Mechanistic Basis for Reduced Viral and Enzymatic Fitness of HIV-1 Reverse Transcriptase Containing Both K65R and M184V Mutations*

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HIV-1 drug resistance mutations are often inversely correlated with viral fitness, which remains poorly described at the molecular level. Some resistance mutations can also suppress resistance caused by other resistance mutations. We report the molecular mechanisms by which a virus resistant to lamivudine with the M184V reverse transcriptase mutation shows increased susceptibility to tenofovir and can suppress the effects of the tenofovir resistance mutation K65R. Additionally, we report how the decreased viral replication capacity of resistant viruses is directly linked to their decreased ability to use natural nucleotide substrates and that combination of the K65R and M184V resistance mutations leads to greater decreases in viral replication capacity. All together, these results define at the molecular level how nucleoside-resistant viruses can be driven to reduced viral fitness.

Nucleoside analogue drugs are core constituents of antiretroviral regimens to control HIV-1 infection (1). Once activated to the 5'-triphosphate state in the infected cell, they target the viral reverse transcriptase (RT), a specialized DNA polymerase encoded by the viral pol gene. In the clinic, however, treatment failures can occur that result in the development of drug-resistant virus. Both the high adaptive capacity of HIV-1 and the incomplete suppression of viral replication are responsible for the selection of drug-resistant viruses (2, 3). Controlling the appearance and spread of drug-resistant viruses requires novel drugs of increased potency that do not readily elicit resistance, and that are active against resistant viruses.

The potency of a nucleoside RT inhibitor (NRTI) as an inhibitor of viral replication depends on several factors. First, the NRTI must be activated to the 5'-triphosphate state by cellular kinases. Second, the analogue 5'-triphosphate must be an efficient substrate for RT that competes significantly at its cellular concentration with its natural dNTP counterpart to terminate viral DNA chain extension. Third, it is increasingly apparent that stability of the analogue-terminated DNA and inefficient excision by RT are key factors in sustained inhibition. Viral drug resistance has been shown to involve the second and third steps listed above (4).

There is intense ongoing research aimed at elucidating resistance mechanisms at the molecular level. In most cases, the corresponding substituted RT (referred to as mutant, mutated, or resistant therein) has altered biochemical properties accounting for viral resistance. At present, two classes of resistance mechanisms for NRTIs have been characterized. The first class involves an increased discrimination of the nucleotide analogue 5'-triphosphate at the RT active site relative to the natural substrate dNTP. Discrimination can be achieved either through selective decreased binding of the 5'-triphosphate analogue (reflected by an increase in the binding equilibrium constant $K_d$) or at the catalytic step of incorporation of the analogue 5'-monophosphate into viral DNA (reflected by a decrease of $k_{cat}$/value, the catalytic constant of incorporation of the nucleotide analogue into DNA). Lamivudine (3TC) and 2'-3' dideoxynucleosides (ddNs, e.g. ddI and ddC) typically select resistance mutations conferring increased discrimination properties to the resistant RT (5–7).

The other class of resistance mechanisms involves repair of the analogue-terminated DNA chain. In this mechanism, the resistant RT exhibits enhanced repair (also named excision or unblocking) of the analogue monophosphate-terminated DNA chain. The repair reaction utilizes pyrophosphate (PP$\prime$) or a nucleoside 5'-triphosphate (NTP) as a co-substrate to unblock the analogue 5'-monophosphate terminated DNA chain in a reaction chemically similar to pyrophosphorolysis, the reversal of the polymerization reaction (8, 9). Zidovudine (AZT) or stavudine (d4T) can select for RT mutations such as M41L, D67N, K70R, L210W, T215Y/F, or K219(Q/E/N/R) that act by decreasing $k_{cat}$ or RT burst rate.

