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The Amino Acid Asn136 in HIV-1 Reverse Transcriptase (RT) Maintains Efficient Association of Both RT Subunits and Enables the Rational Design of Novel RT Inhibitors

Jan Balzarini, Joeri Auwerx, Fátima Rodríguez-Barrios, Allel Chedad, Viktor Farkas, Francesca Ceccherini-Silberstein, Carlos García-Aparicio, Sonsoles Velázquez, Erik De Clercq, Carlo-Federico Perno, María-José Camarasa, and Federico Gago

Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium (J.B., J.A., E.D.C.); Department of Pharmacology, University of Alcalá, Alcalá de Henares, Spain (F.R.-B., F.G.); Faculty of Sciences, Katholieke Universiteit Leuven, Kortrijk Campus, Kortrijk, Belgium (A.C.); Department of Organic Chemistry, Eötvös Loránd University, Budapest, Hungary (V.F.); Department of Experimental Medicine, University of Rome "Tor Vergata", Rome, Italy (F.C.-S., C.-F.P.); and Instituto de Química Médica, Consejo Superior de Investigaciones Cientificas, Madrid, Spain (C.G.-A., S.V., M.-J.C.)

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ABSTRACT

The highly conserved Asn136 is in close proximity to the non-nucleoside reverse transcriptase (RT) inhibitor (NNRTI)-specific lipophilic pocket of human immunodeficiency virus type 1 (HIV-1) RT. Site-directed mutagenesis has revealed that the catalytic activity of HIV-1 RT mutated at position Asn136 is heavily compromised. Only 0.07 to 2.1% of wild-type activity is retained, depending on the nature of the amino acid change at position 136. The detrimental effect of the mutations at position 136 occurred when the mutated amino acid was present in the p51 subunit but not in the p66 subunit of the p51/p66 RT heterodimer. All mutant enzymes could be inhibited by second-generation NNRTIs such as efavirenz. They were also markedly more sensitive to the inactivating (denaturating) effect of urea than wild-type RT, and the degree of increased urea sensitivity was highly correlated with the degree of (lower) catalytic activity of the mutant enzymes. Replacing wild-type Asn136 in HIV-1 RT with other amino acids resulted in notably increased amounts of free p51 and p66 monomers. Our findings identify a structural/functional role for Asn136 in stabilization of the RT p66/p51 dimer and provide hints for the rational design of novel NNRTIs or drugs targeting either Asn136 in the β7–β8 loop of p51 or its anchoring point on p66 (the peptide backbone of His96) so as to interfere with the RT dimerization process and/or with the structural support that the p51 subunit provides to the p66 subunit and which is essential for the catalytic enzyme activity.

HIV-1 reverse transcriptase (RT) is a key enzyme in HIV replication and therefore is an attractive target for HIV chemotherapy (De Clercq, 2002). The enzyme consists of two subunits. The p51 subunit is derived from p66 after removal of the p15 RNase H part of the p66 subunit. Although the substrate binding site is located in the p66 subunit, the p51 subunit of HIV RT is essential for loading the p66 subunit on the template primer (Harris et al., 1998). Three classes of anti-HIV compounds have been approved for the treatment of HIV infection that target the virus-encoded RT (De Clercq, 2000). They consist of the group of nucleoside RT inhibitors (NRTIs), a nucleotide RT inhibitor, and the non-nucleoside RT inhibitors (NNRTIs) (De Clercq, 2000). Drug resistance is a major problem for long-term chemotherapy targeting HIV-1 RT (Balzarini, 1999; Schinazi et al., 2001). In particular, NNRTIs easily select for drug-resistance mutations in this enzyme. The NNRTIs are specific for HIV-1 strains and bind to a lipophilic pocket in HIV-1 RT at an allosteric site that is close to, but distinct from, the substrate binding site (Kohlstaedt et al., 1992; Ding et al., 1995; Esnouf et al., 1995; Ren et al., 1995; Hopkins et al., 1996). The NNRTI-specific pocket mainly consists of amino acids that belong to the p66 subunit, but the bottom of the pocket is located close to a

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ABBREVIATIONS: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; dGTP, 2′-deoxy-GTP; ddGTP, 2′,3′-dideoxy-GTP; RDDP, RNA-dependent DNA polymerase; FPLC, fast-performance liquid chromatography; GW420867, quinoxaline; Ni-NTA, nickel-nitrilotriacetic acid; WT, wild type; TCA, trichloroacetic acid; GST, glutathione *S*-transferase.

peptide stretch that is contributed by the p51 subunit (Kohlstaedt et al., 1992; Ding et al., 1995; Esnouf et al., 1995; Ren et al., 1995; Hopkins et al., 1996). Indeed, Glu138 and Thr139, as well as Ser134, Ile135, Asn136, and Asn137 from the p51 subunit, line the outer part of the NNRTI binding pocket in the p66 subunit and make up a portion of the dimerization interface (Fig. 1).

To date, two NNRTIs are known to select for amino acid mutations that are located at the p66/p51 interface. One is *tert*-butyldimethylsilyl aminooxathiole dioxide-m^{3}T, which selects for the E138K mutation (Balzarini et al., 1993, 1994; Boyer et al., 1994; Jonckheere et al., 1994), and the other one is (+)-calanolide A, which selects for the T139I mutation in HIV-1 RT (Buckheit et al., 1995). Modeling studies and experimental results revealed that *tert*-butyldimethylsilyl aminooxathiole dioxide-m^{3}T and its derivatives may interfere with the association of the p66 and p51 subunits, thereby destabilizing the HIV-1 RT heterodimeric enzyme (Arion et al., 1996; Sluis-Cremer et al., 2000; Rodriguez-Barrios et al., 2001; Camarasa et al., 2004). The fact that both Asn136 and Asn137 are highly conserved among the (heterodimeric) RTs of all HIV-1, HIV-2, simian immunodeficiency virus, feline immunodeficiency virus, and several other lentivirus strains that have been characterized so far (Table 1) points to a defined, but as yet unidentified, functional and/or structural role for these residues. Both Asn136 and Asn137 in the p66 subunit are exposed to the solvent, but the side-chain carboxamide group of Asn136 in the p51 subunit is engaged in two hydrogen bonds with the peptide backbone of His96 in the p66 subunit (Fig. 1). It is interesting that the monomeric RT of Moloney murine leukemia virus has no asparagine conservation at the 136 and 137 sites (Table 1).

No mutations at amino acid position 136 of RT have ever been detected in NNRTI-exposed HIV-1–infected cell cultures. In patients infected with HIV-1 who have never received antiretroviral drugs, the presence of mutations at this position was completely absent (0 of 457 patients) and less than 1% in patients infected with HIV-1 who were treated using highly active antiretroviral therapy with NNRTIs (15 of 1556 patients) (Ceccherini-Silberstein et al., 2005). Thus, because of its highly conserved nature, Asn136 seems to play a crucial role in the integrity of HIV RT and/or its catalytic function.

Our studies revealed that Asn136 is essential to preserve the catalytic activity of HIV RT and plays a crucial role in the structure and stabilization of the enzyme heterodimer. We also provide evidence that the NNRTI pocket site in which the amino acid Asn136 from p51 binds can be a potential target in the design of novel NNRTIs endowed with higher antiviral potency and/or with a more favorable resistance profile than the current NNRTIs in clinical use.

Materials and Methods

Compounds. Delavirdine, efavirenz, and quinoxaline (GW420867) were kindly provided by Drs. R. Kirsh and J.-P. Kleim (Hoechst AG, Frankfurt, Germany). Nevirapine was from Boehringer Ingelheim GmbH (Ingelheim, Germany), and ddGTP was obtained from Sigma-Aldrich (St. Louis, MO).

