

In Vitro Selection of the T215Y Mutation by Stavudine (d4T) in Viruses Carrying 215D/C From Drug-Naïve Persons



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ABSTRACT

Background: The T215Y mutation has long been associated with resistance to AZT and, more recently, has been seen in patients failing d4T therapy. We have previously identified a distinct group of viruses that have a unique set of mutations at codon 215 among newly diagnosed persons. These mutations include 215D and 215C and do not confer resistance to AZT or d4T. However, HIV-1_{215D/C} have higher ability than WT viruses to select 215Y in vitro in the presence of AZT. In the present study, we evaluated the capacity of these viruses to acquire 215Y in the presence of d4T.

Methods: Nine recombinant viruses were studied: 6 had cloned RT sequences containing 215C or D, and 3 had control WT sequences. All viruses were cultured in the presence of increasing concentrations of d4T. Mutations at codon 215 and other positions of the RT were monitored by sequence analysis after each passage. Fold-changes in IC₅₀ values for d4T-TP and d4T were determined by an RT assay and by a replication-based assay (MT4/MTT).

Results: Of the 6 recombinant viruses carrying 215D or C, 4 acquired 215Y after a mean of 57 days (range=32-89) in culture. In contrast, the 215Y mutation was not seen in the 3 control WT viruses. Interestingly, 7 of 9 viruses acquired the K65R mutation. Among these, 4 had K65R only, 2 had K65R and 215Y, and 1 had K65R and V75A (HXB2). Reduced susceptibility to d4T-TP was seen by an RT-based assay in 7 isolates carrying either 215Y, K65R/215Y, or K65R/V75A, with a mean increase in IC₅₀ of 3.3-fold (range=1.2-6.8). However, resistance to d4T in the MT4/MTT assay was low or undetectable, with a mean increase in IC₅₀ of 1.4-fold (range=0.9-2.4), likely reflecting the inability of this assay to detect d4T resistance in these mutants.

Conclusions: Our results document for the first time the selection of 215Y by d4T in vitro. These findings suggest that 215Y confers a selective advantage in the presence of d4T, and may explain the selection of 215Y by d4T observed in vivo. The observed selection of K65R suggests that mutations at codons other than 215 and 75 may also contribute to d4T resistance. Our findings suggest that patients infected with HIV-1_{215D/C} and treated with antiretroviral regimens containing d4T may be at increased risk for development of 215Y.

INTRODUCTION

Through surveillance of drug-resistant HIV-1 in 603 treatment-naïve, recently diagnosed HIV-1-infected persons, we recently identified a distinct group of viruses that have mutations at codon 215 of the reverse transcriptase (RT) that are different from either the wild type (WT) T or the zidovudine (AZT)-selected T215Y/F (García-Lerma *et al.*, *PNAS* 2001;98:13907-13912).

These mutations are mainly 215D, 215C and 215S and were found in 20 patients (3.3%). They all differ from 215Y by 1-nt change and likely represent revertants of 215Y.

The 215D, 215C, and 215S mutations do not affect susceptibility to AZT, d4T or other nucleoside analogs.

However, viruses having 215D or 215C have increased ability for selecting the 215Y mutation and become AZT-resistant in vitro, thus raising concerns about the potential of these viruses to compromise the efficacy of antiretroviral therapy containing AZT.

OBJECTIVES

To evaluate the capacity of HIV-1_{215D} and HIV-1_{215C} to acquire 215Y in the presence of d4T.

To characterize genotypic changes in these viruses following in vitro selection of d4T resistance.

To better understand the basis of phenotypic resistance to d4T.

METHODS

VIRUSES

Seven recombinant viruses generated using plasma HIV-1 RT sequences from treatment-naïve, recently diagnosed HIV-1-infected patients were used. Recombinants were obtained in MT-4 cells using cloned RT sequences and the RT-deleted HXB2-based proviral molecular clone pHIV/RTBstEII.

