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Mutants of Feline Immunodeficiency Virus Resistant to 2′,3′-Dideoxy-2′,3′-Didehydrothymidine

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We selected mutants of feline immunodeficiency virus (FIV) that are resistant to 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T). Two mutants were selected in cultured cells with a stepwise increase in d4T concentration, resulting in mutants able to replicate in 100 μM d4T. These mutants were three- to sixfold more resistant to d4T than wild-type FIV. They were also cross-resistant to 3′-azido-3′-deoxythymidine (AZT), 3′-fluoro-2′,3′-dideoxythymidine, 2′,3′-dideoxycytidine, 2′,3′-dideoxyinosine, and 9-(2-phosphonylmethoxyethyl)adenine, and they were highly resistant to phosphonoformic acid (PFA). Plaque-purified mutants were isolated from each of the mutant populations. The mutant phenotype was stable, because both of the plaque-purified mutants remained d4T resistant even after three passages in the absence of d4T. One of the plaque-purified mutants, designated D4R-3c, was further characterized. Compared with wild-type reverse transcriptase (RT), RT purified from D4R-3c was 3-fold resistant to inhibition by the 5′-triphosphate of d4T, 10-fold resistant to inhibition by the 5′-triphosphate of AZT, and 6-fold resistant to PFA. D4R-3c had a single point mutation in the RT-encoding region of the *pol* gene at position 2474, resulting in a Val to Ile mutation at codon 47 of the FIV RT. The role of this mutation in d4T resistance was confirmed by site-directed mutagenesis.

The rapid emergence of drug-resistant variants of human immunodeficiency virus type 1 (HIV-1) is a significant barrier to the treatment of AIDS (43, 49). The most widely used therapeutic agents are nucleoside analogs whose active forms are nucleotide analogs that inhibit reverse transcriptase (RT) (8). Several nucleoside analogs have been approved and used clinically to treat AIDS, namely, 3′-azido-3′-deoxythymidine (AZT), 2′,3′-dideoxyinosine (ddI), 2′,3′-dideoxycytidine (ddC), 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T), and (−)-β-2′,3′-dideoxy-3′-thiacytidine (3TC). However, variants of HIV-1 that are resistant to these drugs or combinations of these drugs emerge in treated patients and are believed to be responsible for drug failures (10, 23, 43, 46, 47, 49). The problem is not limited to nucleoside analogs or even to inhibitors of RT. HIV-1 mutants resistant to nonnucleoside inhibitors of RT, such as nevirapine (44), or to protease inhibitors (6, 17) have been isolated from treated patients. Numerous HIV-1 mutants resistant to RT or protease inhibitors have also been selected in cell culture systems (cf. references 11, 12, 18, 22, and 35). The mechanisms of resistance of HIV-1 to RT and protease inhibitors have been traced to mutations in genes encoding the HIV-1 RT and protease, respectively (45). In order to develop successful AIDS therapy, it will be necessary to understand the mechanisms of resistance and to develop strategies to combat it.

We have developed model systems using feline immunodeficiency virus (FIV) that have been useful for studies of viral resistance to AIDS therapy (32). FIV is a lentivirus that causes a naturally occurring acquired immunodeficiency syndrome in domestic cats that is strikingly similar to AIDS in humans (36, 37). FIV also causes immunosuppressive disease and neuropathogenesis in experimentally infected specific-pathogen-free cats (1, 5, 9, 38, 39, 50), which affords an excellent opportunity for in vivo experimentation. FIV has been particularly useful for studies of viral resistance to nucleoside analogs because its RT is similar to the HIV-1 RT in its physical properties, catalytic activity, and sensitivity to the active forms of AZT, ddI, ddC, and d4T (7, 29, 30). The first reported lentivirus mutants with drug resistance phenotypes that were selected in vitro were AZT-resistant mutants of FIV (41). These mutants were phenotypically similar to AZT-resistant mutants of HIV-1 isolated from patients (23, 41). We have subsequently reported mutants of FIV that are resistant to ddI (13), ddC (28), or the combination of AZT plus ddI (13).

