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Biochemistry

Selective pressure of a quinoxaline nonnucleoside inhibitor of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) on HIV-1 replication results in the emergence of nucleoside RT-inhibitor-specific (RT Leu-74 → Val or Ile and Val-75 → Leu or Ile) HIV-1 mutants

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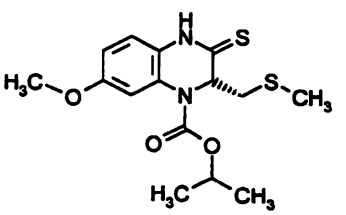
ABSTRACT **The quinoxaline nonnucleoside RT inhibitor (NNRTI) (*S*)-4-isopropoxycarbonyl-6-methoxy-3-(methylthiomethyl)-3,4-dihydroquinoxaline-2(1*H*)-thione (HBY 097) was used to select for drug-resistant HIV-1 variants *in vitro*. The viruses first developed mutations affecting the NNRTI-binding pocket, and five of six strains displayed the RT G190→E substitution, which is characteristic for HIV-1 resistance against quinoxalines. In one variant, a new mutant (G190→Q) most likely evolved from preexisting G190→E mutants. The negative charge introduced by the G190→E substitution was maintained at that site of the pocket by simultaneous selection for V179→D together with G190→Q. After continued exposure to the drug, mutations at positions so far known to be specific for resistance against nucleoside RT inhibitors (NRTIs) (L74→V/I and V75→L/I) were consistently detected in all cultures. The inhibitory activities of the cellular conversion product of 2′,3′-dideoxyinosine (ddI, didanosine), 2′,3′-dideoxyadenosine (ddA) and of 2′,3′-didehydro-3′-deoxythymidine (d4T, stavudine) against these late-passage viruses were shown to be enhanced with the L74→V/I RT mutant virus as compared with the wild-type (wt) HIV-1_{MN} isolate. Clonal analysis proved linkage of the codon 74 and codon 75 mutations to the NNRTI-specific mutations in all RT gene fragments. The nonnucleoside- and nucleoside-resistance mutation sites are separated by approximately 35 Å. We propose that the two sites "communicate" through the template-primer which is situated in the DNA-binding cleft between these two sites. Quinoxalines cause high selective pressure on HIV-1 replication *in vitro*; however, the implication of these findings for the treatment of HIV-1 infection has yet to be determined.**

Viral resistance against both nucleoside reverse transcriptase (RT) inhibitors (NRTIs) and nonnucleoside RT inhibitors (NNRTIs) of human immunodeficiency virus type 1 (HIV-1) replication develops *in vitro* (cell culture) and *in vivo* (patients). NRTIs select for RT amino acid substitutions at positions that can be allocated to different structural elements of the protein in the range of residue numbers 41–219 (1–5). A molecular mechanism for some alterations can be assumed on the basis of the RT crystal structure (6–8). In contrast, NNRTI-specific substitutions map exclusively to those protein secondary structure elements that together form a lipophilic pocket within the palm domain of the p66 RT subunit. All NNRTIs are believed to bind to this region, and mutations selected for by these drugs usually occur in the segments composed of RT amino acids 98–108, 179–190, and 230–236 (9–16). Especially the Y181→C RT mutant appears with many chemically distinct compounds, including different NNRTI combinations (12).

6-Chloro-3,3-dimethyl-4-(isopropenyloxycarbonyl)-3,4-dihydroquinoxaline-2(1*H*)-thione (S-2720) and other quinoxalines select for a characteristic G190→E RT mutant when drug-resistant viruses are generated in cell culture (17, 18). However, the interesting feature of the latter mutant is a clearly decreased enzymatic activity of the resulting recombinant G190→E enzyme *in vitro* (17, 19, 24).

We report here the results of *in vitro* dose-escalation experiments with the clinical candidate (*S*)-4-isopropoxycarbonyl-6-methoxy-3-methylthiomethyl-3,4-dihydroquinoxaline-2(1*H*)-thione (HBY 097):



HBY 097

(*i*) In all virus strains, amino acid 190 of the RT could be shown to be varied after several passages, and in five of six virus populations the G190→E RT mutant developed. (*ii*) In one strain, a G190→Q variation evolved together with a V179→D substitution. (*iii*) When the resistant viruses were further subcultivated, alterations in RT codon 74 (L74→V/I) and codon 75 (V75→L/I) consistently appeared in all lineages.

