Selection and characterization of 2',3'-dideoxy-2',3'-didehydrothymidine-resistant mutants of feline immunodeficiency virus

Ya Qi Zhu

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SELECTION AND CHARACTERIZATION OF 2’,3’-DIDEOXY-2’,3’-DIDEHYDROTHYMIDINE-RESISTANT MUTANTS OF FELINE IMMUNODEFICIENCY VIRUS

by

Ya Qi Zhu

B.S., Beijing Hygiene School

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Chairman, Board of Examiners

Dean, Graduate School

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Selection and Characterization of 2',3'-Dideoxy-2',3'-Didehydrothymidine (d4T)-Resistant Mutants of Feline Immunodeficiency Virus (80 pp)

Director: Thomas W. North

A step-wise selection protocol was used to obtain two mutants of feline immunodeficiency virus (FIV) resistant to 2',3'-dideoxy-2',3'-didehydrothymidine (d4T). These mutants were selected by passage of FIV 34TF10 (a molecular clone of FIV Petaluma) in increasing concentrations of d4T, beginning at 12.5 µM in the initial round and increasing the concentration 2-fold in each subsequent round. Two mutants able to replicate in 100 µM d4T (8 times of the IC50) were obtained and plaque-purified.

Both mutants were 3 to 4-fold resistant to d4T, relative to FIV 34TF10. These mutants were cross-resistant to 3'-azido-3'-deoxythymidine (AZT), 3'-fluoro-2',3'-dideoxythymidine (FLT), 2',3'-dideoxycytidine (ddC), 2',3'-dideoxyinosine (ddI), 9-(2-phosphonylmethoxyethyl)-adenine (PMEA), and were highly resistant to phosphonoformic acid (PFA). The mutant phenotype was stable, as the virus remained d4T-resistant even after passage in the absence of d4T for three rounds. Reverse transcriptase (RT) was purified from one of these mutants, D4R-3c. This RT was 5-fold resistant to the 5'-triphosphate of d4T (d4TTP), relative to wild-type FIV RT. Sequence analysis of the RT-encoding region of the pol gene revealed a point mutation at position 2474 resulting in a Val to Ile mutation at amino acid 47 of the FIV RT. Site-directed mutagenesis was performed and showed that this amino acid change was responsible for the d4T-resistance.
ACKNOWLEDGMENTS

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INTRODUCTION

GENERAL INTRODUCTION

In the mid-1980s, announcements in Paris and in Bethesda, Maryland, declared that a retrovirus had been isolated that was the etiologic agent of the acquired immune deficiency syndrome (AIDS) which poses a serious challenge to modern medicine. The retrovirus is known today as the human immunodeficiency virus type-1 (HIV-1). The virus targets cells of the immune system, specifically the helper T lymphocytes. HIV infection results in immunosuppression, rendering the patient susceptible to opportunistic infections. These infections lead to a high incidence of morbidity and mortality (40). HIV-1 is currently the number two killer of people between the ages of 25 and 45 years. Over 13 million people are presently infected with the virus and predictions for the future are frightening (50). To conquer this disease, two strategies have been undertaken: a protective vaccine or effective drugs able to block the life cycle of the virus. It is widely agreed that an AIDS vaccine is necessary, but this virus is in a state of antigenic flux, and consequently,
protective vaccine development has been difficult. To date, no protective vaccine has been developed. In contrast, modest success has been achieved with chemotherapy. Antiviral agents are usually targeted at a receptor, enzyme or another viral component that is required for replication. There are various events in the replicative cycle of HIV that could be considered as targets for chemotherapeutic intervention: 1) virus adsorption to the cell membrane; 2) fusion between the viral envelope and the cell membrane; 3) uncoating of the viral nucleocapsid; 4) reverse transcription of the viral RNA to proviral DNA; 5) integration of the proviral DNA into the cellular genome; 6) transcription of the proviral DNA to RNA, a process that is regulated by transcriptional transactivators (i.e., TAT); 7) conversion of the viral precursor mRNA to mature mRNA, a process that is regulated by posttranscriptional transactivators (i.e., REV); 8) posttranslational processing of the viral precursor proteins by proteolysis, glycosylation, and myristoylation; and 9) virion assembly and release ("budding") (12, 44). To date, numerous compounds have been reported to inhibit the replication of HIV in vitro, yet only relatively few agents have at this time been formally licensed (in the United
States) for clinical use in the treatment of AIDS. These are zidovudine (3'-azido-2',3'-dideoxythymidine, AZT) (34), didanosine (2',3'-dideoxyinosine, ddl) (17), zalcitabine (2',3'-dideoxycytidine, ddC) (73), stavudine (2',3'-didehydro-2',3'-dideoxythymidine, d4T) (2) and (-)-β-2',3'-dideoxy-3'-thiacytidine (3TC) (55). Several protease inhibitors have been approved also. 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 (18,35,59,63,64,71). The problem of resistance is not limited to nucleoside analogs. Mutants of HIV-1 resistant to non-nucleoside inhibitor of RT, such as nevirapine (60), or to protease inhibitors (10,28) have been isolated from treated patients. Numerous HIV-1 mutants resistant to RT or protease inhibitors have also been selected in cell culture systems (19,20,30,36,51).

Resistance of HIV-1 to RT and protease inhibitors has been traced to mutations in genes encoding the HIV-1 RT and protease, respectively (62). In order to develop successful AIDS therapy, it will be necessary to understand mechanisms of resistance and to develop strategies to combat it.
Animal models of AIDS are essential for understanding the pathogenesis of retrovirus-induced immune deficiency and encephalopathy, and for development and testing of new therapies and vaccines. Many viruses and animal models have been evaluated for use as AIDS models, including simian immunodeficiency virus (SIV), avian retroviruses, murine retroviruses, severe combined-immunodeficiency (SCID) mouse models, lentiviruses in sheep, goats, horses and cows, HIV type-2 (HIV-2) infection in macaques, and feline immunodeficiency virus (FIV) infection of cats. Since its discovery in 1987 (53), FIV has become recognized as a valuable animal model for AIDS studies. The advantages of this model are 1) the FIV biology, infection cycle, and disease closely mimic those of HIV and human AIDS, 2) cats are relatively inexpensive compared to nonhuman primates, 3) FIV is safe to work with since it is not infectious to human, and 4) in vitro and in vivo systems are available and their versatility facilitates studies of pathogenesis, antiviral therapies, protective vaccines, and drug resistance.
FELINE IMMUNODEFICIENCY VIRUS

Feline immunodeficiency virus (FIV) is a lentivirus that causes an immune deficiency in domestic cats that is very similar to human AIDS and which can also be induced experimentally in specific-pathogen-free cats (SPF) (70,74). FIV was first described by Neils Pedersen and co-workers in 1987. Subsequent investigations have shown that FIV is an important health problem for domestic cats throughout the world. The expectation that FIV would become a leading animal model for HIV studies has been fulfilled.

The virion. The mature extracellular FIV virion is spherical to ellipsoidal, 100 to 125 nm in diameter, and bordered by an outer envelope with poorly defined short projections or knobs. The elongated core is composed of a conical shell that surrounds an eccentric electron-dense nucleoid. A polygonal electron-lucent halo is often visible between the core and a granular layer found just inside the envelope (45,53,69,75). Each FIV virion contains: the envelope (Env) glycoproteins: surface (SU) protein, and transmembrane (TM) glycoprotein; the internal protein (Gag) components: the matrix (MA) protein, capsid (CA) protein and
nucleocapsid (NC) protein; and the Pol proteins including protease (PR), RT, dUTPase (DU) and integrase (IN). FIV virions also contain two copies of the positive-stranded, polyadenylated RNA genome (about 9200 bases long). As is typical of retroviruses, in the FIV virion Gag proteins are about 20-fold more abundant than Pol proteins (16) (Fig.1).

