevirapine (BI-RG-587; dipyridodiazepinone) was obtained from Boehringer Ingelheim (Ridgefield, CT). Delavirdine [bis(heteroaryl)piperazine (BHAP)] (U-90152) and efavirenz (DMP-266) were provided by Dr. R. Kirch (at that time at Hoechst AG, Frankfurt, Germany) and Dr. J.-P. Kleim (currently at GlaxoSmithKline, Stevenage, UK). The thiocarboxanilide derivative UC-781 was obtained from W.G. Brouwer (Middlebury, CT, and Guelph, Ont., Canada). The quinoxaline GW420867X was provided by Dr. J.-P. Kleim (GlaxoSmithKline, Stevenage, UK). 2′,3′-Dideoxyguanosine-5′-triphosphate (ddGTP), 2′,3′-didehydro-2′,3′-dideoxythymidine-5′-triphosphate (d4TTP) and phosphonoformic acid (PFA, foscarnet) were obtained from Sigma Chemical Ltd. (St. Louis, MO). (+)-Calanolide A was delivered by Sarawak MediChem Pharmaceuticals Inc. (Sarawak, Malaysia).

2.2. Site-directed mutagenesis of HIV-1 RT

Mutant RT-enzymes containing the N137A, N137Q, N137Y, N137K, N137T, N137E, N137D, N137H, N137S, P95A or P140A mutation in both p66 and p51 subunits were derived from the RT sequence cloned in pKRT2His [14]. Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Westburg, Leusden, The Netherlands), as described before [6]. The two synthetic oligonucleotide primers (Invitrogen Life Technologies, Merelbeke, Belgium) used contained the desired mutation at amino acid position 137, 140 or 95 of HIV-1 RT. The presence of the desired mutation was confirmed by sequencing the RT gene on an ABI Prism 3100 sequencer (Applied Biosystems, Foster City, CA), using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

2.3. Construction of mutant recombinant HIV-1 reverse transcriptases

Recombinant HIV-1 RT enzymes were expressed from a two-plasmid coexpression system as previously described [15]. The p66 subunit of RT was expressed from pACYC66His and the p51 subunit from pKRT51. To construct wild-type and mutated pACYC66His, wild-type and mutated pKRT2His were digested with *Eco*RI and *Avi*II and the RT-containing fragments were ligated into pACYC184 digested with *Eco*RI and *Sca*I. To construct wild-type and mutated pKRT51, wild-type and mutated pKRT2His were digested with *Nco*I and *Kpn*I and the RT-containing fragment was ligated into pKRT51 digested with *Nco*I and *Kpn*I.

2.4. Preparation of E. coli extracts

Expression of recombinant RT was performed as described previously [16]. LB medium (800 ml) containing 100 μg/ml ampicillin and 10 μg/ml tetracycline was inoculated with an overnight culture of *E. coli* JM109 transformed with both plasmids of the coexpression system and started at an OD_{600} of 0.1. The culture was grown at 37 °C, induced with 1 mM final concentration of IPTG for expression of RT, and after centrifugation the pellet was stored at −20 °C. Later, the bacterial cell pellet was resuspended in 15 ml lysis buffer (50 mM Na-phosphate buffer, 5 mM β-mercapto-EtOH, 0.9% glucose, 100 mM NaCl, 1 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin and 10% glycerol) and passed through a SLM Aminco French Pressure Cell Press (Beun de Ronde, La Abcoude, The Netherlands). The lysate was centrifuged for 20 min at 17000*g*.

2.5. Purification of wild-type and mutant recombinant HIV-1 RT

The purification of RT was performed as described previously [16]. Briefly, the supernatant of the lysed bacterial cell culture was incubated with Ni–NTA resin (Qiagen). After sedimentation of the Ni–NTA resin with the bound $(His)_6$-tagged proteins, a column was formed and washed twice with sodium phosphate buffer containing 10 mM imidazole. Then, the RT was eluted from the column with sodium phosphate buffer containing 125 mM imidazole. The imidazole-containing buffer was exchanged by a Tris–HCl buffer and the eluate was concentrated to 2 ml using Ultrafree-15 centrifugal filtration devices (Millipore, Brussels, Belgium). The $(His)_6$-tagged RT was further purified to about 98% purity over a Hitrap Heparin column (Amersham Biosciences, Roosendaal, The Netherlands). All fractions containing heterodimer RT were pooled and stored in a 50% glycerol buffer at −20 °C. Protein concentrations in these stock solutions were determined using the Bio-Rad Protein Assay (Bio-Rad, Nazareth Eke, Belgium) with bovine serum albumin (BSA) as standard.

