Biochemical Pharmacology, Vol. 55, pp. 617–625, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00506-6

Retention of Marked Sensitivity to (*S*)-4-Isopropoxycarbonyl-6-Methoxy-3-(Methylthiomethyl)-3,4-Dihydroquinoxaline-2(1*H*)-Thione (HBY 097) by an Azidothymidine (AZT)-Resistant Human Immunodeficiency Virus Type 1 (HIV-1) Strain Subcultured in the Combined Presence of Quinoxaline HBY 097 and 2′,3′-Dideoxy-3′-Thiacytidine (Lamivudine)

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ABSTRACT. An azidothymidine (AZT)-resistant virus strain (HIV-1/AZT) (containing the 67 Asp → Asn, 70 Lys → Arg, 215 Thr → Phe and 219 Lys → Gln mutations into its reverse transcriptase) was grown in the combined presence of 2′,3′-dideoxy-3′-thiacytidine (3TC, lamivudine) and the nonnucleoside reverse transcriptase inhibitor (*S*)-4-isopropoxycarbonyl-6-methoxy-3-(methylthiomethyl)-3,4-dihydroquinoxaline-2(1*H*)-thione (quinoxaline HBY 097). Replication of HIV-1/AZT was inhibited to a significantly greater extent by the combination of 3TC and quinoxaline HBY 097 than by either drug alone. Virus breakthrough was markedly delayed in the combined presence of 3TC and HBY 097 at drug concentrations as low as 0.05 μg/mL and 0.0025 μg/mL, respectively. The virus that was recovered after exposure to the compounds (3TC and HBY 097) individually had acquired, in the genetic AZT-resistance background of HIV-1/AZT, 103 Lys → Glu and 106 Val → Ala mutations. The 103 Lys → Glu mutation had not been observed before. However, both virus mutants retained marked sensitivity to HBY 097. In all cases, the genotypic AZT-resistance mutations were maintained in the mutant virus RT genomes, and the viruses also remained phenotypically resistant to AZT. Given the exquisite potency of a concomitant combination of 3TC and HBY 097 in suppressing virus replication, this drug combination should be further pursued in clinical trials in HIV-1-infected individuals. BIOCHEM PHARMACOL 55;5:617–625, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. 3TC; quinoxalines; AZT; nonnucleoside reverse transcriptase inhibitors (NNRTI); resistance; HIV-1

Two important classes of drugs that are targeted at the HIV-1 reverse transcriptase (RT)§ are currently approved for treatment of HIV infections [i.e. the nucleoside RT inhibitors (NRTI) zidovudine (AZT), zalcitabine (DDC), didanosine (DDI), stavudine (D4T) and lamivudine (3TC) and the nonnucleoside RT inhibitors (NNRTIs) nevirapine and BHAP U-90152 (delavirdine)] or are subject of clinical trials in HIV-1 seropositive individuals [i.e. the NNRTIs, α-APA (loviride), 8-chloro-TIBO R091767 (trivirapine)] [for an overview, see refs. 1 and 2]. The most recent NNRTI submitted to clinical trials is the quinoxaline derivative (*S*)-4-isopropoxycarbonyl-6-methyl-3-(methylthiomethyl)-3,4-dihydro-quinoxaline-2(1*H*)-thione (HBY 097) (Fig. 1) [3, 4]. This drug was selected from a series of quinoxaline derivatives as a second-generation NNRTI. HBY-097, as well as its predecessor 6-chloro-3,3-dimethyl-4-(isopropenyloxycarbonyl)-3,4-dihydroquinoxaline-2(1*H*)-thione (S-2720) [5–7], were shown to inhibit at nanomolar concentrations

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§ *Abbreviations:* AZT, 2′,3′-dideoxy-3′-azidothymidine, azidothymidine, zidovudine; BHAP, bis(heteroaryl)piperazine; $CCID_{50}$, cell culture infective dose-50; DDC, 2′,3′-dideoxycytidine, zalcitabine; DDI, 2′,3′-dideoxyinosine, didanosine; HBY 097, (*S*)-4-isopropoxycarbonyl-6-methoxy-3-(methylthiomethyl)-3,4-dihydroquinoxaline-2(1*H*)-thione; HIV, human immunodeficiency virus; NNRTI, nonnucleoside RT inhibitor; NRTI, nucleoside RT inhibitor; PCR, polymerase chain reaction; RT, reverse transcriptase; 3TC, 2′,3′-dideoxy-3′-thiacytidine; TIBO, tetrahydroimidazo[4,5,1-jk][1,4]benzodiazepin-2(1*H*)-one; TSAO-m^3T, [2′,5′-bis-O-(*tert*-butyldimethylsilyl)-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide]-N^3-methylthymidine; UC-10, N-{[4-chloro-3((1,1-dimethylethogy)imino)methyl]phenyl}-3-furancarbothiamide.

Received 27 May 1997; accepted 26 August 1997.