**Life cycle.** FIV is an enveloped, single-stranded RNA virus that belongs to the lentivirus family. FIV and HIV-1 share the prototypical life cycle of a retrovirus (Fig. 2). The virus is first adsorbed by a receptor on the host cell membrane. HIV binds to T cells surface receptor, the CD4 molecule (CD9 in FIV), and by interacting with this and at least one other cell surface molecule, a conformational change occurs in the envelope glycoproteins to reveal the hydrophobic sequence of the transmembrane envelope protein. This leads to a fusion of the viral envelop with the cell membrane and delivery of the viral capsid into the cytoplasm of the cell (38). Within the capsid structure the diploid RNA genome is reverse transcribed by the viral RT. RT is the viral-encoded RNA-dependent DNA polymerase responsible for
synthesis of a DNA copy of the viral RNA genome. This generates an RNA/DNA heteroduplex containing a complete DNA copy of the viral genes. Subsequently, the RNA is degraded by the RNase H activity of RT, and a DNA duplex is synthesized by RT. The double-stranded DNA genome is then integrated into the host genome as a provirus (68). The provirus may remain dormant until some, as of yet unknown, factor(s) stimulates the provirus into active replication to produce infectious virus particles. The cell will then produce viral RNA and proteins. These progeny viruses assemble and are released from the cell surface by budding. The virus can then infect other cells.

**Genome structure and organization.** FIV diverges from other lentiviruses throughout the genome. Its sequence organization, however, is similar in complexity to that of other lentiviruses (Fig.3). Three large open reading frames (ORFs), gag, pol, and env, encode the internal structural proteins, the RT and other viral enzymes, and the envelope proteins, respectively. FIV also has several small ORFs that encode regulatory proteins. The provirus contains two long terminal repeat (LTR) elements, one at each end of the genome, which accommodate multiple regulatory sequences.
Fig 1. Molecular anatomy of the FIV virion.
Fig 2. The retroviral life cycle.
Fig 3. Organization of the FIV genome.

The *gag* gene, spanning approximately nucleotides 600 to 2000, is initially translated to yield a precursor polypeptide of about 50 kDa that is posttranslationally cleaved by the viral protease (PR) (14,65) to yield (amino to carboxy order) three functional core proteins. They are mature MA, CA, and NC proteins. The MA is approximately 15 kDa, which has recently been shown to be myristoylated (16). The CA protein is the largest *gag* product. It is the major core protein, a 25 kDa group-associated antigen. The NC protein is
approximately 10 kDa, which contains motifs characteristic of retroviral nucleic acid binding proteins and is believed to be associated with the genomic RNA to form the ribonucleoprotein (14,65).

The pol gene is an enzyme cassette, which spans nucleotides 1900 to 5200 and, thus, overlaps the gag gene by 109 nucleotides. Pol is in a -1 reading frame with respect to that of the gag gene and is translated as a Gag-Pol fusion polyprotein produced by ribosomal frameshifting (8), as in other retroviruses. The Gag-Pol fusion precursor protein is eventually processed into the functionally mature enzymes during virus assembly. The Pol portion of the precursor polyprotein is eventually cleaved into PR, RT, DU, and an endonuclease or integrase, most likely due to autodigestion (15,16).

The primary translation product of the env gene (which spans nucleotides 6250 to 8850) is a precursor glycoprotein of 145 to 150 kDa (gp145) that is rapidly reduced in size to a molecule of 139 kDa (gp130). gp130 is subsequently processed into the mature
glycoproteins SU (gp95) and TM (gp40) of the viral envelope by proteolytic cleavage in the Golgi complex (66).

Moreover, FIV also contains regulatory genes. The rev and vif are the only two that have been characterized to some extent.

**Biochemical features of RT.** The RT of FIV has been purified and extensively characterized by North, et al. FIV RT consists of two polypeptides with common N-termini, a 66-kDa (p66), and a 51-kDa subunit. Both subunits are present in equimolar amounts (46, 49). FIV RT p51 is generated by cleavage of the RNase H domain (p15) at the C-terminus of FIV RT p66 by the virus-encoded protease. FIV RT is very similar to HIV-1 RT in physical properties, catalytic activity and sensitivity to several RT inhibitors (11, 47, 48, 49). Both enzyme have comparable requirements for Mg$^{2+}$ and similar template specificities (49). From sequence analysis, HIV-1 RT and FIV RT share 63% identity at the nucleotide level and 48% identity and 67% similarity at the amino acid level, respectively (1).

The first five antivirals approved for AIDS therapy are targeted
to RT. RT has become an excellent target for attempts to arrest the proliferation of HIV-1 for several reasons: a) it is a crucial enzyme in the viral replication cycle; b) its properties are quite different from those of the other cellular DNA polymerases; and c) it is active in the cytoplasmic compartment of the infected cell, separate from the nuclear and mitochondrial DNA polymerases. FIV and HIV-1 RTs are nearly identical in susceptibility to several nucleotide analogs that are active forms of antiviral drugs, including 2',3'-dideoxythymidine 5'-triphosphate (ddTTP), AZTTP, 2',3'-dideoxycytidine 5'-triphosphate (ddCTP), 2',3'-dideoxy-2',3'-didehydrothymidine 5'-triphosphate (d4TTP). They are also similar in susceptibility to 3'-fluoro-3'-deoxythymidine 5'-triphosphate (3'-F-dTTP), 3'-amino-3'-deoxythymidine (3'-NH₂-dTTP) and two noncompetitive inhibitors PFA and phosphonoacetate (PAA) (48). Because of the similarities of FIV and HIV-1 RTs, FIV has been extremely useful as a model for RT-targeted antivirals.

**Transmission and pathogenesis.** In contrast to HIV-1, FIV is not efficiently transmitted sexually (54). FIV is usually spread through an infected cat biting or scratching an uninfected cat.
Experimentally-infected female cats have been shown to transmit the virus to their offspring (72), although naturally-infected cats transmitting virus to offspring has not been reported. Interspecies transmission does not occur with FIV. This species specificity of FIV is characteristic of retroviruses. No FIV antibodies have been detected in humans who have had extensive contact with FIV-infected cats, including individuals who have been bitten. This shows that FIV is a safe model for AIDS studies.

The pathological effects observed in FIV-infected cats may be both a direct consequence of FIV infection and secondary to the immunodeficiency state caused by the virus. From observations of field and experimentally-infected SPF cats, it has become evident that FIV infections can be divided into several sequential steps. On the basis of the type and severity of clinical signs, a staging of FIV-infected cats into five phases which parallels the classification systems used for HIV-induced pathology. a) Acute phase. In some cases primary infection is clinically silent. More commonly, it manifests itself as a transient illness (1 to 4 weeks) with generalized lymphadenopathy, mild pyrexia dullness, neutropenia,
acute diarrhea, and mild upper respiratory symptoms (8). b) Asymptomatic phase. During this latent stage of infection, virus can be isolated from the blood, but no clinical signs are apparent. c) Persistent generalized lymphadenopathy (PGL). In this stage the animal exhibits vague signs of disease, including recurrent fevers, anorexia, weight loss, or nonspecific behavioral changes but without any obvious indications of secondary or opportunistic infections. d) AIDS-related complex (ARC). Cats in this category usually present with chronic secondary infections of the oral cavity, upper respiratory tract, and other body sites but no opportunistic infections. Most cases diagnosed as ARC progress to feline AIDS (FAIDS) after variable time intervals (26,27,52). e) FAIDS. It is similar in many respects to full-blown AIDS of humans. Cats suffer from severe secondary infections listed above and from neoplastic and neurologic disorders. Infections are often multiple, sustained by opportunistic agents, and resistant to treatment. Most animals present with a marked loss of body weight as well as severe anemia and leukopenia. The clinical picture worsens rapidly, and once the diagnosis is made, the mean survival time is usually less than 1 year, in spite of supportive therapy (27,52). In addition to the above
stages, Pedersen (52) has proposed a sixth group to include cats presenting with miscellaneous disorders (neurological, neoplastic, ocular, and immunologically mediated, etc.) in the absence of other manifestations that, if present, would allow their inclusion in the previous classification.

