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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

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ABSTRACT

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are specific for human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) and do not inhibit HIV-2. Given that the amino acids lining the NNRTI-specific pocket of HIV-1 RT display higher similarity to the corresponding feline immunodeficiency virus (FIV) RT amino acids than to HIV-2 RT, the susceptibility of FIV RT and chimeric HIV-1/FIV RTs to NNRTIs and the role of the p51 subunit in the inhibitory action of NNRTIs were investigated. We found that the wild-type FIV RT and the FIVp66/HIVp51 chimeric enzyme showed no susceptibility for NNRTIs. On the other hand, the chimeric HIVp66/FIVp51 RT retained a sensitivity spectrum for NNRTIs similar to that of the wild-type HIV-1 RT. The noncompetitive nature of inhibition of HIV-1 RT by nevirapine was also observed with the HIVp66/FIVp51 chimeric enzyme. Inhibition of the chimeric RTs by nucleoside reverse transcriptase inhibitors and foscarnet was in the same range as observed for the corresponding HIVp66/HIVp51 and FIVp66/FIVp51 wild-type enzymes. The chimeric RTs had an affinity (K_m) for their dNTP substrate and template/primer comparable with that of the wild-type HIV-1 and FIV RTs, but their catalytic efficacy (k_{cat}) was markedly decreased. This decreased catalytic efficacy of the RT chimeras may suggest suboptimal interactions between p66 and p51 in the chimeric enzymes. Our results point to a minor role of the p51 subunit in the sensitivity to RT inhibitors.

Human immunodeficiency virus type 1 (HIV-1) and feline immunodeficiency virus (FIV) reverse transcriptases (RT) are responsible for the replication of the lentiviral genomic single-stranded RNA to double-stranded DNA (Hottiger and Hubscher, 1996). Both RTs consist of two polypeptides with common N termini, a 66-kDa subunit (p66) and a 51-kDa subunit (p51) (North et al., 1990, 1994). Both subunits are present in equimolar amounts and form a heterodimer. The p51 subunit is generated by cleavage of the RNase H domain (p15) at the C terminus of p66 by a virus-encoded protease. The heterodimeric form is the most stable and active form of the RT enzyme found in vivo (Lightfoote et al., 1986; Lowe et al., 1988). Sequence comparisons between HIV-1 RT and FIV RT revealed 63% identity at the nucleotide level and 48% identity and 67% similarity at the amino acid level (Amacker et al., 1995).

The structure of HIV-1 RT is highly asymmetric; the polymerase domain (consisting of the four subdomains: fingers, palm, thumb, and connection) of the p66 and p51 subunits are arranged in a strikingly different manner (Kohlstaedt et al., 1992). The p66 subunit forms a DNA binding cleft with the active site residues and encodes both the polymerase and RNase H activity of the enzyme, whereas the p51 subunit is catalytically inactive (Cheng et al., 1991; Le Grice et al., 1991; Boyer et al., 1992; Hostomsky et al., 1992). The role of the p51 is still uncertain, and several possible functions have been suggested: a role in processivity (movement of enzymes on the template) of the p66 subunit (Huang et al., 1992), involvement in tRNA primer binding (Mishima and Steitz,

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ABBREVIATIONS: HIV, human immunodeficiency virus; FIV, feline immunodeficiency virus; RT, reverse transcriptase; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; 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, phosphonophormic acid (foscarnet); GST, glutathione-*S*-transferase; PAGE, polyacrylamide gel electrophoresis.

1995; Dufour et al., 1998), loading of the p66 subunit onto the template primer (Amacker and Hubscher, 1998), enhancement of the strand displacement DNA synthesis (Hottiger et al., 1994; Amacker et al., 1995), and a role in induction and maintenance of an optimal structural conformation (Tasara et al., 1999).