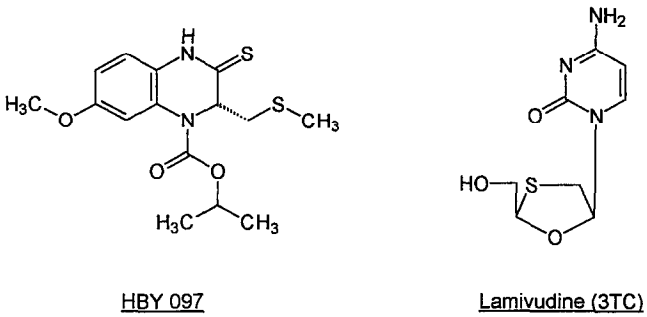

FIG. 1. Structural formulae of the quinoxaline HBY 097 and 3TC.

the replication of several HIV-1 strains, including clinical isolates, in different laboratory cell lines and monocyte/macrophages and peripheral blood lymphocytes [3, 4]. Both HBY 097 and S-2720 also proved highly inhibitory to various AZT-resistant HIV-1 strains. HBY 097 was chosen for clinical trials in HIV-1-infected individuals based on its potent antiviral efficacy, its favorable toxicity profile, and its good oral bioavailability in animals.

The most recent NRTI approved for AIDS treatment (in combination with AZT) is 2',3'-dideoxy-3'-thiacytidine (3TC, lamivudine) [8–12]. This NRTI, in which the 3'-carbon of 2'-deoxycytidine has been replaced by a sulfur atom, represents the only β-L-(−) isomer of the dideoxynucleoside series that has been approved for the treatment of AIDS (Fig. 1). 3TC has a low toxicity profile in patients [13]. However, in contrast to other NRTIs and much like NNRTIs, 3TC rapidly selects for highly resistant mutant virus strains in cell culture [13–16]. It predominantly selects for the 184 Met → Ile/Val mutation in the HIV-1 RT. Interestingly, 3TC remains highly active against mutant HIV-1 strains that contain amino acid changes conferring resistance to the NNRTIs [for an overview, see refs. 1 and 17]. Another interesting feature of 3TC is the finding that the 184 Met → Val mutation restores phenotypic AZT sensitivity of AZT-resistant HIV-1 strains when the 184-Val mutation is introduced in an AZT-resistance background [18, 19].

In vitro, the quinoxalines S-2720 and HBY 097 characteristically generate a 190 Gly → Glu substitution in the reverse transcriptase of drug-treated HIV-1 strains, which results in marked resistance to virtually all NNRTIs [5, 20, 21]. Interestingly, the presence of the Gly-190 → Glu mutation in the HIV-1 reverse transcriptase severely affects the catalytic efficiency of the enzyme [7], which may result in the selection of replication-attenuated HIV-1 strains.

Since a majority of AIDS patients are currently under AZT treatment or have in the past been treated with AZT, treatment of such patients with HBY 097 will necessarily imply that in many cases the drug will have to interact with mutant virus strains that already contain at least one or several characteristic AZT mutations (i.e. 41 Met → Leu, 67 Asp → Asn, 70 Lys → Arg, 215 Thr → Tyr/Phe and/or 219 Lys → Gln [for an overview, see ref. 17]).

We have examined herein the effects of different combinations of HBY 097 and 3TC on an AZT-resistant virus strain containing the 67 Asp → Asn, 70 Lys → Arg, 215 Thr → Phe and 219 Lys → Gln mutations in its RT. The aim was to assess whether HBY 097 would suppress or generate the emergence of drug-resistant virus strains under these different experimental conditions starting from a genetic AZT-resistance background, and whether different amino acid mutations may emerge under these treatment conditions than those observed for wild-type HIV-1 treatment. The selection for HIV-1 strains resistant against HBY 097 and 3TC was carried out both in the absence and in the continuous presence of AZT. We found that the combination of HBY 097 with 3TC allowed the emergence of low-level resistance mutations while preserving the AZT-resistance mutations in the mutant virus genomes.

MATERIALS AND METHODS

Test Compounds

The synthesis of the TSAO derivative [2',5'-bis-O-(*tert*-butyldimethylsilyl)-3'-spiro-5''-(4''-amino-1'',2''-oxathiole-2'', 2'' - dioxide]-N^3-methylthymidine] (TSAO-m^3T) has been described elsewhere [22–24] and was kindly provided by Dr. M.-J. Camarasa (Madrid, Spain). The thiocarboxanilide *N*-[4-chloro-3-(t-butyloximeether)phenyl]-2-methyl-3-furancarbothioamide (UC-10) [25] was synthesized by chemists at Uniroyal Chemical. 3TC was kindly provided by Dr. R. Schinazi. TIBO R82913 was provided by Dr. D. G. Johns and Dr. Zhang Hao [National Institutes of Health (NIH)] and was also obtained from Pharmatech International Inc. Nevirapine (BI-RG-587) and BHAP (U-90152) were provided by Dr. P. Ganong (Boehringer Ingelheim) and Dr. R. Kirsch (Hoechst AG), respectively. Quinoxaline HBY 097 was synthesized at Hoechst AG. AZT, DDC and DDI were obtained from Sigma Chemical Co., Dr. D. G. Johns (NIH) and Dr. M. Hitchcock (Bristol Myers Squibb, now at Gilead Sciences), respectively. The structural formulae of lamivudine and HBY 097 are shown in Fig. 1.