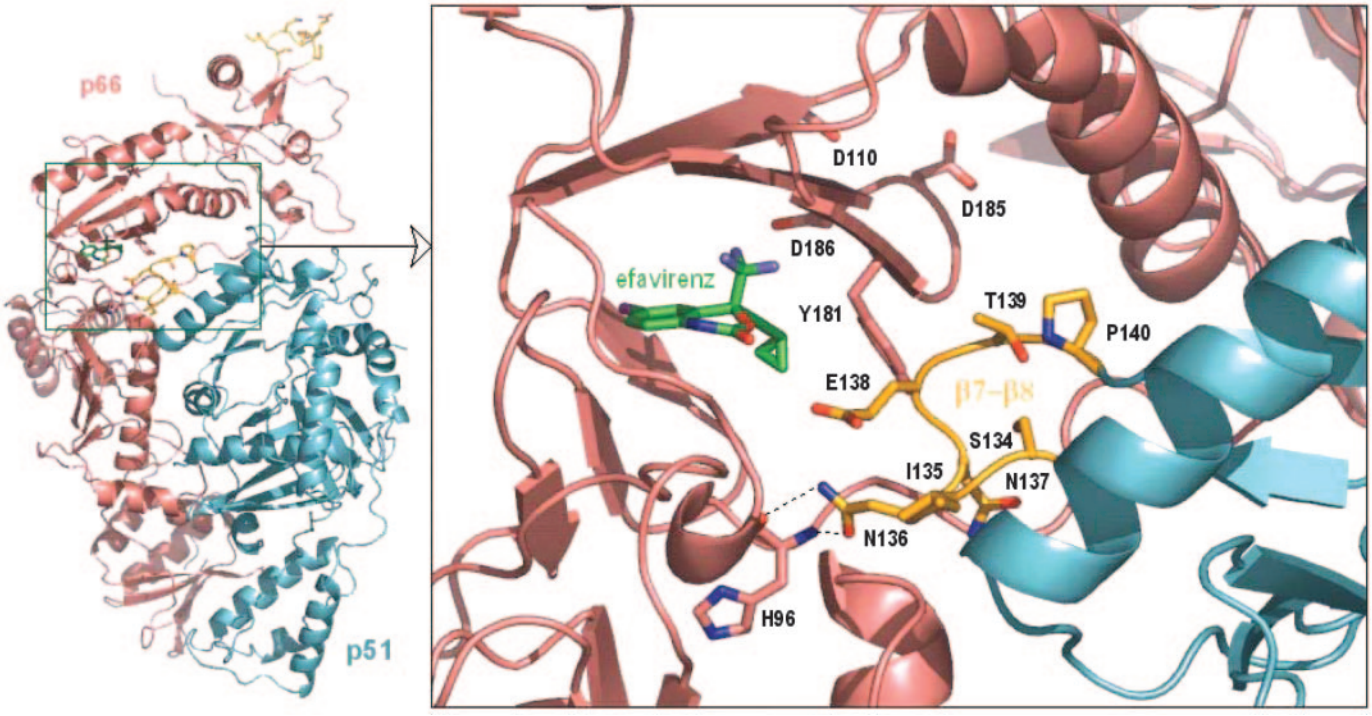

Fig. 1. Left, schematic representation of the dimeric structure of HIV-1 RT as found in the complex with the NNRTI efavirenz and solved by X-ray crystallography (Ren et al., 2000). The coordinates were taken from the Protein Data Bank (http://www.rcsb.org/pdb; Protein Data Bank code 1FK9) and were visualized and pictured using the PyMOL Molecular Graphics System (DeLano Scientific LLC, San Carlos, CA; http://www.pymol.org). The protein Cα trace of each subunit is shown as a ribbon, colored pink for p66 and cyan for p51, whereas efavirenz is displayed as sticks with carbon atoms colored in green. The 134 to 140 stretch (SINNETP) in both subunits has been colored orange, and the side chains of these amino acids are also displayed as sticks. Right, enlarged view of the framed area shown on the left providing detail of the location of the β7–β8 loop of p51 at the subunit interface in relation to both the active site (catalytic aspartates Asp110, Asp185, and Asp186) and the NNRTI binding site (represented by Tyr181). The highly directional hydrogen bonds established between the side-chain carboxamide of Asn136 in p51 and the backbone carbonyl and amide groups of His96 in p66 are displayed as broken lines.

Site-Directed Mutagenesis of HIV-1 RT. Mutant RT enzymes containing the N136A, N136Q, N136Y, N136K, N136T, N136S, N136L, or N136D mutation were derived from the RT sequence cloned in pKRT2His (D'Aquila and Summers, 1989; Pelemans et al., 1998). Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene, Westburg, Leusden, The Netherlands) as described previously (D'Aquila and Summers, 1989). The two synthetic oligonucleotide primers (Invitrogen, Merelbeke, Belgium) that were used contained the desired mutation at amino acid position 136 of HIV-1 RT. The presence of the desired mutation was confirmed by sequencing of 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).

Construction of Mutant Recombinant HIV-1 Reverse Transcriptases. Recombinant HIV-1 RT enzymes were expressed from a two-plasmid coexpression system described previously by Jonckheere et al. (1994). The p66 subunit of RT was expressed from pACYC66His and the p51 subunit from pKRT51. To construct wild-type and 136-mutated pACYC66His, wild-type and 136-mutated pKRT2His were digested with EcoRI and AviII, and the RT-containing fragments were ligated into pACYC184 digested with EcoRI and ScaI. To construct wild-type and 136-mutated pKRT51, wild-type and 136-mutated pKRT2His were digested with NcoI and KpnI, and the RT-containing fragment was ligated into pKRT51 digested with NcoI and KpnI. For all mutant enzymes, the mutation was introduced in both p66 and p51 subunits. Only for the mutant N136T RT, the mutation was introduced solely in p66, solely in p51, or in both p66 and p51 subunits.

Preparation of *Escherichia coli* Extracts. Expression of recombinant RT was performed as described previously (Jonckheere et al., 1994). Luria broth (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 optical density (at 600 nm) of 0.1. The culture was grown at 37°C, induced with 1 mM final concentration of isopropyl β-D-thiogalactoside for expression of RT, and after centrifugation the pellet was stored at −20°C. Later, the bacterial cell pellet was resuspended in 15 ml of lysis buffer (50 mM sodium phosphate buffer, 5 mM β-mercaptoethanol, 0.9% glucose, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 10% glycerol) and passed through an SLM Aminco French Pressure Cell Press (Beun de Ronde, La Abcoude, The Netherlands). The lysate was centrifuged for 20 min at 17,000*g*.

Purification of Wild-Type and Mutant Recombinant HIV-1 RT. The purification of RT was performed as described previously (Pelemans et al., 1998). In brief, the supernatant of the lysed bacterial cell culture was incubated with Ni-NTA resin (QIAGEN, Westburg, Leusden, The Netherlands). After sedimentation of the Ni-NTA resin with the bound His_6-tagged proteins, a column was formed and washed twice with a sodium phosphate buffer containing 10 mM imidazole. Then, the RT was eluted from the column with a sodium phosphate buffer containing 125 mM imidazole. The imidazole-containing buffer was exchanged with 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 approximately 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 as standard.

Reverse Transcriptase Assay. For the determination of IC_{50} values for 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, 5 mM dithiothreitol, 300 μM glutathione, 500 μM EDTA, 150 mM KCl, 5 mM $MgCl_2$, 1.25 μg of bovine serum albumin, a fixed concentration of the labeled substrate [^{3}H]dGTP (1.6 μM, 1 μCi; specific activity, 12.6 Ci/mmol; Amersham Biosciences), a fixed concentration of the template/primer poly(rC) · oligo$(dG)_{12-18}$ (0.1 mM; Amersham Biosciences), 0.06% Triton X-100, 5 μl of inhibitor solution [containing various concentrations (10-fold dilutions) of the compounds], and 5 μl of the RT preparations that correspond to 0.85, 17, 12, 162, 51, 7, 27, 26, and 29 ng of enzyme (protein) for the wild-type and the N136A, N136Q, N136Y, N136K, N136T, N136S, N136L, and N136D mutant RTs, respectively. 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 (5%, v/v) in water were added. The solutions were kept on ice for at least 30 min, after which the acid-insoluble material was filtered over Whatman GF/C glass-fiber filters and washed with 5% trichloroacetic acid in H_2O and ethanol. The filters were then analyzed for radioactivity in a liquid scintillation counter (Canberra Industries, Zellik, Belgium). The IC_{50} value for each test compound was determined as the compound concentration that inhibited HIV-1 RT activity by 50%.

To determine the K_m value of the template/primer against HIV-1 RT in the presence of different concentrations of urea, the RT activity was measured as described above in the presence of 10, 14, 20, 33, 50, 67, 100, and 140 μM poly(rC) · oligo(dG) for HIV-1 RT (WT), 14, 20, 33, 50, 67, and 100 μM poly(rC) · oligo(dG) for mutant N136T HIV-1 RT, and 20, 33, 50, 67, 100, and 140 μM poly(rC) · oligo(dG) for mutant N136L HIV-1 RT. The amounts of urea varied between 0.3 and 1.5 M.

