VIROLOGY 231, 112–118 (1997)
ARTICLE NO. VY978513

In Vitro Selection for Different Mutational Patterns in the HIV-1 Reverse Transcriptase

View metadata, citation and similar papers at core.ac.uk
brought
provided by Elsevier

Reverse Transcriptase Inhibitor HBY 097

Jörg-Peter Kleim,*[,1] Irvin Winkler,* Manfred Rösner,* Reinhard Kirsch,* Helga Rübsamen-Waigmann,† Arno Paessens,† and Günther Riess*

*Hoechst AG, Central Pharma Research, D-65926 Frankfurt; and †Bayer AG, Institute of Virology, D-42096 Wuppertal, Germany

Received December 10, 1996; returned to author for revision January 3, 1997; accepted February 24, 1997

In vitro resistance of HIV-1 against high levels of HBY 097 ((*S*)-4-isopropoxycarbonyl-6-methoxy-3-(methylthiomethyl)-3,4-dihydro-quinoxaline-2(1*H*)-thione) and other quinoxaline nonnucleoside reverse transcriptase inhibitors (NNRTIs) is characterized by a specific amino acid substitution in the reverse transcriptase (RT), Gly190Glu. This change results in decreased RT polymerase activity and in reduced growth properties of the corresponding viral variant. Here we show that the appearance of the crippling mutation at codon 190 can be prevented by lowering the selective pressure exerted by HBY 097. Under low selective pressure an accumulation of other NNRTI-specific mutations is observed. Up to five NNRTI-specific substitutions were detected in some of these virus lineages. In addition, we report novel RT amino acid changes which were not observed previously, including Val106Ile, Val106Leu, and Gly190Thr. HBY 097 selects for different mutational patterns under high and low selective pressure conditions, respectively. Thus, the type of mutations which appear in HIV-infected patients undergoing therapy may be determined by the levels of the selecting drug. © 1997 Academic Press

INTRODUCTION

HIV-1 resistance against nonnucleoside reverse transcriptase inhibitors (NNRTIs) is mediated through mutations affecting the hydrophobic NNRTI binding pocket within the polymerase domain of the p66 subunit (Tantillo *et al.,* 1994; De Clercq, 1995; Schinazi *et al.,* 1996). Though nucleoside RT inhibitors (NRTIs) have different molecular target sites at the same enzyme, the resistance profiles of these two classes of drugs have been shown to interact. For example, it has been shown that the type of *pol* gene mutations which appear with NNRTIs is influenced by preexisting NRTI resistance mutations: (+)-*S*-4,5,6,7-tetrahydro-9-chloro-5-methyl-6-(3-methyl-2-butenyl)imidazo [4,5,1-jk][1,4]-benzodiazepin-2(1*H*)-thione (TIBO R82913) and nevirapine select mainly for the Tyr181Cys substitution both *in vitro* and *in vivo.* In contrast, the presence of a zidovudine-resistant background causes the frequent appearance of Lys103Asn, Val106Ala, Gly190Ala, and other substitutions affecting the NNRTI binding pocket of the RT when the same drugs are used (Richman *et al.,* 1991, 1994; Larder, 1992).

Little is known about the influence of other parameters related to the selective pressure of a certain drug (or drug combination) on the qualitative generation of viral resistance. We describe here the results of comparative *in vitro* selection experiments with the quinoxaline NNRTI HBY 097 (Kleim *et al.,* 1995; Fig. 1), applying defined different experimental protocols to create resistant mutants. Statistically significant evidence is presented proving the concept that lowering the selective pressure of HBY 097 results in different viral genotypes compared to those found under high selective pressure conditions.

Our results may help to explain the appearance of different resistant mutants detected with HBY 097 or other inhibitors under *in vivo* conditions.

MATERIALS AND METHODS

Inhibitors of the HIV reverse transcriptase

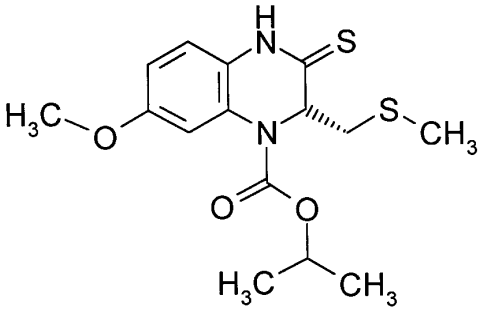
HBY 097 ((*S*)-4-isopropoxycarbonyl-6-methoxy-3-(methylthiomethyl)-3,4-dihydro-quinoxaline-2(1*H*)-thione) and S-2720 (6-chloro-3,3-dimethyl-4-(isopropenyloxycarbonyl)-3,4-dihydroquinoxaline-2(1*H*)-thione) were synthesized as described (Kleim *et al.,* 1993, 1995). Zidovudine (3′-deoxy-3′-azidothymidine; AZT) was obtained from Burroughs Wellcome (Research Triangle Park, NC). Delavirdine (1-(5-methanesulfonamido-1*H*-indol-2-yl-carbonyl)-4-(3-1-methylethyl-aminopyridinyl)-piperazine; U-90152) and nevirapine (6,11-dihydro-11-cyclopropyl-4-methyldipyrido-(1,4)diazepin-6-one; BI-RG-587) were prepared according to published methods (Hargrave *et al.,* 1991; Romero *et al.,* 1993).