Cells and Viruses

CEM cells were obtained from the American Tissue Cell Culture Collection. The AZT-resistant HIV-1 clone was generously provided by Dr. B. Larder (Glaxo Wellcome). HIV-1/AZT represents the RTMC strain containing the AZT-resistance mutations 67 Asp → Asn, 70 Lys → Asn, 215 Thr → Phe and 219 Lys → Gln in its RT [19, 26].

Selection of Mutant HIV-1/AZT Strains in the Presence of HBY-097 and 3TC

A high multiplicity of infection (MOI) [>1,000 50% cell culture infective dose ($CC_{ID_{50}}$)] of the AZT-resistant HIV-1 strain was subjected to several passages in 5 mL CEM cell cultures (~3.5 × 10^5 cells per mL) in the presence of fixed concentrations of 3TC (0.02 or 0.05

μg/mL) or HBY 097 (0.001 or 0.0025 μg/mL) in 25 cm^2 culture flasks (Corning) to produce virus strains that were able to grow in the presence of the individual compounds. Each culture was maintained both in the presence and in the absence of AZT (0.03 μg/mL). The initial virus input in each cell culture consisted of 0.5 or 1.0 mL of supernatant that was obtained from an HIV-1-infected cell culture at the time at which the virus was most abundantly present (~4 days postinfection). The culture medium consisted of RPMI-1640 containing 10% fetal bovine serum, 2 mM L-glutamine and 0.075% $NaHCO_3$. Passages were performed every 3 to 4 days by adding 750 μL of the infected culture supernatant to 4 to 5 mL of a suspension containing ~3.5×10^5 uninfected CEM cells per mL. The supernatants of the treated HIV-1-infected cell cultures were frozen in aliquots at −70° after the syncytium formation became abundant in the cell cultures, and the virus was further passaged for at least 4 to 5 additional subcultivations in the presence of the test compounds.

In the drug combination experiments (3TC with HBY 097), the paired compounds were combined at the same initial concentrations as used for single-drug treatment (i.e. 0.001 μg/mL HBY 097 + 0.02 μg/mL 3TC; 0.001 μg/mL HBY 097 + 0.05 μg/mL 3TC; 0.0025 μg/mL HBY 097 + 0.02 μg/mL 3TC and 0.0025 μg/mL HBY 097 + 0.05 μg/mL 3TC). The individual drug concentrations were chosen in such a way that the combination of both drugs still resulted in (delayed) virus breakthrough. Higher drug concentrations are not toxic, but would result in knock-out of the virus replication in the cell cultures [20, 21]. Again, the treatment of the HIV-1/AZT-infected cell cultures with the paired drug combinations was performed both in the presence and absence of continuous exposure of AZT at 0.03 μg/mL. In addition, the AZT concentration was further increased to 0.13 μg/mL starting at day 23 postinfection. HBY 097 and 3TC concentrations were not increased during subcultivations, and the treated HIV-1-infected CEM culture supernatants were frozen under similar conditions as described above. Those cell cultures that did not show visible giant cell formation after 12 subcultivations were passaged for at least an additional 5 to 10 subcultivations in the absence of the test compounds. Then, p24 determinations were performed on the culture supernatant fluids by p24 ELISA (duPont de Nemours) according to the manufacturer's procedure.

Sensitivity of Several HIV-1 Mutant Strains to Various Test Compounds in CEM Cells

CEM cells were suspended at 250,000 cells per mL of culture medium and infected with the mutant HIV-1 strains at 100 times the 50% cell culture infective dose ($CC_{ID_{50}}$) per mL. One $CC_{ID_{50}}$ is defined as the amount of virus that is infective to 50% of a series of identical cell cultures. Then, 100 μL of the infected cell suspensions were added to 200 μL microtiter plate wells containing 100 μL of serial (5-fold) dilutions of the test compounds, starting at 50 μg/mL as the highest drug concentration. After 4 days incubation at 37°, the cell cultures were examined for syncytium formation by microscopic examination. Quantification of giant cell formation was made by giving each culture an individual score on a scale with at least 8 gradations. Control HIV-1-infected cell cultures contained approximately 200 giant cells per microscopic view. The EC_{50} was determined as the compound concentration required to inhibit syncytium formation by 50%.

