In Vitro Induction of Human Immunodeficiency Virus Type 1 Variants Resistant to 2′-β-Fluoro-2′,3′-Dideoxyadenosine

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2′-β-Fluoro-2′,3′-dideoxyadenosine (F-ddA) is an acid-stable purine dideoxynucleoside analog active against a wide spectrum of human immunodeficiency virus type 1 (HIV-1) and HIV-2 strains in vitro. F-ddA is presently undergoing a phase I clinical trial at the National Cancer Institute. We induced HIV-1 variants resistant to F-ddA by exposing wild-type HIV-1 (HIV-1$_{LAI}$) to increasing concentrations of F-ddA in vitro. After 18 passages, the virus was fourfold less sensitive to F-ddA than HIV-1$_{LAI}$. Sequence analyses of the passage 18 virus revealed changes in three amino acids in the reverse transcriptase (RT)-encoding region of the *pol* gene: P to S at codon 119 (P119S; present in 3 of 13 and 28 of 28 molecular clones before and after F-ddA exposure, respectively), V179D (0 of 13 and 9 of 28, respectively), and L214F (9 of 13 and 28 of 28, respectively). Drug sensitivity assays using recombinant infectious clones confirmed that P119S was directly responsible for the reduced sensitivity of HIV-1 to F-ddA. Various infectious clones with single or multiple amino acid substitutions conferring viral resistance against nucleoside RT inhibitors, including HIV-1 variants with multi-dideoxynucleoside resistance, were generally sensitive to F-ddA. The moderate level of resistance of HIV-1 to F-ddA, together with the lack of conferment of significant cross-resistance by the F-ddA-associated amino acid substitutions, warrants further investigation of F-ddA as a potential antiviral agent for use in treatment of HIV-1 infection.

To date, a number of nucleoside reverse transcriptase (RT) inhibitors have been developed for the treatment of patients with human immunodeficiency virus type 1 (HIV-1) infection. Five compounds, zidovudine (AZT), didanosine (ddI), stavudine (d4T), zalcitabine (ddC), and lamivudine (3TC), are now clinically available in the United States. However, the emergence of HIV-1 variants resistant to these RT inhibitors is thought to be a factor in the limited efficacy of antiviral therapy (2, 8, 15, 21, 23, 28, 33). Indeed, HIV-1 variants resistant to these drugs have been linked to clinical deterioration in patients receiving antiviral therapy (14, 22, 36). Thus, the development of novel compounds that are active against HIV-1 variants resistant to the currently available RT inhibitors is urgently needed.

2′-β-Fluoro-2′,3′-dideoxyadenosine (F-ddA), an acid-stable 2′-fluoro purine dideoxynucleoside (ddN), has recently been developed and is active against a wide spectrum of HIV-1 and HIV-2 strains in vitro (Fig. 1) (18, 19). It is noteworthy that F-ddA falls within the cell activation-independent ddN class (5, 6) and exhibits greater anti-HIV activity in resting cells than in activated, dividing cells (30). Recently, it was reported that F-ddA exhibited potent anti-HIV activity that appeared to be superior to that of AZT, as examined in severe combined immunodeficiency mice reconstituted with human peripheral blood leukocytes (hu-PBL-SCID mice) (27). F-ddA is currently undergoing a National Cancer Institute phase I clinical trial as a potential therapeutic agent for treatment of HIV-1 infection.

In this study, we induced, in vitro, HIV-1 variants resistant to F-ddA by continuously exposing the virus to increasing concentrations of F-ddA and defined the virological properties of the infectious HIV-1 clones carrying F-ddA-associated *pol* gene mutations. We also examined the activity of F-ddA against a variety of HIV-1 clones carrying *pol* gene mutations which are associated with resistance to various therapeutic ddNs.

MATERIALS AND METHODS

Cells and viruses. MT-2 cells were grown in an RPMI-1640-based culture medium, and Cos-7 cells were grown in Dulbecco's modified Eagle medium; each of these media was supplemented with 10% fetal calf serum, 4 mM L-glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. A laboratory HIV-1 strain, HIV-1$_{LAI}$, was used as the source of wild-type infectious virions for induction of variants with reduced sensitivity to F-ddA.

