Amino Acid Substitutions in HIV-1 Reverse Transcriptase with Corresponding Residues from HIV-2

EFFECT ON KINETIC CONSTANTS AND INHIBITION BY NON-NUCLEOSIDE ANALOGS*

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Nevirapine is a highly potent and specific inhibitor of human immunodeficiency virus type 1 (HIV-1) polymerase, but is inactive against HIV-2 and other polymerases. Previous studies demonstrated that residues 176–190 of HIV-1 reverse transcriptase (RT) can confer nevirapine sensitivity to HIV-2 RT. To better characterize the role of this sequence in HIV-1 RT, we have progressively substituted residues 176–190 of HIV-2 RT for those of HIV-1 RT and monitored the impact on the kinetic properties; inhibitory activity of nevirapine (11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido[2,3-b:2',3'-e][1,4]diazepin-6-one), EBPU (5-ethyl-1-benzylxomethyl-6-(phenethyl)uracil), and TIBO-R82150 ((+)-S-4,5,6,7-tetrahydro-5-methyl-6-(5-methyl-2-butenyl)imidazo[4,5,1-jk][1,4]benzodiazepin-2(1H)-thione); and inhibitor-induced fluorescence changes of the mutant enzymes.

The study revealed that in addition to Tyr-181 and Tyr-188, a new amino acid residue (Gly-190) plays an important role in determining susceptibility to nevirapine and E-BPU, but not to TIBO-R82150. These data argue that these non-nucleoside inhibitors fit differently, even though they share a common binding pocket. Nevirapine was seen to exert inhibitory activity by altering the interaction of the enzyme with the template-primer. Kinetic parameters were modulated by the template (DNA versus RNA) as well as by some of the mutations.

Reverse transcriptase is a multifunctional enzyme that catalyzes the incorporation of deoxyribonucleotides using both RNA and DNA templates. The enzyme also possesses an RNAase H activity that removes ribonucleotides from RNA-DNA hybrids. This combination of functions allows the synthesis of a duplex DNA starting from a single-stranded RNA genomic template (see review by Goff (1990)). Human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) is an asymmetric heterodimer with subunits of 51 (p51) and 66 (p66) kDa. p51 results from a proteolytic cleavage that removes the RNase H domain, which is located at the COOH terminus in the p66 polypeptide. HIV-1 RT shares common features with the Klenow fragment of Escherichia coli polymerase I, including a large cleft that accommodates the template and the spatial localization of 3 acidic residues, Asp-110, Asp-185, and Asp-186 (which correspond to Asp-705, Asp-882, and Glu-883 in the Klenow fragment, respectively), believed to be part of the polymerase active site (Delarue et al., 1990; Kohlstaedt et al., 1992).

There has been an intense effort to inhibit HIV-1 RT in an attempt to develop chemotherapeutic agents for AIDS treatment (see review by Mitsuya (1992)). Inhibitory compounds identified thus far fall into two categories: nucleoside analogs, of which 3′-azido-3′-deoxythymidine is a member, and non-nucleoside analogs (see review by De Clercq (1992)). Nevirapine is a member of this latter group (Meruzzi et al., 1990; Hargrave et al., 1991; Klunder et al., 1992) and is being tested in clinical trials. Previous chemical cross-linking and mutational analyses have demonstrated that nevirapine binds near Tyr-181 and Tyr-188 in HIV-1 RT and that mutation of either of these residues causes reduced sensitivity to nevirapine in vitro (Cohen et al., 1991; Shih et al., 1991). More recently, the mutation Tyr-181 → Cys of HIV-1 RT has also been reported to cause loss of drug susceptibility in cell culture studies (Richman et al., 1991; Nunberg et al., 1991; Mellors et al., 1992).

In the crystal structure of HIV-1 RT complexed with nevirapine, Tyr-181 and Tyr-188 are seen in proximity to the inhibitor and appear opposite to each other in an antiparallel β-sheet that forms one face of the binding pocket (Fig. 1). Significantly, the catalytic residues, Asp-185 and Asp-186, are situated in the β-turn that connects the β-strands containing these Tyr residues (Kohlstaedt et al., 1992).

