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High Prevalence of the K65R Mutation in Human Immunodeficiency Virus Type 1 Subtype C Isolates from Infected Patients in Botswana Treated with Didanosine-Based Regimens▽

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We analyzed the reverse transcriptase genotypes of human immunodeficiency virus type 1 subtype C viruses isolated from 23 patients in Botswana treated with didanosine-based regimens. The K65R mutation was selected either alone or together with the Q151M, S68G, or F116Y substitution in viruses from seven such individuals. The results of in vitro passage experiments were consistent with an apparent increased propensity of subtype C viruses to develop the K65R substitution.

Non-subtype-B human immunodeficiency virus (HIV) is prevalent throughout sub-Saharan Africa and in eastern Asia (12) and may now represent ≈15 to 20% of all new infections in Western countries (4, 17). Although current evidence suggests that all HIV subtypes probably respond in an equivalent fashion to antiretroviral drugs (ARVs), there is increasing evidence that distinct pathways toward the development of drug resistance may emerge among viruses of different subtypes. In part, this may be based on differences in codon usage and baseline sequences that can vary extensively among viral subtypes. This is an issue that is of importance to patients but which also has public health importance, given that drug-resistant variants of HIV can be sexually transmitted (16).

Subtype C is the most prevalent non-B subtype worldwide and is the most common subtype in Botswana (12). Previous work has shown that distinct patterns of codon usage can result in the appearance of specific mutations associated with resistance to nonnucleoside reverse transcriptase (RT) inhibitors. For example, a V106M mutation appears to be more prevalent among subtype C viruses than among subtype B viruses, for which a V106A substitution is more common at this position (3). Studies on mother-to-child transmission of HIV type 1 (HIV-1) reported that viruses from a higher proportion of women infected with HIV-1 subtype C or D developed resistance to nevirapine (NVP) than was found for women infected with subtype A viruses (7). In addition, an L89M/I substitution may be preferentially associated with resistance to protease inhibitors for subtype C viruses (1, 6).

Although the K65R substitution can cause extensive cross-resistance among currently used nucleoside reverse transcriptase inhibitors (NRTIs), this mutation has been observed relatively rarely among viruses from subtype B-infected individuals who received antiviral drugs (9, 10, 15, 18). For example, K65R was observed in viruses from a relatively small proportion of individuals treated with tenofovir (TDF) together with lamivudine (3TC) and efavirenz (EFV) over long periods (8). Interestingly though, d4T was shown to select for K65R in culture with subtype C viruses (9). Recent increases in the frequency of K65R in Western countries are attributed to extensive TDF use in recent years (11, 13). We recently demonstrated that subtype C clinical isolates, unlike those of subtype B, can select for the K65R substitution within 12 weeks when exposed to increasing concentrations of TDF in tissue culture (2). These observations led us to evaluate the incidence of K65R in Botswana patients who received ARV therapy in the context of both first- and second-line regimens provided by the National Antiretroviral Treatment Program.

In January 2002, Botswana officially initiated a national antiretroviral treatment program providing highly active antiretroviral therapy (HAART) within the public health system. The government of Botswana offers free ARVs to all citizens if they have a CD4 cell count of less than 200 cells/μl or an AIDS-defining condition. HIV-infected adults who qualify for treatment begin with the three-drug regimen of zidovudine (ZDV) plus 3TC plus EFV if male or ZDV plus 3TC plus NVP if female, due to potential teratogenicity associated with EFV. In cases of therapeutic failure, second-line regimens that include various combinations of didanosine (ddI), stavudine (d4T), and lopinavir/ritonavir, which has now replaced nelfinavir (NFV), and third-line regimens containing saquinavir/ritonavir are available.