The nonnucleoside inhibitor binding pocket is close to the DNA-binding cleft and contains structural elements in common with the "primer grip" (7, 20, 21). Mutations at position 190 could distort the conformation of the primer grip or other nearby DNA-binding elements, and the NRTI-specific mutations (L74→V/I and V75→L/I) possibly compensate for the changes across the DNA-binding cleft.

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Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; NNRTIs, nonnucleoside inhibitors of the HIV-1 RT; HBY 097, (*S*)-4-isopropoxycarbonyl-6-methoxy-3-(methylthiomethyl)-3,4-dihydroquinoxaline-2(1*H*)-thione; NRTIs, nucleoside RT inhibitors; ddI, didanosine, 2′,3′-dideoxyinosine; ddA, 2′,3′-dideoxyadenosine; d4T, stavudine, 2′,3′-didehydro-3′-deoxythymidine; AZT, zidovudine, 3′-azido-3′-deoxythymidine; wt, wild type.

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MATERIALS AND METHODS

Compounds. HBY 097 and S-2720 were synthesized as described (17, 22). 3′-Azido-3′-deoxythymidine (zidovudine, AZT) was obtained from Burroughs Wellcome. 2′,3′-Dideoxyadenosine (ddA), 2′,3′-didehydro-3′-deoxythymidine (stavudine, d4T), 6,11-dihydro-11-cyclopropyl-4-methyldipyrido-(1,4)-diazepin-6-one (nevirapine), 3-(4,7-dichloro-1,3-benzoxazol-2-yl-methylamino)-5-ethyl-6-methylpyridin-2(1*H*)-one (L-697661), and 1-(5-methanesulfonamido-1*H*-indol-2-yl-carbonyl)-4-[3-(1-methylethylamino)pyridinyl]piperazine (U-90152) were synthesized in house according to published methods.

Cells and Viruses. HIV-1_{MN} was propagated on H9 cells. These reagents were obtained through the AIDS Research and Reference Reagent program of the National Institute of Allergy and Infectious Diseases, Bethesda, Md. For determination of 50% inhibitory concentrations (IC_{50}s) of test compounds, 1×10^6 cells were infected for 30 min with HIV-1_{MN} at 2–4 $\times$ 10^5 tissue culture infective units (TCIU). The supernatant was removed and the infected cells were transferred to 24-well dishes (2.5×10^5 cells per ml); drugs in serial dilutions were then added immediately. Upon 72 hr of incubation, virus replication was evaluated by measurement of the p24 antigen concentration in the supernatants by an antigen capture assay (Innotest).

Selection of mutant HIV-1 strains resistant to inhibition by HBY 097 was done as described (17). A dose-escalation protocol was used, starting with HBY 097 at 1 ng/ml at passage 1.

Population-Based Sequence Determination of the HIV-1 RT Gene. Purified total cellular DNA from infected cell lysates was eluted in 10 mM Tris·HCl/1 mM EDTA, pH 8.0. A 0.5-μg sample of DNA was used for a first PCR with primers JA99 and RIT137 (23). Reaction conditions were 3 min at 94°C, 35 to 50 cycles with 30 sec at 96°C, 30 sec at 60°C, and 60 sec at 72°C

