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Lamivudine-Resistant Human Immunodeficiency Virus Type 1 Variants (184V) Require Multiple Amino Acid Changes to Become Co-Resistant to Zidovudine In Vivo

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Exposure of human immunodeficiency virus to the nucleoside analogue lamivudine (3TC) rapidly selects for resistant variants with a valine at codon 184 (M184V) in the catalytic site of reverse transcriptase. *In vitro*, 184V demonstrated increased enzyme fidelity and suppressed zidovudine resistance. Clinical trials demonstrated that 3TC-zidovudine combination therapy results in a strong and sustained antiviral response. To investigate the role of 184V on *in vivo* virus evolution, the effect of zidovudine addition in 3TC-pretreated patients harboring 184V was studied. *In vivo*, no significant change in fidelity was observed with 184V, shown by generation of the classical pattern of zidovudine mutations. Of interest, in contrast to zidovudine monotherapy, in which just one substitution is sufficient for *in vivo* development of significant zidovudine resistance, multiple substitutions are required for the same level of zidovudine resistance in strains harboring 184V. This need for multiple substitutions may be one of the mechanisms explaining the sustained antiretroviral response of the 3TC-zidovudine combination.

In vitro and *in vivo* exposure of human immunodeficiency virus type 1 (HIV-1) to the reverse transcriptase (RT) inhibitor lamivudine (3TC) results in the selection of viruses with a 500- to 1000-fold reduced susceptibility [1–6]. This reduced susceptibility is caused by mutations at codon 184 of the RT gene: The wild type methionine residue is replaced by either an isoleucine (M184I) or a valine residue (M184V) [1–6]. Codon 184 is located within the active site of RT (YMDD motif) [7–9]. Changes at this codon affect the enzymatic properties of the enzyme [10–12]. An increased fidelity was described for the 184V-containing RT [13]. It has been speculated that changes in fidelity may influence the evolution of the virus and may prevent or delay the generation of drug-resistant variants [13]. Additionally, 184V has been described to suppress zidovudine resistance *in vitro* [3, 4]. *In vivo*, combination therapy with 3TC and zidovudine results in a strong and sustained antiviral response [14, 15] (van Leeuwen R, unpublished data). To determine to what extent the increased fidelity and

the zidovudine resistance-suppressing effect could help to explain the *in vivo* synergistic result of this combination, we studied the effect of addition of zidovudine on 4 patients continuously treated with 3TC and harboring a virus population encoding the 184V.

Materials and Methods

Study population. The original study population consisted of 40 HIV-1-infected patients treated with 3TC [16]. Zidovudine was added to 3TC therapy when patients had been treated with 3TC for at least 24 weeks, had a CD4 cell count decline of at least 50% of the baseline values, or had been diagnosed with an AIDS-defining illness according to the 1987 Centers for Disease Control and Prevention criteria (van Leeuwen R, unpublished data).

Four patients were selected for further studies (C0001, C0011, C0020, and C0034). These patients, randomly selected from a subset of patients who were antiretroviral therapy-naïve before receiving 3TC monotherapy, were subsequently treated with a combination of 3TC and zidovudine. The 3TC monotherapy period consisted of 0.5 mg/kg/day for 78 weeks for patient C0001, 1 mg/kg/day for 71 weeks for patient C0011, 2 mg/kg/day for 84 weeks for patient C0020, and 12 mg/kg/day for 46 weeks for patient C0034. During the combination therapy period, patients C0001, C0011, and C0020 were treated with 200 mg/day 3TC and 600 mg/day zidovudine for 101, 101, and 81 weeks, respectively. Patient C0034 received 600 mg/day 3TC and 600 mg/day zidovudine for 103 weeks. Patient C0034 developed an acute cytomegalovirus retinitis at week 56 of the combination therapy period and was continuously treated with foscarnet (6000 mg/day) until the end of the observation period.

Plasma (heparinized) and peripheral blood mononuclear cells (PBMC) were collected from patients at the start of 3TC treatment, at the moment zidovudine was added, and at regular intervals

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Written informed consent was obtained from all subjects for their participation in the formal 3TC protocols that they subsequently enrolled in, and all of the major amendments to these protocols.