Catalytic Activity of Wild-Type and Mutant Heterodimer HIV-1 RTs in the Presence of Urea and Acetonitrile. Denaturation curves were plotted by preincubation of RT with different

TABLE 1

Alignment of amino acids Ser134 to Gly141 in reverse transcriptase enzymes from different retrovirus strains

The alignment for MMLV was generated automatically using T-COFFEE (http://igs-server.cnrs-mrs.fr/~cnotred/Projects_home_page/t_coffee_home_page.html) but manually corrected on the basis of 3D molecular superposition.

Lentivirus	Amino Acid Position in the RT							
	134	135	136	137	138	139	140	141
HIV-1 (HXB2)	Ser	Ile	Asn	Asn	Glu	Thr	Pro	Gly
HIV-2 (ROD)	Ser	Val	Asn	Asn	Ala	Glu	Pro	Gly
SIV (Rhesus mac)	Ser	Val	Asn	Asn	Ala	Glu	Pro	Gly
SIV (Sun tailed)	Ser	Val	Asn	Asn	Gln	Ala	Pro	Gly
FIV (Petaluma)	Arg	Lys	Asn	Asn	Ala	Gly	Pro	Gly
BIV (Cl 127)	Pro	Val	Asn	Arg	Glu	Gly	Pro	Ile
EIAV (Cl 22)	Ser	Ile	Asn	His	Gln	Glu	Pro	Asp
Visna (Evi)	Ser	Pro	Asn	Asn	Leu	Gly	Pro	Cys
CAEV (CORK)	Ser	Pro	Asn	Asn	Leu	Gly	Pro	Cys
MMLV	Asp	Pro	Glu	Met	Gly	Ile	Ser	Gly

SIV, simian immunodeficiency virus; FIV, feline immunodeficiency virus; BIV, bovine immunodeficiency virus; EIAV, equine infectious anemia virus; CAEV, caprine arthritis and encephalitis virus; MMLV, Moloney murine leukemia virus.

concentrations of urea ranging from 0.0625 to 2.0 M or acetonitrile ranging from 2 to 14% for 30 min at 37°C in 50 μl of reaction buffer (containing 50 mM Tris-HCl, pH 7.8, 0.06% Triton X-100, 5 mM dithiothreitol, 150 mM KCl, 0.3 mM glutathione, 1.25 mg/ml bovine serum albumin, 0.5 mM EDTA, 5 mM $MgCl_2$, and 1.4 mM poly(rC) · oligo(dG); Amersham Biosciences). The polymerase reaction was initiated by adding [8-^{3}H]dGTP (0.1 mM, 1 mCi/ml) (Amersham Biosciences) as substrate. After incubating for 10 min at 37°C, the reactions were terminated by addition of 1 ml of ice-cold trichloroacetic acid (TCA) 5% in 200 mM $Na_4P_2O_7$ and 200 μl of yeast RNA (2 mg/ml, pH 8.0). Reaction products were incubated on ice for 30 min and were precipitated on a Whatman GF/C filter (Whatman, Clifton, NJ). The filters were washed with 20 ml TCA 5% and dried with 2 ml of ethanol. The amount of incorporated radioactive substrate was analyzed in a TR-2500 liquid scintillation counter (PerkinElmer NV Life Sciences, Zaventem, Belgium) by adding 4 ml of HiSafe2 (PerkinElmer). 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 of 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., Chicago, IL) to determine the concentration of urea at the midpoint of the denaturation curve.

Construction and Expression of Wild-Type and Mutant HIV-1 RT Fusion Plasmids in Bacterial Expression Vectors. Glutathione *S*-transferase (GST)-tagged p51 mutant subunits containing either the N136Q, N136T, N136Y, or N136K substitutions were constructed by subcloning an NsiI/KpnI restricted fragment from the p51-encoding portion of the above-described pKRT51 containing the desired mutation (glutamine, threonine, tyrosine, or lysine) at position 136 into the NsiI/KpnI-digested pGEX51H [previously constructed by Auwerx et al. (2002)]. Expression and purification of the wild-type and mutant N-terminal GST-tagged p51 subunits and N-terminal His_6-tagged p66 were performed as described previously (Auwerx et al., 2002).

FPLC Size-Exclusion Chromatography. Size-exclusion chromatography was performed using a 10 × 300-nm Superdex 200 HR 10/30 column (Amersham Biosciences). Freshly prepared RT samples or RT samples that were left in elution buffer for 24, 72, or 336 h after elution from a Ni-NTA column and that contain 5 to 10 μg of protein were applied on the size-exclusion column and eluted with 200 mM potassium phosphate, pH 7.0, at the flow rate of 0.5 ml as described previously (Restle et al., 1990).

CD Spectroscopy. CD spectra of wild-type and mutant N136T and N136L RTs in the far-UV region (190–260 nm) were acquired at 25°C on a Jasco J-600A spectropolarimeter (Jasco, Tokyo, Japan) using a cuvette of 1 ml. Protein solutions were 0.17 and 0.25 μM in 0.75 mM EDTA, 0.75 mM DDT, 2% glycerol and 7.5 mM Tris at pH 7.8. The data were expressed as residual ellipticity [Θ] (deg cm^2 · $dmol^{-1}$) using 114.75 as the mean residue weight for HIV-1 RT. The spectra were obtained with a 1-nm bandwidth, a 1-s time constant, and a data density of 10 points/nm. To estimate the fractions of the different types of secondary structure, analysis of CD data was performed with the CDNN program (Böhm et al., 1992).

Results

Catalytic DNA Polymerase Activity of HIV-1 RT Mutated at Amino Acid Position Asn136. To investigate the influence of changes at the amino acid residue Asn136 of HIV-1 RT activity, we constructed eight recombinant RTs by site-directed mutagenesis: 136A, 136L, 136Y, 136K, 136D, 136S, 136T, and 136Q. In this way, the different types of amino acid side chains were represented: an aliphatic side chain in alanine and leucine, an aromatic side chain in tyrosine, a protonated amino group in the positively charged lysine, a carboxylate in the negatively charged aspartic acid, and a polar uncharged group in serine, threonine, and glutamine. The above-mentioned mutations were introduced in both p66 and p51 subunits of the RT heterodimer, and all mutant recombinant RTs were purified to ≥98% homogeneity through Ni-NTA- and heparin-containing affinity columns.

To perform RT assays that could reliably measure the catalytic activity of the different mutant enzymes, the following enzyme (protein) amounts were used in the 50-μl reaction mixture: wild-type Asn136 RT, 0.87 ng; N136A RT, 17 ng; N136Q RT, 12 ng; N136Y RT, 162 ng; N136K RT, 51 ng; N136T RT, 7 ng; N136S RT, 27 ng; N136L RT, 26 ng; and N136D RT, 29 ng. Under the experimental conditions, between 5000 dpm (for the highest amounts of RT protein) and 60,000 dpm (for the lowest amounts of RT protein) [8-^{3}H]dGTP was incorporated into the template/primer poly(rC) · oligo(dG) after a 30-min incubation period. As a rule, the RNA-dependent DNA polymerase (RDDP) activities of all mutant enzymes were impaired severely (Fig. 2). The catalytic activity of the mutant enzymes ranged between 0.07 and 2.1% of wild type. The catalytically most active mutant RT contained the N136T mutation (2.1% activity of wild type), and the presence of the N136Y, N136L, and N136D amino acid mutations in HIV-1 RT resulted in mutant enzymes endowed with the poorest catalytic activity (≤0.1%). Thus, mutating Asn136 of HIV-1 RT to other amino acids results in severely compromised RTs possessing a very low catalytic RDDP activity.

The mutant N136T RT had the highest catalytic activity among all the mutant Asn136 RTs studied. In this enzyme, the N136T mutation was present in both p66 and p51 subunits. To assess the role of the subunit location of the N136T mutation in the decreased catalytic activity of the mutant RT, two additional mutant RTs were constructed in which the N136T mutation was introduced in either solely the p66 or solely the p51 subunit of the heterodimeric RT enzyme. Although the mutant RT enzyme in which N136T was solely present in the p51 subunit had a catalytic activity that was 8.4 ± 3.7% of wild-type enzyme, the heterodimeric enzyme at which N136T was solely present in p66 had a catalytic activity of 71 ± 23% of the wild-type enzyme. Thus, the exclusive presence of the N136T mutation in p51 had a much more deleterious effect on the catalytic activity of the mutant enzyme than when it was solely present in p66.

