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Characteristics of the Pro225His Mutation in Human Immunodeficiency Virus Type 1 (HIV-1) Reverse Transcriptase That Appears under Selective Pressure of Dose-Escalating Quinoxaline Treatment of HIV-1

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Treatment of human immunodeficiency virus type 1 (HIV-1)-infected CEM cell cultures with escalating concentrations of the quinoxaline S-2720 resulted in an ordered appearance of single and multiple mutant virus strains that gradually became resistant to the quinoxaline and other nonnucleoside reverse transcriptase (RT) inhibitors (NNRTIs). A novel mutation, Pro225His, consistently appeared in a Val106Ala RT-mutated genetic background. The contribution of this mutation to the resistance of the mutant HIV-1 RT to NNRTIs was additive to the resistance caused by the Val106Ala mutation. Interestingly, site-directed mutagenesis studies revealed that the Pro225His-mutated RT had acquired markedly greater sensitivity to bis(heteroaryl)piperazine (BHAP U-90152) (delavirdine) but not to any of the other NNRTIs. The kinetics of inhibition of the Pro225His mutant RT by the NNRTIs (including BHAP U-90152) was not substantially different from that observed for the wild-type RT. The hypersensitivity of the mutant enzyme and virus to BHAP U-90152 could be rationally explained by the molecular-structural determinants of the RT-BHAP complex, which has recently been resolved by X-ray crystallography.

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is important for antiviral therapy. Four different categories of RT inhibitors can be distinguished: (i) 2′,3′-dideoxynucleoside analogs (designated nucleoside RT inhibitors or NRTIs), (ii) acyclic nucleoside phosphonate analogs, (iii) nonnucleoside RT inhibitors (NNRTIs), and (iv) phosphonoformic acid (for an overview, see references 5 and 6). An important class of NNRTIs is represented by the quinoxalines, such as S-2720 and HBY 097. The quinoxaline derivatives are highly potent and specific inhibitors of HIV-1 replication in cell culture and of cell-free HIV-1 RT activity (2, 14, 16, 17). They are markedly active against a variety of mutant HIV-1 strains that are resistant to other HIV-1-specific RT inhibitors (2, 16, 17). Both S-2720 and HBY 097 have a very low cytotoxic potential. Also, HBY 097 has good oral bioavailability and is undergoing clinical trials (16).

The rapid emergence of viral variants resistant to HIV-1-specific RT inhibitors is a major disadvantage of this category of HIV-1 inhibitors. Drug resistance is primarily due to mutations in the RT gene. The mutation pattern that appears upon treatment with NNRTIs depends to a certain extent on the nature of the drug used. For example, under high selective quinoxaline pressure, the Gly190Glu mutation preferentially appears, thus resulting in a markedly diminished polymerase activity of the mutant RT and, consequently, retarded replication of the corresponding mutant virus (14, 15). Lowering the drug pressure gave rise to a different set of mutations, in which the Gly190Glu mutation was rarely seen (2, 18).

In the present study, we found that the novel Pro225His mutation consistently appears in the HIV-1(III_B) RT gene under dose-escalating treatment with quinoxaline. This mutation emerges early in drug treatment, following the appearance of the Val106Ala mutation in the RT. The impact of the Pro225His mutation, as a single mutation and in combination with the Val106Ala mutation, in resistance against the quinoxalines and other NNRTIs has been extensively investigated. Most remarkably, we found that the Pro225His mutation sensitized the HIV-1 RT to bis(heteroaryl)piperazine (BHAP U-90152) but not to other NNRTIs.

MATERIALS AND METHODS

Test compounds. The synthesis of the TSAO {[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) has been described elsewhere (3, 21, 22). 9-Chloro-TIBO [4,5,6,7-tetrahydro-5-methylimidazo(4,5,1-*jk*)(1,4)-benzodiazepin-2(IH)-one; R82913] was kindly provided by Zhang-Hao (National Cancer Institute, National Institutes of Health [NIH], Bethesda, Md.) and also was obtained from Pharmatech International Inc. (West Orange, N.J.). Nevirapine (BI-RG-587) and pyridinone L-697,661 were kindly provided by P. Ganong (Boehringer Ingelheim, Ridgefield, Conn.) and M. Goldman (Merck, Sharp & Dohme, West Point, Pa.), respectively. 8-Chloro-TIBO (R86183) and α-anilinophenylacetamide (α-APA) (Loviride) were provided by K. Andries (Janssen Pharmaceutica, Beerse, Belgium). BHAP U-90152, quinoxaline S-2720 [6-chloro-3,3-dimethyl-4-(isopropenyloxycarbonyl)-3,4-dihydroquinoxalin-2(1H)-thione], and HBY 097 [(*S*)-4-isopropoxycarbonyl-6-methoxy-3-(methylthiomethyl)-3,4-dihydroquinoxaline-2 (1H)-thione] were provided by R. Kirsch and M. Rösner (Hoechst AG, Frankfurt, Germany). The 1-(2-hydroxyethoxymethyl)-6-(phenylthio)thymine (HEPT) derivative MKC-442 was kindly provided by M. Baba (Fukushima Medical College, Fukushima, Japan). The oxathiin carboxanilide derivatives UC-10 and UC-781 were obtained from Uniroyal Chemical Ltd. (Middlebury, Conn., and Guelph, Ontario, Canada). Lamivudine (3TC) was

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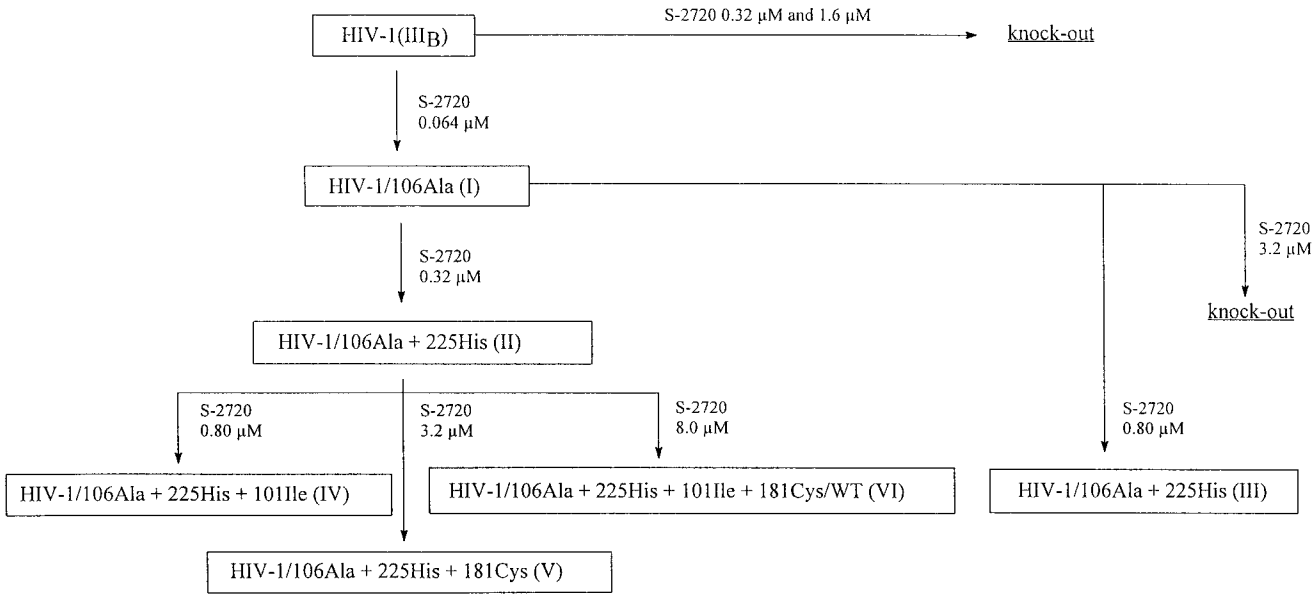