Antiviral agents. AZT, ddC, and ddI were purchased from Calbiochem (La Jolla, Calif.). F-ddA, 2′-β-fluoro-2′,3′-ddATP (F-ddATP), and 2′-β-fluoro-2′,3′-dideoxyinosine (F-ddI) were prepared as previously described (18, 19). 3TC and 9-(2-phosphonylmethoxyethyl)adenine (PMEA) were kind gifts from R. F. Schinazi, Emory University, Atlanta, Ga. and M. J. M. Hitchcock, Gilead Sciences, Inc., Foster City, Calif., respectively.

Selection of resistant HIV-1$_{LAI}$ variants. HIV-1$_{LAI}$ was serially passaged in MT-2 cells in the presence of increasing concentrations of F-ddA. Briefly, MT-2 cells were infected with an inoculum of HIV-1$_{LAI}$ standardized to contain 1 RT cpm/cell as previously described (7). The culture supernatant was harvested on day 7, assayed for RT activity, and used to infect fresh MT-2 cells for the next round of culture. The concentration of F-ddA used at the first passage was 20 μM. HIV-1 was serially passaged in medium with a given concentration of F-ddA until the virus started to replicate in the presence of the drug, at which time the F-ddA concentration was increased; the selection was continued in this manner.

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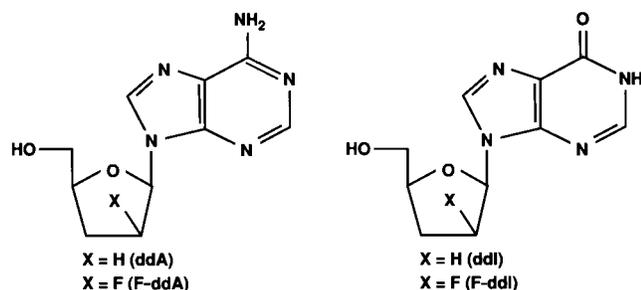
FIG. 1. Structures of F-ddA and related compounds.

Virus-containing supernatants were harvested after passage 18 and stored at −70°C until used as a source of F-ddA-resistant HIV-1 (HIV-1$_{P18}$). Concentrations were increased up to 200 μM since F-ddA was cytotoxic above that concentration.

Drug susceptibility assay. The drug sensitivities of HIV-1 clones were determined as previously described with minor modifications (25, 31). Briefly, MT-2 cells (2×10^4/ml) were pretreated with each drug, individually, for 2 h at 37°C, exposed to each virus preparation at 40 or 100 50% tissue culture infectious doses (TCID$_{50}$s), and cultured in 200-μl aliquots of culture medium containing various concentrations of the drug in 3- or 10-fold serial dilutions in 96-well culture plates. All assays were performed in triplicate or quadruplicate, and the amounts of p24 antigen produced by the cells were determined on day 7 in culture by using a commercially available radioimmunoassay kit (Du Pont NEN, Boston, Mass.). Drug concentrations which resulted in 50% inhibition (IC$_{50}$s) were determined by comparison with the p24 production level in drug-free control cell cultures as previously described (25, 31).

Determination of the nucleotide sequence of the *pol* gene. The nucleotide sequence of cDNA obtained from viral particles following reverse transcription with AS-89 (5′-AAT CTG GCT AGC CCA ATT CAA TTT-3′) or proviral DNA was determined. Briefly, the *Xma*I-*Nhe*I site was excised following PCR amplification of cDNA or proviral DNA with the primer pair AS-88 (5′-TAA AAT TAA AGC CCG GGA TGG ATG-3′) and AS-89. The 763-bp PCR product containing the *Xma*I-*Nhe*I site was inserted into a pTZ19R-based vector, pTZT, created by introducing *Xma*I and *Nhe*I restriction sites into the multiple cloning site of pTZ19R. Competent *Escherichia coli* cells (strain DH5α; GIBCO/BRL, Gaithersburg, Md.) were then transformed with the *pol*-inserted pTZT. Plasmid DNAs were purified and sequenced by the dideoxy-chain termination method with dye-labelled primers in an automated DNA sequencer (model 373; Applied Biosystems, Foster City, Calif.).

