The HIV-1 Reverse Transcriptase M184I Mutation Enhances the E138K-Associated Resistance to Rilpivirine and Decreases Viral Fitness

Rima Kulkarni, BS, Kerim Babaoglu, PhD,† Eric B. Lansdon, PhD,† Laurence Rimsky, PhD,‡ Veerle Van Eygen, MSc,‡ Gaston Picchio, PhD,§ Evguenia Svarovskaia, PhD,* Michael D. Miller, PhD,* and Kirsten L. White, PhD**

Background: The registrational phase III clinical trials of the nonnucleoside reverse transcriptase (RT) inhibitor (NNRTI) rilpivirine (RPV) in combination with two nucleoside/nucleotide RT inhibitors (NRTIs) found a unique genotypic resistance pattern involving the NNRTI mutation E138K with the NRTI mutation M184I. Eighty percent of subjects used emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF); a single tablet regimen of FTC/RPV/TDF is in development.

Methods: HIV-1 with E138K and/or M184V or I mutations were constructed and phenotyped in MT-2 cells and the PhenoSense and Antivirogram assays. Viral fitness was determined using growth competitions. Molecular models of the mutants were constructed from the RT-RPV crystal structure.

Results: The E138K mutant showed low-level reduced susceptibility to RPV (2.4-fold), but full susceptibility to FTC and tenofovir (TFV). Viruses with M184V or M184I showed high-level resistance to FTC and full susceptibility to RPV and TFV. Addition of M184I, but not M184V, to E138K, further decreased susceptibility to RPV and maintained FTC resistance. The E138K and M184V or I single and double mutants showed decreased replication fitness compared with wild type. M184V outcompeted M184I when compared directly and in the background of E138K. E138K + M184I was less fit than either E138K or M184I alone. Removing a salt bridge between E138/K101 is implicated in resistance to RPV.

Conclusions: The higher frequency of E138K and M184I among RPV + FTC/TDF virologic failures is due to reduced susceptibility of the single mutants to RPV and FTC and the enhanced resistance to RPV for the double mutant at the cost of decreased viral fitness.

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From the *Clinical Virology, Gilead Sciences, Inc, Foster City, CA; †Structural Chemistry, Gilead Sciences, Inc, Foster City, CA; ‡Tibotec BVBA, Beerse, Belgium; and §Tibotec, Inc, Titusville, NJ.

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The authors R.K., K.B., E.B.L., E.S., M.D.M. and White are employees of Gilead Sciences Inc. The remaining authors L.R., V.V.E., and G.P. are employees of Tibotec BVBA and Tibotec Inc., respectively.

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Correspondence to: Dr Kirsten L. White, PhD, Gilead Sciences, Inc, 333 Lakeside Drive, Foster City, CA, 94404 (e-mail: kwhite@gilead.com).

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INTRODUCTION

The emergence of human immunodeficiency virus type 1 (HIV-1) drug resistance mutations is a major limitation to duration of efficacy. Drug resistance mutations are often coselected and induce resistance to the inhibitors in the regimen. Generally, mutations that increase drug resistance lead to decreased viral fitness. As suggested in a recent report,[1] decreased viral fitness may impact the viral load and transmissibility of the virus. However, additional mutations can arise that result in fitness compensation,[2] which has been very well characterized for HIV-1 protease inhibitor resistance-associated mutations,[3] the NRTI resistance-assisted mutations S68G and K65R,[4] and the integrase inhibitor resistance-associated mutations Q148K and G140S.[5,6]

Rilpivirine (RPV, TMC278) is a recently approved NNRTI for the treatment of HIV-1 infection in antiretroviral-naive patients. In the 2 completed 96-week phase III trials (ECHO and THRIVE), treatment-naive HIV-infected adults were randomized to RPV or efavirenz, both given once daily with the following: (1) background NRTIs tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC) (ECHO); or (2) TDF/FTC, zidovudine/lamivudine (3TC), or abacavir/3TC (THRIVE).[7,8] The overall response of confirmed viral load $<$50 copies per milliliter (Intent to Treat-Time to Loss of Virologic Response) at week 48 with RPV was 84% and was noninferior to that with efavirenz (82%) ($P < 0.0001$ for noninferiority, 12% margin).[9] Furthermore, RPV had improved tolerability but more virologic failures versus EFV.

