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Genotypic and phenotypic characterization of HIV-1 isolated from patients receiving (−)-2′,3′-dideoxy-3′-thiacytidine

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Abstract

We attempted to determine whether HIV-1 developed resistance to (−)-2′,3′-dideoxy-3′-thiacytidine ((−)-3TC or 3TC, lamivudine) in patients with advanced human immunodeficiency virus type 1 (HIV-1) infection during therapy with 3TC. Genotypic analysis of HIV-1 strains isolated from 6 patients receiving 3TC revealed that as early as 2 months of therapy, HIV-1 developed a Met to Val amino acid substitution at codon 184 ($Met^{184} \rightarrow Val$) in the reverse transcriptase-coding region of the *pol* gene. A detailed study of a series of HIV-1 strains isolated from a patient demonstrated that Met at codon 184 was first substituted with Ile by 2 weeks of 3TC therapy, followed by the substitution with Val by 8 weeks. All HIV-1 strains with the $Met^{184} \rightarrow Val$ substitution were profoundly less susceptible to 3TC (1800- to 5500-fold decreased sensitivity) as compared to pretherapy virus strains. These strains were also moderately less sensitive to 2′,3′-dideoxycytidine (4.5- to 9-fold), but more sensitive to 3′-azido-2′,3′-dideoxythymidine (2- to 14-fold). A decrease in viremia levels and an increase in CD4 counts were observed early in therapy; however, these changes were only transient. Our data suggest that reversal of such beneficial changes is associated with the $Met^{184} \rightarrow Val$ substitution of the *pol* gene of HIV-1. The

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data also suggest that 3TC, as a single agent, may induce virologic and immunologic improvement in patients with advanced HIV-1 infection, but only transiently.

Keywords: Human immunodeficiency virus type 1 (HIV-1); (−)-2′,3′-dideoxy-3′-thiacytidine (3TC); Drug resistance

1. Introduction

The agent (−)-2′,3′-dideoxy-3′-thiacytidine ((−)-3TC or 3TC, sometimes abbreviated as (−)-SddC; lamivudine) is an antiretroviral 2′,3′-dideoxypyrimidine analog in which the 3′-carbon of the ribose ring is replaced with a sulfur atom and the ribose ring is inverted as compared to physiological nucleosides. 3TC has potent activity against a wide range of retroviruses in vitro, including strains of human immunodeficiency virus type 1 (HIV-1) that are resistant to 3′-azido-2′,3′-dideoxythymidine (AZT, zidovudine) (Soudeyns et al., 1991; Coates et al., 1992a,b; Schinazi et al., 1992). This potency is comparable to that of 2′,3′-dideoxycytidine (ddC, zalcitabine) as tested in several in vitro assay systems (Coates et al., 1992b; Schinazi et al., 1992). Animal toxicology studies and certain in vitro studies further suggested that 3TC would be well tolerated (Chang et al., 1992; Hart et al., 1992; Sommadossi et al., 1992). Thus, a North American phase I/II trial to assess toxicity, pharmacokinetics, and activity of 3TC was initiated at 3 sites: the National Cancer Institute (NCI), Bethesda, Maryland; Boston City Hospital, Boston, Massachusetts; and St. Paul's Hospital, Vancouver, British Columbia (Pluda et al., 1992, 1995).

Since AZT-resistant HIV-1 variants were first isolated in 1989 (Larder et al., 1989) and a potential correlation of the emergence of HIV-1 variants with reduced susceptibility to antiretroviral drugs with clinical deterioration was proposed (Tudor-Williams et al., 1992; Montaner et al., 1993), the identification of mutations known to be associated with drug-resistance has become important in the development both of single anti-HIV agents and combination treatment regimens. In this regard, Gao et al. developed in vitro HIV-1 variants that were resistant to the racemic mixture of 3′-thia-2′,3′-dideoxycytidine (BCH-189) (Gao et al., 1993a). Subsequently, Schinazi and his coworkers reported that a 3TC-resistant HIV-1 variant was isolated from a patient receiving 3TC and that the variant had an amino acid substitution from Met to Val at codon 184 in the *pol* region ($Met^{184} \rightarrow Val$) (Schinazi et al., 1993). In the present study, we studied a subset of patients enrolled in the phase I/II trial of 3TC in the NCI to determine whether HIV-1 rapidly developed resistance to 3TC during antiretroviral therapy with 3TC. We also attempted to study whether the emergence of such HIV-1 variants was associated with changes in selected laboratory endpoints.