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The abbreviations used are: HIV-1, human immunodeficiency virus; type I NRTI, nucleoside reverse transcriptase inhibitor; RT, reverse transcriptase; 3TC, 2'-3' dideoxycytidine; 3TC-TP, 2'-3'-dideoxycytidine 5'-triphosphate; tenofovir-PP, tenofovir diphosphate; dNTP, 2'-dideoxynucleoside 5'-triphosphate; Inorganic pyrophosphate, PP$\prime$; $k_{cat}$/value, catalytic constant; $k_{cat}$/$k_{cat}$, catalytic constant; $K_d$, dissociation constant; $K_m$, Michaelis constant; $V_{max}$, maximum velocity.
such as 3TC and AZT (14, 15) select mutant RTs that have antagonistic resistance mutations with respect to 3TC decrease resistance to AZT. Treatment combining AZT with 3TC leads to a more sustained efficacy than treatment with either alone, although virus resistant to both drugs will eventually evolve without adequate viral load suppression. Consequently, it is possible that certain combinations of drugs might deliberately lead to a genetically defined virus with altered fitness and a resistance profile such that the virus remains fully or partially susceptible to drugs in the current or next regimen.

Tenofovir disoproxil fumarate is an orally bioavailable form of tenofovir that has shown potent and durable efficacy in phase III clinical trials (16). Tenofovir is an acyclic nucleotide phosphonate analogue of AMP that is active on RT in its diprophosphate form (tenofovir-PP). The RT mutation K65R appears to be the only mutation selected for by tenofovir in vitro and in vivo, and results in low level resistance to tenofovir (17, 18). This resistance is partially to fully suppressed when present in combination with the 3TC/emtricitabine (FTC)/resistance mutation M184V (17–19). In this study, we focused on RT resistance mutations acting through discrimination of the nucleotide analogue relative to its natural counterpart, specifically K65R, M184V, and the multidrug-resistant Q151M complex mutations, and their effects on drug susceptibility, RT enzymatic function, and viral replication capacity. We give a mechanistic basis for the reduced resistance, as well as the impaired viral and enzymatic fitness of HIV-1 reverse transcriptase containing both K65R and M184V mutations.

**EXPERIMENTAL PROCEDURES**

**Phenotypic Assays of Drug Susceptibility**—Phenotypic data for tenofovir, zidovudine (AZT), lamivudine (3TC), didanosine (ddI), stavudine (d4T), and abacavir were obtained for patient-derived recombinant viruses present in the Virco library using the Antivirogram assay (Virco Central Virological Laboratory, Mechelen, Belgium). The antiretroviral history of patients from whom these viruses were obtained is unknown. Viruses were chosen based on their RT genotype of M184V alone (n = 10), K65R alone (n = 5), K65R in combination with M184V (n = 5), and the combination of Q151M, V75I, F77L, and F116Y (n = 5).

**Assay of Viral Replication Capacity**—Replication capacity was measured using a single cycle virus growth assay as previously described with patient-derived recombinant viruses present in the Virco library using the Antivirogram assay (Virco Central Virological Laboratory, Mechelen, Belgium). The antiretroviral history of patients from whom these viruses were obtained is unknown. Viruses were chosen based on their RT genotype of M184V alone (n = 10), K65R alone (n = 5), K65R in combination with M184V (n = 5), and the combination of Q151M, V75I, F77L, and F116Y (n = 5).

**Molecular Modeling of Tenofovir-PP in the RT Active Site**—The molecular modeling was performed with GenMol (23–25) with its all-atom force field, from the x-ray crystal structure of wild type ternary complex RT-DNA-dTTP (26). We extracted amino acids surrounding the catalytic domain of the enzyme, as described for the crystal structure of HIV-RT, and the residues in the 3′ region of HIV-RT included the residues of both p66 and p51 domain, DNA template/primer, two catalytic magnesium ions, and dTTP. Close attention was given for both magnesium ions, because they have a key role in positioning the incoming dNTP or NRTI and in catalysis. In order to prepare the closed RT-DNA-dTTP complex (26), the authors used a ddGTP primer terminus positioned at the so-called “Priming site” (P site). We replaced ddGTP by dGTP and interactions of its 3′-OH with Mg2+ were optimized. In the crystal structure, both magnesium ions were complexed only with residues Asp185, Asp110, and Val111. By a conformational analysis, we optimized the complexation process of both Mg2+. The complexation process of Mg2+, with the RT-DNA-dTTP ternary complex, was performed in the radius of 5 Å around the magnesium. This radius of 5 Å around the magnesium included the residues of both p66 and p51 domain, DNA template/primer, two catalytic magnesium ions, and dTTP. Close attention was given for both magnesium ions, because they have a key role in positioning the incoming dNTP or NRTI and in catalysis.
position 5 of the template was mutated to the counterpart of the incoming nucleotide dATP and tenofovir-PP (A to T). The conformation of 5th template nucleotide and the side chain of selected amino acid were minimized within the Biopolymer module of GenMol, using a conformational analysis method. Finally, the incoming dATP or tenofovir-PP were docked into the DNA-polymerase active site from the positioning template nucleotide and the side chain of selected amino acid were using GenMol All-Atom force field.