The resistance findings from the Week 48 analysis of ECHO and THRIVE showed that 10% and 6% of patients in the RPV and EFV groups, respectively, met the definition of virologic failure for resistance analyses.[9] Thirty-three percent of these virologic failure subjects had no NNRTI or NRTI resistance–associated mutations. The proportion of RPV virologic failures with treatment-emergent resistance-associated mutations in patients with baseline VL $\leq$100,000 copies per milliliter was lower than in those with $>$100,000 copies per milliliter.[10] In those with resistance, the most prevalent treatment-emergent NNRTI and NRTI mutations in RPV virologic

failures were E138K and M184I, respectively. E138K plus M184I was the most frequently detected mutation combination and was often present with 1 or more additional NNRTI mutations.[10] E138K has been previously selected in vitro by RPV[11] and etravirine (ETR)[12] and has a low prevalence in samples submitted for routine clinical resistance testing (<1%).[13]

To understand the high frequency of co-occurrence of the NNRTI E138K and the NRTI M184V or I mutations among patients failing RPV in ECHO and THRIVE, this study sought to identify the impact of these mutations on RPV, FTC, and TFV resistance and on viral replication fitness. Molecular models were evaluated to generate hypotheses for resistance mechanisms.

MATERIALS AND METHODS

Reagents

TFV and FTC were synthesized by Gilead Sciences (Foster City, CA), RPV by Tibotec (Beerse, Belgium), lamivudine (3TC) was from Moravek Biochemicals (Brea, CA), zidovudine (AZT) was from Sigma-Aldrich (St Louis, MO), and EFV from Toronto Research Chemicals (North York, Ontario, Canada).

Construction of Site-Directed Mutant Viruses and Phenotypic Analyses

The HIV-1 single or double mutations E138K, M184V, and M184I were constructed using the QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) and a version of the pETxx-LAI p66 shuttle vector[14] that contained reverse transcriptase (RT) amino acids 15–490 from HXB2D. The RT XmaI/XbaI fragment was cloned into the full-length xxLAI plasmid as described.[4] The nucleotide sequences of the resulting plasmids and viruses were confirmed by population sequencing (Elim Pharmaceuticals, Hayward, CA). The susceptibilities of the wild-type and mutant HIV-1 viruses to NRTIs and NNRTIs were determined in a 5-day multiple cycle assay in MT-2 cells,[15,16] with the exception that cytopathic effect was measured using Cell Titer-Glo (Promega, Madison, WI). Effective concentrations which inhibited 50% of viral replication (EC_{50}) were determined using Prism (GraphPad Software, La Jolla, CA). The wild-type and mutant LAI backbone viruses were sent for phenotyping in 2 commercial assays [the PhenoSense Assay[17] (Monogram Biosciences, Inc.) and the Antivirogram Assay[18] (Virco BVBA, Mechelen, Belgium], and the resulting fold-change values were normalized to the wild-type LAI backbone fold-change value for cross-study comparisons and to control for effects of backbone polymorphisms. The wild-type LAI virus used in these studies showed 0.68-fold change to RPV in the PhenoSense Assay compared with its NL4-3 control and 1.8-fold for the wild-type LAI virus in the Antivirogram Assay compared with the HXB2D control.

Growth Competition and MultiCode RTx Polymerase Chain Reaction Assay

The growth competition and MultiCode RTx polymerase chain reaction (PCR) assay was described.[4] Briefly, mutant viruses were generated by transfecting plasmids encoding mutant or wild-type virus into 293T cells using TransIT-LT1 Transfection Reagent (Mirus Bio Corporation, Madison, WI). Viral stocks were titered using the MAGI assay.[19] Viral stocks were mixed at 1:1 ratio to 3×10^3 infectious units to infect 6×10^6 MT-2 cells in 1 mL for 2 hours with a multiplicity of infection of 0.0005. To remove cell-free viruses, cells were pelleted and washed 3 times with 5 mL of phosphate-buffered saline, resuspended in 10 mL of medium, and cultured at 37°C in the absence or presence of drug. On days 3, 6, and 9, cells were pelleted and 10 μL of the supernatant was collected and transferred to a new flask containing 6×10^6 MT-2 cells in a total volume of 10 mL. The multiplicity of infection was kept very low throughout the entire experiment to avoid any potential recombination between competing viruses. Viral RNA was extracted from 200 μL of the supernatant at each time point using the EZ1 Virus Card v2.0 on the BioRobot EZ1 workstation (Qiagen, Valencia, CA). Viral RNA samples were subjected to DNaseI digestion for 60 minutes using the TURBO DNAfree kit (Ambion, Austin, TX).