Preparation of Mutant HIV-1-Infected CEM Cell Cultures for Polymerase Chain Reaction (PCR) Analysis and Sequencing of the Pol Gene of the Mutant HIV-1 Strains

CEM cells infected with the HIV-1 mutant strains were incubated for 3 days, centrifuged, washed twice with PBS, and suspended in 10 μL PCR buffer [10 × concentrated: 100 mM Tris · HCl, pH 8.3, 800 mM KCl, 15 mM $MgCl_2$ and 0.01% (w/v) gelatin (Cetus-Vanderheyden), 8 μL $MgCl_2$ (25 mM), 72 μL Milli-Q water and 10 μL proteinase K (10 μg) (Calbiochem) in 0.5% Tween-20 and 0.5% NP-40 in H_2O]. The cell suspension was then incubated at 56° for 1 hr, and subsequently heated at 95° for 10 min. Amplification of proviral DNA was performed using a nested PCR protocol. In a first PCR reaction, 10 pmol/50 μL of primers JA99 and RIT137 [27] were used. Conditions for amplification reactions were 3′ at 94° followed by 35 to 50 cycles with 30″ at 96°, 30″ at 60° and 60″ at 72°. An aliquot of the first PCR was used for a second amplification with primers JA100 (27) and RIT138 (5′-Biotin-CT-GTCTTTTTCTGGTAGCACTATAGG), using the same protocol as above, but omitting the initial denaturation step. Single-stranded DNA obtained using streptavidin-coated magnetic beads (Dynal) served as a template for the dideoxy chain termination sequencing reaction. 5′-fluoresceinated oligonucleotides RT1SEQ2F(5′-CAATG-GCCATTGACAGAAG) and RT8KF(5′-CTGCATT-TACCATACCTAG) allowed the determination of HIV-1 provirus sequences corresponding to RT amino acids 35–242. Analysis of sequencing reactions was done with an Activated Laser Fluorescence (A.L.F.) automated DNA sequencer (Pharmacia).

Site-Directed Mutagenesis of HIV-1 Recombinant Reverse Transcriptase Leading to Insertion of NNRTI-Specific Amino Acid Mutations in an AZT-Resistance Background

Cloning and expression of HIV-1 (NY-5 strain) reverse transcriptase in *E. coli* was done as described elsewhere [28]. Point mutations were introduced in the RT gene either by gapped-duplex or polymerase chain reaction-mediated mutagenesis [29]. Purification of the recombinant RT mutants was essentially done as described by Sardana *et al.* [30] by a two step procedure using DE-52 cellulose (Whatman), and Mono S fast performance liquid chromatography (Pharma-

TABLE 1. Sensitivity of mutant HIV-1/III$_B$ strains to 3TC and NNRTI derivatives

Compound	EC_{50} (μg/mL)[a,b]							
	W.T.	100 Leu → Ile	103 Lys → Asn	106 Val → Ala	181 Tyr → Cys	190 Gly → Glu	184 Met → Ile	184 Met → Val
HBY 097	0.001	0.002	0.003	0.002	0.002	>10	0.001[c]	0.003[c]
TSAO-m^{3}T	0.024	0.042	0.138	2.2	2.0	>50	0.025	0.019
BHAP U-90,152	0.006	0.46	0.27	0.16	0.25	2	0.006	0.003
Nevirapine	0.030	0.10	0.50	2.3	2.5	>50	0.021	0.070
TIBO R82913	0.02	1.7	≥5	0.50	2.0	≥5	0.020	0.011
UC-10	0.05	0.085	≥1	0.130	0.075	>2	0.009	0.007
3TC	0.04	0.05	0.03	0.01	0.01	0.01	0.70	≥50

[a] 50% effective concentration, or compound concentration required to inhibit HIV-1(III$_B$)-induced giant cell formation in CEM cell cultures by 50%.
[b] Data are the means of at least 3 independent experiments.
[c] Data for quinoxaline S-2720.

cia). Fractions greater than 80% pure were used in the enzyme assays. The NNRTI-specific substitutions were 100 Leu → Ile, 103 Lys → Asn, 181 Tyr → Cys and 188 Tyr → His. These amino acid substitutions were introduced in an AZT-resistance background, i.e. 67 Asp → Asn + 70 Lys → Arg + 215 Thr → Tyr + 219 Lys → Gln, as described before [31].

RT Enzyme Assays

The RT assays contained in a total reaction mixture volume (50 μL) 50 mM Tris · HCl (pH 7.8), 5 mM dithiothreitol, 300 mM glutathione, 500 μM EDTA, 150 mM KCl, 5 mM $MgCl_2$, 1.25 μg of bovine serum albumin, appropriate concentrations of labeled substrate [8-^{3}H]dGTP (specific radioactivity 15.6 Ci/mmol) (Moravek Biochemicals), a fixed concentration of the template/primer poly(C) · oligo(dG)$_{12-18}$ (0.1 mM), 0.06% Triton X-100, 10 μL of inhibitor at various concentrations, and 1 μL of the RT preparation. The reaction mixtures were incubated at 37° for 15 min, at which time 100 μL of calf thymus DNA (150 μg/mL), 2 mL of $Na_4P_2O_7$ (0.1 M in 1 M HCl) and 2 mL of trichloroacetic acid (10% v/v) were added. The solutions were kept on ice for 30 min, after which the acid-insoluble material was washed and analyzed for radioactivity. In the experiments where the IC_{50} values of the test compounds were determined with respect to [8-^{3}H]dGTP, a fixed concentration of the natural substrate [8-^{3}H]dGTP (2.5 μM) was used.

