0026-895X/04/6501-244–251$20.00
MOLECULAR PHARMACOLOGY
Vol. 65, No. 1
2818/1120482
Mol Pharmacol 65:244–251, 2004
Printed in U.S.A.

Susceptibility of Feline Immunodeficiency Virus/Human Immunodeficiency Virus Type 1 Reverse Transcriptase Chimeras to Non-Nucleoside RT Inhibitors

Joeri Auwerx, Robert Esnouf, Erik De Clercq, and Jan Balzarini

Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium (J.A., E.D.C., J.B.); and the Division of Structural Biology, Henry Wellcome Building for Genomic Medicine, University of Oxford, Oxford, United Kingdom (R.E.)

Received July 14, 2003; accepted October 1, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

To map the determinants of the lack of susceptibility of feline immunodeficiency virus (FIV) reverse transcriptase (RT) to anti human immunodeficiency virus type 1 (HIV-1) non-nucleoside RT inhibitors (NNRTIs), a variety of chimeric HIV-1/FIV RTs were constructed. The majority of chimeric RTs had an affinity (K_m) for their natural substrates comparable with that of the wild-type HIV-1 and FIV RTs, but their catalytic efficacy was decreased. Whereas HIV-1 RT could be made entirely insensitive to NNRTIs by exchanging the amino acid sequence 97 through 205 of FIV RT, none of the reverse FIV/HIV-1 RT chimeras gained susceptibility to NNRTIs. The amino acids that are thought to be involved in NNRTI susceptibility and that are different from those in HIV-1 RT have also been introduced in FIV RT. These mutant RTs gained virtually no susceptibility to efavirenz or capravirine. Vice versa, when these HIV-1–specific amino acids were replaced by their FIV RT counterparts in HIV-1 RT, susceptibility to the NNRTIs was lost. Thus, replacing segments or substituting relevant amino acids in FIV RT by their HIV-1 RT counterparts did not suffice to make FIV RT sensitive toward NNRTIs and was often accompanied by a decrease or even total loss of polymerase activity. It is postulated that, in contrast to the results found for HIV-1/HIV-2 RT chimeras and supported by the crystal structure of HIV-2 RT, there exist significant differences in the structure and/or flexibility of FIV RTs that may prevent NNRTIs from interacting with the FIV RT.

Non-nucleoside reverse transcriptase (RT) inhibitors (NNRTIs) are highly specific for human immunodeficiency virus type 1 (HIV-1), and they are not active against HIV-2 or any other retrovirus, including simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV). This is in contrast to the nucleoside class of inhibitors, the nucleoside reverse transcriptase inhibitors, which are not specific inhibitors of HIV-1 and are broadly effective to other lentiviruses as well, including FIV (North et al., 1990). NNRTIs act non-competitively with respect to both template/primer and nucleotide substrate and fit in a binding pocket that is located in the p66 subdomain near, but distinct from, the polymerase active site. Several crystal structures of HIV-1 RT in complex with different classes of NNRTIs show that all NNRTIs share a common binding site (Arnold et al., 1992; Kohlstaedt et al., 1992; Stammers et al., 1994). Structurally diverse classes of NNRTIs used in this study, including the three clinically approved NNRTIs (nevirapine, delavirdine, and efavirenz), all have significant potential to suppress HIV-1 replication in cell culture and in HIV-1–infected persons. Yet, a limited factor is the emergence of drug-resistant virus strains. When the virus was exposed to the drugs for a number of serial passages in cell culture, or when HIV-1-infected persons are treated with these drugs for varying time periods, a wide variety of amino acid mutations in the HIV-1 RT may occur depending on the nature, duration and dose of the NNRTIs administered. The mutations that lead to partial or full resistance of the virus to the NNRTIs have been reported (for review, see Schinazi et al., 2000).

In many cases, one point mutation in the NNRTI-specific pocket of HIV-1 RT is sufficient to substantially decrease the susceptibility of the mutated enzyme (and virus) to the first-

This research was supported by the European Commission (grant QRLT 2000-0291 and the Réné Descartes Prize 2001 grant HPAW-2002-90001), the "Geconcerteerde Onderzoeksacties van de Vlaamse Gemeenschap" (GOA 00-12) and the "Fonds voor Wetenschappelijk Onderzoek Vlaanderen" (FWO G-0104-98). J.A. is a recipient of the Flemish Institute supporting the Scientific-Technological Research in Industry (IWT).

ABBREVIATIONS: RT, reverse transcriptase; NNRTI, non-nucleoside reverse transcriptase inhibitors; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; FIV, feline immunodeficiency virus; TSAO-m^{3}T, [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; ddGTP, 2′,3′-dideoxyguanosine-5′-triphosphate; PFA, phosphonoformic acid (foscarnet); GW420867X, (*S*)-2-ethyl-7-fluoro-3-oxo-3,4-dihydro-2*H*-quinoxaline-carboxylic acid isopropylester; UC-781, 2-methylfuran-3-carbothioic acid[4-chloro-3-(3-methyl-but-2-enyloxy)-phenyl]-amide.

generation NNRTIs (such as nevirapine and delavirdine). The appearance of concomitant multiple amino acid mutations are required to markedly compromise the inhibiting potential of second-generation NNRTIs (such as efavirenz, quinoxaline, thiocarboxanilides, and capravirine). An understanding of the structural basis for HIV-1 RT sensitivity and resistance to NNRTIs is important for monitoring and anticipating the development of clinical resistance of HIV-1 to NNRTIs. In addition, studies on the role of the individual amino acids in the sensitivity of HIV-1 RT toward double-stranded NNRTIs may provide novel insights into RT function.

Interestingly, the majority of amino acids in the NNRTI pocket of HIV-1 RT that are instrumental in displaying marked sensitivity to NNRTIs (Balzarini, 1999; Schinazi et al., 2000) are also present in FIV RT except for (the corresponding amino acids in FIV RT are in parentheses) Lys101 (Gln101), Glu138 (Ala138), Val179 (Asp179), and Phe227 (Tyr227) (Fig. 1). In this respect, FIV RT is surprisingly more similar to HIV-1 RT than HIV-2 RT, which differs in at least 8 amino acids, including Ala101, Ile106, Ala138, Ile179, Ile181, Leu188, Ala190, and Tyr227. The specific parts containing amino acids lining the NNRTI pocket and important for NNRTI sensitivity are residues 98 to 110, 179 to 190, and 225 to 236. In fact, exchanging these amino acid chains between HIV-1 and HIV-2 RT rendered HIV-2 RT susceptible to NNRTIs such as nevirapine, whereas, conversely, HIV-1 RT containing the amino acids present in HIV-2 RT lost their susceptibility to these NNRTIs (Shih et al., 1991; Yang et al., 1996). These observations clearly suggest that these amino acids stretches in the NNRTI-specific pocket are of crucial importance for recognition of NNRTIs by the HIV-1 RT. In an attempt to allow NNRTI-testing in the in vivo (i.e., monkey) setting, hybrid SIV strains in which the entire RT gene was replaced by the HIV-1 RT gene (designated RT-SHIV) have been constructed (Überla et al., 1995). Construction of an RT-FHIV strain in which the FIV RT gene has been replaced by the HIV-1 RT gene has not been reported.