HIV-2 RT is closely related to HIV-1 RT in amino acid sequence (~60% identity), and both enzymes contain several aliphatic residues that flank the β-hairpin formed by Tyr-183, Met-184, Asp-185, and Asp-186 (see Table I). HIV-2 RT has Ile and Leu instead of Tyr at positions 181 and 188, respectively, even though they share a common binding pocket. Nevirapine and E-BPU, but not to TIBO-R82150. These data argue that these non-nucleoside inhibitors fit differently, even though they share a common binding pocket.
respectively, and, as expected, does not bind nevirapine (see Table I). Even though mutation of Ile-181 and Leu-188 to Tyr in HIV-2 RT does not confer complete sensitivity to nevirapine, a chimeric HIV-2 RT containing amino acids 176–190 of HIV-1 RT recovers most of the susceptibility to nevirapine inhibition (Shih et al., 1991; Condra et al., 1992). This region thus appears to be of considerable interest because of its ability to bind non-nucleoside inhibitors, close proximity to the catalytic center, and sequence homology to other polymerases.

In this study, we progressively substituted residues 176–190 of HIV-2 RT for the corresponding residues from HIV-1 RT. We monitored susceptibility to nevirapine inhibition and to two other non-nucleoside reverse transcriptase (NNRT) inhibitors and the effects of these mutations on the kinetic parameters under steady-state conditions. The mechanism of inhibition by nevirapine was also studied by inhibitor-induced fluorescence changes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Poly(rC)–oligo(dG)12–14, poly(rA)–oligo(dT)12–14, poly(dC)–oligo(dG)12–14, poly(dA)–oligo(dT)12–14, poly(rA), and oligo(dT)12–14 were from Pharmacia LKB Biotechnology Inc.; (rC)100 and (dG)10 were from The Midland Certified Reagent Co. (Midland, TX). Template-primer stocks for fluorescence were prepared by annealing a 1:5 molar ratio of poly(rA) to oligo(dT)12–14 in 50 mM Tris, Ethidium bromide was purchased from Molecular Probes, Inc. Heparin-agarose was prepared by the covalent linkage of heparin to dGTP (–10 Ci/mmol) and [3H]dTTP (–20 Ci/mmol) were from Du Pont–New England Nuclear. CDPK was obtained from Calbiochem. Ethidium bromide was purchased from Molecular Probes, Inc. Heparin-agarose was prepared by the covalent linkage of heparin to dGTP (–10 Ci/mmol) and [3H]dTTP (–20 Ci/mmol) were from Du Pont–New England Nuclear. CDPK was obtained from Calbiochem. Ethidium bromide was purchased from Molecular Probes, Inc.

**Purification of Recombinant Enzymes**—Two-liter cultures of E. coli were grown in fermentors (MBR, Wetzikon, Switzerland) using 2 × YT-based medium (Sambrook et al., 1989), pH 7.8, containing glucose (1 g/liter) after inoculation with a 40-ml overnight culture of strain JM109. Isopropyl-β-D-thiogalactopyranoside (0.5 mM final concentration) was added at A260 = 1.5–1.8, and the fermentation was continued for an additional 6 h. Oxygen was kept <1%; pH and glucose were not maintained after the fermentation had started. Biomass yield ranged from 8 to 17 g of cell paste, which was lysed and applied to a DEAE-Sephacel column (~300-ml bed volume; Pharmacia) according to Muller et al. (1989). Fractions containing polymerase activity were pooled and applied to a heparin-agarose column (30-ml bed volume) pre-equilibrated in buffer A (50 mM Tris-HCl, pH 8.2 (at 4 °C), 1 mM dithiothreitol, 1 mM EDTA, 25 mM NaCl, 6% glycerol) at a flow rate of 0.5 ml/min using a fast protein liquid chromatography apparatus (Pharmacia). The column was developed with a 300-ml linear gradient of 25–300 mM NaCl in buffer A with reverse transcriptase eluting near 150 mM NaCl. The fractions containing the peak of polymerase activity were pooled, concentrated (Stirred Cells Model 8010, YM-30 membranes, Amicon), and chromatographed by hydrophobic interaction HPLC to remove unprocessed p66 as reported by Warren et al. (1992). For this, concentrated heparin-agarose pools were combined with equal volumes of 2.0 M (NH4)2SO4 in 0.1 M K3PO4, pH 6.0, and injected onto a 7.5 × 75-mm TSK phenyl-5PW HPLC column (Bio-Rad). Two-ml samples were chromatographed (1 ml/min) using a two-step gradient. Buffers were as follows: buffer HIC-A, 1.0 M (NH4)2SO4 in 0.1 M K3PO4, pH 6.0; buffer HIC-B, 0.1 M K3PO4, pH 6.0; and buffer HIC-C, 50% ethylene glycol in 0.1 M K3PO4, pH 6.0. The following gradient parameters were used: 0–3 min, 100% buffer HIC-A; 3–7 min, 100% buffer HIC-A to 60% buffer HIC-A, 40% buffer HIC-B; 7–17 min, 60% buffer HIC-A, 40% buffer HIC-B to 100% buffer HIC-B; and 17–20 min, 100% buffer HIC-B to 100% buffer HIC-C. The major peak eluting between 19 and 21 min containing heterodimeric reverse transcriptase was collected (Fig. 2), diluted with equal volumes ice-cold 2× storage buffer (100 mM MES, 600 mM K3PO4, pH 6.0, 350 mM KCl, 10% glycerol, 0.04% NaN3), concentrated (Centricon YM-30 membranes, Amicon) ~10-fold, and stored at 4 °C. For quantification, the purified enzyme was subjected to reverse-phase HPLC analysis on an RP-304 column (C4) at a flow rate of 1 ml/min, monitoring absorbance at 220 nm. Solvent System A was H2O, 0.05% trifluoroacetic acid; Solvent System B was acetonitrile, 0.05% trifluoroacetic acid, with gradient parameters as follows: 0–4 min, 0–
30% Solvent System B; 7–22 min, 30–60% Solvent System B; and 22–30 min, hold at 60% Solvent System B. Protein concentration was determined by injecting 20 µl of a purified enzyme preparation and comparing integrated peak area to that of a standard of known concentration. The concentration of the standard reverse transcriptase sample was determined by total amino acid compositional analysis using Waters Pico-Tag methodology (Waters, 1986).

