JOURNAL OF VIROLOGY, Mar. 1997, p. 2357–2362 Vol. 71, No. 3
0022-538X/97/$04.00+0

A Novel Met-to-Thr Mutation in the YMDD Motif of Reverse Transcriptase from Feline Immunodeficiency Virus Confers Resistance to Oxathiolane Nucleosides

ROBERT A. SMITH,[1] KATHRYN M. REMINGTON,[1]† ROBERT M. LLOYD, JR.,[2,3] RAYMOND F. SCHINAZI,[2,3]* AND THOMAS W. NORTH[1]*

Division of Biological Sciences, University of Montana, Missoula, Montana 59812,[1] and Georgia VA Research Center for AIDS and HIV Infections, Veterans Affairs Medical Center,[2] and Department of Pediatrics, Emory University School of Medicine,[3] Decatur, Georgia 30033

Received 13 August 1996/Accepted 25 November 1996

Variants of feline immunodeficiency virus (FIV) that possess a unique methionine-to-threonine mutation within the YMDD motif of reverse transcriptase (RT) were selected by culturing virus in the presence of inhibitory concentrations of (−)-β-L-2′,3′-dideoxy-5-fluoro-3′-thiacytidine [(−)-FTC]. The mutants were resistant to (−)-FTC and (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (3TC) and additionally exhibited low-level resistance to 2′,3′-dideoxycytidine (ddC). DNA sequence analysis of the RT-encoding region of the *pol* gene amplified from resistant viruses consistently identified a Met-to-Thr mutation in the YMDD motif. Purified RT from the mutants was also resistant to the 5′-triphosphate forms of 3TC, (−)-FTC, and ddC. Site-directed mutants of FIV were engineered which contain either the novel Met-to-Thr mutation or the Met-to-Val mutation seen in oxathiolane nucleoside-resistant HIV-1. Both site-directed mutants displayed resistance to 3TC, thus confirming the role of these mutations in the resistance of FIV to β-L-3′-thianucleosides.

The emergence of drug-resistant mutants of human immunodeficiency virus type 1 (HIV-1) in infected patients is believed to result in the failure of current antiviral strategies (47, 57). The most common therapeutic agents for the treatment of AIDS are nucleoside analogs which are phosphorylated to their corresponding nucleotides and which inhibit reverse transcriptase (RT) (11, 13). The nucleoside analogs currently approved for use in the treatment of AIDS are 3′-azido-3′-deoxythymidine (AZT), 2′,3′-dideoxyinosine (ddI), 2′,3′-dideoxycytidine (ddC), 2′,3′-didehydro-3′-deoxythymidine (d4T), and (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (3TC). Drug-resistant HIV-1 mutants arise in response to the selection pressure generated by treatment of infected individuals with these compounds (15, 28, 29, 47, 51–53, 57). Resistance also arises in response to nonnucleoside inhibitors of RT, such as nevirapine (48), and to protease inhibitors (9, 23, 42). In addition, numerous mutants have been selected in vitro which are resistant to RT or protease inhibitors (16, 17, 18, 21, 24, 27, 39). In laboratory and clinical isolates, resistance has been correlated with mutations in the RT- or protease-encoding regions, respectively, of the *pol* gene (32).

We have developed systems which use feline immunodeficiency virus (FIV) as a model to examine mechanisms of resistance to HIV-1 inhibitors (36). FIV is a lentivirus which causes immune deficiency and neuropathogenesis in domestic cats which are remarkably similar to those of AIDS in humans (1, 3, 12, 40–42, 44, 58). FIV is well suited as a model for studies of viral resistance to nucleoside compounds because of the similarity of FIV RT to HIV-1 RT in physical properties, catalytic activities, and sensitivity to the active forms of AZT, ddC, ddI, d4T, and 3TC (10, 33, 34). Also, the first drug-resistant lentivirus mutants selected in vitro were AZT-resistant mutants of FIV (45), and we have subsequently reported FIV mutants resistant to ddI (19), ddC (31), d4T (59), and the combination of AZT and ddI (19).

Recent reports have demonstrated the efficacy of 3TC (also known as epivir or lamivudine), both alone and in combination with AZT, in the inhibition of HIV-1 replication in vitro and in vivo (8, 20, 30, 43, 51). The 3TC-AZT combination has been approved for clinical treatment of HIV-1 infection and represents a significant improvement over conventional AZT monotherapy in delaying the resurgence of virus titers associated with the emergence of drug resistance (30). Mutants resistant to 3TC arise rather rapidly during therapy through the acquisition of a Met-to-Val or a Met-to-Ile mutation at position 184 (Met184Val or Met184Ile) of RT, as first reported by Schinazi et al. (49). However, these Met184Val mutants have been demonstrated to phenotypically suppress AZT resistance mutations (30, 55) and may enhance the fidelity of nucleotide insertion by RT in vitro (56). This introduces the possibility that the use of 3TC in generating Met184Val mutants may represent a favorable event in the clinical treatment of HIV-1 infection.

Here we report the selection and characterization of FIV mutants which possess a unique Met-to-Thr mutation in the highly conserved YMDD motif of RT (2, 6, 38, 49, 54). These mutants were generated in response to selection with (−)-β-L-2′,3′-dideoxy-5-fluoro-3′-thiacytidine [(−)-FTC]. (−)-FTC is the 5-fluoro-derivative of 3TC and is a potent inhibitor of HIV-1 and hepatitis B virus replication (14, 50).

* Corresponding author. For Raymond F. Schinazi: Phone: (404) 728-7711. Fax: (404) 728-7726. For Thomas W. North: Phone: (406) 243-2118. Fax: (406) 243-4304.

† Present address: Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709.

