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Clonal Resistance Analyses of HIV-1 after Failure of Therapy with Didanosine, Lamivudine, and Tenofovir

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Abstract

Background—The rapid failure of initial therapy with combinations of nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) that exclude zidovudine has not been fully explained by standard virus population analyses of HIV-1 drug resistance. We therefore investigated HIV-1 genotype and phenotype at the single genome level in samples from patients on a failing regimen of tenofovir (TNV), didanosine (ddI), and lamivudine (3TC).

Methods—Single genome sequencing was performed on nine failure samples containing both K65R and M184V mutations by standard genotype, either as wild-type/mutant mixtures (6/9) or as mutant only (3/9). Recombinant clones with different combinations of observed mutations were generated and tested for NRTI susceptibility.

Results—Of the 204 single genome sequences analyzed, 50% were K65R/M184V double-mutants, 38% were M184V single-mutants, 10% were M184I single-mutants, and only 1% (2 sequences) were K65R single-mutants. Phenotypic testing of recombinant clones showed a significant increase in resistance for double-mutants: mean fold-resistance to ABC, ddI, and TNV was 6.5, 4.3, and 1.6 for K65R/M184V double-mutants versus 2.5, 1.9, and 0.6 for M184V single-mutants, respectively ($p < 0.001$).

Conclusions—Mutants with K65R and M184V linked on the same genome were the most common HIV-1 variants in samples analyzed from patients failing TNV, ddI, and 3TC with both mutations detected by standard genotype. The double-mutant exhibited reduced susceptibility to all three NRTI in the regimen. This resistant phenotype, resulting from just two linked point mutations, likely contributes to rapid failure of NRTI combinations that exclude zidovudine.

Background

Combinations of three nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) have been studied as initial therapy of HIV-1 infection with goals of regimen simplification and sparing of other drug classes. Unfortunately, such combinations, especially those excluding zidovudine (AZT), have been associated with rapid viral rebound [1–4].

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One such study [1] tested a combination of once-daily tenofovir (TNV), didanosine (ddI), and lamivudine (3TC) in treatment-naïve participants. No patient had $>2.0 \log_{10}$ decreases in plasma HIV-1 RNA by week 12 and 91% (20/22) went off therapy before week 24 because of inadequate virologic response or viral rebound. Standard population phenotyping of samples at treatment discontinuation showed 3TC resistance in 100% (20/20), no change in TNV susceptibility, and reduced susceptibility to ddI in only 16%. Standard population genotyping identified M184V/I in all participants, but K65R in only 10/20. These findings didn't adequately explain rapid and consistent regimen failure. We used single genome sequencing (SGS) to better characterize viral population at failure and to assess linkage between resistance mutations. We also compared population and clonal phenotypes to investigate the observed preservation of TNV and ddI sensitivity.

Methods

Patient Samples

Plasma samples were obtained at the time of treatment discontinuation from a trial of once-daily TNV/ddI/3TC [1]. Samples were available for 9/10 patients who had both M184V and K65R mutations detected by standard genotype, either with or without wild-type as a mixture. This study was approved by the Institutional Review Board of the University of Pittsburgh.

Standard Genotype/Phenotype

Standard genotypes and phenotypes were obtained by the PhenoSense GT assay (Monogram Biosciences, South San Francisco, CA).

Single Genome Sequences

SGS was performed as published [5]. Amplicons from cDNA dilution having 30% positive PCR reactions were sequenced using ABI Prism BigDye terminator kit version 3.1 (Applied Biosystems, Foster City, CA). Genomes were assembled using Sequencher version 2.5 (Gene Codes Corp., Ann Arbor, MI) and analyzed for resistance mutations using Stanford Drug Resistance Database algorithm (<http://hivdb.stanford.edu>). Sequences showing more than one genome were excluded from analysis (51 in total).

Generation of Recombinant Clones

Recombinant viruses were generated from plasma samples as reported [6]. Plasmids from individual bacterial clones were purified using the Wizard Plus Miniprep system (Promega, Madison, WI) and sequenced using ABI Prism BigDye terminator kit version 3.1. Clones were transfected into MT-2 cells by electroporation to produce infectious recombinant virus [7].

Clonal Phenotype Analyses

Single replication-cycle drug susceptibility assays were performed on cloned recombinant viruses as reported [8]. Susceptibility assays were performed at least three times for each virus. Differences in mean IC_{50} values were assessed for statistical significance using the Mann-Whitney test.

Results

Standard Population Genotypes

Table 1 shows population genotypes from the time of treatment discontinuation for the nine patients studied. Each sample had both K65R and M184V; six samples had 65R present as a mixture with wild-type K65, and three of these six contained a mixture of 184V with wild-type

M184. The only other NRTI resistance mutation detected was 70E in three samples, each as a mixture with wild-type K70.

