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Concomitant combination therapy for HIV infection preferable over sequential therapy with 3TC and non-nucleoside reverse transcriptase inhibitors

(lamivudine/HIV-1/resistance/combination therapy)

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ABSTRACT **Exposure to 3TC of HIV-1 mutant strains containing non-nucleoside reverse transcriptase inhibitor (NNRTI)-specific mutations in their reverse transcriptase (RT) easily selected for double-mutant viruses that had acquired the characteristic 184-Ile mutation in their RT in addition to the NNRTI-specific mutations. Conversely, exposure of 3TC-resistant 184-Val mutant HIV-1 strains to nine different NNRTIs resulted in the rapid emergence of NNRTI-resistant virus strains at a time that was not more delayed than when wild-type HIV-1(III_B) was exposed to the same compounds. The RTs of these resistant virus strains had acquired the NNRTI-characteristic mutations in addition to the preexisting 184-Val mutation. Surprisingly, when the 184-Ile mutant HIV-1 was exposed to a variety of NNRTIs, the 188-His mutation invariably occurred concomitantly with the 184-Ile mutation in the HIV-1 RT. Breakthrough of this double-mutant virus was markedly accelerated as compared with the mutant virus selected from the wild-type or 184-Val mutant HIV-1 strain. The double (184-Ile + 188-His) mutant virus showed a much more profound resistance profile against the NNRTIs than the 188-His HIV-1 mutant. In contrast with the sequential chemotherapy, concomitant combination treatment of HIV-1-infected cells with 3TC and a variety of NNRTIs resulted in a dramatic delay of virus breakthrough and resistance development.**

The clinical use of selective HIV inhibitors targeted at the virus-encoded reverse transcriptase (RT) or protease has been hampered by the ability of the virus to become drug resistant within a relatively short time. There is now accruing evidence that future treatment modalities of HIV-1 infection should obligatorily include a combination of compounds, preferably those drugs that contain a complementary resistance spectrum against the target enzyme and/or that are directed against different targets in the viral replication cycle.

In fact, both the non-nucleoside RT inhibitors (NNRTIs) and the nucleoside RT inhibitor (NRTI) (−)2′,3′-dideoxy-3′-thiacytidine (3TC, lamivudine) rapidly select for drug-resistant virus strains in HIV-1-infected cell cultures and in drug-treated HIV-1-infected patients (1–9). NNRTIs select for a variety of amino acid mutations (6–12). The rate of appearance and level of (cross)-resistance to the NNRTIs highly depend on the location and the nature of the amino acid changes in the RT (12, 13). In contrast, 3TC consistently selects for either 184-Ile or (predominantly) 184-Val in the RT (normally 184-Met) (1–4, 10). In patients, the 184-Ile substitution in the RT appears first, but is replaced by 184-Val during continued 3TC chemotherapy (11).

The 184-Val RT has also been shown to have a higher fidelity of nucleotide incorporation. This was thought to incapacitate HIV-1 to acquire additional resistance-conferring mutations specific for other drugs such as NNRTIs (5). Hence, the concept of sequential therapy starting with 3TC as a first line therapy was introduced (5). Here we report data that strongly support simultaneous combination therapy with 3TC and NNRTIs. We found that the sequential therapy with 3TC and NNRTIs allowed the virus to more rapidly accumulate mutations to both 3TC and NNRTIs. On the contrary, concomitant combination of 3TC with NNRTIs led to a marked delay of HIV drug-resistance development.

MATERIALS AND METHODS

Test Compounds. TIBO R82913 was kindly provided by Zhang Hao (National Institutes of Health, Bethesda) or obtained from PharmaTech International (West Orange, NJ). 8-Chloro-TIBO and α-APA were supplied by Janssen. Both 3TC and its 5′ triphosphate-derivative 3TC-TP were kindly provided by J. Cameron (Glaxo-Wellcome, Stevenage, U.K.). Nevirapine (BI-RG-587) and pyridinone L-697,661 were provided by P. Ganong (Boehringer Ingelheim) and M. Goldman (Merck Sharp & Dohme), respectively. BHAP U-90152 and quinoxaline HBY 097 were synthesized by R. Kirsch (Hoechst Pharmaceuticals, Frankfurt). MKC-442 was kindly provided by M. Baba (Fukushima Medical College, Fukushima, Japan) and TSAO-m^3T was a kind gift from M.-J. Camarasa (Consejo Superior de Investigaciones Cientificas, Madrid). The oxathiin carboxanilide derivatives UC-10 and UC-781 were obtained from Uniroyal Chemical Ltd. (Middlebury, CT).

