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K65R, TAMs and Tenofovir

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Abstract

The management of drug resistance has become part of the management of HIV disease in the treated individual. As two or more nucleoside reverse transcriptase inhibitors (NRTIs) are generally part of each antiretroviral regimen, there is a need to fully understand resistance and cross-resistance within this class of drugs. Broad cross-resistance to NRTIs caused by the group of HIV RT mutations associated with zidovudine and stavudine therapy (thymidine analogue mutations or TAMs) has been well established. The response to tenofovir disoproxil fumarate (TDF) therapy is also limited by certain patterns of TAMs (≥3 TAMs with M41L or L210W). The K65R mutation can result from tenofovir DF, abacavir, stavudine, zalcitabine or didanosine therapy. From* in vitro *phenotypic analysis, the K65R mutation shows no cross-resistance to zidovudine, but low-level resistance to tenofovir and the other NRTIs. Based on clinical cut-offs established for the individual NRTIs, the phenotypic results with K65R suggest full-to-partial drug activity for multiple NRTIs, including tenofovir, against the K65R mutant. Similar to the M184V mutation, the K65R mutation is also associated with reduced* in vitro *viral replication capacity, hallmarks of which can be demonstrated at the enzymatic level. From cross-sectional genotypic analyses, the K65R mutation and TAMs appear to represent separate patterns of NRTI resistance. Among treatment-naive patients who developed the K65R mutation in clinical trials, successful second line regimens were established. Thus, the K65R mutation appears manageable for the sequencing of treatment regimens in the case of its development. **(AIDS Rev 2004;6:22-33)**

Key words

Tenofovir. HIV. Resistance.

Introduction

Nucleoside reverse transcriptase inhibitors (NRTIs) were the first antiretroviral drugs introduced for the treatment of HIV-1 infection. Eight NRTIs have been approved for use: zidovudine, zalcitabine, stavudine, didanosine, lamivudine, abacavir, tenofovir and, most recently, emtricitabine. Tenofovir is unique among the NRTIs in that it is an acyclic nucleoside phosphonate, analogous to the monophosphate form of the other NRTIs[1]. Tenofovir disoproxil fumarate (tenofovir DF, Viread®) is an oral prodrug of tenofovir that is rapidly converted to tenofovir upon absorption[2,3]. Tenofovir has *in vitro* activity against all subtypes of HIV-1 and against HIV-2 and human hepatitis B virus[4-7].

Current antiretroviral therapy for HIV-1 infection generally combines two or more NRTIs with a protease inhibitor (PI) and/or a non-nucleoside reverse transcriptase inhibitor (NNRTI), and such combinations can achieve full suppression of HIV-1 RNA (<50 copies/ml) in most HIV-1 infected patients. The advent of successful combination therapy has resulted in a dramatic decrease in AIDS mortality since 1996[8]. Despite these advances in AIDS therapy, HIV resistance to antiretroviral drugs can emerge during antiretroviral therapy, resulting in further failure of the current regimen to suppress viral replication and potentially compromising future treatment options[9]. There are multiple reasons why resistance develops, but the root cause is continued viral replication that allows viral mutants to replicate. Some of these mutant forms of HIV may have reduced susceptibility to one or more of the antiretroviral drugs in the regimen and this leads to the selec-

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tion or out-growth of a resistant virus. Successful combination therapy results in strong suppression of viral replication, and combines multiple drugs that would require multiple genetic mutations for a fully resistant virus to emerge.

Resistance to all of the approved NRTIs has been observed both *in vitro* and *in vivo*. Each NRTI induces a relatively defined set of resistance mutations that are located in the target enzyme RT. Two mechanisms of NRTI resistance have been defined to date. The first mechanism involves steric hindrance in which the resistance mutation directly interferes with the binding and incorporation of the NRTI, as observed for lamivudine and its signature RT mutation M184V[10]. The second mechanism involves ATP-mediated excision of the newly incorporated NRTI that is subsequently removed by RT in a reaction that is the reverse of the incorporation reaction[11]. The resistance mutations known as 'thymidine analogue mutations' or TAMs (M41L, D67N, K70R, L210W, T215F/Y and K219Q/E/N/R) that occur with zidovudine or stavudine exposure appear to mediate resistance via this mechanism[12].

In addition to their effects on zidovudine and stavudine susceptibility, the TAMs can mediate cross-resistance to all other NRTIs, even if those other NRTIs do not themselves select for TAMs. For example, cross-resistance to lamivudine in the presence of TAMs has been documented in lamivudine-naive patients despite the absence of the M184V mutation[13]. Susceptibility to abacavir is also reduced in the presence of TAMs and resistance rises notably with the addition of M184V[14]. The clinical significance of these reductions in abacavir susceptibility has been confirmed in several clinical trials[15,16]. Other multinucleoside resistance pathways such as the Q151M complex and T69 insertions, although rare, cause high-level resistance to most NRTIs[17,18].

From *in vitro* analyses, tenofovir has shown full activity against a wide variety of NRTI-resistant strains, including viruses with some TAMs (D67N + K70R + T215Y), didanosine (L74V) or zalcitabine (T69D) resistance mutations[19,20]. Susceptibility to tenofovir is enhanced in the presence of the M184V mutation that is selected by lamivudine or emtricitabine[19,21]. Unlike other NRTIs, tenofovir retains activity against the Q151M complex of mutations, whereas isolates carrying the T69 insertion mutations show high-level resistance to tenofovir[22]. Tenofovir can select for the K65R mutation *in vitro*, as can zalcitabine, didanosine, stavudine and abacavir[14,19,23-25].