Selection conditions for the generation of HBY 097-resistant HIV-1 variants

The HIV-1_{MN} laboratory strain was used and viruses were propagated on H9 cells as described (Kleim *et al.,*

[1] To whom reprint requests should be addressed.

0042-6822/97 $25.00
Copyright © 1997 by Academic Press
All rights of reproduction in any form reserved.



HBY 097

FIG. 1. HBY 097 ((*S*)-4-isopropoxycarbonyl-6-methoxy-3-(methylthiomethyl)-3,4-dihydro-quinoxaline-2(1*H*)-thione).

1995). These reagents were obtained through the AIDS Research and Reference Reagent Program, AIDS Program, National Institute of Allergy and Infectious Diseases, Bethesda, MD. Virus lineages were divided in two categories: group A mutants (high selective pressure; 7 individual lineages) were obtained by raising the HBY 097 concentration by a factor of 4–5 with every subcultivation step. The amount of drug present in the passage 1 culture medium was 0.5 ng/ml, and the last cultures analyzed contained 10,000 ng/ml of HBY 097. Group B mutants (low selective pressure; 12 different lineages) were treated in the same way, except that the concentration of drug was raised only 2- to 2.5-fold with every subsequent passage. Within group B the initial and final compound concentrations were 0.2, 0.4, or 0.5 ng/ml and 10,000 or 20,000 ng/ml, respectively.

Genotypic and phenotypic analyses of HIV-1_{MN} group A versus group B variants

The appearance of RT gene mutations was monitored at different time points for all virus lineages from group A and group B: Total DNA from infected H9 cells was purified using a silica-membrane-based spin column system (Qiagen). DNA was eluted in 10 m*M* Tris–HCl, 1 m*M* EDTA, pH 8.0. For a first PCR analysis 0.5 μg of DNA was used with 10 pmol/50 μl of primers JA99 and RIT137 (Albert *et al.,* 1994), respectively. Reaction conditions were 3 min, 94°; 35 to 50 cycles for 30 sec, 96°; 30 sec, 60°; and 60 sec, 72° in 0.2 m*M* each dNTP, 10 m*M* Tris–HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, and 2 units/50 μl of Taq polymerase (Boehringer Mannheim).

An aliquot of the first PCR was used for a nested amplification with primers JA100 and RIT138 (Albert *et al.,* 1994; Kleim *et al.,* 1995) using the same protocol as above, omitting the initial denaturation step. The biotinylated DNA strand of the 817-bp product was first separated from the unlabeled complementary strand using streptavidin-coated magnetic beads (Dynal) and the single-stranded DNA was then served as a template for the dideoxy chain termination sequencing reaction. Primers RT1SEQ2F and RT8KF (Kleim *et al.,* 1995) allowed the determination of HIV-1 provirus sequences corresponding to RT amino acids 35–242. Analysis of the reactions was performed with the aid of an ALF automated DNA sequencer (Pharmacia).

Phenotypic resistance of late passage viruses from individual lineages against HBY 097 and four other RT inhibitors was done by evaluation of the 50% inhibitory concentrations (IC_{50}s): 1×10^6 cells were infected for 30 min with HIV-1_{MN} or one of the described group A/B variants at $2–4 \times 10^5$ tissue culture infective units (TCIU). Supernatants were then removed and infected cells were transferred to 24-well plates (Greiner; 2.5×10^5 cells/ml/well). Drugs in serial fivefold dilutions were added immediately, followed by 72 hr of incubation at 37° in a 5% CO_2 humid atmosphere. For the calculation of IC_{50} values, the p24 antigen concentrations in the culture supernatants were measured by an antigen capture assay (Organon Teknika).

RESULTS

Generation of highly resistant mutant viruses using defined different experimental procedures

We examined the development of *in vitro* resistance against the NNRTI HBY 097 under different selective pressure conditions. The aim was to find out whether the HIV-1_{MN} parental strain would genotypically evolve in a different way in those cell cultures, allowing the virus to adapt more slowly to higher concentrations of the inhibitor. Virus lineages were divided into two categories: group A was characterized by high selective pressure; i.e., the concentration of HBY 097 was raised by a factor of five with every new subcultivation step. In group B, the factor was two, resulting in low selective pressure conditions.