The HIV-1 RT is an important target for the chemotherapy of AIDS because of its key role in virus replication (De Clercq, 1995a,b). The non-nucleoside RT inhibitors (NNRTIs) represent a large and chemically diverse group of compounds inhibiting HIV-1. Although the NNRTIs are potent and selective HIV-1 inhibitors with low toxicity, their use for anti-AIDS therapy is compromised by rapid emergence of drug-resistant viruses (De Clercq, 1996). Although very similar to HIV-1 RT, the HIV-2 RT shows no susceptibility to NNRTIs. However, when an alignment was made for the primary amino acid sequences of the NNRTI-specific pocket of HIV-1 RT with the corresponding amino acids in HIV-2 RT and FIV RT, a higher sequence similarity was found for FIV RT than for HIV-2 RT (Fig. 1). Chimeric enzymes involving HIV-1 RT and other lentiviral RTs have been constructed by other groups, contributing to a further understanding of the function of the individual subunits within the HIV-1 RT heterodimer, the mapping of the catalytic sites, and the NNRTI-binding pocket. Indeed, the construction of HIV-1/HIV-2 (Howard et al., 1991; Shih et al., 1991; Yang et al., 1996), simian immunodeficiency virus/HIV-1 (Isaka et al., 1998), and HIV-1/murine leukemia virus RT chimeras (Hizi et al., 1993; Misra et al., 1998) have already been reported. Amacker and Hubscher (1998) made a chimeric FIV/HIV-1 to investigate the role of the p51 subunit in the heterodimer. The HIVp66/FIVp51 chimera in their study was found to be resistant to the NRTI 3′-azido-3′-deoxythymidine triphosphate and the NNRTI nevirapine. FIVp66/HIVp51 RT and even the wild-type FIV RT were reported to be susceptible to the inhibitory activity of nevirapine. To investigate the NNRTI sensitivity to FIV RT in more detail and to gain further insights in the potential role of the p51 subunit in the sensitivity to and/or inhibition by NNRTIs, we also constructed, besides wild-type FIVp66/FIVp51 RT, stable and functionally active chimeric HIVp66/FIVp51 and FIVp66/HIVp51 RTs and compared their sensitivity spectrum with a variety of different classes of NNRTIs.

We found in our kinetic studies that the chimeric RT enzymes had a affinity toward their deoxynucleoside triphosphate substrate and template comparable with that of wild-type HIV-1 and FIV RTs, but their catalytic efficacy was markedly decreased. Inhibition by nevirapine or any other NNRTI was not observed for wild-type FIV RT or the chimeric FIVp66/HIVp51 RT. Instead, the chimeric HIVp66/FIVp51 RT retained marked sensitivity to the inhibitory effects of all NNRTIs investigated. Inhibition of the chimeric RTs by NRTIs was in the same range as observed for the HIV-1 RTs and FIV RTs.

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

Cloning of P66 and P51 Subunits. The complete FIV RT coding sequence of the Petaluma isolate was ligated into the *Eco*RI-*Pst*I digested expression vector pKK223-3 with inducible *tac* promoter (Amersham Biosciences, Roosendaal, the Netherlands) creating the pFIV66-WT. An analogous construct, pFIV51-WT, was created for expression of the p51 FIV RT subunit. HIV-1 RT was expressed by the pKRT2 expression vector (D'Aquila and Summers, 1989) under the control of the *trc* promoter. The p51 subunit of HIV-1 RT was expressed by pKRT51, like pKRT2, based on pKK233-2 (Amersham Biosciences).

For further purification of the HIV-1 and FIV RT enzymes, we used the glutathione-*S*-transferase (GST) fusion system. To make sure that the p51 subunit of the chimeric RTs was not derived from eventual bacterial proteolysis, we purified the enzymes by a GST tag on the p51 amino terminus. Therefore, we cloned the FIV p51 and HIV-1 p51 RT sequences into pGEX 4T-1 (Amersham Biosciences). The p51 RT sequences were amplified from pFIV51-WT and pKRT51, respectively, by means of PCR with *Pfu* polymerase. The primers used for PCR contained add-on sequences for restriction endonuclease sites. The 5′ primer contained an *Eco*RI restriction site, and the 3′ primer contained a *Not*I restriction site. The desired fragment was digested with *Eco*RI and *Not*I and then purified using QIAquick gel purification kit (QIAGEN, Westburg, Leusden, the Netherlands). This fragment was ligated into the *Eco*RI-*Not*I digested pGEX 4T-1 vector creating pGEX51F and pGEX51H, which express, respectively, the FIV p51 subunit and the HIV-1 p51 subunit together with the GST fusion protein at the amino terminus.

Recombinant FIV RT enzymes were expressed from a two-plasmid coexpression system. The *Sal*I-*Dra*I portion of pFIV66-WT was subcloned into the *Sal*I-*Sma*I- digested pREP4 (QIAGEN) for the construction of pREP66. This plasmid was compatible with pGEX51H and pGEX51F in *Escherichia coli* and contains the kanamycin resistance gene.

HIV-1 RT was expressed from the two-plasmid coexpression system as described by Jonckheere et al. (1996). The p66 subunit was subcloned into pACYC184 containing the p15A *ori* (Chang and Cohen, 1978) and a tetracycline resistance gene (pACYC66).

The chimeras were formed by different plasmid combinations. In this way, we constructed, besides the wild-type expression systems, a pREP66-pGEX51H and pACYC66-pGEX51F system expressing, respectively, FIVp66/HIVp51 and HIVp66/FIVp51.