MATERIALS AND METHODS

Chemicals. Phosphonoformic acid (PFA), dCTP, dATP, dGTP, dTTP, and ddC were purchased from Sigma Chemical Co., St. Louis, Mo. AZT was provided by Glaxo Wellcome Co., Research Triangle Park, N.C.; d4T was provided by Bristol-Myers Squibb Co., Wallingford, Conn.; and 9-(2-phosphonylmethoxyethyl)adenine (PMEA) was provided by Gilead Sciences, Inc., Foster City, Calif. ddI was provided by the Developmental Therapeutics Branch, Division of AIDS,

National Institute of Allergy and Infectious Diseases. 3TC, (−)-FTC, 3TC triphosphate (3TCTP), and (−)-FTC 5′-triphosphate [(−)-FTCTP] were synthesized and fully characterized by mass spectroscopy, nuclear magnetic resonance imaging, and high-pressure liquid chromatography as described previously (4, 7, 22). [*Methyl*-^{3}H]dTTP was obtained from Dupont-New England Nuclear, Boston, Mass. GeneAmp PCR core reagents were purchased from Perkin-Elmer Cetus, Norwalk, Conn. The *Taq* DyeDeoxy terminator cycle sequencing kit was purchased from Applied Biosystems, Foster City, Calif. Restriction enzymes *Pst*I, *Hin*dIII, *Nsi*I, and *Pac*I were obtained from Boehringer Mannheim, Indianapolis, Ind., and T4 DNA ligase was obtained from Gibco BRL, Grand Island, N.Y. All other chemicals were reagent grade or better.

Cells and virus. Virus produced from a molecular clone of the Petaluma strain of FIV, 34TF10 (54), was used as wild-type FIV for these studies. Wild-type and mutant strains of FIV were grown and maintained in Crandell feline kidney (CrFK) cells with L&M medium supplemented with 10% fetal bovine serum as previously described (37). All CrFK cells used for these experiments were passaged no more than six times after they were obtained from the American Type Culture Collection (Rockville, Md.), as higher-passage cells lose their ability to be infected by FIV (26). FIV mutants were maintained in medium containing (−)-FTC, and all cultures were refed with fresh medium and drug every 2 days.

FIA. Inhibition of FIV infection by RT inhibitors was quantified by a focal infectivity assay (FIA) as described previously (45). Resulting data were plotted as the percentage of control foci (no drug) versus inhibitor concentration. Concentrations of drug required to inhibit focus formation by 50% (EC_{50}) were obtained directly from the linear portion of these plots with a computer-generated regression line (45). Within an experiment, each value represents the mean of four determinations. Results from three or more independent experiments were used to derive the mean EC_{50} ± the standard error.

Selection and plaque purification of (−)-FTC-resistant mutants. FIV mutants resistant to oxathiolane nucleosides were obtained by passage of FIV in CrFK cells maintained in the continuous presence of 10 μM (−)-FTC. For the first round of selection, two 25-cm^2 flasks of freshly seeded, low-passage CrFK cells (approximately 10^5 cells total) were preincubated for 1 h in the presence of 10 μM (−)-FTC in order to convert the drug to the active triphosphate form. One milliliter of culture supernatant containing approximately 690 focus-forming units of FIV grown from the molecular clone, 34TF10, was adsorbed directly onto the monolayer of each flask for 1 h. Following adsorption, 3 ml of L&M medium containing (−)-FTC at a final concentration of 10 μM was added to each culture. Infected cells were maintained in the continuous presence of 10 μM (−)-FTC by replacing media and drugs every 48 h, and cells were removed with trypsin and subcultured as necessary. Cultures were monitored weekly by FIA for the presence of virus, which was detectable approximately 4 weeks postinfection. Virus from each of the two cultures was used to initiate a second round of infection as described above, but only one of these cultures produced detectable levels of virus. This virus was carried to a third and final round of infection and was then assayed for sensitivity to (−)-FTC. The resulting population, designated FTR-1c, demonstrated approximately fourfold resistance to (−)-FTC, with an EC_{50} of 4.6 μM (data not shown). This stock was then plaque purified as previously described (46) to obtain FTR-2c and FTR-3c, which were then used for further analysis.

Enzymes and enzyme assays. RT was purified from virions of mutant FIV by methods developed previously by North et al. (34). Briefly, mutant virions were harvested by centrifugation of supernatants from infected cell cultures and then lysed with a detergent buffer. Viral lysates were then purified by DEAE-cellulose and phosphocellulose column chromatography. Assays for RT activity with poly(rI)-oligo(dC) as template were performed as previously described (33, 34). Kinetic parameters were determined as previously described (10, 33), with intercept values calculated from double-reciprocal plots. Recombinant FIV RT (35) was used as the wild-type control.

Nucleic acid preparation and sequence analysis. Total cellular DNA containing proviral DNA was extracted from infected CrFK cells as previously described (46). Following ethanol precipitation, DNA was resuspended in sterile distilled water and used for amplification by PCR as previously described (31, 46). Gel-purified PCR product was sequenced at the Murdock Molecular Biology Facility with a *Taq* DyeDeoxy terminator sequencing kit and was analyzed on a model 373A automated DNA sequencer (Applied Biosystems). Sequencing was performed in the forward and reverse directions with two or more primers covering each 250-bp section of the RT-encoding region of the *pol* gene.

Site-directed mutagenesis. In order to construct the mutants at codon 183 of RT, a 2,076-bp *Pst*I-*Hin*dIII fragment, corresponding to nucleotides 2817 to 4893 of pFIV-34TF10, was cloned into the pAlter-1 mutagenesis vector (Promega) and mutagenized with the Altered Sites II in vitro mutagenesis system (Promega). Mutagenesis primers 5′-TTACCAATATACGGATGACAT-3′ and 5′-TTACCAATATGTGGATGACAT-3′ were used to introduce the Met183Thr and Met183Val mutations, respectively (mutations underlined). Following sequence analysis to confirm the presence of the desired mutation, an 874-bp *Nsi*I-*Pac*I fragment corresponding to nucleotides 3582 to 4456 of pFIV-34TF10 was cloned back into pFIV-34TF10, resulting in full-length, mutant molecular infectious cloned DNA. All resulting clones were sequenced in order to verify the presence of the mutation and the integrity of the *pol* gene. These constructs were then introduced into *Escherichia coli* JM109, and 2 μg of the resulting plasmid DNA was purified and used to transfect CrFK cells as previously described (46) for the production of virus.