Inhibitory Activity of NNRTIs and ddGTP against Wild-Type and Mutant Asn136 Recombinant HIV-1 RTs. The mutant enzymes were evaluated for their sensitivity to the inhibitory activity of a variety of NNRTIs and the NRTI derivative ddGTP (Table 2). The inhibitory activity of both NNRTIs and ddGTP against wild-type RT, expressed as an IC_{50} value, ranged between 0.022 and 0.45 μM, depending on the nature of the compound. The N136T RT mutant that was endowed with the highest catalytic activity among all RT mutants tested kept pronounced sensitivity to all NNRTIs and ddGTP. In addition, the mutant N136D RT reasonably kept its sensitivity to the drugs (decrease of the inhibitory activity of the NNRTIs ranged between 1.4- to 6-fold). In contrast to the susceptibility of the mutant N136T and N136D RTs to the inhibitory effect of the NNRTIs, the other mutant Asn136 RT enzymes substantially lost between 10- and >25-fold sensitivity to some of the evaluated drugs, depending on the nature of the drug and the introduced amino acid mutation. It is interesting that efavirenz, which

was among the most potent inhibitors of the wild-type RT enzyme, only lost its inhibitory potential by 6-fold at most. It is remarkable that the inhibitory potential of the NRTI derivative ddGTP against one or several of the mutant RT enzymes in most cases decreased at least 10-fold and in some cases even 50- to 100-fold. The N136L RT mutant was quite resistant to most of the NNRTIs and to ddGTP. It should be noted that different enzyme (protein) concentrations were required to reliably obtain the above-mentioned drug-inhibition values. The protein amounts used in the assays ranged between 0.87 ng for wild-type RT and 162 ng for mutant N136Y RT (see *Materials and Methods*). However, when expressed in nanomolarity, wild-type enzyme was present at ~0.15 nM, and the (least catalytically efficient) mutant N136Y RT was present at ~30 nM in the assay mixture. Thus, the enzyme concentrations used were well below those of the compounds that showed an inhibitory effect against the wild-type and mutant enzymes. Moreover, under these experimental conditions, it was ascertained that the IC_{50} values of the test compounds were not affected when enzyme concentrations (i.e., wild-type RT) in the assay had been varied by at least 100-fold (data not shown). Thus, although the drug inhibition data were obtained in the presence of a wide variety of mutant enzyme concentrations, the IC_{50} values depicted in Table 2 can be reliably compared with one another.

When the NNRTIs and ddGTPs were evaluated for their inhibitory activity against the HIV-1 RT heterodimers that contained the N136T mutation solely in either the p66 or the p51 subunit, no marked differences were noted compared with the wild-type or mutant RTs that contained this mutation in both subunits (Fig. 3).

Effects of Exposure of Urea and Acetonitrile on Mutant Asn136 RT Activity. Wild-type and four mutant Asn136 HIV-1 RTs (i.e., N136T, N136K, N136Y, and N136L) were exposed to a wide variety of urea (or acetonitrile for N136T and N136L) concentrations, and the catalytic activity of the urea- (and acetonitrile) exposed enzymes was measured (Fig. 4, A and B). For the wild-type enzyme, the RT activity gradually decreased in the presence of increasing urea concentrations (Fig. 4A). A urea concentration as low as 0.20 M slightly decreased the catalytic activity of wild-type RT, and 2.0 M urea abolished its catalytic activity almost completely. Half of the RT catalytic activity was retained at around 1.0 M urea. However, when the mutant Asn136 RT enzymes were exposed to the different concentrations of urea, the enzymes had invariably gained a mark-

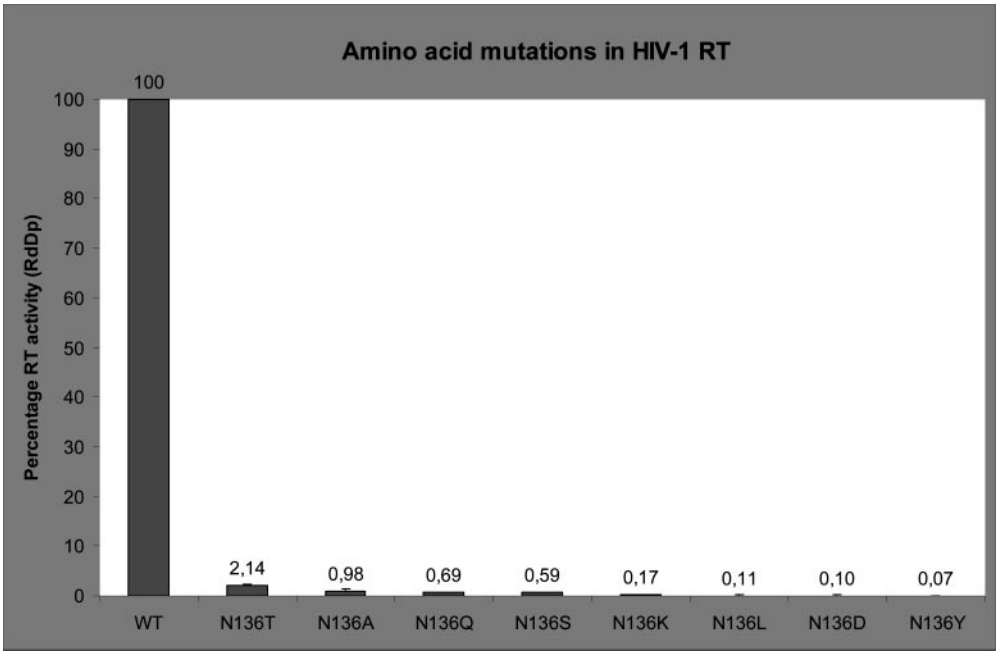

Fig. 2. Catalytic RNA-dependent DNA polymerase activity of mutant Asn136 HIV-1 RT enzymes. Poly(rC)/oligo(dG) was used as the template/primer and [^{3}H]dGTP as the radiolabeled substrate. The following enzyme (protein) concentrations were used in the enzyme assays: WT, 0.87 ng; N136A, 17 ng; N136Q, 12 ng; N136Y, 162 ng; N136K, 51 ng; N136T, 7 ng; N136S, 27 ng; N136L, 26 ng; and N136D, 29 ng.

TABLE 2
Inhibitory activity of test compounds against mutant HIV-1 RTs (N136X)
Data are the mean (± S.D.) of at least three to four independent experiments.

RT Mutation	IC_{50}				
	Nevirapine	Delavirdine	Efavirenz	Quinoxaline	ddGTP
			μM		
WT	0.45 ± 0.25	0.31 ± 0.025	0.027 ± 0.006	0.022 ± 0.006	0.051 ± 0.009
N136T	1.0 ± 0.29	0.90 ± 0.16	0.035 ± 0.006	0.033 ± 0.004	0.065 ± 0.007
N136A	>10	6.6 ± 2.4	0.21 ± 0.20	0.28 ± 0.01	0.28 ± 0.48
N136Q	≥10	4.5 ± 1.7	0.051 ± 0.018	0.39 ± 0.21	1.4 ± 0.20
N136S	5.1 ± 2.6	3.1 ± 1.7	0.052 ± 0.044	0.35 ± 0.32	0.58 ± 0.51
N136K	>10	≥10	0.16 ± 0.021	≥1	0.93 ± 0.64
N136L	>10	9.1 ± 1.3	0.13 ± 0.014	0.80 ± 0.021	6.1 ± 1.0
N136D	2.7 ± 2.3	1.3 ± 1.2	0.036 ± 0.0007	0.037 ± 0.002	0.19 ± 0.021
N136Y	>10	>10	0.12 ± 0.01	≥1	1.6 ± 0.32

Template/primer, poly(rC) · oligo(dG); substrate, 1.6 μM [^{3}H]dGTP.

edly higher sensitivity to the denaturing activity of urea. Although the urea IC_{50} value shifted from 1 M for wild-type enzyme to 0.6 M for the N136T mutant RT, the urea IC_{50} was further decreased to 0.25 and 0.20 M for the mutants N136Y and N136K RT, respectively. The N136Q RT mutant showed an intermediate sensitivity to urea ($IC_{50} \sim 0.4$ M). An equally increased sensitivity to urea was observed for mutant N136T RT when the N136T mutation was solely introduced in p51 (Fig. 4A, □ curve compared with ■ curve), whereas the sensitivity to urea was not markedly increased when the N136T mutation was solely introduced in the p66 subunit of the enzyme (data not shown). Such a markedly increased sensitivity of the Asn136 RT mutants toward urea was not observed for mutant Y318H, Y318L, or Y318W RTs whose catalytic activity was also compromised compared with wild-type RT (i.e., 1.6, 4.4, and 73% of the wild-type RT, respectively) (Pelemans et al., 1998) but for which the amino acid mutation is located in the p66 thumb area of and not at the p66/p51 interface (data not shown).