**Treatment.** Currently available anti-HIV therapies are only partially effective in controlling virus replication. Since RT plays an important role in replication of both HIV-1 and FIV, it is an attractive target for antiviral chemotherapy. RT inhibitors can be divided into two groups, depending on whether they are targeted at the substrate binding site or nonsubstrate binding site (13). To the first group belong the 2',3'-dideoxynucleosides (i.e., ddC, ddl), 3'-azido-2',3'-dideoxynucleosides (i.e., AZT), 3'-fluoro-2',3'-dideoxynucleosides (i.e., FLT), 2',3'-didehydro-2',3'-dideoxynucleosides (i.e., d4T, d4C) and carbocyclic derivatives thereof (i.e., carbovir), 2'-fluoro-ara-2',3'-dideoxynucleosides, 1,3-dioxolane derivatives (i.e., 2',3'-dideoxy-3'-thiacytidine), oxetanocin analogues and carbocyclic derivatives thereof (i.e., cyclobut-G) and the PMEA and 9-(3-fluoro-2-
phosphonylmethoxypropyl)adenine (FPMPA) derivatives. These compounds need to be phosphorylated intracellularly to their triphosphate forms before they act as competitive inhibitors or alternate substrates (chain terminators) of RT (Fig.4). The second group includes the tetrahydro-imidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)one (TIBO), 1-[(2-hydroxyethoxy)-methyl]-6-(phenylthio)thymine (HEPT), dipyrido[3,2-b:2',3'-e]-[1,4]diazepin-6-one (nevirapine) and pyridin-2(1H)one derivatives, which interact as such, noncompetitively, with a specific allosteric binding site of HIV-1RT. Members of this group do not inhibit FIV.

In addition, several protease inhibitors are also being studied. To date, the FDA has approved five nucleoside analogs (AZT, ddI, ddC, d4T and 3TC) and two protease inhibitors for treatment of humans with AIDS. Since both HIV-1 and FIV RT are susceptible to the nucleoside analog inhibitors, FIV is an excellent choice as an animal model for drug resistance studies of viral mutants resistant to these drugs.
Fig 4. Nucleoside and pyrophosphate analogs with antiviral activities.
Drug resistance. The rapid emergence of drug-resistant variants of HIV-1 is a significant impediment to the treatments of AIDS. Clinically, drug-resistant mutants arise in AIDS patients treated with nucleoside analogs, protease inhibitors, etc. The first reported lentivirus mutants with drug-resistant phenotypes that were selected in vitro were AZT-resistant mutants of FIV (57). These mutants were phenotypically similar to AZT-resistant mutants of HIV-1 isolated from patients (35,57). Mutants of FIV that are resistant to ddl (21), ddC (43) or the combination of AZT plus ddl (21) have been subsequently reported. Resistance to AZT, ddC have been traced to mutations within the RT-encoding region of the pol gene (58,43).

The d4T is a thymidine analogue with an unsaturated bond between the 2′- and 3′-carbons of the sugar moiety. Like AZT, it is a potent inhibitor of HIV replication in several cell lines and prevents the cytopathic effects of the viral infection (4,5,6,25,39,40,41). d4T is metabolized in cells to the triphosphate form (d4TTP), which is a potent inhibitor of the viral RT in a manner that is competitive with respect to the natural substrate, dTTP. D4TTP also serves as a
substrate for incorporation into DNA, which causes chain
termination (24). d4T has shown efficacy in patients with AIDS and
is less toxic to host cells than AZT (22,40,41,42). Cells take up d4T
by nonfacilitated diffusion (3) and then phosphorylate it by cellular
enzymes to its mono-, di-, and triphosphates. Unlike AZT, d4T has a
low affinity to thymidine kinase in several cell lines (6,42). Thus,
the conversion to its monophosphate by this enzyme is the rate-
limiting step in its metabolic activation pathway by triphosphate
(25,42).

There are no clinical reports in the literature of documented
mutations in the HIV-1 RT conferring decreased sensitivity to d4T.
Clinical isolates highly resistant to AZT were found to remain
sensitive to d4T (35). Furthermore, recombinant viruses resistant to
AZT, ddl, ddC or 3TC are not cross-resistant to d4T (33). In view of
the clinical potential of d4T, it is important to determine how
readily viral resistance is likely to arise and what cross-resistance
to other inhibitors might be expected. Lacey and Larder have
selected mutants of HIV-1 that are resistant to d4T in vitro (33).
These variants have a mutation at codon 75 in the RT-encoding
region of the HIV-1 pol gene that results in substitution of Thr for Val-75 of the HIV-1 RT. This mutation confers moderate (seven-fold) d4T-resistance and cross-resistance to both ddl and ddC.

In order to study drug resistance, it is important to have a defined system that is safe and easily manipulated. It is also useful to have a system which allows for timely selection of drug-resistant mutants. FIV has a well-defined cell culture system and drug-resistant mutants can be readily selected (59). In addition, there is a molecular clone of FIV, FIV 34TF10, with a known sequence (67). This molecular clone provides a defined genetic system to study mutations. Using FIV as an animal model, many questions pertaining to drug resistance can be addressed. Not only can drug-resistant mutants be selected and characterized in a cell culture system, but the pathogenicity and infectivity of drug-resistant mutants can be determined in cats (7). In the work described here we have utilized the FIV system to characterize resistance to d4T.
SPECIFIC GOALS

The goal of this study was to select and characterize d4T-resistant mutants of FIV 34TF10. Specific objectives were to select variants of FIV that appear when virus replication occurs in the presence of d4T, to determine whether these mutants are cross-resistant to other antivirals, and to determine whether d4T-resistance is due to alterations in RT by characterization of RT purified from the mutant and by DNA sequence analysis and site-directed mutagenesis.
MATERIALS AND METHODS

CHEMICALS. Triton X-100, dTTP, aminoethyl carbazole, 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, NC. The d4T and the 5'-triphosphate of d4T (d4TTP) were provided by Bristol-Myers Squibb Co., Wallingford, CT. PMEA was provided by Gilead Sciences, Inc., Foster City, CA. The ddl was provided by the Developmental Therapeutics branch, Division of AIDS, National Institute of Allergy and Infectious Diseases. The 3'-fluoro-2',3'-dideoxythymidine (FLT) was obtained from the Lederle Laboratories Division of American Cyanamid Co., Pearl River, NY. Poly (rA)-oligo(dT)_10 was purchased from Pharmacia LKB, Piscataway, NJ. Nuclease-free bovine serum albumin (BSA) was obtained from Boehringer Mannheim, Indianapolis, IN. DEAE-cellulose DE52 and phosphocellulose P11 were purchased from Whatman, Hillsboro, OR. International Bio Technologies (IBI) phenol for DNA extractions was purchased from VWR Scientific. GeneAmp PCR Core
Reagents were purchased from Perkin Elmer Cetus, Norwalk, CT. The Taq DyeDeoxy™ Terminator Cycle Sequencing Kit was purchased from Applied Biosystems, Foster City, CA. EcoR1 was purchased from Promega, Madison, WI. and Nsi1 was from American Applied Biotechnology, Aurora, CO. T4 DNA ligase was from New England Biolabs, Beverly, MA. All other chemicals were reagent grade or better.