In this review, the effects of pre-existing resistance mutations on the activity of tenofovir DF in treatment-experienced patients, and on the development of resistance to tenofovir DF in both treatment-experienced and treatment-naive patients from controlled clinical studies, will be summarized. Moreover, the currently available *in vitro* data on the K65R mutant, including its effects on HIV replication capacity and its potential for cross-resistance, will be reviewed and linked to the *in vivo* results in an attempt to provide a better understanding of the optimum use of tenofovir DF in antiretroviral therapy.

Clinical response to tenofovir DF in patients with pre-existing resistance

The clinical efficacy of tenofovir DF has been shown in phase II (GS-98-902) and phase III (GS-99-907) clinical trials in highly treatment-experienced patients[26-28]. These studies were randomized, double-blind, placebo-controlled, intensification studies of adding tenofovir DF to a patient's existing failing antiretroviral regimen. The design of these studies provided the opportunity to examine the specific activity of the single new drug in the regimen. Such an intensification protocol can only be used for drugs that do not develop resistance quickly. Previous studies of up to 28 days of tenofovir DF monotherapy had not shown any detectable development of resistance[29].

Patients in these studies had plasma HIV-RNA levels >400 copies/ml and <100,000 copies/ml (study 902) or <10,000 copies/ml (study 907) and had been on a failing antiretroviral regimen for ≥8 weeks prior to entry. Baseline HIV-1 genotypic data revealed that 94% of patients from both trials had plasma HIV-1 expressing one or more primary NRTI-associated resistance mutations in RT[30]. A similar percentage of patients from both studies had HIV-1 expressing various patterns of NRTI-associated mutations. Most patients (71%) had HIV-1 with TAMs at RT codons 41, 67, 70, 210, 215, or 219 (mean of 2.8 mutations), and 67% had HIV-1 with M184V/I mutations. Few patients at study entry had HIV-1 expressing the K65R mutation (1.4%, n = 6).

Despite the presence of RT resistance mutations at baseline, patients adding tenofovir DF 300 mg to their existing failing regimen demonstrated a significant decline in plasma HIV-RNA from baseline to week 24 ($DAVG_{24}$) of –0.58 $\log_{10}$ copies/ml in study 902 ($p < 0.001$ *vs* placebo, intent-to-treat) and –0.59 $\log_{10}$ copies/ml in study 907 ($p < .001$ *vs* placebo, intent-to-treat)[27,28]. Given the similar study designs, similar study populations, and nearly identical treatment responses in these studies, further analyses combined the tenofovir DF 300 mg arms (n = 222) and the placebo arms (n = 110) from each study.

Patients with HIV containing TAMs or M184V at baseline demonstrated statistically significant reductions in HIV-RNA compared to placebo[26,28]. However, patients without TAMs showed a significantly stronger HIV-RNA response (–0.80 $\log_{10}$ copies/ml) than patients with TAMs (–0.50 $\log_{10}$ copies/ml). Patients with just one or two TAMs had responses that were not significantly different than those without TAMs (Fig. 1)[31]. Patients whose HIV had the M184V mutation in the absence of TAMs had the strongest HIV-RNA response (–0.96 $\log_{10}$ copies/ml) and this was significantly superior to patients without M184V. Patients who entered these trials with a baseline K65R mutation did not show a treatment response to tenofovir DF (–0.01 $\log_{10}$ copies/ml).

Among patients with multiple TAMs (>2), two distinct TAM patterns were observed in these studies. There were highly significant positive correlations for the M41L, L210W and T215Y mutations to occur together[32]. Another set of positive correlations was observed for the D67N, K70R, K219Q/E/N/R and T215F mutations. Strongly negative correlations were observed for the K70R-M41L, K70R-L210W, and K70R-T215Y mutation pairs. Similar observations of two distinct patterns of TAMs have been previously described[33,34].

Response to treatment in patients with ≥3 TAMs differed markedly depending on which pattern of TAMs was present[31]. In the absence of the M41L and L210W mutations, patients with ≥3 TAMs (e.g. D67N, K70R, K219Q/E/N/R, ± T215F) showed an HIV-RNA response of –0.67 $\log_{10}$ copies/ml as compared to –0.21 $\log_{10}$ copies/ml in the presence of M41L or L210W (Fig. 1). The M41L and L210W mutations appeared to be the best predictor of reduced response since, in the absence of these mutations, patients with the T215Y mutation in their HIV-1 showed a –0.70 $\log_{10}$ HIV-RNA response.

Development of resistance mutations to tenofovir DF

Treatment-experienced patients

Patients in both studies 902 and 907 were monitored over 48 weeks for the emergence of new resistance mutations[26,28]. In study 902, 135 patients elected to continue study therapy through 96 weeks and they were also monitored for resistance development[35]. Overall, there was a high rate of new TAM development (40%, Fig. 2) during these studies, reflective of the fact that 90% of patients were taking either zidovudine or stavudine along with tenofovir DF in their regimen, and most patients had detectable viral replication. These results show that, even in cases of low-level viremia (median baseline HIV-RNA 3,200 copies/ml), there was a significant accumulation of TAMs over this course of study. Although a possible contribution of tenofovir DF toward selection of these TAMs can not be ruled out, more patients in the placebo arm had developed TAMs than in the tenofovir DF arm,