Cells and Viruses. CEM cells were obtained from the American Type Culture Collection. HIV-1(III_B) was originally obtained from the culture supernatant of persistently HIV-1-infected H9 cells and was provided by R. C. Gallo and M. Popovic (National Institutes of Health).

Sensitivity of Several HIV-1 Mutant Strains to the Various Test Compounds in CEM Cell Cultures. CEM cells were suspended at 250,000 cells per ml of culture medium and infected with wild-type HIV-1(III_B) or mutant HIV-1 strains at 100 cell cuture infective dose-50 ($CCID_{50}$) per ml. Then 100 μl of the infected cell suspensions was added to 200-μl microtiter plate wells containing 100 μl of an appropriate dilution of the test compounds. After 4 days incubation at 37°C, the cell cultures were examined for syncytium formation. The 50% effective concentration (EC_{50}) was determined as the compound concentration required to inhibit syncytium formation by 50%.

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Abbreviations: RT, reverse transcriptase; NNRTI, non-nucleoside RT inhibitor; NRTI, nucleoside RT inhibitor.

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Selection of 3TC-Resistant Virus Strains Containing NNRTI-Specific Amino Acid Mutations in Their RT. HIV-1(III_B) and mutant HIV-1 strains derived thereof were 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 (i.e., 0.5 and 2.5 μg/ml) in 25-cm^2 culture flasks to produce mutant virus strains that were able to grow in the presence of 3TC. The initial virus input in each cell culture consisted of $\approx$500 μl of supernatant, which was obtained from an HIV-1-infected CEM cell culture at the time that full cytopathicity was present. 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 0.5–1.0 ml of the infected culture supernatant 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°C after the syncytium formation became abundant in the cell cultures, and the virus was further passaged for at least five additional subcultivations in the presence of 3TC.

Selection of NNRTI-Resistant Virus Strains Starting from Wild-Type, 184-Ile RT, and 184-Val RT Mutant Virus. The selection procedure was essentially the same as described above. The initial virus input in the 5-ml cell culture bottles was 1.5 μg p24 for HIV-1/III_B, 1.2 μg p24 for 184-Ile HIV-1, and 3.7 μg p24 for 184-Val HIV-1, respectively. The virus was derived from the supernatant of freshly infected CEM cell cultures at day 4 post infection when full cytopathicity was recorded.

Drug Combination Assays. In the drug combination experiments (3TC with either BHAP, TSAO-m^3T, UC-10, or MKC-442), the compounds were combined at the same initial concentrations as used for single-drug treatment. Drug concentrations were not increased during subcultivations and the treated HIV-1-infected CEM cell culture supernatants were frozen in aliquots at −70°C after abundant syncytium formation became evident. Those cell cultures that did not show visible giant cell formation after 12 subcultivations were further passaged for at least an additional 10 subcultivations in the absence of the test compounds. Then, p24 determinations were performed on the culture supernatant fluids by p24 ELISA (DuPont) according to the manufacturer's instructions.

Preparation of Mutant HIV-1-Infected CEM Cell Cultures for PCR Analysis and Sequencing of the DNA Polymerase 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 phosphate-buffered saline, and suspended in 10 μl polymerase chain reaction (PCR) buffer [10× concentration: 100 mM Tris·HCl, pH 8.3/800 mM KCl/15 mM $MgCl_2$/0.01% (wt/vol) gelatin (Cetus–Vanderheyden, Brussels), 8 μl $MgCl_2$ (25 mM), 72 μl Milli-Q water, 10 μl proteinase K (10 μg) (Calbiochem) in 0.5% Tween 20 and 0.5% Nonidet P-40 in H_2O]. The cell suspension was then incubated at 56°C for 1 hr and subsequently heated at 95°C for 10 min. The method to amplify part of the RT gene of the mutant viruses that appeared in cell culture, and to determine the amino acid sequence of the mutant RTs, was as follows. In a first PCR reaction 10 pmol/50 μl of primers JA99 and RIT137 were used (14). Conditions for amplifications were 3′ 94°C, 35–50 cycles with 30″ 96°C, 30″ 60°C, and 60″ 72°C in 0.2 mM of each dNTP, 10 mM Tris·HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$. An aliquot of the first PCR was used for a nested amplification with primers JA100 (14) and RIT138 (5′-Biotin-CTGTCTTTTTCTGGTAGCACTATAGG), using the same protocol as above, omitting the initial denaturation step. Single-stranded DNA obtained using streptavidin-coated magnetic beads (Dynal, Oslo) served as a template for the dideoxy chain-termination sequencing reaction, 5′-fluoresceinated oligonucleotides RT1SEQ2F (5′-CAATGGCCATTGACAGAAG) and RT8KF (5′-CTGCATTTACCATACCTAG) allowed the determination of HIV-1 provirus sequences corresponding to RT amino acids 35–242. Analysis of sequencing reactions was done with an automated laser fluorescence DNA sequencer (Pharmacia).