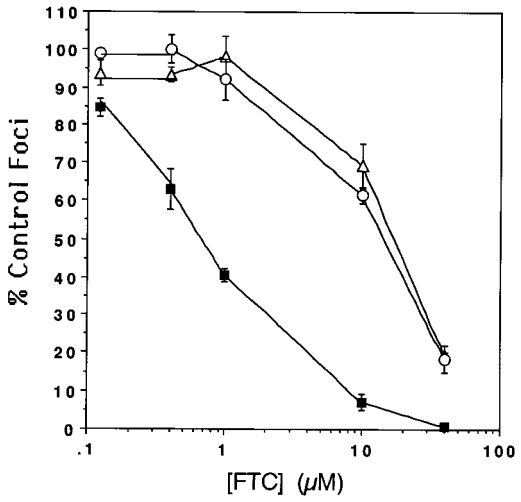

FIG. 1. Inhibition of FIV 34TF10 (■) and (−)-FTC-resistant mutants FTR-2c (△) and FTR-3c (○) by (−)-FTC. Results are from four experiments with four determinations per experiment. Bars represent standard errors of the means and are omitted when the standard error was too small to be shown.

RESULTS

Selection and plaque purification of (−)-FTC-resistant mutants. The original mutant population, designated FTR-1c, was selected by passaging virus produced by the 34TF10 molecular clone of FIV for three rounds of infection in the presence of 10 μM (−)-FTC as described above. FTR-1c displayed approximately fourfold resistance to (−)-FTC, with an EC_{50} of 4.6 μM (data not shown).

To minimize potential heterogeneity within the mutant population, plaque purification of FTR-1c was performed to obtain virus stocks derived from a single focus-forming unit. Two plaque-purified mutants, designated FTR-2c and FTR-3c, were 11- to 15-fold resistant to (−)-FTC (Fig. 1) and were chosen for further phenotypic characterization. FTR-2c and -3c were six- to eightfold resistant to 3TC and additionally displayed low-level resistance (2.5- to 3.5-fold) to ddC. Both mutants showed wild-type susceptibility to AZT, PMEA, ddI, d4T, and PFA (Table 1).

RT. RT purified from FTR-2c was compared to wild-type FIV RT with respect to susceptibility to inhibition by ddC

TABLE 1. Susceptibilities of FIV 34TF10 and plaque-purified (−)-FTC-resistant mutants to antiviral compounds as determined by FIA

Compound	Mean EC_{50} (μM) ± SEM[a] for:			Fold increase[b] for:	
	34TF10	FTR-2c	FTR-3c	FTR-2c	FTR-3c
(−)-FTC	0.9 ± 0.2	13.9 ± 0.9	9.8 ± 0.7	15	11
3TC	1.2 ± 0.1	7.6 ± 0.9	9.3 ± 2.1	6.3	7.8
AZT	1.0 ± 0.3	1.0 ± 0.3	1.1 ± 0.2	1.0	1.1
ddC	1.9 ± 0.3	7.2 ± 0.8	5.2 ± 0.5	3.8	2.7
ddI	1.9 ± 0.4	1.5 ± 0.3	1.6 ± 0.1	0.8	0.8
PMEA	0.6 ± 0.1	0.5 ± 0.0	0.6 ± 0.0	0.8	1.0
PFA	150 ± 0.7	150 ± 4.4	110 ± 2.8	1.0	0.7
d4T	14 ± 1.9	11 ± 2.2	11 ± 0.6	0.8	0.8

[a] Values are from three or more experiments, with four determinations per experiment.
[b] Increase over 34TF10 level.

TABLE 2. Kinetic constants for RTs from wild-type FIV and FTR-2c mutant

Inhibitor	K_i (nM)[a] for RT of:		K_i/K_m for RT of:		Fold increase of K_i/K_m[b]
	Recombinant FIV	FTR-2c	Recombinant FIV	FTR-2c	
ddCTP	112 ± 5.2	1400 ± 160	0.024	0.189	7.9
(−)-FTCTP	138 ± 12	380 ± 32	0.030	0.054	1.8
3TCTP	138 ± 5.2	391 ± 44	0.030	0.056	1.9

[a] Values are the means ± standard errors of the means of at least two experiments with three determinations per experiment. The mode of inhibition by ddCTP, FTCTP, and 3TCTP was competitive with respect to substrate. The K_m for dCTP was 4.6 ± 0.3 μM for recombinant FIV RT and 7.0 ± 0.7 μM for FTR-2c RT.
[b] Increase of K_i/K_m for FTR-2c over K_i/K_m for recombinant (wild-type) FIV.

5′-triphosphate (ddCTP), (−)-FTCTP, and AZT 5′-triphosphate (AZTTP) (Table 2). RTs from both FTR-2c and wild-type FIV were inhibited by (−)-FTCTP, 3TCTP, and ddCTP in a manner that was competitive with respect to dCTP. K_m values for dCTP were 7.0 ± 0.7 μM for FTR-2c RT and 4.6 ± 0.3 μM for wild-type FIV RT. K_i values for inhibition of the mutant enzyme by (−)-FTCTP and 3TCTP were threefold greater than the corresponding K_i values for wild-type FIV RT. Additionally, the K_i value for the inhibition of FTR-2c RT by ddCTP was 12.5-fold greater than the K_i value for the wild-type enzyme.