Construction of recombinant HIV-1 clones. To generate recombinant infectious HIV-1 clones with various mutations in the *pol* gene, mutant and wild-type molecular clones were constructed as previously described (31). Briefly, a desired mutation was introduced into the *Xma*I-*Nhe*I region (759 bp) of pTZNX1, which encoded Gly-15 to Ala-267 of HIV-1 RT (strain BH 10), by the oligonucleotide-based mutagenesis method (37). The *Xma*I-*Nhe*I fragment was inserted into a pHXB2RIP7 (a kind gift from M. Reitz, Jr., National Institutes of Health, Bethesda, Md.)-based plasmid, pSUM9, generating various molecular clones with the desired mutation. Additional mutations were introduced by the same method when deemed necessary. To generate an HIV-1 clone containing three mutations (P119S, V179D, and L214F), the *Xma*I-*Nhe*I fragment of pTZT containing the *pol* region (vide supra) with the three amino acid substitutions was inserted into pSUM9. Each molecular clone (20 μg as DNA) was transfected into Cos-7 cells (4×10^5/dish) by the calcium phosphate method (Promega, Madison, Wis.). After 24 h, MT-2 cells (10^6/dish) were added and cocultured with the Cos-7 cells for an additional 24 h. When an extensive cytopathic effect was observed, cell-free supernatants were harvested and the virus was further propagated in H9 cells. The culture supernatant was harvested and stored at −70°C until use. The presence of intended mutations and the absence of unintended mutations in infectious clones were confirmed by determination of the nucleotide sequence of the proviral DNA isolated from the virus-producing H9 cells. An infectious clone, HIV-1$_{HXB2D}$, was generated by using pSUM9 (31) and served as a wild-type infectious clone.

Expression of HIV-1 RTs and assays of their sensitivity to ddNTPs. Wild-type and mutant RT expression vectors were constructed as previously described (37). Briefly, an *Xma*I-*Nhe*I fragment carrying one of the mutations described above was inserted into the XmNh linker region of pKRT07. *E. coli* JM109 (Promega, Madison, Wis.) was transformed with the resulting RT expression vector and cultured for overexpression of the enzyme, which was monitored and confirmed by both sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the RT activity assay as previously described (37).

Susceptibilities of bacterially expressed wild-type and mutant RTs to various ddN 5′-triphosphates (ddNTPs) were determined as previously described (37). Briefly, the reaction mixture was prepared to contain 50 mM Tris-Cl (pH 7.8), 6 mM MgCl$_2$, 150 mM KCl, 0.01% Triton X-100, 20 μM [^{3}H]dATP, 200 μM each

TABLE 1. Selection of HIV-1$_{LAI}$ clones less susceptible to F-ddA

Passage no.	Concn of F-ddA (μM)	Duration (wk)	IC$_{50}$[a] (μM)
1	20	1	5 (1×)
2–3	60	2	
4–7	100	4	
8	120	2	
9–13	150	5	
14–17	200	5	
18	200	1	20 (4×)

[a] For determination of IC$_{50}$s, MT-2 cells (2×10^3) were exposed to 100 TCID$_{50}$s of HIV-1$_{LAI}$ or HIV-1$_{P18}$, cultured in the presence of various concentrations of F-ddA, and the amount of p24 antigen produced was measured on day 7 of culture. The numbers in parentheses indicate fold changes. The sensitivities of HIV-1$_{LAI}$ and HIV-1$_{P18}$ were examined on four independent occasions. All assays were performed in quadruplicate. The IC$_{50}$ of F-ddA for HIV-1$_{P18}$ was higher than that for HIV-1$_{LAI}$ in all four experiments (the range of the fold difference was 4 to 10; the mean fold difference [± standard deviation] was 5.9 ± 3.4).

remaining deoxynucleoside triphosphate, 0.1 μM MS2/22A (a heteropolymeric template-primer consisting of phage MS2 genomic RNA [Boehringer Mannheim, Indianapolis, Ind.] as the template and the synthetic oligonucleotide 22A (5′-CGT TAG CCA CTC CGA AGT GCG T-3′) as the primer), approximately 1 nM RT, and various concentrations of ddNTP. Reactions were initiated by raising the reaction temperature from 0 to 37°C. The reaction mixtures were incubated for 30 min at 37°C, and the reactions were quenched by the addition of 25 μl of 0.5 M EDTA. Products were then subjected to a DE81 filter binding assay as previously described (37).