FIG. 1. Selection scheme and genotypic analysis of the RTs of mutant virus strains.

kindly provided by J. Cameron (Glaxo-Wellcome, Stevenage, United Kingdom). The dideoxyinosine and dideoxycytosine derivatives were a kind gift of D. G. Johns (National Cancer Institute, NIH). The zidovudine and dideoxyguanosine derivatives and 2′,3′-dideoxyguanosine-5′-triphosphate (ddGTP) were from Sigma Chemical Co. (St. Louis, Mo.).

Cells and viruses. CEM cells were obtained from the American Type Culture Collection (Rockville, Md.). HIV-1(III_B) was originally obtained from the culture supernatant of persistently HIV-1-infected H9 cells and was kindly provided by R. C. Gallo and M. Popovic (at that time at NIH).

Selection of HIV-1(III_B) mutant strains. The selection sequence is depicted in Fig. 1. (Virus designations are also indicated in Table 1.) Five-milliliter CEM cell cultures (4×10^5 cells/ml) were infected by HIV-1(III_B) in the presence of 0.013, 0.064, 0.32, and 1.6 μM quinoxaline S-2720. The culture medium consisted of RPMI 1640 containing 10% fetal bovine serum, 2 mM L-glutamine, and 0.075% $NaHCO_3$. The initial multiplicity of infection was >1,000 times the 50% cell culture infective dose. Cell culture passages were performed every 3 to 4 days by adding 1.0 ml of the virus-infected cell cultures to 4 ml of a cell suspension containing 4×10^5 uninfected CEM cells per ml. The concentrations of the inhibitors were kept constant throughout the initial experiment. Virus recovered from the cells in the presence of 0.064 μM S-2720 (designated strain I) was then exposed to 0.32, 0.80, and 3.2 μM S-2720. Finally, virus recovered from these cells in the presence of 0.32 μM S-2720 (designated strain II) was then exposed to fixed concentrations of 0.80, 3.2, and 8.0 μM S-2720, and the viruses emerging from these cultivations were designated strains IV, V, and VI, respectively. The virus-infected cell cultures that had been passaged in the presence of S-2720 and that did not show any microscopically visible virus-induced cytopathicity (giant-cell formation) were prepared for p24 antigen (NEN, Dupont) and proviral DNA determinations.

Activity assay for the various test compounds against wild-type HIV-1(III_B) and mutant HIV-1(III_B) strains in CEM cell cultures. The procedure to measure the sensitivity of wild-type HIV-1 and mutant HIV-1 strains was performed as described previously (2). The 50% effective concentration (EC_{50}) was determined as the concentration of compound required to inhibit syncytium formation by 50%.

Preparation of HIV-1-infected CEM cell cultures for PCR analysis and sequencing of the RT gene. Preparation of HIV-1-infected CEM cell cultures for PCR was performed as previously described (2).

Amplification of proviral DNA and sequencing of the 1,754-bp fragment covering amino acid residues 1 to 580 was done essentially as described by Schmit et al. (24, 25). Briefly, a first PCR amplified a 1,754-bp fragment by using primers RT01 (sense, 5′-GTAGAATTCTGTTGACTCAGATTGG) and RT02 (antisense, GATAAGCTTGGGCCTTATCTATTCCAT) (12). Then, 2 μl of the outer PCR product was used in a nested PCR (100-μl reaction volume) with primers M13-USP-A35 (sense, 5′-GTAAAACGACGGCCAGTTTGGTTGC ACTTTAAATTTTCCCATTAGTCCTATT) and M13-RSP-NE-1(35) (antisense, 5′-biotinyl-AGGAAACAGCTATGACCTTACTAACTTCTGTATGTC ATTGACAGTCCAGCT), covering the first 804 bp of the RT gene, or with primers M13-USP-AV47 (sense, 5′-GTAAAACGACGGCCAGTGAACTCCA TCCTGATAAATGGAC) and M13-RSP-AV48 (antisense, 5′-biotinyl-AGGA AACAGCTATGACGTTCTTCTTGGGCCTTATCTAT), covering the next 1,017 bp. These primers introduce into the inner PCR product the sequence of the M13 universal and reverse sequencing primers (underlined), allowing use of a fluorescence-labeled M13 universal or reverse sequencing primer for the sequencing reaction. To sequence both DNA strands completely, other internal fluorescence-labeled sequencing primers were used in addition to the M13 sequencing primers. The biotinyl label introduced by the M13-RSP-NE-1(35) and M13-RSP-AV48 primers made possible easy separation of both DNA strands with streptavidin-coated magnetic beads (Dynal). The PCR products were purified on Microspin S-400 HR columns (Pharmacia, Montreal, Quebec, Canada) and sequenced with an Autoread T7 Sequencing kit (Pharmacia). Sequence analysis was performed on an Automated Laser Fluorescent (ALF) DNA sequencer (Pharmacia).

Site-directed mutagenesis of HIV-1 RT. All mutant enzymes used in this study were derived from the RT sequence cloned in plasmid pKRT2 (4). Site-directed mutagenesis was performed by using the Altered Sites II *in vitro* Mutagenesis Systems kit (Promega). Briefly, the RT-coding region of pKRT2, an *Nco*I-*Hin*dIII fragment, was amplified by PCR with ULTma polymerase and primers RT(*Sma*I) (sense, TTCACCCGGGAAACAGACCAT) and RT(*Hin*dIII) (antisense, GCCAAAACAGCCAAGCTTTATAGT). The PCR product was digested with *Sma*I and *Hin*dIII and then inserted into the *Sma*I-*Hin*dIII cloning sites of the pALTER-1 mutagenesis vector, which is included in the kit, to create pALTER-1(RT). The single-stranded DNA of this plasmid was prepared as described in the protocol. After annealing of the mutagenic oligonucleotide, the Ampicillin Repair oligonucleotide, and the Tetracycline Knockout oligonucleotide to the single-stranded DNA, a mutant DNA strand was synthesized with T4 DNA polymerase and T4 DNA ligase. *Escherichia coli* ES1301 *mutS* was then cotransformed with pALTER-1(RT) and R408 helper DNA. After a 3-h incubation period, a fresh JM109 culture was infected with the supernatant of the cotransformation culture and incubated for another hour before the JM109 culture was plated on ampicillin plates to select for mutants. The presence of the desired mutation was determined and confirmed by sequencing the RT gene of pALTER-1(RT) on an ALF DNA sequencer (Pharmacia) by the protocol provided by Pharmacia. The RT gene was then reinserted in the *Nco*I-*Hin*dIII sites of the pKRT2 expression vector.