2. Materials and methods

2.1. Patients

The 6 patients studied in this work were a subset of those enrolled in the National Cancer Institute on a phase I/II, non-randomized, dose-escalating study of 3TC to

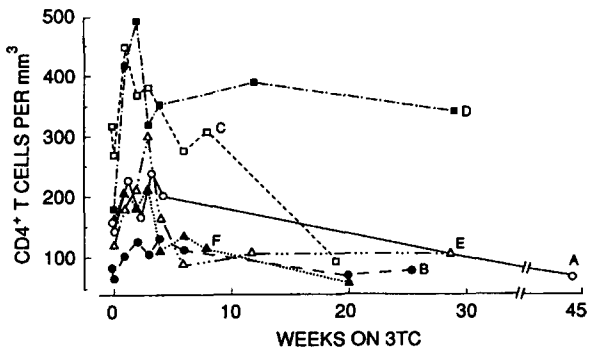

Fig. 1. Numbers of circulating $CD4^+$ T-lymphocytes in patients receiving 3TC.

determine its toxicity, antiviral activity, and pharmacokinetics (Pluda et al., 1992, 1995). This trial was conducted during the period from September 1991 until October 1992. 3TC for the study was provided by Glaxo, Inc. In the present study, a subset of 6 patients enrolled in the phase I/II trial were examined for the development of resistance to 3TC. Three patients (A, B, and E) were selected for study because they had received 3TC for relatively long periods of time (3–10 months). Two patients (C and F) were selected for study because of the suspected development of 3TC-resistant HIV-1 variants due to changes in CD4 and p24 levels (Fig. 1). Patient D was selected for study because $CD4^+$ levels higher than baseline were observed throughout 3TC treatment. Clinical, immunologic and virologic profiles of the 6 patients are illustrated in Table 1. Of the 6 patients studied, two patients were diagnosed as having acquired immunodeficiency syndrome (AIDS) and 4 as having AIDS-related complex (ARC) upon entry to this study. All were male, aged 32–56 years. All patients had CD4 counts at entry below 300 cells/mm^3. 3TC was administered twice daily at the following dosages: 4 mg/kg/day (patient A); 8 mg/kg/day (patients B and F); and 12 mg/kg/day (patients C, D, and E). All but one patient had received prior anti-HIV therapy with AZT or AZT plus other drugs; however, these patients had ended such therapy at least 1 month prior to the initiation of 3TC therapy.

2.2. *Isolation, titration, and drug sensitivity testing of clinical HIV-1 strains*

HIV-1 strains were isolated from 6 patients at various time points during therapy with 3TC. Briefly, peripheral blood mononuclear cells (PBMC) from each patient were cocultured with PBMC which had been obtained from HIV-1 seronegative volunteers and stimulated with phytohemagglutinin (PHA-PBMC) in recombinant interleukin-2 (rIL-2; 10 U/ml, Amgen, Thousand Oaks, CA)-containing culture media (RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 4 mM L-glutamine, 50 U/ml of penicillin, and 50 μg/ml of streptomycin). The coculture was terminated on day 9 when the level of p24 Gag protein produced in the culture supernatant reached $\geqslant$ 10 ng/ml as assessed by p24 Gag protein radioimmunoassay (Du Pont, Boston, MA). The supernatant was collected, and stored at $-70°$ C until it served as a source of