2.6. Reverse transcriptase assay

For determination of the 50% inhibitory concentration (IC_{50}) of the test compounds against HIV-1 RT, the RNA-dependent DNA polymerase assay was performed as follows: the reaction mixture (50 μl) contained 50 mM Tris–HCl (pH 7.8), 0.06% Triton X-100, 5 mM DTT, 0.3 mM glutathione, 150 mM KCl, 5 mM $MgCl_2$, 1.25 mg/ml BSA, 0.5 mM EDTA, 0.1 mM template/primer poly(rC)·oligo$(dG)_{12–18}$ (Amersham Biosciences), a fixed concentration of the labeled substrate [8-^{3}H]dGTP (1.6 μM, 1 μCi; specific activity, 12.6 Ci/mmol; Amersham Biosciences), 5 μl of inhibitor solution [containing various concentrations (10-fold dilutions) of the compounds], and 5 μl of the RT preparations. In the case, where d4TTP (or PFA) were evaluated for their inhibitory activity, 0.15 mM poly(rA)·oligo$(dT)_{12–18}$ was used as the template/primer, and 1.6 μM [^{3}H]dTTP as the radiolabeled substrate. The reaction mixtures were incubated at 37 °C for 30 min, at which time 200 μl of yeast RNA (2 mg/ml) and 1 ml of trichloroacetic acid (TCA) (5% in 20 mM $Na_4P_2O_7$) were added. The solutions were kept on ice for at least 30 min, after which the acid-insoluble material was precipitated on Whatman GF/C glass-fibre filters and washed with 20 ml TCA (5% in water) and 2 ml ethanol. The amount of incorporated radioactive substrate was analyzed in a liquid scintillation counter (Canberra Packard, Zellik, Belgium). The IC_{50} for each test compound was determined as the compound concentration that inhibited HIV-1 RT activity by 50%.

2.7. Stability of wild-type and mutant heterodimer HIV-1 RTs in the presence of different urea concentrations

Denaturation curves were plotted by preincubation of RT with different concentrations of urea ranging from 0.0625 M up to 2.0 M for 10 min at 37 °C in 50-μl reaction buffer as described above. The polymerase reaction was initiated by adding 1 μl [8-^{3}H]dGTP (1 mCi/ml) (Amersham Biosciences) as substrate. After incubating for 10 min at 37 °C the reactions were terminated, precipitated, washed and analyzed as described above. Polymerase activity was determined as the amount of nucleotide incorporated at each urea concentration relative to the amount of nucleotide incorporation in the absence of denaturant. The percentage polymerase activity was plotted versus the urea concentration and the data were fitted to a curve using the program SigmaPlot Version 8.0 (SPSS Inc.) to determine the concentration of urea at the midpoint of the denaturation curve.

2.8. Three-dimensional structure visualization

The RT structure was visualized using the ViewerLite 5.0 software (Accelrys Inc.; http://www.accelrys.com) and the X-ray coordinates of a covalently trapped catalytic complex with a DNA template:primer deposited in the Protein Data Bank (PDB, http://www.rcsb.org/PDB/) with code 1RTD [17].

3. Results

3.1. RNA-dependent DNA polymerase activities of wild-type and mutant N137X, P95A and P140A HIV-1 RTs

Nine recombinant mutant RTs were constructed by site-directed mutagenesis at amino acid position 137, namely N137A, N137Q, N137Y, N137K, N137T, N137E, N137S, N137H and N137D. Also two mutant RT enzymes in which the prolines at positions 95 and 140 were each replaced by an alanine were made. The mutations were introduced in both p66 and p51 subunits of the RT heterodimer, and all recombinant mutant RTs were purified to $\geqslant 98\%$ homogeneity through Ni–NTA- and heparin-containing affinity columns.