Expression and purification of mutant HIV-1 RT. *E. coli* JM109 was transformed with the plasmids for the expression of the p66 forms of wild-type RT and the Val106Ala mutant, the Pro225His mutant, and the Val106Ala Pro225His double mutant. Transformed bacteria were grown overnight at 37°C in 50 ml of 2× YT medium containing 100 μg of ampicillin per ml. Aliquots of these overnight cultures (25 ml) were used to inoculate 500 ml of fresh 2× YT medium containing 100 μg of ampicillin per ml. The cells were grown for 6 h at 37°C with vigorous shaking, and then the expression of RT was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM. After 2 h of induction, the cells were harvested by centrifugation (5,000 × *g*, 15 min). The cell pastes were suspended in lysis buffer (50 mM Tris-HCl [pH 7.9, 4°C] containing 60 mM NaCl and 1 mM EDTA), using a 1:1 ratio of lysis buffer to cell paste wet weight. Bacterial lysates were then prepared by using the lysozyme-

DNase I protocol described by Sambrook et al. (23). The lysates were clarified by centrifugation (10,000 × *g*, 20 min).

The lysates were applied at 4°C onto columns of DEAE-Sephacel (Pharmacia) equilibrated with lysis buffer, using a 1-ml bed volume of DEAE-Sephacel for each 3 ml of clarified lysate. Columns were washed with 2 ml of lysis buffer, and the flowthrough and the wash (which contained the p66 RT) were combined, concentrated to 1 ml with Ultrafree-15 centrifugal filtration devices (Millipore, Nepean, Ontario, Canada), and then incubated at 4°C for 16 to 20 h to allow processing of the p66 RT to the p51-p66 heterodimer by bacterial proteases present in the eluates.

The RT was then purified at room temperature by using a modification of the rapid high-pressure liquid chromatography method that we have recently described (11).

RT assay. For determination of the 50% inhibitory concentrations (IC_{50}s) of the test compounds, the RT assay was performed as described before (1). A fixed concentration of the labeled substrate [^{3}H]dGTP (specific radioactivity, 3.6 Ci/mmol) (5.6 μM, 1 μCi) and a fixed concentration of the template-primer poly(C) · oligo(dG_{12-18}) (0.1 mM) were used. The IC_{50}s of the test compounds were determined as the compound concentration that inhibited the recombinant RT activity by 50%.

For the experiments in which the K_i values of the test compounds with respect to the substrate [^{3}H]dGTP were determined, appropriate substrate and inhibitor concentrations (see the legend to Fig. 2) were used in the presence of a fixed concentration of 0.1 mM poly(C) · oligo(dG_{12-18}). For the experiments in which the K_i values of the test compounds with respect to the template-primer poly(C) · oligo(dG_{12-18}) were determined, appropriate template-primer and inhibitor concentrations (see the legend to Fig. 2) were used in the presence of a fixed concentration of 5.6 μM [^{3}H]dGTP. For the experiments in which the reversibility of the inhibition of Pro225His RT by BHAP U-90152 was determined, BHAP U-90152 at the IC_{50} and at one-half the IC_{50} was exposed to a variety of mutant enzyme dilutions (i.e., 100, 80, 60, 40, 20, and 10% of the normal enzyme concentration) in the presence of fixed concentrations of [^{3}H]dGTP (5.6 μM) and poly(C) · oligo(dG_{12-18}) (0.1 mM).

RESULTS

In vitro selection of mutant HIV-1 strains resistant to quinoxaline S-2720. Quinoxaline S-2720-resistant virus strains were selected under dose-escalating drug treatment conditions. An overview of the selection procedure for the quinoxaline-resistant HIV-1 strains that emerged under S-2720 pressure is shown in Fig. 1. HIV-1(III_B)-infected CEM cell cultures were treated with quinoxaline S-2720 at initial fixed concentrations of 0.013 μM (approximately the EC_{50}), 0.064 μM (~5 times the EC_{50}), 0.32 μM (~25 times the EC_{50}), and 1.6 μM (~125 times the EC_{50}). At the highest drug concentrations, i.e., 0.32 and 1.6 μM, no virus-induced cytopathicity was detected for more than 64 days (approximately 18 subcultivations) postinfection. Since no HIV-1 p24 or proviral DNA could be detected in these cell cultures (data not shown), it was assumed that the cell cultures were cleared of HIV-1 (Fig. 1).

At quinoxaline S-2720 concentrations of 0.013 and 0.064 μM, virus breakthrough (50% cytopathicity) was observed at 11 and 25 days, respectively (Table 1). Quinoxaline-resistant HIV-1 that emerged from the HIV-1(III_B)-infected cell culture treated with 0.064 μM S-2720 (designated strain I) was then further grown in the presence of fixed 0.32, 0.80, and 3.2 μM quinoxaline S-2720 concentrations. Again, no virus breakthrough was observed in the presence of quinoxaline at its highest concentration (3.2 μM) after 64 days. However, quinoxaline-resistant HIV-1 emerged at the lower S-2720 concentrations after 14 days (0.32 μM S-2720) and 42 days (0.80 μM S-2720), and 50% cytopathicity was noted on day 20 for 0.32 μM S-2720 (virus designated strain II) and on day 44 for 0.80 μM S-2720 (virus designated strain III) (Table 1; Fig. 1). Quinoxaline-resistant HIV-1 from the cell culture treated with quinoxaline at a concentration of 0.32 μM (virus strain II) was finally exposed to quinoxaline concentrations of 0.80, 3.2, and 8.0 μM. A rapid breakthrough of quinoxaline-resistant virus was observed in the presence of all three quinoxaline concentrations used (viruses designated strains IV, V, and VI, respectively).

TABLE 1. Delay of virus breakthrough in the presence of the quinoxaline S-2720

Expt	Virus strain	Compound concn (μM)	First microscopic sign of virus breakthrough (days)	Appearance of 50% cytopathicity (days)	Designation of new virus strain
1	III_B	0.013	8	11	
		0.064	21	25	I
		0.32	>64	>64	NA[a]
		1.6	>64	>64	NA
2	I	0.32	14	20	II
		0.80	42	44	III
		3.2	>64	>64	NA
3	II	0.80	1	7	IV
		3.2	6	12	V
		8.0	7	13	VI

[a] NA, not applicable.