RESULTS

Cross-Resistance/Sensitivity Spectrum of Mutant HIV-1 Strains against NNRTIs and 3TC

As a rule, the 3TC-resistant HIV-1 strains (containing the 184 Met → Ile/Val mutations) retained full sensitivity to the inhibitory effects of the NNRTIs, whereas the NNRTI-resistant HIV-1 strains were inhibited by 3TC at similar concentrations at which wild-type virus was inhibited (Table 1). However, among the NNRTI-resistant HIV-1 strains, the sensitivity/resistance spectrum against the NNRTIs was highly dependent on the nature of the amino acid change in the RT (Table 1). In general, HBY 097 proved superior to all other NNRTIs in inhibiting both wild-type and a series of mutant (resistant) HIV-1 strains. These observations together with our findings that 3TC and HBY 097 showed a complementary sensitivity/resistance spectrum against mutant HIV-1 strains, and the fact that many AZT-treated HIV-1-infected individuals may harbor virus strains that contain one or several AZT-specific mutations, led us to evaluate the activity of combinations of HBY 097 and 3TC against an HIV-1 strain containing several characteristic AZT mutations in its RT.

Inhibitory Effect of Several Combinations of HBY 097 and 3TC on AZT-Resistant Virus Breakthrough in the Presence or Absence of AZT

The HIV-1/AZT clone containing the 67 Asp → Asn, 70 Lys → Arg, 215 Thr → Phe and 219 Lys → Gln mutations was used in our study. When 3TC was added as a single drug to HIV-1/AZT-infected CEM cell cultures at 0.02 μg/mL, that is at a concentration that is inferior but close to its EC_{50}, no marked delay of virus replication was found (Table 2). However, at 0.05 μg/mL, 3TC delayed virus appearance in the drug-treated cell cultures by 2 to 4 days. HBY 097, added at concentrations as low as 0.001 (the EC_{50} value) and 0.0025 μg/mL (a drug concentration close to its EC_{90} value), delayed virus-induced cytopathicity by 1 to 2 days, and 14 to 16 days, respectively. Thus, a marked delay of virus breakthrough was obtained by 3TC (at 0.05 μg/mL), and, even more so by HBY 097 (at 0.0025 μg/mL). There was no statistical difference in the delay of virus breakthrough when the virus-infected cell cultures were treated with 3TC or HBY 097 in the continuous presence or absence of 0.03 μg/mL AZT (Table 2).

Combination of both drugs at their lowest concentrations (i.e., 3TC at 0.02 μg/mL and HBY 097 at 0.001 μg/mL) did not result in an additional delay of virus breakthrough. However, when HBY 097 at 0.001 μg/mL was combined with 3TC at 0.05 μg/mL, virus breakthrough as measured by the appearance of 50% cytopathicity was

TABLE 2. Inhibitory effect of double and triple combinations of lamivudine, HBY-097 and AZT on AZT-resistant virus breakthrough in CEM cell cultures

Compound	Concentration (μg/mL)	Mean day of HIV-1/AZT[a] breakthrough (50% cytopathicity) without AZT	Mean day of HIV-1/AZT[a] breakthrough (50% cytopathicity) +AZT (0.03 μg/mL)[b]
None	0	≤3	≤3
3TC	0.02	4	4
3TC	0.05	5	7
HBY 097	0.001	5	4
HBY 097	0.0025	17	19
3TC + HBY 097	0.02		
	0.001	8	4
3TC + HBY 097	0.05		
	0.001	17	19
3TC + HBY 097	0.02		
	0.0025	23	23
3TC + HBY 097	0.05		
	0.0025	38	59[c]

[a] AZT-resistant virus strain containing four mutations (i.e. 67 Asp → Asn, 70 Lys → Arg, 215 Thr → Phe and 219 Lys → Gln) in the RT.
[b] The AZT concentration was kept constant for all subcultivations.
[c] 63 days if AZT concentration was increased to 0.13 μg/mL after 23 days.

markedly delayed (Table 2). Where 3TC at 0.02 μg/mL was combined with HBY 097 at 0.0025 μg/mL, or HBY 097 at 0.001 μg/mL was combined with 3TC at 0.05 μg/mL, the continuous presence or absence of AZT did not affect the delay of virus breakthrough (Table 2).

In the HIV-1/AZT-infected cell cultures, the combination of HBY 097 at 0.0025 μg/mL with 3TC at 0.05 μg/mL did not result in full clearance of the virus from the cultures, but caused a further delay of virus breakthrough (5 to 7 days for 3TC and 17 to 19 days for HBY 097 as single drugs, versus 38 and 59 days for the drug combination). When the AZT concentration was increased from 0.03 to 0.13 μg/mL at day 23 postinfection, virus breakthrough was not markedly more delayed [63 days (in the presence of 0.13 μg/mL AZT) vs. 59 days (in the presence of 0.03 μg/mL AZT)] (Table 2).