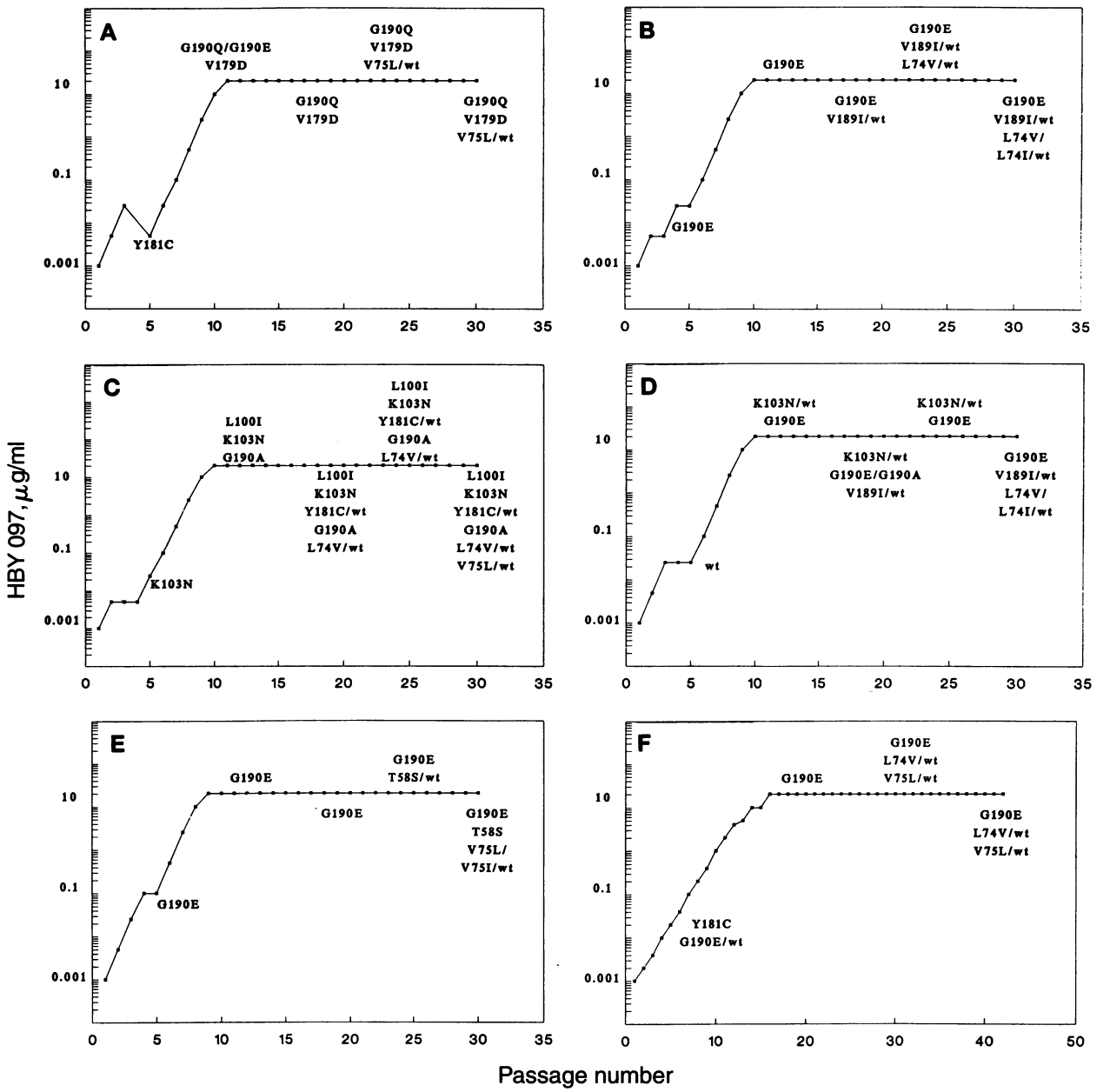

FIG. 1. Evolution of HIV-1_{MN} RT genes during selection for resistance against HBY 097. H9 cell cultures infected with HIV-1_{MN} contained increasing amounts of HBY 097 in the culture medium. Sequence analysis was performed at passages 5, 12, 20, 25, and 30 (cultures *A–E*) or at passages 5, 18, 31, and 42 (culture *F*). Amino acid alterations deduced from the DNA sequences are given. Slashes identify mixtures of different amino acids; wt, wild type.

in 0.2 mM each dNTP/10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$.