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during 2 years of 3TC and zidovudine combination therapy. Heparinized plasma was stored at -70°C , and PBMC were stored in liquid nitrogen.

CD4 cell count. CD4 cell counts in peripheral blood were determined by flow fluorocytometry, as previously described [17].

Viral RNA analysis. RNA was extracted from 100 μL of heparinized plasma according to the method described by Boom et al. [18]. Subsequently, the isolated RNA was quantified by use of the prototype Roche assay [19], and the N-terminal part of the RT gene (nucleotides 2453–3461) was reverse-transcribed and amplified by use of a one-tube RT–polymerase chain reaction (PCR) procedure [20].

Proviral DNA analysis. DNA was extracted from 2×10^6 patient PBMC according to the method described by Boom et al. [18]. Subsequently, the RT gene was amplified using 5% of the purified DNA sample, 10 pmol of the oligonucleotide RT18 (5'-GGAAAC-CAAAAATGATAGGGGAATTGGAGG-3'; bp 2376–2406), 14 pmol of 3'RTout (5'-TCTACTGTCCATGCATGGCTTC-3'; bp 4392–4369) (Pharmacia Biotech, Roosendaal, Netherlands), 200 μM each of the four dNTPs (Pharmacia, Piscataway, NJ), 2 mM MgCl_2 , 50 mM TRIS, pH 8.3, 25 mM KCl, 100 $\mu\text{g}/\text{mL}$ bovine serum albumin (Boehringer Mannheim, Mannheim, Germany), and 1.25 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). All reactions were performed in a 50- μL volume, covered by paraffin oil, in heat-stable microplates (HI TEMP; Techne, Princeton, NJ) by use of a thermal cycler (PHC-3; Techne). The reactions were incubated for 5 min at 95°C and amplified for 35 cycles according to the following protocol: 1 min at 95°C , 1 min at 55°C , and 2 min at 72°C . Subsequent to this procedure, the amount of amplified product was further increased in a second (nested) amplification reaction consisting of 25 cycles. Five microliters of the first PCR product was transferred to a fresh (50- μL) amplification reaction containing 14 pmol of the oligonucleotide RT19 (5'-GGACATAAAGCTATAGGTACAG-3'; bp 2453–2474) in combination with either 11 pmol of primer RT22 (5'-AGGTTAAAATCACTAGCCATTGCTCTCC-3'; bp 4311–4284) for amplification of the complete RT gene or 14 pmol of RT20 (5'-CTGCCAGTTCTAGCTCTGCTTC-3'; bp 3461–3440) for amplification of the N-terminal part of the RT gene (Pharmacia Biotech) and the same reaction buffer as described for the first PCR. The same amplification conditions as described for the first PCR were used.

Sequence analysis. The amplified products of the N-terminal part of the viral RT gene were cloned by use of the TA cloning system (Invitrogen, Leek, Netherlands). Recombinant plasmids were purified and sequenced by use of a cycle sequencing kit (Taq Dye Deoxy Terminator; Applied Biosystems, Foster City, CA) and oligonucleotides SP6, T7, KRT (5'-CAGGATGGAGTTCAT-AAC-3'; bp 3257–3240), and H34 (5'-CTTCAGGAAGTATAC-TGCATTACCATACCTAGT-3'; bp 2917–2950) (Pharmacia Biotech). All sequencing reactions were analyzed on a DNA sequencer (model 373; Applied Biosystems).