RESULTS

Induction of HIV-1 variants with reduced sensitivity to F-ddA. We exposed a wild-type HIV-1 isolate, HIV-1$_{LAI}$, to increasing concentrations of F-ddA in MT-2 cells as illustrated in Table 1. Every 7 days, the culture supernatant was harvested, assayed for RT activity, and used to initiate the next round of cell-free infection of fresh MT-2 cells. The culture was initiated at a concentration of 20 μM F-ddA and ended at 200 μM. Phenotypic evidence of drug resistance (a ca. twofold increase in the IC$_{50}$) was first detected at around passage 10; resistance increased to ca. threefold by passage 14 and stabilized at approximately a fourfold increase by passage 18. When both HIV-1$_{LAI}$ and passage 18 HIV-1 (HIV-1$_{P18}$) were examined with F-ddA, the IC$_{50}$s were 5 and 20 μM, respectively (Table 1). Although this fourfold difference was rather modest compared to the difference observed in cases of AZT or 3TC resistance (15, 16), it was reproducibly seen in four independent sets of experiments (Table 1).

Amino acid substitutions associated with F-ddA exposure. We determined the nucleotide sequences of the RT-encoding

TABLE 2. Amino acid substitutions in RT of HIV-1$_{P18}$

Virus	Amino acid substituted at codon[a]:			No. of substituted clones/total clones sequenced (%)
	119 (P)	179 (V)	214 (L)	
Wild type (HIV-1$_{LAI}$)	—[b]	—	—	3/13 (23)
	S	—	—	1/13 (8)
	—	—	F	7/13 (54)
	S	—	F	2/13 (15)
HIV-1$_{P18}$	S	—	F	19/28 (68)
	S	D	F	9/28 (32)

[a] The letters in parentheses indicate the original amino acids in those positions (i.e., in HIV-1$_{HXB2D}$).
[b] —, the amino acid was the same as in HIV-1$_{HXB2D}$.

TABLE 3. Sensitivities to ddNs of infectious HIV-1 clones containing F-ddA-associated amino acid substitutions

Infectious clone	IC$_{50}$ (μM) of ddN[a]:						
	F-ddA	F-ddI	AZT	ddI	ddC	PMEA	3TC
HIV-1$_{HXB2D}$	5.0 (1×)	11 (1×)	0.013 (1×)	1.4 (1×)	0.41 (1×)	1.0 (1×)	1.2 (1×)
HIV-1$_{P119S}$	23 (4.6×)	15 (1.4×)	0.012 (0.9×)	1.3 (0.9×)	0.36 (0.9×)	1.1 (1.1×)	1.0 (0.8×)
HIV-1$_{L214F}$	4.4 (0.9×)	16 (1.5×)	0.009 (0.7×)	1.2 (0.9×)	0.48 (1.2×)	0.92 (0.9×)	1.2 (1.0×)
HIV-1$_{P119S/L214F}$	14 (2.8×)	38 (3.5×)	0.018 (1.4×)	1.5 (1.1×)	1.0 (2.4×)	1.3 (1.3×)	1.4 (1.2×)
HIV-1$_{P119S/V179D/L214F}$	20 (4.0×)	29 (2.6×)	0.015 (1.2×)	1.6 (1.1×)	0.85 (2.1×)	1.2 (1.2×)	1.7 (1.4×)