An aliquot of the first PCR was used for a nested amplification with primers JA100 (23) and RIT138 (5′-biotinyl-CTGTCTTTTTCTGGTAGCACTATAGG), using the same protocol as above. The biotinylated DNA strand of the 817-bp product was obtained by using streptavidin-coated magnetic beads (Dynal) to serve as a template for the sequencing reaction. Sequencing primers RT1SEQ2F (5′-fluorescein-CAATGGCCATTGACAGAAG) and RT8KF (5′-fluorescein-CTGCATTTACCATACCTAG) allowed the determination of HIV-1 provirus sequences corresponding to RT amino acids 35–242. Analysis of the reactions was performed with an A.L.F. automated DNA sequencer (Pharmacia).

Expression of Recombinant HIV-1 RTs and *in Vitro* RT Assays. RT gene inserts 1.7 kbp long were transferred to the expression vector pMalcRI (New England Biolabs), using *Bam*HI/*Xba* I recognition sequences within the RT1/RT2B PCR primer sequences (17). Recombinant HIV-1 RT fused C-terminally to the *Escherichia coli* maltose-binding protein (MBP) was obtained and purified upon induction of the Ptac promoter as described (17, 19). RT preparations were up to 90–95% pure, as judged by examination of stained gels.

For determination of enzyme IC_{50} values, dilutions of compounds in dimethyl sulfoxide were incubated with scintillation proximity (SPA) reagents (Amersham). RNA-dependent DNA synthesis with a heteropolymeric primer/template (5′-GTCATAGCTGTTTCCTG-3′/5′-UCUGCGGCAUUGCGAGCGGAUAACAAUUUCACACAGGAAACAGCUAUGAC-3′) was allowed to occur for 20 min at 37°C in the presence of [^{3}H]dTTP. Enzymes were diluted in 20 mM Tris·HCl, pH 7.2, and inhibitor solutions were tested in duplicate.

RESULTS

Analysis of the HIV-1 RT Gene During *in Vitro* Selection for Resistance of HIV-1_{MN} Against HBY 097. H9 cells were infected with HIV-1_{MN} and cultured in the presence of HBY 097. NNRTI-specific mutations were detected in all but one culture at passage 5 (Fig. 1), and the G190→E RT was already predominant in strains B and E. At passages 12 and 18 the Y181→C mutants had disappeared from cultures A and F, respectively, and five of six cultures displayed G190→E RTs. In addition, lineage A was a mixture of G190→Q/G190→E mutants, accompanied by a V179→D substitution, leaving V179→D/G190→Q RT changes at passage 20. In culture C, an accumulation of NNRTI-specific mutations was observed, and in cultures B and D a V189→I (GTA → ATA) change appeared, which was sustained to passage 30.

Evolutionary changes continued in the RT gene during later passages, affecting the fingers domain of the RT molecule. Surprisingly, mutations in codons 74 and 75 (L74→V/I, V75→L/I) were detected in all cultures.

Determination of Mutations in Cloned Proviral RT Genes. At first, we were interested in the passage 12 viruses of culture A. There was a mixture of two mutations in RT codon 190, together with an alteration in position 179 (Fig. 1). The majority (13/18) of the clones carried a V179→D/G190→Q combination, and 0 of 18 RT genes harbored V179→D along with G190→E. Likewise, there were no (0/18) V179→D single mutants and only 2 of 18 clones encoded the G190→Q RT in the absence of V179→D.

In the late-passage viruses of cultures A, D, E, and F L74→V, L74→I, V75→L or V75→I changes were always linked to a codon 190 mutation (26/26 clones). Although there were mixtures of mutations observed in both codon 74 and 75 in cultures B, C, D, and F (Fig. 1), none of the clones sequenced displayed a codon 74 mutation together with a codon 75 variation (0/26 clones).

Inhibitor Sensitivities and Polymerase Activities of Recombinant HIV-1 RTs. All NNRTIs tested were shown to be inactive against G190→E or G190→Q substituted RTs (Table 1). The V179→D-containing double mutants exhibited a very low enzymatic activity, whereas the V179→D single mutant was found to be as active as the wt RT. Interestingly, the V179→D alteration alone only resulted in a 3-fold loss of inhibitor potency of HBY 097. The V75→I/G190→E RT displayed a substantially enhanced polymerase activity, as compared with the G190→E single mutant.