Recombinant Virus	Mutations	CCID50/ml
RD 01	215C	25,281
RD 02	210W, 215C	32,340
RD 03	41L, 210W, 215C	12,383
RD 04	210W, 215D	197,461
RD 05	41L, 215D	15,625
RD 22	-	78,125
RD 23	-	78,736

The following recombinant viruses were generated using the RT of HXB2: HXB2_{wt}, HXB2_{215D}, HXB2_{K65R}, HXB2_{K65R/T215Y}, and HXB2_{T215Y}. The T215D, K65R, and T215Y mutations were introduced in the pHXB2RIP7-based infectious clone pSUM9 by site-directed mutagenesis. The K65R mutation was also introduced in RD 22wt to generate RD22_{K65R}.

DRUGS

d4T and AZT were obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH. The purity of the preparation of d4T was further confirmed by LC/MS/MS analysis. d4T-triphosphate (d4T-TP) was kindly provided by Anne-Mieke Vandamme, Rega Institute, Luven, Belgium.

IN VITRO SELECTION OF DRUG RESISTANCE

Inocula of 1.5 x 10⁶ MT-4 cells were exposed to 1,500 CCID₅₀ (MOI = 0.001) of each virus for 2 h at 37°C. After two washes with PBS, cells were resuspended in complete medium containing d4T at a concentration close to the IC₅₀ value for each virus (0.7). Cultures were then incubated at 37°C, and media containing d4T was changed every 3-4 days as required. Virus production was monitored by microscopic assessment of syncytium formation through all the culture. Once virus production was evident at a given concentration of drug, 500 ul of clarified supernatant was added to 1.5 x 10⁶ fresh cells and cultured in the presence of a higher concentration of drug (two-fold) or at the same concentration if the virus titer was low.

SEQUENCE ANALYSIS OF HIV-1 RT

Full-length HIV-1 RT (amino acids 7-546) was amplified by RT-nested PCR and sequenced using primers AV36, AV44, A35, NE(1)35, AV180, and AV181.

PHENOTYPIC TESTING

MT-4/MTT assay. MT-4 cells were exposed to 200 CCID₅₀ of each virus in the absence or in the presence of d4T. After 5 days in culture, the viability of cells was examined by the MTT assay. The concentration of d4T required to inhibit 50% virus-induced cell killing [50% effective concentration (EC₅₀)] was then calculated.

RT-based drug susceptibility assay. Susceptibility of RTs to d4T-TP was determined enzymatically by measuring IC₅₀ values using the Amp-RT assay. Amp-RT measures the ability of HIV-1 RT to produce a cDNA copy of an exogenous RNA template. The RT-generated cDNA is amplified by PCR amplification and quantitated by an ELISA-based, non-radioactive oligoprobing system. For analysis of RT susceptibility to d4T-TP, duplicate Amp-RT reactions were done in the absence and presence of several concentrations of d4T-TP. The concentration of dTTP in the RT step of Amp-RT was 15µM. The other three dNTPs were used at 20µM each.