In cultures of both group A (A1–A3, A5, A7) and group B (B7–B10, B12), it was necessary to use the same amount of HBY 097 at two or more subsequent passages, since no virus replication was observed in the corresponding wells at a given drug concentration. However, once a limit of $>$50 ng/ml HBY 097 was reached, it was easily possible to proceed to the next dose level in all of the 19 lineages analyzed. Under lowered selective pressure (group B) this 50 ng/ml threshold was reached at passage 9 in most cultures, whereas under high selective pressure (group A) the majority of cultures arrived at the same concentration of HBY 097 after five subcultivations. The final concentrations were 10,000 or 20,000 ng/ml, equaling up to 40,000-fold the initial amount of compound used in the first passage.

Evaluation of genotypic changes in RT genes of group A and group B mutants

In order to determine the lower limit of detection of variants at a given nucleotide position, PCR products

TABLE 1

Genotypic Analyses of Group A Strains (High Selective Pressure) versus Group B Lineages (Low Selective Pressure)

Strain	Passage No., [HBY 097 (ng/ml)] genotype[a]							
Group A viruses								
A1	1, 0.5 wt	⇒	5, 2.5 Gly190Ala/wt	⇒	10, 5,000 Leu100Ile/wt, *Gly190GLu*/Ala	⇒	15, 20,000 *Gly190Glu*	⇒	20 *Gly190Glu*
A2	1,0.5 wt	⇒	5, 2.5 Val106Ala/wt, Tyr181Cys/wt, Tyr188His/wt	⇒	10, 20,000 Leu74Val, *Gly190Glu*	⇒	15 Leu74Val, *Gly190Glu*	⇒	20 Leu74Val, *Gly190Glu*
A3	1, 0.5 wt	⇒	5, 50 Val106Ile, Tyr181Cys	⇒	10, 20,000 *Gly190Glu*	⇒	15 Leu74Val/wt, *Gly190Glu*	⇒	20 Leu74Val/wt, *Gly190Glu*
A4	1, 0.5 wt	⇒	5, 250 Leu100Ile, Tyr188His/wt, Gly190Ala	⇒	10, 20,000 Leu100Ile/wt, *Gly190Glu*/Ala/ Val	⇒	15 Leu74Val/wt, Leu100Ile/wt, *Gly190Glu*/Ala	⇒	20 Leu74Val/wt, Leu100Ile, Val106Ala, *Gly190Glu*/Ala, Phe227Leu
A5	1, 0.5 wt	⇒	5, 10 Leu100Ile/wt, Val106Ile/wt, Tyr181Cys/wt	⇒	10, 20,000 *Gly190Glu*	⇒	15 *Gly190Glu*	⇒	20 Valy75Leu/wt, *Gly190Glu*
A6	1, 0.5 wt	⇒	5, — ND	⇒	10, 5,000 Gly190Val, Glu194Gln/wt[b]	⇒	15, 20,000 *Gly190Glu*Val, Glu194GLn/wt,[b] Lys219Glu/wt[c]	⇒	20 Leu74Val/wt, *Gly190Glu*Val, Glu194Gln/wt,[b] Lys219Glu[c]
A7	6, 60 ND	⇒	11, 20,000 ng/ml *Gly190Glu*	⇒	15 Val106Leu/wt, Tyr181Cys, Tyr188His/Leu, *Gly190Glu*/wt	⇒	20 Val106Leu, Tyr181Cys, Tyr188Leu	⇒	30 Val106Leu, Tyr181Cys, Tyr188Leu
Group B viruses									
B1	1, 0.2 wt	⇒	5, 4 wt	⇒	10, 200 *Gly190Glu* Val179Gly/wt[b]	⇒	15, 10,000 *Gly190Glu,* Val179Gly[b]	⇒	20 *Gly190Glu*
B2	1, 0.2 wt	⇒	5, 4 Leu100Ile/wt	⇒	10, 200 Leu100Ile, Tyr181Ile	⇒	15, 10,000 Leu100Ile, Tyr181Ile	⇒	20 Leu100Ile, Tyr181Ile, Gly190Ala
B3	1, 0.2 wt	⇒	5, 4 Leu100Ile/wt	⇒	10, 200 Leu100Ile/wt, Lys103Asn, Tyr181Cys/wt, Gly190Ala/wt	⇒	15, 10,000 Leu100Ile/wt, Lys103Asn, Tyr181Cys, Gly190Ala/wt	⇒	20 Leu100Ile, Lys103Asn, Tyr181Cys/wt, Val189Ile/wt, Gly190Ala
B4	1, 0.2 wt	⇒	5, 4 Leu100Ile/wt	⇒	1, 200 Leu100Ile/wt, Lys103Asn/wt, Tyr181Cys/wt, Gly190Ala/wt	⇒	15, 10,000 Leu100Ile, Lys103Asn, Tyr181Cys/wt, Tyr188Phe/Leu/His	⇒	20 Leu100Ile, Lys103Asn, Tyr181Cys/wt, Tyr188Leu/His, Gly190Ala/wt
B5	1, 0.2 wt	⇒	5, 4 Leu100Ile/wt	⇒	10, 200 Leu100Ile/wt, Lys103Asn, Tyr181Cys/wt	⇒	15, 10,000 Leu100Ile/wt, Lys103Asn, Tyr181Cys/wt	⇒	20 Leu100Ile, Lys103Asn, Tyr181Cys, Gly190Ala/wt
B6	1, 0.2 wt	⇒	5, 4 wt	⇒	10, 200 Leu100Ile/wt, Gly190Ala	⇒	15, 10,000 Leu100Ile/wt, *Gly190Glu*/Ala	⇒	20 Leu100Ile/wt, *Gly190Glu*/Ala