**Polymerase Assays and Steady-state Kinetics**—RNA-directed DNA polymerase activity in the presence of nevirapine (11-cyclopentyloxy-5,11-dihydropyrido[2,3-b:2',3'-e][1,4]diazepin-6-one) was performed in buffer P1 (50 mM Tris-HCl, pH 7.8, 60 mM NaCl, 2 mM MgCl₂, 8 mM dithiothreitol, 5 µg/ml (rC)₉₀ (dG)₂₀, 30 pmol of [³H]dGTP) in a 50-µl reaction containing 50–100 fmol of enzyme for 30 min at 23 °C. RNA-directed DNA polymerase activity in the presence of TIBO-R82150 (++)-S,4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)imidazo[4,5-1,6]-[1]benzodiazepin-2(1H)-thione) and E-BPU (5-ethyl-1-benzopyrrolyl-6-(phenylthio)uracil) was performed according to Tramontano and Cheng (1992). Acid-insoluble radioactivity was counted as described (Cheng et al., 1987). Assays for steady-state kinetic measurements were done with 5–10 fmol of enzyme (diluted in buffer A) incubated in buffer P2 (50 mM Tris-HCl, pH 7.8, 50 mM KCl, 6 mM MgCl₂, 0.1 mg/ml heat-inactivated bovine serum albumin, 1 mM dithiothreitol) containing an appropriate amount of [³H]dNTP and 0.5 A₅₅₀ unit of template-primer in a 50-µl reaction for 15 min at 37 °C. The amount of incorporated radioactivity was kept below 10%. Michaelis constants and rate constants were derived using the Enzfitter program (Elsevier-Biosoft, version 1.05), with a proportional weighting setting. Manual interpolations using Lineweaver-Burk and Eadie-Hofstee plots also were done in many instances.

**Fluorescence Spectroscopy and Calculation of Dissociation Constants**—The interaction of nevirapine with the enzymes was followed by measuring the fluorescence change of ethidium bromide-poly(rA)₉₀-enzyme complexes upon addition of nevirapine. Solutions containing 50 nM enzyme, 50 nM poly(rA)-oligo(dT)₁₂₋₁₈, and 3 µM ethidium bromide in 50 mM Tris-HCl, pH 7.8, 100 mM KCl, 2 mM MgCl₂, 0.02% CHAPS were prepared for fluorescence measurements. CHAPS was added to prevent the enzyme from adhering to the sides of the fluorescence cuvette during the course of the experiment and had no adverse effects on enzyme activity. Intercalated ethidium bromide was at a concentration below saturation. A nevirapine titration solution was prepared from a concentrated stock in dimethyl sulfoxide at 100 µM in the ethidium bromide-poly(rA)₉₀-oligo(dT)₁₂₋₁₈ enzyme complex to avoid dilution corrections to the fluorescence intensity measurements. Aliquots of this solution were titrated into ethidium bromide-poly(rA)₉₀-enzyme samples, and fluorescence intensity was measured in the slow time base mode after 2 min of stirring. In all cases, the final concentration of dimethyl sulfoxide was <1%. Fluorescence measurements were made on an SLM-8000C photon-counting spectrofluorometer equipped with a magnetically stirred and temperature-controlled cell. All measurements were made at 23°C in 1-cm fluorescence cuvettes. Ethidium bromide fluorescence intensity was measured at 595 nm using an excitation wavelength of 360 nm to optimize detection of bound over free fluorophore (Le Peccq, 1971). Excitation and emission bandwidths were set at 4 nm.

