In Vitro Selection and Analysis of Human Immunodeficiency Virus Type 1 Resistant to Derivatives of β -2',3'-Didehydro-2',3'-Dideoxy-5-Fluorocytidine

Jennifer L. Hammond,¹ Urvi M. Parikh,¹ Dianna L. Koontz,¹ Susan Schlueter-Wirtz,²
Chung K. Chu,³ Hengameh Z. Bazmi,¹ Raymond F. Schinazi,²
and John W. Mellors^{1*}

Department of Medicine, Division of Infectious Diseases, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261¹; Department of Pediatrics, Emory University School of Medicine, and the Georgia VA Research Center on AIDS and HIV Infection, Veterans Affairs Medical Center, Decatur, Georgia 30033²; and College of Pharmacy, The University of Georgia, Athens, Georgia 30602³

Received 19 January 2005/Returned for modification 10 March 2005/Accepted 15 June 2005

Serial passage of human immunodeficiency virus type 1 in MT-2 cells in increasing concentrations of the D- and L-enantiomers of β -2',3'-didehydro-2',3'-dideoxy-5-fluorocytidine (d4FC) resulted in the selection of viral variants with reverse transcriptase substitutions M184I or M184V for L-d4FC and I63L, K65R, K70N, K70E, or R172K for D-d4FC. Phenotypic analysis of site-directed mutants defined the role of these mutations in reducing susceptibility to L- or D-d4FC.

Derivatives of β -2',3'-didehydro-2',3'-dideoxy-5-fluorocytidine (d4FC) (Fig. 1) are potent and selective inhibitors of both wild-type and nucleoside reverse transcriptase inhibitor-resistant human immunodeficiency virus type 1 (HIV-1) in clinical development (2, 6, 10, 12, 15). A phase I-II study with HIV-infected patients having virus with the M184V resistance mutation showed that the substitution of L-d4FC (elvucitabine or ACH-126,443) for lamivudine produced a 0.67 to 0.78 log₁₀ decline in plasma HIV RNA (copies/ml) over a 28-day period, although dose-dependent hematological toxicity was evident (L. M. Dunkle, J. C. Gathe, D. E. Pedevillano, H. G. Robison, W. G. Rice, J. C. Pottage, Jr., and the ACH-006 Study Team, Abstr. XII Int. HIV Drug Resist. Workshop: Basic Princ. Clin. Implic., abstr. 2, 2003). A phase I-II study in which D-d4FC was given once daily at 50 mg, 100 mg, or 200 mg for 10 days to 30 antiretroviral naïve, HIV-infected subjects showed marked reductions in plasma HIV-1 RNA averaging 1.7 log₁₀ copies/ml without discernible toxicity (R. L. Murphy, D. Schürmann, A. Beard, L. Cartee, R. F. Schinazi, and M. J. Otto, Abstr. 11th Conf. Retrovir. Opport. Infect., abstr. 137, 2004). The potency and favorable safety profile of D-d4FC have led to its accelerated clinical development. Because of the interest in both enantiomers of d4FC, the goal of the current study was to evaluate the influence of stereochemistry on resistance to d4FC resistance by performing in vitro selections.

Selection experiments were conducted as previously described (1). A total of 10⁶ MT-2 cells (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health) were pretreated for 2 h with L-d4FC or D-d4FC (synthesized by R. Schinazi) (12) and then infected with HIV-1_{LAI} at a multiplicity

of infection of 0.1 50% tissue culture infective doses/cell. A 100- μ l aliquot was used to initiate a new cycle of infection and the selective pressure (i.e., drug concentration) was generally doubled every two to three passages. The passaged virus was regularly monitored for a reduction in susceptibility to the compounds by determining the 50% inhibitory concentration (IC₅₀) of the passaged virus relative to that of the unpassaged HIV-1_{LAI} (7). Student's *t* test was used to determine the statistical significance of differences between log-transformed IC₅₀ values.