RESULTS

The interplay between suppressive mutations and viral fitness bears potential to increase the efficacy of antiretroviral drug regimens. The aim of our study was to determine the potential for tenofovir in such a strategy. We investigated the mechanism of drug resistance to tenofovir by its most relevant mutation K65R in order to identify which drug would be best combined with tenofovir in terms of resistance suppression, as well as resistance mutations that would maximize the alteration of viral replication capacity.

**HIV-1 Genotypes Associated with Reduced Susceptibility to Tenofovir**—Table I shows antiviral resistances determined using the Virco Antivirogram assay with tenofovir, zidovudine (AZT), lamivudine (3TC), didanosine (ddI), stavudine (d4T), and abacavir.

### Table I

<table>
<thead>
<tr>
<th>Resistance group</th>
<th>N</th>
<th>Tenofovir</th>
<th>Mean fold decrease in susceptibility from wild-type (range)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>AZT</th>
<th>3TC</th>
<th>d4T</th>
<th>Abacavir</th>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>K65R</td>
<td>5</td>
<td>2.7 (1.8-3.8)</td>
<td>1.0 (0.7-1.9)</td>
<td>7.7 (1.1-15.6)</td>
<td>2.0 (0.9-3.2)</td>
<td>1.6 (1.0-2.0)</td>
<td>2.9 (2.3-3.5)</td>
</tr>
<tr>
<td>M184V</td>
<td>10</td>
<td>0.7 (0.3-1.3)</td>
<td>0.9 (0.2-2.5)</td>
<td>&gt;50 (&gt;50)</td>
<td>1.0 (0.3-2.4)</td>
<td>1.4 (0.4-2.8)</td>
<td>1.3 (0.9-2.4)</td>
</tr>
<tr>
<td>K65R + M184V</td>
<td>5</td>
<td>1.7 (0.4-3.5)</td>
<td>0.7 (0.6-1.1)</td>
<td>&gt;50 (&gt;50)</td>
<td>2.5 (1.9-2.9)</td>
<td>1.0 (0.4-1.7)</td>
<td>7.6 (2.3-14.3)</td>
</tr>
<tr>
<td>Q151M complex</td>
<td>5</td>
<td>1.8 (1.1-3.0)</td>
<td>43 (9.6-85)</td>
<td>21 (1.3-2.6)</td>
<td>13 (6.4-91)</td>
<td>20 (6.1-57)</td>
<td>11 (3.0-24)</td>
</tr>
</tbody>
</table>

* Fold change versus wild-type control using the Virco Antivirogram assay with tenofovir, zidovudine (AZT), lamivudine (3TC), didanosine (ddI), stavudine (d4T), and abacavir.

<sup>b</sup> Statistically significant decrease in susceptibility as compared to a panel of 10 viruses with wild-type HIV sequence (p < 0.01, Student’s t test).

Mechanisms of RT-mediated Tenofovir-PP Resistance—A nucleotide analogue 5'-triphosphate is characterized by its efficiency of incorporation (k_

\text{pol}/K_d) into DNA as compared with that of its natural counterpart. For a given RT, comparing k_

\text{pol}/K_d values between a natural dNTP and its corresponding analogue is a convenient manner to evaluate selectivity for (or discrimination of) the analogue. Comparing selectivity between RTs defines in vitro resistance (or susceptibility) at the enzymatic level. The results are presented in Table II and Fig. 1. K65R was found to be the most tenofovir-PP resistant RT, in accordance with the fact that this is the only tenofovir-selected mutation that has been shown to develop in vitro and in tenofovir-treated patients to date (17, 18). This mutation is known to occur at low frequency during regimens making use of dideoxynucleosides (ddC and ddI) as well as abacavir. The molecular mechanism of resistance to ddN by K65R is known in great detail: ddNTPs bind to K65R RT with the same affinity as dNTPs, but their incorporation rate is decreased selectively, accounting for resistance (6).