Construction of an HXB2 molecular clone lacking the complete RT gene. To determine the drug susceptibility of the entire RT gene, a novel HXB2 deletion clone lacking aa 2–561 of the RT gene (HXB2 \blacktriangle 2-561RT) was constructed. This clone was based on HXB2 \blacktriangle 2-261RT (HXB2 molecular clone lacking aa 2–261 of the RT gene) [21], which was digested with *Sma*I and *Bal*II, thereby removing the C-terminus of the RT gene and the N-terminal part

of the integrase gene. To introduce an *Xba*I restriction site at the 3' end of the RT gene and to restore the N-terminal part of the integrase gene, a PCR reaction using oligonucleotides 5'endRT-SmaNotXba (5'-ATCCCCGGGGCGGCCGCTCTAGATGGG-ATA-3'; bp 4214–4244), and 3'polM (5'-CTTGGATCCACT-ACGATGTCCTATTATCTTG-3'; bp 5016–4982) (Pharmacia Biotech) was performed. Subsequent cloning of the PCR fragment into HXB2 \blacktriangle 2-261RT (both digested with *Sma*I and *Bal*II) resulted in the RT-deleted HXB2 molecular clone (lacking aa 2–561) with an intact integrase gene (confirmed by sequence analysis).

Analysis of the biologic sensitivity to 3TC and zidovudine. For the analysis of the biologic sensitivity, patient-derived RT sequences were introduced into an RT deletion clone via homologous recombination [22]. Therefore, amplified proviral DNA (bp 2453–4311) was cotransfected with linearized HXB2 \blacktriangle 2-561RT, and amplified proviral DNA or viral RNA (bp 2453–3461) was cotransfected with linearized HXB2 \blacktriangle 2-261RT [21] in SupT1 cells. The transfected cell cultures were subsequently monitored for the appearance of syncytia, and when full-blown syncytia were observed, cell-free virus was obtained. 3TC and zidovudine drug susceptibilities were determined in duplicate using the HeLa-CD4 plaque reduction assay [23].

Results

Baseline profiles. Four patients who were antiviral therapy-naïve were pretreated with 3TC monotherapy. Viral RT sequences amplified from plasma were analyzed after 3TC pretherapy to determine the presence of the M184V substitution and the appearance of additional amino acid changes (figure 1). The M184V substitution was present in all patients. Several additional codon changes were observed, most of them in individual patients, except for a T69N observed in 2 patients (C0020 and C0034) and a K122R or K122E substitution in patient C0011 and C0020, respectively. At baseline, all patients' isolates possessed a 3TC-resistant phenotype (IC_{50} values $>100 \mu\text{M}$; figure 2). This high IC_{50} value, exceeding the upper limit of the assay, can be the result of the M184V substitution on its own, and therefore we were unable to investigate if any of the additional amino acid changes affect 3TC susceptibility.

Zidovudine and 3TC combination therapy. Because of 3TC pretherapy, all patients harbored the M184V substitution at the moment zidovudine was added. The effects of zidovudine addition on viral RNA load in plasma in relation to the development of zidovudine resistance (by phenotypic and genotypic methods) was studied. To be able to directly relate the virus load response to changes in zidovudine sensitivity, we amplified, cloned, and sequenced part of the RT gene obtained from plasma and placed it in a virus reference strain to determine the drug susceptibility.

HIV-1 RNA load and CD4 cell counts. Changes in plasma HIV-1 RNA levels and CD4 cell counts were determined after addition of zidovudine to 3TC. Individual longitudinal responses are depicted in figure 2. All patients demonstrated a