RESULTS

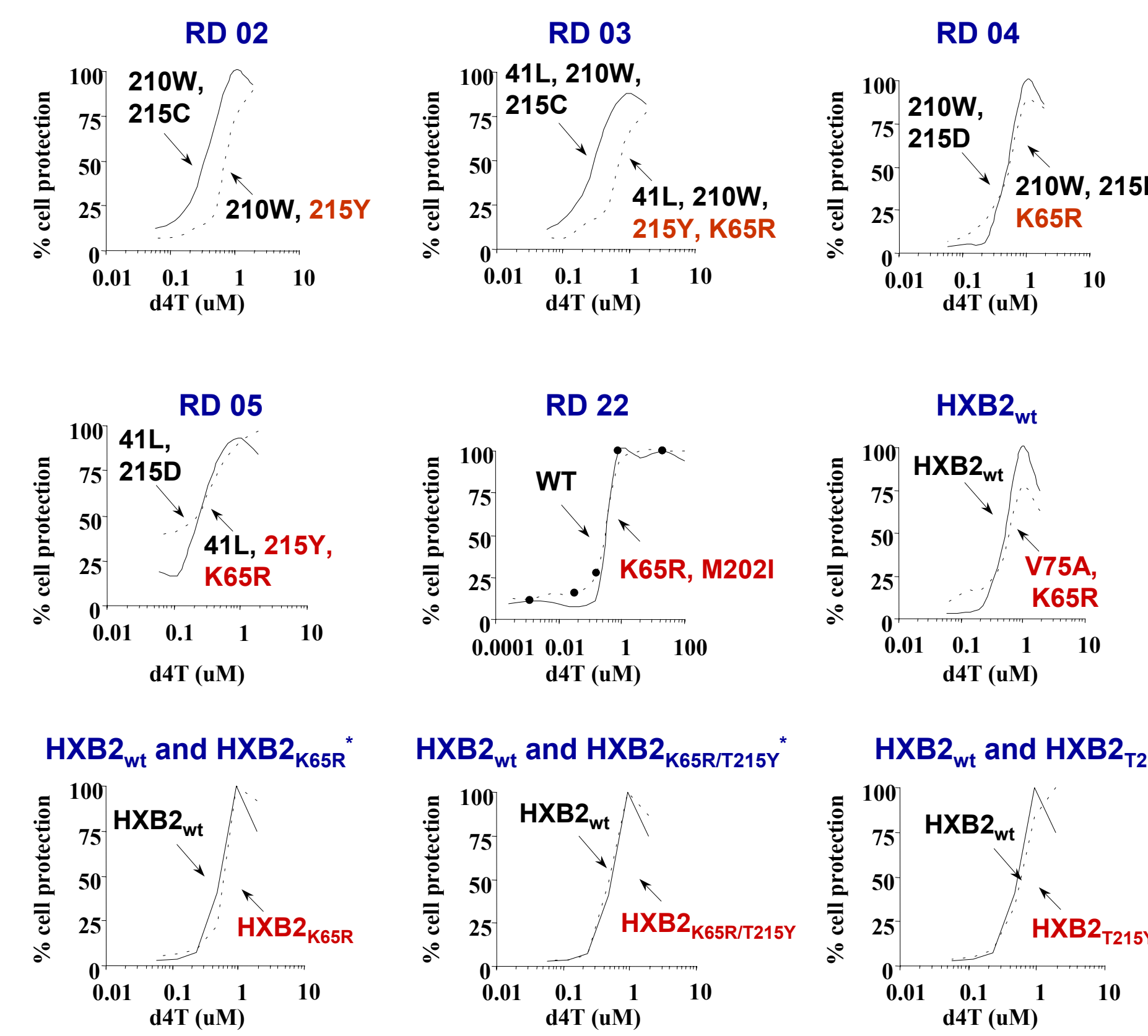
1. In vitro selection of the 215Y mutation by d4T in viruses carrying 215D/C and comparison with the kinetics of emergence of 215Y with AZT