Acetonitrile also had a more pronounced effect on inactivation of the catalytic activity of the Asn136 mutant enzymes than on that of the wild-type RT enzyme. Indeed, whereas ~6.7% acetonitrile was required to decrease the wild-type enzyme activity by 50%, lower concentrations of acetonitrile (i.e., 5.0 and 3.2%) were sufficient to afford a comparable (50%) decrease in catalytic activity for the mutant N136T and N136L RT enzymes, respectively (Fig. 4B).

It was also striking that there was a very close correlation between the catalytic activity of the mutant Asn136 RTs and the urea concentration required to decrease RT activity by 50%. The r^2 value of the regression line was as high as 0.996 (Fig. 5). Such correlation was absent for the Tyr318 mutant RTs in which the Tyr318 is located near the thumb domain of RT, and that also results in compromised catalytic activity when mutated (Pelemans et al., 1998).

Determination of K_m Values of Template/Primer for Wild-Type and Mutant HIV-1 RTs in the Presence or Absence of Urea. The K_m values of the template/primer poly(rC) · oligo(dG) for wild-type and mutant N136T and N136L HIV-1 RTs were determined in the presence or absence of different urea concentrations (Fig. 6 and Table 3). The K_m value of template/primer was lowest for wild-type RT (10 μM) and increased for the mutant enzymes as a function of their more pronounced compromised catalytic activity; thus, the K_m value was 1.5-fold higher than wild-type for mutant N136T RT (15 μM) and was highest for mutant N136L RT (33 μM). It is interesting that in all cases (wild-type, N136T RT, and N136L RT), the presence of urea further dose-dependently increased the K_m value of the enzymes for

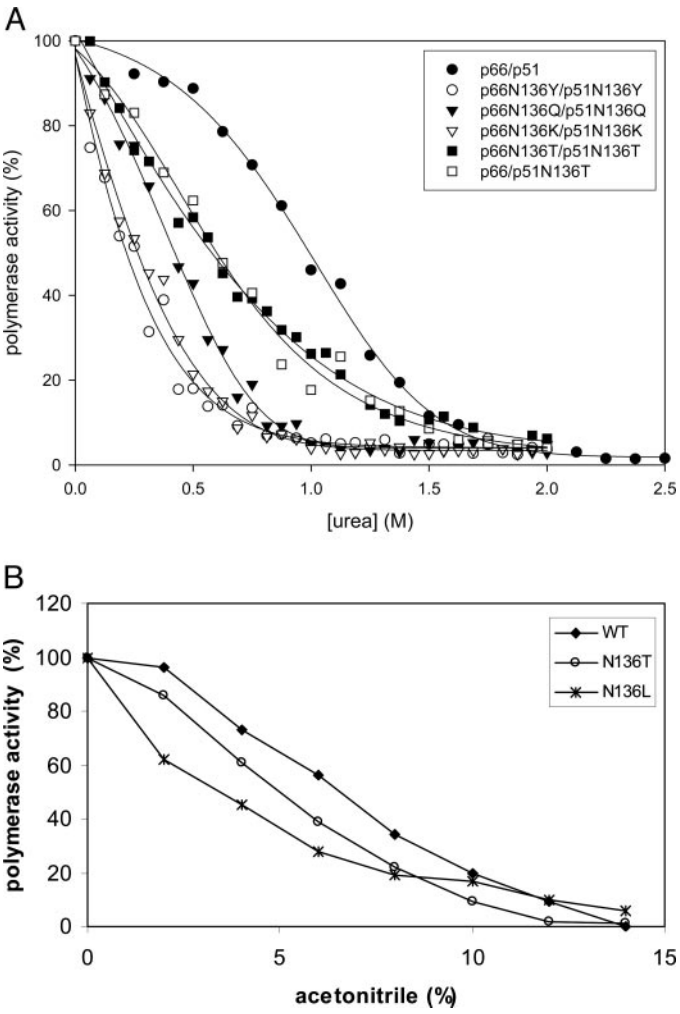

Fig. 4. Effect of urea (A) and acetonitrile (B) on the catalytic activity of mutant Asn136 HIV-1 RTs. A variety of different urea concentrations or acetonitrile concentrations was added to the RT reaction mixture, and the assay was started by the addition of radiolabeled substrate. After a 30-min incubation period, the reaction mixture was precipitated, washed with TCA, and the radiolabel present in the TCA-insoluble material was determined in a liquid scintillation counter.

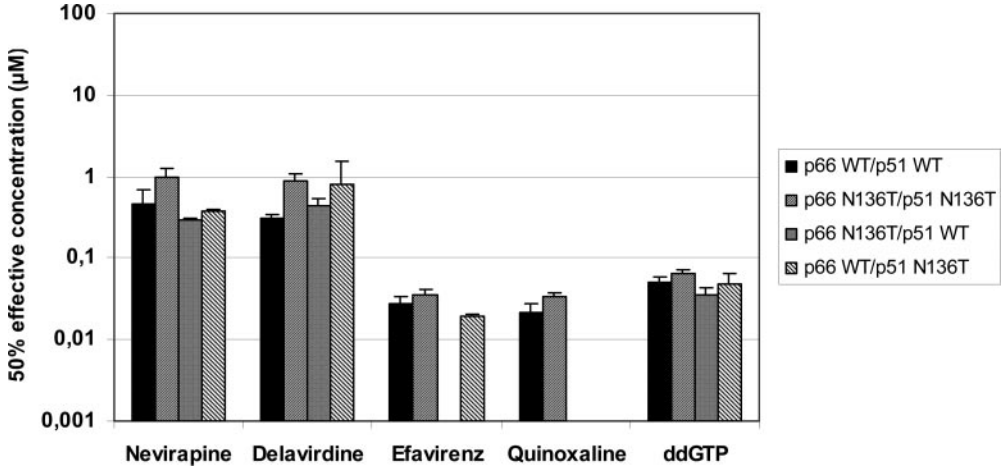

Fig. 3. Inhibitory activity of test compounds against the catalytic activity of mutant HIV-1 RT enzymes that contain the Asn136 threonine mutation solely in the p66 (▨), solely in the p51 (▧), or in both the p66 and the p51 (▨) subunits of RT. Wild-type enzyme is represented by ■.

the template/primer (4.7-, 2.5-, and 3-fold, respectively) at the highest urea concentrations tested (1.5 M for WT RT, 0.9 M for N136T RT, and 0.45 M for N136L RT, respectively). The different urea concentrations chosen for the individual (wild-type and mutant) enzymes corresponded to comparably inactivating effects of these urea concentrations on the wild-type and mutant enzymes.

Analysis of Monomer Content of Wild-Type and Mutant Asn136 Heterodimer RTs at Different Time Points after Isolation. Wild-type and mutant N136T and N136Y HIV-1 RT heterodimers [consisting of N-terminal His_6-tagged p66 and GST-tagged p51 mutant subunits] were isolated from an Ni-NTA column. The fraction that corresponded to each p66/p51 heterodimer (as ascertained by subsequent gel electrophoresis) was then left in the elution solution at −20°C and analyzed on a size-exclusion FPLC column after 24, 72, and 336 h (14 days). Whereas >99% of wild-type RT enzymes still existed as a p66/p51 heterodimer at all time points measured, the mutant N136T RT heterodimer consisted of a mixture of p66/p51 heterodimer plus p66 and p51 monomers after 24 h. After 72 h, the formation of monomers in the mixture had proceeded still further (Fig. 7). The ratio of monomer/heterodimer content of the mutant N136Y RT was even higher than that observed for the mutant N136T RT after 24 and 72 h (Fig. 7). Thus, the lower the catalytic activity of the enzyme, the higher the ratio of free monomer/heterodimer in the enzyme preparation at ≥24 h after isolation of the enzyme. The earlier apparent retention time (~9.3 min) of WT RT heterodimer than mutant heterodimer RT (retention time, ~10.2 ml) is probably an artifact in the FPLC running. The nature of the peak that seems to appear between the heterodimer and monomer fraction upon longer incubation times is currently unknown. However, its appearance might be caused by the action of a specific contaminating protease activity, because the samples do not contain any protease inhibitor.

CD Spectra of Wild-Type and Mutant HIV-1 RTs. To reveal whether the mutations at amino acid 136 had an effect on the general structure and conformation of the HIV reverse transcriptase, the CD spectra of wild-type and the mutant N136T and N136L RTs were determined and compared (Fig. 8).