**ISOTOPE.** The [Methyl-3H]dTTP was purchased from Dupont-New England Nuclear, Boston, MA.

**CELLS AND VIRUS.** Virus produced from a molecular clone of the Petaluma strain of FIV, 34TF10 (67), 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. Uninfected and infected cells were grown in L & M medium which was composed of equal parts of Leibovitz L-15 and Dulbecco's Modified Eagle Medium (Gibco BRL Life Technologies, Inc. Grand Island, NY.), supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2.0 mM L-glutamine and 10% fetal bovine serum (FBS). FBS was heat
inactivated for 30 min. at 56°C. All cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. The CrFK cells used were passaged 6 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 ability to support FIV replication (32). 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.

Virus stocks were prepared from medium containing extracellular virus released from CrFK cells that had been infected with FIV for more than 21 days. Medium was removed from cultures that were near confluence and cells were removed from the medium by centrifugation at 500 X g for 10 min. The supernatant was stored at -80°C in medium containing 10% dimethyl sulfoxide. Aliquots of these stocks were thawed and used to infect CrFK cells for subsequent studies.
FOCAL INFECTIVITY ASSAY (FIA). The FIA was developed in this lab by Remington et al. (57) and is a modification of the FIA developed by Chesebro and Wehrly to quantitate HIV-1 (9). In this assay, CrFK cells were seeded into 24-well plates at a density of 1.5 X 10⁴ cells per well and incubated for 1 h at 37°C in growth medium or growth medium containing appropriate concentration of drug. This incubation was to allow the cells to convert drugs to their active forms. These cells were infected with 30 to 60 focus-forming units (FFU) of wild-type or mutant FIV per well, then incubated at 37°C for 4-5 days to allow cells to grow to confluence. The medium was replaced with fresh medium and appropriate drug concentration every 2 days. When the cells reached confluence, the medium was aspirated and the cells were fixed with methanol for five minutes. The cells were then washed twice with TNE (0.01 M Tris-HCl, pH 7.5, containing 0.15 M NaCl and 0.002 M EDTA), and once with TNE that contained 1% adult bovine serum. Immunostaining was performed by incubating the fixed cells for 30 min with 0.2 ml of a 1/200 dilution of polyclonal antiserum that had been obtained from FIV-infected specific-pathogen-free cats. Cells were washed twice with TNE that contained 1 % serum to remove excess antiserum and
were then incubated for 40-60 min with 0.2 ml of a 1/600 dilution of horseradish peroxidase-conjugated goat anti-cat IgG (Organon-Teknika, Durham, NC.). The cells were again washed with TNE and foci of infected cells were then stained by reacting the antibody-bound monolayers for 20 min in the dark with a solution of aminoethyl carbazole (1 part 4 mg/ml aminoethyl carbazole in dimethylformamide per 19 parts 0.05 M sodium acetate buffer, pH 5), containing 30% H₂O₂ (1 μl per 2 ml). After incubation, the cells were rinsed three to five times with water and allowed to dry. Foci appeared as groups of 4 or more infected cells (red), which is defined as a focus of infection, against an unstained background. Foci were examined and counted under a dissecting microscope at 10 to 40 X magnification. For visualization, light was reflected off a piece of ground glass to reduce the refractivity of the infected cells. For dose response curves with drugs, data were plotted as percentage of control plaques (no drug) versus inhibitor concentration. Concentrations required to inhibit focus formation by 50% (IC₅₀ values) were obtained directly from the linear portion of these plots.
SELECTION OF d4T-RESISTANT MUTANT. A step-wise selection protocol that is similar to that used by Gao et al. (19,20) and by Larder et al. (36) to select drug-resistant variants of HIV-1 was chosen. The initial round of selection was carried out with 12.5 μM d4T, which is equivalent to the IC₅₀ of d4T for FIV (58). The concentration of d4T was then increased 2-fold in each subsequent round. For the first round, two 25-cm² flasks of CrFK cells at 60% confluence (about 2 X 10⁶ cells) were pre-treated for one hour with 12.5 μM d4T to enable conversion of the drug to the active form. Then CrFK cells were infected with a cell-free culture supernatant of FIV 34TF10 at an input multiplicity of approximately 2000 focus forming units (FFU). Medium and drug were replaced every 48 hours, and cells were removed with trypsin and subcultured as necessary. The cultures were monitored weekly for the presence of virus by the FIA. By 2 to 3 weeks post-infection 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. Using 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 post-
infection. After passage in 100 μM d4T (which is approximately 8 times the IC₅₀ for FIV), two d4T-resistant mutants, D4R-1c and D4R-2c were obtained. These mutants were subsequently plaque-purified.

Another selection protocol called “blast” selection was attempted. It is essentially as described by Remington et al. (57). Infections were carried out in the continuous presence of d4T at 100 μM, which is 8 times the IC₅₀ for inhibition of FIV replication. Using this approach, AZT, ddl and ddC-resistant mutants of FIV have been successfully selected in the presence of drugs at concentrations 5 to 10 times higher than the IC₅₀.

PLAQUE-PURIFICATION OF d4T-RESISTANT MUTANTS. To minimize potential heterogeneity within the d4T-resistant mutant populations, plaque-purified isolates were obtained from D4R-1c and D4R-2c. Plaque-purification of the mutant virus was carried out with a modification of the procedure described by Remington et. al. (58). Virus of both D4R-1c and D4R-2c were plaque-purified using limiting dilutions of virus in the FIA. Limiting dilutions of virus
were used to infect CrFK cells in 24-well plates in the presence of 100 μM d4T. After 5 to 6 days, each supernatant was transferred to a well of a new plate which had been seeded with uninfected CrFK cells at 30% confluence in the presence of 100 μM d4T. The original plate was stained and examined for those wells that contained a single focus. Corresponding wells of the subculture plate were incubated until the cells reached confluence. The cells and supernatant from each well which contained a single focus were transferred to flasks and maintained in medium containing 100 μM d4T. Cultures were maintained by replacing medium and drug every two days. When they reached 100% confluence, cultures were monitored for virus production by FIA. Several cultures yielded virus. One from each of D4R-1c and 2c, designated D4R-3c and D4R-4c, were used for further characterization.