Determination of the RNA-dependent DNA polymerase (RDDP) activity of the mutant enzymes, using poly(rC)·oligo(dG) as the template and [^{3}H]dGTP as the radiolabeled substrate, revealed that the mutant N137H RT retained 64% of the catalytic activity (Fig. 1). The N137S, N137A and N137Q RTs showed markedly compromised catalytic activity (11–14% of that of wild-type RT) among the mutated N137X RTs. Seriously impaired catalytic activities were noted for the other five mutant N137X RTs. They only retained 3% (for N137D) to less than 0.1% (for N137T and N137K) catalytic activity. The proline to alanine mutations at positions 95 and 140 of HIV-1 RT also resulted in markedly decreased RT activity (4% for mutant P95A RT enzyme and 0.3% for mutant P140A RT enzyme) (Fig. 1).

3.2. Inhibitory activities of NNRTIs, PFA, ddGTP and d4TTP against wild-type, N137X, P95A and P140A mutant HIV-1 RTs

The mutant N137X, P95A and P140A enzymes were evaluated for their sensitivity to the inhibitory activity of a variety of NNRTIs and ddGTP (Tables 1 and 2). Most of the mutant N137X RTs kept full sensitivity to the NNRTIs tested. Only the mutant N137K RT showed moderate resistance to (+)-calanolide A (5-fold) but other NNRTIs retained their inhibitory activity against this enzyme. Interestingly, all mutant N137X RTs significantly gained sensitivity towards the thiocarboxanilide UC-781 (Tables 1 and 2). Also the P95A and P140A RTs were 3- to 10-fold more sensitive to the inhibitory effect of UC-781. In contrast, substantial resistance to (+)-calanolide A was observed for the mutant P95A RT enzyme (30-fold). Surprisingly, all mutant RT enzymes showed less susceptibility and often even relatively high resistance towards the nucleotide RT inhibitor ddGTP (up to 130-fold for the mutant N137K RT), and, to a lesser extent, also to d4TTP (Table 1). Also the pyrophosphate analog PFA showed significant resistance (up to 15-fold) depending the nature of the mutation in RT.

3.3. Effects of urea on wild-type and mutant N137A, N137E and N137D RT activity

Wild-type and mutant N137A, N137E and N137D RTs were exposed to a variety of urea concentrations and their catalytic activity was measured under these experimental conditions (Fig. 2). For the wild-type enzyme, the RT activity gradually decreased in the presence of increasing concentrations of urea. Half of the wild-type catalytic RT activity was retained at ~0.75 M urea (i.e., the urea-IC_{50}). When mutant N137 RTs were exposed to different concentrations of urea, the enzymes showed increased sensitivity towards the denaturing effect of urea. Whereas the urea-IC_{50} shifted from 0.75 to 0.55 M for the mutant N137A and N137D RTs, the urea-IC_{50} was further decreased to 0.35 M for the mutant N137E RT enzyme.

3.4. Structural role of amino acids N137, P140 and P95 in HIV-1 RT

The segment from I135 to P140 is part of the so-called β7–β8 loop that is present in both RT subunits (Fig. 3). In p66, this loop is located on the top of the so-called finger domain of RT and is exposed to the solvent, but in p51 the β7–β8 loop has been identified as a binding energy "hot spot" for dimerization [9,18]. This is in good agreement with the finding that the β7–β8 loop is essential for the catalytic activity of the p66 subunit [10,11]. Accordingly, whereas the side chain of N137 in the p66 subunit is facing the solvent, in the p51 subunit it is found lying on the peptide backbone of p66 in a stretch comprising L92–G93–I94. It is worth mentioning that the region encompassing amino acids 83–99 displays a different conformation in both subunits despite having identical composition. Thus, a short α-helix from P97 to G99 is present in the larger (p66) subunit but it is absent in the smaller (p51) subunit. Furthermore, the backbone of these two segments can