Despite the high similarity of the NNRTI pocket between HIV-1 RT and FIV RT, no inhibitory effect of NNRTIs against FIV RT has ever been reported, even at drug concentrations that are by several orders of magnitude higher than those required to fully suppress HIV-1 RT activity (Auwerx et al., 2002). Therefore, single, double, and triple amino acid substitutions were introduced into FIV RT and HIV-1 RT to study the influence of these amino acids on the NNRTI susceptibility of the RTs. In addition, we constructed a variety of FIV/HIV-1 chimeras in which relevant well defined parts of the p66 subunit of FIV RT were substituted by the corresponding HIV-1 p66 amino acid stretches. These amino acid stretches in FIV RT were also introduced into the p66 subunit of HIV-1 RT. Recombinant wild-type, chimeric and mutant enzymes were expressed and purified using a $(His)_6$-tag, and their sensitivity spectrum and kinetic properties were analyzed.

These studies revealed that FIV RT must be profoundly different from HIV-1 and HIV-2 RT in terms of structure and/or flexibility, so as to prevent interaction of FIV RT with NNRTIs, even after this enzyme has been equipped with those amino acids that are proven to engender susceptibility of HIV-1 RT toward NNRTIs.

Materials and Methods

Test 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^3T) were provided by Dr. M.-J. Camarasa (Consejo Superior de Investigaciones Científicas, Madrid, Spain). Nevirapine (BI-RG-587; dipyridodiazepinone) was obtained from Boehringer Ingelheim (Ridgefield, CT). Delavirdine (bis(heteroaryl)piperazine; U-90152) and efavirenz (DMP 266) and capravirine were provided by Dr. R. Kirch (Hoechst AG, Frankfurt, Germany) and Dr. J.-P. Kleim (GlaxoSmithKline, Stevenage, UK). Emivirine (MKC-442) was kindly provided by Dr. Ph. A. Furman (when at Triangle Pharmaceuticals, Research Triangle Park, NC). The thiocarboxanilide derivative UC-781 was obtained from W.G. Brouwer (Guelph, Ontario, Canada). The quinoxaline GW420867X was provided by Dr. J.-P. Kleim (GlaxoSmithKline, Stevenage, UK). 2′,3′-Dideoxyguanosine-5′-triphosphate (ddGTP) and phosphonoformic acid (PFA; foscarnet) were obtained from Sigma Chemical Ltd. (St. Louis, MO).

Construction of Plasmids for the Expression of Intramolecular Chimeras. HIV-1 RT and FIV RT were expressed as described previously by Jonckheere et al. (1996) and Auwerx et al. (2002). For the enzyme expression the *Escherichia coli* strain JM109 was used. Exchanges of amino acid stretches between HIV-1 and FIV RTs were made by the use of six restriction sites, NcoI, SstI, SstII, NsiI, NheI, and PacI. Of these sites, NcoI, NheI, and PacI were present only in wild-type FIV RT and NsiI was present in both RTs. The SstI and SstII restriction sites were absent from both RT sequences. Each of these sites and combinations of the sites were created by site-directed mutagenesis, if necessary. Creation of the restriction sites did not lead to undesirable amino acid substitutions because the nucleotide replacements resulted in silent mutations. The restriction sites allowed exchanges between or within the amino acid subdomains in RT. The locations of the sites with respect to codon positions are shown in Fig. 2.

The chimeras were constructed with pKRT2 (D'Aquila and Summers, 1989) and pFIV66 (North et al., 1994) as the parental plasmids, which contain both a $(His)_6$-tag sequence at the ATG start codon of the RT gene that is translated into an N-terminal His-tag used in the protein purification procedure.

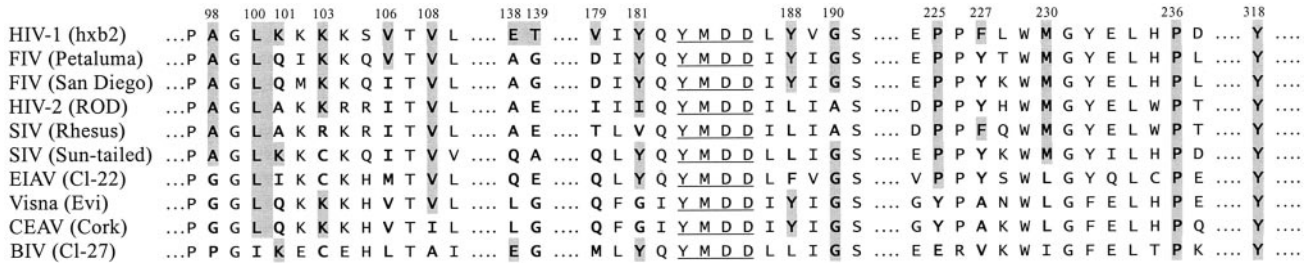

Fig. 1. Alignment of important amino acid stretches in the NNRTI-binding pocket of HIV-1 compared with the corresponding amino acids in other lentiviral RTs. The amino acids that are instrumental for susceptibility of the RT to NNRTIs are bold and shaded. The underlined sequence is conserved between the lentiviral RTs and includes the Asp185 and Asp186 amino acid residues critical for polymerase activity.

Introduction of Point Mutations in FIV and HIV-1 RT. Besides the construction of chimeras, we also introduced single, double, and triple amino acid substitutions in the FIV and HIV-1 RTs. Because we focused on the NNRTI pocket (Fig. 1), only those amino acids that are known to be of major importance in recognizing the NNRTIs by HIV-1 RT were changed. Therefore, in HIV-1 RT, the K101Q, V179D, and F227Y substitutions were made, whereas, conversely, in FIV RT, Q101K, D179V, and Y227F mutations were introduced. The mutations were introduced using the site-directed mutagenesis method (Stratagene, Westburg, Leusden, the Netherlands), as described previously by Pelemans et al. (1998).