Nucleotide sequence analysis. DNA sequence analysis of the entire RT-encoding region of the *pol* gene from FTR-2c was performed in both the forward and reverse directions (Fig. 2), and the resulting sequence was compared to the sequence from FIV 34TF10 (54). Two point mutations were observed in FTR-2c. The first of these is a T-to-C transition at position 2883 which results in a Met-to-Thr mutation, encoded by codon 183, in FIV RT. Codon 183 is located in the conserved YMDD motif of FIV RT and corresponds to Met-184 of HIV-1 RT (Fig. 2). The second mutation found in FTR-2c is an A-to-C transversion at position 3053 which results in a change of isoleucine to leucine at position 240 of RT. Sequence analysis of the other mutant, FTR-3c, was also performed in the area surrounding the position 183 and 240 regions in both the forward and reverse directions. This mutant contained the same Met-to-Thr change at the amino acid encoded by codon 183 but displayed the wild-type sequence at codon 240. Since FTR-2c and FTR-3c displayed nearly identical susceptibilities to 3TC, we conclude that codon 240 is not necessary for the acquisition of 3TC resistance.

In order to determine whether FTR-2c and -3c were genetically stable, both mutants were passaged for three rounds of infection in the absence of (−)-FTC by protocols described previously (46). Both plaque-purified mutants remained significantly resistant to (−)-FTC even after three rounds of infection in the absence of drug. However, resistance to (−)-FTC decreased slightly by the third round of infection, with FTR-2c and FTR-3c displaying EC_{50} of 6.4 and 5.9 μM, respectively.

Site-directed mutagenesis. In order to confirm the role of mutations at codon 183 of the FIV RT gene in FTC and 3TC resistance, the Met183Val and Met183Thr mutations were introduced into the molecular clone FIV 34TF10 by site-directed mutagenesis. Both of the resulting mutants displayed seven- to eightfold resistance to 3TC (Fig. 3), with EC_{50} of 1.4 μM for 34TF10, 10.7 μM for FIVMet183Val, and 9.5 μM for FIVMet183Thr. These data substantiate the role of these mutations at the Met codon of the YMDD motif in the resistance of FIV to (−)-FTC and 3TC.

A

```
      178 179 180 181 182 183 184 185 186 187 188//

      Ile Val Ile Tyr Gln Tyr Met Asp Asp Leu Tyr//  HIV-1

      Asp Ile Tyr Gln Tyr Met Asp Asp Ile Tyr Ile//  FIV 34TF10
2867  GAT ATT TAC CAA TAT ATG GAT GAC ATT TAT ATA//  2899

                          Thr
      --- --- --- --- --- -C- --- --- --- --- ---//  FTR-2C

                          Thr
      --- --- --- --- --- -C- --- --- --- --- ---//  FTR-3C
```

B

```
      \\235 236 237 238 239 240 241 242 243 244 245

      \\His Pro Asp Lys Trp Thr Val Gln Pro Ile Val  HIV-1

      \\Pro Leu Trp Trp Thr Ile Gln Gln Lys Gln Leu  FIV 34TF10
3038  \\CCA TTA ACA TGG ACA ATA CAA CAG AAA CAG TTA  3070

                            Leu
      \\--- --- --- --- --- C-- --- --- --- --- ---  FTR-2C

      \\--- --- --- --- --- --- --- --- --- --- ---  FTR-3C
```

FIG. 2. Nucleotide and deduced amino acid sequences of the regions of the FIV *pol* gene surrounding positions 2883 (A) and 3053 (B). The corresponding sequences from HIV-1 are also shown for comparison. Note that HIV-1 and FIV RTs exhibit extensive homology in the positions surrounding position 184 (A) and that the FIV sequence is displaced by one residue relative to the HIV-1 sequence. Nucleotide sequence data for FTR-2c and FTR-3c were compared to the sequence data for FIV 34TF10. Mutations are shown in boldface.

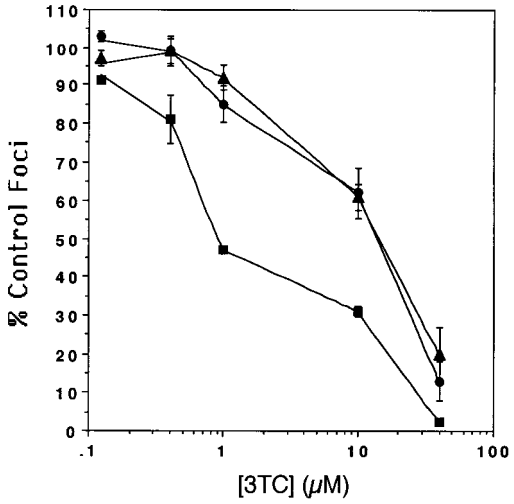

FIG. 3. Susceptibility to inhibition by 3TC of FIV 34TF10 (■) and the two FIV mutants made by site-directed mutagenesis, FIVMet184Val (▲) and FIVMet184Thr (●). Results are from three experiments with four determinations per experiment. Bars represent standard errors of the means and are omitted when the standard error was too small to be shown.

DISCUSSION

We have selected FIV mutants resistant to two β-L-oxathiolane nucleosides, (−)-FTC and 3TC (the absolute configuration of these compounds is 2*R*,5*S*), and these mutants carry a unique Met183Thr mutation in the YMDD motif of RT. Previous studies of HIV-1 have shown that Met-to-Val and Met-to-Ile mutations in the YMDD motif (position 184 in HIV-1) confer resistance to these oxathiolane nucleosides (5, 17, 49, 55). Here we have identified a drug resistance mutation in FIV which is localized to the same codon within the highly conserved YMDD motif as that associated with 3TC resistance in HIV-1 but which leads to a novel amino acid substitution.