Inhibitory Effect of Combination of 3TC-TP and NNRTIs Against HIV-1 RT. The assay procedure for measuring the inhibitory effect of test compounds against HIV-1 RT has been described (15). Poly(I)·oligo(dC) (0.015 mM) was used as the template/primer and [5-^{3}H]dCTP [2 μCi/assay (1 Ci = 37 GBq) in 50 μl] (2.5 μM) as the radiolabeled substrate. Inhibition of HIV-1 RT activity by the combination of different concentrations of 3TC-TP (0.8, 0.4, 0.2, 0.1, and 0 μg/ml) and various concentrations of nevirapine (50, 20, 8, and 0 μg/ml), BHAP U-90152 (5, 2, 0.8, and 0 μg/ml), MKC-442 (1, 0.5, 0.25 and 0 μg/ml), quinoxaline HBY 097 (0.01, 0.005,

Table 1. Breakthrough of mutant HIV-1/III_B strains in CEM cell cultures in the presence of 3TC

Mutant RT virus strain	3TC concentration, μg/ml	Mean day of virus breakthrough (50% cytopathicity)	Additional amino acid mutation in the RT
103-Asn	0.5	17	ND
	2.5	28	103-Asn + 184-Ile/Thr
106-Ala	0.5	26	ND
138-Lys	0.5	13	138-Lys + 184-Ile
	2.5	19	138-Lys + 184-Ile
181-Cys*	0.5	36	181-Cys + 184-Ile
	2.5	>45†	NA
181-Cys*	0.5	22	181-Cys + 184-Ile
	2.5	>45	NA
190-Glu	0.05	>45	NA
	0.2	>45	NA
	0.5	>45	NA
	2.5	>45	NA
Wild type	0.5	22	ND
	2.5	32	184-Val
	0	2.5	NA

ND, not determined; NA, not applicable.

*These 181-Cys mutant viruses represent two independent isolates.

†At day 45 of the experiment (13th passage), no cytopathicity was detectable. Therefore, the value of mean day of virus breakthrough was indicated as >45.

Table 2. Breakthrough of wild-type HIV-1(III_B) and 184-Ile mutant HIV-1 and 184-Val mutant HIV-1 strains in CEM cell cultures in the presence of a variety of NNRTIs