**REVERSE TRANSCRIPTASE (RT).** Reverse transcriptase (RT) was purified from virions of FIV 34TF10 and mutant FIV by a method developed by North et al. (49). FIV-infected CrFK cells were grown in 225-cm² tissue culture flasks to confluence. Culture medium containing virus was collected, cells were removed by
centrifugation at 500 X g for 5 min, then the virus pelleted by centrifugation at 41,000 X g for 60 min. The supernatant was discarded and the pellet resuspended in 50 mM Tris-HCl, pH 7.9, 20 mM dithiothreitol (DTT), 160 mM KCl, 0.05% Triton X-100 to disrupt virions. Nuclease-free BSA was added to a final concentration of 500 μg/ml, and this virus lysate was stored at -80°C until purification. For purification, the virus lysate was dialyzed three times against 500 ml of 50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM DTT, 5% glycerol (buffer A) and then applied to a 1.5 X 30-cm column of DEAE-cellulose. The column was washed with buffer A, and then reverse transcriptase was eluted with a 150-ml linear gradient of 0-500 mM NaCl in buffer A. Fractions containing peak RT activity were pooled, nuclease-free BSA was added to a final concentration of 500 μg/ml, and this was dialyzed three times against 500 ml of 50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM DTT, 20% glycerol, 50 mM NaCl (buffer B). This sample was then applied to a 1.5 X 30-cm column of phosphocellulose; the column was washed with buffer B, and then RT was eluted with a 150-ml linear gradient of 50-500 mM NaCl in buffer B. Peak fractions were pooled and stored at -80°C.
**RT ACTIVITY ASSAY.** During purification, RT was assayed for activity as reported previously (48,49) with poly(rA)-oligo(dT)\textsubscript{10} as the template-primer. Reactions were typically carried out in a volume of 50 µl and contained 50 mM Tris-HCl, pH 8.5, 10 mM DTT, 0.05% Triton X-100, 250 µg of bovine serum albumin (nuclease-free) per ml, 6 mM MgCl\textsubscript{2}, 80 mM KCl, 20 µM [methyl-\textsuperscript{3}H]dTTP (33 µCi/ml), 0.5 A\textsubscript{260} units (U) of poly(rA)-oligo(dT)\textsubscript{10} per ml, and appropriate amounts of RT. Samples containing 40 µl were taken at 30 min and spotted onto filters (no. 3, 2.3-cm diameter; Whatman, Inc., Clifton, NJ) that had been presoaked with 5% trichloroacetic acid (TCA)-1% sodium pyrophosphate (NaPPi). Filters were dried in an oven then washed four times (at least 1 hour each) with 5% TCA-1% NaPPi at 4°C, twice with 95% ethanol at room temperature, dried in an oven and then counted in Liquifluor (Dupont-NEN Research Products, Boston MA). One unit of RT is defined as the amount of enzyme required to catalyze the incorporation of 1 nmol of dTMP per hour into poly (rA)-oligo(dT)\textsubscript{10} at 37°C.

**RT INHIBITION ASSAY.** Inhibition of RT from the d4T-resistant mutant (D4R-3c) by d4TTP was compared to inhibition of wild-type
recombinant FIV RT. Double-reciprocal plots were used to determine kinetic constants (Km and Ki) with the template-primer poly(rA)-oligo(dT)$_{10}$ and d4TTP as the inhibitor. Reactions were typically carried out in a volume of 50 µl and contained 50 mM Tris-HCl, pH 8.5, 10 mM DTT, 0.05% Triton X-100, 250 µg of bovine serum albumin (nuclease-free) per ml, 6 mM MgCl$_2$, 80 mM KCl, varying amounts of [methyl-$^3$H]-dTTP (16.5, 8.25, 4.95, 3.3, 2.45, and 1.65 µCi/ml) corresponding to varying concentrations of dTTP (20, 10, 6, 4, 3, and 2 µM), 0.5 A$_{260}$ U of poly(rA)-oligo(dT)$_{10}$ per ml, varying concentrations of d4TTP which were 0, 5, 10, 20 nM, and appropriate amounts of RT. Samples containing 40 µl were taken at 1 hour and spotted onto filters (no. 3, 2.3-cm diameter; Whatman, Inc., Clifton, NJ) that had been presoaked with 5% TCA-1% NaPPi (4°C). Filters were washed with TCA four times (15 min each) and twice with 95% ethanol, dried and then counted in Liquifluor (Dupont-NEN Research products, Boston, MA).

**NUCLEIC ACID PREPARATION.** Total cellular DNA containing provirus was extracted from CrFK cells that were infected with d4T-resistant FIV. Confluent monolayers of infected cells were
washed three times with TBS (25 mM Tris-HCl, pH 7.4, containing 5 mM KCl and 137 mM NaCl) and removed from flasks (cells peel from flask wall during the third wash). Cells were pelleted by centrifugation at 500 X g for 10 min. The cells were washed twice with TE (10 mM Tris-HCl, pH 7.6, containing 1 mM EDTA) then suspended in this TE buffer. Cell suspensions were treated with 0.1 mg/ml proteinase K and 0.5% SDS overnight at 37° C. The cell lysates were then extracted three times with phenol that had been equilibrated with 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and 1 mM EDTA. The aqueous phase was collected and dialyzed against 10 mM HCl, pH 7.5, containing 100 mM NaCl and 1 mM EDTA until the A_{260} of the dialysate was less than 0.1. The dialyzed aqueous phase was treated with RNase A (0.1 mg/ml) at 37° C for 3 hours. The RNase A-treated aqueous phase was extracted with one-half volume equilibrated phenol and one-half volume of chloroform:isoamyl alcohol (24:1). The aqueous layer was removed and extracted again with one volume of chloroform:isoamyl alcohol (24:1). The salt concentration was adjusted by adding 1/30 volume of 3 M sodium acetate, pH 5 to the aqueous phase. The DNA was precipitated with three volumes of cold absolute ethanol at -20° C.
overnight. The DNA was removed from the ethanol and dried in air. The DNA was dissolved in sterile distilled water and used for amplification by the polymerase chain reaction (PCR).

**PCR AMPLIFICATION AND NUCLEOTIDE SEQUENCE ANALYSIS.**

Amplification of the RT-encoding region of the *pol* gene was performed using the Perkin-Elmer/Cetus *GeneAMP* PCR protocol. Each 100 µl reaction contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 µM of each dNTP (dATP, dCTP, dGTP and dTTP), 4 mM MgCl₂, 0.2 µM of each primer, 2.5 Units of *AmpliTaq* DNA polymerase, and 10 to 20 µg of target DNA. Reactions were overlaid with 50-100 µl of light mineral oil. The sense primer (5'-GTA ATG TTT GTG TGT TAG AAG ATA AGT G-3') and the reverse complement primer (5'-ATG ATA TGG TGG ATG TTG TGA GGT-3') were synthesized in the Murdock Molecular Biology Facility, University of Montana, Missoula, MT. These primers were chosen to amplify a 1763-base pair fragment that contained nucleotides 2268 through 4031. The PCR was run for 30 cycles, each cycle comprised of 30 seconds of denaturation at 94° C, 30 seconds of annealing at 62° C and two minutes of extension at 72° C. After PCR, product was visualized on an agarose
gel. For this, the mineral oil was separated from the PCR products and an aliquot of the mixture was run on a 0.8% low-melting agarose electrophoresis gel in 40 mM Tris acetate, pH 7.6, containing 1 mM EDTA (61). The agarose gel and running buffer contained 0.5 μg/ml ethidium bromide. A 1763-base pair band was identified by comparing the electrophoresed PCR reaction to a 1 Kb DNA ladder (Gibco BRL, Gaithersburg, MD). The remaining PCR product was purified using the QIAquick PCR Purification Kit, Qiagen Inc., Chatsworth, CA. Sequencing was performed in the forward and reverse directions with two or more primers covering each 250-base pair section of the RT-encoding region along with small flanking regions on either end of RT. Four different primers were used to sequence the 2474 region containing the point mutation. This DNA was directly sequenced by Joan Strange, The Murdock Molecular Biology Facility, The University of Montana, Missoula, MT, with a Taq Dye Deoxy™ Terminator sequencing kit and analyzed on a Model 373A automated DNA sequencer (Applied Biosystems, Foster City, CA).