Reverse Transcriptase Characterisation of Mutant HIV-1/AZT Strains that Emerged Under 3TC and HBY 097 Therapy

The HIV-1/AZT strain did not lose its AZT-specific resistance mutations during the selection process in the presence of 3TC and/or HBY 097 (Table 3). HBY 097 at 0.0025 μg/mL selected for an HIV-1/AZT mutant containing the 106 Val → Ala mutation both in the absence and in the continuous presence of 0.13 μg/mL AZT. In both cases, the mutant virus pool was not pure but contained a mixture of wild-type valine and mutant alanine at position 106 of the RT.

When in the drug combination the 3TC concentration was 0.02 μg/mL and that of HBY 097 0.0025 μg/mL, the 103 Lys → Glu HIV-1/AZT mutant (in the presence of 0.13 μg/mL AZT) arose in the virus-infected cell cultures.

In the HIV-1/AZT cell cultures treated with combinations of 3TC and HBY 097 in the presence of 0.03 and 0.13 μg/mL AZT, the 106 Val → Ala mutation appeared. The HIV-1/AZT strain did not acquire any additional mutations when the combination of 3TC and HBY 097 was administered in the absence of AZT (Table 3).

TABLE 3. Identification of additional mutations in the AZT-resistant[a,b] virus strains that emerged under single, double or triple drug combination therapy

Compound	Concentration (μg/mL)	Additional mutations Without AZT	Additional mutations +AZT (0.03 μg/mL)	Additional mutations +AZT (0.13 μg/mL)
3TC	0.02	none	none	none
3TC	0.05	none	none	none
HBY 097	0.001	none	none	none
HBY 097	0.0025	WT/106-Val → Ala[c]	none	WT/106-Val → Ala[c,d]
3TC + HBY 097	0.02			
	0.001	none	none	none
3TC + HBY 097	0.05			
	0.001	none	none	none
3TC + HBY 097	0.02			
	0.0025	none	none	103 Lys → Glu[e]
3TC + HBY 097	0.05			
	0.0025	none	106 Val → Ala	106 Val → Ala[f]

[a] AZT-resistant virus strain, containing 67 Asp → Asn, 70 Lys → Arg, 215 Thr → Phe and 219 Lys → Gln in the RT.
[b] The mutations in the RT gene were determined for those virus strains that were derived after full cytopathicity was obtained for at least 5 subsequent passages (3 to 4 days for each passage) in the presence of the indicated (single or combined) drug concentrations.
[c] Mixture of wild-type (WT) and 106 Val → Ala.
[d] Mutant virus strain isolated at day 40.
[e] Mutant virus isolated at day 40.
[f] Mutant virus isolated at day 80.

TABLE 4. Sensitivity of mutant HIV-1/AZT strains to NRTI and NNRTI derivatives

Compound	EC_{50}[a] (μg/mL)		
	Mutant virus strains		
	67 Asp → Asn 70 Lys → Arg 215 Thr → Phe 219 Lys → Gln	67 Asp → Asn 70 Lys → Arg 215 Thr → Phe 219 Lys → Gln 103 Lys → Glu (b)	67 Asp → Asn 70 Lys → Arg 215 Thr → Phe 219 Lys → Gln 106 Val → Ala (c)
HBY 097	0.002 ± 0.0005	0.005 ± 0.0024	0.006 ± 0.0
TSAO-m^3T	0.12 ± 0.06	1.0 ± 0.3	0.155 ± 0.35
BHAP U-90,152	0.015 ± 0.007	0.065 ± 0.021	0.075 ± 0.007
Nevirapine	0.065 ± 0.021	2.0 ± 0.7	5.0 ± 1.4
TIBO R82913	0.075 ± 0.035	0.55 ± 0.35	0.65 ± 0.21
UC-10	0.025 ± 0.007	0.15 ± 0.0	0.33 ± 0.11
3TC	0.11 ± 0.08	0.065 ± 0.021	0.08 ± 0.0
DDC	0.025 ± 0.013	0.017 ± 0.0	0.012 ± 0.001
AZT	≥25	≥25	18.7 ± 11.3
DDI	2.9 ± 0.83	3.5 ± 0.0	2.9 ± 0.83

[a] 50% effective concentration, or compound concentration required to inhibit HIV-1-induced giant cell formation by 50%. Data are the means of at least 2 to 3 independent experiments.
[b] Selected in the presence of AZT at 0.13 μg/mL + 3TC at 0.02 μg/mL + HBY 097 at 0.0025 μg/mL.
[c] Selected in the presence of AZT at 0.13 μg/mL + 3TC at 0.05 μg/mL + HBY 097 at 0.0025 μg/mL.