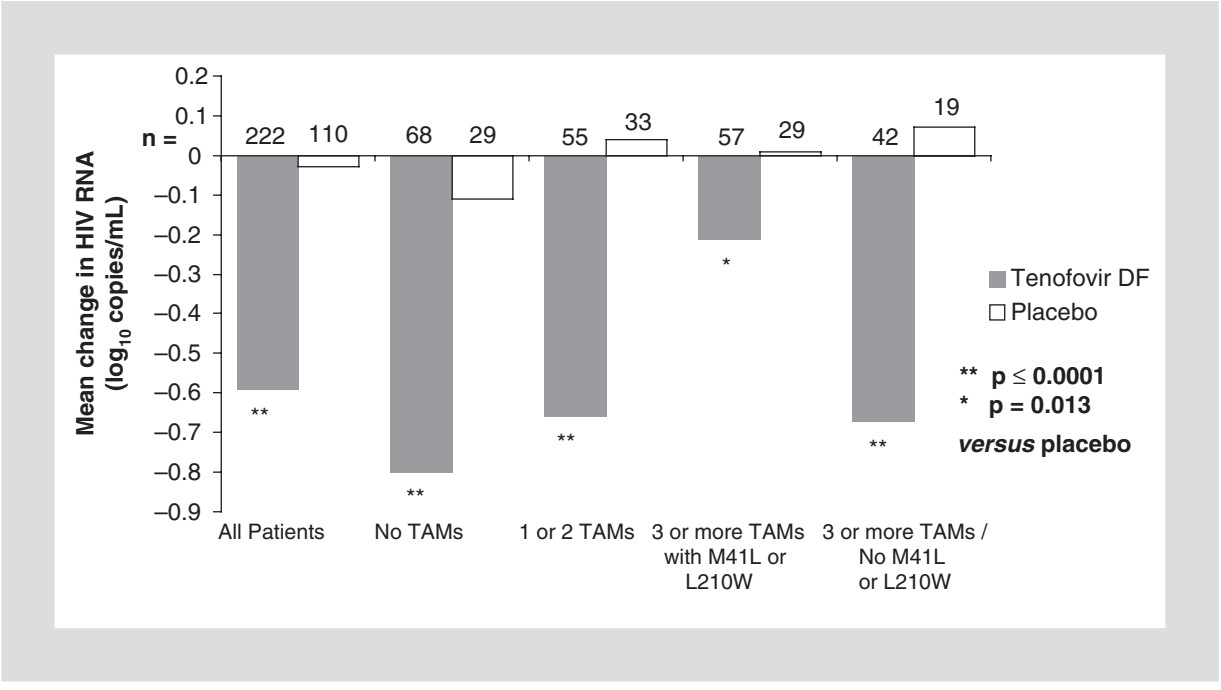

Figure 1. *Effect of type and number of TAMs on HIV-RNA response.*

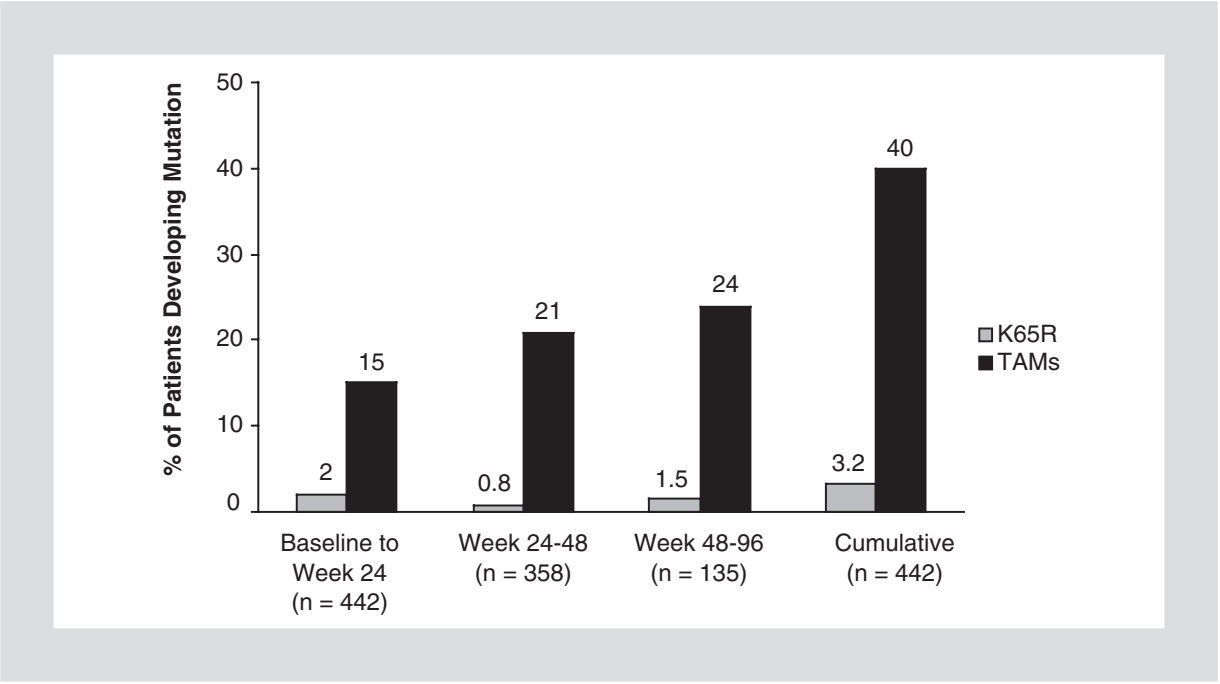

Figure 2. *Development of TAMs or K65R in treatment-experienced patients treated with TDF and other NRTIs.*

suggesting that the background therapy was predominantly driving TAM development[36].

In contrast, there was a relatively low rate of K65R development (3.2%) through 96 weeks of follow-up[37]. Also in contrast to TAM development, all patients who had developed K65R were in the tenofovir DF arm and none in placebo arm. Among the patients that developed K65R, 7 of 14 were taking a thymidine analogue concomitantly, and the remaining patients were taking either didanosine, abacavir or both. Thus, it is likely that tenofovir DF was primarily responsible for the development of K65R, but didanosine and/or abacavir may have contributed in some patients. The median time to develop the K65R mutation among these patients was 27 weeks. A surprising observation from these studies was that there was no HIV-RNA rebound associated with the development of the K65R mutation among these patients. The possible reasons for this observation will be discussed later.