To extend this work we have selected mutants of FIV that are resistant to d4T. This drug is a thymidine analog which, like AZT, is a potent inhibitor of HIV-1 (2–4, 15, 25–27) and FIV (28, 42) replication. Mutants of HIV-1 that are resistant to d4T have been reported (21). These variants have a mutation at codon 75 in the RT-encoding region of the HIV-1 *pol* gene that results in substitution of a Thr for a Val at amino acid 75 of the HIV-1 RT. This mutation confers moderate d4T resistance and cross-resistance to ddI and ddC. In the work reported here, we have selected and characterized a d4T-resistant mutant of FIV with a mutation that maps to a unique domain of RT.

MATERIALS AND METHODS

Chemicals. Triton X-100, dTTP, aminoethylcarbazole, ddC, and phosphonoformic acid (PFA) were purchased from Sigma Chemical Co., St. Louis, Mo. AZT and the 5′-triphosphate of AZT (AZTTP) were provided by the Burroughs Wellcome Co., Research Triangle Park, N.C.; d4T and the 5′-triphosphate of d4T (d4TTP) were provided by Bristol-Myers Squibb Co., Wallingford, Conn.; 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; and 3′-fluoro-2′,3′-dideoxythymidine (FLT) was obtained from the Lederle Laboratories Division of American Cyanamid Co., Pearl River, N.Y. Poly(rA)-oligo$(dT)_{10}$ was purchased from Pharmacia LKB, Piscataway, N.J. Nuclease-free bovine serum albumin was obtained from Boehringer Mannheim, Indianapolis, Ind. DEAE-cellulose DE52 and phosphocellulose P11 were pur-

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chased from Whatman, Hillsboro, Oreg. [*methyl*-^{3}H]dTTP was purchased from Dupont-New England Nuclear, Boston, Mass. The International Bio Technologies phenol for DNA extractions was purchased from VWR Scientific. 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. *Eco*RI was purchased from Promega, Madison, Wis., and *Nsi*I was from American Applied Biotechnology, Aurora, Colo. T4 DNA ligase was from New England Biolabs, Beverly, Mass. All other chemicals were reagent grade or better.

Cells and virus. Virus produced from a molecular clone of the Petaluma strain of FIV, 34TF10 (48), 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 as described previously (33). The CrFK cells used were passaged six or fewer times after they were obtained from the American Type Culture Collection (Rockville, Md.) because we have observed that at high passage number in L&M medium these cells lose their ability to support FIV replication (20). The d4T-resistant mutants of FIV were maintained in medium containing d4T, and the medium was replaced with fresh medium containing the appropriate concentration of d4T every 2 days.

Focal infectivity assay. FIV infectivity in the presence or absence of various inhibitors was determined by a focal infectivity assay, as described previously (41). Data were plotted as a percentage of control foci (no drug) versus inhibitor concentration. Concentrations required to inhibit focus formation by 50% (IC_{50}s) were obtained directly from the linear portion of these plots by using a computer-generated regression line (41). Within an experiment, each value represents the mean of four determinations. Results from two or more independent experiments were used to derive the mean ± standard error IC_{50}.

Plaque purification of d4T-resistant FIV. The methods of plaque purification were performed as described previously (42).

Enzymes and enzyme assays. RT was purified from virions of mutant FIV by methods developed in our laboratory (30). Recombinant FIV RT (31) was used as the wild-type control. Assays for RT with poly(rA)-oligo(dT) as the template-primer were performed as reported previously (29–31). Double-reciprocal plots were used to determine kinetic constants (K_m and K_i), as reported previously (7, 29).

Nucleic acid preparation and DNA sequence analysis. Total cellular DNA containing proviral DNA was extracted from CrFK cells that were infected with wild-type or d4T-resistant FIV by procedures that we have described previously (42). After ethanol precipitation, DNA was resuspended in distilled water and was used for amplification by PCR. Amplification of the RT-encoding region of the *pol* gene was performed by the Perkin-Elmer Cetus GeneAmp PCR protocol. Each 100-μl reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μM (each) deoxynucleoside triphosphate (dATP, dTTP, dCTP, and dGTP), 4 mM $MgCl_2$, 0.2 μM (each) primer, 2.5 U of AmpliTaq DNA polymerase, and 10 to 20 μg of target DNA. The reaction mixtures were overlaid with 50 to 100 μl of light mineral oil. The sense primer (5′-GTA ATG TTT GTG TCT TAG AAG ATA ACT C-3′) and the reverse complement primer (5′-ATC ATA TCC TGC ATC TTC TGA CCT-3′) were synthesized on an ABI 394 DNA synthesizer in the Murdock Molecular Biology Facility, University of Montana, Missoula. The primers were chosen to amplify a 1,763-bp fragment of the FIV genome that contained nucleotides 2268 through 4031. PCR was run for 30 cycles; each cycle comprised 30 s of denaturation at 94°C, 30 s of annealing at 62°C, and 2 min of extension at 72°C. After PCR, the product was run on a 0.8% low-melting-point agarose gel and was visualized with ethidium bromide. A 1,763-bp fragment was purified with the QIAquick PCR Purification Kit, Qiagen Inc., Chatsworth, Calif. DNA was sequenced in 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 and flanking regions.