To differentiate between 2 competing viruses, silent changes were present at amino acids 6 and 7 of the RT gene of xx-LAI and F-xx-LAI.[4,14] For each growth competition experiment, viruses with the 2 different marker mutations were combined. The percentage of mutant viral RNA at each time point during the competition experiment was determined using MultiCode RTx allele-specific PCR (EraGen, Madison, WI) as described but with modifications.[20,21] Briefly, the PCR conditions involved 20 μL reaction mixtures in 1X ISOlution buffer (EraGen), 5 mM dithiothreitol, Titanium Taq DNA polymerase (Clontech, Palo Alto, CA), and SuperScript III RT (Invitrogen, Carlsbad, CA) at the manufacturer's recommended concentration. The MultiCode RTx reverse transcriptase–polymerase chain reaction assays were carried out using the Roche LightCycler 480 (Roche, Indianapolis, IN) with the following cycling conditions: 5 minutes at 54°C for reverse transcription, 2 minutes denaturing at 95°C, and 1 cycle of 5 seconds at 95°C, 5 seconds at 47°C, and 20 seconds at 72°C, followed by 65 cycles of 5 seconds at 95°C, 5 seconds at 57°C, and 20 seconds at 72°C with optical read. A thermal melt with a 0.4°C step from 60°C to 95°C was performed directly after the last 72°C step of thermal cycling. Allele-specific primers were as follows: the F-xx-LAI (GG)-specific primer was FAM-isoC-TGCTGACATTAGTCCTATTGAGACG; the xx-LAI (AT)-specific primer was HEX-isoC-GACGAGACCATTAGTCCTATTGAAACT; and the reverse primer was TGTCAATGGCCATTGTTTAACTTTTGG. PCR primers were used at the following final concentrations: 2 forward allele-specific primers at 200 nM and reverse primer at 400 nM. The percentage of a mutant was determined based on standard curves generated using SigmaPlot (Systat Software, San Jose, CA).

Relative Fitness Calculations

The relative fitness value (1 + s) was calculated as previously described $(1 + s) = \exp[\frac{1}{t} X \ln (M_t/W_t \; X \; W_{t0}/M_{t0})]$, where s is the selection coefficient, t is time in days, M_{t0} and W_{t0} are initial fractions of mutant virus and wild-type virus, and

M_t and W_t are fractions of mutant and wild-type virus at time of measurement.[22] Resulting 1 + s values <1.0 indicates that virus 2 was outcompeted by virus 1. A 1 + s value of 1.0 indicates that both viruses grew with the same fitness. The *t* test was used for statistical comparison.

Molecular Modeling

To assess effects of the E138K mutation, the crystal structure of HIV RT with bound RPV (PDB 3MEE)[23] was prepared using the Protein Preparation Workflow in the Schrödinger Maestro modeling package version 9.2 (Schrödinger, LLC, New York, NY). Bond orders were assigned, hydrogens added, and a restrained minimization was done to remove any severe strain. The E138K mutation was then introduced manually, and the side chains of both K138 and K101 were optimized using Prime.[24] For both the wild-type and the mutant structures, the ligand and the shell of residues 6.5 Å around it were subjected to energy minimization using the OPLS-2005 forcefield in a generalized born solvent model with Macromodel (Schrödinger, LLC) under default parameters; the only exception was the maximum number of iterations being increased to 5000 to allow full convergence. All images were produced using PyMOL version 1.3 (Schrödinger, LLC).

RESULTS

M184I Increases E138K-Based Resistance to RPV

The phenotypes of HIV-1 with RT mutations E138K, M184V, M184I and the combinations E138K + M184V and E138K + M184I were evaluated using 3 different assays: (1) a 5-day, multicycle assay in MT-2 cells, (2) the 3-day, single-cycle PhenoSense Assay (Monogram Biosciences, Inc) in 293T cells, and (3) the 3-day, multicycle Antivirogram Assay (Virco) in MT-4 E-GFP cells.

First, using the 5-day, multiple-cycle assay in MT-2 cells, HIV-1 with E138K showed low-level reduced susceptibility to RPV (2.4-fold compared with wild type), but full susceptibility to FTC and TFV (Table 1). The single mutants M184V and M184I showed high-level reduced susceptibility to FTC, but remained fully susceptible to RPV and TFV. The double mutants E138K + M184I and E138K + M184V showed reduced susceptibility to both RPV and FTC, but remained susceptible to TFV. The combination of E138K + M184I showed further reduced susceptibility to RPV (4.1-fold) than E138K alone (2.4-fold).

Second, the viruses were also phenotyped using the PhenoSense Assay (Table 2). The reverse transcriptase/protease region from the site-directed mutant LAI viruses was transferred into the PhenoSense NL4-3–based vector. The overall resistance trends observed using the first assay were confirmed. The E138K mutant showed 2.3-fold reduced susceptibility to RPV, and the M184V and I mutants showed high-level reduced susceptibility to FTC. The double mutant E138K + M184I showed reduced susceptibility to RPV and FTC and remained susceptible to TFV. The trend toward further reduced susceptibility to RPV for E138K + M184I compared with E138K was also observed for the structurally related NNRTI ETR.