Table 1
Profile of patients at entry

Patient	Sex	Diagnosis	Age	p24 [a] (pg/ml)	$CD4^+$ ($/mm^3$)	3TC dosage (mg/kg/day)	Prior anti-HIV therapy [b]
A	M	ARC	34	< 32	143	4	AZT −22 to −13 months IFN −11 to −7 months IFN + IL-2 −7 to −5 months AZT −5 to −1 months
B	M	ARC	32	< 32	66	8	AZT −19 to −1 months
C	M	AIDS	56	< 32	269	12	AZT −2 to −1 months
D	M	ARC	43	< 32	179	12	No prior therapy
E	M	AIDS	39	< 32	122	12	AZT −31 to −8 months ddC −4 to −3 months ddI −3 to −2.5 months AZT −2.5 to −1 months
F	M	ARC	34	106	163	8	AZT −15 to −14 months ddC −14 to −11 months L697.661 −8 to −5 months

[a] Serum p24 antigen levels (pg/ml) were determined by the enzyme-linked immunosorbent assay (ELISA, Abott).
[b] Relative to initiation of 3TC therapy; e.g., AZT −5 to −1 months, denotes that the patient received 4 months of AZT therapy ending 1 month prior to start of 3TC therapy.

infectious virions. Each pair of pre- and post-therapy HIV-1-containing supernatant preparations to be compared was simultaneously titrated for their 50% tissue culture infectious doses ($TCID_{50}$) using a single PHA-PBMC batch and the p24 Gag protein radioimmunoassay. Titration was performed in 8 replicates.

The sensitivity of HIV-1 strains to drugs was determined as previously described (Shirasaka et al., 1993) with minor modifications. Briefly, PHA-PBMC (6×10^5) were exposed to a 40 $TCID_{50}$ dose of each virus isolate in the presence of various concentrations of 3TC, AZT, 2′,3′-dideoxyinosine (ddI, didanosine), or ddC, performed in triplicate. The sensitivity of a given HIV-1 strain to a drug was defined as the drug concentration that yielded a reduction in p24 Gag protein production by 90% (IC_{90}) as compared to control cultures similarly exposed to virus, but not exposed to drug.

2.3. Quantification of viral RNA copies

Viral RNA copy in serum or plasma was determined as previously described (Aoki-Sei et al., 1992; Kojima et al., 1993; 1995). Briefly, serum or plasma samples (0.1 ml) were diluted with 1% bovine serum albumin-containing PBS to 1 ml and centrifuged at 30,000 *g* for 1 h at 4°C in a Heraeus Sepatech high speed centrifuge (no. 3753 rotor). Viral RNA was extracted from the pelleted virus using the acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Nicoletta, 1987). Following phenol–chloroform extraction, the nucleic acids were precipitated with ethanol and then

dissolved in diethylpyrocarbonate (DEPC)-treated water. This solution was treated with RNase-free DNase I and subsequently subjected to ethanol precipitation. The resultant purified RNA, dissolved in 50 μl DEPC-treated water, was subjected to reverse transcription to produce cDNA. A portion of the cDNA-containing reaction mixture was subjected to polymerase chain reaction (PCR), yielding a 581-bp fragment in the *pol* region. For detection of amplified products, ^{32}P-end labeled oligomers were used as probes, the PCR product was electrophoresed on 10% polyacrylamide gel, and the dried gel was exposed to Kodak X-OMAT film. The density of the band was measured by densitometry using the software package for the analysis of 1-D gels and films (Quantity One; PDI, Huntington Station, NY) and particle numbers were determined against a standard, whereby HIV-1 RNA is extracted from a serially diluted HIV-1_{LAI} stock preparation containing known numbers of virus particles per ml as determined by electron microscopy, as previously described (Kojima et al., 1995).