Expression and Purification of Reverse Transcriptase Enzymes. LB medium (800 ml) containing the appropriate antibiotics

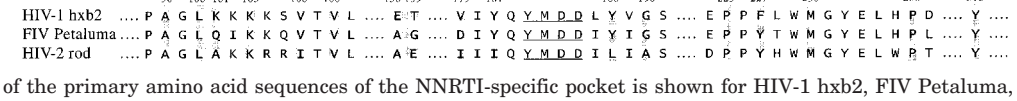

Fig. 1. Alignment of the primary amino acid sequences of the NNRTI-specific pocket is shown for HIV-1 hxb2, FIV Petaluma, and HIV-2 rod. The amino acids that are instrumental in retaining sensitivity to NNRTIs are indicated in bold and shaded. The underlined sequence is conserved between the lentiviral RTs and includes D185 and D186 residues critical for polymerase activity.

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 the 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 12,000 rpm using the SS34 rotor in a Sorval centrifuge (Goffin-Meyvis, Kappelen, Belgium). The supernatant was incubated with 1 ml of pre-equilibrated glutathione-*S*-Sepharose beads (Amersham Biosciences) at 4°C while rotating for at least 1 h. After incubation, the beads were washed three times with 20 ml of buffer (50 mM sodium phosphate buffer, pH 7.8, 0.5 mM NaCl, 5 mM β-mercaptoethanol, and 10% glycerol). The RT was eluted from the beads by bulk incubation with elution buffer [containing 20 mM reduced glutathione (Sigma)] at 4°C while rotating for 15 min. The beads were recovered by centrifugation at 750 rpm, and the supernatant was collected. This elution procedure was repeated at least four times. The elution fractions were pooled and afterward analyzed by SDS-PAGE. The pooled sample was concentrated to a volume of ~2 ml, and the elution buffer was exchanged by Hep A buffer (20 mM Tris-HCl, pH 7.8, 0.05 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol) to remove high concentrations of reduced glutathione using the Vivaspin 15 centrifugal filtration devices (Vivascience; Van der Heyden, Brussels, Belgium). The protein underwent fast-performance liquid chromatography to about 98% purity over a Hitrap Heparin column (Amersham Biosciences). After the binding of the RT to the heparin column, elution was accomplished by a linear salt gradient of 0.05–1 M NaCl. Heterodimer RT eluted at approximately 0.3 M NaCl, as determined by SDS-PAGE. All fractions with the same relative amounts of the p51 and p66 subunits were pooled (Fig. 2) 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.

Preparation of *E. Coli* Lysates. LB 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, pH7.8, 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 (12,000 rpm, 20 min), and the supernatant was stored at −80°C in aliquots of 80 μl.

Reverse Transcriptase Assay. For determination of the 50% inhibitory concentrations (IC_{50}) of the test compounds, the RT assays were performed as described previously (Balzarini et al., 1992). A fixed concentration of the labeled 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%.

To assess the processivity of chimeric HIVp66/FIVp51 RT compared with wild-type HIVp66/HIVp51 RT, the enzyme activity was determined at different time points. The amount of dGTP incorporated by a fixed concentration of RT enzyme (8 ng HIVp66/HIVp51 and 670 ng HIVp66/FIVp51 RT) was examined at a variety of different incubation periods ranging from 15 to 150 min.

Determination of the relative specific activity of chimeric HIVp66/FIVp51 RT and wild-type HIVp66/HIVp51 RT was also performed by measuring the RT activity in a 30-min RT assay as described above, using various enzyme concentrations ranging from 3.2 ng to 10.7 μg in the enzyme assays (50 μl).

Steady-state kinetic assays were performed as described previously (Balzarini et al., 1992), except that the reaction mixtures were incubated for 30 instead of 60 min during the assays with variable substrate (dGTP or dTTP) or template/primer [poly(rC)·oligo(dG_{12-18}) or poly(rA)·oligo(dT_{12-18})]. 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 poly(rC)·oligo(dG_{12-18}) and dGTP were determined in the presence of 1.4 μM (1 μCi) [2.8-^{3}H] dGTP (specific radioactivity, 14.1 Ci/mmol) and 0.1 mM poly(rC)·oligo(dG_{12-18}), respectively. 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) or template/primer [poly(rC)·oligo(dG_{12-18})] versus the velocities of dGTP incorporation at each substrate or template/primer concentration. In the assays using [^{3}H]dTTP as the labeled substrate and poly(rA)·oligo(dT_{12-18}) as the template/primer, the K_m and k_{cat} values for dTTP were determined in the presence of 0.015 mM poly(rA)·oligo(dT_{12-18}).

To determine the K_i value of nevirapine ($K_{i,\ nev}$) and the kinetic mechanism (competitive/noncompetitive) of wild-type HIV-1, FIV, and the chimeric RTs, the assays [using [2,8-^{3}H]dGTP and poly(rC)·oligo(dG_{12-18})] were performed in the presence of different concentrations of nevirapine ranging from 0.4 to 2 μg/ml, respectively.