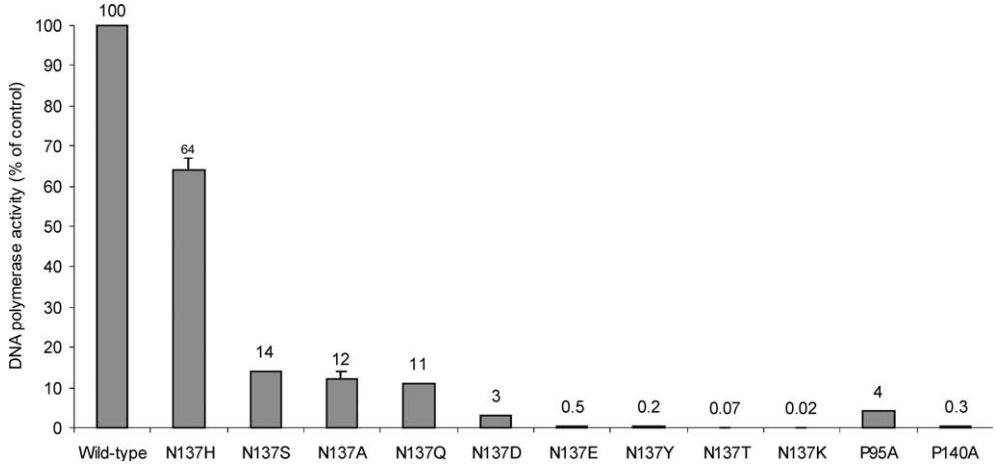

Fig. 1. RNA-dependent DNA polymerase activity of mutant N137X, P95A and P140A HIV-1 RT enzymes. Data are means of three to four independent experiments. S.D. is usually well-below 15% of the average activity values.

Table 1
Inhibitory activity (IC_{50}[b], μM) of test compounds against mutant N137X HIV-1 RTs

RT[a] mutation	Nevirapine	Delavirdine	Efavirenz	UC-781	GW420867X	TSAO-m^3T	TSAO-T	(+)-Calanolide A	ddGTP	d4TTP	PFA
WT	1.1 ± 0.1	0.5 ± 0.3	0.011 ± 0.003	0.11 ± 0.09	0.011 ± 0.000	1.6 ± 0.6	1.6 ± 1.0	0.03 ± 0.01	0.05 ± 0.02	0.19 ± 0.02	3.4 ± 0.4
N137Y	0.61 ± 0.04	0.6 ± 0.2	0.008 ± 0.000	0.020 ± 0.005	0.008 ± 0.003	0.3 ± 0.1	0.41 ± 0.03	0.02 ± 0.01	1.2 ± 0.3	2.6 ± 0.3	39 ± 15
N137K	1.3 ± 0.3	0.8 ± 0.2	0.019 ± 0.004	0.04 ± 0.01	0.02 ± 0.01	3.3 ± 0.0	2.1 ± 0.8	0.155 ± 0.007	6.4 ± 1.1	3.3 ± 1.8	50 ± 0
N137T	1.1 ± 0.3	0.8 ± 0.3	0.011 ± 0.005	0.03 ± 0.01	0.03 ± 0.02	0.3 ± 0.2	0.7 ± 0.1	0.08 ± 0.01	2.2 ± 0.6	1.7 ± 0.0	15 ± 8
N137D	0.92 ± 0.02	0.3 ± 0.1	0.007 ± 0.001	0.020 ± 0.003	0.011 ± 0.003	1.2 ± 0.2	1.1 ± 0.7	0.07 ± 0.03	0.13 ± 0.07	1.6 ± 0.3	33 ± 7
N137A	1.0 ± 0.0	0.6 ± 0.5	0.009 ± 0.002	0.030 ± 0.001	0.010 ± 0.002	0.9 ± 0.4	1.8 ± 0.4	0.04 ± 0.03	0.2 ± 0.1	0.21 ± 0.05	28 ± 3
N137E	1.0 ± 0.1	0.7 ± 0.3	0.02 ± 0.01	0.032 ± 0.002	0.013 ± 0.001	1.7 ± 0.6	2 ± 1	0.09 ± 0.05	0.083 ± 0.002	0.04 ± 0.02	1.7 ± 0.5
N137Q	0.6 ± 0.2	0.4 ± 0.2	0.005 ± 0.001	0.027 ± 0.004	0.007 ± 0.000	0.8 ± 0.3	0.66 ± 0.00	0.07 ± 0.03	0.5 ± 0.1	0.24 ± 0.07	39 ± 15
N137H	0.5 ± 0.3	0.18 ± 0.04	0.015 ± 0.009	0.048 ± 0.001	0.011 ± 0.003	1.0 ± 0.4	1.1 ± 0.2	0.034 ± 0.000	0.70 ± 0.04	–	–
N137S	0.36 ± 0.02	0.27 ± 0.01	0.005 ± 0.000	0.050 ± 0.001	0.021 ± 0.001	1.4 ± 0.4	1.0 ± 0.2	0.055 ± 0.007	0.39 ± 0.01	–	–
P140A	0.8 ± 0.2	0.9 ± 0.1	0.009 ± 0.002	0.045 ± 0.009	0.010 ± 0.002	0.6 ± 0.4	1.07 ± 0.00	0.04 ± 0.01	1.3 ± 0.3	–	–
P95A	1.0 ± 0.2	0.39 ± 0.00	0.008 ± 0.001	0.014 ± 0.001	0.012 ± 0.000	0.9 ± 0.7	0.9 ± 0.8	0.9 ± 0.1	1.4 ± 0.1	–	–