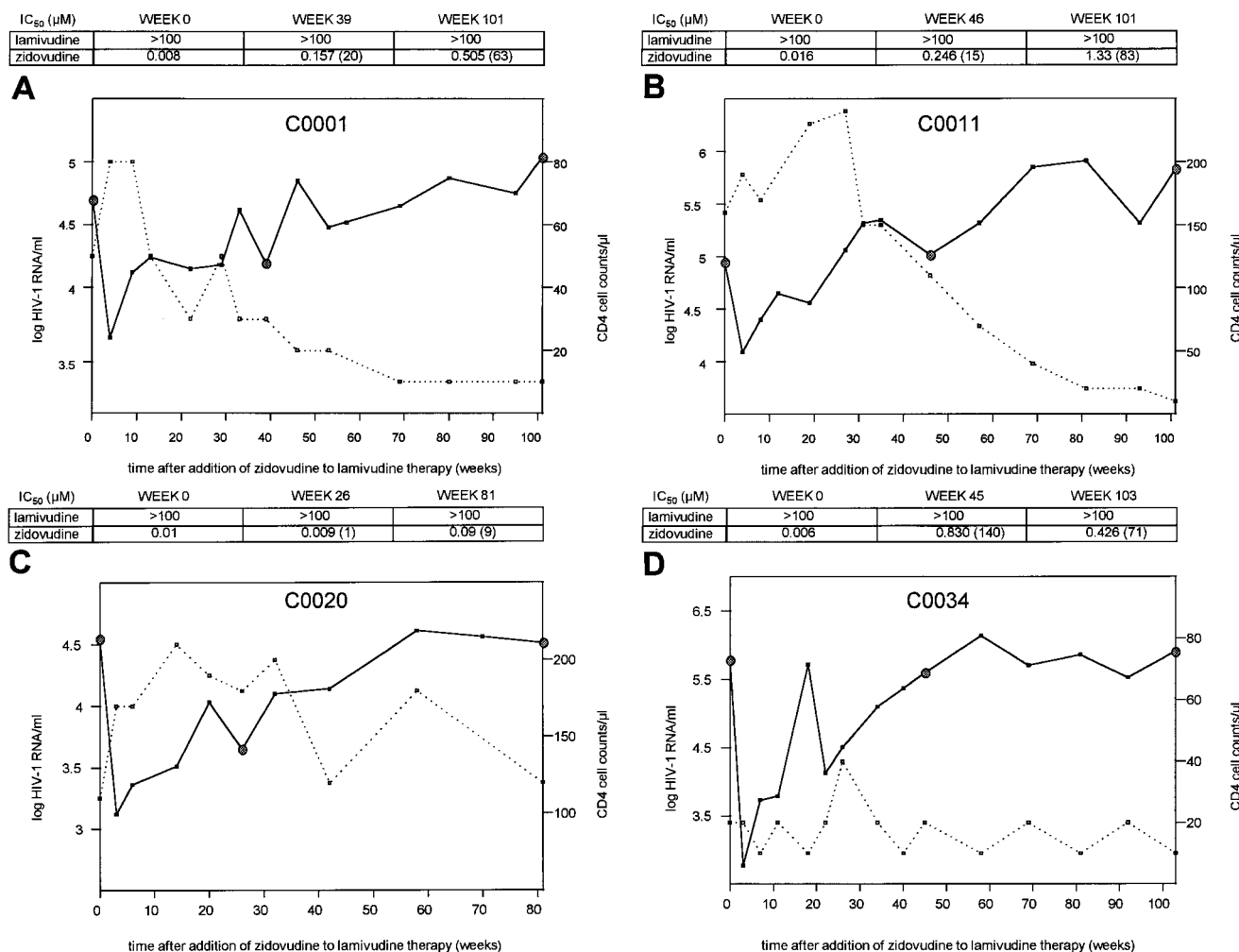


Figure 2. Plasma HIV RNA levels (solid rules) and CD4 cell counts (dotted rules) from moment of addition of zidovudine to 3TC therapy in patients C0001 (A), C0011 (B), C0020 (C), and C0034 (D). IC₅₀ values (μM) for 3TC and zidovudine of N-terminal part of the viral reverse transcriptase, determined in duplicate using recombinant viruses in HeLa-CD4 plaque reduction assay, are shown above. Fold changes in IC₅₀ values are in parentheses.

significant decline in HIV-1 RNA concentration (0.9–3.0 log). However, ultimately in patients C0001, C0020, and C0034, the HIV-1 RNA concentrations returned to their baseline level after ~1 year. The HIV-1 RNA concentration in patient C0011 increased to the baseline level at week 27. After reaching baseline values, HIV-1 RNA levels remained stable in patients C0001, C0020, and C0034, whereas in patient C0011 an addi-

tional increase was observed. In most patients, the responses in HIV-1 RNA load were mirrored by the changes in CD4 cell counts (figure 2).