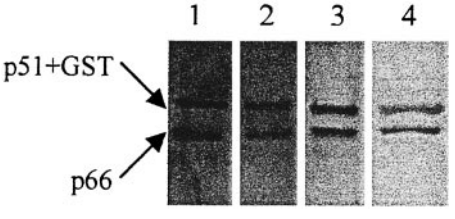

Fig. 2. Purification of chimeric and wild-type RT heterodimers. A silver-stained 12% SDS-polyacrylamide gel of the pooled RT fraction is shown. Lane 1, HIVp66/FIVp51; lane 2, FIVp66/HIVp51; lane 3, FIVp66/FIVp51; lane 4, HIVp66/HIVp51 RT heterodimer.

Results

Kinetic Analysis of the Wild-Type (HIVp66/HIVp51 and FIVp66/FIVp51) and Chimeric (HIVp66/FIVp51 and FIVp66/HIVp51) RTs. Kinetic analysis of the reverse transcriptases was performed with both the substrates (i.e., dGTP or dTTP) and the template-primer [i.e., poly(rC)·oligo(dC)] as variables. The kinetic parameters for the different RT enzymes with dGTP and dTTP as the variable substrate are summarized in Tables 1 and 2, respectively. The K_m value of the HIVp66/FIVp51 chimera for dGTP was 3.2-fold higher than the K_m value for wild-type HIV-1 RT. The K_m values of wild-type FIV

TABLE 1
Kinetic analysis of HIV-1 and FIV wild-type and chimeric RT enzymes with dGTP as the variable substrate
The data are means of at least two to three independent experiments (means ± S.D.).

RT	$K_{i,nev}$	K_m	k_{cat}	k_{cat}/K_m
		μM	*pmol / μg of protein / s*	
HIVp66/HIVp51	0.52	2.7 ± 0.7	1.0 ± 0.2	0.40
HIVp66/FIVp51	0.47	8.6 ± 1.2	0.043 ± 0.009	0.005
FIVp66/FIVp51	>50	3.1 ± 0.6	1.1 ± 0.1	0.36
FIVp66/HIVp51	>50	4.2 ± 0.2	0.036 ± 0.001	0.008

RT, and the chimeric FIVp66/HIVp51 RT were similar as observed for wild-type HIV-1 RT. The k_{cat} values of the wild-type HIV-1 and FIV RT enzymes were about 1 pmol/μg of protein/s. In contrast, the k_{cat} values for the two chimeras were substantially lower (23- to 30-fold) than for the wild-type RT enzymes, indicating that the chimeras allow fewer substrate molecule incorporations per unit time than the wild-type RTs. The lower k_{cat} values of FIVp66/HIVp51 and HIVp66/FIVp51 combined with the slightly higher K_m values resulted in a catalytic efficiency (k_{cat}/K_m) that was only 2.3% and 1.2%, respectively of the catalytic efficiency of the wild-type RTs.

To confirm these results found for dGTP as the variable substrate, we determined also the K_m values of wild-type HIV-1 RT and chimeric HIVp66/FIVp51 RT with dTTP as variable substrate, finding K_m values of 8.5 μM and 7.9 μM, respectively. These values were comparable with those found for chimeric HIVp66/FIVp51 (8.6 μM) with dGTP as the variable substrate. In this assay we found a k_{cat} value for the chimeric HIVp66/FIVp51 that was, as in the assay with dGTP as the variable substrate, also considerably lower than the k_{cat} value found for wild-type HIV-1 RT. This results in a catalytic efficiency of HIVp66/FIVp51 RT that is ~100-fold lower than the wild-type catalytic RT activity.

The kinetic parameters of the different RT enzymes with poly(rC)·oligo(dG) as the variable substrate are summarized in Table 3. Wild-type HIV-1 RT had a K_m value of 2.9 μM for poly(rC)·oligo(dG). The K_m value of the chimeric HIVp66/FIVp51 RT containing the HIV-1 p66 subunit was ~3.5 times higher. The K_m value of FIVp66/HIVp51 RT subunit was 4.2 μM and thus ~2-fold lower than that of wild-type FIV RT. Thus, the k_{cat} values of the RT chimeras are both in the same range and are 10 to 60 times lower than the k_{cat} values of the corresponding wild-type RTs. This might indicate that the bound template/primer is not in an optimal position to allow efficient catalysis, probably because of subtle differences in dimerization of both subunits.

Processivity and Relative Activity of Wild-Type HIVp66/HIVp51 RT and Chimeric HIVp66/FIVp51 RT. To understand the low catalytic efficiency of the chimeric enzymes, in particular the NNRTI-sensitive chimeric HIVp66/FIVp51 RT, we investigated the processivity of these enzymes over a broad incubation time period (Fig. 3). We observed a linear progression of the reaction for wild-type HIV-1 RT up to 2 h after initiation of the reaction. In contrast, the chimeric HIVp66/FIVp51 RT did not proceed linearly anymore after ±45 min incubation. To obtain a comparable incorporation of [^{3}H]dGTP in these reactions, 80-fold more chimeric HIVp66/FIVp51 RT than wild-type HIVp66/HIVp51 RT was required.