As shown in Table II and Fig. 1, this mechanism was also found to be valid in the case of tenofovir-PP which binds to K65R RT with the same affinity (K_

\text{d}_\text{a} = 18 \mu M) as wild-type RT (K_

\text{d}_\text{a} = 23 \mu M) whereas the rate of insertion of the analogue into DNA (k_

\text{pol}) is decreased 20-fold (from 7 to 0.32 s⁻¹). The comparison with dATP provides an overall enzymatic resistance to tenofovir-PP of 4.4-fold, in accordance with the low-level of viral resistance shown in Table I and as previously published (17, 19). When these types of experiments were performed using M184V RT, an affinity constant similar to that of wild-type (K_

\text{d}_\text{a} = 30 \mu M) was found and the catalytic constant k_

\text{pol} also remained unchanged at 6.9 s⁻¹. The remarkably similar kinetic profiles between wild-type RT and M184V RT for tenofovir-PP can be visualized on Fig. 1A. Notably, as further described below, dATP is a 3.7-fold poorer substrate for M184V RT than for wild-type RT (k_

\text{pol}/K_d drops from 6.7 to 1.8 s⁻¹), whereas tenofovir-PP is equally effective for these two RTs. Thus, relative to dATP, M184V RT discriminates tenofovir-PP less effectively than wild-type RT (8 versus 22-fold, respectively). As a result, M184V RT is actually 2.5-fold more sensitive to tenofovir-PP than wild-type RT. When the two mutations are combined, the susceptibility of K65R/M184V RT to tenofovir-PP is similar to that of wild-type RT. The presence of M184V in a K65R background increases the k_

\text{pol} for tenofovir-PP but also increases its K_d resulting in a similar k_

\text{pol}/K_d ratio as K65R alone. However, relative to dATP, which shows a 3-fold decrease in k_

\text{pol}/K_d for the double mutant versus K65R, the discrimination capacity is reduced 2.6-fold for tenofovir-PP accounting for a susceptibility to tenofovir-PP that is near wild-type RT. M184V thus has a suppressive effect on K65R-mediated tenofovir resistance in agreement with the phenotypic results presented above.

This observation prompted us to examine the effect of K65R on 3TC resistance. K65R is able to promote 3TC-TP resistance as judged by 8.6-fold resistance relative to wild-type RT that is mediated by decreased k_

\text{pol} (Table II). In contrast, M184V RT promotes 30-fold 3TC-TP resistance that is mediated by increased K_d as previously described (5, 7). Interestingly, the presence of both K65R and
M184V combines both types of resistance, i.e., decreased $k_{pol}$ and increased $K_a$ (illustrated on Fig. 1B) for 3TC-TP. The double mutant K65R/M184V is thus a “3TC super resistant” RT (see Fig. 1C), but its clinical relevance to 3TC resistance is likely to be low since M184V alone already provides the highest measurable levels of 3TC-resistance, >50-fold.

**Molecular Modeling of Tenofovir-PP in the RT Active Site—**Molecular modeling was used to visualize how mutations can affect the positioning of nucleotides and tenofovir in the HIV-1 RT DNA polymerase catalytic domain (Fig. 2). We first evaluated the positionning of tenofovir-PP in the active site of the wild-type RT. Tenofovir-PP binds to the N-site of RT through electrostatic and hydrophobic interactions. The propyl chain of the tenofovir-PP stacks to the side chain of Tyr-115, making hydrophobic interactions (Fig. 2A). The oxygen atom of the tenofovir-PP linker makes electrostatic interaction with the N-H main chain of Tyr-115 (not shown). This orientation of the methyl group in the acyclic linker of the tenofovir-PP avoids an electrostatic interaction with the residues 114, 115 and 116 of the hydrophilic 3'-OH binding pocket. However, with this orientation, the methyl group is in close contact with the Gln-151 side chain (Gln-151 in pink, Fig. 2A). In order to avoid any steric hindrance while keeping a correct positioning of tenofovir-PP in the N-site, the Gln-151 rotates slightly in the ternary complex (Fig. 2A). Similar observations of a rotated Gln-151 side chain have been made from a recent crystal structure of tenofovir-PP bound in a ternary complex. The existence of these specific interactions allow tenofovir-PP to have favorable interaction with the N-site of HIV-1 RT and a correct orientation of its α-phosphonate for catalysis, in agreement with the relatively high incorporation efficiency of tenofovir-PP by wild-type RT observed with pre-steady state kinetics (Table II).