Template/primer: 0.1 mM poly(rC)·oligo(dG); substrate: 1.6 μM [^{3}H]dGTP for all inhibitors except for d4TTP and PFA where 0.15 mM poly(rA)·oligo(dT) was used as the template/primer and 1.6 μM [^{3}H]dTTP as the radiolabeled substrate.
Data are means for two to three independent experiments ± S.D.
[a]The amino acid mutations are present in both p66 and p51 RT subunits.
[b]50% Inhibitory concentration, or compound concentration required to inhibit RT activity with 50%.

Table 2
Fold resistance[a] of mutant N137X, P140A and P95A HIV-1 RTs towards compounds tested in Table 1

RT mutation	Nevirapine	Delavirdine	Efavirenz	UC-781	GW420867X	TSAO-m^3T	TSAO-T	(+)-Calanolide A	ddGTP	d4TTP	PFA
WT	1	1	1	1	1	1	1	1	1	1	1
N137Y	0.55	1.2	0.73	0.18	0.73	0.19	0.26	0.67	24	14	11
N137K	1.2	1.6	1.73	0.36	1.8	2.1	1.3	5.2	128	17	15
N137T	1	1.6	1	0.27	2.7	0.19	0.44	2.7	44	9	4.4
N137D	0.84	0.6	0.64	0.18	1	0.75	0.68	2.3	2.6	8.4	9.7
N137A	0.91	1.2	0.82	0.27	0.91	0.56	1.1	1.3	4	1.1	8.2
N137E	0.91	1.4	1.82	0.29	1.2	1.1	1.3	3	1.7	0.21	0.5
N137Q	0.55	0.8	0.45	0.25	0.64	0.5	0.41	2.3	10	1.3	11
N137H	0.45	0.36	1.36	0.44	1.0	0.63	0.69	1.1	14	–	–
N137S	0.33	0.54	0.45	0.45	1.8	0.88	0.63	1.8	7.8	–	–
P140A	0.73	0.82	0.82	0.41	0.91	0.38	0.67	1.3	26	–	–
P95A	0.91	0.78	0.73	0.13	1.1	0.57	0.56	30	28	–	–

[a]Fold-resistance is expressed as the ratio of the IC_{50} of the test compounds against the mutant RT enzymes versus their IC_{50} against the wild-type RT enzyme.