We also determined the relative specific enzyme activity over a broad range of RT concentrations to assess the linearity of the reaction in the presence of these enzyme concentrations (Fig. 4). We observed for HIVp66/HIVp51 RT a linear [^{3}H]dGTP incorporation up to ~1000 ng of enzyme. Higher enzyme concentrations probably caused an extensive consumption of template/primer or substrate in the reaction mixture, leading to staggering of the enzyme reaction. For chimeric HIVp66/FIVp51 RT, the enzyme activity is much lower than in the case of HIVp66/HIVp51 RT, and resulted in a linear incorporation of [^{3}H]dGTP in function of all enzyme concentrations tested (down to 3.2 ng). Thus, there was no indication that there occurred reduced subunit association at the lowest enzyme concentrations that may have resulted in substantial amounts of potential p66/p66 homodimer formation (and concomitantly lower enzyme activity).

Inhibitory Activities of NNRTIs, ddGTP, and PFA Against Wild-Type (HIVp66/HIVp51 and FIVp66/FIVp51) and Chimeric (HIVp66/FIVp51 and FIVp66/HIVp51) RTs. The wild-type HIV-1 and FIV RTs and the two RT chimeras were evaluated for their sensitivities to the inhibitory activity of a variety of NNRTIs, ddGTP and PFA (Table 4).

HIV-1 RT showed a pronounced sensitivity to the inhibitory effect of a variety of NNRTIs. The extent of inhibition

TABLE 2
Kinetic analysis of HIV-1 wild-type and chimeric HIVp66/FIVp51 RT enzymes with poly(rA) · oligo(dT) as template/primer and dTTP as the variable substrate

RT	K_m	k_{cat}	k_{cat}/K_m
	μM	*pmol / µg protein / s*	
HIVp66/HIVp51	8.5	1.1	0.14
HIVp66/FIVp51	7.9	0.01	0.001

TABLE 3
Kinetic analysis of HIV-1 and FIV wild-type and chimeric RT enzymes with poly(rC) · oligo(dG)[a] as the variable template/primer
The data are means of at least two to three independent experiments (means ± S.D.).

RT	$K_{i,nev}$	K_m	k_{cat}	k_{cat}/K_m
		μM	*pmol / µg protein / s*	
HIVp66/HIVp51	0.55	2.9 ± 0.5	3.4 ± 0.7	1.2
HIVp66/FIVp51	1.2	10 ± 1.9	0.05 ± 0.02	0.005
FIVp66/FIVp51	>50	8.8 ± 1.7	0.76 ± 0.03	0.086
FIVp66/HIVp51	>50	4.2 ± 0.4	0.06 ± 0.002	0.015

[a] dGTP, 1 μCi.

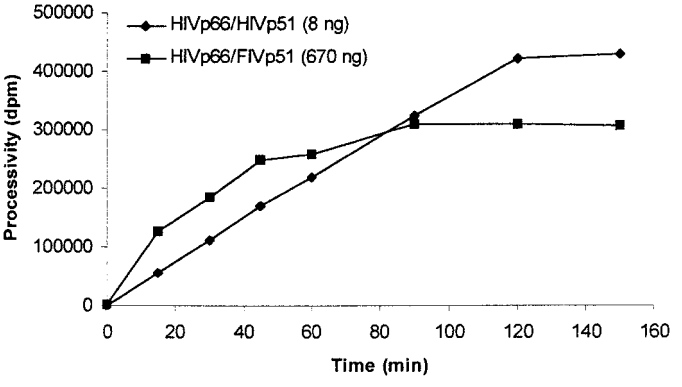

Fig. 3. Relative processivity of HIVp66/HIVp51 RT and HIVp66/FIVp51 RT as a function of incubation time.

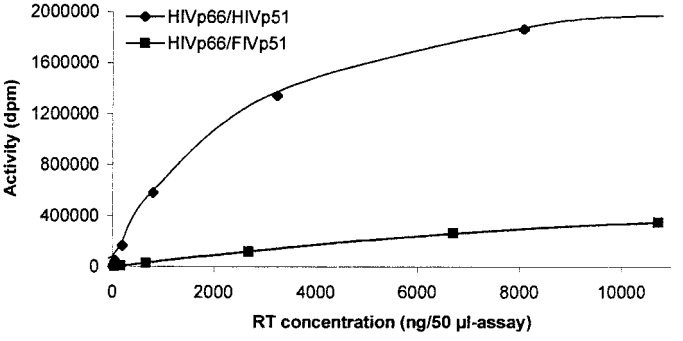

Fig. 4. Relative specific activities of HIVp66/FIVp51 and HIVp66/FIVp51 RTs as a function of different enzyme concentrations.