Amino acid changes in passaged virus were identified by extracting RNA (TRIZOL reagent; Gibco BRL, Grand Island, NY) from pelleted virions (25,000 $\times$ g for 1 h at 4°C). The near full-length coding region of reverse transcriptase (RT) (amino acids [aa] 1 to 511) was amplified by RT-PCR (1), purified (Wizard PCR purification system; Promega, Madison, WI), and sequenced (aa 1 to 350) by using an ABI 377 sequencer (Applied Biosystems, Foster City, CA). To confirm the role of these changes, recombinant mutants were made by using oligonucleotide-directed mutagenesis (Altered Sites II; Promega) or by cassette cloning of the XmaI-to-XbaI RT fragment from passaged virus into the pxxHIV-1_{LAI} vector (14). Infectious recombinant HIV-1 was generated as previously described (14).

In two independent experiments, virus resistant to L-d4FC was isolated after 4 (selection no. 1) or 10 (selection no. 2) passages. Both selections were initiated at an L-d4FC concentration of 0.5 μ M (~5 times the IC₅₀ of the wild type). The selective pressure could not be increased above 1.1 μ M because of cytotoxicity for MT-2 cells. Phenotypic analyses of viruses from passages 4 and 10 showed >20-fold (selection no. 1) and >11-fold (selection no. 2) levels of resistance to L-d4FC, respectively; and genotype analyses of these viruses identified mutations at codon 184 (M to I or V) (Table 1). A recombinant HIV-1 encoding the M184V mutation showed >46-fold resistance to L-d4FC. The M184V mutation was also selected in

* Corresponding author. Mailing address: 818 Scaife Hall, University of Pittsburgh School of Medicine, 3550 Terrace Street, Pittsburgh, PA 15261. Phone: (412) 624-8512. Fax: (412) 383-7982. E-mail: mellors@dom.pitt.edu.

β -L-2',3'-didehydro-2',3'-dideoxy-5-fluorocytidine (L-d4FC)

β -D-2',3'-didehydro-2',3'-dideoxy-5-fluorocytidine (D-d4FC)

FIG. 1. Structures of 2',3'-didehydro-2',3'-dideoxy-5-fluorocytidine derivatives.

HIV-1_{LAI} passaged with L-d4FC in human peripheral blood mononuclear cells (13).

Viruses resistant to D-d4FC were isolated in three independent passage experiments. The first selection was initiated at a concentration of 0.75 μ M (~6 times the IC₅₀ of D-d4FC) and increased to a final concentration of 4.0 μ M over the course of 37 passages. Phenotypic analysis of the virus from passage 37 showed a 14-fold decrease in susceptibility to D-d4FC (Table 1). Genotype analysis of virus from this passage identified mutations at codons 70 (K to N), 90 (V to I), and 172 (R to K). All three mutations are novel and, with the exception of R172K, are in highly conserved regions of HIV-1 RT. Site-directed mutants encoding K70N or V90I/R172K mutations showed a 2.1-fold reduction in susceptibility. Both the site-directed mutant encoding K70N/V90I/R172K mutations and the p37 RT recombinant virus also showed significant reduc-

TABLE 1. Selection of HIV-1 resistant to D- and L-d4FC

Compound	Selection attempt	Range of drug concn (μ M)	Virus passage	Resistance (<i>n</i> -fold)	Codon change ^b	aa change ^b
L-d4FC	1	0.5–1.1	4	>20	ATG→ATA ATG→GTG	M184I/V
	2	0.5–1.1	10	>11	ATG→ATA	M184I
D-d4FC	1	0.75–4.0	37	14	AAA→AAT GTT→ATT AGA→AAA	K70N V90I R172K
	2	0.2–4.8	20	8.1	AAA→AGA GTT→GAT	K65R V179D
	3	0.2–1.6	12 16	13 16	ATA→TTA AAA→GAA	I63L K70E

^a Resistance relative to wild-type virus (HIV-1_{LAI} passage 0).