Single Genome Sequences

Multiple (12-46) single genome sequences were obtained from each of nine plasma samples (Table 1). Of the 204 sequences, 179 (88%) contained M184V and 22 (11%) contained M184I. K65R was detected in 105 (51%) sequences. The most common sequence was the K65R/M184V double-mutant in 102/204 (50%), followed by M184V single-mutant in 77/204 (38%), and M184I single-mutant in 21/204 (10%). The K65R/M184V double-mutant was detected in 8/9 patients. The K65R single-mutant was detected in only 2/204 (1%) sequences. K65R/M184I double-mutant and wild-type were detected only once each.

Other NRTI mutations were found at low frequency. Five of 13 sequences had K70E from Patient #5. Other NRTI mutations were detected in only 1-2 sequences (not shown).

Standard Population Phenotypes

Table 2A shows standard population susceptibilities to 3TC, ddI, TNV and ABC. ABC was studied as an indicator of resistance to other NRTI regimens that exclude AZT. Each of the nine samples had the maximum possible IC₅₀ for 3TC defined by the assay. The average fold-resistance of the nine samples was 1.81±0.55 for ddI, 0.63±0.20 for TNV, and 3.69±1.69 for ABC. Samples were further categorized as having “pure” M184V and K65R (three samples) or as M184V and a “mixture” of K65R and wild-type K65 (six samples). Average fold-resistance for the “pure” group was 2.45±0.15 for ddI, 0.86±0.20 for TNV, and 5.41±1.49 for ABC. Average fold-resistance for the “mixture” group was 1.49±0.31 for ddI, 0.52±0.05 for TNV, and 2.83±1.01 for ABC. Fold-resistance values were significantly higher than for “pure” group (Mann-Whitney *p*-value for ddI=0.025, TNV=0.024, and ABC=0.025).

Clonal Phenotypes

Recombinant infectious viral clones were generated from five patient samples to compare phenotypic effects of different combinations of mutations. Of twelve clones studied, five were M184V single-mutants, one was a M184I single-mutant, and six were K65R/M184V double-mutants. No clone contained other known NRTI resistance mutations. Table 2B shows results of clonal phenotype analyses. For 3TC, the IC₅₀ for all clones was above the maximum possible value for the assay (>270 μM). For ddI, TNV, and ABC, the mean IC₅₀ values for K65R/M184V double-mutants were significantly higher than for M184V single-mutants (*p* < 0.001). Specifically, for K65R/M184V double-mutants versus M184V single-mutants, the mean IC₅₀ for ddI was 14.29±4.93 μM (fold-resistance=3.8) versus 5.11±2.68 μM (fold-resistance=1.4), for TNV 7.10±3.37 μM (fold-resistance=1.6) versus 2.37±0.91 μM (fold-resistance=0.5) and for ABC 68.25±18.37 μM (fold-resistance=7.7) versus 20.78±7.41 μM (fold-resistance=2.4).

Discussion

This is the first study to perform clonal genotype and phenotype analyses of plasma from patients failing triple NRTI therapy. K65R and M184V were linked on the same genome in 8/9 patients with both mutants detected by standard genotype, and double-mutants comprised 50% of all sequences. M184V/I single-mutants comprised 48% of all sequences, whereas only two sequences (1%) were K65R single-mutants. Frequent linkage of K65R and M184V suggests an advantage for double-mutants.

Phenotypic analysis of twelve recombinant clones showed a significant decrease in ddI, TNV, and ABC susceptibility for K65R/M184V double-mutants compared to M184V single-mutants

($p < 0.001$). The observed reversal of TNV hypersusceptibility and increased ddI and ABC resistance with the addition of K65R to M184V is consistent with site-directed mutant studies [9,10]. However, patient-derived double-mutants showed greater resistance to TNV compared to that reported for site-directed mutants [10], possibly due to different polymorphisms in patient-derived recombinants. Population phenotype analyses failed to accurately measure combined phenotypic effects of K65R and M184V. This is likely because population phenotypes represent an average of the variants present. Indeed, the population phenotype of samples with “pure” K65R and M184V by standard genotype was more similar to that of individual double-mutant clones, compared to the phenotype of samples with mutant/wild-type “mixtures”, which were more like single M184V clones.

Not all of the single genome sequences contained K65R. Furthermore, 10/20 patients had only M184V detected by standard genotype, indicating that double-mutants were infrequent or not present. Escape of M184I/V single-mutants remains unexplained because these viruses were fully susceptible or hypersusceptible to TNV. It may be that TNV doesn’t penetrate or isn’t phosphorylated to its active metabolite in certain target cells, allowing the M184V single-mutant to replicate. This possibility is supported by observations that TNV alone isn’t fully suppressive of HIV-1 replication [11,12] and its tissue penetration is variable [13].