was dependent on the nature of the NNRTIs, the quinoxaline GW867420X and thiocarboxanilide UC-781 representing the most potent inhibitors of HIV-1 RT. Whereas HIVp66/FIVp51 RT showed a marginal decrease in sensitivity to capravirine (4.6-fold) and TSAO-m^{3}T (3.6-fold) compared with wild-type HIV-1 RT, all the other NNRTIs including nevirapine, delavirdine, efavirenz, emivirine, the quinoxaline GW867420X and the thiocarboxanilide UC-781 showed similar inhibitory activity against this chimeric enzyme compared with wild-type RT. Also, the NRTI ddGTP and PFA had comparable inhibitory effects on both enzymes. Thus, overall, there was a close correlation between the inhibitory effects of NNRTIs, ddGTP, and PFA on the HIVp66/HIVp51 and HIVp66/FIVp51 RT enzymes, containing the HIV-1 p66 subunit in common. This was shown in a linear regression plot with the IC_{50} values of the different NNRTIs, ddGTP, and PFA for the wild-type HIVp66/HIVp51 RT and the chimeric HIVp66/FIVp51 RT (Fig. 5). The correlation coefficient (r) was 0.96, which points to a remarkable similarity between the two enzyme constructs with regard to their ddGTP and NNRTI binding sites.

In contrast, the wild-type FIVp66/FIVp51 RT showed full resistance to all NNRTIs that were included in the study (that is, at drug concentrations that are at least more than 100 to 10,000-fold higher than reported to efficiently inhibit HIV-1 p66 containing wild-type and chimeric RT enzymes). ddGTP had ~8-fold less inhibitory effect on FIV RT compared with HIV-1 RT. Also FIV RT was ~5-fold less sensitive to PFA compared with HIV-1 RT. The chimeric FIVp66/HIVp51 RT also showed full resistance to all NNRTIs studied as did wild-type FIV RT. The inhibitory values of ddGTP and PFA against FIVp66/HIVp51 were also in the same range as observed for wild-type FIV RT.

Kinetic Analysis of the Nature of Inhibition of Wild-Type HIVp66/HIVp51 and Chimeric HIVp66/FIVp51 RTs by Nevirapine. The K_i value for the NNRTI nevirapine ($K_{i,\ nev}$) is shown in Tables 1 (against dGTP) and 3 (against the template/primer). The $K_{i,\ nev}$ value with dGTP as the variable substrate was 0.52 μM for wild-type HIV-1 RT and 0.47 μM for chimeric HIVp66/FIVp51 RT. These nearly identical $K_{i,\ nev}$ values confirm the results found for the determination of the IC_{50} values of nevirapine, which were very similar for wild-type HIV-1 and chimeric HIVp66/FIVp51 RT (see above and Table 4). If poly(rC)·oligo(dG) was used as the variable template, the $K_{i,\ nev}$ value for wild-type HIV-1 RT was 0.55 μM, and 1.2 μM for HIVp66/FIVp51 RT. The $K_{i,\ nev}$ value for wild-type FIV and FIVp66/HIVp51 RT could not be determined because of full resistance of these enzymes to NNRTIs, including nevirapine (IC_{50}>50 μM).

To investigate the kinetic inhibition mechanism of nevirapine, we analyzed the mode of inhibition of both enzymes in the presence of various concentrations of the NNRTI (Fig. 6). Double-reciprocal Lineweaver-Burk plots for the inhibition of RT by nevirapine with respect to dGTP as variable substrate, or poly(rC)·oligo(dG) as variable template/primer, revealed noncompetitive inhibition in all cases, indicating a binding of the drug that was independent from the binding of the substrate or template/primer to the RT.

Discussion

In lentiviral virions, the viral protease cleaves the p66 subunit between amino acids Phe440 and Tyr441 to yield the p51 and p15 subunits (Graves et al., 1990). Two models have been proposed for the generation of the mature p66/p51 heterodimer. Davies et al. (1991) suggested a model in which first a homodimer of two p66 molecules is formed. In this homodimer, one p66 subunit resembles the tertiary structure of p51 to allow proteolytic cleavage by the viral protease. Another model proposes the formation of a catalytically inactive heterodimer that becomes fully active after a slow conformational change (Divita et al., 1995). In vivo, however, a combination of both mechanisms might occur (Morris et al., 1999). The total structure in the HIV-1 RT is highly asymmetric and the polymerase regions of p66 and p51 subunits are divided into four subregions (i.e., fingers, palm, thumb, and connection) (Kohlstaedt et al., 1992). The contact between the p66 and p51 subunit occurs between the p66 palm and the p51 fingers, the connection domain and the p66 RNase H and the p51 thumb domains (Ding et al., 1994).