^b Amino acid change relative to wild-type virus (HIV-1_{LAI} passage 0).

tions in susceptibility to D-d4FC, 3.9- and 2.4-fold, respectively (Table 2).

The second and third selections for D-d4FC-resistant virus were initiated at a lower concentration, 0.20 μ M (~1.5 times the IC₅₀). The selective pressure was increased to a concentration of 4.8 μ M over the course of 20 passages (selection no. 2) and to 1.6 μ M over the course of 16 passages (selection no. 3). Passage 20 virus from selection no. 2 showed an 8.1-fold decrease in susceptibility to D-d4FC, and genotypic analysis identified mutations at codons 65 (K to R) and 179 (V to D) in RT (Table 1). In site-directed mutants, the K65R mutation alone decreased D-d4FC susceptibility 6.2-fold; the addition of the V179D mutation did not increase the level of resistance (5.5-fold) (Table 2), nor did it affect the replicative fitness of virus with the K65R mutation as determined in competition experiments in MT-2 cells (not shown). These results are consistent with findings from Geleziunas et al., who recently reported that K65R is the sole mutation after in vitro selection with D-d4FC (2).

In the third selection, virus with the I63L mutation was identified at passage 12 (13-fold resistance) and further selec-

TABLE 2. D-d4FC Susceptibility of recombinant viruses encoding mutations selected by serial passage in D-d4FC

HIV-1 variant ^a	D-d4FC IC ₅₀ ^b (μ M)	Resistance (<i>n</i> -fold) ^c	<i>P</i> value ^d
Wild type	0.19 $\pm$ 0.13		
K70N	0.40 $\pm$ 0.28	2.1	<0.05
V90I/R172K	0.39 $\pm$ 0.26	2.1	<0.05
K70N/V90I/R172K	0.72 $\pm$ 0.63	3.9	<0.005
xp37 ^e	0.45 $\pm$ 0.17	2.4	<0.005
K65R	1.15 $\pm$ 0.43	6.2	<0.0005
K65R/V179D	1.03 $\pm$ 0.28	5.5	<0.0005
I63L	0.27 $\pm$ 0.13	1.5	0.25
K70E	0.49 $\pm$ 0.22	2.6	<0.05

^a HIV-1_{LAI} encoding the indicated resistance mutations.

^b IC₅₀ (means $\pm$ standard deviations from at least three independent experiments) determined by inhibition of p24 antigen production in MT-2 cells.

^c Resistance relative to wild-type virus.

^d For the comparison of log-transformed mean IC₅₀s by using Student's *t* test.

^e Mutant derived by cassette cloning of the RT gene from D-d4FC p37 virus into the xHIV_{LAI} vector (see Materials and Methods).

tion resulted in virus having greater resistance (16-fold) and the K70E mutation (Table 1). A site-directed mutant with the I63L mutation was not significantly resistant to D-d4FC (1.5-fold; $P = 0.25$), but the K70E mutation significantly decreased susceptibility to D-d4FC by 2.6-fold (Table 2). We were unable to select D-d4FC-resistant viruses in human peripheral blood mononuclear cells after more than 20 passages (data not shown).

The site-directed mutant encoding the M184V substitution showed no reduction in susceptibility to D-d4FC (0.9-fold) (data not shown). Conversely, the K65R mutant showed 41-fold resistance to L-d4FC (not shown).

Selection of the M184I/V mutation by L-d4FC is consistent with previous studies of lamivudine. The crystal structure of HIV-1 RT with a bound deoxynucleotide triphosphate predicts that replacing M184 with a β -branched amino acid, such as I or V, will create a "steric clash" between residue 184 and the pseudosugar ring of nucleoside analogs in the L configuration, including L-d4FC (3, 11).

