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# Rapid *in vitro* selection of human immunodeficiency virus type 1 resistant to 3′-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase

(acquired immunodeficiency syndrome/combination therapy/antiviral drug)

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ABSTRACT **Resistant variants of human immunodeficiency virus type 1 (HIV-1) have been selected by limited passage in MT4 cells of both wild-type and 3′-azido-3′-deoxythymidine (AZT, zidovudine)-resistant strains with the nucleoside analogues (−)-2′-deoxy-3′-thiacytidine (3TC) and (−)-2′-deoxy-5-fluoro-3′-thiacytidine (FTC). Virus variants selected independently were crossresistant to both inhibitors. This rapid *in vitro* selection of resistant virus has not previously been seen with nucleoside analogues but is reminiscent of that observed with the nonnucleoside reverse transcriptase inhibitors. However, passage of wild-type virus with a combination of AZT and FTC appreciably delayed emergence of FTC-resistant virus. DNA sequence analysis of the reverse transcriptase coding region from FTC-resistant virus revealed changes at codon 184 in the highly conserved Tyr, Met, Asp, Asp (YMDD) region. When the mutation Met$^{184}$ → Val was introduced into the infectious clone HXB2, this change alone accounted for the resistance (>1000-fold) seen with both 3TC and FTC, and for a 5- to 15-fold reduction in sensitivity to their (+) enantiomers. It had no effect on susceptibility to AZT or nevirapine and minimal effect on susceptibility to 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine. To determine the influence of this mutation in a background of mutations conferring resistance to AZT and nonnucleoside reverse transcriptase inhibitors, a series of HIV-1 variants were created by site-directed mutagenesis. All mutants with Met$^{184}$ → Val were crossresistant to 3TC and FTC. The Met$^{184}$ → Val mutation did not influence nevirapine resistance, but resistance to AZT was suppressed. Similar suppression of AZT resistance was seen with Tyr$^{181}$ → Cys. Interestingly, when both Met$^{184}$ → Val and Tyr$^{181}$ → Cys substitutions were present, highly resistant virus reverted to complete AZT sensitivity. Assessment of the interactive effects of multiple drug-resistance mutations may help to establish a rationale for using these drugs in the future therapy of HIV disease.**

The development and clinical use of selective inhibitors to treat human immunodeficiency virus (HIV) infection have been marred by the ability of the virus to become drug resistant (1). This first became apparent during analysis of clinical isolates from individuals with acquired immunodeficiency syndrome (AIDS) receiving long-term 3′-azido-3′-deoxythymidine (AZT, zidovudine) therapy (2). Resistant variants have also emerged during prolonged monotherapy with the two other "first-generation" nucleoside analogue HIV inhibitors: 2′,3′-dideoxyinosine (ddI) (3, 4) and 2′,3′-dideoxycytidine (ddC) (5). Interestingly, it has proved difficult or impossible to derive HIV-1 strains highly resistant to nucleoside analogues by exposure of virus in cell cultures to these inhibitors (6–8). In contrast, however, resistance develops rapidly in culture to the newly described group of HIV-1 specific nonnucleoside reverse transcriptase (NNRT) inhibitors, including the tetrahydroimidazobenzodiazepinones and -thiones (TIBOs) (9), pyridinones (10), and dipyridodiazepines (e.g., nevirapine; ref. 11). This is mirrored during the clinical use of NNRT inhibitors, where rapid emergence of resistant strains occurs with nevirapine (D. Richman, personal communication) and with the pyridinones (E. A. Emini, personal communication). These observations underline the value of *in vitro* selection studies for predicting the relative ease with which resistant viruses are likely to appear during therapy.