Recombinant	SELECTION WITH d4T				SELECTION WITH AZT			
	Passage (d4T, uM)	Cumulative time (days)	Codon 215	Other	Passage (AZT, uM)	Cumulative time (days)	Codon 215	Other
RD 01 (215C)	1 (0.7) 2 (1.4) 3 (2.8) 4 (6) 5 (6) 6 (12)	8 20 28 64 93	n.d. C C C/Y Y	n.d. - - V75A V75I/H481Y	1 (0.03) 2 (0.06) 3 (0.12) 4 (0.24)	6 12 18 24	n.d. n.d. C/Y Y	n.d. n.d. - -
RD 02 (210W, 215C)	1 (0.7) 2 (1.4) 3 (2.8) 4 (6) 5 (6) 6 (12)	6 12 19 46 67	n.d. n.d. C Y Y	n.d. n.d. - - -	1 (0.03) 2 (0.06) 3 (0.12) 4 (0.24) 5 (0.48)	6 12 18 24 28	n.d. n.d. C C/Y Y	n.d. n.d. - - -
RD 03 (41L, 210W, 215C)	1 (0.7) 2 (1.4) 3 (2.8) 4 (6) 5 (12) 6 (12)	6 13 20 41 69 79	n.d. n.d. C C/Y Y/C Y	n.d. n.d. K65R K65R K65R K65R	1 (0.03) 2 (0.06) 3 (0.12) 4 (0.24) 5 (0.48)	6 12 18 24 28	n.d. n.d. C Y/C Y	n.d. n.d. - - -
RD 04 (210W, 215D)	1 (0.7) 2 (1.4) 3 (2.8) 4 (6) 5 (12) 6 (12)	6 12 20 64 86 112 141	n.d. n.d. D D D D	n.d. n.d. - - K65R K65R K65R	1 (0.03) 2 (0.06) 3 (0.12) 4 (0.24) 5 (0.48)	6 12 18 24 28	n.d. n.d. D D Y	n.d. n.d. - - -
RD 05 (41L, 215D)	1 (0.7) 2 (1.4) 3 (2.8) 4 (6) 5 (12) 6 (12)	7 14 22 53 67 111	n.d. n.d. D D D D	n.d. n.d. - - K65R K65R	1 (0.03) 2 (0.06) 3 (0.12) 4 (0.24) 5 (0.48)	6 12 18 22 27	n.d. n.d. D D Y	n.d. n.d. - - -
HXB2 _{215D} (T215D)	1 (0.7) 2 (1.4) 3 (2.8) 4 (6) 5 (12) 6 (12)	6 12 20 38 64 82 111	n.d. n.d. n.d. D D D	n.d. n.d. - - - K65R K65R	1 (0.03) 2 (0.06) 3 (0.12) 4 (0.24) 5 (0.48) 6 (0.96) 7 (3) 8 (10)	6 12 18 22 27 34 39 45 53 60	n.d. n.d. n.d. D T T T F F	n.d. n.d. n.d. - - - - K70K/R K70R
RD 22 (wild type)	1 (0.7) 2 (1.4) 3 (2.8) 4 (6) 5 (12) 6 (12)	7 14 22 42 81 110	n.d. n.d. T T T T	n.d. n.d. D67G D67G K65R, M202I K65R, M202I K65R, M202I	1 (0.03) 2 (0.06) 3 (0.12) 4 (0.24) 5 (0.48) 6 (0.96) 7 (3) 8 (10)	6 12 18 22 27 34 39 45 53 60	n.d. n.d. n.d. T T T T T T F F	n.d. n.d. n.d. - - - - K70K/R K70R
RD 23 (wild type)	1 (0.7) 2 (1.4) 3 (2.8) 4 (6) 5 (12) 6 (12)	7 15 27 51 77 94	n.d. n.d. T T T T	n.d. n.d. K65R K65R K65R K65R	1 (0.03) 2 (0.06) 3 (0.12) 4 (0.24) 5 (0.48) 6 (0.96) 7 (3) 8 (10)	6 12 18 28 34 39 45 53 60	n.d. n.d. n.d. T T T T T T F F	n.d. n.d. n.d. - - - - D67N D67N, K70K/R D67N, K70R
HXB2 (wild type)	1 (0.7) 2 (1.4) 3 (2.8) 4 (6) 5 (12) 6 (12)	6 12 20 55 66 87 109	n.d. n.d. T T T T	n.d. n.d. V75V/A V75V/A, K65R V75V/A, K65R V75A, K65R	1 (0.03) 2 (0.06) 3 (0.12) 4 (0.24) 5 (0.48) 6 (0.96) 7 (3) 8 (10)	6 12 18 30 44 57 61 74	n.d. n.d. n.d. T T T T T T T T	n.d. n.d. n.d. - - - - D67D/N D67N/D