Treatment-naive patients

Study GS-99-903 (study 903) was a 144-week, double blind, randomized phase III clinical trial evaluating the activity of tenofovir DF or stavudine in combination with lamivudine and efavirenz in treatment-naive patients (n = 600). At the week 96 interim analysis, 82% of patients in the tenofovir DF arm and 78% of patients in the stavudine arm had HIV-RNA below 400 copies/ml (intent-to-treat, missing = failure)[38]. Patients who failed to achieve viral load suppression of <400 copies/ml, or who demonstrated virologic rebound in HIV-RNA, were genotypically analyzed for the development of resistance mutations. A total of 74 patients met these criteria for analysis and had plasma samples available for analysis (36/299 in the tenofovir DF arm and 38/301 in the stavudine arm)[39]. Development of resistance mutations to the NNRTI class was the most common (6.5% of all patients) with no significant difference between treatment groups (Table 1). Among NRTI-associated resistance mutations, the most common mutation that developed was the M184V or I mutation, which is associated with resistance to lamivudine therapy. M184V/I occurred in 4.5% of patients with no difference between treatment groups. The K65R mutation occurred more frequently in the tenofovir DF arm (2.7%) as compared to the stavudine arm (0.7%, p = 0.06). The observation of K65R development in the stavudine arm demonstrates that treatment with either stavudine or lamivudine may also result in the development of this mutation on an infrequent basis. In all cases of K65R development, development of NNRTI or lamivudine resistance either preceded or was coincident with the development of K65R. From these analyses there were no other patterns of resistance attributable to tenofovir DF.

Phenotypic susceptibility data were obtained for all tenofovir DF-treated patients who developed the

Table 1. Development of resistance mutations through week 96 in study 903

	TDF + 3TC + EFV (n = 299)			d4T + 3TC + EFV (n = 301)		
	N	% of Total	% of Failures	N	% of Total	% of Failures
Virologic failures	36[2]	12%		38	12.6%	
Any EFV-R[1]	22[2]	7.4%	61.1%	17	5.6%	44.7%
Any M184V/I	14	4.7%	38.9%	13	4.3%	34.2%
Any K65R	8	2.7%	22.2%	2	0.7%	5.2%
Wild-type or as baseline	11	3.7%	30.6%	18	6.0%	47.3%

1. K103N, V106M, Y188C/L or G190A/S/E/Q (K103N in 28/39; others >50 fold EFV-R with other mutations).
2. Three patients (all in TDF arm) had >4-fold EFV-R at baseline and developed additional EFV-R.

K65R mutation (n = 8)[39]. Development of the K65R mutation was associated with minimal changes in tenofovir susceptibility (mean 1.3-fold, range 0.9 to 2.2-fold change from wild-type, ViroLogic PhenoSense™ HIV assay). There were no reductions in susceptibility to zidovudine or stavudine (mean 0.5-fold and 0.9-fold, respectively). There were changes in susceptibility for didanosine, abacavir, and lamivudine in patients who developed K65R (mean fold changes of 1.4, 2.3, 11-fold without M184V and 2.4, 4.8 and >50-fold with M184V, respectively); the magnitude of these changes appeared dependent on the presence of M184V. Phenotypic analyses of HIV from patients in the tenofovir DF treatment group who did not develop the K65R mutation showed no changes in tenofovir susceptibility upon virologic failure.

Follow-up data on the tenofovir DF-treated patients who developed K65R showed that five of these eight patients achieved < 50 copies/ml of HIV-RNA on their subsequent PI-based regimen (Table 2)[39]. Of the remaining patients, one patient was lost to follow-up, one patient was non-adherent and the third is still on study with no available follow-up. There was a wide range of NRTIs chosen, with five patients switching to zidovudine and two patients maintaining tenofovir DF therapy in addition to adding zidovudine or didanosine. Thus, it appears that multiple NRTIs have partial or full activity against the K65R mutant virus consistent with the *in vitro* data.

The development of the K65R mutation along with M184V and NNRTI-resistance among these eight patients was associated with HIV-RNA rebound. However, prior to beginning their new drug regimen, the HIV-RNA viral load showed a mean 0.9 $\log_{10}$ reduction with respect to the patient's pre-treatment value[39]. These clinical observations are in agreement with the *in vitro* observation that the K65R mutant viruses from these patients had evidence of a replication capacity defect. The mean replication capacity was 45% of wild-type among these patients (range 2 to 82%, ViroLogic PhenoSense™ assay). These results suggest that, among these patients with virologic failure and resistance mutations, either partial drug activity and/or some degree of replication defect within their mutant virus contributed to continued viral load suppression of approximately 1 log. These patients also had maintained a mean CD4 cell increase of 49 cells/mm^3 from baseline.

Table 2. Follow-up of tenofovir DF-treated patients who developed K65R

Patient	Next Regimen[1]	Response[2]	Follow-up[2]
1	TDF/AZT/LPV/r	<50, W32	<50, W120
2	TDF/3TC/ddI/LPV/r	<50, W36	<50, W108
3	ddI/d4T/IDV/r	<50, W72	<50,W120
4	ddI/IDV	<50, W44	<50, W120
5	AZT/3TC/SQV/r	<50, W48	D/C W48, lost to follow-up
6	AZT/ddI/NFV	423, W68	D/C W68; lost to follow-up
7	AZT/3TC/APV	1905, W32	Developed M184V at W48, non-adherence
8	AZT/3TC/LPV/r	Not available	On study; no additional follow-up available

1. TDF, tenofovir disoproxil fumarate; AZT, zidovudine; 3TC, lamivudine; ddI, didanosine; d4T, stavudine; LPV, lopinavir; IDV, indinavir; SQV, saquinavir; NFV, nelfinavir; APV, amprenavir; r, low dose ritonavir boosting
2. HIV-RNA (copies/ml) at indicated study week; D/C, study discontinuation