TABLE 1—*Continued*

Strain	Passage No., [HBY 097 (ng/ml)] genotype[a]							
B7	6, 16 Gly190Ala	⇒	11, 500 ND	⇒	15, 8,000 Leu74Val/wt, Leu100Ile, Gly190Thr/Ala	⇒	20, 20,000 Leu74val, Leu100Ile, Gly190Thr	⇒	30 Leu74Val, Leu100Ile, Gly190Thr
B8	5, 2 Tyr181Cys/wt	⇒	10, 60 Lys103Asn, Tyr181Cys	⇒	15, 2,000 Lys103Asn, Tyr181Cys	⇒	31, 20,000 Lys101Ile, Lys103Asn, Tyr181Cys, Gly190Ala	⇒	40 Lys101Ile, Lys103Asn, Tyr181Cys, Gly190Ala
B9	5, 4 Gly190Ala	⇒	10,120 Leu100Ile, Gly190Ala	⇒	15, 4,000 Leu100Ile, Val106Ala/wt Gly190Ala	⇒	31, 20,000 Leu100Ile, Val106Ala, Gly190Ala	⇒	40 Leu100Ile, Val106Ala, Gly190Ala
B10	5, 8 Leu100Ile/wt, Tyr181Cys/wt	⇒	10, 240 ND	⇒	15, 8,000 Leu100Ile, Thyr181Cys, Tyr188His	⇒	31, 20,000 Leu100Ile, Val106Ile, Tyr181Cys, Tyr188Leu	⇒	40 Asp86Asn,[b] Leu100Ile, Val106Ile, Tyr181Cys, Tyr188Leu
B11	5, 16 Leu100Ile	⇒	10, 500 Leu100Ile, Val106Ala	⇒	15, 16,000 Leu100Ile, Val106Ala, Pro225His	⇒	31 Leu74Ile, Leu100Ile, Lys103Asn, Val106Ala/wt, Pro225His	⇒	40 Leu74Ile, Leu100Ile, Lys103Asn, Tyr181Ile/ Asn/Lys Pro225His
B12	5, 2 wt	⇒	10, 60 Lys103Arg/wt	⇒	15, 2,000 Leu100Ile/wt, Tyr181Ile, Gly190Ala/wt	⇒	31, 20,000 Leu100Ile, Tyr181Ile, Gly190Ala	⇒	40 Leu100Ile, Tyr181Ile, Gly190Ala

Note. Slashes indicate mixed populations apparent at a given codon position. ND, not determined; wt, wild type.

[a] For better clarity, drug concentrations are not indicated once 10,000 ng/ml are reached. The Gly190Glu change is printed in italics.

[b] Asp86Asn, Val179Gly, and Glu194Gln have not been reported to be associated with drug resistance (Schinazi *et al.* (1996); the significance of these changes is not clear.

[c] Lys219Glu is a substitution originally observed in zidovudine-resistance HIV-1 RT (Larder *et al.,* 1991).

derived from HIV-1_{MN} and RT Gly190Glu mutant virus were mixed at different ratios and submitted to sequencing reactions. The limit of detectability of the G → A transition was found to be at a proportion of 0.875:0.125 (wt:Gly190Glu; data not shown).

Each of the individual lineages of both groups was analyzed for resistance-conferring mutations for at least four different time points (passages) during the dose escalation procedure. A summary of the results of the comprehensive mutational analyses is given in Tables 1 and 2.