Genetic studies have shown that one to three mutations in the RT coding region give rise to partial AZT resistance (<100-fold, $IC_{50} < 1$ μM; refs. 12–14). However, four to five amino acid substitutions (codons 41, 67, 70, 215, and 219) are required to produce highly resistant virus (>100-fold, $IC_{50} > 1$ μM; refs. 12–14). A RT mutation at codon 74 was identified in HIV isolates during ddI therapy which gave an 8-fold reduction in sensitivity to ddI and a 15-fold reduction with ddC (3). In the presence of AZT mutations this Val$^{74}$ substitution had a suppressive effect on AZT resistance. During ddC therapy a single RT mutation at codon 69 also resulted in partial resistance (5-fold) (5). Commonly, resistance to the NNRT inhibitors is due to a single substitution at codon 181 in RT (Tyr → Cys), although mutations in other regions of RT, particularly between residues 100 and 108, have been described (9–11, 15). The codon 181 mutation also suppresses AZT resistance when present in a genetic background of AZT-resistance mutations (9).

A second generation of nucleoside analogue inhibitors has been described recently that have more diverse modifications than previous compounds, particularly to the sugar, in an attempt to improve selectivity for greater safety during long-term therapy. These include the 3′-thiacytidine or 3′-oxathialane cytosine analogues (−)-2′-deoxy-3′-thiacytidine (3TC) (16) and (−)-2′-deoxy-5-fluoro-3′-thiacytidine (FTC) (17) (Structure I). In both cases, the "unnatural" L sugar configuration, or (−) enantiomer, is the most potent and selective form (17, 18). The triphosphate derivatives of these nucleosides are inhibitors of HIV-1 RT, presumably accounting for the antiviral activity (17, 19).

As part of the preclinical development of FTC, we decided to assess the potential for the development of resistance to the inhibitor *in vitro* as a possible predictor for what may happen in the clinic. In these studies we describe the isolation in MT-4 cells of resistant variants to both FTC and 3TC. To explore further what may occur in combination therapy with

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Abbreviations: HIV, human immunodeficiency virus; RT, reverse transcriptase; NNRT inhibitor, nonnucleoside RT inhibitor; AZT, 3′-azido-3′-deoxythymidine; ddC, 2′,3′-dideoxycytidine; ddI, 2′,3′-dideoxyinosine; 3TC, (−)-2′-deoxy-3-thiacytidine; FTC, (−)-2′-deoxy-5-fluoro-3′-thiacytidine.

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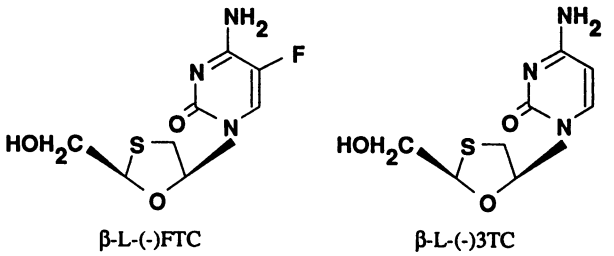


Structure I

these inhibitors, we examined the effect of AZT on development of FTC resistance. In addition, after determining the genetic basis of this resistance, we examined the influence of the FTC/3TC-resistance mutation on virus susceptibility to inhibitors when incorporated into virus variants with AZT- and/or NNRT inhibitor-resistance mutations.

## MATERIALS AND METHODS

**Cells and Virus.** Two human lymphoblastoid T-cell lines were used: MT-4 to propagate HIV-1 for *in vitro* selection of resistant virus and MT-2 (20) for electroporation experiments. Cells were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum plus antibiotics. Human CD4+ HeLa cells, HT4LacZ-1 (21) were used to assess virus sensitivity to inhibitors by plaque reduction (22). This cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum plus antibiotics. The HIV-1 strains used in these studies were the wild-type virus HXB2, derived from the molecular clone pHXB2-D (23), and two AZT-resistant mutants and one NNRT inhibitor-resistant mutant, constructed by site-directed mutagenesis of the RT coding region in pHXB2-D. The AZT-resistant mutants were HIVRTMC (12), with the RT substitutions $Asn^{67}$, $Arg^{70}$, $Phe^{215}$, and $Gln^{219}$, and HIVRTMN (14), with $Leu^{41}$ and $Tyr^{215}$. The NNRT inhibitor-resistant mutant was HXB2(181C) (9), with $Cys^{181}$. Cell-free virus supernatants were stored at −70°C.