		Wild-type HIV-1		184-Ile mutant HIV-1		184-Val mutant HIV-1	
Compound	Initial concentration, μg/ml	Virus breakthrough*†	Mutations in the RT	Virus breakthrough*†	Mutations in the RT	Virus breakthrough*†	Mutations in the RT
Pyridinone L-697661	0.05	5 (3)	ND	5 (3)	ND	3 (3)	ND
	0.25	5 (3)	None	5 (3)	184-Ile	5 (3)	184-Val
Nevirapine	0.1	7 (7)	ND	5 (5)	ND	5 (5)	ND
	0.5	20 (14)	106-Ala	9 (7)	184-Ile	23 (11)	184-Val + 188-Cys/WT(Tyr) + 190-Ala/WT(Gly)
BHAP U-90152	0.1	23 (11)	236-Leu	7 (7)	ND	17 (11)	184-Val + 236-Leu
	0.5	—‡	NA‡	15 (11)	184-Ile + 188-His	34 (32)	184-Val + 236-Leu
8-chloro-TIBO	0.025	25 (18)	101-Glu	9 (7)	ND	27 (18)	184-Val + 106-Ala
	0.1	—	NA	13 (7)	184-Ile + 188-His	30 (25)	184-Val + 106-Ile
α-APA (Loviride)	0.025	16 (7)	ND	5 (3)	ND	13 (7)	ND
	0.1	23 (11)	181-Cys	13 (7)	184-Ile + 188-His	23 (18)	184-Val + 181-Cys
Quinoxaline HBY 097	0.005	33 (21)	101-Glu	13 (7)	184-Ile + 188-His	—	NA
	0.02	—	NA	20 (11)	184-Ile + 188-His	—	NA
TSAO-m^3T	0.1	9 (3)	ND	5 (3)	ND	5 (3)	ND
	0.5	16 (11)	138-Lys	9 (7)	184-Ile	13 (7)	184-Val + 138-Lys/WT(Glu)
Thiocarboxanilide UC-10	0.1	20 (11)	179-Asp	9 (7)	ND	18 (7)	184-Val + 101-Arg/WT(Lys) + 103-Asn/WT(Lys)
	0.5	—	NA	20 (11)	184-Ile + 188-His	—	NA
Thiocarboxanilide UC-781	0.01	20 (11)	106-Ala/WT(Val)	11 (7)	184-Ile + 188-His	18 (14)	184-Val + 181-Cys
	0.05	—	NA	18 (11)	184-Ile + 188-His	—	NA
MKC-442 (I-EBU)	0.05	23 (11)	181-Cys	9 (7)	ND	25 (14)	184-Val + 188-His
	0.25	—	NA	13 (7)	184-Ile + 188-His§	48 (35)	184-Val + 188-His

ND, not determined; NA, not applicable.
*Data indicate the mean day required for 50% cytopathicity to develop (after initiation of the experiment).
†Values in parentheses represent the first day of microscopically visible virus breakthrough.
‡Virus breakthrough was completely suppressed in the presence of the drugs, even after further subcultivation of the cell cultures in the absence of the drugs.
§This virus strain was also shown to contain a 204-Asp mutation in its RT.

0.0025, and 0 μg/ml), α-APA (20, 10, 5, 2.5, and 0 μg/ml), UC-10 (5, 2, 0.8, and 0 μg/ml), and UC-781 (0.08, 0.04, 0.02, 0.01, 0.005, and 0 μg/ml) was examined.

RESULTS

Treatment of Mutant HIV-1 Strains Containing NNRTI-Specific Mutations in Their RT with 3TC. CEM cell cultures were infected by a variety of mutant virus strains that had emerged in the presence of different NNRTIs. The cells were then treated with 3TC at 0.5 or 2.5 μg/ml. The breakthrough of the wild-type and mutant virus strains was suppressed in a concentration-dependent manner in the presence of 3TC (Table 1). At a concentration of 0.5 μg/ml, 3TC delayed virus breakthrough for 17–36 days. At 2.5 μg/ml, 3TC suppressed the replication of the mutant 181-Cys HIV-1 strains and the mutant 190-Glu HIV-1 strain for at least 45 days post infection. When starting with wild-type HIV-1, 3TC selected for the 184-Val mutation in the RT. However, when starting with virus strains that already contained the NNRTI-specific mutations in their RT, 3TC treatment gave rise to the 184-Ile, but not the 184-Val mutation. Meanwhile, the preexisting NNRTI-specific resistance mutations were retained (Table 1). Thus, none of the NNRTI-specific mutations disappeared when the virus was exposed to the 3TC-resistance selection process. The double-mutated virus strains became resistant to 3TC, while maintaining their original resistance/sensitivity profile for the different NNRTIs (data not shown). The additionally acquired resistance to 3TC was apparently due to the appearance of the 184-Ile mutation in the RT. Interestingly, at concentrations as low as 0.2 and 0.05 μg/ml, 3TC fully suppressed the 190-Glu mutant virus in CEM cell cultures (Table 1).

Treatment of Mutant HIV-1 Strains Containing the 184-Ile or 184-Val Mutation in Their RT with a Variety of NNRTIs. CEM cell cultures were exposed to wild-type HIV-1, 184-Ile mutant HIV-1, or 184-Val mutant HIV-1 strains and treated with 10 different NNRTIs belonging to 9 different structural classes (Table 2). It has been previously ascertained that there is no marked difference in the EC_{50} (50% effective concentration) of the individual NNRTI drugs against wild-type, 184-Ile, and 184-Val mutant virus strains (10). In all cases, virus broke through in the cell cultures, in the presence of the drugs at 3 times their EC_{50}, and, in most cases, also in the presence of the drugs at 15 times their EC_{50} (Table 2). The mean day of breakthrough of the 184-Val mutant virus in the presence of the NNRTIs did not markedly differ from that of the wild-type HIV-1(III_B).