**REVERSION STUDIES.** The stability of the d4T-resistant phenotype was determined for D4R-3c and D4R-4c. These mutants
were passaged in the absence of d4T. CrFK cells were infected with D4R-3c, or D4R-4c and maintained without d4T. When virus production was apparent (about two weeks), an IC$_{50}$ for d4T was determined by FIA. The resulting viruses were used to initiate a subsequent round of infection. Each virus was passaged three times in the absence of d4T.

**SITE-DIRECTED MUTAGENESIS.** To facilitate mutational analysis, we constructed a 34pol cassette virus from the infectious molecular clone, FIV 34TF10 (56). This was constructed by modification of the EcoR1 site in the polylinker of FIV 34TF10, enabling use of the natural EcoR1 site of the virus for the pol cassette. The EcoR1 site in the polylinker was changed from GAATTC to GAATTG. This modification was achieved by PCR using four synthetic primers. Primer #144 was the most 5' and spanned the DralI site of the pUC119 portion of 34TF10 clone (5'-GAT TGG GTG TGA TGG TTC AGG TAG TGG G-3'). Primer #145 was an antisense primer that spanned the Eco R1 site of the vector's polylinker and changed this site (5'-GAA TTG ACT GGC CGT CGT CGT TTT AC-3', base change underlined). Primer #146 was a sense primer that matched primer #145 at the
Eco R1 site and was also designed to alter the natural EcoR1 site (5'-GTA AAA CGA CGG GCC AGT ÇAA TTC-3', base change underlined). Primer #147 was the most 3' and spanned the SacI site of the virus (5'-CAA CTT GAT TAT GGA GOT CGA TG-3'). Primer pairs #144 / #145 and #146 / #147 were used for PCR to amplify DNA from the 34TF10 target. Amplified DNA products were gel-purified, combined, and reamplified using primers #144 and #147. The resulting PCR product and 34TF10 were cleaved with DralII and SacI and the digests were gel-purified then ligated. The desired mutation was confirmed by sequence analysis. After transfection into CrFK cells, 34pol cassette produced FIV that was indistinguishable from FIV 34TF10.

To introduce the mutation at codon 47 of the FIV RT, an 1164-bp fragment corresponding to nucleotide 1740-2904 was amplified using primer #16 (GAT CCT ATA TAA ATG TCA TCC) and primer #41 (GGA TCA GGA CCA GTG TGT). Amplification was performed using the Perkin-Elmer/Cetus *GeneAMP* PCR protocol. Each 100 µl reaction contained 10 mM Tris-HCl, pH8.3, 50 mM KCl, 200 µM of each dNTP (dATP, dCTP, dGTP and dTTP), 4 mM MgCl2, 0.2 µM of each primer, 2.5
Units of *AmpliTaq*® DNA polymerase, and 10 to 20 μg of target DNA. Reactions were overlaid with 50-100 μl of light mineral oil. The PCR was run for 30 cycles, each cycle comprised of 30 seconds of denaturation at 94° C, 30 seconds of annealing at 52° C and two minutes of extension at 72° C. After PCR, product was visualized on an agarose gel. For this, the mineral oil was separated from the PCR products and an aliquot of the mixture was run on a 0.8% low-melting agarose electrophoresis gel in 40 mM Tris acetate, pH 7.6 containing 1 mM EDTA (61). The agarose gel and running buffer contained 0.5 μg/ml ethidium bromide. The PCR product and 34pol cassette were each cleaved with EcoR1 and NsiI. The reaction contained 10X NsiI buffer, 24 U EcoR1, 30 U NsiI and 3.6 mg of the PCR product (including FIV 34TF10 or D4R-3c DNA) and then incubated 3 hours at 37° C. The fragments were gel-purified by gene cleaning. First, the gel slices were cut, and weighed. Then 3 volume of sodium iodide were added and incubated 5 min at 45-55 °C. 5 μl of glassmilk was added and incubated at room temperature for 30 min. After centrifugation, pellets were washed three times with NEW wash (ethanol, both glassmilk and NEW wash are from BIO 101 gene-clean kit) and eluted with 20 μl of water. OD260 was measured.
The resulting 803-bp fragment from D4R-3c, corresponding to nucleotide 1871-2674 of the FIV genome, was ligated into the cleaved 34pol cassette by using vector:insert ratio 1:4, adding T4 DNA ligase and incubating overnight at 16°C. DNA sequence analysis was used to confirm the presence of the desired mutation. Only one ligation product contained the 2474 point mutation. This construct was transformed into the J5 strain of *E. coli* JM109 and the resulting plasmid DNA was used to transfect CrFK cells for 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 8 times the IC₅₀ for FIV). This approach, which we call “blast” selection, had been successful for selection of FIV mutants resistant to AZT (57,58), ddl (21) or ddC (43). However, in attempts to select with 100 μM d4T, we were unable to obtain any d4T-resistant mutants. Virus obtained after this selection had wild type susceptibility to d4T. Therefore, we switched to a step-wise selection protocol that is similar to that used by Gao et al. (19,20) and by Larder et al. (36) 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 IC₅₀ of d4T for FIV (58). The concentration of d4T was then increased 2-fold in each subsequent round. For the first round of selection, two 25 cm² flasks of 60% confluent CrFK cells were pre-treated with 12.5 μM d4T for 1 h prior to infection to ensure that the drug had been
converted to its active form by the cells. Then the drug pre-treated CrFK cells were infected with a cell-free culture supernatant of FIV 34TF10 at an input multiplicity of approximately 2000 focus forming units (FFU). Media and drug were replaced every 48 hours, and cells were removed with trypsin and subcultured as necessary (usually every week). Cultures were monitored weekly for the presence of virus by the FIA. By 2 to 3 weeks post-infection virus production was apparent.

For the second round of selection, culture supernatants (cell-free) from each of the first-round cultures (approximately 2000 FFU also) were used to infect flasks of CrFK cells in the presence of 25 \( \mu \text{M} \) d4T. They were maintained with 25 \( \mu \text{M} \) d4T and monitored for virus production as before. Using these protocols, virus was subsequently passaged in 50 and 100 \( \mu \text{M} \) d4T. In each round of selection, virus production was apparent by 2 to 3 weeks post-infection. After passage in 100 \( \mu \text{M} \) d4T, dose response curves were performed for preliminary screening of the d4T-resistant phenotype. Two d4T-resistant mutants, designated D4R-1c and D4R-2c, were obtained. These mutants were resistant to d4T (Fig. 5). At this point
d4T DOSE RESPONSE CURVE FOR D4R-1c, AND 2c

Fig 5. Inhibition of FIV 34TF10, D4R-1c and D4R-2c by d4T as determined by FIA. Results are from two experiments and each point represent four determinations. Bars represent standard error of the mean, and are omitted where the standard error was too small to accurately show using error bars.
plaque-purification of mutant viruses were initiated.

**PLAQUE-PURIFICATION OF d4T-RESISTANT MUTANTS.** To minimize potential heterogeneity within the d4T-resistant mutant populations, the FIA was used to isolate a clone of virus that had descended from a single infectious particle. A focus of infection represents an infection by a single virion (Fig. 6).

**Fig 6.** Focus of FIV-infected CrFK cells detected with the immunostaining procedure described in methods.
CrFK cells were seeded at a density of 1.0 X 10^4 cells per well in TC24 plates, with 100 μM d4T, and were infected with virus stocks (D4R-1c and D4R-2c) that had been diluted to contain approximately one infectious particle per well. After six days, culture supernatants were transferred to uninfected cells with 100 μM d4T in TC24 plates, and the original plates were immunostained to identify those wells that contained a single focus of infection. Cells infected with progeny from a single infection event were transferred along with supernatant to a flask and maintained with 100 μM d4T and monitored for virus production by the FIA. Two plaque-purified mutants, designated D4R-3c and D4R-4c (obtained from D4R-1c and D4R-2c, respectively) were selected for further characterization. Both plaque-purified mutants retain the d4T-resistant phenotype of the parent populations (Fig. 7).