Drug susceptibility testing and genotypic characterization of HIV-1 RT at return to baseline. The susceptibility of HIV-1 RT to zidovudine and 3TC was studied in relation to the virus load response (figure 2). The plasma population of all

Figure 1. Amino acid sequence of N-terminal part of HIV reverse transcriptase (RT) (aa 1–261) in patients C0001, C0011, C0020, and C0034 at start of 3TC monotherapy (a), at start of 3TC and zidovudine combination therapy (b), after increase in HIV-1 RNA concentration (c), and after 2 years of combination therapy (d) compared with amino acid sequence of HIV consensus B RT. For patients C0001, C0020, and C0034, 2 clones were sequenced at start of 3TC monotherapy and at start of combination therapy, whereas 4 clones were sequenced during combination therapy. For patient C0011, 7 clones were sequenced at start of 3TC monotherapy and at start of combination therapy, 13 clones were sequenced at moment HIV-1 RNA load returned to baseline, and 8 clones were sequenced after 2 years of combination therapy. Upper-case letters refer to amino acid changes observed in all clones, whereas heterogenous amino acid positions are indicated with lower-case letters. When ≥3 amino acids are present at particular position, these are indicated by 1 (mixture of E/G/D), 2 (mixture of I/V/T), or 3 (mixture of L/M/I/T).

Table 1. Zidovudine susceptibility and genotypic characterization of plasma-derived HIV-1 reverse transcriptase (RT) at return to baseline.

Patient	Week	RT residue							IC ₅₀ , μ M (fold change)
		Classical zidovudine resistance–conferring substitutions					L210	I/V135	
		M41	D67	K70	T215	K219			
C0001	39	L	N		Y		W		0.157 (20)
C0011	46	L	N	R	Y		W	L/T/M	0.246 (15)
C0020	81	L	N	R	Y				0.090 (9)
C0034	45	L/Q	N	R	F	E		T	0.830 (140)

patients remained resistant to 3TC (IC₅₀, >100 μ M). The virus populations at start of combination therapy were susceptible to zidovudine (IC₅₀, 0.006–0.016 μ M). Drug susceptibility determinations and sequencing of at least 4 clones per patient were performed after their RNA concentration had returned to the baseline level (figure 1; table 1). Clearly, in 3 of 4 patients, zidovudine IC₅₀ values had only increased <20-fold, and in all 4 patients, at least three zidovudine resistance–conferring mutations (M41L + D67N + T215Y/F) were present. The M41L, D67N, and T215Y/F amino acid changes were selected in all patients, though not in all clones. The K70R amino acid change was selected in 3 of 4 patients and a K219E substitution in 1 patient. Two additional substitutions (I135M/L/T and L210W) were observed in 2 of the 4 patients. In 1 of these 2 patients, an I135V amino acid change was observed at start of combination therapy.

To investigate the potential role of the C-terminal part of RT, we performed a control experiment in which we analyzed the HIV-1 proviral DNA population after 2 years of combination therapy. Recombinant viruses were made, introducing either the full RT gene (aa 2–561) or the N-terminal part (aa 2–261), both derived from the proviral DNA population. The additional effect of the C-terminal part of RT on zidovudine resistance was limited to <2-fold in all 4 patients, indicating that changes in the C-terminal part of RT may not play a major role in vivo (table 2).

Table 2. Zidovudine susceptibility of N-terminal part of reverse transcriptase (RT) and complete RT, obtained from proviral DNA population after 2 years of 3TC and zidovudine combination therapy.

Patient	IC ₅₀ , μ M (fold change)	
	N-terminus, aa 2–261	Complete RT, aa 2–561
C0001	0.38	0.57 (1.5)
C0011	0.75	1.2 (1.6)
C0020	0.12	0.22 (1.8)
C0034	0.33	0.46 (1.4)

Relationship of phenotypic and genotypic changes. To directly relate the effect of observed amino acid changes on zidovudine and 3TC susceptibility, RT clones obtained longitudinally from patient C0011 were analyzed (figure 1; table 3). In the presence of wild type codon 184, the combination M41L + T215Y has been demonstrated to confer a 50- to 60-fold increase in IC₅₀ (table 3). In the background of the 184V, however, M41L + T215Y conferred only a small increase (5- to 14-fold) in IC₅₀, and even for this small increase, additional substitutions were required: L210W (clone 1.1) or I135L + D192G (clone 1.2) for a 5-fold increase and I135M + M245T (clone 1.3) for a 14-fold increase.