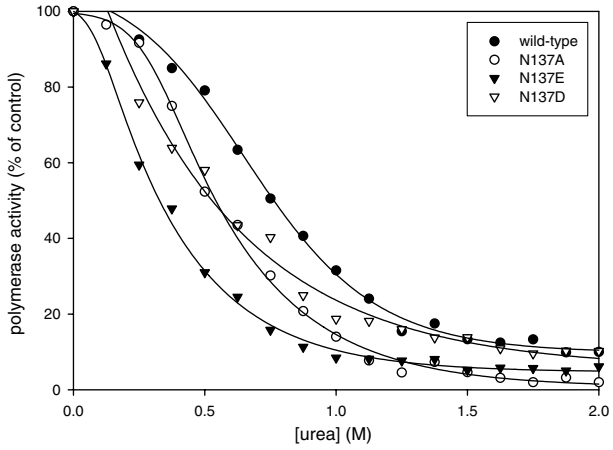

Fig. 2. Effect of urea on the catalytic activity of mutant N137A, N137D, N137E and wild-type HIV-1 RTs.

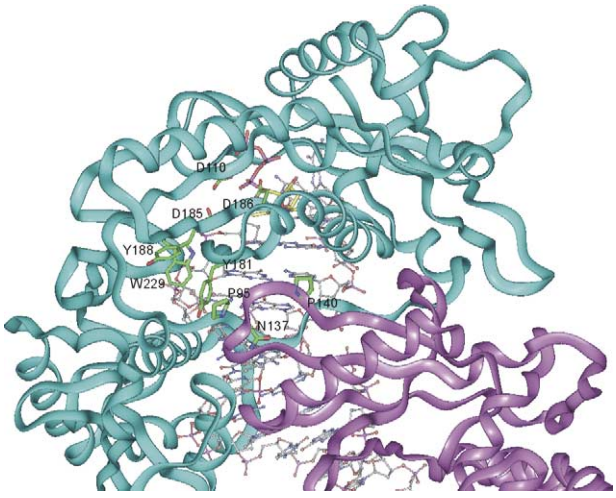

Fig. 3. Detail of the HIV-1 RT subunit interface showing as sticks the side chains of N137 and P140 in the β7–β8 loop of p51 (pink ribbon) and of P95 in the p66 subunit (cyan ribbon). Carbon atoms of the DNA template:primer are colored grey whereas those of the incoming nucleotide triphosphate are colored yellow. Residues Y181, Y188 and W229 making up the NNRTI binding pocket and the catalytic D110, D185 and D186 are shown as thick and thin sticks, respectively.

be superimposed only on a relatively short stretch made up of G93, I94, P95, H96 and P97, which presents a highly invariant amino acid region even in the presence of NNRTI (i.e., nevirapine, delavirdine and efavirenz) drug pressure (Ceccherini-Silberstein et al., data not shown).

4. Discussion

Because of its essential role in the HIV-1 replication cycle, RT is an attractive target for anti-HIV therapies. All of the current clinically used RT inhibitors are either competitive (i.e., NRTIs and NtRTIs) or allosteric (i.e., NNRTIs) inhibitors of HIV-1 RT. The catalytic activity of the RT enzyme is only observed when it is in its (hetero)dimeric form [8]. The NRTIs and NtRTIs interact with the substrate-binding site, whereas the NNRTIs bind to an NNRTI-specific lipophilic pocket at a close distance from the NRTI-binding site. The p66/p51 dimerization interface represents a potential novel target on HIV-1 RT that may lead to the development of new classes of antiretrovirals [7,9,19–21]. Indeed, an important region for dimerization of the RT is an amino acid stretch (comprising P134 to P140), which forms the β7–β8 loop in the p51 subunit [18]. It has an essential role in maintaining the catalytic activity of the p66 subunit [10]. When the highly conserved N137 in this amino acid stretch was replaced by other amino acid residues, a strong decrease in DNA polymerase activity was noted. Thus, replacement by either the closely related Q or by an A that would not be expected to give rise to steric hindrance at the dimer interface resulted in enzymes that retained just about 10% of the activity of the wild-type counterpart. However, substitutions involving bulkier (e.g., T or Y) or more charged side chains (e.g., E, D and K) probably influence the interaction of wild-type N137 with the carbonyl oxygen of the main peptide chain of I94 in the p66 subunit in a more dramatic manner (catalytic activity between 3% and 0.02% of wild-type RT).