As a rule, the 184-Ile mutant virus was much less efficiently suppressed in the CEM cell cultures by the individual NNRTIs than were the corresponding 184-Val mutant or parental wild-type virus strains. The markedly accelerated breakthrough of the 184-Ile mutant virus strain in the presence of NNRTIs in the CEM cell cultures was confirmed in an independent set of experiments with either CEM or C8166 cell cultures (data not shown). Moreover, whereas a number of NNRTIs (i.e., quinoxaline and thiocarboxanilides) were able to fully suppress ("knock-out") wild-type and mutant 184-Val HIV-1 at higher concentrations, they failed to do so for the 184-Ile HIV-1 mutant (Table 2).

When the wild-type, 184-Ile, or 184-Val mutant virus-infected cell cultures were incubated in the concomitant presence of the NNRTIs and 3TC (0.5 μg/ml), the breakthrough of 184-Val HIV-1 was not affected and the breakthrough of 184-Ile HIV-1 was slightly delayed, whereas the breakthrough of wild-type virus was markedly delayed (data not shown). These observations can be explained by the strong resistance of 184-Val HIV-1 to 3TC ($EC_{50} > 50$ μg/ml), the moderate susceptibility of 184-Ile HIV-1 to 3TC (EC_{50}: ≈1-5 μg/ml), and the high sensitivity of wild-type HIV-1 to 3TC (EC_{50}: 0.008–0.05 μg/ml). Thus, the concomitant presence of 3TC during the incubation of the 184-Val HIV-1- and 184-Ile HIV-1-infected cell cultures with the NNRTIs did not markedly alter resistant virus breakthrough in the cell cultures, as compared with that noted for the NNRTIs in the absence of 3TC (Table 2).

When the mutant virus strains that emerged in the NNRTI-treated CEM cell cultures were analyzed for the presence of amino acid changes in their RT, the following observations were made (Table 2). (*i*) The 184-Val and the 184-Ile mutations were retained in the RTs when the corresponding mutant viruses were exposed to the different NNRTIs. (*ii*) The characteristic NNRTI-specific amino acid changes (i.e., at amino acid positions 106, 138, 179, 181, and 236 in the RT) were found following exposure of wild-type HIV-1 to the NNRTIs. (*iii*) One or several NNRTI-specific mutations were added to the 184-Val mutation during the NNRTI selection procedure. (*iv*) Surprisingly, however, the 188-His substitution was consistently and invariably added to the 184-Ile RT mutation, irrespective of the nature of the NNRTI.

Resistance/Sensitivity Spectrum of Mutant HIV-1 Strains to NNRTIs and NRTIs. BHAP, TSAO-m^3T, MKC-442 and α-APA became 167- to 960-fold less inhibitory, and HBY 097, nevirapine, UC-781, and pyridinone 19- to 58-fold less inhibitory to the double-mutant 188-His + 184-Ile virus strain, whereas 3′-azido-3′-deoxythymidine (AZT) and dideoxyinosine kept their full inhibitory effect (Table 3). Thus, the resistance of the double-mutated 184-Ile + 188-His virus to the NNRTIs was significantly higher than that of the single-

Table 3. Inhibitory activity of NNRTIs and NRTIs against mutant HIV-1/III_B strains in CEM cell cultures

Compound	Fold increase of EC_{50} versus wild-type virus					
	Wild type*	184-Ile	184-Val	188-His	184-Ile + 188-His	184-Val + 188-His
Nevirapine	1 (0.03)	0.67	2.3	1.7	28	6.7
BHAP U-90152	1 (0.006)	1.0	0.50	10	167	83
Pyridinone L-697661	1 (0.017)	0.76	0.41	—	58	2.9
TSAO-m^3T	1 (0.021)	1.2	0.71	71	381	38
9-chloro-TIBO	1 (0.02)	1.0	0.50	45	—	—
α-APA	1 (0.005)	2.8	0.80	100	960	70
MKC-442	1 (0.002)	1.0	1.0	—	390	25
UC-10	1 (0.029)	0.30	0.28	6.9	7.9	2.7
UC-781	1 (0.002)	—	—	8.0	37	8.0
HBY 097	1 (0.001)	1.0	3.0	2.0	19	5.0
AZT	1 (0.001)	1.7	1.0	1.3	1.2	1.5
ddI	1 (0.92)	2.8	2.6	0.49	1.3	1.3
3TC	1 (0.01)	70	≥5,000	0.60	280	>2,000