It is interesting to note that during 3TC monotherapy of HIV-1-infected patients, drug-resistant variants appear which initially carry the Met184Ile mutation. As therapy continues, these mutants are subsequently replaced by variants carrying the Met184Val substitution. In addition, it has recently been reported that the Met184Thr mutation has been observed in 3TC-resistant HIV-1 isolates selected in vitro, although both the replication capacity and the RT activity of this variant were markedly reduced (25).

The 10- to 15-fold resistance to (−)-FTC and 6- to 8-fold resistance to 3TC exhibited by these FIV mutants differs from the 100-fold or greater resistance seen previously in oxathiolane nucleoside-resistant HIV-1 mutants obtained both from clinical isolates (51) and from in vitro selections with either (−)-FTC (49) or 3TC (17, 49, 55). In addition, these FIV mutants display slight cross-resistance (threefold) to ddC but wild-type sensitivity to ddI. This again is in contrast to oxathiolane nucleoside-resistant HIV-1 mutants, which possess either low-level resistance to both ddC and ddI (17, 55) or no resistance to either 2′,3′-dideoxy compound (49). These phenotypic differences may result from basic differences between the two lentiviruses, or alternatively they may be due to differences in selection protocols, cell culture systems, or phenotypic assays used to determine the drug sensitivities of mutant isolates. Further selections with 3TC are being performed to determine if FIV mutants can be obtained which display a higher level of 3TC resistance than reported here.

Reverse transcriptase purified from our (−)-FTC-resistant mutant was resistant to ddCTP, (−)-FTCTP, and 3TCTP. The mutant enzyme had K_i values and K_i/K_m ratios for (−)-FTCTP and 3TCTP which were about three- and twofold higher, respectively, than those of the wild-type FIV RT. Additionally, the mutant enzyme displayed a higher level of resistance to ddCTP, with K_i values and K_i/K_m ratios 13- and 8-fold higher, respectively, than those of the wild-type enzyme. A comparison of these data to data on the ability of the corresponding nucleosides to inhibit FIV replication demonstrates that the degree of resistance to these three inhibitors at the enzyme level did not correlate with the level of viral resistance to (−)-FTC, 3TC, and ddC. Previous studies of HIV-1 have shown similar discrepancies between inhibition at the phenotypic level and inhibition of the enzyme. An HIV-1 variant resistant to (−)-FTC showed an EC_{50} 300 times greater than the EC_{50} of the wild-type parent at the phenotypic level. However, the 50% inhibitory concentration for the inhibition of RT by (−)-FTCTP for this mutant was only 25-fold higher than that for the parent strain (49).

We have previously described AZT-resistant mutants of FIV which revert very rapidly (within one round of infection) when passaged in the absence of AZT (46). In contrast, both of the oxathiolane nucleoside-resistant FIV mutants described here remained significantly resistant to 3TC following three rounds of infection in the absence of drug. However, the decrease in EC_{50} for (−)-FTC by the third round of infection suggests that some wild-type virus may have begun to emerge in the population. This emergence may be due to a selective disadvantage of the mutant virus when replicating in the absence of (−)-FTC. In support of this, we have observed that FTR-2c replicates more slowly than wild-type FIV in the absence of drug (data not shown).

Other FIV mutants resistant to AZT (45), ddI (19), d4T (59), ddC (31), or the combination of ddI and AZT (19) display phenotypes similar to those of drug-resistant HIV-1 mutants. However, none of these FIV mutants carry point mutations which map to homologous locations within the RTs of similar HIV-1 mutants (19, 31, 45, 59). The (−)-FTC-resistant variants of FIV reported here have a mutation at the corresponding codon of the HIV-1 RT gene which confers resistance to (−)-FTC and 3TC. Additionally, when the Met-to-Val change seen in HIV-1 is introduced into wild-type FIV, the resulting virus is also resistant to 3TC at about the same level as that seen with the FIV Met-to-Thr mutant.

The FIV variants described above represent two plaque-purified mutants derived from the same original mutant population. On the basis of the phenotypic similarities of the Met-to-Val and Met-to-Thr FIV mutants, it is likely that subsequent selections with oxathiolane nucleosides will generate the Met-to-Val FIV mutant in vitro. The data presented here suggest that FIV represents an attractive model for evaluating aspects of 3TC or (−)-FTC resistance which can only be addressed in an animal model, including pathogenesis of codon 184 mutants, and the ability of these mutants to acquire resistance to other inhibitors in combination chemotherapy.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI28189 from the National Institute of Allergy and Infectious Diseases (T.N.) and by the Department of Veterans Affairs and the Georgia Research Center on AIDS and HIV Infection (R.F.S.).

We thank Doug McBroom for excellent technical assistance and Joan Strange and the Murdock Molecular Biology Facility for DNA sequence analysis and oligonucleotide synthesis.