The M184V mutation was also introduced in the model in order to evaluate its effects. When a cyclic deoxyribonucleotide is modeled (here dATP), the oxolane ring of the ribose comes in the close vicinity of the van der Waals surface (VDW) of the methyl group of Val-184 side chain, providing a nonfavorable interaction (Fig. 2B, red arrow) between a hydrophobic surface (Val-184 side chain, surface in blue) and a hydrophilic polar surface corresponding to the oxygen atom of the dATP ribose ring (polar surface in red). Similar observations of steric clash with Val-184 and the sulfur atom of the oxolane ring of 3TC-TP have been made by Sarafianos et al. (27). Interestingly, relative to Met-184, the presence of the bulkier Val-184 (a β-branched methyl amino acid) does not alter the specific binding mode of the acyclic tenofovir-PP. These observations are in agreement with our kinetic data showing that tenofovir-PP is not affected in its binding affinity to M184V RT, whereas dATP, dCTP, and 3TC-TP all bind with less affinity to M184V RT than to wild-type RT. These results prompted us to examine whether a decreased affinity of all natural nucleotides for RT could be measured in vitro and whether this would have an impact on HIV replication capacity.

### Table II

Pre-steady state kinetic constants of dATP/tenofovir-PP and dCTP/3TC-TP incorporation by HIV-1 RTs

<table>
<thead>
<tr>
<th>Nucleotide HIV-1 RT</th>
<th>dATP</th>
<th>Tenofovir-PP</th>
<th>Selectivity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Resistance fold&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{pol}$</td>
<td>$K_d$</td>
<td>$k_{pol}/K_d$</td>
<td>$k_{pol}$</td>
</tr>
<tr>
<td>WT</td>
<td>50&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.7</td>
<td>7.0</td>
</tr>
<tr>
<td>K65R</td>
<td>12</td>
<td>6.9</td>
<td>1.7</td>
<td>0.32</td>
</tr>
<tr>
<td>M184V</td>
<td>23</td>
<td>18</td>
<td>1.8</td>
<td>6.9</td>
</tr>
<tr>
<td>K65R/M184V</td>
<td>20</td>
<td>37</td>
<td>0.56</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nucleotide HIV-1 RT</th>
<th>dCTP</th>
<th>3TC-TP</th>
<th>Selectivity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Resistance fold&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{pol}$</td>
<td>$K_d$</td>
<td>$k_{pol}/K_d$</td>
<td>$k_{pol}$</td>
</tr>
<tr>
<td>WT</td>
<td>7.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.93</td>
<td>0.047</td>
</tr>
<tr>
<td>K65R</td>
<td>2.7</td>
<td>2.8</td>
<td>0.96</td>
<td>0.0056</td>
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<tr>
<td>M184V</td>
<td>9.5</td>
<td>21</td>
<td>0.45</td>
<td>0.026</td>
</tr>
<tr>
<td>K65R/M184V</td>
<td>5.7</td>
<td>19</td>
<td>0.3</td>
<td>0.003</td>
</tr>
</tbody>
</table>

<sup>a</sup> $K_a$ and $k_{pol}$ were determined as described under “Experimental Procedure.” S.D. were <20%.

<sup>b</sup> Selectivity and resistance were determined as described under “Results.” The selectivity is the ratio of $[k_{pol}/K_a]$ (nucleotide analogue)/$[k_{pol}/K_d]$ (nucleotide). The resistance is determined by the ratio of selectivity<sub>WT</sub>/selectivity<sub>mutant</sub>.