Purification of RT. Luria broth medium (1 liter) containing the appropriate antibiotics were inoculated with an overnight culture of *E. coli* JM109 transformed with both plasmids of the expression system. The culture was started at an A_{600} of 0.1 and incubated at 37°C with vigorous shaking. Expression of recombinant RT was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. After 4 h, the cells were harvested, washed, and kept frozen overnight at −20°C. Cell lysis was accomplished by mechanical lysis in a SLM Aminco French Pressure Cell Press (Thermo Spectronic, Beun de Ronde, La Abcoude, the Netherlands). The cell paste was resuspended in 15 ml of lysis buffer (50 mM sodium phosphate buffer, pH 7.8, 100 mM NaCl, 5 mM β-mercaptoethanol, 0.9% glucose, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 10% glycerol) and subsequently placed in the French Press unit, which was kept at 4°C. After lysis, the cell lysate was centrifuged for 25 min at 17,200*g*. The supernatant was incubated with 2 ml of pre-equilibrated nickel-nitrilotriacetic acid beads (QIAGEN, Westburg, Leusden, the Netherlands) at 4°C, while rotating for at least 1 h. After incubation and sedimentation of the nickel-nitrilotriacetic resin with the bound $(His)_6$-tagged proteins, and the column was formed and washed three times with 20 ml of buffer (50 mM sodium phosphate buffer, pH 7.8, 0.5 mM NaCl, 5 mM β-mercaptoethanol, 25 mM imidazole, and 10% glycerol). The HIV-1 and FIV RTs were eluted from the column with the same phosphate buffer containing 125 mM imidazole. The imidazole-containing elution buffer was exchanged by heparin buffer (20 mM Tris-HCl, pH 7.8, 0.05 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol), and the eluate was concentrated to 2 ml using Vivaspin 15 centrifugal filtration devices (Vivascience, Van der Heyden, Brussels, Belgium). The protein was further fast performance liquid chromatography-purified to about 98% purity over a Hitrap Heparin column (Amersham Biosciences, Roosendaal, the Netherlands). After the binding of RT to the heparin column, elution was accomplished by a linear salt gradient of 0.05 to 1 M NaCl. RT eluted at approximately 0.3 M NaCl, as determined by SDS-polyacrylamide gel electrophoresis of the eluted peak fractions. All fractions containing RT were pooled and stored in buffer containing 0.3 M NaCl and 25% glycerol at −20°C. Protein concentrations in the stock solutions were determined with the Pierce Protein Assay (Polylab, Antwerp, Belgium), using bovine serum albumin as a standard.

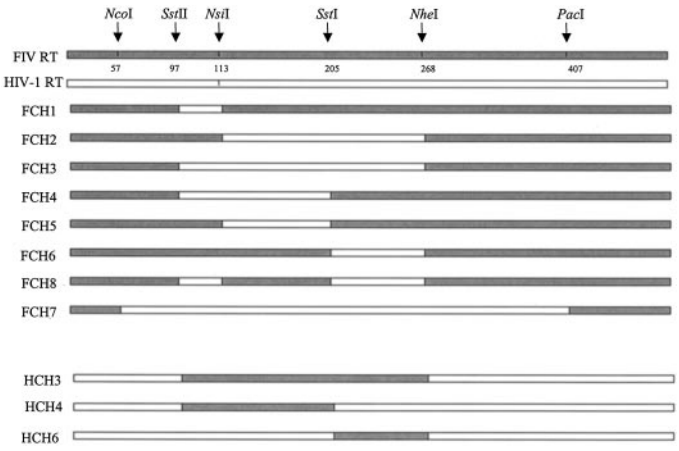

Fig. 2. Overview of the different chimeras. The numbers under the upper bar indicate the position of the amino acids where each restriction enzyme recognizes its site. The open bar represents HIV-1 RT and the gray bar represents FIV RT p66. The gray or open segments in each chimeric construct represent the parental [FIV (gray) or HIV-1 (open)] RT part from which the sequences were derived. A restriction site that was already present in the genome of the RT gene is marked with a vertical line. If the site was not present, it was introduced by site-directed mutagenesis.

Preparation of *E. coli* Lysates. Luria broth medium (25 ml) containing the appropriate antibiotics were inoculated with an overnight culture of *E. coli* JM109 transformed with both plasmids of the expression system at an A_{600} of 0.1. The culture was grown at 37°C, induced with isopropyl-β-D-thiogalactopyranoside, and stored as described in the previous section. The cell pellet was resuspended in 1 ml of lysis buffer (500 mM NaCl, 50 mM Tris-HCl, pH 7.8, 2 mM EDTA, 5 mM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100, 1 mg/ml lysozyme, and 10% glycerol) and sonicated for 5 to 10 min. The lysate was centrifuged at 17,200*g* for 20 min and the supernatant was stored at −80°C in aliquots of 80 μl.

Reverse Transcriptase Assay. For determination of the 50% inhibitory concentration (IC_{50}) values of the test compounds against the HIV-1 and FIV RTs, the enzyme assays were performed as described previously (Balzarini et al., 1992). A fixed concentration of the radiolabeled substrate [2,8-^{3}H]dGTP (specific radioactivity, 14.1 Ci/mmol; Amersham Biosciences) (1 μCi or 1.4 μM) and a fixed concentration of the template primer poly(rC) · oligo$(dG)_{12-18}$ (0.1 mM; Amersham Biosciences) were used in the reaction mixture containing a variety of drug concentrations. The IC_{50} of each compound was determined as the compound concentration that inhibited recombinant RT activity by 50%.

Steady-state kinetic assays were performed as described previously (Balzarini et al., 1992), except that the reaction mixtures were incubated for 30 min instead of 60 min during the assays with variable substrate (dGTP). Under these experimental conditions, the catalytic reaction of the different enzymes proceeded linearly and proportionally with time. The K_m and k_{cat} values for dGTP were determined in the presence of 0.1 mM poly(rC) · oligo(dG_{12-18}). The K_m and V_{max} (k_{cat}) values were derived from the double-reciprocal Lineweaver-Burk plots of the concentrations of the variable substrate (dGTP) versus the velocities of dGTP incorporation in the template/primer complex at each substrate concentration.

Results

Catalytic Activity of the Chimeric RT Enzymes. The expression plasmids were examined for their ability to encode stable chimeric RT proteins. The bacterial lysates and the purified enzymes were analyzed by SDS-polyacrylamide gel electrophoresis to verify the accumulation of stable protein. Only FCH1 and FCH5 of the chimeras showed significant activity that was almost comparable with their corresponding wild-type enzymes. HCH6 and FCH6 had a markedly reduced activity but were still sufficiently active to perform RT assays for determination of the IC_{50} values (Table 1). The chimeras in which larger amino acid parts were exchanged (i.e., FCH7 and FCH8 and HCH4) were only active in a crude lysate. No catalytic activity in the other chimeric RT constructs (i.e., FCH2, FCH3, FCH4, and HCH3) could be detected, in either the crude bacterial lysates or with the purified enzymes.