TABLE 1. Susceptibilities to NNRTIs and NRTIs for HIV-1 With Drug Resistance Mutations in MT-2 Cells

	Average Fold Change in EC_{50} Value					
	NNRTI		NRTI			
Virus	RPV	EFV	FTC	3TC	TFV	AZT
Wild type	1.0	1.0	1.0	1.0	1.0	1.0
M184V	1.0	1.0	>177	>60	0.8	0.9
M184I	1.4	0.9	>102	>60	0.8	0.5
E138K	2.4	1.2	0.6	0.7	0.7	1.0
E138K+M184V	2.0	1.2	>177	>60	0.6	0.5
E138K+M184I	4.1	1.5	>96	>60	0.7	0.5

5-day HIV-1 assay in MT-2 cells using site-directed mutants constructed in xxLAI. Data are mean values from 4 to 6 replicates.

Finally, the viruses were studied in the Antivirogram Assay. The reverse transcriptase/protease region from the site-directed mutant LAI viruses was transferred into the assay's standard HXB2D backbone (Table 2). The M184V and M184I single mutants showed reduced susceptibility to FTC, but full susceptibility to RPV and TFV. The E138K mutant showed low-level reduced susceptibility to RPV (2.3-fold). The double mutant E138K + M184I showed reduced susceptibility to RPV and FTC and was susceptible to TFV. The addition of M184I to E138K showed a slight increase in reduced susceptibility to RPV (and ETR) compared with E138K alone.

In all assays, viruses with M184V and M184I were resistant to FTC. E138K showed low-level reduced susceptibility to RPV. The E138K + M184V or I showed reduced susceptibility to both FTC and RPV, and only the combination of E138K + M184I further reduced susceptibility to RPV. Overall, E138K + M184I showed the greatest reduction in susceptibility to RPV among all the mutants tested in this series.

E138K + M184I Shows No Compensatory Effect for Replication Fitness of the Single Mutants

To investigate the effects of the E138K, M184V, M184I, and double mutations on viral replication fitness, direct growth competition experiments were conducted. Two viruses were mixed at equal proportions of infectious units and used to infect MT-2 cells. Viruses were propagated for 9 days and quantified by real-time allele-specific PCR at days 0, 3, 6, and 9 (Fig. 1). The fitness of each mutant was quantified by calculating relative fitness (1 + s) values (Table 3). Control experiments demonstrated that construction of the wild-type virus with the silent mutations did not affect the viral fitness (wild-type xx-LAI virus vs wild-type F-xx-LAI virus; 1 + s value = 1.00) (Table 3). For the wild-type versus M184V competition, the proportion of M184V compared with wild-type virus decreased from 50% to 20% by day 9, indicating reduced replication fitness of the M184V virus in the absence of drug (Fig. 1A). Decreased fitness of M184V is consistent with published studies.[25–28] The viruses with E138K, M184I,

TABLE 2. Susceptibilities to NNRTIs and NRTIs for HIV-1 With Drug Resistance Mutations in the PhenoSense and Antivirogram Assays

	Fold Change in EC_{50} Values*										
	NNRTI				NRTI						
Virus	RPV	EFV	ETR	NVP	ABC	ddI	FTC	3TC	d4T	TFV	AZT
PhenoSense assay†											
Wild type	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
M184V	0.84	0.80	0.77	0.77	3.4	2.0	>96	>79	1.0	0.63	0.51
M184I	1.0	0.77	0.92	0.55	2.1	2.3	>96	>79	1.0	0.59	0.39
E138K	2.3	1.4	2.2	1.0	1.2	1.3	1.2	1.3	1.2	1.2	1.5
E138K + M184V	2.0	1.2	2.0	0.90	4.2	2.2	>96	>79	0.79	0.79	0.92
E138K + M184I	2.5	1.5	2.8	0.75	2.3	2.2	>96	>79	0.66	0.66	0.64
Antivirogram assay‡											
Wild type	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
M184V	0.8	0.9	1.0	0.7	2.0	2.3	>18	>34	0.6	0.6	0.6
M184I	0.9	0.8	1.1	0.5	1.6	2.4	>18	>34	0.8	0.6	0.5
E138K	2.3	1.3	2.3	0.7	0.7	1.1	1.1	0.9	0.7	0.7	1.1
E138K + M184V	2.0	1.1	2.1	0.7	1.8	1.9	>18	>34	0.4	0.4	0.5
E138K + M184I	2.6	1.4	3.4	0.7	2.0	3.0	>18	>34	1.0	0.7	0.7

*Data normalized for wild-type xxLAI to 1-fold.