The CD spectrum of wild-type HIV-1 RT exhibits a broad and prominent band of negative ellipticity between 260 and 202 nm, with a peak at 212 nm and a shoulder at 226 nm. The ellipticity becomes positive in the region of 202 to 190 nm. The CD spectrum of the mutant N136T RT is also dominated by a negative ellipticity band, but compared with the WT HIV-1 RT, it is considerably blue-shifted, with a peak at 204 nm, a shoulder at 220 nm, and a crossover at 196 nm. The overall shape of the spectrum of the mutant N136L RT is similar to that of the mutant N136T RT but with a slightly increased negative and positive ellipticity and a crossover at 198 nm (Fig. 8). Both mutant RTs display CD spectra typical of a considerable amount of α-helical structures, with a positive band at 190 nm and a double minimum in the region of 205 to 220 nm. These spectra, therefore, show a similar secondary structure, however, that is clearly different from that of the wild-type RT. To quantify the observed spectral changes, the contribution of the various secondary structural elements to the measured CD spectra was determined using the CDNN program of Böhm et al. (1992). The results obtained with this deconvolution program are listed in Table 4 and confirm the increased α-helical content in the mutants. Thus, the substitution of Asn136 by threonine or leucine induces structural effects in HIV-1 RT that are observed as an increase in the number of α-helices (11–13%) and a slight decrease of both random coil (8%) and β-sheet (3%).

Discussion

X-ray crystallographic analyses have revealed subtle structural differences in a large number of complexes of HIV-1 RT

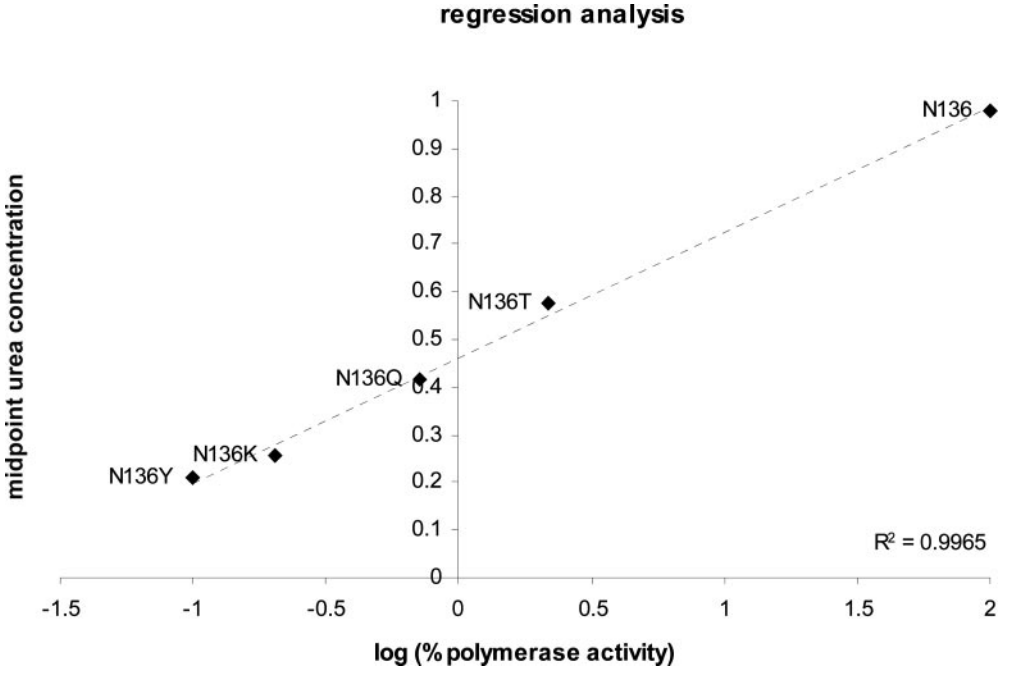

Fig. 5. Correlation between the catalytic activity of mutant Asn136 HIV-1 RT enzymes and the IC_{50} value of urea against these mutated enzymes. The log percentage of RT activity was taken from Fig. 2, and the midpoint of the urea concentration at which urea resulted in 50% inhibition of enzyme activity was calculated from the curves depicted in Fig. 4.

with different NNRTIs (Kohlstaedt et al., 1992; Ding et al., 1995; Esnouf et al., 1995; Ren et al., 1995; Hopkins et al., 1996). The energetics of dimerization have also been studied computationally, and the contributions of individual residues to the surface area that is buried upon dimer association have been dissected (Rodriguez-Barrios et al., 2001). The segment from Ile135 to Pro140 in p51, which is part of the β7–β8 loop and essential for the catalytic activity of the p66 subunit (Pandey et al., 2001, 2002), has been identified as a "hot spot" of binding energy (Rodriguez-Barrios et al., 2001) (Fig. 9). Of these amino acids, Asn136 is the most buried (Rodriguez-Barrios et al., 2001) and engages its side-chain carboxamide group in two hydrogen bonds to the backbone of His96 in the p66 subunit (Fig. 1). It is interesting that Asn136 is highly conserved among all known lentiviral RTs (Table 1), and our site-directed mutagenesis studies now reveal that none of the mutant Asn136 enzymes shows significant catalytic RT activity. It is noteworthy that the N136T RT mutant, which was endowed with the highest catalytic activity among all HIV-1 RT mutants tested in this study, is the most prevalent enzyme mutant found in patients infected with HIV-1 who have been treated with NNR-

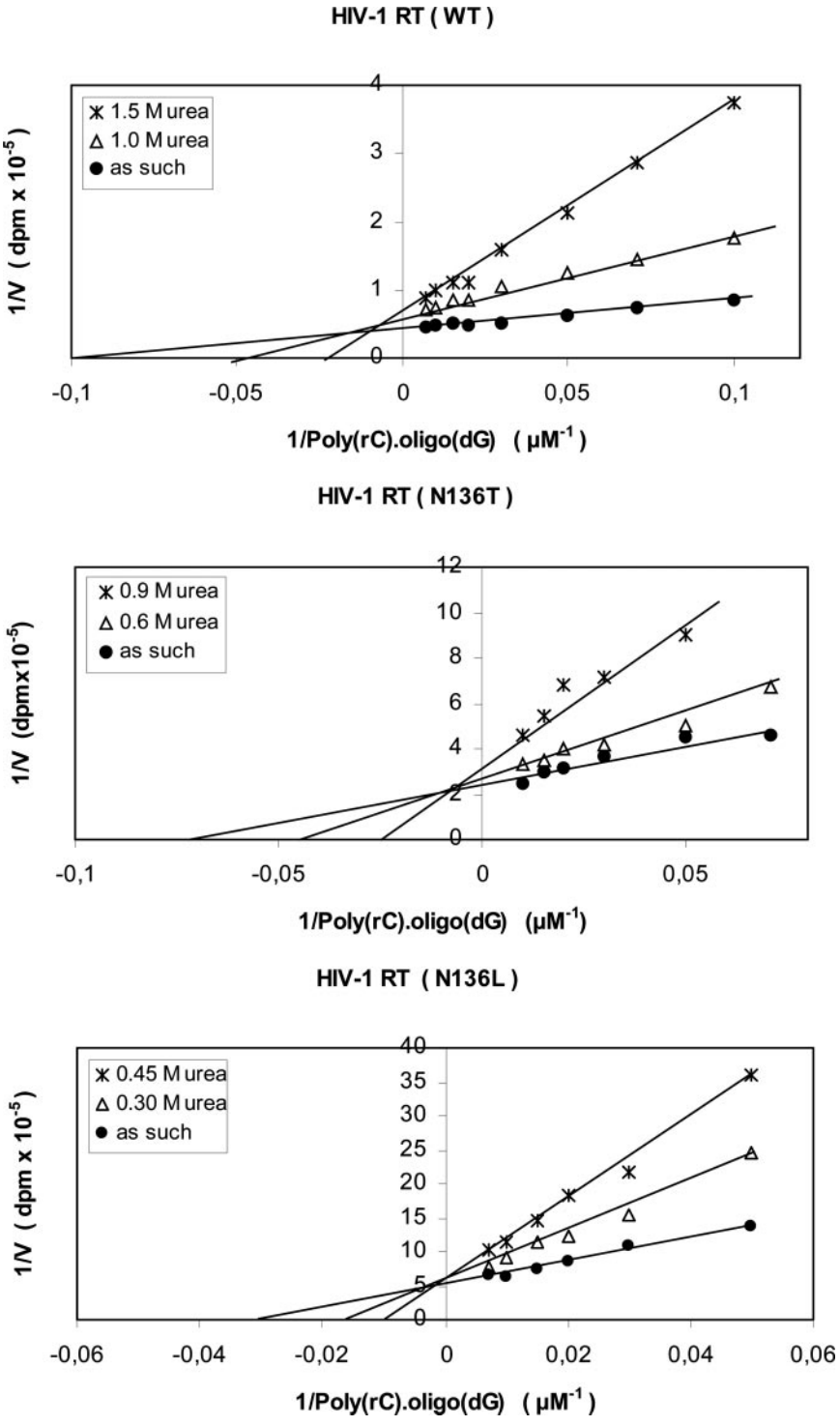

Fig. 6. Lineweaver-Burk plots (reciprocal of S versus V) for the kinetics of wild-type and mutant N136T and N136L HIV-1 RTs in the absence (●, as such; i.e., without urea) or in the presence (*, △) of different urea concentrations. Poly(rC)/oligo(dG) was used as template/primer and [^{3}H]dGTP as the radiolabeled substrate.