<sup>c</sup> Value from Selmi et al. (6).

### Resistance and HIV-1 Fitness with K65R and M184V RT Mutations

<table>
<thead>
<tr>
<th>Nucleotide HIV-1 RT</th>
<th>dATP</th>
<th>Tenofovir-PP</th>
<th>Selectivity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Resistance fold&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
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<td>$k_{pol}$</td>
<td>$K_d$</td>
<td>$k_{pol}/K_d$</td>
<td>$k_{pol}$</td>
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<tr>
<td>WT</td>
<td>50&lt;sup&gt;c&lt;/sup&gt;</td>
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</tr>
<tr>
<td>K65R/M184V</td>
<td>20</td>
<td>37</td>
<td>0.56</td>
<td>1</td>
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</table>

### Experimental Procedure

- **K65R and M184V Impair Natural Nucleotide Binding and Catalysis, Respectively**—There are reports in the literature showing that M184V and K65R mutations affect the “enzymatic fitness” of HIV-1 RT by decreasing the processivity of the enzyme (19, 28). However, processivity is a general term and many altered biochemical properties of RT may account for a defect in processivity along the DNA polymerization pathway, such as primer/template binding ($k_{pol}$ and/or $k_{off}$), nucleotide binding ($K_a$), nucleotide incorporation ($k_{pol}$), pyrophosphate release, or translocation to the next template base. In order to characterize the relationship between RT processivity, viral replication capacity and drug resistance mutations, the incorporation of the four natural nucleotides was individually investigated in terms of catalysis and binding and compared between wild-type RT and the three mutants K65R, M184V, and K65R/M184V RT.

We observe that the K65R and M184V mutants incorporate natural dNTPs with an overall lower efficiency than wild-type RT (Fig. 3A). The K65R mutant RT exhibits a marked difference in purine nucleotide incorporation efficiency relative to pyrimidines. The causes of the reduced dNTP incorporation efficiency of drug-resistant RT were investigated further. Catalytic rates of incorporation $k_{pol}$ and binding affinity constants ($K_a = 1/K_d$) are reported in Fig. 3, B and C, respectively, as a percent of wild-type. Interestingly, the nature of the deficiency is closely linked to the corresponding mechanism of drug resistance. Indeed, K65R RT displays reduced $k_{pol}$ values for all of the combined dNTPs relative to wild-type RT, ranging from 22% (dATP) to 66% (dCTP) (Table III and Fig. 3B). However, binding constants of nucleotides for K65R are generally not affected (Fig. 3C). In the case of M184V RT, it is the other way around: the catalytic step is nearly unchanged compared with wild-type RT (Fig. 3B), but binding affinities are reduced relative to wild-type RT (Fig. 3C), in agreement with previous observations (7, 29). Interestingly, the characteristics brought by each individual substitution appear additive in the K65R/M184V double mutant RT to confer the lowest incorporation efficiency to wild-type RT (Table III).
efficiency among the RTs studied here (Fig. 3A). Indeed, the most striking value is the 12-fold reduction of nucleotide incorporation efficiency for dATP relative to wild-type (k_{pol}/K_d decreases from 6.7 to 0.56 s^{-1}μM^{-1}, Table II). At the level of kinetic constants, an altered binding affinity mainly accounts for this polymerization deficiency with a 5-fold decrease in nucleotide affinity for dATP and dGTP, and a 2-fold decrease for dTTP and dCTP (Fig. 3C) relative to wild-type RT. For the catalytic step, K65R/M184V RT is somewhat intermediate between K65R and M184V RT. Table III also summarizes the overall incorporation efficiency (written as percent k_{pol}/K_d dNTPs) for all RTs, where the efficiencies of incorporation of each individual dNTP were averaged (Table III), and plotted in Fig. 4A. As a control, the multidrug-resistant Q151M RT is included in this table for the purpose of comparison. Q151M RT exhibits a comparable (104%) nucleotide efficiency as wild-type RT, whereas K65R RT and M184V RT display 42 and 41% incorporation efficiency of wild-type RT, respectively. K65R/M184V RT is the least efficient, conferring an overall 27% nucleotide incorporation efficiency relative to that of wild-type RT. In conclusion, we found that the incorporation efficiency of natural nucleotides into DNA follows the order: wild-type RT ≫ K65R RT > M184V RT > K65R/M184V RT.