All lineages in both groups displayed the parental genotype at HBY 097 concentrations below 2 ng/ml. At higher drug levels, alterations at various positions appeared, with Leu100Ile being the most abundant change (13/19 lineages). Above 60 ng/ml of HBY 097, either the codons of two amino acids changed in the RT gene of each of the strains or the Gly190Glu substitution was present. The crippling Gly190Glu mutant RT was detected in all lineages of the high selective pressure group A, but the same change was noted in only 2/12 of the RT genes derived from low selective pressure viruses (group B). This difference is highly statistically significant ($P < 0.001$, χ^2 test). In group B, an accumulation of other NNRTI-specific mutations was observed, also resulting in high-level HBY 097 resistance: a total of five different positions relevant for NNRTI resistance were mutant in strains B3 and B4. Secondary changes at NRTI resistance positions were identified in 5/7 strains of group A and in 2/12 strains belonging to group B ($P < 0.05$). Novel mutations at sites with defined importance for RT inhibitor resistance are Val106Ile, Val106Leu, Gly190Thr, and Gly190Val. The Val106Leu substitution was present only in lineage (A7) where a replacement of Gly190Glu was evident: a variant carrying a triple mutant RT had completely displaced Gly190Glu at passage 20.

TABLE 2

Quantitative Evaluation of Individual Amino Acid Changs Observed in Both Groups

HIV-1RT amino acid No.	Parental phenotype	Resistance-conferring mtuations: Mutant 1	Mutant 2	Mutant 3	Mutant 4
100	Leu	Ile (3/10)			
101	Lys	Ile (0/1)			
103	Lys	Asn (0/5)	Arg (0/1)		
106	Val	Ala (2/2)	Ile[a] (2/1)	Leu[a] (1/0)	
179	Val	Gly[a] (0/1)			
181	Tyr	Cys (4/5)	Ile (0/3)	Lys[a,b] (0/1)	Asn[a,b] (0/1)
188	Tyr	His (3/2)	Leu (1/2)	Phe[a] (0/1)	
189	Val	Ile (0/1)			
190	Gly	Ala (2/9)	Glu (7/2)	Val (1/0)	Thr[a] (0/1)
225	Pro	His[a] (0/1)			
		Secondary changes			
74	Leu	Val (3/1)	Ile (0/1)		
75	Val	Leu (1/0)			
219	Lys	Glu (1/0)			

Note. Each mutant amino acid is counted once per individual lineage. Numbers in parentheses, number of appearances in group A/group B.
[a] Change occurred at a position relevant for NNRTI resistance; specific influence of this particular amino acid not yet determined.
[b] Tyr181Lys/Asn codons were detected as possible genotypes in only one mixed population (B11).

Phenotypic resistance of individual group A and group B viral variants

Late passage viruses from different lineages of both groups, carrying up to four mutations in their RT genes, were tested for their susceptibilities to inhibition by various RT inhibitors (Table 3). All of the NNRTIs tested were completely inactive against mutants A1, A2, B8, and B9 (i.e., lost up to 1670-fold of inhibitory potency compared to activities seen with the parental virus).

DISCUSSION

In this study, we describe for the first time the defined manipulation of HIV-1 genotypes using an inhibitor of HIV-1 replication. Resistance against the quinoxaline HBY 097 was accomplished by two general pathways in a well-characterized *in vitro* system, using a permanent cell line and a laboratory strain of HIV-1. Alternative selective pressure conditions applied result in two different ways followed by HIV-1_{MN} to become resistant against the quinoxaline derivative. These are characterized by different raise factors of the drug concentration in the virus cultures, resulting in high (group A) and low (group B) selective pressure. Though viral variants of both groups acquired indistinguishable high-level NNRTI resistance (Table 3), statistically significant differences were noted with respect to the mutations which led to the observed phenotypes (Tables 1, 2): (i) The crippling Gly190Glu alteration, which is derived from a single G → A transition, appeared preferentially under high selective pressure. In contrast, attenuation of the selective pressure exerted by the inhibitor yielded multiple mutations at various codons. Interestingly, Gly190Ala — which does not confer complete resistance to HBY 097 and retains wt level polymerase activity — was also observed more frequently in group B. (ii) Secondary mutations affecting codons relevant for NRTI resistance were previously described to appear with HBY 097 in dose escalation experiments, and the resulting amino acid changes have been partly demonstrated to have compensatory effects on RT polymerase activity (Kleim *et al.,* 1996). These mutations were more likely to show up under high selective pressure conditions.