Determination of the mutations in the RT genes of the quinoxaline-resistant HIV-1 strains I to VI. In the RT gene of HIV-1 strain I, which emerged under treatment with 0.064 μM S-2720, a mutation causing an amino acid change from Val to Ala at position 106 was found (Fig. 1). This mutation was retained in all other S-2720-resistant HIV-1 strains. Upon further exposure of the Val106Ala RT HIV-1 strain to 0.32 μM S-2720, a Pro225His mutation was added to the Val106Ala mutation (strain II). The quinoxaline-resistant virus that emerged after treatment of the Val106Ala RT HIV-1 strain with 0.80 μM S-2720 (strain III) also acquired the Pro225His mutation in its RT in addition to the Val106Ala mutation (Fig. 1). Treatment of the RT double-mutant virus (strain II) with S-2720 at 0.80, 3.2, and 8.0 μM resulted in the addition of Lys101Ile (strain IV), Tyr181Cys (strain V), and Lys101Ile Tyr181Cys/WT (strain VI) mutations, respectively (Fig. 1).

Sensitivities of quinoxaline-resistant HIV-1 strains to HIV-1-specific RT inhibitors. The quinoxaline-resistant HIV-1 strains were evaluated for their sensitivities to different NNRTIs and to NRTIs (Table 2). The Val106Ala RT HIV-1 mutant strain I showed marked resistance to nevirapine (≥230-fold), MKC-442 (87-fold), and S-2720 (80-fold) compared to wild-type HIV-1(III_B). TSAO-m^{3}T, 9-chloro-TIBO, and BHAP U-90152 showed 47-, 26-, and 20-fold decreases, respectively, in inhibitory activity against the Val106Ala RT mutant virus strain I. Pyridinone L-697,661 showed only a moderate decrease (10-fold) in antiviral potency. The quinoxaline clinical candidate, HBY 097, showed only twofold-decreased inhibitory activity against the Val106Ala RT-mutated virus. The addition of a Pro225His mutation in the RT of the Val106Ala RT mutant virus upon selection with 0.32 or 1.6 μM S-2720 (mutant virus strains II and III) led to a further decrease in the sensitivity of the virus to quinoxaline S-2720 (4- to 7-fold), HBY 097 (~10-fold), 9-chloro-TIBO (R82913) (5- to 8-fold), and MKC-442 (12- to 15-fold), but, surprisingly, led to an increased sensitivity to BHAP U-90152 (7-fold) (Table 2). The inhibitory activities of pyridinone L-697,661 and TSAO-m^{3}T against this double-mutated virus strain were not significantly altered.

The mutant virus strains IV, V, and VI, which emerged from the same HIV-1 Val106Ala Pro225His strain II but under a higher S-2720 selective pressure, had a markedly more pronounced resistance than the mutant strain II (Table 2). The most striking differences were seen for S-2720 and HBY 097,

TABLE 2. Sensitivity of mutant HIV-1 strains to NNRTIs and NRTIs

Compound[a]	EC_{50}[b] (μM) for HIV-1 strain[c]:						
	III_B	I (Val106Ala)	II (Val106Ala Pro225His)	III (Val106Ala Pro225His)	IV (Val106Ala Pro225His Lys101Ile)	V (Val106Ala Tyr181Cys Pro225His)	VI (Lys101Ile Val106Ala Pro225His Tyr181Cys/WT)
MKC-442	0.00248 ± 0.00023	0.215 ± 0.023	3.28 ± 2.42	2.65 ± 0.93	13.2 ± 4.6	≥17	>17
9-Chloro-TIBO	0.06 ± 0.03	1.55 ± 0.0	12.4 ± 5.3	7.8 ± 2.2	≥16	≥16	>16
Nevirapine	0.074 ± 0.047	≥17	>17	>17	>17	>17	>17
Pyridinone L-697,661	0.0143 ± 0.012	0.143 ± 0.040	0.086 ± 0.040	0.071 ± 0.0	0.6 ± 0.0	1.51 ± 1.14	≥1
BHAP U-90152	0.018 ± 0.007	0.35 ± 0.13	0.055 ± 0.0	0.050 ± 0.009	0.186 ± 0.046	0.4 ± 0.0	3.3 ± 1.5
TSAO-m^3T	0.030 ± 0.007	1.4 ± 0.48	2.5 ± 1.2	≥5	≥8	>8	>5
S-2720	0.0013 ± 0.0005	0.103 ± 0.036	0.39 ± 0.0	0.68 ± 0.42	8.1 ± 2.3	14 ± 3.42	14 ± 6.8
HBY 097	0.003	0.006 ± 0.0013	0.058 ± 0.0	0.058 ± 0.0	1.06 ± 0.19	1.1 ± 0.16	0.9 ± 0.1
AZT	0.008 ± 0.0	0.004	0.004 ± 0.001	0.007 ± 0.001	0.003 ± 0.001	0.003 ± 0.0	0.002 ± 0.0
ddC	0.05 ± 0.02		0.028 ± 0.004	0.025 ± 0.0	0.025 ± 0.0	0.03 ± 0.007	0.018 ± 0.004
3TC	0.0364 ± 0.0057		0.16 ± 0.12	0.093 ± 0.016	0.101 ± 0.0	0.17 ± 0.101	0.081 ± 0.028
ddI	8.3 ± 5.8	1.3	8.0 ± 2.8	6.0 ± 0.0	3.5 ± 0.7	4.5 ± 2.1	4.0 ± 0.0
ddG	1.3 ± 0.9	1.4 ± 0.0	2.7 ± 1.6	1.1 ± 0.5	0.65 ± 0.07	0.65 ± 0.07	0.55 ± 0.21

[a] AZT, zidovudine; ddC, dideoxycytosine; ddI, dideoxyinosine; ddG, dideoxyguanosine.
[b] Concentration required to inhibit HIV-1-induced cytopathicity in CEM cell cultures by 50%. Data are means and standard deviations.
[c] Origins of mutant virus strains: I, selected from HIV-1(III_B) in the presence of 0.064 μM S-2720; II, selected from strain I in the presence of 0.32 μM S-2720; III, selected from strain I in the presence of 0.80 μM S-2720; IV, selected from strain II in the presence of 0.8 μM S-2720; V, selected from strain II in the presence of 3.2 μM S-2720; VI, selected from strain II in the presence of 8.0 μM S-2720.

which were ~20- to 35-fold less inhibitory for these mutant strains than for mutant strain II. The mutant virus strains IV, V, and VI showed only moderately or slightly higher resistance to the other NNRTIs (i.e., 9-chloro-TIBO, nevirapine, and TSAO-m^3T) (Table 2). The resistance profiles of the three quinoxaline-resistant viruses that arose from the Val106Ala Pro225His RT mutant virus strain II upon further exposure to 0.8 μM S-2720 (strain IV, containing, in addition, the Lys101Ile mutation), 3.2 μM S-2720 (strain V, containing, in addition, the Tyr181Cys mutation), and 8.0 μM S-2720 (strain VI, containing, in addition, the Lys101Ile and the Tyr181Cys/WT mutations) were quite comparable for all NNRTIs except BHAP U-90152 (Table 2). Indeed, BHAP U-90152 showed a 3-fold-decreased inhibitory activity against virus strain IV, which had acquired an additional Lys101Ile mutation; a 7-fold-decreased inhibitory activity against virus strain V, which had acquired an additional Tyr181Cys mutation, and a 60-fold-decreased inhibitory activity against virus strain VI, which had acquired the additional Lys101Ile and Tyr181Cys/WT mutations (Table 2).