2.4. Determination of nucleotide sequences of polymerase-encoding pol region of the HIV-1 genome

The cDNA preparation described above for the method of viral quantification was also used to determine the nucleotide sequence of the *pol* gene as previously described (Shirasaka et al., 1993). Briefly, cDNA preparations were subjected to PCR amplification using primer pairs which incorporated restriction enzyme sites. The primer pairs used were AS71 (5′-GTA CCA GTA GAA TTC AAG CCA GGA-3′) and SA012 (5′-CTG GCA GCT CTA GAG GCT GTA CTG-3′), generating a 721-base PCR product which includes codons 13–243 of reverse transcriptase, or AS71 and SA011 (5′-TTC ATA ACC CAT CTA GAG GAA TGG-3′), generating a 678-base PCR product which includes codons 13–227 of reverse transcriptase. The amplified *pol* fragment was extracted with phenol–chloroform–isoamyl alcohol, precipitated with ethanol, and resuspended in distilled water. The *pol* fragment was then digested with *Eco*RI and *Xba*I, subjected to electrophoresis in an agarose gel, retrieved using glass beads, and inserted into the pTZ19R vector (United States Biochemical Corp., Cleveland, OH). Competent *Escherichia coli* (strain DH5α) were transformed with the *pol*-inserted pTZ19R and plasmid DNA was purified and sequenced by the dideoxy chain termination method.

3. Results

3.1. Changes in CD4$^+$ during 3TC therapy

All of the 6 patients examined in this study had an increase in circulating CD4$^+$ counts within 1 week of initiating 3TC therapy (Fig. 1). The mean rise in the first week was 102 CD4$^+$ cells/mm^3 (range 30–239). Overall, CD4 counts remained greater than the entry level through the third week of 3TC therapy, followed by a decline in subsequent weeks. By weeks 19–44, CD4 counts fell below the entry level with a mean

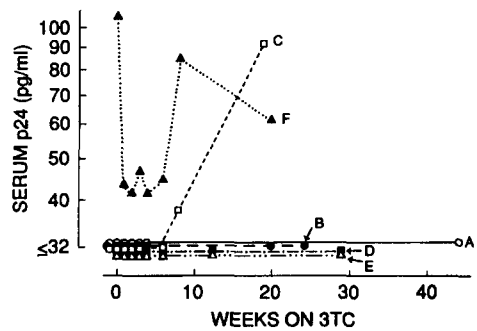

Fig. 2. p24 antigen levels in serum of patients receiving 3TC.

decrease of 38 $CD4^+$ cells/mm^3 (range $-199 \sim 164$). Only one of the 6 patients (patient D) had CD4 counts above the entry level throughout the study period.

3.2. Changes in serum p24 antigen levels during 3TC therapy

Prior to 3TC therapy, only one patient (patient F) had detectable p24 (Table 1 and Fig. 2). His serum p24 level dropped from 106 to 44 pg/ml after 1 week of 3TC therapy. The level of p24 in this patient remained below 50 pg/ml until the 8th week of therapy, when it rose approaching the entry level (Fig. 2). Another patient (patient C) showed undetectable p24 through 6 weeks of 3TC therapy, then became positive for p24 at 8 weeks, and the p24 level subsequently rose further later on therapy. The remaining 4 patients did not show detectable serum p24 throughout the study period.

3.3. Changes in viral particle numbers in plasma and serum

Virus particle numbers in plasma and serum were determined using the PCR-based HIV-1 quantitative assay (RT-PCR) (Aoki-Sei et al., 1992; Kojima et al., 1993; 1995), before and at various time points during 3TC therapy (Fig. 3). Plasma viremia levels decreased by an average 2.4-fold relative to the entry level in 5 patients when assessed at week 4 of therapy (Fig. 3A). Later in therapy (weeks 17–43) plasma viremia levels averaged close to baseline for all 6 patients. We, therefore, further studied viremia changes at earlier time points of 3TC therapy. Using patient serum (plasma at early time points was not available), by 1 week of therapy, viremia levels decreased by an average 10-fold in 4 patients (patients A, B, C, and F in Fig. 3B) as compared to those in pretherapy serum samples. Viremia levels in serum remained at least 6.5-fold lower than entry level through the 6th week of therapy in these 4 patients. However, by weeks 17–43, the viremia approached the entry level with only a 1.8-fold decrease in viremia relative to baseline. Thus, it appears that a reduction of viremia mostly occurred during the first week of 3TC therapy to a level of about 10% of entry level. The reduction was, however, only transient.