**Compounds.** The 3'-thiacytidine nucleosides were synthesized at Wellcome Laboratories, and the (−) and (+) enantiomers were purified to >99% purity as described (17). Nevirapine was a gift from Boerhinger Ingelheim, and ddI and ddC were purchased from Sigma. All compounds were dissolved in dimethyl sulfoxide at 40 mM and diluted in culture medium immediately before use.

**Sensitivity Assays by Plaque Reduction in HT4LacZ-1 Cells.** These were performed as described (22), and the concentration of compound which inhibited virus plaque formation (foci of multinucleated giant cells) by 50% ($IC_{50}$) was determined. The values presented here are means from at least two replicate assays.

**Passage of HIV-1 in MT4 Cells.** Cells ($2 \times 10^6$ per culture) were infected with cell-free HIV supernatants at low multiplicity (<0.1 plaque-forming unit per cell) and virus allowed to adsorb for 1 hr at 37°C. The cells were diluted in RPMI 1640 medium containing 10% (vol/vol) fetal bovine serum plus antibiotics, and test compounds were added at 2–4 times their $IC_{50}$ values (1 and 2 μM FTC, 6 and 12 μM 3TC). Cultures were incubated at 37°C until an extensive cytopathic effect was present and then were clarified by low-speed centrifugation. The virus supernatants were used for further passage and sensitivity testing. Infected cell pellets were used for analysis of viral DNA. The concentration of FTC or 3TC was increased 2- and 4-fold at each passage, and the highest concentration showing complete cytopathic effect was used for the next passage. In combination studies with HXB2, the concentration of AZT was maintained at 50 nM throughout, with an initial FTC concentration of 0.5 μM.

**Genetic Analysis of RT by Sequencing of PCR Products and M13 Clones.** DNA was extracted from HIV-infected MT-4 cells and about 800 base pairs of the RT coding region (amino acids 1–265) was amplified by the polymerase chain reaction (PCR). The product was sequenced directly between codons 40 and 108 and between codons 170 and 225 by using specific oligonucleotide primers as described (9). In addition, the entire RT coding region was obtained by PCR from HXB2 passaged six times in FTC, cloned into M13, and sequenced around the codon 184 region as described (12).

**Construction of Recombinant HIV-1 Variants.** Site-directed mutagenesis using synthetic oligonucleotides (7) introduced mutations into the RT coding region of wild-type virus and a number of AZT- or NNRT inhibitor-resistant variants cloned in M13. Clones were analyzed for expression of functional RT in *Escherichia coli* (7). Recombinant HIV-1 variants were obtained after cotransfection of RT mutant M13 DNA and the RT-deleted provirus clone pHIVΔRTBstE11 into MT-2 cells (14).

## RESULTS AND DISCUSSION

**Selection of Resistant HIV-1 Variants in MT-4 cells.** Initially, MT-4 cells were infected with either wild-type HIV-1 (HXB2) or an AZT-resistant strain derived from it (HIVRTMC) and exposed to low concentrations of FTC. Progeny virus was recovered and serially passaged in MT-4 cells in the presence of increasing FTC concentrations. The FTC sensitivity of virus recovered after each passage, assessed by plaque reduction assay in CD4+ HeLa cells, is shown in Fig. 1*A*. Remarkably, we observed extremely rapid emergence of resistant virus, which reflected the rapid virus growth during each passage. By the fourth passage of HXB2 and only the second of HIVRTMC, $IC_{50}$ values exceeded 50 μM. When tested at higher compound concentrations, the $IC_{50}$ values of passage 6 virus were in excess of 250 μM, representing a >1000-fold shift in sensitivity. These variants were also highly resistant to 3TC but showed no crossresistance to AZT, ddI, or nevirapine. Further virus passage studies, similar to the above but made with 3TC, also resulted in rapid development of highly resistant virus (Fig. 1*B*) that was crossresistant to FTC.

If such rapid resistance also occurs in the clinic, this might severely limit the therapeutic value of these inhibitors, except possibly in combination with other HIV-1 inhibitors such as AZT. Therefore, to test this, we studied the effects of a combination of AZT and FTC on emergence of resistant virus during *in vitro* passage. AZT at 50 nM was able to delay, but not prevent, development of FTC-resistant virus (Fig. 1*A*). This delay in emergence of resistance was also reflected in slower growth of the virus, which may be due in part to synergy observed *in vitro* between AZT and FTC (M. St. Clair, personal communication). These data support the possible use of these compounds in combination therapy.