Site-directed mutagenesis. To introduce the mutation at codon 47, a fragment containing this region from mutant D4R-3c was amplified by PCR and was introduced into the molecular clone, 34pol cassette (28). An 1,164-bp fragment of FIV RT, corresponding to nucleotides 1740 to 2904, was amplified with primer 16 (GATCCTATATAAATGTCATCC) and primer 41 (GGATCAGGACCAG TGTGT). The PCR product and 34pol cassette were each cleaved with *Eco*RI and *Nsi*I, and the fragments were gel purified. The resulting 803-bp fragment from D4R-3c, corresponding to nucleotides 1871 to 2674 of the FIV genome, was ligated into the cleaved 34pol cassette. DNA sequence analysis was used to confirm the presence of the desired mutation and the absence of any other mutations arising from a PCR error (only one of five clones analyzed had the correct sequence). This construct was introduced into the J5 strain of *Escherichia coli* JM109, and the resulting plasmid DNA was used to transfect CrFK cells for the production of virus.

RESULTS

Selection of d4T-resistant mutants. In initial experiments, we attempted to obtain d4T-resistant mutants of FIV by selection in the continuous presence of a high concentration of d4T (100 μM, which is approximately eight times the IC_{50} for FIV).

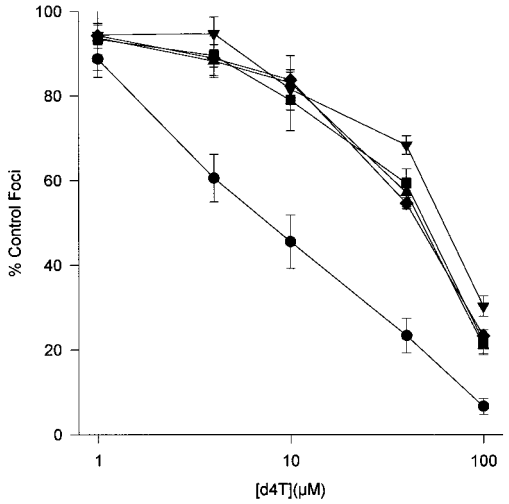

FIG. 1. Inhibition of FIV 34TF10 (●), D4R-1c (◆), D4R-2c (▼), D4R-3c (■), and D4R-4c (▲) by d4T. Results are from three experiments, with four determinations per experiment. Bars represent standard errors of the means and are omitted if the standard error was too small to be shown.

This approach had been successful for the selection of FIV mutants resistant to AZT (41, 42), ddI (13), or ddC (28). However, in attempts to select mutants with 100 μM d4T we were unable to obtain any d4T-resistant mutants. Therefore, we switched to a stepwise selection protocol that is similar to that used by Gao et al. (11, 12) and by Larder et al. (22) to select drug-resistant variants of HIV-1. The initial round of selection was carried out with 12.5 μM d4T, which is equivalent to the IC_{50} of d4T for FIV (42). The concentration of d4T was then increased twofold in each subsequent round. For the first round, two 25-cm^2 flasks of CrFK cells were pretreated for 1 h with 12.5 μM d4T and were then infected with a cell-free culture supernatant of FIV 34TF10 at an inoculum of approximately 2,000 focus-forming units. Medium and drug were replaced every 48 h, and the cells were removed with trypsin and subcultured as necessary. Cultures were monitored weekly for the presence of virus by the focal infectivity assay. By 2 to 3 weeks postinfection virus production was apparent. Culture supernatants from each of the first-round cultures were used to infect flasks of CrFK cells for the second round of selection in the presence of 25 μM d4T. By these protocols, virus was subsequently passaged in 25, 50, and 100 μM d4T. In each round of selection, virus production was apparent by 2 to 3 weeks postinfection. After passage in 100 μM d4T, two d4T-resistant mutants, mutants D4R-1c and D4R-2c, were obtained. These mutants were five- to sixfold resistant to d4T relative to the wild type (Fig. 1).