D-d4FC selected several different mutation patterns: (i) a combination of three novel mutations (K70N, V90I, and R172K); (ii) a mutation known to confer broad nucleoside reverse transcriptase inhibitor resistance (K65R) (1, 9); and (iii) two other mutations in the fingers subdomain of RT (I63L and K70E). Although the contribution of the I63L mutation to D-d4FC resistance is uncertain, the K70E mutation, which replaced the I63L mutation at higher drug concentrations, is located near the incoming deoxynucleotide triphosphate and could affect the position of the bound analog triphosphate (3). It is likely that the mutation confers some level of discrimination against incorporation of D-d4FC triphosphate, although the specific molecular interactions that underlie this are not yet defined.

The selection of several different genotypes by D-d4FC indicates that there is more than one genetic route to D-d4FC resistance. It is notable, however, that each genotype conferred only low-level resistance (≤ 6.2 -fold) to D-d4FC in site-directed mutants. The reason different genotypes were selected may have been related to the variation in starting drug concentration (4, 5) or the stochastic nature of in vitro resistance selection (i.e., chance). Although our results suggest multiple genetic mechanisms of viral escape from D-d4FC, clinical studies are needed to determine which, if any, of these occur in vivo.

Our finding of multiple mutations in the fingers subdomain of RT suggests that resistance to D-d4FC is due to discrimination rather than excision. This concept is supported by the observation that D-d4FC retains potency against virus containing the zidovudine resistance mutations D67N, K70R, T215Y, and K219Q, which confer excision competence (8, 12). Thus, D-d4FC may be a potent inhibitor for patients with virus having zidovudine resistance mutations, although clinical studies are needed to confirm this.

In summary, the results of this study should prove useful in directing resistance testing of viruses from patients treated with L- or D-d4FC and in choosing combinations of nucleoside analogs for treatment and prevention of drug-resistant HIV-1.

This work was supported by grants from the National Cancer Institute (SAIC contract 20XS190A) and from the National Institute of Allergy and Infectious Diseases, Division of AIDS (AACTG Central Group grant U01AI38858). R.F.S. is supported by National Institutes of Health grant R37-AI-41980 and the Department of Veterans Affairs. R.F.S. is the principal founder and shareholder in Pharmasset, Inc., and will receive future royalties from sales of D-d4FC (Reverset). J.W.M. is a consultant to and holds share options in Pharmasset, Inc.