The NRTIs retain virtually full activity against all mutant HIV-1 strains (Table 2), irrespective of the nature of the amino acid mutations in the HIV-1 RT.

Inhibitory activities of NNRTIs against wild-type and mutant recombinant HIV-1 RTs. The Val106Ala and Pro225His mutations were introduced into the wild-type HIV-1 RT by site-directed mutagenesis. By combining these two mutations through ligation of the appropriate RT fragments, the Val106Ala Pro225His double-mutant RT was also constructed. The Val106Ala and the Pro225His mutant RTs exhibited enzyme activities that were comparable to wild-type polymerase activity, whereas the Val106Ala Pro225His double-mutant RT showed a slightly lower activity compared to the wild type (data not shown).

A variety of NNRTIs were evaluated for their inhibitory activities against the wild-type, Val106Ala single-mutant, Pro225His single-mutant, and Val106Ala Pro225His double-mutant HIV-1 RTs. The Val106Ala RT showed resistance against all NNRTIs, but to various degrees. The highest levels of resistance were noted for nevirapine (65-fold), TSAO-m^3T (>250-fold), and the thiocarboxanilide UC10 (66-fold) (Tables 3 and 4). BHAP U-90152, quinoxaline HBY 097, MKC-442, α-APA, the thiocarboxanilide UC781, 9-chloro-TIBO, and 8-chloro-TIBO were 6- to 24-fold less effective against the Val106Ala mutant RT. The Pro225His RT showed much less resistance than the Val106Ala RT. Most NNRTIs were only two- to threefold less inhibitory to the Pro225His mutant RT than to wild-type RT. Only MKC-442 lost more (eightfold) of its inhibitory activity against the Pro225His RT.

Surprisingly, the Pro225His RT was ~eightfold more sensitive to BHAP U-90152 than the wild-type RT (Tables 3 and 4). When the Val106Ala mutation was combined with the Pro225His mutation in the RT, the levels of resistance to all NNRTIs suggested that the effects of each single mutation were simply additive in the double mutant. Indeed, the experimentally obtained degrees of resistance against NNRTIs for the double mutant are virtually identical to the values that would be expected if the contribution of each mutation to resistance was independent (Table 4), with the calculated linear regression coefficient between the two series being 0.85. Interestingly, BHAP U-90152 had equal inhibitory activities against the Val106Ala Pro225His double-mutant RT enzyme and the wild-type RT enzyme, due to the compensation of resistance due to the Val106Ala mutation by the higher sensitivity conferred by the Pro225His mutation (Table 4).

TABLE 3. Inhibitory effects of HIV-1-specific inhibitors and ddGTP on recombinant mutant HIV-1 RTs

Compound	IC_{50}[a] (μM) for:			
	Wild-type RT	Val106Ala mutant RT	Pro225His mutant RT	Val106Ala Pro225His mutant RT
MKC-442	0.106 ± 0.00	1.3 ± 0.00	0.79 ± 0.17	12 ± 1.9
9-Chloro-TIBO	1.5 ± 0.65	9.5 ± 3.4	2.4 ± 0.18	≥10
8-Chloro-TIBO	0.248 ± 0.120	5.3 ± 1.98	0.67 ± 0.11	8.9 ± 0.98
Nevirapine	4.3 ± 1.2	275 ± 24	12 ± 4.49	807 ± 338
BHAP U-90152	0.70 ± 0.02	11 ± 3.0	0.090 ± 0.007	0.90 ± 0.04
TSAO-m^3T	3.0 ± 0.15	>828	6.6 ± 1.37	>828
α-APA	1.0 ± 0.09	12 ± 6.1	1.9 ± 0.17	15 ± 2.51
UC10	0.148 ± 0.040	9.8 ± 3.6	0.74 ± 0.11	31 ± 6.0
UC781	0.023 ± 0.001	0.57 ± 0.03	0.074 ± 0.006	2.41 ± 0.12
S-2720	0.076 ± 0.08	1.6 ± 1.5	0.20 ± 0.09	12 ± 8.8
HBY 097	0.012 ± 0.003	0.073 ± 0.001	0.018 ± 0.006	0.213 ± 0.029
ddGTP	0.13 ± 0.06	0.16 ± 0.10	0.086 ± 0.003	0.097 ± 0.004

[a] Concentration required to inhibit the RT reaction by 50%. Data are means and standard deviations.

Kinetic analysis of mutant Pro225His RT. The modes of inhibition of Pro225His RT by BHAP U-90152 (which showed an eightfold-increased activity against the mutant enzyme compared with wild-type RT), quinoxaline HBY 097 (which retained its inhibitory potency against the mutant enzyme), and MKC-442 (which markedly lost inhibitory activity against the mutant enzyme) were investigated and compared with that of inhibition of the wild-type RT. Dixon plots of the kinetic data revealed that the K_i values of the compounds against the Pro225His RT mutant were similar to their IC_{50} values (Table 3). For wild-type RT the K_i values were 0.65, 0.01, and 0.08 μM for BHAP U-90152, HBY 097, and MKC-442, respectively, and for the mutant RT they were 0.13, 0.028, and 0.73 μM, respectively. The kinetics of inhibition of the wild-type and mutant RTs were similar for the three drugs. Indeed, Lineweaver-Burk diagrams revealed a noncompetitive type of enzyme inhibition with respect to the radiolabeled substrate dGTP (Fig. 2A, B, and C) and against the template-primer poly(C) · oligo(dG) (Fig. 2D, E, and F). Also, we could show that inhibition of both the wild-type and Pro225His mutant RTs by BHAP U-90152 was reversible (data not shown).

DISCUSSION

Upon selection for viral resistance with low quinoxaline S-2720 concentrations in cell culture, the Val106Ala RT mutant virus appeared. However, increased S-2720 drug pressure consistently resulted in the addition of the Pro225His mutation to the Val106Ala RT genetic background. Recently, in an independent set of experiments, Kleim et al. (18) observed the same sequential appearance of a Val106Ala mutation followed by addition of a His mutation at position 225 in the RT in the presence of escalating concentrations of HBY 097, a close analog of S-2720 and the clinical candidate chosen from the quinoxaline class of NNRTIs. Interestingly, the Pro225His mutation was also found in a patient receiving HBY 097 treatment (18a). Thus, our in vitro observations may prove relevant for quinoxaline treatment in the clinic. To the best of our knowledge, the Pro225His mutation has not been previously reported to appear in the presence of NNRTIs, in either cell culture or drug-treated patients.