[a] MT-2 cells (2 × 10^3) were exposed to 100 TCID$_{50}$s of each infectious HIV-1 clone. The amount of p24 antigen produced was measured on day 7 of culture. The experiment was conducted at least twice, and comparable data were obtained; representative data are shown here. All experiments were conducted in triplicate. The numbers in parentheses indicate fold changes compared to HIV-1$_{HXB2D}$. The IC$_{50}$s of F-ddA for HIV-1 clones with amino acid substitutions were higher than those of HIV-1$_{HXB2D}$ in all three experiments (the values for range and mean fold difference [± standard deviation] were 2.6 to 4.6 and 3.3 ± 1.1, 0.9 to 1.6 and 1.3 ± 0.5, 2.2 to 2.8 and 2.5 ± 0.4, and 2.0 to 4.0 and 3.0 ± 1.4 for HIV-1$_{P119S}$, HIV-1$_{L214F}$, HIV-1$_{P119S/L214F}$, and HIV-1$_{P119S/V179D/L214F}$, respectively).

regions of HIV-1$_{LAI}$ and HIV-1$_{P18}$ (Table 2). Using the primer AS89, the RT-encoding region of the virus was reverse transcribed, and the cDNA obtained was subjected to PCR amplification, cloning, and nucleotide sequencing. In each of the 28 HIV-1$_{P18}$ clones examined, Ser (TCC) was substituted for Pro (CCC) at codon 119 (P119S) and Phe (TTT) was substituted for Leu (CTT) at codon 214 (L214F). In addition, in 9 of the 28 HIV-1$_{P18}$ clones sequenced, Asp (GAT) was substituted for Val (GTT) at codon 179 (V179D). It should be noted, however, that the P119S and L214F substitutions were also found in some of the HIV-1$_{LAI}$ clones (3 and 9 of 13, respectively) while the V179D substitution was not identified (Table 2).

Drug susceptibility of HIV-1 containing F-ddA-associated substitutions. In order to determine which amino acid substitutions are associated with the observed reduced sensitivity of HIV-1 to F-ddA, various recombinant infectious clones were generated. In a representative experiment, the IC$_{50}$ of F-ddA for an infectious clone carrying P119S (HIV-1$_{P119S}$) was 4.6-fold higher than that for HIV-1$_{HXB2D}$ (Table 3). However, the IC$_{50}$ of F-ddA for an infectious clone containing L214F (HIV-1$_{L214F}$) was comparable to that for HIV-1$_{HXB2D}$. Both HIV-1$_{P119S/L214F}$ and HIV-1$_{P119S/V179D/L214F}$ exhibited reduced sensitivity to F-ddA (ca. three- and ca. fourfold, respectively) compared with HIV-1$_{HXB2D}$, suggesting that neither L214F nor V179D affects the modest resistance conferred by P119S.

We also asked whether HIV-1 variants with reduced sensitivity to F-ddA were resistant to other ddNs, namely, F-ddI, AZT, ddI, ddC, PMEA, and 3TC. It was found that HIV-1$_{P119S/L214F}$ and HIV-1$_{P119S/V179D/L214F}$ were moderately resistant to F-ddI, but none of the HIV-1 clones examined exhibited discernible resistance to any other ddN (Table 3).

Activity of F-ddA against ddN-resistant HIV-1. We then asked whether F-ddA was active against HIV-1 variants with reduced susceptibilities to other therapeutic ddNs in vitro. The infectious clones examined included HIV-1$_{K65R}$ (less susceptible to ddC, ddI, and 3TC than HIV-1$_{HXB2D}$) (8, 38), HIV-1$_{L74V}$ (less susceptible to ddI and ddC) (1, 16, 33), HIV-1$_{M184V}$ (less susceptible to 3TC, ddI, and ddC) (3, 4, 12, 28, 35), and HIV-1$_{T215Y}$ (less susceptible to AZT) (15). A K65R, M184V, or T215Y substitution did not appreciably reduce sensitivity to F-ddA (less than a twofold reduction) (Table 4). HIV-1$_{L74V}$ had a moderately reduced susceptibility to F-ddA (ca. three-fold).