† Single cycle HIV-1 assay (PhenoSense, Monogram) using resistance test vectors with the reverse transcriptase/protease from the site-directed mutants constructed in xxLAI into NL4-3.

‡Multiple cycle HIV-1 assay (Antivirogram, Virco) using the reverse transcriptase/protease region from the site-directed mutants constructed in xxLAI into HXB2D. Values are means from 2 to 4 experiments.

E138K + M184V, and E138K + M184I also showed reduced replication fitness compared with wild type (Figs. 1B–E).

The relative replication fitness of mutant versus mutant viruses with M184V or M184I alone or in combination with E138K were investigated (Figs. 1F, G). The M184I virus grew poorer than M184V (1 +s value = 0.89) in a competitive setting. The E138K + M184I also grew poorer than E138K + M184V (1 + s value = 0.87) in competition. These results are consistent with the previous findings that M184V has a growth advantage over M184I.[29–31]

The replication fitness of M184I, E138K, and E138K + M184I determined from the mutant versus wild-type competition experiments suggested that the double mutant E138K + M184I was less fit than E138K or M184I. To test this directly, the competition experiments between M184I and E138K + M184I (Fig. 1H) and E138K versus E138K + M184I were conducted (Fig. 1I). The E138K + M184I mutant was consistently less fit than M184I alone (1 + s value = 0.83) and E138K alone (1 + s value = 0.90). Therefore, the E138K + M184I double mutant was less fit than E138K or M184I alone.

The replication fitness in the presence of RPV was determined for E138K and E138K + M184I (Figs. 1J–L). This assay is influenced by both the replication fitness of each virus and the susceptibility of each virus to RPV. In the ECHO and THRIVE studies, the mean minimum plasma concentration (Ctrough) of RPV was 80 ng/mL (0.2 μM).[32] In the presence of 0.8 nM RPV, E138K maintained the fitness advantage over E138K + M184I. At 2 nM RPV, the 2 viruses grew similarly. Confirming the further reduction in susceptibility to RPV when M184I is added to E138K, E138K + M184I was found to outcompete the E138K virus in the presence of 8 nM RPV (Fig. 1L).

The Salt Bridge Between E138 and K101 is Important for RPV Positioning

Molecular models were studied to gain a structural understanding of the interaction between E138K (RT p51 subunit) and M184V or I (RT p66 subunit) that leads to increased RPV resistance. In the crystal structure of RT bound to RPV, the positions of the amino acid residues surrounding RPV bound to RT in the NNRTI-binding site were examined.[23] In this structure, RPV resides in its horseshoe-shaped conformation. At the solvent-exposed region of RT, the negatively charged E138 forms a salt bridge with the positively charged K101 (Fig. 2A). In the case of *K* replacing the *E* at position 138, the salt bridge is absent, and the 2 positively charged side chains repel one another to cause an increased opening of the NNRTI-binding pocket to solvent and decreased van der Waals contact between RPV and the protein (Figs. 2C, D). This observation is consistent with the finding that alternative residues at E138 (A, G, K, R, or Q) lack a negative charge and result in 2-fold to 4-fold decreased phenotypic susceptibilities to RPV and ETR.[11,33] The M184 residue is a key residue in the conserved YMDD motif in the RT polymerase active site, and substitutions with beta-branched amino acids such as isoleucine or valine cause dramatic steric clashes with 3TC-triphosphate (and FTC-triphosphate).[34] From the perspective of the NNRTI-binding pocket, M184 (RT p66) is located distal to the E138 residue (RT p51) on a type I beta turn between beta strands β9 and β10, that, together with β11, form the wall of the NNRTI-binding pocket that displays the key hydrophobic side chains of Y181 and Y188 (Fig. 2A). Based on the full sensitivity to RPV by viruses with only M184V or I, and when the E138/K101 salt bridge is intact, RPV can tolerate small