For the calculation of dissociation constants, the fluorescence intensity at [nevirapine] = 0 was normalized to 1 for convenience. Assuming a one-binding site model, the data were then fit to Equation 1 by a nonlinear least-squares algorithm:

$$
\Delta F = \Delta F_{\text{max}} (K_d + [K_N] - [K_d])/(4K_dE_i^{1/2})/E_i^2 \tag{1}
$$

where \( \Delta F \) = measured fluorescence change at given total nevirapine concentration, \( \Delta F_{\text{max}} \) = maximum fluorescence change at saturation of binding site, \( E_i = \) total enzyme concentration, \( L_N = \) total ligand concentration, and \( K_d = \) dissociation constant. \( K_d \) and \( \Delta F_{\text{max}} \) were determined from the fitting procedure.

**Student's t Test**—Probability values were calculated from Student's two-tailed t test performed against wild-type reverse transcriptase (wtRT-1).

**RESULTS**

**Inhibition by Nevirapine, TIBO-R82150, and E-BPU**—RNA-directed DNA polymerase activity of the purified heterodimeric enzymes (Fig. 2) was assayed in the presence of nevirapine using poly(rC)-oligo(dG)₁₂₋₁₈ as template-primer (Table I). Mutants in which Tyr-181 and/or Tyr-188 were substituted with Ile and Leu, respectively (mutants A–E), were not inhibited by concentrations of nevirapine up to 250 µM. One exception was mutant F, which showed an IC₅₀ of ~250 µM. Back-substitution to Tyr-181 and Tyr-188 in the otherwise HIV-2 RT sequence (mutant G) restored most, but not all, of the susceptibility to nevirapine inhibition. This mutant, in fact, had an IC₅₀ that was still 20-fold higher than that of wtRT-1 (1.37 versus 0.07 µM). These data confirmed that Tyr-181 and Tyr-188 are necessary (Cohen et al., 1991; Shih et al., 1991), but not sufficient, to mediate full inhibition by nevirapine. Mutation of Lys-176 back to Pro (to generate mutant J) did not have a significant effect (0.95 µM). However, when Ala-190 was further mutated back to Gly to (to generate mutant I), the susceptibility approached that of wtRT-1 (0.14 µM). Other back-mutations to wtRT-1 in mutants J–L did not substantially change the IC₅₀ values. These data demonstrated that besides Ile-181 → Tyr and Leu-188 → Tyr, conversion of Ala-190 back to Gly contributed to the recovery of nevirapine susceptibility. We next tested whether the Gly-190 → Ala mutation would confer resistance to nevirapine in the absence of other substitutions. The Gly-190 → Ala mutation conferred an increase in IC₅₀ of ~24-fold (1.68 µM), thus confirming that Gly-190 plays a critical role in mediating inhibition of HIV-1 RT by nevirapine. In another mutant containing the Gly-190 → Val substitution in crude bacterial lysate, we observed an increase in IC₅₀ of ~1000-fold over that of wtRT-1 (data not shown).

The Gly-190 → Ala mutant and mutant H, along with the respective control enzymes wtRT-1 and mutant I, were then tested for their susceptibility to inhibition by TIBO-R82150 and E-BPU. Both mutants displayed a 30-fold increase in IC₅₀ with E-BPU, but were sensitive to TIBO-R82150 (Table II). This suggested that TIBO-R82150 does not interact with all of the same residues as do nevirapine and E-BPU.

**FIG. 2. Absorbance profile of hydrophobic interaction HPLC for wtRT-1.** Inset, Coomassie Blue stain of denaturing polyacrylamide gel containing 1 µl of a 1:2 dilution of peak 1 (lane 1) and 1 µl of a 1:1.5 dilution of peak 2 (lane 2). See “Experimental Procedures” for details.
Interaction of Nevirapine with Mutant HIV-1 RT

**Table I**

Fifty percent inhibition of RNA-directed DNA polymerase activity by nevirapine on mutant enzymes of HIV-1 RT