Natural Nucleotide Insertion Efficiency Correlates with Viral Replication Capacity—The drug-resistant viruses harboring
mean replication capacity of patient-derived viruses that lacked any NRTI- or PI-associated mutations (wild-type, \( n = 1307 \)) or contained only K65R (\( n = 17 \)), only M184V (\( n = 291 \)), K65R plus M184V (\( n = 12 \)), or Q151M complex plus M184V (\( n = 8 \)) were determined (Fig. 4B). There were insufficient patient viruses for analysis that had the Q151M complex but did not include M184V. As expected, the wild-type viruses replicated efficiently (95% of the control virus) whereas the viruses that contained K65R or M184V had diminished replication capacity (58 and 65%, respectively). Viruses containing both K65R and M184V displayed further decreases in replication capacity (38%) compared with the single mutants. Finally, viruses that contained both Q151M plus M184V replicated with similar efficiency to the M184V alone viruses (59%). Therefore, unlike the case for the addition of K65R to M184V, the addition of the Q151M complex to M184V did not appear to have a significant effect on replication capacity, which is consistent with its efficient nucleotide incorporation shown in Fig. 4A. In previous studies, HIV-1 with Q151M complex mutations also did not show a replication disadvantage relative to wild-type HIV-1 (31, 32). Hence, in the case of the K65R and M184V mutations, the decreases in nucleotide incorporation efficiency correlate with the decrease in HIV-1 replication capacity such that the reductions conferred by the two mutations individually are seemingly additive for the combination of K65R plus M184V in the same virus.

**DISCUSSION**

In the treatment of AIDS, the prolonged use of nucleoside analogues under conditions of suboptimal therapy can select for drug-resistant and often multidrug-resistant viruses. To optimize antiretroviral regimens it is important to understand and anticipate gene mutations that confer drug resistance. Drug resistance mutations may also affect viral fitness, and much is still to be learned on the interplay of resistance and viral fitness for the long term management of patients with HIV-1. Our results give a molecular basis for the observed loss of both resistance and replication capacity of viruses containing K65R or M184V RT substitutions.

In the first part of the study, patient-derived viruses were tested for their capacity to resist tenofovir and other nucleoside analogues. We show that the K65R mutation gives HIV-1 a moderate reduction in susceptibility to tenofovir while the additional presence of the M184V mutation restores the sensitivity to tenofovir, as described before (19). Our investigation with pre-steady state kinetics and molecular modeling gives mechanistic support to these viral resistance patterns. We observe that K65R impairs tenofovir-PP incorporation and propose that the positioning of the \( \alpha \)-phosphate is affected with this mutation resulting in the decreased catalytic rate of reaction through a \( "K_{pol}\)-dependent" pathway. This property is characteristic of the K65R mutation and occurs in the same way for dideoxynucleotide resistance (6). The two mutations K65R and M184V when put together appear to combine their effects such that there are further reductions in dATP incorporation. This loss of efficiency appears responsible for the partial loss of resistance of the K65R/M184V virus to tenofovir. Interestingly, our experiments with 3TC-TP give the opposite result: K65R and M184V have together an additive or synergistic effect on 3TC resistance. Hence, we show that two single mutations can have either an antagonistic or synergistic effect over HIV-1 resistance in vitro, essentially due to their intrinsic chemical properties. The clinical significance, if any, of the increased level of resistance for the K65R/M184V double mutant relative to M184V alone for lamivudine is unknown.

The second part of the study describes the loss of replication capacity of the K65R/M184V virus. Our single cycle replication
analysis highlights the low replication capacity of K65R/M184V virus, the latter retaining only 38% of that of wild type. This finding is in agreement with our observation regarding the impaired incorporation of natural nucleotides by K65R/M184V RT. We find that both K65R and M184V single mutations impair the incorporation of natural nucleotides by RT, each of them through their respective characteristics closely linked to their mode of drug discrimination as described above. These impairments brought by each individual mutation (and cumulated in K65R/M184V RT) turn into a 40% loss of replication capacity with K65R and M184V containing viruses.