The shapes of the dose-response curves for d4T-resistant mutants were different from the dose-response curves obtained with wild-type FIV (Fig. 1). We do not know the reason for this, but it is not due to the toxicity of d4T to CrFK cells. Even at the highest concentration of d4T used in the selections, 100 μM, we observed no cytotoxicity or reduction in the growth rate of CrFK cells.

Plaque purification of d4T-resistant mutants. To minimize potential heterogeneity within the d4T-resistant mutant populations, plaque-purified isolates were obtained from mutants D4R-1c and D4R-2c. Two plaque-purified mutants, designated D4R-3c and D4R-4c (obtained from D4R-1c and D4R-2c, respectively), were selected for further characterization. Both

TABLE 1. Susceptibilities of FIV 34TF10 and d4T-resistant mutants to antiviral compounds determined by the focal infectivity assay

Compound	Mean ± SE IC_{50} (μM)[a]		
	FIV 34TF10	D4R-3c	D4R-4c
d4T	9 ± 2	34 ± 8	32 ± 7
AZT	0.3 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
FLT	0.2 ± 0.05	1.1 ± 0.1	1.3 ± 0.1
ddC	4.4 ± 0.3	9.1 ± 1.5	9 ± 2.1
ddI	1.1 ± 0.04	2.4 ± 0.4	1.5 ± 0
PFA	207 ± 44	>10,000	>10,000
PMEA	0.1 ± 0.01	0.4 ± 0.02	0.4 ± 0.02

[a] Values are from two or more experiments, with four determinations per experiment.

plaque-purified mutants retained the d4T resistance phenotype of the parent populations (Fig. 1).

Mutants D4R-3c and D4R-4c were cross-resistant to several other antiviral compounds (Table 1). Compared with the wild type, both mutants were 2- to 2.5-fold resistant to AZT, 5- to 6-fold resistant to FLT, 2-fold resistant to ddC, 1.5- to 2-fold resistant to ddI, and 4-fold resistant to PMEA. They were also highly resistant (>50-fold) to the nonnucleoside inhibitor PFA.

RT. RT was purified from D4R-3c, and its susceptibility to inhibition by d4TTP, AZTTP, and PFA was compared with that of wild-type FIV RT. As expected, with respect to dTTP, inhibition by d4TTP or AZTTP was competitive and inhibition by PFA was noncompetitive (Fig. 2). The kinetic parameters obtained from these experiments are summarized in Table 2. The two enzymes had nearly identical K_ms for dTTP. However, the K_i for inhibition of RT from D4R-3c by d4TTP, 4.4 nM, was three times higher than that of wild-type FIV RT. The K_i values for inhibition of this mutant enzyme by AZTTP and PFA were 10 and 6 times higher, respectively, than those for inhibition of the wild-type FIV RT.

Nucleotide sequence analysis. DNA sequence analyses of the entire RT-encoding region of the *pol* gene from D4R-3c and FIV 34TF10 were performed in the forward and reverse directions. These analyses revealed only a single point mutation, a G-to-A base change, at position 2474 of the FIV *pol* gene. This substitution results in the replacement of a Val residue with an Ile residue at codon 47 (Fig. 3). In order to confirm the role of this mutation in d4T resistance, we introduced the G-to-A mutation at position 2474 into the FIV 34pol cassette by site-directed mutagenesis. The resulting mutant displayed 2.5-fold resistance to d4T compared with the wild type, confirming the role of this mutation in d4T resistance.

In order to determine whether this mutation was genetically stable, we passaged mutants D4R-3c and D4R-4c each for three rounds in the absence of d4T using protocols that we have described previously (42). The resulting viruses remained d4T resistant at a level indistinguishable from those of D4R-3C and D4R-4c, even after three rounds of passage without d4T (data not shown). These data demonstrate that the d4T-resistant phenotype is stable.