The amino acid sequence of HIV-1 RT from residues 176 to 190 is shown. In boldface type are the residues of the HIV-2 RT sequence, where dots indicate residues identical to HIV-1 RT. The underlined sequence is conserved between HIV-1 and HIV-2 RTs and includes Asp-185 and Asp-186 residues critical for polymerase activity (see text). Values were obtained using poly(rC)-oligo(dG) as the template-primer. See "Experimental Procedures" for details.

| Enzyme   | Amino acid sequence | IC₅₀ (mean ± S.D., n = 3) |
|----------|---------------------|----------------------------|
| wtRT-1   | 176 P D I V I Y Q Y M D D L Y V G 188 190 I I I A | 0.07 ± 0.02 |
| wtRT-2   | K V I I I I I I I I I I A | >>250 |
| A⁺       | K V I I I I I I I I I I A | >>250 |
| B        | V I I I I I I I I I I I A | >>250 |
| C        | V I I I I I I I I I I I A | >>250 |
| D        | V I I I I I I I I I I I A | >>250 |
| E        | V I I I I I I I I I I I A | >>250 |
| F        | V I I I I I I I I I I I A | >>250 |
| G        | K V I I I I I I I I I I A | 1.37 ± 0.15⁺ |
| H        | V I I I I I I I I I I I A | 0.95 ± 0.14⁺ |
| I        | V I I I I I I I I I I I A | 0.14 ± 0.01⁺ |
| J        | V I I I I I I I I I I I A | 0.29 ± 0.01⁺ |
| K        | V I I I I I I I I I I I A | 0.06 ± 0.04 |
| L        | V I I I I I I I I I I I A | 0.11 ± 0.01⁺ |
| Gly-190→Ala |                | 1.68 ± 0.62⁺ |
| Tyr-181→Ile |                | 73 ± 4⁺ |
| Tyr-181→Cys |                | 4.83 ± 0.71⁺ |
| Leu-187→Ala |                | 0.02 ± 0.01⁺ |

* This mutant was referred as RT-1 176–190 in Shih et al. (1991).
⁺ p < 0.001 (mutant enzyme versus wtRT-1 in Student's t test).
⁻ p < 0.025.
⁻⁻ p < 0.050.
⁻⁻⁻ p < 0.010.
⁻⁻⁻⁻ From Shih et al. (1991).
⁻⁻⁻⁻⁻ From Richman et al. (1991).

**Table II**

Fifty percent inhibition of RNA-directed DNA polymerase activity by TIBO-R82150 and E-BPU

Values were obtained using poly(rC)-oligo(dG) as the template-primer. See "Experimental Procedures" for details.

| Enzyme   | IC₅₀ (mean ± S.D., n = 3) TIBO-R82150 E-BPU |
|----------|-------------------------------------------|
| wtRT-1   | 404 ± 27 228 ± 21                        |
| Gly190→Ala | 838 ± 92⁺ 7100 ± 9²            |
| I        | 440 ± 89 336 ± 31                       |
| H        | 419 ± 8 8888 ± 555⁺                    |

¹ p < 0.005.
⁻⁻⁻⁻⁻ p < 0.001.

Fluorescence—Nevirapine binding to the poly(rA)-oligo(dT)₁₂₋₁₈-enzyme complex in the presence of ethidium bromide resulted in changes in the emission intensity of the fluorophore, indicating a perturbation in the interaction between the enzyme and the polynucleotide. Titration with different concentrations of inhibitor were used to calculate dissociation constants. As this method relies on the fluorescence change associated with the polynucleotide, and not the enzyme directly, we refer to the dissociation constants obtained as apparent $K_d$ values. Fluorescence intensity was unchanged with enzymes containing mutations at Tyr-181 and/or Tyr-188 (mutants A-F), confirming that no nevirapine binding occurred (Table III). On the other hand, mutants in which Tyr-181, Tyr-188, and Gly-190 (mutants I-L as well as Leu-187→Ala) were present demonstrated $K_d$ values comparable to that of wtRT-1. Mutants containing the Gly-190→Ala mutation (mutants G, H, and Gly-190→Ala) displayed a moderate increase in $K_d$; however, a poor fit of the data to

**Table III**

Apparent dissociation constants for nevirapine

Values were obtained using poly(rA)-oligo(dT) as the template-primer. See "Experimental Procedures" for details.

| Enzyme   | $K_d$ (mean ± S.D., n = 2) µM |
|----------|-------------------------------|
| wtRT-1   | 0.323 ± 0.049                 |
| A        | No binding                    |
| B        | Weak/no binding               |
| C        | Weak/no binding               |
| D        | Weak/no binding               |
| E        | No binding                    |
| F        | No binding                    |
| G        | 1.810 ± 0.170⁺                |
| H        | 0.815 ± 0.211⁺                |
| I        | 0.402 ± 0.034                 |
| J        | 0.340 ± 0.042                 |
| K        | 0.365 ± 0.003                 |
| L        | 0.310 ± 0.096                 |
| Gly-190→Ala | 0.468 ± 0.074⁺                |
| Leu-187→Ala | 0.247 ± 0.065                 |

¹ The nonlinear least-squares fit to a one-binding site model was poor; the $K_d$ reported for these enzymes is the best fit to the data.⁻⁻⁻⁻⁻ p < 0.010.