The addition of the Pro225His mutation and the subsequent appearance of the Lys101Ile and Tyr181Cys mutations led to virus strains with markedly increased resistance to the quinoxa-

TABLE 4. Comparison between calculated and experimental IC_{50} values for the Val106Ala Pro225His double-mutant RT

Compound	IC_{50} (μM) for wild-type RT	IC_{50} for mutant RT/IC_{50} for wild-type RT			
		Val106Ala mutant	Pro225His mutant	Val106Ala Pro225His mutant	
				Exptl	Calculated[a]
MKC-442	0.106	12	7.5	110	89
9-Chloro-TIBO	1.5	6.2	1.5	>6.5	9.49
8-Chloro-TIBO	0.248	21	2.7	36	57
Nevirapine	4.3	65	2.8	189	182
BHAP U-90152	0.70	16	0.13	1	2
TSAO-m^3T	3.0	>250	2.2	>500	>500
α-APA	1.0	12	1.9	16	24
UC10	0.148	66	5.0	210	330
UC781	0.023	24	3.2	104	77
S-2720	0.076	22	2.6	158	57
HBY 097	0.012	6.1	1.5	18	9.1
ddGTP	0.13	1.2	0.7	0.7	1.2

[a] Expected value obtained by assuming an additive effect of the single mutations on the sensitivity of the double-mutant RT to the NNRTI.

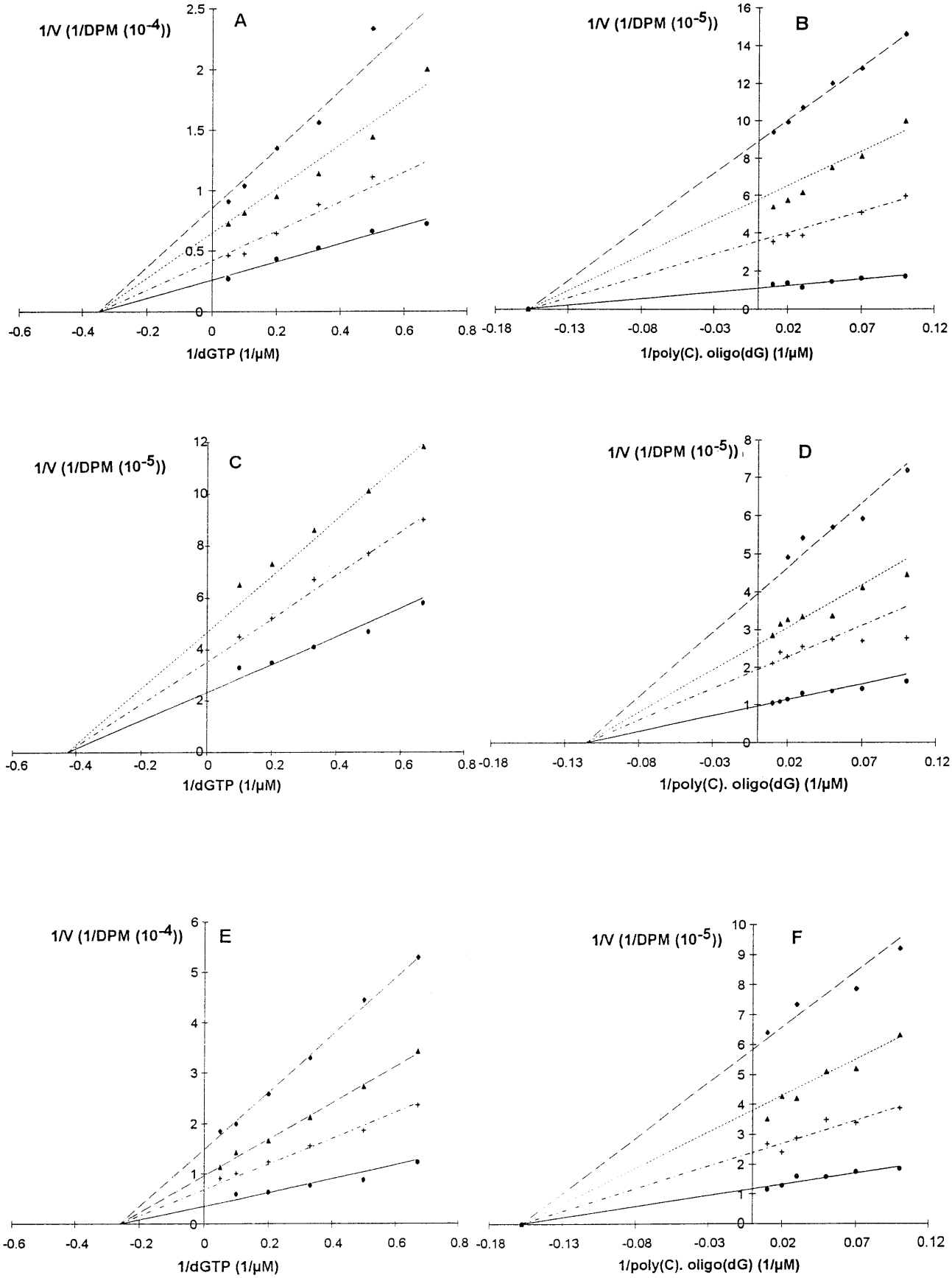
A
1/V (1/DPM (10^{-4}))
2.5
2
1.5
1
0.5
0
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1/dGTP (1/μM)
B
1/V (1/DPM (10^{-5}))
16
14
12
10
8
6
4
2
0
-0.18
-0.13
-0.08
-0.03
0.02
0.07
0.12
1/poly(C). oligo(dG) (1/μM)
C
1/V (1/DPM (10^{-5}))
12
10
8
6
4
2
0
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1/dGTP (1/μM)
D
1/V (1/DPM (10^{-5}))
8
7
6
5
4
3
2
1
0
-0.18
-0.13
-0.08
-0.03
0.02
0.07
0.12
1/poly(C). oligo(dG) (1/μM)
E
1/V (1/DPM (10^{-4}))
6
5
4
3
2
1
0
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1/dGTP (1/μM)
F
1/V (1/DPM (10^{-5}))
10
9
8
7
6
5
4
3
2
1
0
-0.18
-0.13
-0.08
-0.03
0.02
0.07
0.12
1/poly(C). oligo(dG) (1/μM)