In our study, we examined the separate roles of the p66 and p51 subunits in the susceptibility of FIV RT to NNRTIs by reconstituting chimeric HIV-1/FIV RTs (HIVp66/FIVp51 and FIVp66/HIVp51 RTs), which could be expressed in a coexpression system and purified as stable heterodimers as shown by SDS-PAGE (Fig. 2). Although we used a GST tag in our RT purification assays, the presence of this GST tag did not influence the kinetic properties of the enzymes, and we

TABLE 4
Sensitivity of wild-type HIV-1, FIV, and chimeric HIVp66/FIVp51 and FIVp66/HIVp51 RTs to the inhibitory effects of NNRTIs, ddGTP, and PFA
poly(rC) · oligo(dG_{12-18}) was the template/primer and [2,8-^{3}H]dGTP was the radiolabeled substrate.

	IC_{50}			
	HIVp66/HIVp51	HIVp66/FIVp51	FIVp66/FIVp51	FIVp66/HIVp51
	$\mu g/ml$			
NNRTIs				
Nevirapine	0.305	0.42	>50	>50
Delavirdine	0.119	0.072	>50	>50
Efavirenz	0.008	0.003	>50	>50
Emivirine	0.039	0.048	>50	>50
Capravirine	0.005	0.023	>50	>50
GW867420X	0.002	0.006	>50	>50
UC-781	0.008	0.006	>50	>50
TSAO-m^{3}T	0.416	1.5	>50	>50
NRTI				
ddGTP (μM)	0.073	0.107	0.52	0.56
PFA (μM)	28	21	147	147

found IC_{50} values for the NNRTIs similar to those found for wild-type HIV-1 RT in the literature.

Because diverse mutations and even single mutations may change the enzymatic activity of RT and may show different local conformational structures (Tantillo et al., 1994), we presume that a replacement of one or both subunits by a homologous subunit of another lentiviral RT can induce important conformational changes. Moreover, it has been suggested that HIV-1 p51 plays a role in the processivity of the p66 subunit (Huang et al., 1992), in loading the HIV-1 p66 subunit onto the template/primer (Amacker and Hubscher, 1998) and a role in the maintenance of an optimal structural enzyme conformation (Tasara et al., 1999). From this perspective, the relatively low catalytic activity and processivity of the chimeras of HIV-1 and FIV RTs can be ascribed to conformational changes and/or suboptimal interaction of p66 and p51 in the chimeric enzymes. Amacker and Hubscher (1988) showed a 2.5-fold increase in RNase H activity compared with the native HIV-1 RT heterodimer. Because significant portions of the p51 helical structure interact with the p66 RNase H domain, these observations suggest a significant alteration of the p51/p66 interactions in the chimeric enzymes. It would therefore be interesting to reveal whether the HIVp66/FIVp51 heterodimeric chimeric enzymes described in our study have a decreased RNase H activity.

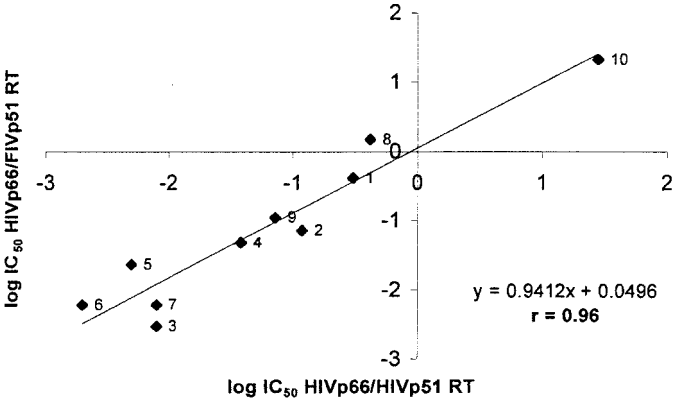

Fig. 5. Regression analysis of IC_{50} values obtained for wild-type HIVp66/HIVp51 and chimeric HIVp66/FIVp51 RTs. Compounds: 1, nevirapine; 2, delavirdine; 3, efavirenz; 4, emivirine; 5, capravirine; 6, GW867420X; 7, UC-781; 8, TSAOm^{3}T; 9, ddGTP; 10, PFA.