Equation 1 was observed. Fig. 3 shows an example of a typical fit of the data for wtRT-1.

Michaelis Constants and Rate Constants—Table IV summarizes the results of steady-state kinetics for dGTP and dTTP with RNA templates. For dGTP, the $K_m$ for wtRT-2 was higher than for wtRT-1 (1.75 versus 0.80 µM), confirming previous data (Hizi et al., 1991), although $k_{cat}/K_m$ ratios were similar for the two enzymes. In most cases, $K_m$ was higher for the mutants than for wtRT-1. The only enzymes whose $K_m$ values were comparable to that of wtRT-1 were mutants J, L,
and Gly-190 → Ala, indicating that mutations at residues 178 (Ile → Val), 179 (Val → Ile), 180 (Val → Ile), and 190 (Gly → Ala) did not influence $K_m$. Inspection of the two pairs of mutants L versus K and J versus I indicated that mutation at residue 187 (Leu → Ile) increased $K_m$. The effect of this residue on $K_m$ was confirmed by the Leu-187 → Ala mutant, in which the less conservative substitution Leu → Ala resulted in an even greater $K_m$. Such a perturbation might not be surprising in view of the fact that Leu-187 is immediately adjacent to aspartic acid residues 185 and 186. Since the postulated role of these residues involves chelating the Mg$^2+$ ions that help orient and bind dNTP molecules, a slight effect on their conformation could be expected to affect binding. Substitution of Tyr-181 with either Ile or Cys also resulted in a significant increase in $K_m$. When $K_m$ values from the mutants containing substitutions at both positions 181 and 187 (mutants A–D) were pooled and the mean (3.07 ± 0.97 μM) was compared with that from mutants containing substitution at position 187, but not at position 181 (mutants E–I and K; mean of 1.87 ± 0.49 μM), the probability value was significant ($p < 0.050$), indicating that substitutions at positions 181 and 187 acted synergistically in decreasing the affinity to dGTP.

A characteristic feature for $k_{cat}$ was the difference between the Tyr-181 → Ile and Tyr-181 → Cys mutants. Although both enzymes contained a substitution at position 181 (to Ile or Cys), only Tyr-181 → Ile resulted in a lower $k_{cat}$. When $k_{cat}$ values from mutant enzymes containing the Tyr-181 → Ile substitution (mutants A–D) were pooled and the mean was compared to that from other mutants (mutants E–I), the difference (0.33 ± 0.05 versus 0.54 ± 0.10 s$^{-1}$) had a very high probability value ($p < 0.005$), confirming that Tyr-181 → Ile affected $k_{cat}$. The $k_{cat}/K_m$ ratio in these enzymes was therefore dramatically impaired (4–10-fold lower than that of wtRT-1).

For dTTP, the $K_m$ for wtRT-2 was also higher than that for wtRT-1. Mutation of Tyr-181 in wtRT-1 to either Ile or Cys (mutants Tyr-181 → Ile, Tyr-181 → Cys, and A–D) increased $K_m$, mimicking the pattern observed with dGTP. While the mutation Leu-187 → Ile did not affect $K_m$, the substitution Leu-187 → Ala did affect $K_m$, suggesting that Tyr-181 and Leu-187 affect the binding of both purine and pyrimidine bases. As was observed for dGTP, $k_{cat}$ was lower when Ile was substituted for Tyr-181 in the Tyr-181 → Ile mutant, but remained normal when Cys was present instead. Most of the other mutants also had low $k_{cat}$ values, indicating that each of the mutations resulted in a less efficient enzyme. Most interestingly, for mutants in which these substitutions were present simultaneously (mutants A–C), $k_{cat}$ for dTTP was restored to normal values. This suggests that these residues interact in a concerted fashion and that the subtle differences in amino acid sequence within this region may be sufficient to impair this interaction and alter the kinetic values. As with dGTP, the mutation Leu-187 → Ala did not affect $k_{cat}$ for dTTP, and Gly-190 → Ala did not affect kinetic parameters.