DISCUSSION

The d4T-resistant mutants that we isolated have relatively low-level resistance to d4T (three- to sixfold). This level of resistance is similar to that reported by Lacey and Larder for a d4T-resistant mutant of HIV-1, which displayed sevenfold resistance (21). Relatively low level resistance has also been observed with mutants of FIV or HIV-1 resistant to ddI or ddC (10–14, 47). In contrast, AZT-resistant mutants of FIV (41, 42) or HIV-1 (22–24) display greater than 40-fold resistance to AZT.

These d4T-resistant mutants of FIV are cross-resistant to ddI and ddC. In this respect they are phenotypically similar to the d4T-resistant mutant of HIV-1 reported by Lacey and

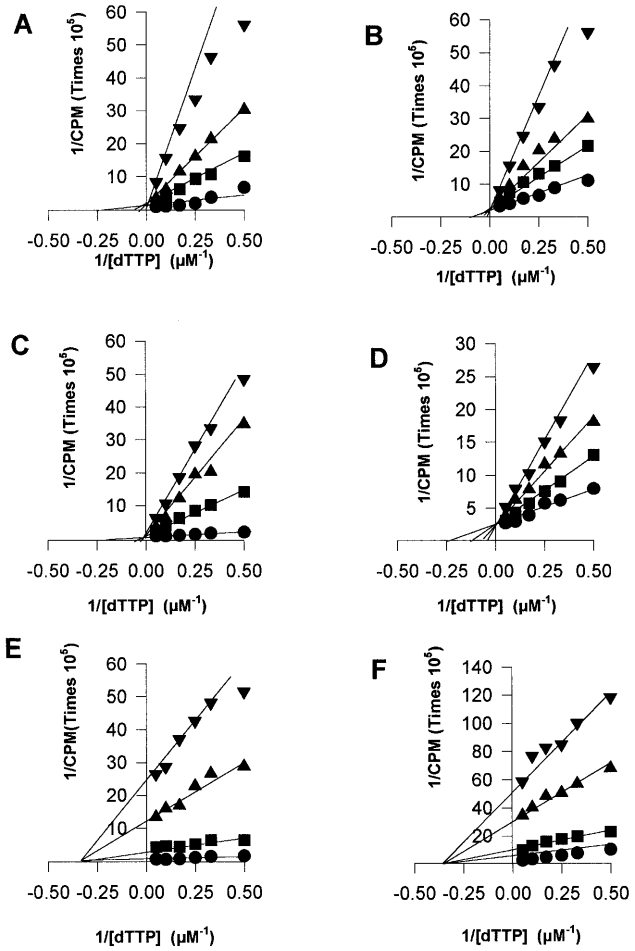

FIG. 2. Kinetics of inhibition of RT from wild-type FIV (A, C, and E) or D4R-3c (B, D, and F) by d4TTP (A and B), AZTTP (C and D), or PFA (E and F). Lineweaver-Burk plots are from reactions with poly (rA)-oligo(dT) as the template and various concentrations of substrate (dTTP). Concentrations of inhibitors were 0 nM (●), 25 nM (■), 50 nM (▲) and 100 nM (▼) d4TTP (A and B); 0 nM (●), 5 nM (■), 10 nM (▲), and 20 nM (▼) AZTTP (C and D); and 0 μM (●), 1 μM (■), 5 μM (▲), and 10 μM (▼) PFA (E and F). Values are averages from duplicate determinations. The specific activity of [^{3}H]dTTP was 300 cpm/pmol.

TABLE 2. Kinetic constants for wild-type FIV and D4R-3c RTs[a]

Inhibitor	K_i (nM)	
	FIV 34TF10 RT	D4R-3c RT
d4TTP	1.4 ± 0.14	4.7 ± 0.16
AZTTP	2.2 ± 0.23	21 ± 1.51
PFA	265 ± 37	1,720 ± 345

[a] Values are reported as the mean ± standard error of the mean of at least five determinations. The mode of inhibition of each enzyme by d4TTP or AZTTP was competitive with respect to substrate. Inhibition of each enzyme by PFA was noncompetitive with respect to substrate. The K_m for dTTP was 5.8 ± 0.6 μM for each enzyme.