Before January 2002 and as per national practice and international guideline recommendations for developing countries, most patients initiated therapy with ddI/d4T-based regimens as the nucleoside backbone. In this study, we evaluated the incidence of K65R in viruses from 23 Botswana patients who received ddI/d4T-based therapy in the context of both first-

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TABLE 1. Treatment history of 23 Botswana patients treated with ddI/d4T-based regimens

Patient no.	CD4 count[b]	VL[b]	Initial regimen (duration[d])	Second and/or third regimen, where applicable (mo)	Last regimen	Duration of treatment with ddI/d4T (mo)	Presence of K65R at genotyping
1	140	5.21	ZDV/3TC/NVP (10)	ddI/d4T/NFV (15)	ZDV/3TC/NFV	15	−
2	158	4.39	ZDV/3TC/NVP (14)	ddI/d4T/NFV (9)	ddI/d4T/NFV	9	−
3	78	4.49	ddI/d4T/EFV (19)	ZDV/3TC/NFV (6)	ABC/3TC/NFV	19	+
4	62	5.87	ZDV/3TC/NVP (36)	ddI/d4T/NFV (5)	SQV/RTV/ABC	5	−
5	214	5.87	ZDV/3TC/NVP (ND)	ZDV/3TC/RTV/SAQ (9)	ddI/d4T/NFV	15	−
6	1	5.87	ZDV/3TC/EFV (2)	ddI/d4T/EFV (4)	ddI/d4T/EFV	4	+
7[a]	7	5.87	ddI/d4T/NVP	ddI/d4T/NVP	ddI/d4T/NVP	4	+
8	NA[c]	NA	ddI/d4T/NVP (7)	ZDV/3TC/NFV (10)	ZDV/3TC/EFV	7	−
9	199	5.70	ZDV/3TC/EFV (12)	ddI/d4T/EFV (9) ZDV/3TC/EFV (6)	ddI/d4T/NFV	13	−
10[a]	0	5.87	ddI/d4T/NVP	ddI/d4T/NVP	ddI/d4T/NVP	4	+
11	45	5.87	ZDV/3TC/NVP (14)	ddI/d4T/NFV (23)	ddI/d4T/NFV	23	−
12	344	5.87	ddI/d4T/EFV (3)	ZDV/3TC/NFV	ZDV/3TC/NFV	3	−
13	2	5.39	ddI/d4T/EFV (15)	ZDV/3TC/NFV(6)	ZDV/3TC/NFV	15	+
14	4	5.46	ZDV/3TC/NVP (16)	ddI/d4T/NFV(6)	ddI/d4T/NFV	6	−
15[a]	4	5.47	ddI/d4T/NVP	ddI/d4T/NVP	ddI/d4T/NVP	15	+
16	20	5.79	ddI/ZDV/NVP (11)	ZDV/3TC/NVP (6)	ZDV/3TC/NVP	11	+
17	9	5.87	ZDV/3TC/EFV (16)	ddI/d4T/NFV (9)	ddI/d4T/NFV	9	−
18	0	5.87	ddI/d4T/EFV (4)	ZDV/3TC/NFV (10)	ZDV/3TC/NFV	4	−
19	NA	5.76	ddI/d4T/NVP	ddI/d4T/NVP (6)	ZDV/3TC/NVP	6	−
20	8	5.79	ZDV/3TC/NVP (18 days)	ddI/d4T/NVP (6), ddI/3TC/NFV (9)	ABC/ddI/LPVr	19	−
21	126	5.82	ZDV/3TC/NVP (22)	ddI/d4T/NFV (8)	ddI/d4T/NFV	8	−
22	2	4.66	ZDV/3TC/NVP (15)	ddI/d4T/NFV (16)	ddI/d4T/NFV	16	−
23	68	5.87	ZDV/3TC/NVP (10)	d4T/3TC/NVP (2)	ddI/ZDV/NFV	14	−

[a] This patient remained on ddI/d4T/NVP throughout the follow-up for 4 months (patients 7 and 10) or 15 months (patient 15).
[b] Viral load (VL) (log) and CD4 counts (no. of cells/μl) evaluated at baseline (before HAART experience). A value of >750,000 HIV-1 RNA copies/ml was considered to be 5.87 logs.
[c] NA, information not available.
[d] Duration of regimen is given in months except where otherwise indicated.

and second-line regimens provided by the National Antiretroviral Treatment Program. All of these patients experienced treatment failure, i.e., rising viral loads and/or diminishing CD4 counts, while on combination regimens that included ddI. Ten of these individuals had initiated treatment with ddI-based regimens (9 with ddI/d4T and 1 with ddI/ZDV) together with either EFV or NVP as nonnucleoside reverse transcriptase inhibitor, while 13 had started therapy with ZDV/3TC/NVP or ZDV/3TC/EFV prior to switching to ddI- and/or d4T-containing regimens. The HAART history was available for all of these patients (Table 1).