Winslow *et al.* (1996) described a limited accumulation of mutations with the NNRTI DMP 266. Consistent with our data, there were different patterns of mutations found with two different experimental settings. However, more than one parameter was varied (i.e., different cell types and different selection procedure), and the data were lacking statistical power. It is important to note that the stepwise acquisition of mutations in the RT genes of HBY 097-treated HIV-1 leads to increasing resistance to the drug. Most of the mutations found (i.e., Leu100Ile, Lys103Asn, Val106Ala, Tyr181Cys, Gly190Ala) were previously tested for their ability to confer resistance to quinoxaline class NNRTIs. Present as single mutants, none of these changes could be demonstrated to cause high-level resistance (Balzarini *et al.,* 1994, 1996a; Kleim *et al.,* 1994, 1995). However, when changes such as Leu100Ile/

TABLE 3

Sensitivity of Late Passage Viruses from Different Group A and Group B Strains to HBY 097 and Other Reverse Transcriptase Inhibitors

Virus	Compound, IC_{50} (ng/ml)				
	HBY 097	S-2720	Nevirapine	Delavirdine	Zidovudine
$HIV\text{-}1_{MN}$	7	6	90	180	<50
A1 (Gly190Glu)	>10000	>10000	>10000	>10000	<50
A2 (Leu 74Val, Gly190Glu)	>10000	>1000	>10000	8700	50
B8 (Lys101Ile, Lys103Asn, Ty181Cys, Gly190Ala)	>10000	>10000	>10000	>10000	<50
B9 (Leu100Ile, Val106Ala, Gly190Ala)	>10000	>10000	>10000	>10000	30

[a] Strain designations correspond to labelling in Table 1.

Val108Ile/Tyr181Ile or Tyr181Cys/Tyr188Leu/Gly190Ala/Met230Leu were expressed as multiple mutant enzymes in *Escherichia coli,* the derived proteins were found to be highly resistant to inhibition by quinoxalines and other NNRTIs. In addition, these enzymes retained wild-type level RNA-dependent DNA polymerase activities *in vitro* (Kleim *et al.,* 1995; and data not shown).

In contrast, NNRTI resistance mediated by the Gly190Glu change is accompanied by a weak RT polymerase activity and retarded *in vitro* growth properties of the corresponding mutant virus (Kleim *et al.,* 1993, 1994; Olmsted *et al.,* 1996). Glu190—as observed in group A—can be obtained by a single favored G → A transition, but is nevertheless avoided under conditions as applied for the generation of strains belonging to group B. Taken together, these data strongly suggest that the described accumulation phenomenon represents an adaptation of HIV-1 to suboptimal drug pressure.

The stepwise addition of mutations to existing mutated *pol* genotypes could originate both from error prone DNA synthesis of HIV-1 RT during *in vitro* replication and, as shown more recently, from nucleic acid recombination processes (Kellam and Larder, 1995; Moutouh *et al.,* 1996). The acquisition of multiple mutations as described here for low selective pressure exerted by HBY 097 does not resemble HIV-1 resistance development induced by other NNRTIs such as nevirapine or delavirdine (Richman *et al.,* 1991, 1994; Dueweke *et al.,* 1993; Demeter *et al.,* 1995) but is instead more related to data obtained from *in vitro* investigations and clinical trials undertaken with inhibitors of the HIV protease (Condra *et al.,* 1995; Tisdale *et al.,* 1995; Molla *et al.,* 1996). In these studies, it was shown that increasing resistance against different protease inhibitors was achieved through the consecutive appearance of up to five substitutions in the protease, leading also to cross-resistance to multiple compounds of that class of drugs.

In conclusion, pharmacokinetic properties are critical parameters for the type of mutations which will appear with HBY 097 and other drugs during therapy of HIV infection. In that way, selective pressure has to be viewed as another determinant of qualitative resistance development in addition to factors such as genetic background or drug combinations. As part of novel combination therapy regimens, NNRTIs such as HBY 097 can be useful to slow down or even suppress the development of viral resistance (Balzarini *et al.,* 1996b; Myers *et al.,* 1996).

ACKNOWLEDGMENTS

We thank B. Bremora, M. Hardt, T. Hofmann, C. Ketter, A. Momberger, C. Lemp, G. Sibenhorn, and S. Stippich for technical assistance and N. Reifsteck for fine editorial help.

REFERENCES

Albert, J., Wahlberg, J., Leitner, T., Escanilla, D., and Uhlén, M. (1994). Analysis of a rape case by direct sequencing of the human immunodeficiency virus type 1 pol and gag genes. *J. Virol.* 68, 5918–5924.

Balzarini, J., Karlsson, A., Meichsner, C., Paessens, A., Riess, G., De Clercq, E., and Kleim, J.-P. (1994). Resistance pattern of human immunodeficiency virus type 1 reverse transcriptase to quinoxaline S-2720. *J. Virol.* 68, 7986–7992.