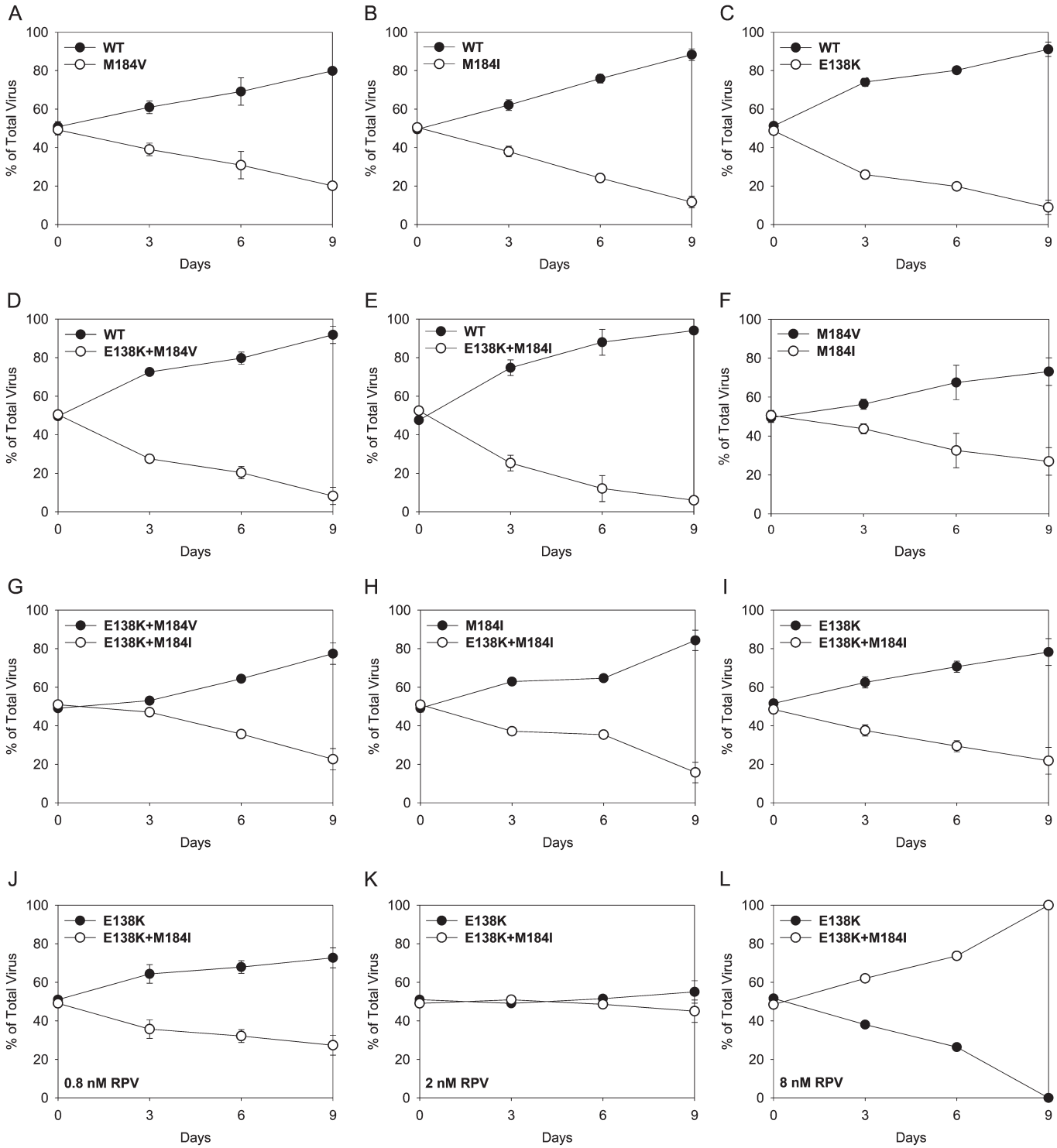

FIGURE 1. Growth competitions of HIV-1 wild-type vs M184V mutant (A), M184I (B), E138K (C), E138K+M184V (D), and E138K + M184I (E). Growth competitions of HIV-1 with M184V vs M184I (F), E138K + M184V versus E138K + M184I (G), M184I vs E138K + M184I (H), and E138K versus E138K + M184I (I). Growth competitions of HIV-1 with E138K vs E138K + M184I in the presence of RPV at 0.8 nM (J), 2 nM (K), and 8 nM (L). Viral stocks were mixed and were used to coinfect MT-2 cells. The proportion of each virus was determined by quantifying the viral RNA by real-time allele-specific reverse transcriptase–polymerase chain reaction. An average of 2–3 independent competition experiments are shown with error bars representing an SD.