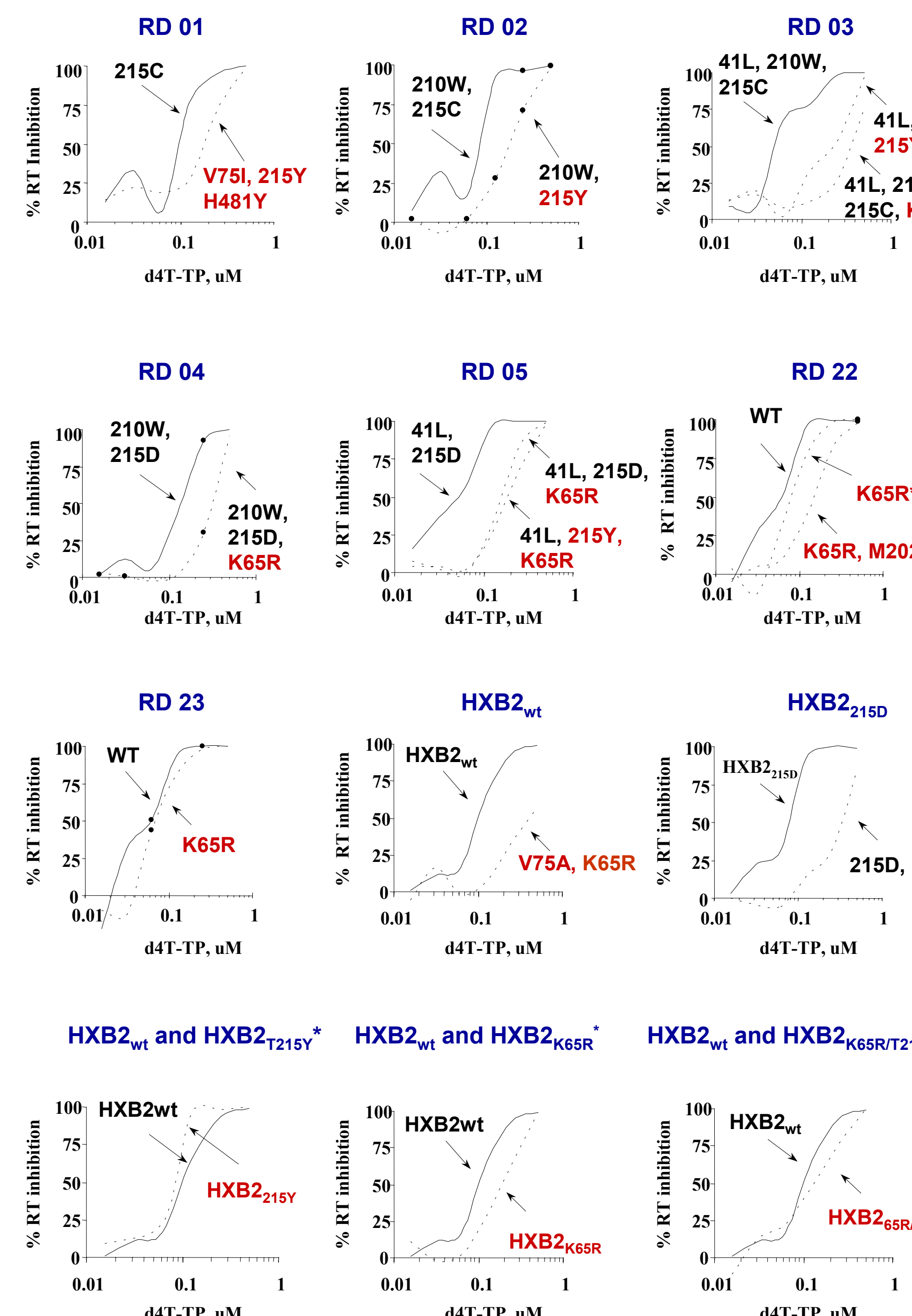
*Mixed genotype. The first amino acid represents the predominant genotype observed in the mixture. N.d.: not done

2. Effect of resistance mutations observed in vitro on susceptibility to d4T

Replication-based assay (MT4/MTT)



RT-based assay (Amp-RT)



*Mutations introduced by site-directed mutagenesis.