Sensitivity of Mutant HIV-1/AZT Strains to Various NNRTIs and NRTIs

HBY 097 retained a very pronounced inhibitory potency against the 103 Lys → Glu and 106 Val → Ala mutant HIV-1/AZT strains (EC_{50}: ~0.005 μg/mL), that is at a drug concentration that is only 2.5- to 3-fold lower than that required to inhibit the HIV-1/AZT strain that lacks the NNRTI-specific mutations in its RT genome (Table 4). BHAP U-90152, the thiocarboxanilide UC-10, TSAO-m^3T, and TIBO R82913 lost their antiviral potency by 5- to 10-fold, whereas nevirapine was 20- to almost 100-fold less inhibitory to the 103 Lys → Glu and 106 Val → Ala RT mutant HIV-1/AZT strains. All NRTIs (except AZT) virtually retained full sensitivity to the mutant HIV-1/AZT strains when compared to the parental HIV-1/AZT strain (Table 4).

Inhibitory Effects of Combinations of 3TC-TP and Quinoxaline HBY 097 Against Recombinant Wild-Type HIV-1 RT

A variety of combinations of various concentrations of 3TC-TP and HBY 097 were evaluated for their inhibitory activity against wild-type HIV-1 RT. Poly(I) · oligo(dC) was used as the template/primer and [^{3}H]dCTP (2.1 μM) as the radiolabeled substrate (Table 5). The triphosphate derivative of 3TC clearly had no synergistic, but rather an additive, inhibitory effect on HIV-1 RT when combined with HBY 097 at concentrations of 3TC-TP that inhibited the enzyme between 18 and 78% when used as a single drug.

Inhibitory Effects of HBY 097, Nevirapine and BHAP U90152 Against Mutant Recombinant HIV-1 RT Enzymes

A series of mutant HIV-1 RT enzymes, containing several NNRTI-specific mutations (i.e., 100 Leu → Ile, 103 Lys → Asn, 181 Tyr → Cys and 188 Tyr → His) in an AZT-resistance background (containing 67 Asp → Asn + 70 Lys → Arg + 215 Thr → Tyr + 219 Lys → Gln) were evaluated for their sensitivity to HBY 097, nevirapine and BHAP U90152 (Table 6). HBY 097 proved 60- to 250-fold more inhibitory to the wild-type HIV-1 and mutant HIV-1/AZT RTs than nevirapine and BHAP U90152. Interestingly, HBY 097 showed only 2- to 13-fold reduction of its inhibitory activity against the mutant HIV-1/AZT RTs that contained an additional NNRTI-specific amino acid mutation. The lowest inhibitory activity of HBY 097 was

TABLE 5. Inhibitory effect of combinations of 3TC-triphosphate with quinoxaline HBY 097 on HIV-1 reverse transcriptase

3TC-triphosphate (μg/mL)	HIV-1 RT activity (percent of control)[a,b]			
	HBY 097 (μg/mL)			
	0	0.0025	0.005	0.010
0	100	93 ± 3	71 ± 5	62 ± 2
0.1	82 ± 4	78 ± 1	64 ± 8	44 ± 11
0.2	73 ± 11	66 ± 6	56 ± 4	47 ± 5
0.4	42 ± 1	38 ± 0	28 ± 3	24 ± 8
0.8	22 ± 10	18 ± 7	15 ± 4	13 ± 4

[a] Poly(I) · oligo(dC) was used as the template/primer and 2.1 μM [^{3}H]dCTP as the radiolabeled substrate.
[b] Data are the means of at least 2 independent experiments.

TABLE 6. Sensitivity of mutant HIV-1/AZT RT enzymes to NNRTI derivatives

	IC_{50}[a,b] (μg/mL)					
	Mutant RT enzymes					
Compound	Wild-type	67 Asp → Asn 70 Lys → Arg 215 Thr → Tyr 219 Lys → Gln	67 Asp → Asn 70 Lys → Arg 215 Thr → Tyr 219 Lys → Gln + 100 Leu → Ile	67 Asp → Asn 70 Lys → Arg 215 Thr → Tyr 219 Lys → Gln + 103 Lys → Asn	67 Asp → Asn 70 Lys → Arg 215 Thr → Tyr 219 Lys → Gln + 181 Tyr → Cys	67 Asp → Asn 70 Lys → Arg 215 Thr → Tyr 219 Lys → Gln + 188 Tyr → His
HBY 097	0.006 ± 0.003	0.006 ± 0.001	0.013 ± 0.005	0.077 ± 0.006	0.027 ± 0.007	0.028 ± 0.003
Nevirapine	1.6 ± 1.2	0.77 ± 0.40	3.8 ± 0.7	24 ± 16	266 ± 148	2.3 ± 0.7
BHAP U-90152	0.45 ± 0.01	0.36 ± 0.01	4.9 ± 1.7	25 ± 14	22 ± 15	2.1 ± 0.03

[a] 50% inhibitory concentration, or compound concentration required to inhibit recombinant mutant HIV-1 RT enzymes by 50%.
[b] Data are the means of at least 2 independent experiments.

against the mutant HIV-1/AZT RT that contained an additional 103 Lys → Asn mutation in the AZT-resistance background (IC_{50}: 0.077 μg/mL). In contrast, nevirapine and BHAP U90152 lost at least 30-fold activity against this mutant enzyme. Moreover, nevirapine showed a 400-fold decreased activity against the 181 Tyr → Cys containing HIV-1/AZT RT enzyme, compared to 60-fold for BHAP U90152 and only 4.5-fold for HBY 097 (Table 6). Thus, HBY 097 retained a marked inhibitory activity against the mutant HIV-1/AZT RT enzymes that was by several orders of magnitude more pronounced than noted for nevirapine and BHAP U90152.