RT Inhibitor IC_{50} Values with Late-Passage Viruses. The virus strains which specified mainly V75→I and V75→L RT mutants (cultures E and F) showed no marked difference in their susceptibilities to d4T and ddA, whereas the presence of G190→E in these variants obviously caused resistance to HBY 097. However, there was an unambiguous loss of sensitivity to ddA and d4T with the virus isolated from culture D, showing the L74→V and L74→I RT substitutions (Table 2). AZT inhibited all viruses at IC_{50} values below 0.037 μM.

DISCUSSION

Among the different chemical classes of NNRTIs, certain quinoxaline derivatives exhibit the novel feature of selecting for resistant viruses (RT G190→E) *in vitro* which possess a crippled RT polymerase function (17, 18, 22). The G190→E RT was responsible for viral resistance in five of six HBY 097-resistant virus strains investigated in this study. This substitution occurred early during the experiments, sometimes replacing alterations such as Y181→C, which may help the virus to escape under low drug pressure conditions. The fact that G190→E RT mutants are not selected in the very early course of an experiment provides evidence for the detrimental effects of a weakly active RT with respect to HIV-1 replication. A G190→E mutant RT obtained by large-scale mutagenesis has recently been shown to exhibit similar *in vitro* properties, displaying a clearly decreased RNA-dependent DNA poly-

Table 1. *In vitro* analysis of recombinant RTs resistant to inhibition by NNRTIs

Enzyme (RT amino acid change)*	Inhibitor IC_{50},† μM				Enzymatic activity‡
	HBY 097	S-2720	Nevirapine	U-90152	
RT-1 MN (wt)	0.08	0.06	3.48	1.56	1.00
RT-1 ED2 (V179→D)	0.23 (3×)	0.72 (12×)	61.21 (18×)	10.51 (7×)	1.10
RT-1 ED21 (V179→D/G190→E)	>29.37 (>367×)	>32.18 (>536×)	>37.55 (>11×)	>21.90 (>14×)	<0.1
RT-1 QD (V179→D/G190→Q)	>29.37 (>367×)	>32.18 (>536×)	ND	ND	<0.1
RT-1 E (G190→E)	>29.37 (>367×)	>32.18 (>536×)	ND	ND	<0.1
RT-1 E75I (V75→I/G190→E)	>29.37 (>367×)	>32.18 (>536×)	ND	ND	0.57
RT-1 I4 (V189→I)	0.17 (2×)	0.11 (2×)	19.53 (6×)	1.83 (1×)	0.50

ND, not done.
*RT-1 ED2, RT-1 ED21, RT-1 E, RT-1 E75I, and RT-1 I4 were prepared by using site-directed mutagenesis (17, 19).
†Fold concentration compared with RT-1 MN is given in parentheses.
‡Relative to the RT-1 MN wt enzyme.

Table 2. RT inhibitor activities against replication of late-passage viruses from cultures D, E, and F

Virus*	Compound IC_{50},† μM		
	HBY 097	ddA	d4T
$HIV\text{-}1_{MN}$	0.006 ± 0.001	1.19 ± 0.13	5.84 ± 0.25
$HIV\text{-}1_{MN}$ D-30	>50 (>8333×)	2.89 ± 0.25 (2.4×)	21 ± 0.20 (3.6×)
$HIV\text{-}1_{MN}$ E-30	>50 (>8333×)	1.15 ± 0.06 (1×)	5.40 ± 0.35 (0.9×)
$HIV\text{-}1_{MN}$ F-42	>50 (>8333×)	0.94 ± 0.08 (0.8×)	4.64 ± 0.18 (0.8×)

*Designation of mutant viruses refers to the culture and passage number, respectively.
†Results are presented as ranges, and fold concentrations compared with $HIV\text{-}1_{MN}$ are given in parentheses.

merase activity (24). In the same study, protein folding of the recombinant enzyme was shown to be impaired through the G190→E substitution.

One virus strain developed an accumulation of NNRTI-specific mutations, giving rise to recombinant RTs which did not yield soluble protein upon expression in *E. coli* (L100→I/K103→N/Y181→C/G190→A). Since the same was observed with a L100→I/K103→N mutant RT, we conclude that this combination of mutations may result in a phenotype similar to that seen with the G190→E RTs. Consistent with our data, Boyer *et al.* (25) recently described a close relationship of low solubility and decreased enzymatic activity of certain mutated HIV-1 RTs.