Effects of the K65R mutation on RT function and HIV replication capacity

The K65R mutation is a relatively old resistance mutation in the history of antiretroviral therapy. It was first discovered in 1994 as a result of *in vitro* selection experiments with zalcitabine and then subsequently in patients treated with zalcitabine[23,40]. As such, a number of biochemical and enzymological studies have been carried out with the K65R mutant RT. The mechanism of resistance to tenofovir and other NRTIs appears to be fairly simple – a binding and/or incorporation defect as measured in steady-state enzymatic analyses as an increased inhibitory constant (K_i)[19,41,42]. In pre steady-state enzyme kinetics analyses, the specific enzymatic defect was determined to be at the level of incorporation with an observed decrease in the catalytic rate constant (k_{pol})[43]. There have been multiple publications with regard to the processivity of the K65R mutant enzyme *vs* wild-type, with results ranging from increased processivity to decreased processivity[42,44]. It appears that the concentration of dNTPs used in these experiments significantly alters the results. There was also a study describing the increased fidelity of the K65R mutant relative to wild-type RT[45]. The physiological relevance of many of these observations remains to be established, but the results do show rather notable alterations in the enzymatic properties of the K65R mutant RT.

Analyses of the K65R mutant in the context of HIV replication *in vitro* may provide a more physiological situation with which to evaluate the effects of the K65R RT mutation. Using site-directed recombinant virus expressing the K65R mutation either alone or with the M184V mutation, White, et al. have demonstrated a decreased replication capacity of the mutant viruses in a single-cycle infection assay (ViroLogic PhenoSense™)[42]. The K65R single mutant replicated at 53% of wild-type and the double mutant with M184V replicated at 24% of wild-type. In a larger analysis of patient-derived recombinant viruses expressing a variety of NRTI-associated mutations, the effects of the K65R mutation on replication capacity were confirmed, with K65R mutant viruses demonstrating a mean replication capacity at 56% of wild-type (Fig. 3)[46]. There was a similar decrease in replication capacity observed for the M184V mutation, in agreement with previous analyses of the replication capacity of the M184V mutant HIV[47]. Of note, there were no significant decreases in replication capacity for viruses expressing multiple TAMs. It is possible that mutations such as TAMs that lie outside of the active site of the RT enzyme only minimally impact the replication capacity.

Recent enzymatic analyses of the K65R, M184V and double K65R + M184V mutant RTs have revealed a possible biochemical basis for the observed decreased in HIV replication assay for these mutants[48]. In these pre steady-state enzymatic analyses, a strong effect of the

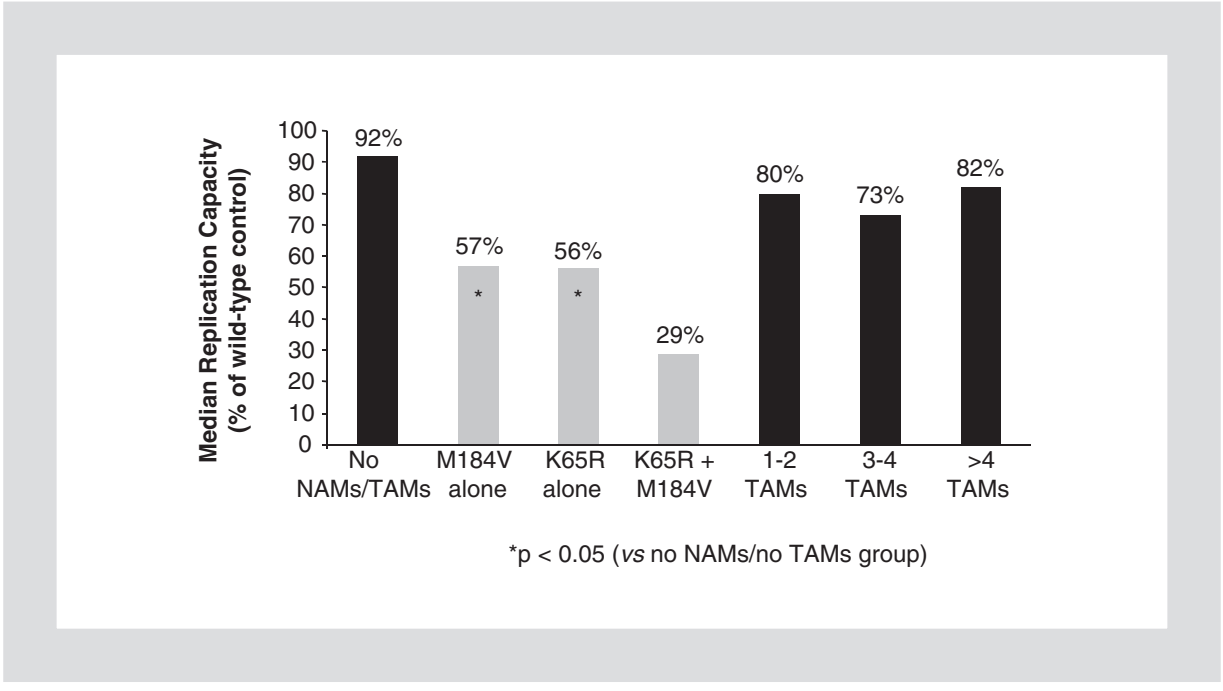

Figure 3. *Replication capacity of HIV from patient-derived virus.*

K65R mutation was observed on the ability of the mutant RT to incorporate natural substrates. The effect was most notable for incorporation of dATP, which demonstrated about a 4-fold decrease in the incorporation rate (k_{pol}). In the same study, an effect for the M184V mutation was also observed that was mediated primarily by a decrease in natural substrate binding (increased K_d). The combination of the two resistance mutations combined both of the debilitating effects of k_{pol} and K_d and resulted in the greatest decrease in the overall incorporation efficiency (k_{pol}/K_d) – 12-fold for dATP. These enzymatic results closely match the results from the single-cycle infection assays that showed defects for single K65R and M184V mutants that appeared additive when the mutations were combined.