We then tested whether mutations Leu-187 → Ile and Tyr-181 → Ile would also increase $K_m$ for dGTP on a DNA template.

### TABLE IV

Michaelis constants and rate constants on RNA template

| Enzyme   | Poly(rA)·olig(dG) template-primer/dGTP substrate | Poly(rA)·olig(dT) template-primer/dTTP substrate |
|----------|-----------------------------------------------|-----------------------------------------------|
|          | $K_m$ (mean ± S.D., n = 2–5) μM                | $k_{cat}$                                      |
|          | $k_m$                                        | $k_{cat}/K_m$ s$^{-1}$                        |
|          |                                               |                                               |
|          | $K_m$ (mean ± S.D., n = 2–3) μM                | $k_m$                                        |
|          |                                               | $k_{cat}/K_m$ s$^{-1}$                        |
| wtRT-1   | 0.80 ± 0.24                                   | 0.65 ± 0.37                                   |
|          |                                               | 0.77 ± 0.23                                   |
|          | 0.12                                         |
| wtRT-2   | 1.75 ± 0.61                                   | 0.83 ± 0.52                                   |
|          |                                               | 0.50 ± 0.35                                   |
| A        | 2.52 ± 0.92                                   | 0.32 ± 0.07                                   |
|          |                                               | 0.13 ± 0.02                                   |
| B        | 3.33 ± 1.64                                   | 0.27 ± 0.07                                   |
|          |                                               | 0.06 ± 0.02                                   |
| C        | 2.10 ± 1.11                                   | 0.37 ± 0.32                                   |
|          |                                               | 0.18 ± 0.12                                   |
| D        | 4.31 ± 1.62                                   | 0.38 ± 0.12                                   |
|          |                                               | 0.10 ± 0.06                                   |
| E        | 1.76 ± 0.03                                   | 0.63 ± 0.37                                   |
|          |                                               | 0.37 ± 0.22                                   |
| F        | 2.77 ± 0.38                                   | 0.70 ± 0.55                                   |
|          |                                               | 0.25 ± 0.17                                   |
| G        | 1.61 ± 0.09                                   | 0.53 ± 0.45                                   |
|          |                                               | 0.33 ± 0.27                                   |
| H        | 1.59 ± 0.45                                   | 0.43 ± 0.23                                   |
|          |                                               | 0.32 ± 0.25                                   |
| I        | 1.43 ± 0.61                                   | 0.37 ± 0.32                                   |
|          |                                               | 0.25 ± 0.13                                   |
| J        | 0.89 ± 0.12                                   | 0.57 ± 0.32                                   |
|          |                                               | 0.62 ± 0.27                                   |
| K        | 2.09 ± 0.12                                   | 0.53 ± 0.28                                   |
|          |                                               | 0.27 ± 0.15                                   |
| L        | 0.93 ± 0.37                                   | 0.55 ± 0.47                                   |
|          |                                               | 0.50 ± 0.53                                   |
| Gly-190 → Ala | 0.73 ± 0.25                                   | 0.75 ± 0.37                                   |
|          |                                               | 0.97 ± 0.17                                   |
| Tyr-181 → Ile | 3.20 ± 0.03                                   | 0.23 ± 0.07                                   |
|          |                                               | 0.07 ± 0.01                                   |
| Tyr-181 → Cys | 2.94 ± 0.03                                   | 0.75 ± 0.38                                   |
|          |                                               | 0.25 ± 0.07                                   |
| Leu-187 → Ala | 3.81 ± 0.01                                   | 0.68 ± 0.01                                   |
|          |                                               | 0.19 ± 0.05                                   |

* $p < 0.010$.
* $p < 0.005$.
* $p < 0.001$.
* $p < 0.050$. 

**Fig. 3.** Change in fluorescence (ΔF) of intercalated ethidium bromide in wtRT-1 and poly(rA)·olig(dT)$_{12-16}$ complex with different concentrations of nevirapine. Data points are denoted by the filled circles. The solid line represents the best fit of the data to Equation 1 in the text.
of the enzyme; we therefore expect that this mutation, which does not appear to have any effect on the corresponding WT enzyme, would result in a higher $K_{\text{m}}$ for dTTP when all of the hydrophobic residues from HIV-2 RT were chosen to be Ala (data not shown).

When kinetic parameters were calculated for poly(rC)-oligo(dG), poly(dC)-oligo(dG), and poly(rA)-oligo(dT) on the wtRT-1 and Gly-190 → Ala mutant enzymes, the Gly-190 → Ala mutation did not affect any of the parameters.