The p51 subunit is involved in the critical step of loading the p66 subunit onto the DNA template/primer [22], and structural evidence points to a tight association between the subunits. P95 in p66 and both N136 and N137 in p51 have been shown to contribute to a large extent to the dimerization binding energy [18]. The binding of small molecule inhibitors like the TSAO derivatives at this interface region can destabilize the RT subunit interaction and inhibits the RT catalytic activity [23]. Mutations in these positions are likely to disrupt the interface in such a way that the resulting conformation of RT is not compatible with optimal catalytic activity. This feature has been observed under our experimental conditions. Moreover, the increased sensitivity of the mutated N137 RTs to the denaturing (inactivating) effect of urea relative to wild-type is in agreement with this hypothesis: a less tight interaction between the p66 and p51 subunits in the mutant RT enzymes decreases RT activity at lower urea concentrations than happens with wild-type enzyme. The increased sensitivity of the mutant enzymes to the denaturing (inactivating) effect of urea is due to an easier conversion of the mutant (heterodimeric) enzymes to their monomeric state, rather than to easier unfolding of their secondary structure by urea. Indeed, Sluis-Cremer et al. [24] and Menéndez-Arias et al. [12] have shown that urea at concentrations less than 2 M (as is the case in our experiments) results in a decreased catalytic activity of the RT enzyme due to dissociation of the heterodimer to monomers, and not to destruction (unfolding) of the secondary structure of the enzyme (which occurs at higher urea concentrations). Our observations are also in agreement with earlier published results that focus on the importance of an intact β7–β8 loop in the p51 subunit to retain efficient catalytic activity of the enzyme [10,11].

Thus, we can conclude that N137 in the β7–β8 loop of the p51 subunit plays a crucial role in maintaining the catalytic activity of heterodimeric RT by favoring an optimal conformation of the p66 substrate active site.

Since p51 is crucial for supporting the binding of the template/primer to the p66 subunit [22], disturbing the p66/p51 interface by a mutation at position N137 in p51 may also (indirectly) hamper the binding efficiency of the substrates (dNTPs) and could account for our observation that the N137 mutant enzyme is markedly resistant to ddGTP and, to a lesser extent,

also to d4TTP and PFA. Such dramatic change in the sensitivity of HIV-1 RT to NRTIs and PFA upon mutations in the neighbourhood of the NNRTI-binding pocket site has, to the best of our knowledge, never been observed before. In fact, we found a close inverse correlation between the catalytic efficiency of the mutant enzymes on the one hand, and their degree of resistance to the inhibitory activity of the substrate analogs ddGTP (Fig. 4) and d4TTP on the other hand ($r = 0.77$ and 0.58, respectively). Indeed, if the substrate active site has been compromised by the N137X mutations in p51, it is not unlikely that also the efficiency of binding of ddGTP (and other NRTIs such as d4TTP) to the active site is affected.

The observed resistance of N137K and P95A mutants towards (+)-calanolide A and not towards other NNRTIs can be explained by the fact that (+)-calanolide A may interact with RT in a manner that is mechanistically different from other NNRTIs, which non-competitively inhibit RT with respect of substrate and template/primer. Indeed, (+)-calanolide A has been reported to be at least partly competitive with respect to dNTP binding [25] and thus may interact differently with the mutant RTs. The observed hypersensitivity towards the thiocarboxanilide UC-781 for the majority of HIV-1 RT mutants is rather puzzling because UC-781 makes direct contacts with K101, V106, Y181 and F227 of p66, but not with the p51 subunit [26,27]. It is possible that mutations at the base of the NNRTI binding pocket induce a perturbation of these amino acids in the NNRTI pocket in such a way that the binding of UC-781 in RT becomes more optimized.