Phenotypic susceptibilities of the K65R mutant HIV

Resistance and cross-resistance for the K65R mutant virus has been published several times using various assay systems[14,19,23,42]. There is general agreement in these results. In the traditional multi-round HIV infection assay, the K65R mutant shows 2- to 4-fold resistance to tenofovir, didanosine, zalcitabine and abacavir. There is a higher level of cross-resistance to lamivudine and emtricitabine (~10-fold). There is no measurable cross-resistance to zidovudine and, in fact, there appears to be a slight degree of hypersusceptibility relative to wild-type for zidovudine (0.5-fold). For stavudine, there is some detectable decrease in susceptibility measurable in some assays (1.8-fold). Recently, a cell-free assay was described that more readily detected resistance for stavudine *in vitro*[25].

Data from recombinant clinical isolates from the two major HIV phenotyping laboratories are largely in agreement with the published reports from site-directed recombinant viruses (Table 3)[49]. In the single-round infection PhenoSense™ assay, slightly lower absolute IC_{50} values are observed, as well as lower fold-changes for most drugs as compared to the multi-round infection assays. For both assays, there are notable changes in the susceptibility of K65R viruses to all NRTIs in the presence of M184V, as has been previously reported in the context of TAMs[50]. Specifically, the fold-resistance for K65R in the presence of M184V increases for abacavir, didanosine, zalcitabine and lamivudine. The fold-resistance decreases for tenofovir, stavudine and zidovudine.

The clinical significance of these susceptibility changes for tenofovir and other NRTIs must be interpreted in the context of clinical cut-offs for the individual drugs. Clinical cut-offs for tenofovir, abacavir, didanosine, lamivudine and stavudine have been established in the PhenoSense™ assay, and for tenofovir and abacavir in the Antivirogram™ assay. There are two types of clinical cut-offs that can be determined from treatment-response data. The first are the points at which a response begins to diminish, and these are the clinical cut-offs that are listed on the PhenoSense™ report. The second are the points at which there is no detectable treatment response, or it is clinically insignificant (e.g. <0.3 $\log_{10}$). This second cut-off can be more difficult to determine and is only available for tenofovir and abacavir. For tenofovir, those clinical cut-offs are 1.4-fold and 4-fold in the PhenoSense™ assay, respectively. For abacavir, they are 4.5-fold and 6.5-fold, respectively. Using the first cut-off for beginning of reduced responses, the proportions of patients with K65R or K65R + M184V that are below the cut-off are listed in table 3. From 90-100% of the K65R patient isolates were below the cut-offs for zidovudine, stavu-

Table 3. Phenotypic susceptibility of K65R and K65R + M184V patient viruses

Drug (cut-off)[1]	K65R (n = 50)		K65R + M184V (n = 58)	
	Median fold change	% below cut-off	Median fold change	% below cut-off
Tenofovir (1.4)	1.6	30%	0.8	88%
Zidovudine (1.9)	0.5	100%	0.3	100%
Stavudine (1.7)	1.2	90%	0.9	100%
Abacavir (4.5)	2.3	100%	3.9	55%
Didanosine (1.7)	1.7	50%	1.9	42%
Zalcitabine (1.7)	2.3	18%	2.6	18%
Lamivudine (3.5)	8.4	16%	>100	3%

1. Phenotypic cut-off established for each drug in PhenoSense™ assay

dine and abacavir; 50% were below the cut-off for didanosine. For K65R + M184V, 100% of isolates were below the cut-off for zidovudine and stavudine, and 42-55% of isolates were below the cut-offs for didanosine and abacavir, respectively. For tenofovir, 30% of K65R isolates were below the 1.4-fold cut-off, and this increased to 88% for isolates with K65R + M184V. Only 1% of samples in the K65R group, and no samples in the K65R + M184V group, were above the 4-fold cut-off for no response to tenofovir DF. Overall, there are low-level susceptibility changes observed for this panel of clinical isolates, and most isolates show full or partial drug susceptibility to multiple NRTIs, including tenofovir.

For lamivudine, on the other hand, the median susceptibility value for the K65R mutant was 8.4-fold, with only 16% of samples below the clinical cut-off of 3.5-fold. Therefore, these results would indicate that most patient viruses with K65R would show a decrease in activity for lamivudine. However, there is no upper cut-off for lamivudine which defines no response. Given the recent observation that there is potential activity of lamivudine even in the context of M184V and much higher fold-resistance values, it is possible that the lower levels of resistance associated with the K65R mutation may also be insufficient for complete resistance to lamivudine[51]. Additional clinical data would be necessary to determine the validity of this hypothesis.

There are several caveats to interpreting the results of phenotyping assays. First, there is an inherent variability in these biological assays that depends on the drug tested and the assay employed. This variability can range from 1.4-fold to up to 2- or 3-fold. For example, an individual fold-change value for a given patient may indicate 1.4-fold for tenofovir on the PhenoSense™ assay. This value is exactly at the clinically determined cut-off for the beginning of reduced response to tenofovir DF. With a 1.4-fold variation in its value, this value could read 1.0 or 2.0 upon a repeat assay, which puts the sample either below or above the first cut-off. Thus, values that are near cut-offs must be interpreted with appropriate caution, and may be conservatively considered to have some degree of reduced susceptibility. Second, effects of genetic mixtures of mutant and wild-type can affect the final fold-change value that is reported. In the case of tenofovir, the effect of K65R mixtures on the resultant phenotype was recently described[52]. For samples with only the K65R mutation, those without evidence of mixture with wild-type showed a mean 1.8-fold reduction in susceptibility to tenofovir (n = 42) *vs* 0.9-fold for samples that had genetic evidence of K65R plus wild-type mixtures (n = 8). In these cases it would be necessary to also obtain the genotype of a patient's virus in order to identify that the K65R mutant was present as a mixture. If mixtures are excluded from the data in table 3, the percentage below the tenofovir cut-offs would be slightly reduced to 28% for K65R viruses and 78% for K65R + M184V viruses. It is likely that a full mutation would develop with continued therapy and, as such, one could assume a phenotype more typically observed for a sample with a full K65R mutation. Such an effect of genetic mixtures on the phenotyping assay has been shown to affect other drugs and other mutations as well[53].