TABLE 3. Relative Fitness of WT and Mutant HIV-1 in Growth Competition Assays

Competing Viruses	Relative Fitness Value (1 + s)*, Mean ± SD	P†
No drug		
WT vs WT	1.00 ± 0.005	1.00
WT vs M184V	0.86 ± 0.008	<0.001
WT vs M184I	0.79 ± 0.01	<0.001
WT vs E138K	0.80 ± 0.005	<0.001
WT vs E138K+M184V	0.79 ± 0.01	<0.001
WT vs E138K+M184I	0.72 ± 0.02	<0.001
M184V vs M184I	0.89 ± 0.02	0.002
E138K + M184V vs E138K + M184I	0.87 ± 0.02	<0.001
M184I vs E138K + M184I	0.83 ± 0.03	0.002
E138K vs E138K + M184I	0.87 ± 0.04	0.007
With drug		
E138K vs E138K + M184I (0.8 nM RPV)	0.90 ± 0.02	0.002
E138K vs E138K + M184I (2 nM RPV)	0.98 ± 0.02	0.44
E138K vs E138K + M184I (8 nM RPV)	1.20 ± 0.01	<0.001

*Relative fitness value versus wild-type HIV-1 was calculated as previously described $(1 + s) = \exp[\frac{1}{t} X \ln (M_t/W_t X W_{t0}/M_{t0})]$, where t, time in days; and M_t, M_{t0}, W_t, and W_{t0}, fractions of mutant virus at initial and time of measurement and fractions of wild-type virus at initial and time of measurement, respectively.[22] Mutant versus mutant competitions were analyzed using the same equation. Relative fitness value of 1.00 represents equivalent levels of viral fitness; values less than 1 represents less efficient growth compared with the competitor. Each competition experiment was conducted a minimum of 2 times.

†The P values were determined using the T test to establish statistical significance of the difference from the wild-type versus wild-type relative fitness.

WT, wild type.

perturbations in the NNRTI-binding pocket from the 184-beta strand side of the pocket. However, when the salt bridge is disrupted, as in the case of E138K, RPV has less tolerance for perturbations from the 2 sides of the NNRTI-binding pocket, and further reduced susceptibility is observed for the double mutant E138K + M184I. No differences between V or I at 184 were investigated. The impact of E138K and M184I was observed for the newer generation NNRTIs RPV and ETR but not for EFV or NVP. An overlay of four NNRTI crystal structures (PDB IDs: 3MEE,[23] 3MEC,[23] 1IKW,[35] and 1LWC[36]) shows overall similar binding of the NNRTI to the same hydrophobic pocket of RT (Fig. 2B). From this comparison, however, RPV and ETR access the area closest to E138 and would be expected to be more sensitive to changes at E138 compared with EFV and NVP that do not reach into this part of the pocket. In addition, RPV has more contact with the β10 strand immediately after the M184 turn, specifically, Y188 (Fig. 2A).

DISCUSSION

In the registrational phase III studies (ECHO and THRIVE) of RPV with 2 NRTIs that include either FTC or 3TC, the most frequent mutation combination that emerged in virologic failures was E138K + M184I, often along with other mutations[9] and reduced susceptibility to RPV and FTC (median of 8.1-fold for RPV and high-level resistance to FTC).[10] When present together with E138K, the isoleucine at position 184 seemed to not readily transition to valine. This was surprising because most subjects in other studies with emergent resistance to FTC or 3TC have the M184V mutation, and if