TABLE 3

K_m values of wild-type and mutant Asu136 RT enzymes for poly(rC) · oligo(dG) in the presence of varying concentrations of urea

Data are the mean of three to four independent experiments. The K_m values were calculated from the average Lineweaver-Burk plots shown in Fig. 7. The amounts of WT, N136T, and N136L RT enzyme used in the 50-μl assays were 0.85, 7.0, and 26 ng of protein, respectively.

RT Enzyme and Urea Concentration	K_m of Template/Primer poly(rC) · oligo(dG)
	μM
Wild-type	
0 M	10
1.0 M	15
1.5 M	47
N136T	
0 M	15
0.6 M	22
0.9 M	38
N136L	
0 M	33
0.3 M	68
0.45 M	98

TIs, with a prevalence of <1% (12 of 1556 patients) (Ceccherini-Silberstein et al., 2005). Although it is somewhat surprising that this mutation appeared in patients treated with NNRTIs because Asn136 does not make direct contact with the exposed drugs, it should be mentioned that the mutations at amino acid 136 are not the sole amino acid mutations present in the RT of the drug-treated patients infected with HIV-1; rather, they are accompanied by several other amino acid changes that may affect not only drug resistance, but also fitness (by increasing replication competence) of these mutant virus strains.

Asn136 in the p66 subunit is located in the solvent-exposed $\beta 7$–$\beta 8$ loop structure that is found markedly distant from both the substrate active site and the NNRTI-binding pocket. Instead, Asn136 in the p51 subunit is located right at the interface between p66 and p51 and contributes to the formation of the bottom of the NNRTI pocket (Fig. 1). Therefore, it was inferred that the heavily compromised catalytic activity

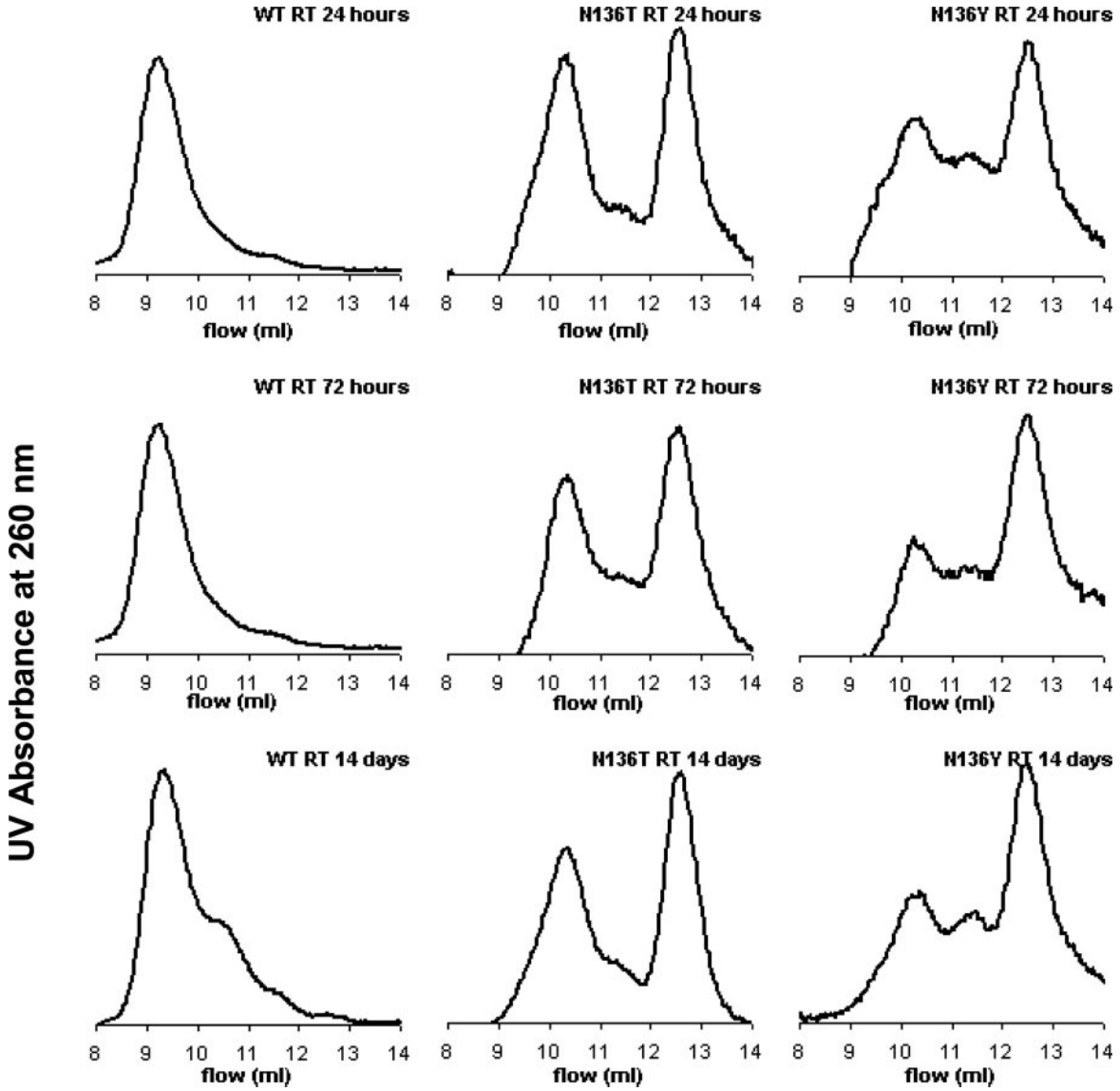

Fig. 7. Determination of p66/p51 heterodimer and free p66 and p51 monomers in wild-type and mutant N136T and N136Y RT heterodimer enzyme preparations by size-exclusion chromatography as a function of incubation time and determined from size-exclusion chromatography. Freshly prepared RT samples were left in elution buffer for 24 (top), 72 (middle), or 336 h (14 days) (bottom) after elution from an Ni-NTA column. Left peaks eluting between 8 and 11 ml represent heterodimer; right peaks eluting between 12 and 14 ml represent a mixture of monomers.

of Asn136-mutated HIV-1 RT is predominantly caused by the effect of the mutated amino acid in the p51 subunit. Indeed, when the N136T mutation was solely introduced in the p66 subunit, the RDDP activity was substantially restored (71% of wild-type), whereas the mutation solely in p51 resulted in only 8% of wild-type RT activity.

The observed effect of the mutated amino acid at position 136 in p51 of the HIV-1 RT catalytic activity is in full agreement with the findings of Pandey et al. (2001), who demonstrated that the β7–β8 loop of p51 is a key structural element for RT dimerization (Pandey et al., 2002). It is clear that Asn136 in HIV-1 RT must fulfill a crucial structural function in the p51 β7–β8 loop to preserve the catalytic activity of the heterodimer. The observed decreased affinity of the mutant enzymes for the template/primer (the lower the catalytic activity of the mutant RT, the higher the K_m value for the template/primer) and the further decreased apparent affinity for the template/primer in the presence of urea are in full agreement with the requirement of an intact β7–β8 loop of p51 for loading the p66 subunit on the template primer (Harris et al., 1998).