### DISCUSSION

Nevirapine, TIBO-R82150, E-BPU, and other NNRT inhibitors of HIV-1, although structurally distinct from each other, appear to share a common binding pocket (Wu et al., 1991; Dueweke et al., 1992). However, each class of NNRT inhibitors interacts differently with amino acid residues within this binding pocket. This is evident from the following:

1. **specific/preferred mutations of HIV-1 RT** in cell culture selection of resistant HIV-1 variants to a given NNRT inhibitor and 2. **unequal cross-resistance of NNRT inhibitors to NNRT-resistant HIV-1 RTs** (Richman et al., 1991; Mellors et al., 1992; Balzarini et al., 1993). Mutations of Tyr-181 and Tyr-188 have been demonstrated to confer resistance to many NNRT inhibitors both in vitro (Shih et al., 1991; De Vreese et al., 1992) and in cell culture selection (Nunberg et al., 1991; Richman et al., 1992). Our finding that the mutation Gly-190 → Ala also confers resistance to nevirapine and E-BPU, but not to TIBO-R82150, identifies the importance of Gly-190 in nevirapine and E-BPU binding to HIV-1 RT and further supports the notion that different inhibitors interact with different residues in the enzyme. The Gly-190 → Ala mutation did not affect the catalytic efficiency of the enzyme; we therefore expect that this mutation, which only requires one change in the codon (GGA → GCA), would be **relevant in vivo**. More recently, we have demonstrated that reconstructed HIV-1 carrying the Gly-190 → Ala mutation is viable in cell culture, and clinical studies indicate that this particular mutation can arise in some patients treated with nevirapine.

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Nevirapine inhibits reverse transcriptase activity allosterically. It appears to exert its inhibitory effect by altering the interaction between the enzyme and template-primer. This is suggested by our fluorescence data as well as by kinetic experiments with different templates (Tramontano and Cheng, 1992). The mechanism by which the Gly-190 → Ala mutation induces resistance, however, seems quite complex. The crystal structure of nevirapine-complexed HIV-1 RT indicates that the pocket in which the ligand is located also includes Gly-190 (Kohlsbach et al., 1992). Because this residue is part of the antiparallel $\beta$-sheet that forms part of the binding pocket, any group connected to it would project toward the same plane as Tyr-181 and Tyr-188 and thereby cause steric hindrance with the ligand (Fig. 1). The even higher $IC_{50}$ value we observed when Val was substituted for Gly supports this interpretation. Additionally, the substitution of Gly-190 with Ala may affect the flexibility of the $\beta$-strand, thereby altering the effect exerted by the ligand on the enzyme/template-primer interaction, and this results in acquired resistance.

Interestingly, the residues on either side of Gly-190 in the adjacent strands of the $\beta$-sheet have different effects on nevirapine binding. The mutation Val-197 → Leu (in mutant $f$ versus $I$) resulted in a higher $K_{\text{m}}$, whereas Leu-187 → Ile (mutant $I$ versus $D$) did not. The $K_{\text{m}}$ values for mutants $D$, $I$, and $J$ were lower than that of wtRT-1. Again, the Gly-190 → Ala mutation did not affect any of the parameters.

When kinetic parameters were calculated for poly(rC)-oligo(dG), poly(dC)-oligo(dG), and poly(rA)-oligo(dT) on the mutants, no differences were seen, indicating that none of the mutations affected template-primer binding or turnover rate (data not shown).

| Enzyme          | $K_{\text{m}}$ (mM) | $k_{\text{m}}$ (s$^{-1}$) | $k_{\text{m}}$/($K_{\text{m}}$) |
|-----------------|---------------------|--------------------------|---------------------------------|
| wtRT-1          | 2.2 ± 0.2           | 0.84 ± 0.02              | 0.88 ± 0.04                     |
| J               | 2.9 ± 0.3           | 0.47 ± 0.02              | 0.75 ± 0.01                     |
| I               | 2.6 ± 0.5           | 0.31 ± 0.04              | 0.12 ± 0.02                     |
| D               | 4.4 ± 0.04          | 0.32 ± 0.06              | 0.07 ± 0.02                     |
| Gly-190 → Ala   | 2.0 ± 0.2           | 0.75 ± 0.11              | 0.39 ± 0.06                     |
| Gly-190 → Ala   | 1.6 ± 0.2           | 0.40 ± 0.02              | 0.25 ± 0.02                     |

* $p < 0.005$.
* $p < 0.025$.
* $p < 0.010$.

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