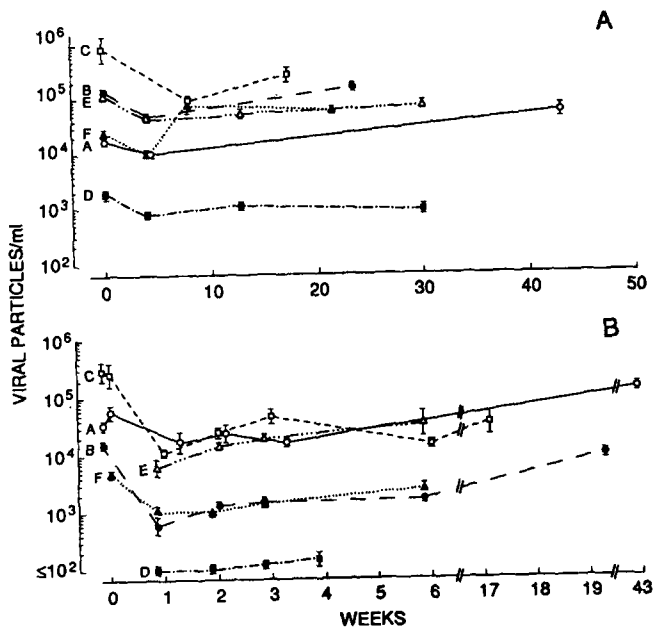

Fig. 3. Effect of 3TC on viral particle numbers in patient plasma and serum. Patient plasma (panel A) and serum (panel B) samples were subjected to centrifugation and viral RNA was extracted from the pelleted virus, treated with DNase I, and cDNA was prepared as described in Materials and methods. The cDNA was amplified through PCR, yielding a 581-bp fragment in the *pol* region. The PCR products were hybridized with ^{32}P-end labeled oligomers and this mixture was separated on a polyacrylamide gel. Band density was measured from autoradiogram images and particle numbers were determined against a standard with known particle concentration. Data represent the geometric means (±S.D.) of duplicate determinations. Note that pre-3TC therapy serum samples were not available for two patients, E and D (panel B).

3.4. Genotypic analysis of circulating HIV-1

Molecular clones containing the polymerase domain-encoding region of the *pol* gene were generated using cDNA produced from HIV-1 RNA purified from plasma or serum samples obtained at various time points on therapy and their nucleotide and deduced amino acid sequences were determined. Table 2 illustrates the deduced amino acids at codons 41, 67, 70, 215, and 219, which are reported to be associated with resistance to AZT (Larder and Kemp, 1989; Kellam et al., 1992); an amino acid at codon 69 which is reported to be associated with resistance to ddC (Fitzgibbon et al., 1992); an amino acid at codon 74 which is reported to be associated with resistance to ddI (St. Clair et al., 1991); and an amino acid at codon 184 which is reported to be associated with resistance to 3TC therapy (Gao et al., 1993b; Schinazi et al., 1993; Tisdale et al., 1993). All pre-therapy clones were wild-type at codon 184, encoding Met (ATG). By contrast, all clones obtained after the initiation of 3TC therapy had amino acid substitutions at codon 184. In particular, all clones generated from virus isolates from patients A–E at

Table 2
Amino acid substitutions at selected codons of clones containing the polymerase domain of HIV-1 isolated from patients receiving 3TC

Patient	Months on 3TC	Number of clones examined	Codon							
			41	67	69	70	74	184	215	219
			Met	Asp	Thr	Lys	Leu	Met	Thr	Lys
A	0	4	Leu (4)	–	–	–	–	–	Tyr (4)	–
	10	4	Leu (4)	–	–	–	–	Val (4)	Tyr (4)	–
B	0	4	–	Asn (4)	–	Arg (4)	–	–	–	Gln (2)
	6	4	–	Asn (4)	–	Arg (2)	–	Val (4)	–	Gln (3)
C	0	4	–	–	–	–	–	–	–	–
	4	4	–	–	–	–	–	Val (4)	Ile (1)	–
D	0	4	–	–	–	–	–	–	–	–
	3	4	–	–	–	–	–	Val (4)	–	–
E	0	4	–	Asn (4)	–	Arg (4)	–	–	Tyr (4)	Gln (4)
	3	4	–	Asn (4)	–	Arg (4)	–	Val (4)	Tyr (4)	Gln (4)
F	0	4	–	–	–	–	–	–	–	Glu (1)
	0.5	12	ND	ND	ND	ND	ND	Ile (7), Val (5)	ND	Glu (6)
	1	12	ND	ND	ND	ND	ND	Ile (4), Val (8)	ND	Glu (5)
	2	6	–	–	–	–	–	Ile (1), Val (5)	–	Glu (6)