After 2 years of combination therapy, substitutions at codons 41, 210, 215, and 245 were conserved. Additional selection of amino acid changes at codons 40, 43, and 219 (clone 2.1) or at codons 40, 43, 71, 114, and 253 (clone 2.2) resulted in viruses with a 38- or 68-fold increase in zidovudine resistance, respectively. Clone 2.3 was identical to clone 2.1 but harbored a wild type codon 184 and, as a result, was susceptible to 3TC (IC₅₀, 1.3 μ M) and >114-fold resistant to zidovudine.

Discussion

This study gives an explanation for the sustained antiviral effects of 3TC and zidovudine combination therapy. Resistance to 3TC is caused by amino acid changes at codon 184 of the HIV-1 RT gene [1–6]. The 184V substitution has been shown to increase the fidelity of RT [13] and, when introduced into the background of a zidovudine-resistant RT gene, to suppress the effect of some zidovudine resistance mutations [3, 4]. We studied the effect of addition of zidovudine in 4 patients continuously treated with 3TC and harboring a virus population encoding the 184V to determine to what extent the increased fidelity and the zidovudine resistance–suppressive effect could help to explain the in vivo synergistic result of this drug combination.

Addition of zidovudine to 3TC resulted, in all patients, in a significant and sustained decrease (0.9–3.0 log) in the HIV-1 RNA concentration. In 3 of 4 patients, it took ~1 year for the

Table 3. Effect of amino acid changes on drug susceptibility in recombinant virus clones containing plasma-derived reverse transcriptase (RT) genes of patient C0011.

	RT residue													IC ₅₀ μM (fold change)	
	Classical zidovudine resistance–conferring substitutions				Additional substitutions									Zidovudine	3TC
	M184	M41	T215	K219	E40	K43	W71	A114	I135	D192	L210	M245	T253		
RTMN*	—	L	Y	—	—	—	—	—	—	—	—	—	—	0.56	ND
RTMM(184V)*	V	L	Y	—	—	—	—	—	—	—	—	—	—	0.05	ND
Baseline															
Clone 0.1	V	—	—	—	—	—	—	—	—	—	—	—	—	0.04	>100
46 weeks															
Clone 1.1	V	L	Y	—	—	—	—	—	—	—	W	—	—	0.19 (5)	>100
Clone 1.2	V	L	Y	—	—	—	—	—	L	G	—	—	—	0.18 (5)	>100
Clone 1.3	V	L	Y	—	—	—	—	—	M	—	—	T	—	0.57 (14)	>100
101 weeks															
Clone 2.1	V	L	Y	T	F	E	—	—	—	—	W	T	—	1.5 (38)	>100
Clone 2.2	V	L	Y	—	F	E	L	T	—	—	W	T	S	2.7 (68)	>100
Clone 2.3	—	L	Y	T	F	E	—	—	—	—	W	T	—	>5 (>114)	1.3

NOTE. ND, not done.

* Data obtained from Tisdale et al. [3].