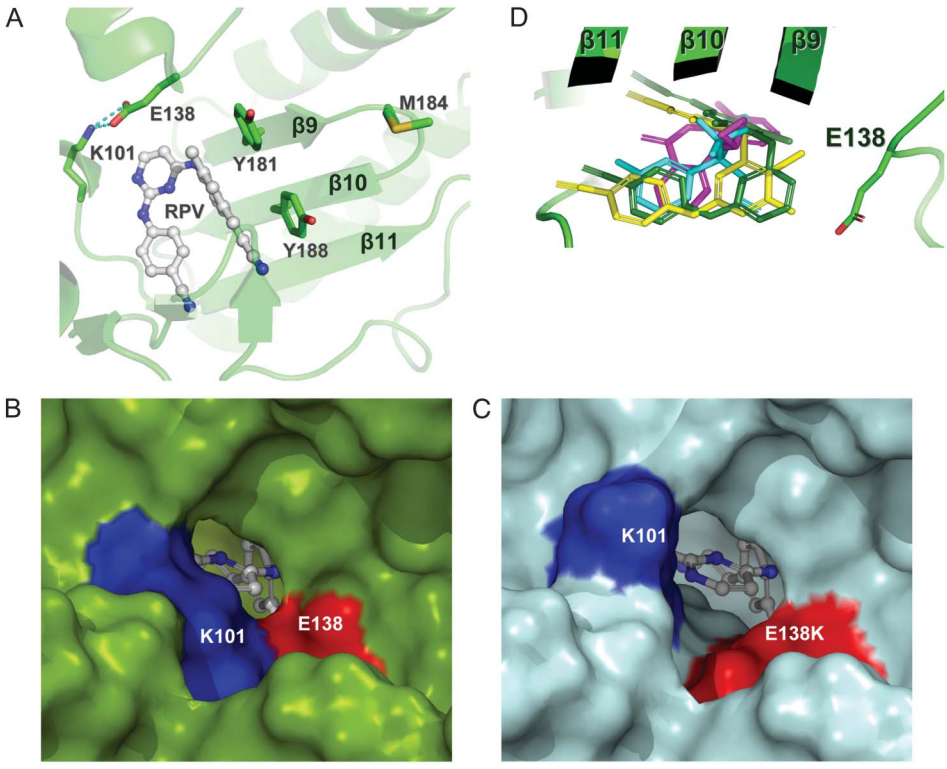

FIGURE 2. The crystal structure of RPV bound to RT is shown (3MEE) with key residues and structural features highlighted (A). Overlay of the crystal structures of RPV (green, 3MEE), ETR (yellow, 3MEC), EFV (cyan, 1IKW), and NVP (magenta, 1LWC) showing the proximity of these inhibitors to E138 (B). Energy minimized models of the wild-type (C) and E138K (D) mutation. The surfaces of residues 101 and 138 are colored blue and red, respectively.

M184I is observed, it is generally replaced by M184V within a short time.[30,31,37] The underlying reasons for the selection of E138K plus a stable M184I mutation were investigated in this study. The results indicate that the E138K + M184I combination is selected due to its dual resistance to RPV and FTC and increased resistance to RPV compared with E138K alone. Our analysis of viral replication fitness in the absence of drug further suggest that the selection of E138K + M184I was not driven by the improvement of viral fitness compared with either single mutant. Studies of the viral sequences from the ECHO and THRIVE patients experiencing virologic failures confirmed these in vitro data,[10] and analysis of the quasispecies present in HIV-1 viral sequences from patients from these trials is underway and may help to further elucidate the evolution of these mutations in vivo.

In contrast to our findings, 2 groups have presented fitness data suggesting that there may be a fitness compensation for the double mutants versus the single mutant in the absence or presence of ETR.[38,39] In these studies, viral growth in the presence of ETR showed that E138K + M184I outcompeted the single mutants, which is consistent with M184I increasing E138K-based ETR resistance. Our data suggest that there is no fitness compensation for E138K and M184I, and this is consistent with clinical data that E138K has not been frequently observed in combination with M184V or M184I.[40] Subtle changes in background viral sequences may contribute to these apparent fitness discrepancies observed in the absence of drug, between these and the present study. In the presence of 8 nM RPV (high dose), we found that the E138K + M184I mutant replicated better than E138K, indicating that under these conditions, the increased resistance to RPV associated with the double mutant allowed for better viral growth which overcame the greater intrinsic replication defect of the double mutant. This viral growth benefit based on increased resistance for the double mutant was not observed in the absence of RPV or in the presence of lower concentrations of RPV (0.8 nM or 2 nM).

Molecular modeling studies were undertaken to gain a structural understanding of the molecular interactions between E138K and M184I. The modeling studies highlight that the impact of mutations at position E138 is higher for RPV and ETR because these compounds bind closer to the E138/K101 salt bridge than other NNRTIs (Fig. 2). When the salt bridge is broken, or in this case actively repelled by the presence of 2 positively charged lysine residues in close proximity, RPV likely loses some binding stability, which confers the low-level reduced susceptibility.[23] K101E + M184I was also observed in ECHO and THRIVE.[10] Interestingly, this mutant would also have the same salt bridge disrupted from the opposite side, and in this case, the 2 negatively charged residues may be causing the repulsion and pocket opening. Residue M184 does not directly interact with RPV, and when M184V or M184I is present alone, the bound RPV can tolerate the subtle changes to the M184 side of the NNRTI-binding pocket. However, in the context of both the double mutants of M184I or M184V with E138K, the coupling of the disrupted salt bridge with the additional mutation may further destabilize the pocket, increasing the reduced susceptibility to RPV. Similar observations were made for ETR (Fig. 2), which are consistent with the similarity of structure and phenotypic resistance profile between these 2 diarylpyrimidine compounds.[10,11,23]

In the RPV arm of ECHO and THRIVE, viruses with the M184I mutation exhibit high-level reduced susceptibility to FTC and 3TC and did not seem to transition to the more stable and fit valine variant. In the ECHO and THRIVE studies, RPV selects for the E138K mutation, FTC (or 3TC) selects for the M184V or M184I mutations, and this resulted in the occurrence of the double mutant E138K + M184I or V with M184I being more frequent than M184V.[10] The data presented in this publication show that the prevalence of E138K + M184I over E138K + M184V is likely due to an increase in reduced susceptibility to RPV when M184I is present together with E138K, and that this reduced susceptibility is associated with a decrease in viral replication fitness in the absence of drug.

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