Dashes indicate amino acid residues conserved with respect to the reference wild-type codons shown at the top. Numbers in parentheses indicate the number of clones containing the designated amino acid substitution. ND, not determined.

3–10 months of 3TC therapy had an amino acid substitution at codon 184 from Met to Val (in most cases GTG; rarely GTA or GTT), designated $Met^{184} \rightarrow Val$.

Next, we investigated when the $Met^{184} \rightarrow Val$ substitution occurred during therapy with 3TC. To this end, HIV-1 was isolated from one patient (patient F) at multiple time points after initiating 3TC and examined for genotypic changes in the *pol* gene. When molecular clones were generated after 2 weeks, a month, and 2 months of therapy, HIV-1 was found to yield a mixture of clones encoding Ile (ATA) or Val (GTG) at codon 184. At 2 weeks of therapy, 7 of 12 clones examined had the $Met^{184} \rightarrow Ile$ substitution while 5 had the $Met^{184} \rightarrow Val$ substitution. When 12 clones were examined at 1 month of therapy, 4 had the $Met^{184} \rightarrow Ile$ substitution, while 8 had the $Met^{184} \rightarrow Val$ substitution. At 2 months of therapy, of 6 clones, only one clone had the $Met^{184} \rightarrow Ile$ substitution, while 5 had the $Met^{184} \rightarrow Val$ substitution. These data suggest that the development of the amino acid substitution at codon 184 can occur within a few weeks. Furthermore, early in therapy, a mixed population of $Met^{184} \rightarrow Ile$ and $Met^{184} \rightarrow Val$ developed, whereby the latter substitution became dominant as 3TC therapy continued.

3.5. AZT-related substitutions persisted as the 3TC-related substitution developed

Five patients (patients A, B, C, E, and F) had received AZT therapy prior to 3TC. Clones from these patients, with the exception of patient C, had at least one of the

AZT-related mutations most commonly seen in patients receiving long-term AZT therapy (Larder and Kemp, 1989; Kellam et al., 1992), both before and after 3TC therapy. Thus, AZT-related substitutions persisted as the 3TC-related substitution developed. No amino acid substitutions were found at codons 69 (ddC-related) and 74 (ddC- and ddI-related) in molecular clones examined in this study.

In addition to the amino acid substitutions determined in Table 2, a portion of the *pol* gene which encodes reverse transcriptase from codons 13–227 (patients D, E, and F) or codons 13–243 (patients A, B, and C) was fully sequenced for a pair of pre- and post-therapy virus from each patient to search for other 3TC resistance-related mutations. This analysis indicated that HIV-1 strains isolated from 5 of the 6 patients had acquired one or more amino acid substitutions that have not been reported to be associated with resistance to any anti-HIV agent (Schinazi et al., 1994). The substitutions were: patient A, $Ala^{200} \rightarrow Ile$, $Lys^{207} \rightarrow Gln$, $Tyr^{208} \rightarrow His$; patient C, $Ile^{142} \rightarrow Val$; patient D, $Ile^{195} \rightarrow Thr$; patient E, $Lys^{20} \rightarrow Arg$; and patient F, $Lys^{166} \rightarrow Arg$. However, because none of the changes were found in common amongst each other, their significance is not clear.