Compatibility and incompatibility of K65R with other RT mutations

As described above in the context of TAMs, there may be specific types of RT mutations that are compatible or incompatible with one another for a given mutant enzyme. Incompatible mutations are most likely due to dominant structural constraints of the enzyme and/or strong functional constraints such that the mutant virus fails to replicate. A recent study by Gonzales, et al. identified three major patterns of NRTI resistance mutations: the two patterns of TAMs mutations as described above and a set of mutations associated with the Q151M complex of multinucleoside resistance[54]. In this database analysis, the K65R mutation clustered with the Q151M mutation along with mutations at RT positions 75, 77, 115 and 116. The M184V mutation did not cluster, but was found with all three groups. Similarly, the L74V did not cluster specifically with any group.

Recently, a set of patient-derived viruses with the K65R mutation submitted to ViroLogic for routine resistance analyses were identified and analyzed (n = 288)[52]. The specific drug histories of these patient samples are not known, but the time of sample acquisition is consistent with increased exposure to tenofovir DF and abacavir as a result of increased prescriptions of these drugs. Among these samples, the most common other mutation observed was the M184V mutation (55%). The Q151M mutation, generally associated with other mutations of the typical multinucleoside resistance complex (62, 75, 77 and 116), was observed in 16% of these samples. Of note, however, the proportion of samples expressing Q151M was highest at the earliest acquisition period prior to the availability of tenofovir DF, and was lowest in the last quarter. Phenotypic resistance to tenofovir among these samples with both Q151M complex and K65R, however, was the highest observed for tenofovir, with a median fold-change of

>4-fold. Thus, most samples of Q151M complex + K65R would lie above the 4-fold upper clinical cut-off for a lack of response to tenofovir DF.

The majority of K65R samples (84%) did not contain the Q151M mutation. Other mutations that were observed to frequently associate with K65R, in addition to M184V, were K20R, A62V, S68G, Y115F, K219E/R and H221Y. Y115F is specifically associated with abacavir therapy and can be observed with either L74V or K65R. K20R, A62V and H221Y have been previously identified as associated with NRTI therapy at a low frequency[40,54]. S68G was recently described in association with K65R in patients treated with abacavir, didanosine and stavudine[55] and previously in patients treated with didanosine[40]. Both A62V and S68G have been observed to develop along with K65R in clinical trials of tenofovir DF. These mutations do not significantly affect the resistance level of K65R to tenofovir with fold-change values changing from 1.7-fold for K65R alone to 1.8- and 1.9-fold for K65R + S62V and K65R + S68G, respectively[39,40]. Given these observations, these mutations more likely represent compensatory mutations for the affects of the K65R mutation on RT replication capacity. These putative compensatory mutations are only partial, however, since the K65R patient-derived samples that showed decreased replication capacity also expressed these mutations.

The L74V mutation, associated with both didanosine and abacavir therapy, was observed to frequently associate with K65R in the entire dataset. However, when specifically analyzing viruses that did not show mixtures of K65R with wild-type, the frequency of L74V in the population declined notably. Moreover, the vast majority of L74V mutations were themselves present as mixtures with wild-type. A published study of patients on didanosine monotherapy reported that, although most patients developed L74V, a minority of patients developed K65R and one patient appeared to have both mutations in their HIV[56]. However, upon clonal analysis of 29 patient-derived HIV clones, each analyzed genome had either K65R or L74V, and none had both. This study also observed the S68G mutation occurring, and only in association with K65R. In early abacavir monotherapy studies, a similar dichotomy between L74V and K65R development was observed[57,58]. Although L74V tends to dominate the resistance profile for both abacavir and didanosine, it is not clear why resistance follows either the L74V or K65R pathways. L74V appears to provide a slightly greater level of resistance to abacavir and this may favor selection of L74V[14]. However, differential effects on the overall fitness of the K65R vs L74V mutants may also play a role. In any case, use of either didanosine or abacavir should be considered to potentially select for either mutation, and possibly low levels of an undetected variant population may be present in patients taking either drug.

Mutations at position K219 in RT were the only TAM mutation observed to frequently associate with K65R. Interestingly, the specific amino acid substitutions were limited to K219E and K219R, whereas among viruses with multiple TAMs K219Q and K219N are much more common. The lack of other TAMs in conjunction with K65R in this analysis may be due to several reasons. First, it could be that the specific drug regimens used by these patients did not include drugs capable of selecting TAMs in addition to K65R. This is unlikely since the vast majority of patients treated during this period were taking either zidovudine or stavudine in their regimens. Second, it is possible that the degree of cross-resistance associated with TAMs to drugs that would on their own select K65R is sufficient such that addition of K65R does not result in greater drug-resistance levels. Third, it is possible that K65R is incompatible with most TAMs for functional or structural reasons in the RT enzyme.