REFERENCES

1. **Ackley, C. D., J. K. Yamamoto, N. Levy, N. C. Pedersen, and M. D. Cooper.** 1990. Immunologic abnormalities in pathogen-free cats experimentally infected with feline immunodeficiency virus. J. Virol. **64:**5652–5655.
2. **Barber, A. M., A. Hizi, J. V. Maizel, and S. H. Hughes.** 1990. HIV-1 reverse transcriptase: structural predictions for the polymerase domain. AIDS Res. Hum. Retroviruses **6:**1061–1072.
3. **Barlough, J. E., C. D. Ackley, J. W. George, N. Levy, R. Acevedo, P. F. Moore, B. A. Rideout, M. D. Cooper, and N. C. Pedersen.** 1991. Acquired immune dysfunction in cats with experimentally induced feline immunodeficiency virus infection: comparison of short-term and long-term infections. J. Acquired Immune Defic. Syndr. **4:**219–227.
4. **Beach, J. W., L. S. Jeong, A. J. Alves, D. Pohl, H. O. Kim, C.-N. Chang, S.-L. Doong, R. F. Schinazi, Y.-C. Cheng, and C. K. Chu.** 1992. Synthesis of enantiomerically pure (2′R,5′S)-(−)-1-[2-(hydroxymethyl)-oxathiolan-5-yl] cytosine as a potent antiviral agent against hepatitis B virus (HBV) and human immunodeficiency virus (HIV). J. Org. Chem. **57:**2217–2219.
5. **Boucher, C. A. B., N. Cammack, P. Schipper, R. Schuurman, P. Rouse, M. A. Wainberg, and J. M. Cameron.** 1993. High-level resistance to (−) enantiomeric 2′-deoxy-3′-thiacytidine in vitro is due to one amino acid substitution in the catalytic site of human immunodeficiency virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. **37:**2231–2234.
6. **Boyer, P. L., A. L. Ferris, and S. H. Hughes.** 1992. Cassette mutagenesis of the reverse transcriptase of human immunodeficiency virus type 1. J. Virol. **66:**1745–1755.
7. **Choi, C. K., J. W. Beach, L. J. Wilson, S. Yeola, D. C. Liotta, and R. F. Schinazi.** 1991. *In situ* complexation directs the stereochemistry of N-glycosylation in the synthesis of oxathiolanyl and dioxythiolanyl nucleoside analogs. J. Am. Chem. Soc. **113:**9377–9379.
8. **Coates, J. A. V., N. Cammack, H. J. Jenkinson, A. J. Jowett, M. I. Jowett, B. A. Pearson, C. R. Penn, P. L. Rouse, K. C. Viner, and J. M. Cameron.** 1992. (−)-2′-Deoxy-3′-thiacytidine is a potent, highly selective inhibitor of

human immunodeficiency virus type 1 and type 2 replication in vitro. Antimicrob. Agents Chemother. **36:**733–739.