3.6. Drug susceptibilities of HIV-1 strains isolated from patient's PBMC

In order to evaluate changes in drug sensitivities of HIV-1 during 3TC therapy, HIV-1 strains were isolated from PBMC obtained from 3 patients. All HIV-1 strains, isolated after 4–10 months of 3TC therapy showed a substantially reduced susceptibility against 3TC (Table 3). When IC_{90} values were determined for pre- and post-therapy HIV-1 strains, the decrease in susceptibility was up to 5500-fold. Interestingly, it was found that these three post-therapy HIV-1 strains became relatively more susceptible to AZT. On the contrary, they were less susceptible to ddI and ddC, although such susceptibility changes were relatively modest in degree (Table 3).

4. Discussion

The study reported here demonstrates that HIV-1 can rapidly develop resistance to 3TC in patients receiving this drug. In agreement with the overall results of this study

Table 3
Drug sensitivities of HIV-1 isolated from patients receiving 3TC

Drug	Patient A			Patient B			Patient C		
	Pre-therapy	Post-therapy (10 months)	Fold-change	Pre-therapy	Post-therapy (6 months)	Fold-change	Pre-therapy	Post-therapy (4 months)	Fold-change
3TC	0.22	400	1800×	0.17	850	5000×	0.10	550	5500×
AZT	2.5	0.29	0.12×	0.59	0.042	0.071×	0.011	0.0068	0.62×
ddI	1.7	2.1	1.2×	1.7	6.8	4.0×	1.5	7.4	4.9×
ddC	0.22	1.0	4.5×	0.17	0.76	4.5×	0.15	1.4	9.3×

The IC_{90} values (μM) were determined by employing PHA-PBMC exposed to HIV-1 (40 $TCID_{50}$ dose/6× 10^5 PBMC) as target cells and using the inhibition of p24 Gag protein production as an endpoint. All drug sensitivities were performed in triplicate.

(Pluda et al., 1993, 1995), the 6 patients studied here had a modest but transient increase in circulating CD4 counts within several weeks of 3TC therapy. Furthermore, PCR-based HIV-1 quantitative assays revealed that all patients had a decrease in viremia levels within several weeks, however it too was transient. It is of note that the magnitude of the viremia reduction seen in the patients studied was relatively modest (2.4-fold), in contrast to the magnitudes of viremia reduction seen with other 2′,3′-dideoxynucleoside analogs such as AZT and ddI (Holodniy et al., 1991; Aoki-Sei et al., 1992; Kojima et al., 1993; 1995). Administration of these latter drugs is also associated with more persistent increases in CD4 counts and decreases in viremia levels (Holodniy et al., 1991; Kojima et al., 1993; Yarchoan et al., 1994). It is conceivable that in the patients studied here, 3TC suppressed the replication of HIV-1 in vivo to some extent; however, the overall effect was not sufficient to bring about a substantial and persistent increase in CD4 counts. It is also possible that the modest and transient increase in CD4 counts is due to the rapid development of HIV-1 variants less susceptible to 3TC.

In each of the 6 patients in this study, HIV-1 developed the $Met^{184} \rightarrow Val$ substitution. Also, post-therapy HIV-1 strains from each of 3 patients assayed in a drug sensitivity assay showed high levels of resistance to 3TC by 1800 ~ 5500-fold using acutely infected PHA-PBMC as target cells. Considering that introduction of the $Met^{184} \rightarrow Val$ substitution into the infectious clone HXB2 resulted in an observed > 1000-fold resistance (Tisdale et al., 1993), it is expected that the appearance of the $Met^{184} \rightarrow Val$ variant in vivo is associated with the development of 3TC-resistant HIV-1 variants. Wainberg and coworkers have also recently indicated such a correlation; however, phenotypic resistance was relatively lower and was detected in only one-third of those study subjects (Wainberg et al., 1995). Such discrepancies, though, may be reflective of variations in determining phenotypic resistance and the different patient populations studied.