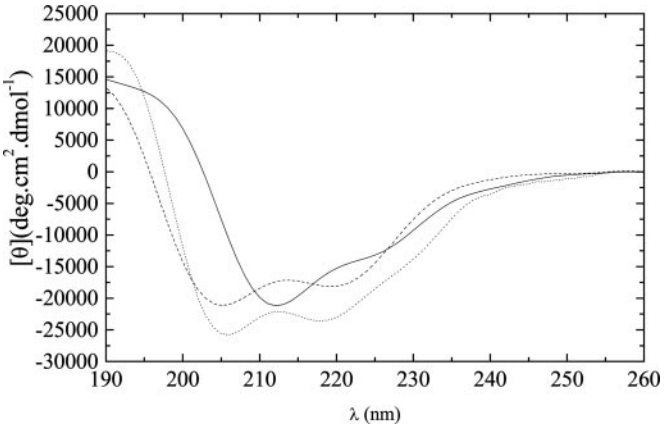

Fig. 8. CD spectra of wild-type and mutant N136T and N136L RTs. Solid line, wild-type HIV-1 RT; dotted line, N136L HIV-1 RT; broken line, N136T HIV-1 RT.

TABLE 4
Secondary structure analysis of the CD of wild-type and mutant N136L and N136T RTs

	Helix	Antiparallel	Parallel	β-Turn	Random Coil	Total
			%			
HIV-1 RT	50.4	5.9	5.3	14.2	24.2	100
N136L	62.7	2.3	4.7	14	16.3	100
N136T	63.6	3.6	3.6	12.8	16.6	100.1

There are three major contact areas between the p66 and the p51 subunits of RT (Becerra et al., 1991b; Wang et al., 1994). The most intensively studied interface area between p66 and p51 is the tryptophan-rich amino acid stretch in the connection subdomain of both RT subunits consisting of six tryptophan residues at codons 398, 401, 402, 406, 410, and 414. Mutagenesis studies at these amino acid locations revealed an important role in RT dimerization. It is interesting that the effect of these amino acids on dimerization proved to be mediated mainly through the p66 subunit. Thus, mutation at Trp401 and Trp414 in p66 resulted in a complete lack of RT activity (Tachedjian et al., 2003). Such mutations impaired RT subunit dimerization by altering the proper positioning of these structural elements against those residues in p51 that make important contacts with p66. A second stretch suggested to be involved in dimerization contains a leucine hepta-repeat motif (Leu282 to Leu310) (Becerra et al., 1991a). This region in p66 has been shown to be instrumental in protein-protein interactions required for p66/p51 RT dimerization (Baillon et al., 1991; Goel et al., 1993). From the crystallographic analyses of HIV-1 RT both in the apo form and in complex with inhibitors, it is apparent that Asn136 is located in the middle of the third major contact area at the interface between p66 and p51. Likewise, we believe that Asn136 in the β7–β8 loop of HIV-1 RT p51 may also perform a function in p66/p51 heterodimerization comparable with that of the tryptophans in the 398 to 414 amino acid stretch of p66 and that of Leu289 in the leucine hepta-repeat stretch of p66. Indeed, it was found by FPLC size-exclusion chromatography that, after mixing equal amounts of p66 and p51 subunits, mutant N136T and N136Y RTs consisted of markedly less intact p66/p51 heterodimer and much more free p66 and p51 subunits than wild-type after 24 h (Fig. 7). These observations are strongly suggestive of a less optimal association of the p66 and p51 subunits in the mutated enzymes than in the wild-type RT. This disturbed binding efficiency between p66 and p51 can be related to the structural changes the mutations at Asn136 afford on RT (increased amount of α-helices and less random coil according to CD analysis of the mutant RT enzymes) (Fig. 8 and Table 4). The pronounced

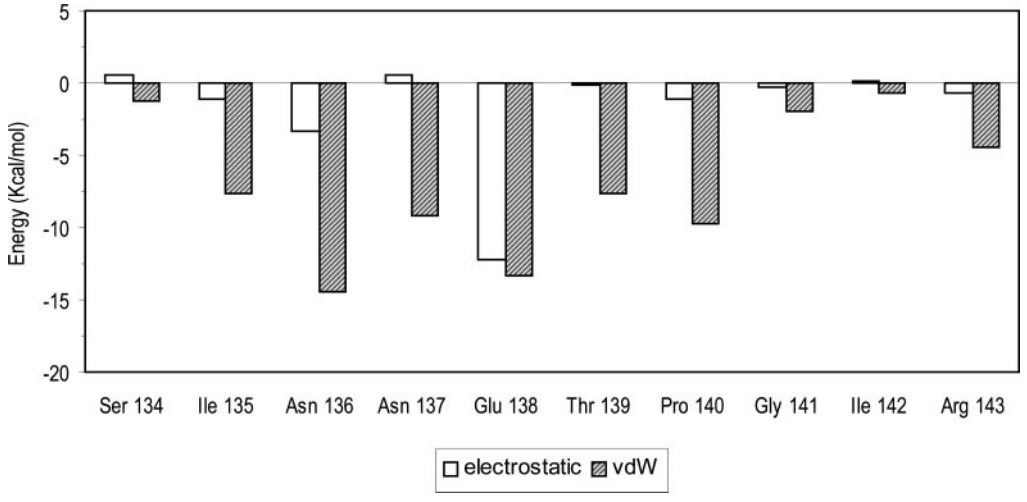

Fig. 9. van der Waals (▨) and electrostatic (□) contributions of the residues making up the tip of the p51 β7–β8 loop to the dimerization energy of HIV-1 RT (modified from Rodriguez-Barrios et al., 2001).

effect of the Asn136 mutation on the RT p66/p51 interface also has a clear effect on the efficiency of DNA binding, because the K_m value of the mutant enzymes for template/primer is higher than the K_m for wild-type enzyme. This observation is in full agreement with the instrumental role of the p51 subunit in the loading of DNA onto the heterodimeric enzyme.

When mutant Asn136 RT enzymes were exposed to a variety of urea concentrations, we found that the lower the catalytic activity of the mutant enzymes, the more easily they were denatured by urea (Fig. 4). These findings may point to a less tight association between both RT subunits at the dimerization interface in the mutant Asn136 enzymes and, again, to the crucial role of this highly conserved asparagine at this location in p51 for maintaining two hydrogen bonds with the main chain of His96 of p66, thus ensuring tight subunit association (Fig. 1). Indeed, Sluis-Cremer and coworkers (2002) reported that in the presence of relatively low urea concentrations (< 2 M), loss of RT activity is a direct result of the dissociation of the heterodimeric RT and is not a result of a conformational change in protein secondary structure. Their studies and also those of Menéndez-Arias et al. (2001) also implied that no significant structural changes (or unfolding events) are associated with the dissociation of the RT heterodimer subunits at the urea concentrations used in our studies.

In contrast with the first-generation NNRTIs (i.e., nevirapine and delavirdine), the second-generation NNRTIs (i.e., efavirenz and quinoxaline) kept a marked inhibitory potential against the mutant enzymes irrespective of the nature of the mutation at amino acid 136. It is interesting that the very potent efavirenz, which is known to enhance RT dimerization (Tachedjian et al., 2001; Tachedjian and Goff, 2003), and the quinoxaline derivative GW420867 retain pronounced inhibitory activity against all Asn136-mutated RT enzymes. Therefore, the impact of a mutation at Asn136 in the p51 of the HIV-1 p66/p51 RT heteroduplex on the sensitivity of the mutant enzymes to second-generation NNRTIs seems to be relatively minor.

Our study now suggests that designing Asn136 mimetics, possibly possessing a similar carboxamide group, might become a novel strategy to develop drugs that interfere with the $\beta7$–$\beta8$ dimerization interface. Another unexplored possibility, given the relative proximity of Asn136 to the NNRTI binding pocket, would be to rationally design modifications of existing NNRTIs to additionally target this highly conserved amino acid in the p51 subunit to improve their resistance profile and suppressive effect on wild-type HIV-1 strains.

In conclusion, Asn136 represents a highly conserved amino acid whose role in HIV-1 RT is shown here to be severely compromised upon mutation to other amino acids. Natural mutations at this amino acid site of RT will lead to a virtually inactive enzyme (and thus poorly replicating virus, if viable at all) that nevertheless will still be sensitive to several second-generation NNRTIs, including the clinically used efavirenz. Therefore, it is very unlikely that mutations at position 136 in HIV RT will appear under selective pressure of these drugs. Asn136 therefore could become an attractive target for the design of novel NNRTIs with improved potency and increased ability to suppress development of drug-resistant viruses.

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Address correspondence to: Prof. J. Balzarini, Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium. E-mail: jan.balzarini@rega.kuleuven.be