Kinetic Properties of the Chimeric RT Enzymes. To further study the characteristics of the chimeric enzymes, we determined their K_m and k_{cat} values with dGTP as substrate (Table 1). The K_m values of the wild-type FIV66 and HIV66 homodimers were 2.5 and 5.5 μM, respectively. The RT chi-

mera FCH1 had a K_m value of 7.6 μM, which was about 3 times higher than that observed for the K_m of wild-type FIV RT. The other active RT chimeras had K_m values that were in the same range as the K_m value of the respective wild-type enzymes, which reflects an affinity of the individual enzymes for their substrate that is comparable with their corresponding wild-type RTs. Instead, the k_{cat} value of the different chimeric enzymes was significantly lower than the values found for the wild-type RT enzymes, which reflects a low catalytic efficacy of the chimeric enzymes. Indeed, for the FCH5 RT, a k_{cat} of only 0.5% of the k_{cat} of wild-type FIV RT was observed, whereas the FCH6 chimeric RT enzyme had a catalytic efficiency that was only 0.01% of that of wild-type FIV RT. HCH6 and FCH1 had k_{cat} values that were about 2% of that of their respective wild-type enzymes (Table 1).

Sensitivity of the Chimeric RT Enzymes to NNRTIs, ddGTP, and PFA. In an attempt to identify the regions in FIV RT that are responsible for the lack of susceptibility to NNRTIs, the enzymatically active FIV/HIV-1 and HIV-1/FIV chimeric RT enzymes and the two parental FIV and HIV-1 RTs were evaluated for their sensitivities to inhibition by the NNRTIs nevirapine, efavirenz, and GW867420X. Nevirapine is a first-generation clinically used NNRTI, whereas both efavirenz and the quinoxaline derivative are second-generation NNRTIs. The IC_{50} values of the NNRTIs for the chimeric RTs are presented in Table 1. As expected, FIV RT was not inhibited by any NNRTI even at concentrations as high as 1000 μM. In contrast, HIV-1 RT proved exquisitely sensitive to efavirenz and GW867420X (IC_{50}, 0.034 and 0.01 μM, respectively) and, to a lesser extent, nevirapine (1.61 μM) (i.e., at compound concentrations 1000- to >100,000-fold lower than the highest concentration of drug that proved inactive against FIV RT). The FIV RT-derived chimeras, which contained several amino acid fragments from HIV-1 RT (Fig. 2) and showed sufficient catalytic activity (i.e., FCH1, FCH5, FCH6, FCH7, and FCH8) did not gain any susceptibility to the NNRTIs (Table 1). Interestingly, the chimeric RT enzyme HCH4, which was derived from the HIV-1 RT and contained the homologous amino acid stretch of FIV RT from amino acids 97 to 205, fully lost susceptibility (IC_{50}, >1000 μM) to the three NNRTIs tested. Its chimeric counterpart FCH4, containing the homologous amino acid stretch derived from HIV-1 RT, nevertheless kept complete resistance toward the NNRTIs.

Besides NNRTIs, ddGTP, and PFA were also evaluated for their inhibitory activities against the chimeric RTs. The values obtained for inhibition of the chimeric enzymes by ddGTP were found to be comparable with the parental RTs, except the chimeric RT enzyme HCH4, which had an IC_{50} value of 1.43 μM [i.e., at a concentration 18-fold higher than that required to inhibit the parental HIV-1 RT, but near the IC_{50} value found for wild-type FIV RT (0.74 μM)]. This observation may suggest that the substrate specificity of RT is predominantly determined by the conformation of the amino acid stretch between 97 and 205 (containing the catalytic aspartic acid triad at amino acid positions 110, 185, and 186). When examining PFA, varying IC_{50} values were found for the FIV RT-derived chimeras; FCH1 and FCH6 became fully resistant to this drug, whereas FCH5 remained susceptible at PFA concentrations that were also required to inhibit the parental enzyme. In contrast to the HIV-1 and HCH6 RTs, the HCH4 RT chimeric enzyme was resistant to PFA. Thus, the HCH4 chimeric RT enzyme seemed to have gained properties that were more comparable with FIV RT than HIV-1 RT.

Introduction of Point Mutations into FIV RT and HIV-1 RT and Sensitivity of the Mutated Enzymes to NNRTIs, ddGTP, and PFA. Besides the construction of the chimeric enzymes, in which relatively large internal segments were exchanged, introduction of single, double, and triple amino acid changes were also made in both HIV-1 and FIV RTs. These mutant enzymes had catalytic activities that were comparable with those of their wild-type enzymes (data not shown). The amino acids of choice represented those that are instrumental for the HIV-1 RT enzyme to keep full sensitivity to NNRTIs but different in FIV RT. We determined whether the complete loss of sensitivity of FIV RT to NNRTIs was caused by the inability of these compounds to bind to the putative pocket in FIV RT in the presence of these amino acids. The introduced amino acid mutations in FIV RT were Q101K, D179V, and Y227F, and all possible combinations derived thereof.

TABLE 1

Inhibitory activity of NNRTIs, PFA and ddGTP against a variety of purified FIV/HIV-1 RT chimeric enzymes

IC_{50} is the 50% inhibitory concentration or compound concentration required to inhibit the RT reaction by 50%. Poly(rC) · oligo(dG) and [^{3}H]dGTP were used as the template/primer and radiolabeled substrate, respectively. Data are presented as the mean ± S.D. for at least two to three independent experiments. 100% FIV RT activity represents 222,250 dpm/ng protein and 100% HIV-1 RT activity represents 2,246,934 dpm/ng protein (100%).