A V179→D/G190→Q [supposed mutations GGA (Gly) → GAA (Glu) → CAA (Gln)] mutant virus strain was observed in only one of six cultures. In this case an escape mechanism from the weakly active G190→E RT was expected, since a G190→Q single-mutant RT was previously shown both to be resistant to inhibition by quinoxalines and to display wt polymerase activity (19). Interestingly, the V179→D/G190→Q recombinant RT also displayed a clearly decreased polymerase activity (Table 1), and the G190→Q single mutants did not take over in lineage A or in one of the other cultures. These findings argue in favor of an important role for the appearance of V179→D and G190→Q at the same time.

The V179 and G190 residues are located in the β9–β10 hairpin, which forms part of the NNRTI-binding pocket (20, 21) (Fig. 2). V179→D and G190→E/Q RT mutants would result in changes of local electrostatic potential and would most likely lead to steric hindrance in binding NNRTIs. The mutations at both of these positions result in increased steric bulk and reduction of pocket size, possibly explaining the observation that the G190→E/Q RT mutants confer high-level resistance to a variety of NNRTIs (this study and ref. 19).

The most surprising result that comes from this study is the consistent occurrence of NRTI-specific mutations in NNRTI-treated virus strains (RT L74→V/I and RT V75→L/I). These additional changes always developed after changes in position 190 were already present in the RT. We did not detect any of the NRTI-specific amino acid changes when we propagated $HIV\text{-}1_{MN}$ on H9 cells in the presence of the pyridinone class NNRTI L-697661 for a total of 30 passages (data not shown).

The L74 and L75 residues are located in the β4 strand, which is a component of the fingers subdomain. These residues in the p66 subunit are about 35 Å distant from residues V179 and G190 in the NNRTI-binding pocket, with the template-primer located in between (Fig. 2). Since a mutation in the RT gene leads to alterations in both the p66 and p51 subunits, it is possible that the substitutions at amino acid positions 74 and 75 in p51 could be important. However, these positions in p51 are at a large distance from the polymerase active site in the RT heterodimer and the changes in p51 are less likely to be involved. Observation of NRTI-specific mutations in the presence of NNRTI-treated virus strains suggests that potential distortion of the conformation of the primer grip or the β9–β10 hairpin by the NNRTI mutations may be compensated by NRTI-specific mutations so as to accommodate the template-primer. Previous studies (8) demonstrated that mutations that confer resistance to dideoxynucleoside analogues, including L74→V, apparently lead to changes at the active site through changing the conformation or position of the template-primer. Analyses of foscarnet-resistant HIV-1 RT also suggest that mutations across the DNA-binding cleft may lead to alterations in binding of foscarnet at the polymerase active site, again "communicating" from the site of mutation through the template-primer (26). We therefore propose a connection between quinoxaline-specific mutations that impair polymerase activity and the NRTI mutations that occur in the fingers subdomain. As a result of the NNRTI-induced interactions between the lipophilic pocket and consequent distortion of the primer grip, NRTI mutations may partly compensate for altered interactions of HIV-1 RT with template-primer.

A 2.4-fold increase in IC_{50} was seen with the 2′,3′-dideoxyinosine (didanosine, ddI) conversion product ddA and the L74→V-containing multiple RT mutant virus that was obtained with HBY 097 (Table 2). The fact that only one-third (4/13 clones) of this provirus population in culture D specified L74→V RTs may account for the difference from the 8.3-fold increase in IC_{50} observed with ddI and a L74→V RT virus construct (2). An influence of the G190→E substitution with respect to the ddA/ddI-resistant phenotype exerted by the L74→V alteration could also be assumed.