Balzarini, J., Pelemans, H., Riess, G., Rösner, M., Winkler, I., De Clercq, E., and Kleim, J.-P. (1996a). AZT-resistant HIV-1 strains subcultured in the combined presence of quinoxaline HBY 097 and lamivudine (3TC), retain marked sensitivity to HBY 097. Submitted for publication.

Balzarini, J., Pelemans, H., Karlsson, A., De Clercq, E., and Kleim, J.-P. (1996b). Concomitant combination therapy for HIV infection preferable over sequential therapy with 3TC and non-nucleoside reverse transcriptase inhibitors. *Proc. Natl. Acad. Sci. USA* 93, 13152–13157.

Buckheit, R. W., Fliakas-Boltz, V., Decker, W. D., Roberson, J. L., Stup, T. L., Pyle, C. A., White, E. L., McMahon, J. B., Currens, M. J., Boyd, M. R., and Bader, J. P. (1995). Comparative anti-HIV evaluation of diverse HIV-1-specific reverse transcriptase inhibitor-resistant virus isolates demonstrates the existence of distinct phenotypic subgroups. *Antiviral Res.* 26, 117–132.

Condra, J. H., Schleif, W. A., Blahy, O. M., Gabryelski. L. J., Graham, D. J.,

Quintero, J. C., Rhodes, A., Robbins, H. L., Roth, E., Shivaprakash, M., Titus, D., Yang, T., Teppier, H., Squires, K. E., Deutsch, P. J., and Emini, E. A. (1995). In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. *Nature* 374, 569–571.

De Clercq, E. (1995). Toward improved anti-HIV chemotherapy: Therapeutic strategies for intervention with HIV infections. *J. Med. Chem.* 38, 2491–2517.

Demeter, L. M., Gerondelis, P., Dexter, A., Berrios, L., Morse, G., Cox, S., Freimuth, W., and Reichman, R. C. (1995). Combination therapy (Rx) with delavirdine (DLV) and ddI, but not AZT, fails to prevent the emergence of Y181C in nucleoside experienced patients (PTS) [Abstract 31]. Program and abstract of the Fourth International Workshop on HIV Drug-Resistance, Sardinia, Italy.

Dueweke, T. J., Pushkarskaya, T., Poppe, S. M., Swaney, S. M., Zhao, J. Q., Chen, I. S. Y., Stevenson, M., and Tarpley, W. G. (1993). A novel mutation in bisheteroarylpiperazine resistant HIV-1 reverse transcriptase confers increased sensitivity to other non-nucleoside inhibitors. *Proc. Natl. Acad. Sci. USA* 90, 4713–4717.

Hargrave, K. D., Proudfoot, J. R., Grozinger, K. G., Cullen, E., Kapadia, S. R., Patel, U. R., Fuchs, V. U., Mauldin, S. C., Vitous, J., Behnke, M. L., Klunder, J. M., Pal, K., Skiles, J. W., McNeil, D. W., Rose, J. M., Chow, G., Skoog, M. T., Wu, J. C., Rosenthal, A. S., and Adams, J. (1991). Novel non-nucleoside inhibitors of HIV-1 reverse transcriptase. 1. Tricyclic pyridobenzo- and dipyridodiazepinones. *J. Med. Chem.* 34, 2231–2241.

Kellam, P., and Larder, B. (1995). Retroviral recombination can lead to linkage of reverse transcriptase mutations that confer increased zidovudine resistance. *J. Virol.* 69, 669–674.

Kleim, J.-P., Bender, R., Billhardt, U. M., Meichsner, C., Riess, G., Rösner, M., Winkler, I., and Paessens, A. (1993). Activity of a novel quinoxaline derivative against human immunodeficiency virus type 1 reverse transcriptase and viral replication. *Antimicrob. Agents Chemother.* 37, 1659–1664.

Kleim, J.-P., Bender, R., Kirsch, R., Meichsner, C., Paessens, A., and Riess, G. (1994). Mutational analysis of residue 190 of human immunodeficiency virus type 1 reverse transcriptase. *Virology* 200, 696–701.

Kleim, J.-P., Bender, R., Kirsch, R., Meichsner, C., Paessens, A., Rösner, M., Rübsamen-Waigmann, H., Kaiser, R., Wichers, M., Schneweis, K. E., Winkler, I., and Riess, G. (1995). Preclinical evaluation of HBY 097, a new nonnucleoside inhibitor of human immunodeficiency virus type 1 replication. *Antimicrob. Agents Chemother.* 39, 2253–2257.

Kleim, J.-P., Rösner, M., Winkler, I., Paessens, A., Kirsch, R., Hsiou, Y., Arnold, E., and Riess, G. (1996). 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. *Proc. Natl. Acad. Sci. USA* 93, 34–38.