9. **Condra, J. H., W. A. Schieif, O. M. Blahy, L. J. Gabryelski, D. J. Graham, J. C. Quintero, A. Rhodes, H. L. Robbins, E. Roth, M. Shivaprakash, D. Titus, T. Yang, H. Teppler, K. E. Squires, P. J. Deutsch, and E. A. Emini.** 1995. In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature (London) **374:**569–571.
10. **Cronn, R. C., K. M. Remington, B. D. Preston, and T. W. North.** 1992. Inhibition of reverse transcriptase from feline immunodeficiency virus by analogs of 2′-deoxyadenosine-5′-triphosphate. Biochem. Pharmacol. **44:**1375–1381.
11. **DeClercq, E.** 1992. HIV inhibitors targeted at the reverse transcriptase. AIDS Res. Hum. Retroviruses **8:**119–134.
12. **English, R. V., P. Nelson, C. M. Johnson, M. Nasisse, W. A. Tompkins, and M. B. Tompkins.** 1994. Development of clinical disease in cats experimentally infected with feline immunodeficiency virus. J. Infect. Dis. **170:**543–552.
13. **Faraj, A., L. A. Agrofoglio, J. K. Wakefield, S. McPherson, C. D. Morrow, G. Gosselin, C. Mathe, J.-L. Imbach, R. F. Schinazi, and J.-P. Sommadossi.** 1994. Inhibition of human immunodeficiency virus type 1 reverse transcriptase by the 5′-triphosphate β enantiomers of cytidine analogs. Antimicrob. Agents Chemother. **38:**2300–2305.
14. **Faraj, A., G. Gosselin, J.-L. Imbach, R. F. Schinazi, and J.-P. Sommadossi.** 1994. Selective inhibition of viral DNA polymerases by the triphosphate of β-L-2′,3′-dideoxycytidine (β-L-DDC) and its 5-fluoro derivative (β-L-FDDC). Antiviral Res. **23**(Suppl. 1)**:**53.
15. **Fitzgibbon, J. E., R. M. Howell, C. A. Haberzettl, S. J. Sperber, D. J. Gocke, and D. T. Dubin.** 1992. Human immunodeficiency virus type 1 *pol* gene mutations which cause decreased susceptibility to 2′,3′-dideoxycytidine. Antimicrob. Agents Chemother. **36:**153–157.
16. **Gao, Q., Z. Gu, J. Hiscott, G. Dionne, and M. A. Wainberg.** 1993. Generation of drug-resistant variants of human immunodeficiency virus type 1 by in vitro passage in increasing concentrations of 2′,3′-dideoxycytidine and 2′,3′-dideoxy-3′-thiacytidine. Antimicrob. Agents Chemother. **37:**130–133.
17. **Gao, Q., Z. Gu, M. A. Parniak, J. Cameron, N. Cammack, C. Boucher, and M. A. Wainberg.** 1993. The same mutation that encodes low-level human immunodeficiency virus type 1 resistance to 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine confers high-level resistance to the (−) enantiomer of 2′,3′-dideoxy-3′-thiacytidine. Antimicrob. Agents Chemother. **37:**1390–1392.
18. **Gao, Q., Z. Gu, M. A. Parniak, X. Li, and M. A. Wainberg.** 1992. In vitro selection of variants of human immunodeficiency virus type 1 resistant to 3′-azido-3′-deoxythymidine and 2′,3′-dideoxyinosine. J. Virol. **66:**12–19.
19. **Gobert, J. M., K. M. Remington, Y.-Q. Zhu, and T. W. North.** 1994. Multiple-drug-resistant mutants of feline immunodeficiency virus selected with 2′,3′-dideoxyinosine alone and in combination with 3′-azido-3′-deoxythymidine. Antimicrob. Agents Chemother. **38:**861–864.
20. **Gosselin, G., R. F. Schinazi, J.-P. Sommadossi, C. Mathé, M.-C. Bergogne, A.-M. Aubertin, A. Kirn, and J.-L. Imbach.** 1994. Anti-human immunodeficiency virus activities of the β-L-enantiomer of 2′,3′-dideoxycytidine and its 5-fluoro derivative in vitro. Antimicrob. Agents Chemother. **38:**1292–1297.
21. **Gu, Z., Q. Gao, H. Fang, H. Salomon, M. A. Parniak, E. Goldberg, J. Cameron, and M. A. Wainberg.** 1994. Identification of a mutation at codon 65 in the IKKK motif of reverse transcriptase that encodes human immunodeficiency virus resistance to 2′,3′-dideoxycytidine and 2′,3′-dideoxy-3′-thiacytidine. Antimicrob. Agents Chemother. **38:**275–281.
22. **Hoong, L. K., L. E. Strange, D. C. Liotta, G. W. Koszalka, C. L. Burns, and R. F. Schinazi.** 1992. Enzyme-mediated enantioselective preparation of pure enantiomers of the antiviral agent 2′,3′-dideoxy-5-fluoro-3′-thiacytidine [(−)-FTC] and related compounds. J. Org. Chem. **57:**5563–5585.
23. **Jacobsen, H., K. Yasargil, D. L. Winslow, J. C. Craig, A. Krohn, I. B. Duncan, and J. Mous.** 1995. Characterization of human immunodeficiency virus type 1 mutants with decreased sensitivity to proteinase inhibitor Ro 31-8959. Virology **206:**527–534.
24. **Kaplan, A. H., S. F. Michael, R. S. Wehbie, M. F. Knigge, D. A. Paul, L. Everit, D. J. Kempf, D. W. Norbeck, J. W. Erickson, and R. Swanstrom.** 1994. Selection of multiple human immunodeficiency virus type 1 variants that encode viral proteases with decreased sensitivity to an inhibitor of the viral protease. Proc. Natl. Acad. Sci. USA **91:**5597–5601.
25. **Keulen, W., A. van Wijk, C. Boucher, and B. Berkhout.** Initial appearance of 184Ile variant in 3TC-treated patients can be explained by the mutation bias of the HIV-1 RT enzyme. Antiviral Ther., in press.
26. **LaCasse, R. A., K. M. Remington, and T. W. North.** 1996. AZT enhances the mutation frequency of feline immunodeficiency virus. J. Acquired Immune Defic. Syndr. Hum. Retrovirol. **12:**26–32.
27. **Larder, B. A., K. E. Coates, and S. D. Kemp.** 1991. Zidovudine-resistant human immunodeficiency virus selected by passage in cell culture. J. Virol. **65:**5232–5236.
28. **Larder, B. A., G. Darby, and D. D. Richman.** 1989. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science **243:**1731–1734.
29. **Larder, B. A., and S. D. Kemp.** 1989. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). Science **246:**1155–1158.
30. **Larder, B. A., S. D. Kemp, and P. R. Harington.** 1995. Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. Science **269:**696–699.
31. **Medlin, H. K., Y.-Q. Zhu, K. M. Remington, T. R. Phillips, and T. W. North.** 1996. Selection and characterization of a mutant of feline immunodeficiency virus resistant to 2′,3′-dideoxycytidine. Antimicrob. Agents Chemother. **40:**953–957.
32. **Mellors, J. W., B. A. Larder, and R. F. Schinazi.** 1995. Mutations in HIV-1 reverse transcriptase and protease associated with drug resistance. Int. Antivir. News **3:**8–13.
33. **North, T. W., R. C. Cronn, K. M. Remington, and R. T. Tandberg.** 1990. Direct comparisons of inhibitor sensitivities of reverse transcriptases from feline and human immunodeficiency viruses. Antimicrob. Agents Chemother. **34:**1505–1507.
34. **North, T. W., R. C. Cronn, K. M. Remington, R. T. Tandberg, and R. C. Judd.** 1990. Characterization of reverse transcriptase from feline immunodeficiency virus. J. Biol. Chem. **265:**5121–5128.
35. **North, T. W., G. L. Hansen, Y.-Q. Zhu, J. A. Griffin, and C.-K. Shih.** 1994. Expression of reverse transcriptase from feline immunodeficiency virus in *Escherichia coli*. Antimicrob. Agents Chemother. **38:**388–391.
36. **North, T. W., and R. A. LaCasse.** 1995. Testing HIV-1 drugs in the FIV model. Nat. Med. **1:**410–411.
37. **North, T. W., G. L. T. North, and N. C. Pedersen.** 1989. Feline immunodeficiency virus: a model for reverse transcriptase-targeted chemotherapy for acquired immune deficiency syndrome. Antimicrob. Agents Chemother. **33:**915–919.
38. **Olmsted, R. A., V. M. Hirsch, R. H. Purcell, and P. R. Johnson.** 1989. Nucleotide sequence analysis of feline immunodeficiency virus: genome organization and relationship to other lentiviruses. Proc. Natl. Acad. Sci. USA **86:**8088–8092.
39. **Otto, M. J., S. Garber, D. L. Winslow, C. D. Reid, P. Aldrich, P. K. Jadhav, C. E. Pattersen, C. N. Hodge, and Y.-S. E. Cheng.** 1993. In vitro isolation and identification of human immunodeficiency virus (HIV) variants with reduced sensitivity to C-2 symmetrical inhibitors of HIV type 1 protease. Proc. Natl. Acad. Sci. USA **90:**7543–7547.
40. **Pedersen, N. C.** 1993. The feline immunodeficiency virus, p. 181–219. *In* J. A. Levy (ed.), The Retroviridae, vol. 2. Plenum Press, New York, N.Y.
41. **Pedersen, N. C., E. W. Ho, M. L. Brown, and J. K. Yamamoto.** 1987. Isolation of a T-lymphotrophic virus from domestic cats with an immunodeficiency-like syndrome. Science **235:**790–793.
42. **Phillips, T. R., O. Prospero-Garcia, D. L. Puaoi, D. L. Lerner, H. S. Fox, R. A. Olmsted, F. E. Bloom, S. J. Henriksen, and J. H. Elder.** 1994. Neurological abnormalities associated with feline immunodeficiency virus infection. J. Gen. Virol. **75:**979–987.
43. **Pluda, J. M., T. P. Cooley, J. S. G. Montaner, L. E. Shay, N. E. Reinhalter, S. N. Warthan, J. Ruedy, H. M. Hirst, C. A. Vicary, J. B. Quinn, G. J. Yuen, W. A. Wainberg, M. Rubin, and R. Yarchoan.** 1995. A phase I/II study of 2′-deoxy-3′-thiacytidine (lamivudine) in patients with advanced human immunodeficiency virus infection. J. Infect. Dis. **171:**1438–1447.
44. **Podel, M. N., M. Oglesbee, L. Mathes, S. Krakowka, R. Olmstead, and L. Lafredo.** 1993. AIDS-associated encephalopathy with experimental feline immunodeficiency virus infection. J. Acquired Immune Defic. Syndr. **6:**758–771.
45. **Remington, K. M., B. Chesebro, K. Wehrly, N. C. Pedersen, and T. W. North.** 1991. Mutants of feline immunodeficiency virus resistant to 3′-azido-3′-deoxythymidine. J. Virol. **65:**308–312.
46. **Remington, K. M., Y.-Q. Zhu, T. R. Phillips, and T. W. North.** 1994. Rapid phenotypic reversion of zidovudine-resistant feline immunodeficiency virus without loss of drug-resistant reverse transcriptase. J. Virol. **68:**632–637.
47. **Richman, D. D.** 1993. Resistance of clinical isolates of human immunodeficiency virus to antiretroviral agents. Antimicrob. Agents Chemother. **37:**1207–1213.
48. **Richman, D. D., D. Havlir, J. Corbeil, D. Looney, C. Ignacio, S. A. Spector, J. Sullivan, S. Cheeseman, K. Barringer, D. Pauletti, C. K. Shih, M. Myers, and J. Griffin.** 1994. Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. J. Virol. **68:**1660–1666.
49. **Schinazi, R. F., R. M. Lloyd, Jr., M.-H. Nguyen, D. L. Cannon, A. McMillan, N. Ilksoy, C. K. Chu, D. C. Liotta, H. Z. Bazmi, and J. W. Mellors.** 1993. Characterization of human immunodeficiency viruses resistant to oxathiolane-cytosine nucleosides. Antimicrob. Agents Chemother. **37:**875–881.
50. **Schinazi, R. F., A. McMillan, D. Cannon, R. Mathis, R. M. Lloyd, A. Peck, J.-P. Sommadossi, M. St. Clair, J. Wilson, P. Furman, G. Painter, W.-B. Choi, and D. Liotta.** 1992. Selective inhibition of human immunodeficiency viruses by racemates and enantiomers of *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine. Antimicrob. Agents Chemother. **36:**2423–2431.
51. **Schuurman, R., M. Nijhuis, R. van Leeuen, P. Schipper, D. de Jong, P. Collis, S. Danner, J. Mulder, C. Loveday, C. Christopherson, S. Kwok, J. Sninsky, and C. A. B. Boucher.** 1995. Rapid changes in human immunodeficiency virus type 1 RNA load and appearance of drug-resistant populations in persons treated with lamivudine (3TC). J. Infect. Dis. **171:**1411–1419.
52. **Shafer, R. W., M. J. Kozal, M. A. Winters, A. K. N. Iversen, D. A. Katzenstein, M. V. Ragni, W. A. Meyer, P. Gupta, S. Rasheed, R. Coombs, M.**