Sequencing of extracted DNA, performed by Bayer TruGene or by Associated Biomedical Systems technology to determine genotypic changes in RT associated with drug resistance, phylogenetic analysis, and confirmation of subtype C infection were performed as previously described (5, 6). Selection of resistance to ddI and d4T (each) using subtype C as well as subtype B clinical isolates was studied with culture, beginning with concentrations of ddI and d4T of 5 and 0.1 μM, respectively, and in combination with 1 μM ddI–0.1 μM d4T. RT assays were performed weekly to assess viral replication. Cells and virus supernatants were harvested for passage, and drug concentrations were increased at each step. Genotyping was performed at select passages to evaluate the time to development of drug resistance.

Frequency of K65R among viruses from ddI-treated patients. The results in Table 2 show that viruses from 7 of the 23 patients who failed ddI-based regimens possessed the K65R substitution after a median exposure to combination ddI/d4T therapy of only 8 months (range, 4 to 18 months). For four of these patients, viruses developed K65R while the patient was still on ddI/d4T at the time of genotyping. For three of the seven patients, viruses developed only K65R, while for four others, viruses also developed Q151M, F116Y, and S68G. The association of K65R and M184V was seen for two patients who had experienced both ddI/d4T- and ZDV/3TC-based regimens. In contrast, for 9 of 13 patients who received 3TC/ZDV as initial therapy, viruses mostly developed thymidine-associated mutations, e.g., M41L, D67N, K70R, T215Y/F, and K219E/Q, while for 3 of 13, viruses had no NRTI resistance (not shown). The presence of thymidine-associated mutations in viruses from patients who first experienced ZDV/3TC may explain the nonemergence of K65R while the patient was subsequently receiving ddI/d4T, due to presumed antagonism among these mutations (13).

In vitro preferential selection of K65R in HIV-1 subtype C viruses. The development of K65R resistance to ddI and/or d4T was confirmed by tissue culture selection using HIV-1 subtype C clinical isolates (Table 3). The K65R and L74V mutations each arose within 15 to 28 weeks in two of five subtype C selections under ddI pressure alone and in three of five selections conducted using combinations of ddI and d4T. In some cases, ddI and/or d4T also selected for the appearance of the D67N and/or V75I substitution in RT. Interestingly, and as previously shown (2), none of the subtype B variants developed the K65R mutation during serial passage (week 28).

The propensity of subtype C viruses to rapidly select K65R may be attributable, in part at least, to differences among

TABLE 2. Association of K65R with other RT mutations in viruses from patients infected with HIV-1 subtype C and treated with ddI/d4T

Patient no.	NRTI mutation(s)	Time of first appearance of K65R (after commencement of ddI/d4T-based therapy) (mo)	Polymorphisms
3	K65R, S68G, F116Y, Q151M, M184V	18	V35T, E36A, T39E, S48T, T69I, D123S, S163C, K166Q, K173T, Q174R, T200A, I202V, E203K, Q207A, H208Y, R211E, L214F, V245M, D250E, R277K, E291D, V292I, I293V, D324E
6	K65R, S68G, K70R, V75I, F116Y, Q151M	4	V35Q, T39D, S48T, V60I, T69I, I135T, E138A, I142T, Q151L, K173A, Q174K, D177E, I178V/L, G196E, T200A, Q207E, R211K, V245K, D250E, R277K, E291D, V292I, I293V, V317A, Q334N
7	K65R	4	V35T, T39K, S48E, T69N, E122K, D123E, I135M, K173T/I, T200A, Q207E, R211K, L214F, V245Q, P272A, R277K, K281R, T286A, E291D, V292I, I293V, E312F, V314P
10	K65R	4	T39E, E40K, D67G, T69N, E122K, K173A, G177G/R, T200A, Q207E, R211K, L214F, H221H, L228L/R, V245E, D250E, E291D, V292I, I293V, I326V, Q334H
13	K65R, S68G, K70R, F77L, Y115F, F116Y, Q151M, K219E	6	V35T, E36A, T39E, T58N, V75A, D123S, R172K, K173T, D177E, I178M, T200A, Q207K, R211K, L214F, P225H, V245Q, E248D, E291D, V292I, I326V, Q334D
15	K65R	12	V35T, T39E, S48T, D123G, K173E, D177G, T200A, Q207K, R211K, L214F, V245Q, E248D, P272S, T286A, L289I, E291D, V292I, Q334N
16	K65R, M184V	11	T27S, V35T, T39E, S48T, D123G, T139K, S162C, K173A, Q174K, D177E, I178L, T200A, Q207D, R211K, L214F, L228R, V245Q, P272S, E291D, V292I, I293V, Q334N