**Genetic Analysis of Resistant HIV Variants Produced During Passage in MT-4 Cells.** To discover the genetic basis of FTC resistance, we performed direct DNA sequence analysis within the RT coding region obtained by PCR amplification from virus-infected cells after six passages in FTC. FTC resistance appeared to be associated with a mutation at codon 184 of RT, since no other amino acid changes were observed in the regions sequenced. A mixture of nucleotides at codon 184 was seen, with both the FTC-passaged viruses, HXB2 and RTMC. The predominant GTG triplet predicts a Met → Val amino acid change (Table 1). Direct sequence analysis of both 3TC- and FTC/AZT-passaged viruses also revealed mixtures at codon 184 similar to those seen with FTC. However, in the case of HXB2/3TC-passaged virus, ATA predominated, which predicts a Met → Ile amino acid change. To confirm data obtained by direct analysis of PCR products we cloned the entire RT coding region from HXB2

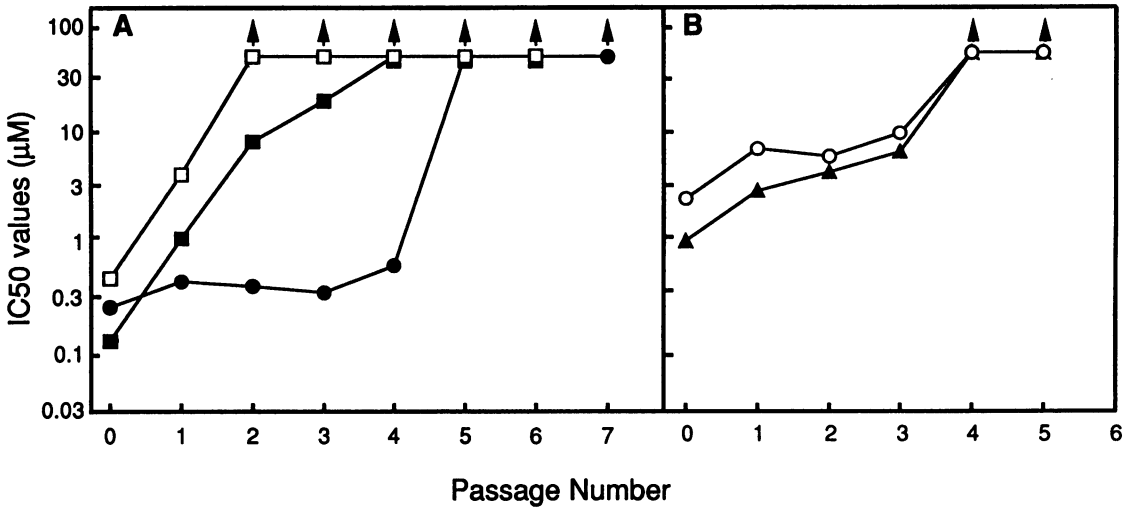


FIG. 1. Sensitivity ($IC_{50}$ values) of HIV strains passaged in MT-4 cells in the presence of the indicated drug(s): FTC (□, RTMC; ■, HXB2) or FTC plus 50 nM AZT (●, HXB2) (*A*) or 3TC (○, RTMC; ▲, HXB-2D) (*B*). MT-4 cells were infected with virus and cultured in the presence of the above compounds. Virus recovered from culture supernatants was serially passaged in increasing concentrations of the compound. Virus was titrated and sensitivity was determined by plaque reduction in the $CD4^+$ HeLa cell line HT4LacZ-1. Arrowheads above data points indicate $IC_{50} > 50$ μM, the highest concentration tested.

passage 6 (FTC) into M13 and sequenced 24 independent clones. This revealed a population of two mutant genotypes with the Met → Val mutation dominant; that is 16 of the 24 clones had GTG (Val) at codon 184, and 8 clones had ATA (Ile). The location of these mutations was unexpected, since they lie in the conserved Tyr, Met, Asp, Asp (YMDD) motif thought to form part of the active site of the RT polymerase domain (24). Previous mutagenesis studies have shown that mutations at this site can severely impair RT function (7, 25, 26). Recently, the $Met^{184}$ → Val mutation has been reported in association with low-level (<5-fold) resistance to ddI and ddC (27). In this study we saw no significant crossresistance with ddI or ddC.