The d4T-resistance of the D4R-1c, D4R-2c, D4R-3c and D4R-4c were confirmed with the FIA by determining the number of foci at varying concentrations of d4T. The dose response curves shown in Fig. 5 and Fig. 7 verify that D4R-1c, 2c and D4R-3c, 4c were considerably less sensitive to inhibition by d4T than the molecular
d4T DOSE RESPONSE CURVE FOR D4R-3c, AND 4c

Fig 7. Inhibition of FIV 34TF10 and D4R-3c and 4c by d4T as determined by FIA. Results are from three experiments and each point represent four determinations. Bars represent standard error of the mean, and are omitted where the standard error was too small to accurately show using error bars.
clone of FIV. The IC$_{50}$ value for inhibition of D4R-1c and 2c by d4T were greater than 36 µM compared to 12 µM for FIV 34TF10. Similar results were determined with D4R-3c and D4R-4c. These data demonstrated that these mutants were at least 3 to 6-fold less susceptible to d4T than FIV 34TF10.

CHARACTERIZATION OF d4T-RESISTANT MUTANTS.

Susceptibility to antiviral compounds. The susceptibility of D4R-3c and D4R-4c to a variety of other antiviral compounds was determined using the FIA and compared to the susceptibilities of FIV 34TF10 (Fig 8. and Table. 1). These data showed that D4R-3c and D4R-4c were cross-resistant to several other antiviral compounds. Both mutants were 2 to 2.5-fold resistant to AZT, 5 to 6-fold resistant to FLT, 2-fold to ddC, 1 to 2 fold to ddl and 4-fold to PMEA. They were also highly resistant (>50-fold) to the non-nucleoside inhibitor, PFA.
Fig 8. Inhibition of FIV 34TF10 and D4R-3c and 4c by various antivirals as determined by FIA. Results are from three experiments and each point represent four determinations. Bars represent standard error of the mean, and are omitted where the standard error was too small to accurately show using error bars.
Table 1. Sensitivities of FIV 34TF10 and d4T-resistant mutants derived from it to antiviral compounds as determined by FIA

<table>
<thead>
<tr>
<th>Compound</th>
<th>FIV 34TF10</th>
<th>D4R-3c</th>
<th>D4R-4c</th>
</tr>
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<tbody>
<tr>
<td>d4T</td>
<td>9 ± 2</td>
<td>34 ± 8</td>
<td>32 ± 7</td>
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<td>AZT</td>
<td>0.3 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.1</td>
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<tr>
<td>FLT</td>
<td>0.2 ± 0.05</td>
<td>1.1 ± 0.1</td>
<td>1.3 ± 0.1</td>
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<tr>
<td>ddC</td>
<td>4.4 ± 0.3</td>
<td>9.1 ± 1.5</td>
<td>9 ± 2.1</td>
</tr>
<tr>
<td>ddl</td>
<td>1.1 ± 0.04</td>
<td>2.4 ± 0.4</td>
<td>1.5 ± 0</td>
</tr>
<tr>
<td>PFA</td>
<td>207 ± 44</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>PMEA</td>
<td>0.1 ± 0</td>
<td>0.4 ± 0.02</td>
<td>0.4 ± 0.02</td>
</tr>
</tbody>
</table>

^Values are from two or more experiments, with four determinations per experiment.
**Reverse transcriptase.** Reverse transcriptase was purified from D4R-3c and from wild-type FIV (recombinant RT) (46). This wild-type FIV RT was produced in *Escherichia coli*, and has been shown to be similar to virion-derived FIV RT in its susceptibility to several antiviral nucleotides. The kinetic parameters of inhibition by d4TTP were determined for D4R-3c RT and compared to wild-type FIV RT. The two enzymes were nearly identical in $K_m$ for dTTP ($5.8 \pm 0.6 \mu M$). However, the $K_i$ for inhibition of RT from D4R-3c by d4TTP, 4.7 nM, was 3-times higher than that of wild-type FIV RT. Furthermore, the $K_i$ values for inhibition of this mutant enzyme and R-RT by AZTTP and PFA were determined. As expected, AZTTP and d4TTP displayed competitive inhibition of the enzymes and PFA displayed non-competitive inhibition (Fig. 9). The inhibition constant of mutant RT by AZTTP, 21 nM, was 10-times higher than that of wild-type FIV RT and by PFA, 1.7 μM, was 6-times higher than for inhibition of the wild-type FIV RT, respectively (Table 2).
Table 2. Kinetic Constants for wild-type FIV and D4R -3c RTs

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>FIV 34TF10 RT</th>
<th>D4R-3c-RT</th>
</tr>
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<tbody>
<tr>
<td>d4TTP</td>
<td>1.4 ± 0.14</td>
<td>4.7 ± 0.16</td>
</tr>
<tr>
<td>AZTTP</td>
<td>2.2 ± 0.23</td>
<td>21 ± 1.51</td>
</tr>
<tr>
<td>PFA</td>
<td>265 ± 37</td>
<td>1720 ± 345</td>
</tr>
</tbody>
</table>

Values are reported as the mean ± SEM of at least five determinations. The mode of inhibition of each enzyme by d4TTP or by AZTTP was competitive with respect to substrate. Inhibition of each enzyme by PFA was non-competitive with respect to substrate. The $K_m$ for dTTP was 5.8 ± 0.6 μM for each enzyme.
Fig 9. Determination of $K_i$ values for inhibition of wild-type FIV RT and D4R-3c RT. Values reported are averages from duplicate determinations.
Nucleotide sequence analysis. DNA sequence analysis of the entire RT-encoding region of the pol gene from D4R-3c and FIV 34TF10 were performed in the forward and reverse directions. FIV 34TF10 DNA had been subcloned into the plasmid pUC119 and was used as the wild-type control. 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 Val-47 with Ile, named I47. The nucleic acid and amino acid sequences of FIV 34TF10 and D4R-3c pol gene are shown in Fig. 10.

Figure 10. (cont. on next page)
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<thead>
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Fig. 10 Nucleotide sequence and deduced amino acid sequence of RT-encoding region of pol gene from FIV 34TF10 clone of FIV in pUC119, virus derived from this molecular clone and D4R-3c. "-" denotes identity.
Reversion studies. In order to determine whether this I47 mutation was genetically stable, CrFK cells were infected with D4R-3c and D4R-4c and maintained in the absence of d4T. When virus production was apparent, an IC50 for d4T were determined, and these viruses were used to initiate a subsequent round of infection. This experiment was carried out for three rounds of infection. Both viruses remained d4T-resistant even after three rounds without d4T (Table. 3). These data demonstrated that the d4T-resistant phenotype is stable.