It is difficult to distinguish between these latter two possibilities and they are not mutually exclusive. Data that support both hypotheses can be derived from the tenofovir DF and abacavir clinical trials. For tenofovir DF, the only patients that developed K65R did not have TAMs in their virus prior to treatment. This was a striking finding, since over 70% of patients enrolled in the treatment-experienced studies had TAMs. Half of the patients who developed K65R in these trials were taking either zidovudine or stavudine concomitantly. In a meta-analysis of abacavir resistance development, there was a strong correlation for K65R development in patients taking abacavir without zidovudine[59]. In the presence of zidovudine, however, patients developed TAMs instead. Overall, the observational data to date are consistent with these two pathways of resistance being separate, and whether this is due to functional constraints on the enzyme, or due to sufficient cross-resistance of the TAMs to the NRTIs class, is not clear. An *in vitro* study offers some insight here. Virus with both multiple TAMs and K65R is replication-viable *in vitro* and, interestingly, addition of the K65R mutation to the TAM background completely resensitized the virus to zidovudine (from 48-fold resistant to 1.3-fold)[60]. A similar observation was made for the addition of the L74V mutation; however the fold-resistance to zidovu-

dine was still 6.3-fold. Thus, acquisition of the K65R mutation in the background of TAMs would reduce resistance to zidovudine significantly while not necessarily increasing resistance to tenofovir. Moreover, the resultant virus would be less fit due to K65R, though clearly still viable in cell culture. Given the different NRTI resistance pathways available to the virus, which particular pathway is taken under conditions of suboptimal therapy will be dictated by the NRTIs in the regimen and the baseline genotype of the patient. From the data thus far, it appears that the TAM pathway dominates when zidovudine is present in the regimen or when TAMs have already been established.

Conclusions and commentary

The convenience of a single pill once-daily with an excellent tolerability profile makes tenofovir DF an attractive component of most regimens. The results from the clinical trials in treatment-experienced patients have demonstrated the efficacy of tenofovir DF in patients with extensive resistance mutations in their HIV. Moreover, in these trials where TAM pathways of resistance were already established in most patients, there was a very low rate of resistance development to tenofovir DF (3.2%) despite the fact that most patients continued to have detectable virus replication. Interestingly, among those patients who did develop K65R, there was no evidence of viral load rebound and generally only low-level changes in tenofovir susceptibility. The simplest explanation for the lack of viral load rebound is reduced viral fitness of the K65R mutant virus. Given the low-level phenotypic changes observed for tenofovir, there is also the possibility of residual tenofovir DF activity in these patients. Clinical trials specifically designed to distinguish between these two possibilities could be easily designed.

The clinical trial results from study 903 demonstrate that the combination of tenofovir DF plus lamivudine and efavirenz is an extremely potent and well-tolerated regimen for treatment-naive patients. In intent-to-treat analyses through 96 weeks, 82% of patients have HIV RNA < 400 copies/ml and 78% have < 50 copies/ml. With this regimen, resistance emergence was infrequent and occurred in 8.4% of patients through 96 weeks. The most common resistance mutations were to efavirenz (7.4%), followed by lamivudine (4.7%), and then tenofovir DF (2.7%). Among the patients who had developed the K65R mutation, all had developed resistance to efavirenz and most to lamivudine as well. Nevertheless, these patients went on to successful second-line regimens of protease inhibitors and various NRTIs. Such successful results are expected from patients who fail an initial NNRTI-based regimen, and the results suggest that the presence of the K65R mutation was not complicating the success of the second-line regimen. Longer term follow-up on these and other patients who fail a regimen and develop a K65R mutation is required, though, to determine the longer term durability of second-line regimens and potential follow-on regimens.

There is a strong desire among patients and physicians to simplify regimens and to preserve future treatment options as much as possible. The best example of this is the simplicity and class-sparing use of Trizivir™, the all-in-one combination of zidovudine plus abacavir plus lamivudine given twice-daily. Although this regimen can be successful in many patients, the regimen has been shown to be inferior to those combining drugs from multiple drug classes, and this has resulted in the closure of one arm of a large clinical trial[61]. Tenofovir DF is also being studied in various class-sparing and simplified regimens. However, recent results from two clinical studies of tenofovir DF plus lamivudine plus abacavir used in a once-daily regimen showed a very poor response rate and resulted in the premature closure of these trials[62,63]. In these regimens, abacavir was being used investigationally as a once-daily drug. With this once-daily regimen, a high proportion of patients developed resistance mutations, with nearly 100% of treatment failures developing the M184V mutation, and approximately half of those patients additionally developing K65R. Since these two mutations should affect all drugs in this regimen, it is understandable how they would develop under conditions of sub-optimal therapy. The underlying factor, however, may be the overall poor regimen efficacy of this once-daily regimen. This is further evidenced by the fact that approximately half of the treatment failures only exhibited the M184V mutation, such that failure of the regimen was not associated with resistance to all drugs in the regimen. The basis for the poor overall efficacy is unknown. Pharmacokinetic studies have shown no interactions resulting in lower plasma drug levels, and *in vitro* antiviral assays have not revealed any antagonistic effects for the drug combinations.

So, which 'simplified' regimens should be used for first-line therapy? Until adequate 'all nuke' regimens have been clearly defined in clinical studies, it appears that caution for these types of regimens is warranted. Combining drugs from multiple drug classes has proven efficacy and, in addition, has the cross-class benefit of establishing a higher resistance barrier in the

case of suboptimal drug levels. Tenofovir DF could be a component in many of these antiretroviral regimens. It is a potent anti-HIV drug that is well tolerated and has a long plasma and intracellular half-life suitable for once-daily dosing. In combination with other licensed once-daily antiretrovirals, it is feasible to create an entirely once-daily regimen for the treatment of HIV that may provide for greater treatment adherence. As treatment adherence is one of the most important aspects of long-term success in antiretroviral therapy, the use of tenofovir DF in regimens may be quite useful for the long-term control of HIV in the infected individual.

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