The drug sensitivity assays also showed that HIV-1 strains carrying the $Met^{184} \rightarrow Val$ substitution were moderately resistant to ddI and ddC, a finding consistent with previous reports by Gao et al. (Gao et al., 1993b). Such a profile of viral resistance to multiple drugs may be related to the fact that the amino acid at codon 184 is located in close proximity to residues thought to be involved in the catalytic activity of reverse transcriptase, i.e., Asp^{185}, Asp^{186}, and Asp^{110} (Kohlstaedt et al., 1992). Further research will be needed to define such relationships. Interestingly, following 3TC therapy, HIV-1 from patient A was shown to retain the AZT-related mutation, $Thr^{215} \rightarrow Tyr$, in addition to acquiring the $Met^{184} \rightarrow Val$ substitution. However, a virus population isolated from this patient was approximately 10-fold more sensitive to AZT following 3TC therapy. This finding is consistent with previous reports (Boucher et al., 1993; Tisdale et al., 1993). The $Lys^{65} \rightarrow Arg$ substitution, which has also been demonstrated to confer resistance to 3TC (Gu et al., 1994), was not detected in the HIV-1 strains isolated from the 6 patients throughout this study.

A detailed study of a series of HIV-1 strains isolated from one patient revealed that Met at codon 184 was substituted with either Ile or Val by as early as 2 weeks of 3TC therapy. This indicates that the $Met^{184} \rightarrow Ile$ substitution was predominant to the $Met^{184} \rightarrow Val$ at 2 weeks of therapy, while the latter prevailed as 3TC therapy continued

(Table 3). The finding that the $Met^{184} \rightarrow Ile$ substitution appears transiently before the $Met^{184} \rightarrow Val$ substitution occurs is consistent with that of others (Schuurman et al., 1993, 1994; Wainberg et al., 1995). Two possible scenarios may explain this phenomenon: (1) HIV-1 actively mutates under drug pressure to simultaneously produce both mutant types (it would seem less likely that the Val^{184} population arose from the Ile^{184} mutant genotype, as this would typically require two separate mutation events); or (2) both $Met^{184} \rightarrow Ile$ and $Met^{184} \rightarrow Val$ variants pre-existed in the absence of 3TC as minute subpopulations which expanded under drug pressure. In either scenario, $Met^{184} \rightarrow Val$ would provide a greater advantage and would eventually outgrow $Met^{184} \rightarrow Ile$.

The second scenario is particularly appealing in light of recent data which support the notion that drug resistance mutations pre-exist within the quasi-species of HIV-1 in patients *not* undergoing therapy (Nájera et al., 1995). This may also help to explain the apparent shift in mutations as follows. In the absence of drug pressure, $Met^{184} \rightarrow Ile$ and $Met^{184} \rightarrow Val$ may both exist as natural viral subpopulations. While both would be minor populations, it is conceivable that HIV-1 carrying $Met^{184} \rightarrow Ile$ is relatively more prevalent than the other. Given this, upon initiation of 3TC therapy, $Met^{184} \rightarrow Ile$ may theoretically become a dominant population over HIV-1 carrying $Met^{184} \rightarrow Val$ by virtue of the former's relatively larger initial subpopulation size. This would result in an initial dominance of the $Met^{184} \rightarrow Ile$ genotype early in therapy. However, because of the suspected greater advantage of $Met^{184} \rightarrow Val$ in terms of 3TC resistance, $Met^{184} \rightarrow Val$ would ultimately prevail as 3TC treatment continues while $Met^{184} \rightarrow Ile$ would diminish.

Taken together, the current data suggest that viral resistance to 3TC develops very rapidly when administered in vivo, is conferred by the $Met^{184} \rightarrow Val$ substitution, and is of great magnitude (> 1000-fold) as assessed in vitro. These genotypic and phenotypic changes appear to be responsible for the limited ability of 3TC to bring about significant clinical benefits when used as a single agent in adults. However, the observations by two groups that 3TC resistance is associated with a decline in resistance to AZT provides a rationale for the clinical evaluation of this drug combination (Boucher et al., 1993; Tisdale et al., 1993). Indeed, recent clinical results have revealed that the 3TC/AZT combination yielded lower viral RNA copies and higher CD4 levels at 24 weeks as compared to the AZT (Katlama et al., 1995) and 3TC monotherapies (Eron et al., 1995).

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