				IC_{50}				
Chimeric RT	RT Activity	K_m	k_{cat}[d]	Nevirapine	Efavirenz	GW867420X	ddGTP	PFA
	%		*pmol / μg protein / s*			*μM*		
FIV66	100	2.5	0.50	>1000	>1000	>1000	0.74 ± 0.11	71 ± 32
FCH1	28	7.6	0.013	>1000	>1000	>1000	1.51 ± 0.06	>500
FCH2								
FCH3								
FCH4								
FCH5	18.7	3.8	0.0025	>1000	>1000	>1000	0.69 ± 0.45	136 ± 27
FCH6	3.3	5.9	0.0005	>1000	>1000	>1000	1.60 ± 0.57	>500
FCH7[a]	1.3	6.1		>1000	>1000	>1000		
FCH8[a]	1.9	4.5		>1000	>1000	>1000		
HIV66	100	5.5	0.57	1.61 ± 0.6	0.034 ± 0.008	0.010 ± 0.002	0.08 ± 0.04	8.5 ± 5.5
HCH3								
HCH4[a]	0.2	8.0		>1000	>1000	>1000	1.43 ± 0.27	>500
HCH6	3.2	6.7	0.018	3.0 ± 2.3	0.016 ± 0.006	0.014 ± 0.007	0.051 ± 0.007	7 ± 3

[a] The RT was not purified. The experiments were performed on 10-fold diluted bacterial RT lysates. RT activity values were expressed as a percentage of the activity found in the wild-type (purified) RT sample

Poor susceptibility to the inhibitory effect of efavirenz (IC_{50} values of 114 μM for the single D179V FIV RT mutant and 143 μM for the triple Q101K+D179V+Y227F mutant) was found (Table 2). In addition, capravirine very slightly inhibited the mutant FIV RT enzymes with IC_{50} values ranging from 329 to 844 μM, but wild-type FIV RT was also slightly inhibited by capravirine at a concentration of 988 μM. Therefore, it should be concluded that the amino acid mutations introduced in the FIV RT did not influence the inhibitory values for capravirine. No other NNRTIs showed any inhibitory activity against any FIV RT mutant. Besides, the different classes of NNRTIs, ddGTP, and PFA were also included in the enzyme assays, and the inhibitory values found for them were highly comparable with those recorded for wild-type FIV RT (i.e., 0.74 μM for ddGTP and 71 μM for PFA) (Table 2).

To assess the importance of the role of the amino acids mentioned above in NNRTI resistance/sensitivity, we introduced the same homologous residues of FIV RT into HIV-1 RT by site-directed mutagenesis, and the results are shown in Table 3. By introducing the K101Q mutation in HIV-1 RT, we found a slight decrease of sensitivity of the enzyme to nevirapine (3-fold) and TSAO-m^3T (10- to 15-fold). Mutating the amino acid position 179 decreased the sensitivity toward the second-generation thiocarboxanilide NNRTI UC781 by 50-fold. The F227Y mutant did not lead to any marked change in the sensitivity of the HIV-1 RT enzyme to most NNRTIs.

The decreased inhibitory activity against the mutated HIV-1 RT enzymes afforded by single amino acid changes were not always additive when multiple mutations derived thereof were combined. For example, we found for delavirdine a 5-fold reduction of drug sensitivity when mutating positions 101 and 179, and an 11-fold reduction when changing residues 179 and 227. However, when the triple RT mutant was compared with wild-type HIV-1 RT, a 5-fold increase of susceptibility to delavirdine occurred. These observations point to a complicated interplay between several amino acids on the conformation of the NNRTI pocket and/or on the affinity to the individual NNRTIs.

Discussion

Although the FIV RT and HIV-1 RT have approximately 48% identity and 67% similarity on the amino acid level, FIV is not susceptible to the inhibitory effects of NNRTIs. This insensitivity of FIV RT to NNRTIs is very intriguing because almost all residues that are instrumental in conferring sensitivity to NNRTIs in HIV-1 RT are also present in FIV RT (Fig. 1). Of 20 relevant amino acids lining the NNRTI pocket in HIV-1 RT, only the NNRTI-characteristic Lys101, Glu138, Val179, and Phe227 amino acids in HIV-1 RT are not present in FIV RT. According to the crystal structure of the binding site for NNRTIs in HIV-1 RT, Tyr181 and Tyr188 are extremely important for sensitivity of HIV-1 RT to NNRTIs, in particular to first-generation NNRTIs (Kohlstaedt et al., 1992; Esnouf et al., 1995; Hsiou et al., 1998). As shown in Fig. 1, these two tyrosine residues at positions 181 and 188 are also present in FIV RT, whereas they are absent in the RTs of SIV and HIV-2, which are, like FIV RT, also insensitive to NNRTIs. Previously, it has been shown that replacing the Ile181 and Leu188 (or even the Leu188 residue alone) in HIV-2 RT by the tyrosine residues present in HIV-1 RT results in a mutated HIV-2 RT enzyme that is highly susceptible to NNRTIs (i.e., nevirapine) (Shih et al., 1991; Isaka et al., 2001).

Other attempts to identify the residues in HIV-2 RT that are responsible for the NNRTI-insensitivity by construction of chimeric enzymes have also been reported (Condra et al., 1992; Bacolla et al., 1993; Hizi et al., 1993; Yang et al., 1996). HIV-2 RT substituted with the amino acid stretch 176 to 190 from HIV-1 RT fully restored sensitivity of HIV-2 RT to NNRTIs (i.e., nevirapine). Given the fact that the putative NNRTI binding pocket in FIV RT is more like that of HIV-1 RT than the corresponding putative binding pocket of HIV-2 RT or SIV RT, we aimed to make FIV RT susceptible to NNRTIs by replacing one or several relevant amino acids in the enzyme or by exchanging important amino acid stretches in the FIV RT enzyme to determine the minimum requirements to restore NNRTI sensitivity in FIV RT.

Therefore, to map the regions of FIV RT that determine the lack of susceptibility to NNRTIs, enzymatically active FIV/HIV-1 chimeric enzymes were constructed and evaluated for sensitivity toward inhibition by a variety of relevant NNRTIs. In contrast to the HIV-1/HIV-2 chimeric RTs, none of our constructed chimeric RT enzymes gained any significant susceptibility, and most chimeras even lost their major enzymatic activities. We interpret the decreased (or lack of) catalytic activity of the FIV/HIV-1 chimeric enzymes as having

TABLE 2

Inhibitory activity of NNRTIs, ddGTP and PFA against mutant FIV RTs

IC_{50} is the 50% inhibitory concentration or compound concentration required to inhibit the RT reaction by 50%. Data are the mean ± S.D. for at least two to three independent experiments.