We also asked whether F-ddA was active against HIV-1 variants with resistance to multiple ddNs (11, 29, 31, 32). The infectious clones examined included HIV-1$_{Q151M}$, HIV-1$_{F77L/F116Y/Q151M}$, and HIV-1$_{A62V/V75I/F77L/F116Y/Q151M}$. All three variants showed reduced susceptibilities to AZT and ddI compared to those of HIV-1$_{HXB2D}$ (Table 5), in agreement with our previous report (31). In contrast, HIV-1$_{Q151M}$ was sensitive to F-ddA while the other two variants showed only moderately reduced susceptibility to F-ddA by ca. three- and ca. twofold, respectively Table 5.

Sensitivity of recombinant RTs to F-ddATP, AZT-TP, and ddATP. The sensitivities of recombinant RTs with various amino acid substitutions were also examined with three ddNTPs: F-ddATP, AZT triphosphate (AZT-TP), and ddATP (Table 5). In agreement with our previous data (37), RT with F77L, F116Y, and Q151M substitutions (RT$_{F77L/F116Y/Q151M}$) and RT$_{A62V/V75I/F77L/F116Y/Q151M}$ were significantly less sensitive to AZT-TP and ddATP than was wild-type RT. In contrast, all RTs were sensitive or only moderately less sensitive to F-ddATP (ca. two- to ca. threefold) than was wild-type RT, although all the IC$_{50}$s of F-ddATP were substantially higher than those of AZT-TP or ddATP (Table 5).

TABLE 4. Sensitivities to ddNs of infectious HIV-1 clones with single, resistance-inducing amino acid substitutions

HIV-1 clone	IC$_{50}$ (μM) for ddN[a]:					
	F-ddA	AZT	ddI	ddC	PMEA	3TC
HIV-1$_{HXB2D}$	5.3 (1×)	0.016 (1×)	1.2 (1×)	0.3 (1×)	1.0 (1×)	1.0 (1×)
HIV-1$_{K65R}$	12 (2.3×)	0.016 (1.0×)	7.9 (6.6×)	1.3 (4.3×)	0.4 (0.4×)	8.1 (8.1×)
HIV-1$_{L74V}$	14 (2.6×)	0.015 (1.0×)	3.2 (2.7×)	1.0 (3.3×)	1.3 (1.3×)	3.0 (3.0×)
HIV-1$_{M184V}$	4.8 (0.9×)	0.009 (0.6×)	2.3 (1.9×)	1.1 (3.7×)	1.1 (1.1×)	>1,000 (>1,000×)
HIV-1$_{T215Y}$	5.0 (0.9×)	0.09 (5.6×)	2.2 (1.8×)	ND[b]	1.8 (1.8×)	ND

[a] MT-2 cells (2 × 10^3) were exposed to 100 TCID$_{50}$s of each infectious clone. The amount of p24 antigen produced was measured on day 7 of culture. The experiment was conducted at least twice, and comparable data were obtained: one set of such data is shown here. All assays were conducted in triplicate. The numbers in parentheses represent fold changes compared to HIV-1$_{HXB2D}$. The IC$_{50}$s of F-ddA for HIV-1 clones with amino acid substitutions were higher than those for HIV-1$_{HXB2D}$ in these two experiments (the values for mean fold difference ± standard deviation were 2.3 ± 0.1, 2.3 ± 0.5, 1.4 ± 0.7, and 1.3 ± 0.5 for HIV-1$_{K65R}$, HIV-1$_{L74V}$, HIV-1$_{M184V}$, and HIV-1$_{T215Y}$, respectively).

[b] ND, not determined.

TABLE 5. Sensitivity of multidrug-resistant HIV-1 to F-ddA and the effect of F-ddATP on recombinant MDR RT

	IC$_{50}$ of F-ddA (μM) vs[a]:						
Amino acid substitution(s)	HIV-1 clone				RT[b]		
	F-ddA	AZT	ddI	PMEA	F-ddATP	AZT-TP[c]	ddATP[c]
None (wild type)	5.8 (1×)	0.013 (1×)	1.5 (1×)	1.4 (1×)	76 (1×)	0.023 (1×)	4.3 (1×)
Q151M	5.0 (0.9×)	0.17 (13.1×)	2.7 (1.8×)	ND[d]	115 (1.5×)	0.19 (8.3×)	11 (2.6×)
F77L/F116Y/Q151M	18 (3.1×)	0.71 (54.6×)	11 (7.3×)	1.9 (1.4×)	178 (2.3×)	0.88 (38.3×)	37 (8.6×)
A62V/V75I/F77L/F116Y/Q151M	14 (2.4×)	2.1 (162×)	20 (13.3×)	1.4 (1.0×)	204 (2.7×)	1.5 (65.2×)	38 (8.8×)