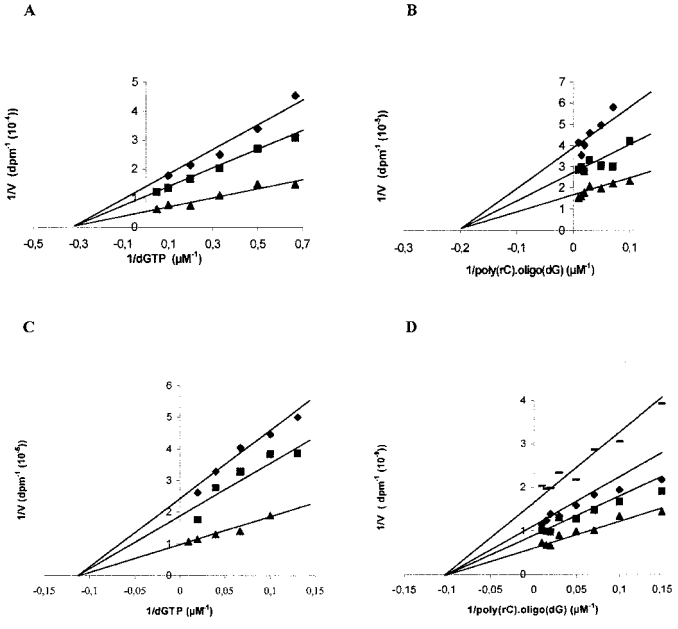

Fig. 6. Double-reciprocal plots for inhibition of wild-type HIV-1 (A and B) and chimeric HIVp66/FIVp51 (C and D) RT by nevirapine. Nevirapine: –, 2 μg/ml; ♦, 0.8 μg/ml; ■, 0.4 μg/ml; and ▲, 0 μg/ml (control). A and C, template/primer [0.1 mM poly(rC)·oligo(dG)] and variable concentrations of [^{3}H]dGTP were used. B and D, [^{2}H]dGTP (5.5 μM) and variable concentrations of template/primer [poly(rC)·oligo(dG)] were used. C and D were performed on RT lysate.

The binding pocket for NNRTIs is located in the p66 subdomain near to, but distinct from, the polymerase active site (Kohlstaedt et al., 1992). Crystal structures of HIV-1 RT complexed with different NNRTIs revealed that all NNRTIs share a common binding site (Ding et al., 1995). Interestingly, the majority of amino acids in the NNRTI pocket of HIV-1 RT that are instrumental in retaining sensitivity to NNRTIs (Schinazi et al., 2000) are identical in FIV RT except (the corresponding amino acids in FIV RT are in parentheses) K101 (Q101), E138 (A138), V179 (D179), and F227 (Y227) (see also Fig. 1).

We could not find any inhibitory effect of NNRTIs on FIV RT, even at drug concentrations that are several orders of magnitude higher than required to fully suppress HIV-1 RT activity. The relatively minor differences in amino acid composition in FIV RT can probably not fully explain the complete resistance of FIV RT against NNRTIs. Therefore, construction of a variety of FIV/HIV-1 chimeric enzymes in which well-defined parts of the p66 subunit are exchanged by the corresponding HIV-1 p66 parts is currently performed to obtain better insights in the resistance of FIV RT to the NNRTIs. According to our data, no major influence of the p51 FIV RT subunit of the chimeric HIVp66/FIVp51 RT on the sensitivity to NNRTIs was observed except for a marginal decrease of the inhibitory potential of capravirine and TSAOm^{3}T. These observations are in agreement with previous observations that the p66 subunit, but not the p51 subunit, predominantly determines the sensitivity of HIV-1 RT to the NNRTIs (Boyer et al., 1994; Jonckheere et al., 1994). Also, the sensitivity (IC_{50}) of HIVp66/FIVp51 RT to the NRTI ddGTP and to PFA was in the same range as that of the wild-type HIV-1 RT.

We have also shown that inhibition of both the wild-type HIV-1 and the chimeric HIVp66/FIVp51 RTs by nevirapine is noncompetitive with respect to the substrate and also noncompetitive with respect to template/primer (Fig. 6), which suggests a similar interaction of this drug with the p66 subunit of wild-type HIV-1 and chimeric HIVp66/FIVp51 RT.

The conclusions of our findings differ from those reported by Amacker and Hubscher (1998). These investigators found that nevirapine was inhibitory toward FIV RT and the chimeric FIVp66/HIVp51 RT whereas our results for nevirapine and all other NNRTIs point to a complete inactivity against FIV p66 containing wild-type or chimeric heterodimer RTs, at least at drug concentrations that are 100- to 10,000-fold higher than the inhibitory values for HIV-1 RT. The K_i values of nevirapine for the wild-type and chimeric enzymes with dGTP as substrate or poly(rC)·oligo(dG) as template/primer was comparable with the value (0.45 μM) these investigators (Amacker and Hubscher, 1998) found with poly(rA)·oligo(dT) as the template/primer. It should be mentioned that none of the NNRTIs included in our studies proved to be inhibitors of the replication of FIV in Crandell feline kidney cells (data not

shown) and these observations are in full agreement with our enzyme data.

In conclusion, the role of the RT p51 subunit is limited to the maintenance of the optimal conformation of the p66 subunit in the heterodimeric RT enzyme. Replacement of the p51 subunit in wild-type HIV-1 or FIV RTs by the FIV or HIV-1 p51 counterpart does not markedly change the sensitivity/resistance profile of RT toward NNRTIs.

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