FIG. 2. Double-reciprocal plots for inhibition of Pro225His mutant HIV-1 RT. (A and B) HBY 097 (0.0192 μg/ml [◆], 0.0096 μg/ml [▲], 0.0048 μg/ml [+], and 0 μg/ml [control] [●]). (C and D) BHAP U-90152 (0.16 μg/ml [◆], 0.08 μg/ml [▲], 0.04 μg/ml [+], and 0 μg/ml [control] [●]). (E and F) MKC 442 (0.8 μg/ml [◆], 0.4 μg/ml [▲], 0.2 μg/ml [+], and 0 μg/ml [control] [●]). (A, C and E) A 0.1 mM concentration of template-primer [poly(C) · oligo(dG)] and variable concentrations of [^{3}H]dGTP were used. (B, D, and F) A 5.5 μM concentration of [^{3}H]dGTP and variable concentrations of template-primer [poly(C) · oligo(dG)] were used.

lines and significant cross-resistance to other NNRTIs. Although the effect of the Pro225His mutation on NNRTI resistance was relatively small for most NNRTIs, the mutation had a significant effect on the inhibitory activity of MKC-442. The addition of the Lys101Ile and/or the Tyr181Cys mutation to the Val106Ala Pro225His RT genetic background may allow the virus to obtain a higher degree of resistance to S-2720 but also to increase its fitness and replicational competence. A similar phenomenon has recently been reported to occur with dose-escalating treatment of HIV-1 with protease inhibitors (20).

Kleim and coworkers (18) have recently found other NNRTI-characteristic mutations (i.e., Leu100Ile, Lys103Asn, and Tyr181Ile/Asn/Lys) appearing in the Val106Ala Pro225His RT genetic background during dose-escalating treatment with the quinoxaline HBY 097. These observations suggest that addition of Lys101Ile and/or Tyr181Cys to the Val106Ala Pro225His RT genetic background during dose-escalating quinoxaline treatment is not obligatory but can be replaced by other NNRTI-characteristic mutations.

Surprisingly, the addition of the Pro225His mutation resensitized the Val106Ala mutant RT to BHAP U-90152. Site-directed mutagenesis studies confirmed the potentiation of the inhibitory action of BHAP U-90152 against the Pro225His-mutated RT. This unexpected result is in agreement with the structures of the RT-BHAP complex as determined by X-ray crystallography (Fig. 3). In the unliganded enzyme, and in RT complexes with all NNRTIs except BHAP U-90152, the position of Pro225 in RT is such that it helps to close the entrance to the NNRTI-binding pocket. In this conformation, Pro225 interacts strongly with the phenyl ring of Phe227, and in most RT-NNRTI complexes it comes close to Pro236 (with Pro226 in the *cis* conformation) (Fig. 3) (9). However, in the RT-BHAP complex the methylsulfonamide group of the inhibitor forces the main chain of Pro225 to swing around by approximately 180°, leaving Pro225 exposed on the surface of the enzyme at the edge of the polymerase active-site binding cleft (Fig. 3) and Pro226 switching to the *trans* conformation. Thus, the change from proline to a more hydrophilic residue (such as histidine) is likely to stabilize the RT-BHAP complex, which is consistent with the observed hypersensitivity. The extra main-chain conformational freedom resulting from the loss of a proline residue is less likely to be significant, since X-ray studies indicate that the structure around amino acid residue 225 is

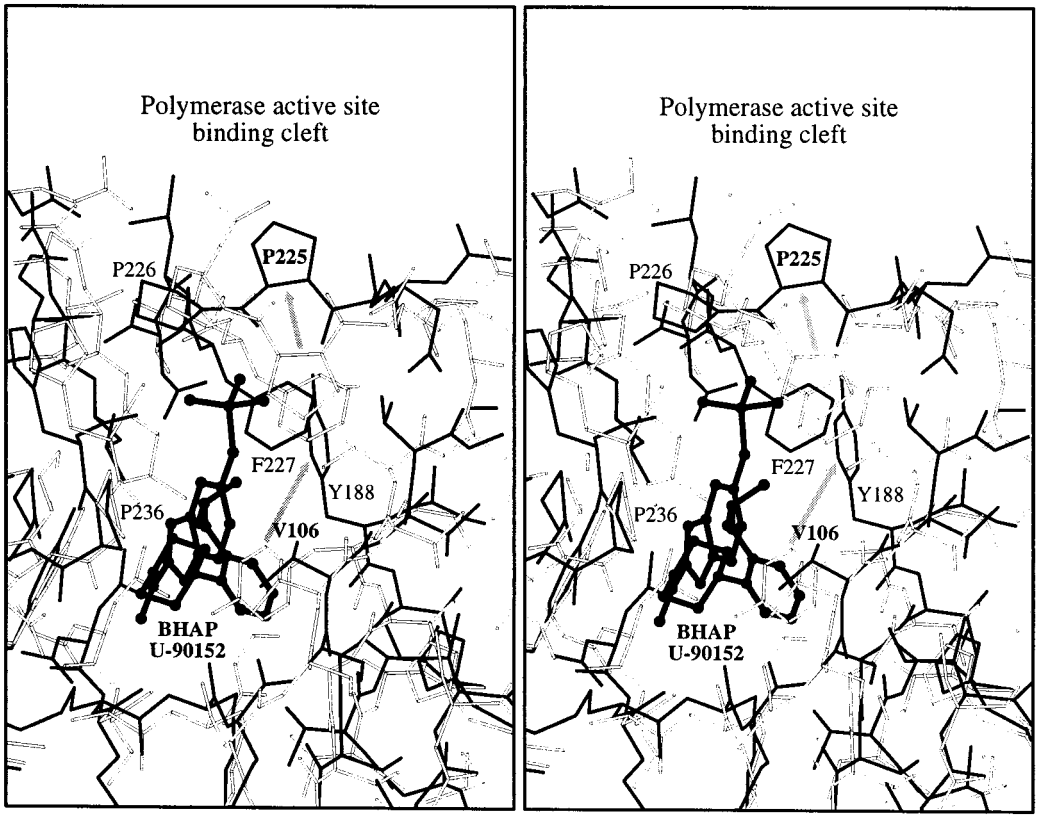

FIG. 3. Stereo diagram showing the interaction between BHAP U-90152 and Pro225 in the RT-BHAP complex (9). BHAP U-90152 is shown as a black ball-and-stick representation, with the surrounding protein structure shown by thin black sticks. The conformation of the protein in the unliganded RT structure (8) is shown by the thin white sticks superimposed onto the model for the complex. Residues Tyr188 and Pro225 (and also Tyr181 [not shown]) are completely repositioned when BHAP U-90152 binds, and this movement is indicated by gray arrows. The figure was drawn by using a modified version of MolScript (10, 19).

already very flexible. Our observations and those of Dueweke et al. (7) that NNRTI-characteristic mutations may confer resistance against certain NNRTIs but produce an increased sensitivity to other members of this family of compounds stress the fact that NNRTI-characteristic mutations do not necessarily afford cross-resistance to all other NNRTIs. Therefore, genotypic identification of such amino acid mutations and/or combinations thereof in plasma samples from NNRTI-treated HIV-1 infected individuals may become increasingly important to allow optimized therapy of such HIV-1 RT mutation-bearing individuals.