coding sequences between subtype B and subtype C viruses. Nucleoside sequences at codons 64 to 66 in the RT of subtype B viruses are commonly AAG (64), AAA (65), and AAA (66), while in subtype C viruses the sequences are AAA (64), AAG (65), and AAG (66). Although single point mutations are likely always involved in selection of K65R in both subtypes B and C, it is possible that polymorphisms that underlie differences between these subtypes may create an environment in which the selection of K65R occurs more easily in viruses of subtype C.

TABLE 3. Selection of RT mutations after sequential passage with ddI and/or d4T

Clinical isolate (subtype)[a]	Expt no.	Wk of passage	Highest drug concn (μM) achieved	Mutation(s) selected
4742 (C)	1	28	40, ddI	K65R
	2	24	5, d4T	None
	3	17	10, ddI; 0.5, d4T	None
4761 (C)	1	24	10, ddI	L74V
	2	22	2.5, d4T	D67N
	3	15	1, ddI; 0.1, d4T	K65R
BG-05 (C)	1	28	30, ddI	K65R, D67N
	2	24	2.5, d4T	None
	3	28	10, ddI; 0.5, d4T	K65R
BG-15 (C)	1	17	10, ddI	None
	2	17	0.5, d4T	None
	3	28	10, ddI; 0.5, d4T	K65R, V75I
Mole 18 (C)	1	28	40, ddI	L74V
	2	22	0.5, d4T	None
	3	22	2.5, ddI; 0.1, d4T	None
5323 (B)	1	28	20, ddI	None
	2	28	2.5, ddI; 0.1, d4T	None
5512 (B)	1	28	20, ddI	None
	2	28	5, ddI; 0.5, d4T	None

[a] GenBank nucleotide sequence accession numbers are as follows: for isolate 4742, AF492595; for isolate 4761, AF492597; for isolate BG-05, AF492600; for isolate BG-15, AF492601; for isolate Mole 18, AF492607; for isolate 5323, DQ917284; for isolate 5512, DQ917283.

In summary, the K65R substitution may emerge at a higher frequency for individuals infected with subtype C viruses who experienced treatment with ddI/d4T. In view of widespread ARV access in sub-Saharan countries, these findings establish a degree of concern in regard to the possibility that certain mutations, such as K65R, may emerge more rapidly in viruses of subtype C.

To be sure, these results do not diminish the importance of access to drugs, such as TDF, that are well tolerated and effective in management of HIV disease. Furthermore, most of the patients followed in this study never fully suppressed viral load due to poor adherence to the regimen, a situation that has now changed due to improved patient management. Conceivably, those patients who initiated therapy with ddI/d4T and whose viruses did not develop K65R may have been more adherent than those whose viruses did develop this substitution, but the reasons for these differences are unclear. ddI/d4T combinations have been shown to be inferior to other regimens in controlled clinical studies (14). Although use of ddI/d4T may be cost-effective in the short term, its continuing use may lead to inferior clinical outcomes and an increased overall cost of patient care.

The first-line regimen currently recommended by the Botswana guidelines on antiretroviral therapy is ZDV/3TC/EFV or NVP, which may explain why the incidence of the K65R mutation is not elevated in viruses from currently treated individuals. We publish these findings to highlight the importance of monitoring drug resistance on as widespread a basis as possible in developing countries so as to better understand the dynamics of selection of specific mutations by viruses of different subtypes and in the interests of public health.

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