V75→I-containing multiple mutant RTs were recently found to be associated with drug-resistance in a few HIV-1 infected patients who underwent NRTI combination therapy

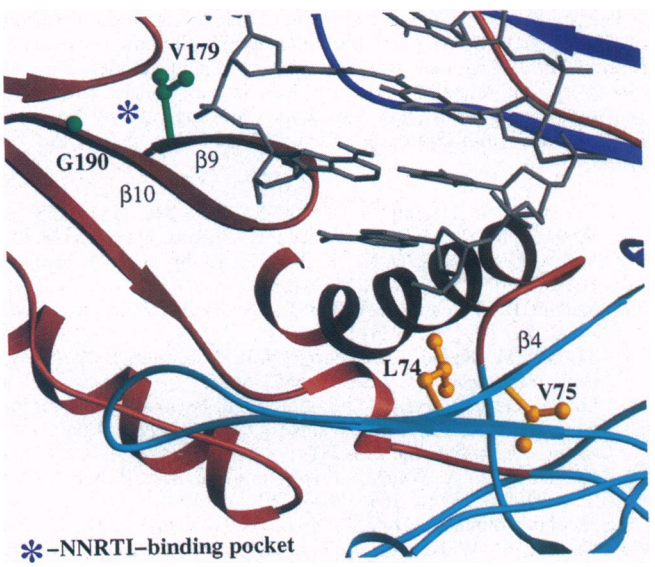

FIG. 2. Structure of the HIV-1 RT/DNA complex (7) showing the relationship between the NNRTI-binding pocket and the NRTI-specific mutation sites. The p66 palm subdomain is indicated in red and the p66 fingers subdomain is shown in blue. Purple represents the p51 fingers subdomain. Double-stranded DNA is shown in grey. The NNRTI mutation sites (V179, G190) are shown in green and the NRTI mutation sites (L74, V75) are shown in gold.

with AZT and ddI or AZT and 2',3'-dideoxycytidine (ddC) (27, 28). However, virus constructs with a V75→I RT single mutation were shown to be highly sensitive to inhibition by AZT, ddI, and other NRTIs (A. K. N. Iversen, R. W. Shafer, K. Wehrly, M. A. Winters, J. I. Mullins, B. Chesebro, and T. C. Merigan, personal communication). Likewise, the quinoxaline-resistant viruses, which contained V75→I or V75→L RT changes in combination with NNRTI-specific substitutions, did not display diminished sensitivity to any of the tested dideoxynucleosides. Neither V75→I alone nor multiple RT mutants containing this change have been reported to be selected for by ddC, ddI, or AZT alone. Therefore, the occurrence of V75→I RT mutants might indicate a higher selective pressure exerted either by combinations of certain NRTIs or by a quinoxaline class NNRTI as compared with NRTI monotherapy.

The most likely explanation for the selective advantage of the mutations in codon 74 and codon 75 is an enhanced polymerase activity of the resulting enzymes, as observed with the V75→I/G190→E RT (Table 1). At this point it could be speculated that, i.e., the nature of the enzyme fusion used could affect the activity of certain recombinant RTs relative to wt. However, all of our numerous control mutants constructed and checked for polymerase activity behaved as expected from published data (Table 1 and refs. 17 and 19).

The fact that both the L74→V/I and V75→L/I changes always appeared after introduction of quinoxaline resistance serves as additional proof of a direct connection of these consecutive events, probably through a molecular mechanism as discussed above.

The data presented here provide a link between HIV-1 resistance against two types of compounds, which inhibit the HIV-1 RT and virus replication through different modes. Therefore, these findings seem at first sight to add just another factor of complexity to the field of resistance to RT inhibitors. However, it also becomes evident that the possibilities for HIV-1 to escape from the effects of carefully selected drugs may be limited. Combination chemotherapy on the level of RT inhibition could possibly make use of the fact that parallels exist in the mutational pathways followed by HIV-1 to become less sensitive to certain nucleoside analogues or quinoxalines.

This study is dedicated to Karl Eduard Schneweis on the occasion of his 70th birthday. We thank Barbara Bremora, Stefanie Dexheimer, Martina Hardt, Elke Lais, Antje Momberger, Claudia Röder, Gertrud Sibenhorn, and Claudia Ketter for technical assistance, and Nicol Reifsteck for editorial help. This work was supported by National Institutes of Health Grants AI 27690 and AI 36144 to E.A. and by Hoechst AG.

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