A negative drug-drug interaction between TNV and other NRTIs has also been proposed as an escape mechanism of M184V/I single-mutants from TNV suppression. However, Hawkins [14] and Lanier [15] didn’t detect pharmacologic antagonism between 3TC, ABC and TNV. Similarly, Kearney [16] didn’t detect an effect of ddI on TNV concentrations. Although these studies failed to identify major negative drug-drug interactions, metabolism at the single cell level wasn’t studied and thus antagonism cannot be entirely excluded.

Delaunay [17] clonally analyzed longitudinal samples from five subjects in the TONUS study of TNV/3TC/ABC. Linkage analysis showed K65R and M184V/I single-mutants were more prevalent at week 4 than at week 12. By week 12, double-mutants containing K65R and M184V/I were predominant in all five subjects. These data are consistent with our finding of frequent double-mutants at treatment discontinuation (week 16 or later).

Failure of triple NRTI regimens [1-4] doesn’t occur as frequently in regimens containing AZT [18-20]. For example, ACTG A5095 [18] compared the efficacy AZT/3TC/ABC, AZT/3TC/efavirenz, and AZT/3TC/ABC/efavirenz. Although the triple NRTI regimen was inferior to efavirenz-containing arms, it did suppress HIV-1 RNA to <50 copies/mL in 61% of patients at week 48. Similarly, TARGET [19], a single-arm trial of twice-daily 3TC/AZT/ABC, showed HIV-1 RNA suppression to <50 copies/mL in 56% of patients. Finally, NZTA4007 [20], a single-arm trial of 3TC/AZT/ABC, achieved HIV-1 RNA suppression to <50 copies/mL in 75% of patients at 24 weeks. The substantially better virologic response of AZT-containing triple NRTI regimens may be due to phenotypic antagonism between the common resistance pathways for AZT (thymidine analog mutations) versus TNV, 3TC, ABC, and ddI (K65R and M184V) [10]. This possibility is supported by the absence of K65R at virologic failure in A5095 and TARGET compared to its high prevalence in studies of NRTI regimens excluding AZT.

In summary, the poor virologic response and rapid viral rebound observed with once-daily TNV/ddI/3TC is explained in some patients by resistance to each component of the regimen resulting from two, linked, single nucleotide mutations in reverse transcriptase (K65R and M184V).

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Table 1
Standard Population Genotype and Single Genome Sequences

Patient	Week Off Treatment	HIV-1 RNA (cps/ml)	Standard Genotype	Single Genomes	K65R/M184V	K65/M184	K65R/M184I	K65R/M184I
1	17	648000	K65R; M184V	12	11	1	0	0
2	23	305000	K65R; M184V	26	25	1	0	0
3	17	20300	K65R; M184V	13	11	1	0	1
4	23	325000	K65K/R; M184V	13	5	8	0	0
5	24	33600	K65K/R; M184V	13	0	13	0	0
6	16	>750000	K65K/R; M184V	21	8	13	0	0
7	16	16400	K65K/R; M184M/V	46	36	4	0	5
8	16	2270	K65K/R; M184M/V/I	14	3	5	2	3
9	21	67000	K65K/R; M184M/V/I	46	3	31	0	12
Total				204	102	77	2	21
				% of Total	50.0%	37.7%	1.0%	10.3%
“Pure” Double Mutants				51	47	3	0	1
				% of Total	92.2%	5.9%	0.0%	2.0%
“Mixture” Samples				153	55	74	2	20
				% of Total	35.9%	48.4%	1.3%	13.1%
								0.7%