	IC_{50}								
	Q101K	D179V	Y227F	D179V + Y227F	Q101K + Y227F	Q101K + D179V	Q101K + D179V + Y227F	FIV RT	HIV-1 RT
					μM				
Nevirapine	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	1.6 ± 0.6
Delavirdine	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	0.52 ± 0.23
Efavirenz	994 ± 63	114 ± 3	>1000	>1000	>1000	>1000	143 ± 22	>1000	0.034 ± 0.008
Emivirine	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	0.16 ± 0.05
UC-781	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	0.016 ± 0.002
GW867420X	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	0.010 ± 0.002
Capravirine	679 ± 184	507 ± 78	677 ± 2	844 ± 9	537 ± 69	755 ± 2	329 ± 62	988 ± 171	0.004 ± 0.001
TSAO-m^3T	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	0.6 ± 0.2
ddGTP	0.63 ± 0.02	0.75 ± 0.16	0.78 ± 0.0	0.81 ± 0.14	0.74 ± 0.13	0.59 ± 0.12	0.74 ± 0.16	0.74 ± 0.11	0.08 ± 0.04
PFA	106 ± 47	118 ± 30	153 ± 29	174 ± 22	129 ± 47	116 ± 20	262 ± 184	71 ± 32	8.5 ± 5.5

TABLE 3
Inhibitory activity of NNRTIs, ddGTP, and PFA against mutant HIV-1 RTs
IC_{50} is the 50% inhibitory concentration or compound concentration required to inhibit the RT reaction by 50%. Data are the mean ± S.D. for at least two to three independent experiments.

	IC_{50}								
	K101Q	V179D	F227Y	V179D + F227Y	K101Q + F227Y	K101Q + V179D	K101Q + V179D + F227Y	HIV-1 RT	FIV RT
	μM								
Nevirapine	3.0 ± 0.1	4.4 ± 1	2.05 ± 0.03	1.39 ± 0.00	4.8 ± 3.7	6.1 ± 0.3	5.0 ± 1.3	1.6 ± 0.6	>1000
Delavirdine	0.9 ± 0.5	2.63 ± 0.55	2.4 ± 1.4	5.8 ± 1.5	2.9 ± 1.7	2.4 ± 1.0	0.10 ± 0.02	0.52 ± 0.23	>1000
Efavirenz	0.06 ± 0.02	0.044 ± 0.009	0.033 ± 0.002	0.06 ± 0.02	0.28 ± 0.06	0.136 ± 0.009	0.10 ± 0.01	0.034 ± 0.008	>1000
Emivirine	0.14 ± 0.01	0.84 ± 0.07	0.19 ± 0.02	0.23 ± 0.04	0.91 ± 0.12	0.62 ± 0.23	0.84 ± 0.12	0.16 ± 0.05	>1000
UC-781	0.16 ± 0.09	0.8 ± 0.2	0.07 ± 0.03	0.06 ± 0.01	0.16 ± 0.08	0.1 ± 0.1	0.8 ± 0.3	0.016 ± 0.002	>1000
GW867420X	0.07 ± 0.03	0.10 ± 0.06	0.015 ± 0.008	0.03 ± 0.01	0.10 ± 0.03	0.08 ± 0.03	0.07 ± 0.05	0.010 ± 0.002	>1000
Capravirine	0.079 ± 0.005	0.08 ± 0.05	0.017 ± 0.004	0.09 ± 0.05	0.08 ± 0.06	0.15 ± 0.02	0.0071 ± 0.0004	0.004 ± 0.001	988 ± 171
TSAO-m^3T	6.1 ± 5.4	6.1 ± 1.2	0.8 ± 0.6	0.395 ± 0.007	6.7 ± 1.0	9 ± 1	6.5 ± 2.0	0.6 ± 0.2	>1000
ddGTP	0.04 ± 0.03	0.03 ± 0.01	0.046 ± 0.001	0.054 ± 0.005	0.018 ± 0.004	0.07 ± 0.02	0.069 ± 0.008	0.08 ± 0.04	0.74 ± 0.11
PFA	3.0 ± 0.7	20 ± 15	9.0 ± 4.6	6.4 ± 2.1	18.3 ± 2.5	8.2 ± 1.8	27 ± 5	8.5 ± 5.5	71 ± 32

been caused by either a change in the overall structure of the chimeric RTs or perturbed dimerization of the subunits of the enzyme and/or an altered structure of the substrate binding site of the RT enzyme. In fact, in a previous study (Auwerx et al., 2002), we found that chimeric heterodimers of HIV-1 and FIV RT (i.e., p66 of HIV-1 RT and p51 of FIV RT or, vice versa, p66 of FIV RT and p51 of HIV-1 RT) had a compromised catalytic activity, which was not observed for HIV-1/HIV-2 heterodimeric RT enzymes. Therefore, we believe that, in contrast with HIV-2, exchanges of amino acid stretches between FIV RT and HIV-1 RT may somewhat compromise the overall folding or conformation of the chimeric subunits, resulting in lowering or even abolition of the enzymatic activity.

Our observations that none of the FIV RT chimeras showed sensitivity to NNRTIs, including the FCH4 chimeric enzyme, which contained the amino acid stretch of HIV-1 RT important for the sensitivity of the HIV-1 RT to NNRTIs, seem to imply that there must exist structural restrictions on the ability to change amino acid parts between both enzymes. In this respect, it is important to note that the HCH4 chimeric HIV-1 RT enzyme fully lost NNRTI susceptibility, pointing to the crucial importance of this part of the enzyme for NNRTI sensitivity. The majority of mutations that are reported to affect NNRTI sensitivity to HIV-1 RT are indeed located within this amino acid stretch (Table 4).

Although the level of sequence identity between FIV and HIV-1 RTs (48%) is somewhat lower than that between HIV-1 and HIV-2 RTs (62%), it is still sufficiently high to suggest an overall similar structure. Indeed, the recent crystal structure of HIV-2 RT confirms its high level of structured similarity with HIV-1 RT (Ren et al., 2002; Bird et al., 2003). The fact that all the chimeric RTs could bind to their substrates with K_m values that are comparable with those of the wild-type enzymes also supports this view. However, the nature of polymerase activity is that the binding site is fairly tolerant to small changes, because the requirement for translocation of the template/primer without dissociation pre-

TABLE 4
Overview of amino acids in HIV-1 RT reported to play a role in sensitivity/resistance of HIV-1 RT against NNRTIs
Data taken from Balzarini (1999) and Schinazi et al. (2000).