3. Fold-changes in IC₅₀/EC₅₀ values for d4T-TP and d4T following in vitro selection of d4T resistance

Recombinant	Mutations	RT-based		MT4/MTT	
		IC ₅₀ , uM (fold-change)	EC ₅₀ , uM (fold-change)	IC ₅₀ , uM (fold-change)	EC ₅₀ , uM (fold-change)
RD 01	215C	0.10	n.d.	n.d.	n.d.
RD 01 _{12uM}	V75I/215Y/H481Y	0.18 (1.8)	n.d.	n.d.	n.d.
RD 02	210W/215C	0.08	0.33	0.08	0.33
RD 02 _{12uM}	210W/215Y	0.18 (2.3)	0.7 (2.1)	0.18 (2.3)	0.7 (2.1)
RD 03	41L/210W/215C	0.05	0.31	0.05	0.31
RD 03 _{3uM}	41L/210W/215C/K65R	0.35 (6.8)	n.d.	0.35 (6.8)	n.d.
RD 03 _{12uM}	41L/210W/215Y/K65R	0.22 (4.4)	0.75 (2.4)	0.22 (4.4)	0.75 (2.4)
RD 04	210W/215D	0.14	0.50	0.14	0.50
RD 04 _{12uM}	210W/215D/K65R	0.31 (2.2)	0.53 (1.1)	0.31 (2.2)	0.53 (1.1)
RD 05	41L/215D	0.05	0.23	0.05	0.23
RD 05 _{6uM}	41L/215D/K65R	0.16 (3.2)	n.d.	0.16 (3.2)	n.d.
RD 05 _{12uM}	41L/215Y/K65R	0.19 (3.7)	0.22 (1.0)	0.19 (3.7)	0.22 (1.0)
HXB2 _{215D}	215D	0.08	n.d.	0.08	n.d.
HXB2 _{215D 6uM}	215D/K65R	0.32 (4)	n.d.	0.32 (4)	n.d.
RD 22	-	0.06	0.30	0.06	0.30
RD 22 _{12uM}	K65R/M202I	0.15 (2.4)	0.28 (0.9)	0.15 (2.4)	0.28 (0.9)
RD 22 _{65R}	K65R	0.08 (1.3)	n.d.	0.08 (1.3)	n.d.
RD 23	-	0.06	n.d.	0.06	n.d.
RD 23 _{12uM}	K65R	0.07 (1.2)	n.d.	0.07 (1.2)	n.d.
HXB2 _{wt}	-	0.10	0.52	0.10	0.52
HXB2 _{12uM}	V75A/K65R	0.41 (4.1)	0.62 (1.2)	0.41 (4.1)	0.62 (1.2)
HXB2 _{65R}	K65R	0.19 (1.9)	0.60 (1.2)	0.19 (1.9)	0.60 (1.2)
HXB2 _{65R/215Y}	K65R/215Y	0.13 (1.3)	0.48 (0.9)	0.13 (1.3)	0.48 (0.9)
HXB2 _{215Y}	215Y	0.08 (0.8)	0.59 (1.1)	0.08 (0.8)	0.59 (1.1)

*Mutations introduced by site-directed mutagenesis. N.d.: not done.

SUMMARY

Viruses having 215D or 215C have increased ability to select for the 215Y mutation in the presence of d4T in vitro. Selection of 215Y by d4T was seen after a mean of 67 days (range = 32-89) in culture in 4/6 viruses carrying 215D/C. In contrast, none of the 3 WT viruses tested acquired 215Y after 104 (range = 94-110) days in culture.

The time required for selection of 215Y by d4T in HIV-1_{215D/C} was longer than that for AZT, probably reflecting a lower selective advantage of 215Y for d4T compared to AZT.

Interestingly, K65R was selected in 7/9 viruses including 4/6 viruses having 215C/D and all three WT HIV-1, demonstrating for the first time the selective advantage of this mutation in the presence of d4T. K65R was the only mutation selected in two viruses with 215D, and was found to precede 215Y in two other viruses with 215C or D. The selection of K65R was far more frequent than V75I/A which was seen in only 2 viruses. M202I, and H481Y were also seen. However, their role in d4T resistance remains undefined.

Reduced susceptibility to d4T was seen biochemically by an RT-based assay in isolates carrying 215Y, K65R/215Y, or K65R/V75A (mean increase in IC₅₀ = 3.3-fold). In contrast, resistance to d4T by the MT4/MTT assay was low or undetectable, probably reflecting the inability of this assay to detect d4T resistance in these viruses.

Differences in the level of d4T resistance among RTs carrying 215Y and/or K65R were seen, suggesting that detectable resistance may be influenced by the RT genetic background, and that different biochemical mechanisms (i.e. decreased affinity of mutant RT for d4T-TP vs. increase ability of mutant RT to remove chain terminators) may contribute to d4T resistance.

CONCLUSIONS

Our results document for the first time the selection of the 215Y mutation by d4T in vitro. The observed selection of 215Y by d4T in vitro suggests that 215Y confers a selective advantage to the virus in the presence of d4T, and may explain the selection of 215Y by d4T observed in vivo.

The frequent selection of K65R by d4T supports a primary role for this mutation in d4T resistance and indicates that mutations at codons other than 215 and 75 may also contribute to d4T resistance.

Our results suggest that patients infected with HIV-1_{215D/C} and treated with antiretroviral regimens containing d4T or AZT may be at increased risk for development of 215Y.

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