Evidence from clinical data supports the view that N137 is a highly conserved amino acid in the HIV-1 RT because in non-treated patients ($n = 457$) mutations at N137 have never been observed and only 0.39% of NRTI/NNRTI-treated patients ($n = 1556$) have mutations at this position (either N137S or N137H) (Table 3). Also, in a cohort of 865 patients that were NRTI-treated but NNRTI-naïve, only two patients (0.23%) contained the N137S mutation in the RT. It is probably no co-incidence that the very few mutations that have been observed in NRTI/NNRTI-exposed HIV-1-infected individuals (four patients with N137S RT and two patients with N137H RT out of 1556 patients) (Table 3) harbour the N137H and N137S mutations that have the least impact on the catalytic RT activity. Moreover, given the partial resistance of the mutant N137S/H RTs to NRTIs (i.e., ddGTP), these mutations can well be selected in the patients cohort by the NRTI pressure rather than by the NNRTI pressure. These observations perfectly fit with our findings that most changes at amino acid residue N137 have a dramatic effect on the catalytic activity. Thus, it is rather unlikely that single-mutant virus strains will easily arise upon exposure of HIV-1 to drugs that are targeted at this amino acid or the site (i.e. P95) on p66 where the side chain of the N137 amino acid binds through two hydrogen bonds.

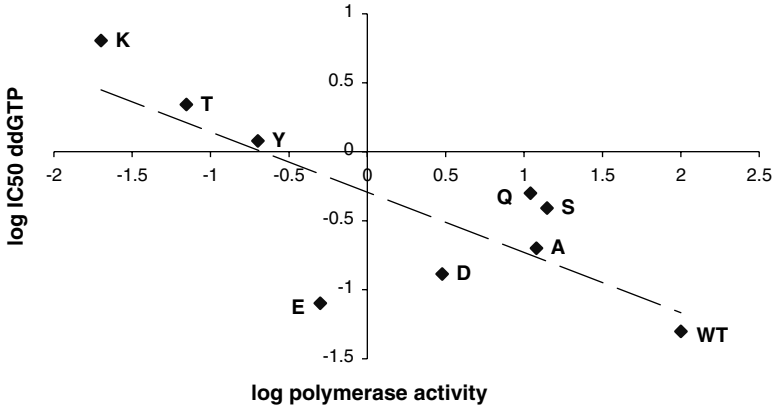

Fig. 4. Inverse correlation between the catalytic DNA polymerase activity of the mutant N137X RT enzymes and their resistance to the inhibitory activity of the NRTI ddGTP. Poly(rC)·oligo(dG) was used as the template primer and [^{3}H]dGTP as the radiolabeled substrate.

Table 3
Mutation profile of the amino acid P95 and the amino acids in the β7–β8 loop of HIV-1 RT from drug-naïve patients (457 isolates) and NRTI/NNRTI-treated patients (=1556 isolates)

Naïve			Wild-type	Treated		
n	%	Mutation		Mutation	%	*n*
0	0		**P95**		0.00	0
1	0.22	S_1	**P133**		0.00	0
3	0.66	R_1C_2	**S134**	T_1R_1	0.13	2
200	43.76	$P_1Q_2K_3M_5L_5R_{14}V_{27}T143$	**I135**	$T_{572}V_{108}L_{92}M_{38}R_{14}K_{10}A2$	52.89	823
0	0.0		**N136**	$T_{12}I_2K_1$	0.96	15
0	0.0		**N137**	S_4H_2	0.39	6
21	4.6	$R_1K_2G_2A_{16}$	**E138**	$A_{79}G_{22}Q_{17}K_{14}T_3S_2D_2R_2$	8.42	131
10	2.2	$V_1M_1A_1R_2I_2P_3$	**T139**	$K_{19}R_{18}A_{13}Q_9M_6I_3S_2V_1P_1$	4.37	68
1	0.22	L_1	**P140**	Q_2T_2	0.26	4

n represents the number of patients with a mutation at a particular amino acid position. The individual number of patients with a specific amino acid mutation is indicated in subscript. The wild-type consensus amino acid is indicated in bold.

Because N137 is (i) highly conserved in HIV-1 RT, (ii) found at the interface between p66 and p51, (iii) difficult to mutate without severe loss of RT activity and also because mutation at position 137 does not result in marked resistance against NNRTIs, we can conclude that N137 is a hot spot instrumental for RT p66/p51 dimerization and enzyme activity. This makes N137 an interesting amino acid to be targeted in structure-based attempts to design novel potential RT dimerization inhibitors. The same reasoning can be applied to the P95 residue (in the p66 subunit) and the P140 residue (in the p51 subunit) of RT. Drug design to test these hypotheses is currently underway.

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