*Values in parentheses represent the 50% effective concentration (EC_{50}) values of the drugs for wild-type HIV-1/III_B (in μg/ml). ddI, dideoxyinosine.

Table 4. Effect of combinations of 3TC and NNRTIs on HIV-1 breakthrough in CEM cell cultures

Compound	Concentration, μg/ml	Mean day of HIV-1 breakthrough (50% cytopathicity)			
		3TC concentration, μg/ml			
		0	0.02	0.05	0.1
BHAP U-90152	0.02	6	27	34	47
	0.04	8	29	64	>52*
TSAO-m^{3}T	0.1	6	19	34	>52†
	0.4	13	23	>52†	>52*
MKC-442	0.02	15	33	>52*	>52*
	0.04	15	50	>52*	>52*
UC-10	0.05	6	33	>52†	>52*
	0.2	21	42	>52*	>52*
None		3	4	6	13

*The cell culture remained p24 negative, even after removal of the drugs at day 52 post infection, and continued cultivation of the cells in the absence of the test compounds for an additional 12 subcultivations.

†When the drugs were washed out at day 52 post infection and the cell cultures were further passaged in the absence of the test compounds, virus breakthrough (50% cytopathicity) occurred on day 57 (for TSAO-m^{3}T) and day 66 (for UC-10).

mutated 188-His HIV-1 and of the double-mutated 184-Val + 188-His HIV-1.

Concomitant Treatment of HIV-1 by 3TC and NNRTIs. Because our data evidently do not support the sequential use of 3TC and NNRTIs in attempts to delay or suppress the emergence of drug-resistant virus strains, a number of NNRTIs were combined with 3TC at different concentrations of each individual drug and simultaneously added to the HIV-1/III$_B$-infected CEM cell cultures (Table 4). Combination of 3TC with NNRTIs resulted in a marked delay of virus breakthrough at relatively low concentrations of the individual test compounds. Moreover, complete suppression of virus breakthrough was obtained in the combination experiments at drug concentrations that were up to 50-fold lower than those required for the drugs used individually (Table 4). This is in sharp contrast with the virus breakthrough that was consistently seen following the sequential use of 3TC and NNRTIs (Table 2).

Anti-RT Activity of Combinations of 3TC and NNRTIs. 3TC-TP, the active triphosphate metabolite of 3TC, has a merely additive, rather than synergistic, inhibitory effect on HIV-1 RT when combined with the NNRTIs nevirapine and BHAP (Table 5), and HBY 097, α-APA, MKC-442, UC-10, or UC-781 (data not shown). Thus, the exquisite antiviral potential of 3TC in combination with the NNRTIs shown in Table 4 cannot be accounted for by a merely synergistic action of the test compounds at the level of HIV-1 RT.

DISCUSSION

It has been reported by several investigators that the 3TC-resistant 184-Val HIV-1 RT is endowed with a higher fidelity than the wild-type virus RT (5, 16). Boyer and Hughes (17) have reported that the RNA-dependent DNA polymerase activity of the 184-Val RT and 184-Ile RT was 75% and 65% that of the wild-type enzyme, respectively, and the 184-Ile RT showed lower processivity than the 184-Val RT. Wainberg and collaborators (5) postulated that the increased fidelity of the RT containing the 184-Val mutation might also explain why the 184-Val mutant virus strains did not gain additional resistance to other drugs such as NNRTIs, AZT, d4T, or protease inhibitors. In our experiments, there was no marked delay in virus breakthrough when CEM cells infected with the 184-Val HIV-1 strain were incubated in the presence of a variety of NNRTIs. We even observed an accelerated virus breakthrough when CEM cells were exposed to 184-Ile HIV-1 and incubated in the presence of the various NNRTIs. Whether the accelerated breakthrough of the double-mutant virus in the presence of the various NNRTIs is due to the acquirement of the 188-His mutation in its RT is now the subject of further investigation.