Larder, B. A. (1992). 3′-azido-3′-deoxythymidine resistance suppressed by a mutation conferring human immunodeficiency virus type 1 resistance to nonnucleoside reverse transcriptase inhibitors. *Antimicrob. Agents Chemother.* 36, 2664–2669.

Larder, B. A., Coates, K. E., and Kemp, S. D. (1991). Zidovudine-resistant human immunodeficiency virus selected by passage in cell culture. *J. Virol.* 65, 5232–5236.

Molla, A., Korneyeva, M., Gao, Q., Vasavanonda, S., Schipper, P. J., Mo, H. M., Markowitz, M., Chernyavskiy, T., Niu, P., Lyons, N., Hsu, A., Granneman, G. R., Ho, D. D., Boucher, C. A. B., Leonard, J. M., Norbeck, D. W., and Kempf, D. J. (1996). *Nature Med.* 2, 760–766.

Moutouh, L., Corbeil, J., and Richman, D. D. (1996). Recombination leads to the rapid emergence of HIV-1 dually resistant mutants under selective drug pressure. *Proc. Natl. Acad. Sci. USA* 93, 6106–6111.

Myers, J. W., Montaner, J. S. G., and the INCAS study group. (1996). A randomised, double-blinded comparative trial of the effects of zidovudine, didanosine, and nevirapine combinations in antiviral naive, AIDS-free, HIV infected patients with CD4 counts 200–600/mm^3. XIth International conference on AIDS, Vancouver, July 7–12, 1996.

Olmsted, R. A., Slade, D. E., Kopta, L. A., Poppe, S. M., Poel, T. J., Newport, S. W., Rank, K. B., Biles, C., Morge, R. A., Dueweke, T. J., Yagi, Y., Romero, D. L., Thomas, R. C., Sharma, S. K., and Tarpley, W. G. (1996). (Alkylamino)piperidine bis(heteroaryl)piperizine analogs are potent, broad-spectrum nonnucleoside reverse transcriptase inhibitors of drug-resistant isolates of human immunodeficiency virus type 1 (HIV-1) and select for drug-resistant variants of HIV-1_{IIIB} with reduced replication phenotypes. *J. Virol.* 70, 3698–3705.

Richman, D., Shih, C. K., Lowy, I., Rose, J., Prodanovich, P., Goff, S. P., and Griffin, J. (1991). Human immmunodeficiency virus mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture. *Proc. Natl. Acad. Sci. USA* 88, 11241–11245.

Richman, D. D., Havlir, D., Corbeil, J., Looney, D., Ignacio, C., Spector, S. A., Sullivan, J., Cheeseman, S., Barringer, K., Pauletti, D., Shih, C. K., Myers, M., and Griffin, J. (1994). Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. *J. Virol.* 68, 1660–1666.

Romero, D. L., Morge, R. A., Genin, M. J., Biles, C., Busso, M., Resnick, L., Althaus, I. W., Reusser, F., Thomas, R. C., and Tarpley, W. G. (1993). Bis(heteroaryl)piperazine (BHAP) reverse transcriptase inhibitors: Structure–activity relationships of novel substituted indole analogues and the identification of 1-[5-methanesulfonamido-1H-indol-2-yl)-carbonyl]-4-[3-[(1-methylethyl)amino]-pyridinyl]piperazine monomethansulfonate (U-90152S), a second-generation clinical candidate. *J. Med. Chem.* 36, 1505–1508.

Schinazi, R. F., Larder, B. A., and Mellors, J. W. (1996). Mutations in retroviral genes associated with drug resistance. *Int. Antiviral News* 4, 95–107.

Tantillo, C., Ding, J., Jacobo-Molina, A., Nanni, R. G., Boyer, P., Hughes, S. H., Pauwels, R., Andries, K., Janssen, P. A. J., and Arnold, E. (1994). Locations of Anti-AIDS drug binding sites and resistance mutations in the three-dimensional structure of HIV-1 reverse transcriptase. *J. Mol. Biol.* 243, 369–387.

Tisdale, M., Myers, M., Maschera, B., Parry, N. P., Oliver, N. M., and Blair, E. D. (1995). Cross-resistance analysis of human immunodeficiency virus type 1 variants individually selected for resistance to five different protease inhibitors. *Antimicrob. Agents Chemother.* 39, 1704–1710.

Winslow, D. L., Garber, S., Reid, C., Scarnati, H., Baker, D., Rayner, M. M., and Anton, E. D. (1996). Selection conditions affect the evolution of specific mutations in the reverse transcriptase gene associated with resistance to DMP 266. *AIDS* 10, 1205–1209.