However, there is an apparent discrepancy between these comparable viral replication capacities and the very different frequencies of selection of these two individual mutations in the clinic: M184V is found in most patients treated with lamivudine while K65R appears less frequently in antiretroviral-experienced patients (33, 34). We propose two rational explanations based on our findings. First, the K65R mutant virus shows low-level resistance to ddNs in vitro (about 2–10-fold). These low level changes in susceptibility may play a role in the low frequency and slow development of K65R; there is not much selective growth advantage for the mutant virus against the drug. We believe the same phenomenon occurs with tenofovir: low levels of resistance (2–3-fold) do not favor a quick selection of the K65R variant, which is found in low frequency (2–5%) under tenofovir treatment (18, 35). This moderate loss of susceptibility contrasts with the high-level of 3TC resistance associated with M184V, which favors the quick and frequent selection of this mutation. Recent observations have shown an increased prevalence of K65R in treated patients in the past years (36). However, the relationship between this trend and the specific use of tenofovir and/or other NRTIs that can also select K65R is unknown.

A second explanation for the difference between K65R and M184V frequency comes from the incorporation rates of natural nucleotides. We observe that the nucleotide incorporation efficiency of K65R is severely imbalanced between purines and pyrimidines whereas it is better equilibrated for all bases in the case of M184V. Although the in vitro replication capacity assays presented here do not show a significant difference between K65R and M184V, natural nucleotide concentrations can vary widely between cell types (37–41). Thus, as a result of the imbalanced incorporation efficiency between purines and pyrimidines for the K65R mutant, there may be a greater defect in replication capacity of the K65R mutant virus with replication impairment does not permit full viral growth in vivo and, thus, viral load remains reduced as a result of the mutation. This may explain the lack of association with viral load increases among treatment-experienced patients who developed K65R in reported clinical trials of tenofovir (18, 35). These two mechanisms of low-level resistance and decreased replication capacity are not mutually exclusive and are likely both operating for K65R and tenofovir. This is similar to what has been reported for patients with detectable resistance to all elements of their regimen: evidence for both residual drug regimen activity and decreased replication capacity of the mutant virus were observed in vivo (20).

Regarding tenofovir resistance, it seems that the K65R mutant virus with replication impairment does not permit full viral growth in vivo and, thus, viral load remains reduced as a result of the mutation. This may explain the lack of association with viral load increases among treatment-experienced patients who developed K65R in reported clinical trials of tenofovir (18, 35). These two mechanisms of low-level resistance and decreased replication capacity are not mutually exclusive and are likely both operating for K65R and tenofovir. This is similar to what has been reported for patients with detectable resistance to all elements of their regimen: evidence for both residual drug regimen activity and decreased replication capacity of the mutant virus were observed in vivo (20).

Although the goal of antiretroviral therapy is the complete suppression of HIV viral load and the prevention of resistance mutations, anticipating the results of possible virologic failure
or resistance development is of major interest. Combination of tenofovir with 3TC or FTC may result in viruses containing both K65R and M184V mutations. These mutations are compatible in the HIV genome and have been observed together in the majority of tenofovir-treated patients who developed K65R and who were also taking 3TC (35, 42). These double mutant viruses, although likely to remain pathogenic, are less fit than wild-type viruses and may be more amenable to control by drugs in the current or subsequent regimen. We believe that the observed reduced fitness could play an important role in the response to antiretroviral therapy, which supports existing information in the field (12, 13, 43, 44). Indeed, the idea of driving HIV-1 to unfitness is not completely new, and the role of several specific mutations in that purpose has already been described for RT (12, 43, 45, 46). However, other studies concerning antagonistic mutations such as M184V in a background of AZT-resistance mutations show that resensitization effects might only be transient, often followed by the evolution of highly mutated isolates with broad resistance patterns (47). Hence, further clinical investigation and in vitro characterization will be needed to fully evaluate the therapeutic relevance and benefit of such strategies based on the combination of antagonistic mutations in HIV-1 RT.

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