**Analysis of HIV Clones Containing the Codon 184 Mutation Alone and with Other Drug-Resistance Mutations.** To prove that the mutation $Met^{184}$ → Val conferred resistance to FTC, we introduced this change into RT of the wild-type clone HXB2 by site-directed mutagenesis. To determine the influence of the $Met^{184}$ → Val mutation on AZT and NNRT inhibitor resistance, we also constructed a series of HIV-1 variants containing various combinations of mutations. Assessment of RT activity from these clones expressed in *E. coli* revealed no significant reductions relative to the parental clones. Recombinant viruses, obtained after transfection of T cells, all proved viable, with growth comparable to that of parent virus. These mutants were tested for sensitivity to a series of RT inhibitors, including AZT, nevirapine, ddI, ddC, and FTC, by plaque reduction assays in $CD4^+$ HeLa cells (Table 2). All mutant viruses with the $Met^{184}$ → Val substitution (alone or in combination with other resistance mutations) were markedly resistant to FTC and 3TC, showing a >1000-fold increase in $IC_{50}$ values (Table 2). Thus, a single amino acid change can confer high-level resistance to a nucleoside analogue. This is in contrast to the single substitutions induced by ddI and ddC, which confer only a ≤10-fold change in sensitivity. Furthermore, at least four amino acid changes in RT are required to confer an ≈100-fold increase in resistance to AZT (12–14).

Table 1. DNA sequence analysis of RT coding region from resistant virus derived by passage in FTC or 3TC or a combination of FTC plus AZT

| HIV isolate | Inhibitor(s) | Codon 184 sequence | Predicted amino acid |
|---|---|---|---|
| HXB2 passage 6 | FTC | GTG | Val |
| | | GTA | Val |
| | | ATA | Ile |
| | | ATG | Met |
| RTMC passage 6 | FTC | GTG | Val |
| | | GTA | Val |
| | | ATA | Ile |
| | | ATG | Met |
| HXB2 passage 7 | FTC + AZT | GTG | Val |
| | | GTA | Val |
| | | ATA | Ile |
| | | ATG | Met |
| HXB2 passage 5 | 3TC | GTG | Val |
| | | GTA | Val |
| | | ATA | Ile |
| | | ATG | Met |
| RTMC passage 5 | 3TC | GTG | Val |
| | | GTA | Val |
| | | ATA | Ile |
| | | ATG | Met |

Nucleotide mixtures at codon 184 are shown, with the strongest nucleotides in large capital letters and the predominant codon and predicted amino acid underlined.

Some crossresistance was also seen with the (+) enantiomers (D-sugar forms) of 3TC and FTC but was much less marked, with only a 5- to 15-fold increase in $IC_{50}$ values. In addition, with nucleoside inhibitors containing normal bases and sugar analogues in the natural D form (ddI and ddC) only minimal changes in susceptibility were seen. Therefore, high-level resistance is associated with the (−) enantiomers or L-sugar forms of these nucleosides, suggesting that the recognition of these analogues by HIV-1 RT is highly specific.

Analysis of recombinant viruses containing combinations of mutations from different drug-resistant variants revealed some interesting effects on sensitivity to inhibitors (Table 2). Despite the proximity of residues 184 and 181 in RT, combined mutations at these codons did not influence resistance caused by either the 181 mutation to nevirapine or the 184 mutation to FTC and 3TC. The situation regarding AZT mutations was different. The $Met^{184}$ → Val change in a background of AZT resistance had a suppressive effect on AZT resistance, which with partially AZT-resistant virus (substitutions at codons 41 and 215) resulted in complete reversion to AZT sensitivity, as seen previously with the