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      42   43   44   45   46   47   48   49   50   51   52
      Glu  Lys  Glu  Gly  Lys  Val  Lys  Arg  Ala  Asp  Ser
2459  GAA  AAA  GAA  GGG  AAA  GTA  AAA  AGA  GCA  GAT  TCA   2491
      ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---   FIV34TF10
      ---  ---  ---  ---  ---  A--  ---  ---  ---  ---  ---   D4R-3c
                              Ile
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FIG. 3. Nucleotide and deduced amino acid sequences of the region of the FIV *pol* gene surrounding position 2474. The entire RT-encoding region was sequenced from FIV 34TF10 and D4R-3c. The indicated sequence is the published sequence for the plasmid containing the 34TF10 molecular clone (39). The only change in the RT-encoding region of D4R-3c was the indicated change in codon 47.

Larder (21). The FIV mutants were also cross-resistant to AZT, PFA, PMEA, and FLT. The d4T-resistant mutant of HIV-1 was not cross-resistant to AZT (21), and its susceptibility to PFA or PMEA was not reported. Because the AZT resistance is low level (two- to threefold), it is likely that these mutants would be masked during selections for AZT-resistant virus. If HIV mutants that are phenotypically similar exist, they would also be missed by the common screens for mutations in codons 41, 67, 70, 215, and 219 of HIV-1 RT.

RT purified from one of the plaque-purified mutants, mutant D4R-3c, was resistant to d4TTP. The mutant enzyme had a threefold higher K_i for d4TTP than that of the wild-type FIV RT, whereas the two enzymes had identical K_ms for dTTP. This threefold resistance at the enzyme level correlates well with the three- to sixfold resistance at the level of virus replication. RT purified from D4R-3c also had decreased susceptibility to inhibition by AZTTP or PFA. However, the level of resistance to these two inhibitors at the RT level did not correlate with the level of resistance to AZT or PFA at the level of virus replication. A lack of correlation between the AZT resistance phenotype of the virus and the AZTTP susceptibility of RT has previously been reported for AZT-resistant mutants of FIV (41) and HIV (23, 24). A similar lack of correlation between virus phenotype and enzyme data was reported by Prasad et al. (40), who described a ddGTP-resistant HIV-1 RT that did not confer ddG resistance when it was introduced into HIV-1. The failure of drug susceptibilities of RTs from these mutants to correlate with drug resistance at the level of virus replication suggests that factors in addition to RT are involved in resistance to AZT, ddG, or PFA. This could occur through the interaction of RT with other viral proteins or with cellular components. Our data suggest that such interactions do not alter the d4TTP susceptibility of the RT from FIV D4R-3c.

Sequence analysis of D4R-3c revealed a single point mutation resulting in an amino acid change from valine to isoleucine at codon 47 of the FIV RT. The mutation in the d4T-resistant mutant of HIV-1 that has been characterized was mapped to codon 75 of the HIV-1 RT (21), and there have been no drug-resistant mutants of HIV-1 or FIV with mutations that map to codon 47 (45). Interestingly, the amino acid found at codon 47 of the RT from the d4T-resistant mutant of FIV (isoleucine) is the amino acid present at codon 47 of the wild-type HIV-1 RT. It will be interesting to determine whether alteration of codon 47 in the HIV-1 RT will affect the susceptibility of HIV-1 to d4T or other RT-targeted antiviral drugs.

On the basis of the X-ray crystal structure of HIV-1 RT (16, 19) and the amino acid sequence homology of the HIV-1 and FIV RTs (34, 48), codon 47 is predicted to be in the "fingers" domain of RT. On the p66 subunit this is on the outside of the fingers domain, whereas the active site of the enzyme is on the opposite, or inner side, of the finger domain. However, position 47 of the p51 subunit is adjacent to the active site of the p66 subunit, in a position where a mutation of this amino acid could affect drug susceptibility. On the basis of the available structural data, we predict that the mutation in codon 47 of the p51 subunit is responsible for the altered susceptibility of RT to several nucleotide analogs.

Because of the emergence of mutants resistant to any of the drugs used in monotherapy, much effort is focused on evaluations of combinations of two or more drugs. The demonstration that d4T-resistant FIV has decreased susceptibility to several other RT inhibitors suggests that mutants resistant to the combination of d4T with another RT inhibitor may arise readily.

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