Table 2A

Standard Population Phenotypes

Patient	Standard Genotype	3TC ^a		ddl ^a		ABC ^a		TNV ^a	
		IC ₅₀ (μM)	Fold-R	IC ₅₀ (μM)	Fold-R	IC ₅₀ (μM)	Fold-R	IC ₅₀ (μM)	Fold-R
1	K65R; M184V	>300	MAX	13.63	2.55	10.25	5.65	0.727	1.03
2	K65R; M184V	>300	MAX	15.91	2.52	13.50	6.77	0.770	0.91
3	K65R; M184V	>300	MAX	12.20	2.28	6.94	3.82	0.447	0.64
4	K65K/R; M184V	>300	MAX	8.22	1.45	6.81	3.42	0.378	0.47
5	K65K/R; M184V	>300	MAX	8.35	1.47	7.20	3.61	0.429	0.53
6	K65K/R; M184V	>300	MAX	9.64	1.80	6.31	3.48	0.377	0.54
7	K65K/R; M184M/V	>300	MAX	9.99	1.87	5.89	3.25	0.395	0.56
8	K65K/R; M184M/V/I	>300	MAX	5.48	1.02	1.92	1.08	0.303	0.43
9	K65K/R; M184M/V/I	>300	MAX	7.05	1.32	3.88	2.14	0.393	0.56
Means of all samples				10.05 ± 3.32	1.81 ± 0.55	6.97 ± 3.36	3.69 ± 1.69	0.469 ± 0.164	0.63 ± 0.20
Means of "mixture" samples				8.12 ± 1.67	1.49 ± 0.31	5.34 ± 2.03	2.83 ± 1.01	0.379 ± 0.042	0.52 ± 0.05
Means of "pure" double-mutants				13.91 ± 1.87	2.45 ± 0.15M ^b	10.23 ± 3.28	5.41 ± 1.49 ^b	0.648 ± 0.175	0.86 ± 0.20b

^a 3TC = lamivudine, ddl = didanosine, ABC = abacavir, and TNV = tenofovir

^b Mean fold-resistance significantly different (p-value = 0.025) between "mixture" and "pure" samples for ddl, ABC and TNV (Mann-Whitney test).

Clonal Genotypes and Phenotypes

Table 2B

Clone ^a	Genotype	N	3TC ^b IC ₅₀ (μM)	Fold-R	ddI ^b IC ₅₀ (μM)	Fold-R	ABC ^b IC ₅₀ (μM)	Fold-R	TNV ^b IC ₅₀ (μM)	Fold-R
1A	K65R/M184V	3	>270	MAX	12.37 ± 3.05	3.3	70.14 ± 10.64	8.0	4.08 ± 1.25	0.9
1B	K65R/M184V	3	>270	MAX	15.17 ± 4.47	4.1	82.80 ± 16.24	9.4	4.29 ± 1.07	1.0
5A	M184V	3	>270	MAX	6.10 ± 0.60	1.6	30.85 ± 11.93	3.5	2.55 ± 1.40	0.6
6A	M184V	5	>270	MAX	3.60 ± 1.16	1.0	16.44 ± 5.20	1.9	1.76 ± 0.60	0.4
6B	M184V	5	>270	MAX	7.30 ± 4.27	2.0	23.22 ± 4.14	2.6	2.40 ± 0.82	0.5
6C	K65R/M184V	5	>270	MAX	13.67 ± 6.09	3.7	66.00 ± 5.60	7.5	7.35 ± 4.56	1.6
6D	K65R/M184V	5	>270	MAX	16.62 ± 7.78	4.5	53.67 ± 23.17	6.1	8.91 ± 2.45	2.0
7A	K65R/M184V	3	>270	MAX	13.54 ± 1.27	3.6	75.67 ± 14.54	8.6	7.26 ± 1.52	1.6
7B	K65R/M184V	3	>270	MAX	13.26 ± 2.41	3.6	69.98 ± 29.97	7.9	9.25 ± 4.28	2.1
9A	M184V	3	>270	MAX	4.42 ± 1.11	1.2	17.23 ± 3.73	2.0	2.78 ± 1.42	0.6
9B	M184V	3	>270	MAX	3.71 ± 0.99	1.0	17.40 ± 0.28	2.0	2.73 ± 0.27	0.6
9C	M184I	3	>270	MAX	5.47 ± 1.29	1.5	15.59 ± 3.79	1.8	2.41 ± 0.41	0.5
Wild-type Control ^c		23	2.56 ± 2.12	---	3.72 ± 2.21	---	8.82 ± 2.77	---	4.50 ± 1.99	---
M184V Single-Mutant		19	>270	MAX	5.11 ± 2.68 ^d	1.4	20.78 ± 7.41 ^e	2.4	2.37 ± 0.91 ^e	0.5
K65R/M184V Double-Mutant		22	>270	MAX	14.29 ± 4.93 ^{f,g}	3.8	68.25 ± 18.37 ^{f,g}	7.7	7.10 ± 3.37 ^{f,g}	1.6

^a Clones are designated by the sample number from which they were derived followed by a letter.

^b 3TC = lamivudine, ddI = didanosine, ABC = abacavir, and TNV = tenofovir

^c Wild-type was xxHIV-1LAI.

^d $p \leq 0.07$ by two tailed Student's t-test for comparison of single mutant wild type IC₅₀s

^e $p \leq 0.05$ by two tailed Student's t-test for comparison of single mutant to wild type IC₅₀s

^f $p < 0.001$ by two tailed Student's t-test for comparison of double mutant to wild type IC₅₀s

^g $p < 0.001$ by two tailed Student's t-test for comparison of double to single mutant IC₅₀s