REFERENCES

- Bazmi, H. Z., J. L. Hammond, S. C. Calvacanti, C. K. Chu, R. F. Schinazi, and J. W. Mellors. 2000. In vitro selection of mutations in HIV-1 reverse transcriptase that decrease susceptibility to $(-)\beta$ -D-dioxolane-guanosine and suppress resistance to 3'-azido-3'-deoxythymidine. *Antimicrob. Agents Chemother.* **44**:1783-1788.
- Geleziunas, R., K. Gallagher, H. Zhang, L. Bachelier, S. Garber, J.-T. Wu, G. Shi, M. J. Otto, R. F. Schinazi, and S. Erickson-Viitanen. 2003. HIV-1 resistance profile of the novel nucleoside reverse transcriptase inhibitor β -D-2,3-dideoxy-2,3-didehydro-5-fluorocytidine (Reverset[™]). *Antivir. Chem. Chemother.* **14**:49-59.
- Huang, H., R. Chopra, G. L. Verdine, and S. C. Harrison. 1998. Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. *Science* **282**:1669-1674.
- Kinjerski, T. L., and R. W. Buckheit, Jr. 1997. The role of genotypic heterogeneity in wild type virus populations on the selection of non-nucleoside reverse transcriptase inhibitor resistant viruses. *Antivir. Res.* **33**:109-115.
- Kleim, J. P., I. Winkler, M. Rosner, R. Kirsch, H. Rubsamen-Waigmann, A. Paessens, and G. Riess. 1997. In vitro selection for different mutational patterns in the HIV-1 reverse transcriptase using high and low selective pressure of the nonnucleoside reverse transcriptase inhibitor HBY 097. *Virology* **231**:112-118.
- Lin, T. S., M.-Z. Luo, C. C. Liu, Y.-L. Zhu, E. Gullen, G. E. Dutshman, and Y.-C. Cheng. 1996. Design and synthesis of 2',3'-dideoxy-2',3'-didehydro- β -L-cytidine (β -L-d4C) and 2',3'-dideoxy-2',3'-didehydro- β -L-5-fluorocytidine (β -L-Fd4C), two exceptionally potent inhibitors of human hepatitis B virus (HBV) and potent inhibitors of human immunodeficiency virus (HIV) in vitro. *J. Med. Chem.* **39**:1757-1759.
- Mellors, J. W., H. Z. Bazmi, R. F. Schinazi, B. M. Roy, Y. Hsiou, E. Arnold, J. Wir, and D. L. Mayers. 1995. Novel mutations in reverse transcriptase of human immunodeficiency virus type 1 reduce susceptibility to foscarnet in laboratory and clinical isolates. *Antimicrob. Agents Chemother.* **39**:1087-1092.
- Meyer, P. R., S. E. Matsuura, A. A. Tolun, I. Pfeifer, A. G. So, J. W. Mellors, and W. A. Scott. 2002. Effects of specific zidovudine resistance mutations and substrate structure on nucleotide-dependent primer unblocking by human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob. Agents Chemother.* **46**:1540-1545.
- Parikh, U. M., D. L. Koontz, C. K. Chu, R. F. Schinazi, and J. W. Mellors. 2005. In vitro activity of structurally diverse nucleoside analogs against human immunodeficiency virus type 1 with the K65R mutation in reverse transcriptase. *Antimicrob. Agents Chemother.* **49**:1139-1144.
- Ray, A. S., R. F. Schinazi, E. Murakami, A. Basavapathruni, J. Shi, S. M. Zorca, C. K. Chu, and K. S. Anderson. 2003. Probing the mechanistic consequences of 5-fluorine substitution on cytidine nucleotide analogue incorporation by HIV-1 reverse transcriptase. *Antivir. Chem. Chemother.* **14**:115-125.
- Sarafianos, S. G., K. Das, A. D. Clark, Jr., J. Ding, P. L. Boyer, S. H. Hughes, and E. Arnold. 1999. Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance with β -branched amino acids. *Proc. Natl. Acad. Sci. USA* **96**:10027-10032.
- Schinazi, R. F., J. Mellors, H. Bazmi, S. Diamond, S. Garber, K. Gallagher, R. Geleziunas, R. Klabbe, M. Pierce, M. Rayner, J.-T. Wu, H. Zhang, J. Hammond, L. Bachelier, D. J. Manion, M. J. Otto, L. Stuyver, G. Trainor, D. C. Liotta, and S. Erickson-Viitanen. 2002. DPC 817: a cytidine nucleoside analog with activity against zidovudine- and lamivudine-resistant viral variants. *Antimicrob. Agents Chemother.* **46**:1394-1401.
- Schinazi, R. F., S. Schlueter-Wirtz, and L. Stuyver. 2001. Early detection of mixed mutations selected by antiretroviral agents in HIV-infected primary human lymphocytes. *Antivir. Chem. Chemother.* **12**(Suppl. 1):61-65.
- Shi, C., and J. W. Mellors. 1997. A recombinant retroviral system for rapid in vivo analysis of human immunodeficiency virus type 1 susceptibility to reverse transcriptase inhibitors. *Antimicrob. Agents Chemother.* **41**:2781-2785.
- Shi, J., J. J. McAtee, S. S. Wirtz, P. Tharnish, A. Juodawlkis, D. C. Liotta, and R. F. Schinazi. 1999. Synthesis and biological evaluation of 2',3'-dideoxy-2',3'-dideoxy-5-fluorocytidine (D4FC) analogues: discovery of carbocyclic nucleoside triphosphates with potent inhibitory activity against HIV-1 reverse transcriptase. *J. Med. Chem.* **42**:859-867.