Amino Acid Position in HIV-1 RT	HIV-1 Wild-Type Amino Acid	NNRTI-Mutated Amino Acid	Corresponding FIV RT Amino Acid
98	Ala	Gly	Ala
100	Leu	Ile	Leu
101	Lys	Glu	Gln
103	Lys	Asn, Thr	Lys
106	Val	Ala	Val, Ile
108	Val	Ile	Val
135	Ile	Met, Thr, Leu	Lys
138	Glu	Lys	Ala
139	Thr		Gly
179	Val	Asp, Glu	Asp
181	Tyr	Cys, Ile	Tyr
184	Met	Ile, Val	Met
188	Tyr	Cys, His, Leu	Tyr
190	Gly	Ala, Glu, Ser	Gly
225	Pro	His	Pro
227	Phe	Leu	Tyr
229	Trp		Trp
233	Glu	Val	Glu
236	Pro	Leu	Pro
238	Lys	Thr	Thr
318	Tyr		Tyr

cludes unnecessarily specific interactions. In addition, no marked preference of HIV-1 RT for a template/primer containing a polymerizable end over one containing a nonpolymerizable end was observed, showing that the specificity of HIV-1 RT is rather low (Huang et al., 2000). There is a large reduction in k_{cat} for all the chimeric RTs that were constructed, implying that within the overall polymerase binding site there are distortions that affect the catalytic residues. Some of these distortions may arise from the nature of the chimeras constructed, because they are based on existing restriction sites (or ones introduced that result in silent mutations) and are not aligned on structural domain boundaries. Chimeras constructed based on structural considerations may lead to fewer distortions, at the expense of point mutations, and thereby greater activity. The chimeras with residual activity do not involve significant changes to the residues implicated in NNRTI susceptibility, so the lack of NNRTI susceptibility is not surprising.

Although structural distortion may affect NNRTI binding in the chimeric RTs with residual activity, this is unlikely to be the case for the FIV RTs bearing point mutations where the activity is close to that of wild-type. Given that the sequence conservation between HIV-1 and FIV RT suggests similarity of structure, the lack of sensitivity of (mutant) FIV RTs to NNRTIs may be ascribed to any of the following factors: 1) Some structural feature of FIV RT makes it impossible for the NNRTI binding pocket to be created. In unliganded HIV-1 RT, the NNRTI binding pocket does not exist but opens up on binding of the inhibitor (Rodgers et al., 1995; Esnouf et al., 1995, 1997; Hsiou et al., 1996). Binding of an NNRTI (such as nevirapine) to RT requires conformational changes of the overall structure as the thumb rotates away from the fingers and also induces a small shift in the fingers-palm unit. In the catalytic complex, the fingers domain closes in toward the palm and the palm itself shifts slightly with respect to the core of the unliganded RT (Huang et al., 1998). Were FIV RT more rigid than its HIV-1 counterpart, this opening might be at too great an energy cost for NNRTIs to penetrate into the pocket. 2) The NNRTIs simply cannot penetrate the (mutant) FIV RT binding pocket, perhaps because of changes in the flexibility of FIV RT or differences in the subunit interactions. 3) The NNRTIs may enter but cannot bind in the enzyme pocket because of conformational hindrance. Although this may well be the case for wild-type FIV RT, in the mutant FIV RTs, the amino acids lining the NNRTI pocket are those of HIV-1 RT, so lack of binding seems unlikely. 4) The NNRTIs may efficiently bind in the enzyme's putative pocket but fail to inhibit the catalytic activity. Whereas this possibility cannot be ruled out, the fact that HIV-2 RT can be made sensitive to NNRTIs by point mutations suggests a similar conformation between HIV-1 RT and HIV-2 RT.

This analysis leads us to the suggestion that structural flexibility may play an important role in NNRTI binding and that FIV RT may be somewhat more rigid that HIV-1 RT but still has the same overall structure. Were this the case, then one might see significant sequence differences between the two RTs for amino acids expected to form domain and subunit interfaces. In several places, this seems to be so. The lack of enzymatic activity of HIV-1/FIV RT heterodimers, unlike HIV-1/HIV-2 RT heterodimers, also points to substantial differences in the subunit interactions (Auwerx et al., 2002).

For the HIV-1 RTs bearing FIV point mutations, there is a complex interplay between the resistance mutations with both resistance and hypersensitivity being observed. Similar observations have been found in numerous NNRTI resistance studies (for an overview, see Bacheler, 1999; Schinazi et al., 2000) and the complexities have been ascribed to the varying abilities of NNRTIs to adapt to the changing shape and interaction possibilities afforded by the mutant NNRTI pocket.

In conclusion, we demonstrated in this study that FIV RT behaves differently from HIV-1 and HIV-2 RT in terms of exchange of individual amino acids and amino acid stretches that determine the NNRTI susceptibility of HIV-1 RT. In contrast with HIV-2 RT, FIV RT cannot be mutated or engineered to acquire susceptibility to the inhibitory effects of the NNRTIs. A crystal structure of FIV RT may resolve the molecular/structural basis of these observations and provide deeper insights in the similarities and differences of this family of RT enzymes that are very closely related.

Acknowledgments

We thank Lizette van Berckelaer for excellent technical assistance.

References

Arnold E, Jacobo-Molina A, Nanni RG, Williams RL, Lu X, Ding J, Clark AD Jr, Zhang A, Ferris AL, Clark P, et al. (1992) Structure of HIV-1 reverse transcriptase/DNA complex at 7 Å resolution showing active site locations. *Nature (Lond)* **357:**85–89.

Auwerx J, North TW, Preston BD, Klarmann GJ, De Clercq E, and Balzarini J (2002) Chimeric human immunodeficiency virus type 1 and feline immunodeficiency virus reverse transcriptases: role of the subunits in resistance/sensitivity to non-nucleoside reverse transcriptase inhibitors. *Mol Pharmacol* **61:**400–406.

Bacheler LT (1999) Resistance to non-nucleoside inhibitors of HIV-1 reverse transcriptase. *Drug Resist Updat* **2:**56–67.

Bacolla A, Shih CK, Rose JM, Piras G, Warren TC, Grygon CA, Ingraham RH, Cousins RC, Greenwood DJ, Richman D, et al. (1993) Amino acid substitutions in HIV-1 reverse transcriptase with corresponding residues from HIV-2. Effect on kinetic constants and inhibition by non-nucleoside analogs. *J Biol Chem* **268:** 16571–16577.

Balzarini J, Péréz-Péréz MJ, San-Félix A, Camarasa MJ, Bathurst IC, Barr PJ, and De Clercq E (1992) Kinetics of inhibition of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase by the novel HIV-1-specific nucleoside analogue [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)thymine (TSAO-T). *J Biol Chem* **267:**11831–11838.

Balzarini J (1999) Suppression of resistance to drugs targeted to human immunodeficiency virus reverse transcriptase by combination therapy. *Biochem Pharmacol* **58:**1–27.

Bird LE, Chamberlain PP, Stewart-Jones GB, Ren J, Stuart DI, and Stammers DK (2003) Cloning, expression, purification and crystallisation of HIV-2 reverse transcriptase. *Protein Expr Purif* **27:**12–18.