HIV-1 RNA concentration to return to the baseline level. At that time, in 3 patients the increase in zidovudine IC₅₀ of the virus population in plasma was only 9- to 20-fold and in the other patient was 140-fold. These data are in agreement with an earlier study in which we used the return-to-baseline criterion to define in vivo significant drug resistance [24]. In this previous study in 24 patients receiving zidovudine monotherapy, it was shown that an 8- to 10-fold increase in zidovudine IC₅₀ conferred by only one amino acid change (K70R) was sufficient to return the virus load to the baseline level. A striking difference was observed when we analyzed the sequences of the RT genes from the patient population receiving 3TC and zidovudine. In patients harboring the 184V, at least three amino acid changes were required for return to baseline, one of them (T215Y/F) consisting of two nucleotide changes (table 1). The selected amino acid changes were in most cases identical to those observed during zidovudine monotherapy (codons 41, 67, 70, 215, and 219), and some additional amino acid substitutions (codons 135 and 210) previously associated with zidovudine resistance were observed [25–30]. Of interest, after prolonged combination therapy, additional amino acid changes contributing to zidovudine resistance were observed in the areas between aa 40–44, 64–74, and 208–219 (neighboring the classical substitutions involved in zidovudine resistance), some of which were previously reported [27, 28, 31].

Larder et al. [15] observed a sustained antiviral effect of 3TC and zidovudine compared with zidovudine monotherapy and theorized that this might be due to the delayed appearance of zidovudine resistance mutations. The observation that multiple amino acid changes are needed to confer significant zido-

vudine resistance in 3TC-resistant patients suggests an alternative mechanism to explain the prolonged suppression of the HIV-1 RNA levels during combination therapy. Even in a highly zidovudine-resistant background, the M184V substitution persisted and continued to have an effect on the level of zidovudine resistance, as illustrated by the difference between a 184 wild type clone with an IC₅₀ value >5 μM and an identical clone harboring the 184V with an IC₅₀ of 1.5 μM.

In the case of the nonnucleoside RT inhibitors, the primary change seen during monotherapy is located at codon 181 (Y181C). Similarly to the 184V, this amino acid change suppresses the effect of zidovudine mutations [32]. However, this effect is lost during zidovudine and nevirapine combination therapy, since alternative nonnucleoside resistance changes are selected that do not suppress zidovudine resistance [33]. The persistence of the 184V during zidovudine and 3TC combination therapy indicates that, unlike the situation with nonnucleoside RT inhibitors, there are no alternatives for the RT enzyme to confer 3TC resistance, pointing at a very specific interaction between RT and this nucleoside analogue. Remarkably, in addition to the 184 substitution, no amino acid changes were observed in or around the catalytic site.

In vitro selection experiments have shown that double-resistance to 3TC and zidovudine could be located in the C-terminal part (between aa 333 and 558) of the RT enzyme [34, 35]. In our patients, we could not find evidence for a role of this region in causing 3TC and zidovudine co-resistance (table 2).

Selection of virus variants with multiple mutations could theoretically be delayed because of increased fidelity of the HIV-1 RT enzyme containing the M184V amino acid change

[13]. This study demonstrated that the 184V does not prevent the generation of multiple zidovudine resistance-conferring mutations. This is in agreement with our *in vitro* observations showing that selection of resistance to nevirapine and ritonavir occurred at the same rate for wild type and 184V mutants [36, 37]. We cannot rule out that the increased polymerase fidelity delays the appearance of resistance mutations, but the data clearly show that the presence of 184V does not prevent the appearance of both classical zidovudine substitutions and additional changes. This is in line with the model of Coffin [38], showing that during virus evolution, small increases in viral fitness (here resistance) are more important than small changes in fidelity. *In vitro* observations have shown that in the absence of 3TC, the 184V-containing viruses may replicate somewhat less efficiently in T cell lines [15]. In primary PBMC, these viruses show a reduced fitness (here replication efficiency) compared with wild type [12]. It cannot be excluded that the reduced replication rate may explain some of the beneficial effects of both 3TC monotherapy and 3TC in combination with other inhibitors [5, 14, 15].

In conclusion, this study demonstrated that in HIV-1-infected patients harboring the 3TC resistance-conferring 184V, selection of zidovudine-resistant HIV-1 variants during 3TC and zidovudine combination therapy could not be prevented. However, unlike the zidovudine monotherapy situation, in the background of 184V, multiple substitutions are required to confer a 10-fold increase in IC_{50} , which is sufficient for a return of HIV-1 RNA concentration to the baseline level. This need for multiple mutations may be one of the mechanisms explaining the sustained antiviral response with the combination of zidovudine and 3TC.

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