Katzman, S. Ficus, and T. C. Merigan. 1994. Combination therapy with zidovudine and didanosine selects for drug-resistant human immunodeficiency virus type 1 strains with unique patterns of *pol* gene mutations. J. Infect. Dis. **169:**722–729.

53. **St. Clair, M. H., J. L. Martin, G. Tudor-Williams, M. C. Bach, C. L. Vavro, D. M. King, P. Kellam, S. D. Kemp, and B. A. Larder.** 1991. Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. Science **253:**1557–1559.
54. **Talbott, R. L., E. E. Sparger, K. M. Lovelace, W. M. Fitch, N. C. Pedersen, P. A. Luciw, and J. H. Elder.** 1989. Nucleotide sequence and genomic organization of feline immunodeficiency virus. Proc. Natl. Acad. Sci. USA **86:**5743–5747.
55. **Tisdale, M., S. D. Kemp, N. M. Parry, and B. A. Larder.** 1993. 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. Proc. Natl. Acad. Sci. USA **90:**5653–5656.
56. **Wainberg, M. A., W. C. Drosopoulos, H. Salomon, M. Hsu, G. Borkow, M. A. Parniak, Z. Gu, Q. Song, J. Manne, S. Islam, G. Castriota, and V. R. Prasad.** 1996. Enhanced fidelity of 3TC-selected mutant HIV-1 reverse transcriptase. Science **271:**1282–1285.
57. **Wainberg, M. A., Z. Gu, Q. Gao, E. Arts, R. Geleziunas, S. Bour, R. Beaulieu, C. Tsoukas, J. Singer, and J. Montaner.** 1993. Clinical correlates and molecular basis of HIV drug resistance. J. Acquired Immune Defic. Syndr. **6**(Suppl. 1)**:**S36–S46.
58. **Yamamoto, J. K., E. Sparger, E. W. Ho, P. R. Andersen, T. P. O'Connor, C. P. Mandell, L. Lowenstine, R. Munn, and N. C. Pedersen.** 1988. Pathogenesis of experimentally induced feline immunodeficiency virus infection in cats. Am. J. Vet. Res. **49:**1246–1258.
59. **Zhu, Y.-Q., K. M. Remington, and T. W. North.** 1996. Mutants of feline immunodeficiency virus resistant to 2′,3′-dideoxy-2′,3′-didehydrothymidine. Antimicrob. Agents Chemother. **40:**1983–1987.