Site-directed mutagenesis. In order to confirm the role of this mutation in d4T-resistance, the G to A mutation at position 2474 was introduced into the FIV 34pol cassette by site-directed mutagenesis. Initial attempts to obtain a 1297-bp fragment from D4R-3c proviral DNA with primers #28 and #16 were not successful. Therefore, we switched to primers #41 (GGATCAGGACCAGTGTGT) and #16 (GATCCTATATAATGTCAATCC) and obtained an 1164-bp fragment containing position 2474. This fragment and 34pol cassette were each cleaved with EcoR1 and Ncol, and ligated. After transforming them into the J5 strain of E. coli JM109, there were no
Table 3. Phenotypic stability of d4T-resistant mutants when passaged in the absence of d4T

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<th>Virus</th>
<th>MEAN IC50 (μM) ± SEM</th>
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<tr>
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<td>34TF10</td>
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<tr>
<td>D4R-3c</td>
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<td>D4R-4c</td>
<td>38 ± 13</td>
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*Values are from two or more experiments with four determinations per experiment.*
colonies. *EcoR1* and *NsiI* were then used in a second attempt, and five colonies were obtained. DNA sequence analysis revealed that one colony had the correct insert containing the I47 mutation. This DNA was transfected into CrFK cells. When virus was apparent, an IC50 for d4T was determined. The mutant made by site-directed mutagenesis was 2.5-fold resistant to d4T, confirming the role of this mutation in d4T-resistance.
DISCUSSION

The results of this study have demonstrated the *in vitro* selection and characterization of d4T-resistant mutants of FIV. This was the first d4T-resistant mutant derived from a molecular clone of either FIV or HIV-1. We initially attempted to select d4T-resistant mutants with a "blast" protocol. Using this method, AZT, ddl and ddC-resistant mutants of FIV were successfully selected. However, we were unable to obtain any d4T-resistant mutants from this type of selection. The virus that was obtained after passaged in 100 μM d4T was quickly apparent after 1 to 2 weeks, and was as susceptible to d4T as the wild-type strain, FIV 34TF10. The reason for this may be incomplete inhibition of wild-type virus. Therefore, we switched to a step-wise selection protocol, and succeeded in selection of d4T-resistant mutants of FIV.

The d4T-resistant mutants we have isolated have relatively low level, i.e. 3- to 6-fold, resistance to d4T. This level of resistance is similar to that reported by Lacey and Larder for a d4T-resistant
mutant of HIV-1, which displayed 7-fold resistance (33). Relatively low level resistance has also been observed with mutants for FIV or HIV-1 resistant to ddl or ddC. For example, a ddl-resistant mutant of FIV displayed a 2 to 4-fold less susceptibility to ddl (21), ddC-resistant mutant of FIV was 3 to 6-fold resistant to ddC (43), and the HIV-1 mutants resistant to ddl or ddC range from 4 to 6-fold resistant (18,19,20,23,64). In contrast, many of the AZT-resistant mutants of FIV (57,58) or HIV-1 (35,36,37) displayed greater than 40-fold resistance to AZT.

The plaque-purified d4T-resistant mutants we have isolated are cross-resistant to ddl and ddC. In this respect they are phenotypically similar to the d4T-resistant mutant of HIV-1 reported by Lacey and Larder (33). The d4T-resistant mutants of FIV were also cross-resistant to AZT, FLT, PMEA and PFA. However, Lacey and Larder reported that the d4T-resistant mutant of HIV-1 remained sensitivity to AZT and they did not determine FLT, PMEA and PFA.
Reverse transcriptases were purified from wild-type and the plaque-purified d4T-resistant mutant (D4R-3c) of FIV. The RT from the mutant virus was resistant to the active form of d4T, d4TTP. The mutant enzyme had a 3-fold higher $K_i$ for d4TTP than the wild-type FIV RT, whereas the two enzymes were identical in $K_m$ for the substrate, dTTP. This 3-fold resistance at the enzyme level correlates well with the 3- to 6-fold resistance at the level of virus replication. Moreover, we found that D4R-3c RT also had decreased susceptibility to inhibition by AZTTP or PFA. Interestingly, the level of the RT resistance to AZTTP was greater than the level of resistance to AZT of virus replication. A similar lack of correlation between virus susceptibility to AZT and RT susceptibility to AZTTP has been shown for many AZT-resistant mutants of HIV-1 and FIV, which have RTs that are not even resistant to AZTTP (36,37,57).

The resistance shown by the RT from D4R-3c is due to a mutation in the RT-encoding region of the FIV pol gene. Sequence analysis of the plaque-purified d4T-resistant mutant of FIV revealed a single point mutation resulting in an amino acid change from valine to
isoleucine at codon 47 of FIV RT. This nucleotide substitution at position 2474 in D4R-3c was the only divergence from the wild-type RT-encoding region. The d4T-resistant mutant of HIV-1 that has been characterized was mapped to codon 75 of the HIV-1 RT (33), and there have been no drug-resistant mutants of HIV-1 or FIV RTs that map to codon 47 (62). Interestingly, the amino acid found at codon 47 of RT from the d4T-resistant mutant of FIV (isoleucine) is the amino acid present at codon 47 of wild-type HIV-1 RT. It will be interesting to determine whether alteration of codon 47 in the HIV-1 RT will affect susceptibility of HIV-1 to d4T or other RT-targeted antiviral drugs.

Some AZT-resistant mutants of FIV that phenotypically revert very rapidly when they replicate in the absence of AZT (58). In contrast, D4R-3c (like a ddC-resistant mutant of FIV, DCR-5c) (43) remained phenotypically stable when passaged in the absence of d4T, even after three passages. This genetic stability makes D4R-3c a better candidate than the genetically unstable mutants for future in vivo studies to evaluate how mutations conferring drug-resistance affect pathogenicity of the virus.
The structure of HIV-1 RT has been determined by X-ray crystallography (29,31). Many reports have shown crystals of RT suitable for structural analysis. HIV-1 RT contained two subunits, p66 and p51. The p66 subunit is folded into five separate subdomains, the RNase H domain and four subdomains of the pol domain, termed fingers, palm, thumb and connection. p51 has the same amino acid sequence and subdomains, but the conformation of the pol domain is astonishingly different from that of p66 (31). Based upon the X-ray crystal structure of HIV-1 RT and the amino acid sequence homology of the HIV-1 and FIV RTs, 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 “fingers”. 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. Based upon 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.
Due to the emergence of mutants resistant to any of the drug used in monotherapy, much effort is currently focused upon evaluation of combinations of two or more drugs. Recently, chemotherapy of HIV infections, as for other chronic infections and malignant diseases, is moving into the direction of multiple drug combinations. The major advantages of this is: non-overlapping toxicities, reduced risk of viral-drug resistance development, and the possibility of synergistic combinations. 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. Thus, d4T may not be suitable for use in these multiple drug combinations.
CONCLUSION

Two d4T resistant mutants (D4R-1c, D4R-2c) were successfully obtained by step-wise selection with 100 µM final d4T concentration. D4R-1c and 2c were plaque-purified and the resulting viruses, designated D4R-3c and 4c. All of these mutants were at least 3-fold resistant to d4T as compared to FIV 34TF10. They were also cross-resistant to AZT, FLT, ddC, ddl, and PMEA, and were highly resistant to PFA. Purified D4R-3c RT was 3-fold resistant to d4TTP, 10-fold to AZTTP and 6-fold to PFA, relative to wild type FIV RT. A single point mutation was found in the d4T-resistant mutant (D4R-3c) that resulted in the amino acid substitution of Ile for Val at codon 47 of the FIV RT. The phenotype of d4T-resistant mutants was stable, as D4R-3c and D4R-4c remained d4T-resistant even after passage in the absence of d4T for three rounds. The role of the V to I mutation at codon 47 in d4T-resistance was confirmed by site-directed mutagenesis.

Future studies should evaluate whether multiple-drug resistant
mutants will arise more rapidly with combinations containing d4T. It will also be important to examine pathogenicity of d4T-resistant FIV, and other FIV drug-resistant mutants, in cats.
REFERENCES