The consistent emergence of the Pro225His mutation subsequent to the Val106Ala mutation may also have a structural explanation, since the Nε2 atom of the mutated amino acid residue, Pro225His, could potentially form a hydrogen bond with the main-chain oxygen of amino acid residue Val106 and may compensate for a structural change induced by the first mutation.

Of the NNRTIs tested, RT bearing the single mutation Pro225His is most resistant to MKC-442 (a member of the HEPT series). A comparative structural study of the binding of HEPT analogs by RT (8) showed that these inhibitors bind in such a manner that their 1-position substituents approach the mouth of the NNRTI-binding pocket (i.e., Pro225). Of the three analogs studied by Hopkins et al. (13), MKC-442 had the smallest 1-position substituent, and thus it might be expected that the activities of the other compounds (i.e., HEPT itself and the HEPT derivative TNK-651) might be more seriously compromised by the Pro225His mutation. Our findings that the kinetics of inhibition of the Pro225His mutant RT by BHAP U-90152, HBY 097, and MKC-442 are essentially identical to that of inhibition of the wild-type enzyme (i.e., noncompetitive inhibition with respect to both substrate and template-primer) are in agreement with the structural model discussed above and indicate that the mutation does not affect the substrate-binding site and template-binding site in the HIV-1 RT.

To assess the individual contributions of the Val106Ala and Pro225His mutations to inhibitor binding and enzymatic activity, we constructed the single Val106Ala, the single Pro225His, and the double Val106Ala Pro225His RT mutants. The data obtained were in full agreement with the antiviral data and showed a clear additive effect of the individual mutations on resistance to the different NNRTIs. In fact, strong correlations were found between the EC_{50} and the IC_{50} values for the NNRTIs against the recombinant mutant enzymes. The correlation coefficients (r) were 0.89, 0.94, and 0.85 for the EC_{50} and IC_{50} values between wild-type HIV-1 and recombinant wild-type HIV-1 RT, Val106Ala mutant HIV-1 and recombinant single-mutant Val106Ala RT, and Val106Ala Pro225His mutant HIV-1 and recombinant Val106Ala Pro225His double-mutant RT, respectively (Fig. 4). Site-directed mutagenesis experiments have also unambigously demonstrated that the Pro225His mutation is responsible for the markedly increased sensitivity of the Val106Ala Pro225His mutant HIV-1 to BHAP U-90152, and these findings are in full agreement with the crystal structure of the RT-BHAP U-90152 complex, which has recently been solved (9). NNRTI-induced sensitization of mutant RTs has so far been observed only for the Pro236Leu mutation, which may appear during BHAP treatment and results in a pronounced resistance to BHAP but sensitization to other NNRTIs such as nevirapine, 9-chloro-TIBO, and pyridinone (7). Thus, the Pro225His mutation represents the second example of an amino acid mutation in HIV-1 RT that appears under selective pressure of one specific NNRTI (i.e., quinoxaline) but which sensitizes the mutant enzyme to inhibition by another NNRTI (i.e., BHAP).

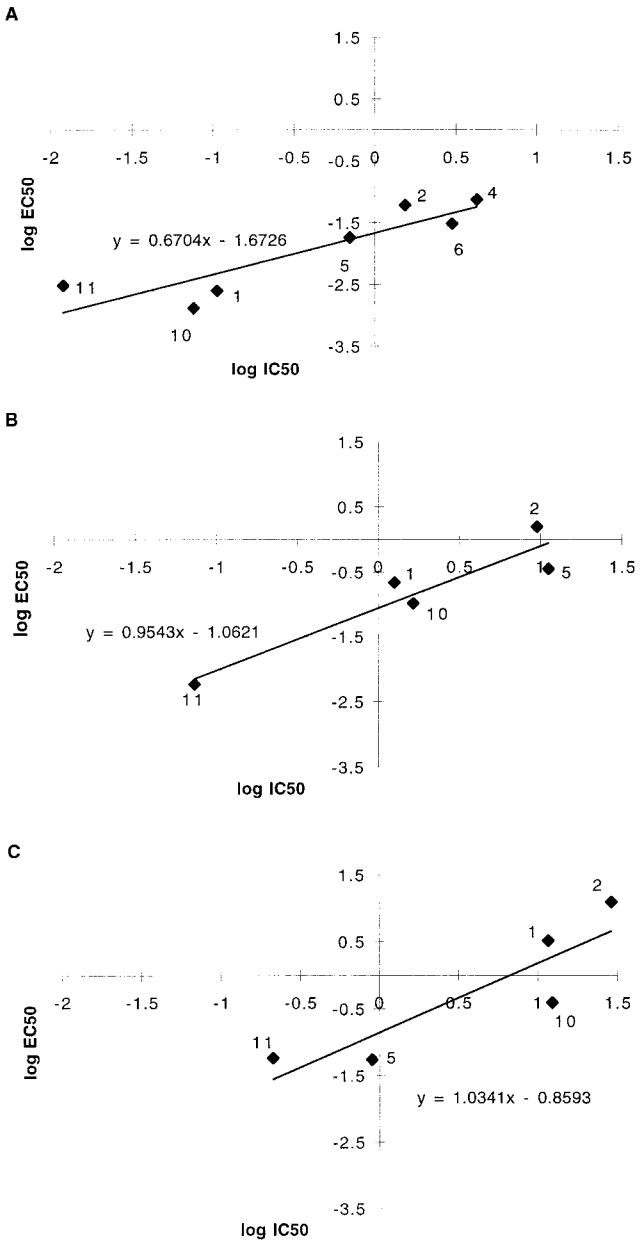

FIG. 4. Regression analysis of antiviral (EC_{50}) and anti-RT (IC_{50}) values obtained for wild-type virus and recombinant wild-type RT (A), Val106Ala mutant virus and recombinant Val106Ala RT (B), and Val106Ala Pro225His double-mutant virus and recombinant Val106Ala Pro225His double-mutant RT (C). Compounds: 1, MKC-442; 2, 9-chloro-TIBO; 3, 8-chloro-TIBO; 4, nevirapine; 5, BHAP U-90152; 6, TSAO-m^3T; 7, α-APA; 8, UC-10; 9, UC-781; 10, S-2720; 11, HBY 097.

In conclusion, we showed that an ordered appearance of NNRTI-specific mutations occurs under the pressure of dose-escalating treatment of HIV-1 with quinoxaline. A novel Pro225His mutation consistently appeared after the Val106Ala mutation. We demonstrated that the Pro225His mutation affords a small, but additive, increase of resistance of RT to NNRTIs but increases the sensitivity of the mutant RT and HIV-1 strains to BHAP U-90152. The increased sensitivity of

the Pro225His RT mutant to BHAP could be explained based on the RT-BHAP complex coordinates that have recently emerged from X-ray crystallographic studies.

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