DISCUSSION

As demonstrated by our present findings, the paired combinations of 3TC with quinoxaline HBY 097 effected a marked suppression of the replication of AZT-resistant HIV-1 strains in CEM cell cultures. These observations are in agreement with our previous findings that paired combinations of NNRTIs, such as nevirapine and BHAP, with 3TC afford a pronounced suppression of wild-type HIV-1 strains [21]. However, the effect of the present paired combination of HBY 097 and 3TC on the suppression of the AZT-resistant HIV-1 strains proved far more pronounced. Moreover, the virus mutant strains that eventually emerged contained either no mutations in their RT or amino acid changes in their RT at sites that have been reported previously to play a role in NNRTI-resistance development (i.e., 103 Lys → Glu, 106 Val → Ala) [5, 17, 32].

It should be mentioned that the nature of the amino acid changes at position 103 of the RT found in this study (103 Lys → Glu) differed from those found for other NNRTIs. Indeed, 103 Lys → Asn, 103 Lys → Gln and 103 Lys → Thr mutations have been reported to appear in the presence of pyridinones [32–34], thiocarboxanilides [25], nevirapine [35], TIBO R82913 [36] and BHAP U-87201 [37]. It is presently not clear whether the AZT-resistance background is responsible for the appearance of the 103 Lys → Glu mutation in the RT, or whether this mutation can equally well appear in a wild-type genetic background under HBY 097 + 3TC drug pressure.

The mutant virus strains that emerged under relatively low HBY 097 selective pressure retained sensitivity to HBY 097 at concentrations that were several orders of magnitude lower than the serum levels that can be achieved in patients on HBY 097 therapy [38]. It should also be noted that the mutant HIV-1/AZT RT enzymes that contained a variety of NNRTI-specific amino acid mutations in addition to the AZT genetic resistance background retained sensitivity to HBY 097 at drug concentrations that are in the nanomolar range. These data are in full agreement with our antiviral data.

The molecular basis for the marked suppression or delay of the emergence of resistant virus strains may reside in the complementarity of the virus sensitivity spectrum of 3TC and HBY 097, rather than a potential synergistic activity of 3TC and quinoxaline against RT. Indeed, the combination of 3TC-triphosphate and HBY 097 achieved only additive inhibition of RT, with poly(I) · oligo(dC) as the template/primer (Table 5).

Cultivation of AZT-resistant HIV-1 strains in the presence of HBY 097 and/or 3TC did not result in loss of AZT resistance-specific mutations during the selection process, regardless of the absence or continuous presence of AZT in the cultures. In all cases, the 67 Asp → Asn, 70 Lys → Arg, 215 Thr → Phe and 219 Lys → Gln mutations in the HIV-1/AZT strain were retained. At the 3TC and HBY 097 drug concentrations used, the NNRTI-specific mutations were simply added to the genetic AZT resistance background. Similar findings were obtained under conditions where another AZT-resistant virus strain that contained the AZT resistance 41 Met → Leu and 215 Thr → Tyr mutation in its RT was used [39].

In conclusion, our observations strongly argue for the concomitant combination of 3TC and quinoxaline HBY 097 to suppress AZT-resistant virus replication and to delay, as long as possible, the emergence of drug-resistant virus strains in HIV-1-infected individuals. HBY 097 combined with 3TC selected for mutant virus strains that showed only minimal resistance (equal or less than 5-fold)

to HBY 097. Higher concentrations of the drugs (i.e. 0.05 μg/mL lamivudine + 0.0025 μg/mL HBY 097) resulted in a marked delay of virus replication and resistance development in the cell cultures. The concomitant combination of HBY 097 with 3TC at their highest possible doses (in the presence and/or absence of AZT) should be further explored in the clinical setting for its potential to suppress (AZT-resistant) HIV-1 replication in HIV-1-infected individuals.

This work was supported in part by the Biomedical Research Programme and the Human Capital and Mobility Programme of the European Commission, the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek (no. 3.3010.91), the Belgian Geconcerteerde Onderzoeksacties (no. 95/5) and Janssen Research Foundation. The authors are grateful to Dr. Vinod Sardana, Dr. John H. Condra and Dr. E. Emini (Merck Research Laboratories, West Point, PA) for generously providing the mutant RT enzymes. The excellent technical assistance of Mrs. Ann Absillis and Lizette van Berckelaer, and the fine editorial assistance of Mrs. Christiane Callebaut is greatly appreciated.

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