Condra JH, Emini EA, Gotlib L, Graham DJ, Schlabach AJ, Wolfgang JA, Colonno RJ, and Sardana VV (1992) Identification of the human immunodeficiency virus reverse transcriptase residues that contribute to the activity of diverse nonnucleoside inhibitors. *Antimicrob Agents Chemother* **36:**1441–1446.

D'Aquila RT and Summers WC (1989) HIV-1 reverse transcriptase/ribonuclease H: high level expression in *Escherichia coli* from a plasmid constructed using the polymerase chain reaction. *J Acquir Immune Defic Syndr* **2:**579–587.

Esnouf R, Ren J, Ross C, Jones Y, Stammers D, and Stuart D (1995) Mechanism of inhibition of HIV-1 reverse transcriptase by non-nucleoside inhibitors. *Nat Struct Biol* **2:**303–308.

Esnouf RM, Ren J, Hopkins AL, Ross CK, Jones EY, Stammers DK, and Stuart DI (1997) Unique features in the structure of the complex between HIV-1 reverse transcriptase and the bis(heteroaryl)piperazine (BHAP) U-90152 explain resistance mutations for this nonnucleoside inhibitor. *Proc Natl Acad Sci USA* **94:** 3984–3989.

Hizi A, Tal R, Shaharabany M, Currens MJ, Boyd MR, Hughes SH, and McMahon JB (1993) Specific inhibition of the reverse transcriptase of human immunodeficiency virus type 1 and the chimeric enzymes of human immunodeficiency virus type 1 and type 2 by nonnucleoside inhibitors. *Antimicrob Agents Chemother* **37:**1037–1042.

Hsiou Y, Ding J, Das K, Clark AD Jr, Hughes SH, and Arnold E (1996) Structure of unliganded HIV-1 reverse transcriptase at 2.7 Å resolution: implications of conformational changes for polymerization and inhibition mechanisms. *Structure* **4:**853–860.

Hsiou Y, Das K, Ding J, Clark AD Jr, Kleim JP, Rosner M, Winkler I, Riess G,

Hughes SH, and Arnold E (1998) Structures of Tyr188Leu mutant and wild-type HIV-1 reverse transcriptase complexed with the non-nucleoside inhibitor HBY 097: inhibitor flexibility is a useful design feature for reducing drug resistance. *J Mol Biol* **284:**313–323.

Huang H, Chopra R, Verdine GL, and Harrison SC (1998) Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. *Science (Wash DC)* **282:**1669–1675.

Huang H, Harrison SC, and Verdine GL (2000) Trapping of a catalytic HIV reverse transcriptase*template:primer complex through a disulfide bond. *Chem Biol* **7:**355–364.

Isaka Y, Miki S, Kawauchi S, Suyama A, Sugimoto H, Adachi A, Miura T, Hayami M, Yoshie O, Fujiwara T, et al. (2001) A single amino acid change at Leu-188 in the reverse transcriptase of HIV-2 and SIV renders them sensitive to non-nucleoside reverse transcriptase inhibitors. *Arch Virol* **146:**743–755.

Jonckheere H, De Vreese K, Debyser Z, Vandekerckhove J, Balzarini J, Desmyter J, De Clercq E, and Anné J (1996) A two plasmid co-expression system in *Escherichia coli* for the production of virion-like reverse transcriptase of the human immunodeficiency virus type 1. *J Virol Methods* **61:**113–125.

Kohlstaedt LA, Wang J, Friedman JM, Rice PA, and Steitz TA (1992) Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science (Wash DC)* **256:**1783–1790.

North TW, Cronn RC, Remington KM, and Tandberg RT (1990) Direct comparisons of inhibitor sensitivities of reverse transcriptases from feline and human immunodeficiency viruses. *Antimicrob Agents Chemother* **34:**1505–1507.

North TW, Hansen GL, Zhu Y, Griffin JA, and Shih CK (1994) Expression of reverse transcriptase from feline immunodeficiency virus in Escherichia coli. *Antimicrob Agents Chemother* **38:**388–391.

Pelemans H, Esnouf RM, Jonckheere H, De Clercq E, and Balzarini J (1998) Mutational analysis of Tyr-318 within the non-nucleoside reverse transcriptase inhibitor binding pocket of human immunodeficiency virus type I reverse transcriptase. *J Biol Chem* **273:**34234–34239.

Ren J, Bird LE, Chamberlain PP, Stewart-Jones GB, Stuart DI, and Stammers DK (2002) Structure of HIV-2 reverse transcriptase at 2.35-Å resolution and the mechanism of resistance to non-nucleoside inhibitors. *Proc Natl Acad Sci USA* **99:**14410–14415.

Rodgers DW, Gamblin SJ, Harris BA, Ray S, Culp JS, Hellmig B, Woolf DJ, Debouck C, and Harrison SC (1995) The structure of unliganded reverse transcriptase from the human immunodeficiency virus type 1. *Proc Natl Acad Sci USA* **92:**1222–1226.

Schinazi RF, Landes BA, and Mellors JN (2000) Mutations in retroviral genes associated with drug resistance: 2000–2001 update. *Int Antivir News* **8:**65–91.

Shih CK, Rose JM, Hansen GL, Wu JC, Bacolla A, and Griffin JA (1991) Chimeric human immunodeficiency virus type 1/type 2 reverse transcriptases display reversed sensitivity to nonnucleoside analog inhibitors. *Proc Natl Acad Sci USA* **88:**9878–9882.

Stammers DK, Somers DO, Ross CK, Kirby I, Ray PH, Wilson JE, Norman M, Ren JS, Esnouf RM, Garman EF, et al. (1994) Crystals of HIV-1 reverse transcriptase diffracting to 2.2 Å resolution. *J Mol Biol* **242:**586–588.

Überla K, Stahl-Hennig C, Bottiger D, Matz-Rensing K, Kaup FJ, Li J, Haseltine WA, Fleckenstein B, Hunsmann G, Öberg B, et al. (1995) Animal model for the therapy of acquired immunodeficiency syndrome with reverse transcriptase inhibitors. *Proc Natl Acad Sci USA* **92:**8210–8214.

Yang G, Song Q, Charles M, Drosopoulos WC, Arnold E, and Prasad VR (1996) Use of chimeric human immunodeficiency virus types 1 and 2 reverse transcriptases for structure-function analysis and for mapping susceptibility to nonnucleoside inhibitors. *J Acquir Immune Defic Syndr Hum Retrovirol* **11:**326–333.

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