Table 2. Inhibitor sensitivity of HIV variants with different combinations of RT mutations conferring resistance to nucleoside and nonnucleoside inhibitors

| Virus | RT genotype | | | | | | | $IC_{50}$, μM | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 41 | 67 | 70 | 181 | 184 | 215 | 219 | (−)-FTC | (+)-FTC | (−)-3TC | (+)-3TC | ddC | ddI | Nev | AZT |
| HXB2 | Met | Asp | Lys | Tyr | Met | Thr | Lys | 0.09 | 19 | 0.24 | 3.5 | 0.24 | 1.26 | 0.09 | 0.02 |
| HXB2(184) | | | | | Val | | | >500 | 300 | >500 | 21.6 | 0.77 | 3.54 | 0.1 | 0.03 |
| HXB2(181) | | | | Cys | | | | 0.02 | | | | | | 15.1 | |
| HXB2(181, 184) | | | | Cys | Val | | | >500 | | | | 0.87 | | 12.6 | 0.01 |
| RTMN | Leu | | | | | Tyr | | 0.34 | | | | 0.71 | 3.02 | 0.1 | 0.56 |
| RTMN(184) | Leu | | | | Val | Tyr | | >500 | | | | 1.57 | 4.90 | 0.06 | 0.05 |
| RTMN(181, 184) | Leu | | | Cys | Val | Tyr | | >500 | | | | 0.94 | 2.60 | 7.9 | 0.04 |
| RTMC | | Asn | Arg | | | Phe | Gln | 0.64 | | | | 0.49 | 2.34 | 0.07 | 1.26 |
| RTMC(184) | | Asn | Arg | | Val | Phe | Gln | >500 | | | | 0.92 | 3.02 | 0.04 | 0.17 |
| RTMC(181, 184) | | Asn | Arg | Cys | Val | Phe | Gln | >500 | | | | 0.95 | 2.04 | 4.6 | 0.04 |

The $Met^{184} \rightarrow Val$ mutation was introduced into a number of AZT- or NNRT inhibitor-resistant variants in M13 by site-directed mutagenesis using synthetic oligonucleotides. All clones were analyzed for expression of functional RT in *E. coli* and found to possess RT activity comparable to (within 2-fold of) wild type. Recombinant HIV-1 variants were obtained after cotransfection of RT mutant M13 DNA and the RT-deleted provirus clone pHIVΔRTBstE11 into MT-2 cells. Virus variants were titrated and compound sensitivity was determined by plaque reduction in the $CD4^+$ HeLa cell line HT4LacZ-1. Some suppression of AZT resistance has been seen previously with the 181 mutation alone: RTMC, $IC_{50}$ 1.5 μM; RTMC(181), $IC_{50}$ 0.5 μM (9). Nev, nevirapine.

$Tyr^{181} \rightarrow Cys$ mutation (9). Remarkably, when substitutions at both codon 181 and codon 184 were present in combination with four AZT-resistance mutations (substitutions at codons 67, 70, 215, and 219), complete suppression of high-level AZT resistance occurred.

In conclusion, these data, together with reports of synergy between the 3′-thiacytidine inhibitors and AZT† and between AZT and nevirapine (28), indicate that combination therapy with these inhibitors may be beneficial in the treatment of HIV-1 infection in AIDS. Such information from preclinical studies should assist in the more rational design of trials in the clinical evaluation of these inhibitors. These studies further substantiate the importance of *in vitro* evaluation of the potential for development of resistance to "new" inhibitors. The $Met^{184} \rightarrow Val$ substitution has already been seen in one patient after 3TC treatment, indicating that the *in vitro* data may be predictive for the clinic (29). Knowledge of the types of virus variants which may be generated during combination therapy and the effects resistance mutations have on drug susceptibility should be of value in adopting the best strategies for control of HIV-1 infection in the future.

†Cammack, N., Coates, J. A. V., Jenkinson, H. J., Rouse, P., Penn, C. R. & Cameron, J. M., 8th International Conference on AIDS, July 19–24, 1992, Amsterdam, abstr. PuA 6036.

**Note Added in Proof.** Schinazi *et al.* (29) have reported isolation of variants resistant to (−)-FTC and to (−)-BCH-189 by passage in human peripheral blood mononuclear cells and in MT2 cells. These mutants also showed the same mutations at codon 184.

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