[a] MT-2 cells (2 × 10^3) were exposed to 40 TCID$_{50}$s of each infectious clone. The amount of p24 antigen produced was measured on day 7. The experiment was conducted at least twice, and comparable data were obtained; one set of such data is shown here. The numbers in parentheses represent fold changes. The IC$_{50}$s of F-ddA for HIV-1$_{F77L/F116Y/Q151M}$ and HIV-1$_{A62V/V75I/F77L/F116Y/Q151M}$ were higher than that for HIV-1$_{HXB2D}$ in three experiments. The range of fold difference values and the mean fold difference (± standard deviation) were 3.1 to 5.0 and 3.9 ± 1.0 and 1.3 to 2.4 and 2.0 ± 0.6, respectively.

[b] Recombinant RTs were not tested against PMEA diphosphate in this study.

[c] Data (from reference 36) shown as a reference.

[d] ND, not determined.

DISCUSSION

In the present work, we examined how HIV-1 develops resistance to the novel ddN analog F-ddA. Wild-type HIV-1 (HIV-1$_{LAI}$) was passaged in the presence of increasing concentrations of F-ddA up to 200 μM. When the sensitivity of HIV-1 to F-ddA at passage 18 (HIV-1$_{P18}$) was examined in comparison to that of HIV-1$_{LAI}$, a reproducible fourfold decrease in sensitivity was observed (Table 1). This suggests that as seen with ddI or ddC, HIV-1 develops only a modest degree of resistance to F-ddA. We have previously shown that HIV-1 develops resistance to AZT in vivo more readily and to a greater degree than to ddI and ddC (10, 32). Reichman et al. have also reported that the difference in ddI sensitivity seen in HIV-1 isolates from patients receiving ddI therapy is minimal and of unknown clinical significance (24). In this respect, the close structural resemblance of ddATP and ddCTP (the active moieties of ddI and ddC, respectively) to their corresponding natural nucleotides (dATP and dCTP) may make it difficult for the virus to mutate to a form which efficiently excludes ddATP or ddCTP while still preserving its RT function. Indeed, molar refraction data indicate that the 3′-azido group of AZT is about three times larger than the corresponding hydroxyl group of 2′-deoxyinosine or 2′-deoxycytidine (13). It is possible that the structure of F-ddATP is close to that of dATP and that HIV-1 RT cannot easily discriminate F-ddATP from dATP. It should be stressed, however, that the relationship between the structural resemblance of nucleosides and the development of drug resistance remains hypothetical, requiring further studies.

Sequence analyses of HIV-1$_{P18}$ revealed that all 28 HIV-1 clones examined had amino acid substitutions at codons 119 and 214 within the RT-encoding region. Nine of 28 clones (32%) also proved to contain a V179D substitution. It should be noted, however, that P119S and L214F substitutions were found in 3 and 9 of 13 clones of HIV-1$_{LAI}$ examined, respectively, while none had the V179D substitution. It also should be noted that the L214F amino acid substitution is recognized as a natural polymorphism in the *pol* gene of HIV-1 (20). When the sensitivities to F-ddA of various infectious clones carrying these amino acid substitutions were examined, the IC$_{50}$ for HIV-1$_{L214F}$ was comparable to that for HIV-1$_{HXB2D}$; however, all infectious clones with the P119S substitution showed reduced sensitivity to F-ddA, although HIV-1$_{P119S}$ was still sensitive to F-ddI (Table 3). The introduction of V179D into HIV-1$_{P119S/L214F}$ did not affect its sensitivity to F-ddA. These results suggest that the P119S substitution was directly responsible for the observed reduced sensitivity of HIV-1 to F-ddA. It is possible, however, that the V179D and/or L214F substitution

was associated with a compensation for a reduction in viral replication brought about by the P119S substitution. It is noteworthy that although a small proportion (3 of 13) of wild-type clones contained the P119S mutation, phenotypic resistance to F-ddA did not occur in cultures until around passage 15. In parallel experiments, a three- to fourfold increase in resistance to AZT occurred by passage 9 (data not shown). Thus, drug resistance to F-ddA appears to arise at a rate not faster than those previously reported for other ddN inhibitors, at least in vitro.