Because the enormous virus replication dynamics and virus plasma load *in vivo* exceed by several orders of magnitude those present in cell culture experiments (18, 19), it is expected that the mutations that emerge during such an intensive virus replication may easily counteract any potentially decreased mutation rate resulting from the higher fidelity of the mutant RT. Moreover, if our observations on the accelerated breakthrough of 184-Ile mutant virus in the presence of NNRTIs can be extrapolated to man, it would seem contraindicated to successively use 3TC and NNRTIs. Such procedure (sequential therapy) may even precipitate virus breakthrough. Also, it has been shown that ratios of $CCID_{50}$ to p24 are at least four times higher in culture fluids of peripheral blood mononuclear cell and MT-4 (5) or CEM cells (personal observations) infected by the 184-Val HIV-1 mutant than in cells infected with HIV-

Table 5. Inhibitory effect of combinations of 3TC-TP with nevirapine and BHAP U-90152 on HIV-1 RT

3TC-TP, μg/ml	HIV-1 RT activity, % of control						
	Nevirapine, μg/ml				BHAP U-90152, μg/ml		
	0	8	20	50	0.8	2.0	5.0
0	100	71 ± 8	48 ± 8	30 ± 8	70 ± 13	53 ± 3	34 ± 5
0.1	82 ± 5	56 ± 4	42 ± 2	25 ± 3	54 ± 2	42 ± 4	32 ± 2
0.2	68 ± 4	51 ± 6	38 ± 4	25 ± 1	52 ± 4	37 ± 7	25 ± 6
0.4	40 ± 2	35 ± 4	26 ± 0	22 ± 4	36 ± 4	30 ± 4	22 ± 0
0.8	25 ± 5	22 ± 2	17 ± 4	13 ± 3	19 ± 2	18 ± 4	14 ± 6

Inhibition of HIV-1 RT activity by the combination of different concentrations of nevirapine and BHAP U-90152 and various concentrations of 3TC-TP was performed as described (15). Poly(I)·oligo(dC) and [^{3}H]dCTP (2.1 μM) were used as template/primer and fixed substrate, respectively.

$1/III_B$ (wild type). One should therefore avoid giving the virus the opportunity to convert into a more infectious form upon treatment with 3TC as a single agent.

It was surprising to note that exposure of NNRTIs to 184-Ile mutant virus strains invariably selected for the appearance of the same amino acid mutation (i.e., 188-His) in the 184-Ile genetic background. These findings are unprecedented in that it has never been observed that the pressure of different RT inhibitors on wild-type or mutant viruses consistently results in the appearance of an identical mutation. Moreover, this double-mutant virus strain proved more resistant to NNRTIs than could have been anticipated from the resistance spectrum of 188-His HIV-1 to the NNRTIs. The underlying mechanism of this phenomenon is currently under investigation.

It has been shown that the 190-Glu-substituted HIV-1 RT has a markedly decreased activity as compared with the wild-type RT (20). It is presently unclear whether combination of the 190-Glu and 3TC resistance (184-Ile /Val) mutations in the RT does affect the activity of the double-mutant enzyme to a greater degree than the combination of the 3TC resistance mutations with other NNRTI-specific amino acid mutations.

Because we found that 3TC is inhibitory to NNRTI-resistant HIV-1 strains, and, vice versa, NNRTIs efficiently inhibit 3TC-resistant viruses (10), the marked suppressive effect noted with the combination of 3TC and NNRTIs may be attributed to a complementary suppression of the NNRTI- and 3TC-specific resistance mutations by 3TC and NNRTIs, respectively. Instead of using 3TC and NNRTIs in sequential order, our studies indicate that 3TC should be simultaneously combined with the NNRTIs. This strategy should be aimed at delaying as long as possible the emergence of drug-resistant virus strains.

The durability of this virus suppression in drug-treated patients remains to be assessed and cannot be predicted from our cell culture data. Indeed, as mentioned before (17, 18), AIDS patients contain virus titers that are by several orders of magnitude higher than the virus titers present *in vitro*. However, these considerations should by no means detract from pursuing the treatment of HIV-infected individuals with combinations of drugs that have a different mechanism of antiviral action such as 3TC and NNRTIs.

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