In the present work, F-ddA was found to be active against all infectious clones carrying various amino acid substitutions conferring ddN-specific reduced susceptibility (IC$_{50}$s differed by less than ca. threefold from those for HIV-1$_{HXB2D}$) (Table 4). F-ddA was also active against infectious HIV-1 clones with multidrug resistance. Two multi-ddN-resistant (MDR) infectious HIV-1 clones, HIV-1$_{F77L/F116Y/Q151M}$ and HIV-1$_{A62V/V75I/F77L/F116Y/Q151M}$, showed greatly reduced susceptibilities to AZT (55- and 162-fold) and ddI (7- and 13-fold), respectively. In contrast, these two clones were only moderately less sensitive to F-ddA (ca. three- and ca. twofold, respectively). Enzymatic analyses of the corresponding recombinant RTs (Table 5) corroborated these drug sensitivity data. It should be noted that PMEA was also active against various drug-resistant HIV-1 variants and the MDR HIV-1 clones. These properties of F-ddA and PMEA may prove to be useful in the treatment of patients with such drug-resistant HIV-1 variants.

F-ddA appears to have several advantages over ddI. Unlike the latter agent, it is resistant to acid hydrolysis (18, 19), greatly simplifying its oral administration and nullifying the need for coadministration of buffers and antacids. It has a high level of oral bioavailability (80% in beagle dogs) (34), and its active moiety, F-ddATP, has a long intracellular half-life (20 h) (9). It is not susceptible to hydrolysis by purine nucleoside phosphorylase, making more of the drug available for activation to its active intracellular metabolite, F-ddATP (19). It should be noted, however, that the IC$_{50}$s of F-ddA and F-ddATP for HIV-1 and RTs, respectively, were substantially higher than those of AZT or ddI (Tables 3 to 5). Specifically, the anti-HIV-1 potency of F-ddA (the mean IC$_{50}$ of F-ddA for HIV-1 is 5.8 μM) was 446-fold lower than that of AZT (Table 5), in agreement with our previous data generated using phytohemagglutinin-treated peripheral blood mononuclear cells (PHA-PBM) (5). In addition, the K_i value of F-ddATP with dATP against wild-type HIV-1 RT was 2.6 μM, ~24-fold higher than that of AZT-TP (36a). These data suggest that F-ddA is less potent than AZT against HIV-1, at least in certain in vitro

assays. In contrast, Ruxrungtham et al. recently showed that F-ddA was superior to AZT in decreasing the infectivity rate of hu-PBL-SCID mice exposed to HIV-1 (27). In this regard, the F-ddATP/dATP ratios in resting PBM and PHA-PBM, which are 13.5 and 1.6, respectively, are much higher than their AZT-TP/dTTP ratios, which are 0.04 and 0.61, respectively. These data suggest that F-ddA is more potent against HIV-1 in resting cells than in activated, dividing cells (5, 30). In hu-PBL-SCID mice, all hu-PBL are activated immune cells, as reported by Rizza et al. (26), although it is possible that they are not fully activated compared to PHA-PBM. If this is the case, one could postulate that F-ddA has the potential to be very effective against HIV-1 in humans in which the majority of lymphocytes are unstimulated and in the resting state (the percentages of CD4$^+$ cells in the proliferative phase (S to G$_2$/M) of the cell cycle are reported to be 12 and 5% in HIV-1-positive patients and healthy controls, respectively) (17). The addition of the present data suggesting that HIV-1 develops only a modest level of resistance to F-ddA in vitro and that the drug maintains its activity against virtually all known HIV-1 variants less susceptible to the currently available therapeutic ddNs further encourages the clinical investigation of F-ddA as a potential antiviral agent for use in treatment of HIV-1 infection.

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