The effects of the W153L substitution in HIV reverse transcriptase on viral replication and drug resistance to multiple categories of reverse transcriptase inhibitors

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A W153L substitution in HIV-1 reverse transcriptase (RT) was recently identified by selection with a novel nucleotide-competing RT inhibitor (NcRTI) termed Compound A that is a member of the benzo[4,5]furo[3,2,d]pyrimidin-2-one NcRTI family of drugs. To investigate the impact of W153L, alone or in combination with the clinically relevant RT-resistance substitutions K65R, M184I, K101E, K103N, E138K and Y181C, on HIV-1 phenotypic susceptibility, viral replication and RT enzymatic function, we generated recombinant RT enzymes and viruses containing each of these substitutions or various combinations thereof. We found that W153L-containing viruses were impaired in viral replicative capacity and were hypersusceptible to Tenofovir (TFV) while retaining susceptibility to most non-nucleoside RT inhibitors. The nucleoside 3TC retained potency against W153L-containing viruses but not when the M184I substitution was also present. W153L was also able to reverse the effects of the K65R substitution on resistance to TFV, and K65R conferred hyper-susceptibility to Compound A. Biochemical assays demonstrated that W153L alone or in combination with K65R, M184I, K101E, K103N, E138K and Y181C impaired enzyme processivity and polymerization efficiency but did not diminish RNase H activity, providing mechanistic insights into the low replicative fitness associated with these substitutions. We show that the mechanism of TFV hypersusceptibility by W153L is mainly due to increased efficiency of TFV-DP incorporation. These results demonstrate that Compound A and/or derivatives thereof have the potential to be important antiretroviral agents that may be combined with Tenofovir to achieve synergistic results.

Keywords
HIV-1, reverse transcriptase, antiretrovirals, NcRTI, W153L, replication capacity, drug resistance.

INTRODUCTION

Current standard anti-HIV-1 therapy termed highly active antiretroviral therapy (HAART) consists of three or more antiretroviral compounds from six distinct classes including nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, entry inhibitors and integrase inhibitors (INIs) (for recent reviews see (1, 2)). HAART commonly includes two NRTIs in combination with either an NNRTI, a PI, or, more recently, an INI (WHO: 2013 Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection. Available at: http://www.who.int/hiv/pub/guidelines/arv2013/en/. Accessed on Jan 10, 2014). HAART has been effective at suppressing HIV-1 replication, partially reversing immunodeficiency, reducing HIV-1-associated complications, and prolonging life (3, 4). However, all antiretrovirals can be compromised by the development of drug resistance (5, 6) that arises from the rapid replication rate of HIV-1 and the error-prone nature of its RT (7, 8). In the absence of an effective vaccine against HIV-1 infection, it is important to develop novel effective antiretrovirals with superior resistance profiles.

There are currently 8 approved NRTIs or nucleos(t)ide reverse transcriptase inhibitors (NtRTI) that all compete with natural dNTP substrates to act as DNA chain terminators. In contrast, NNRTIs act allosterically and non-competitively by inducing conformational changes in RT. Mechanisms of NNRTI resistance are due to specific substitutions in RT that affect the
entry of NNRTIs into the NNRTI binding pocket or decrease NNRTI binding by steric hindrance (6, 9, 10). Two major mechanisms account for resistance to NRTIs, discrimination and excision (for reviews see (11, 12)). Discrimination is based on the decreased incorporation of NRTIs by a mutated RT whereas excision is based on the enhanced ability of a mutated RT to excise an incorporated chain-termination inhibitor from the viral DNA terminus.

Recently, a novel class of HIV-1 RT inhibitors termed nucleotide-competing reverse transcriptase inhibitors (NcRTIs) has been identified (13-17). The indolopyridone compound INDOPY-1 defines this class of compounds that are not nucleotide analogues but rather act as nucleotide-competing inhibitors of RT. NcRTIs inhibit reverse transcription by binding to the RT active site in competition with the next incoming nucleotide to form dead-end-complexes, a distinct mechanism. The potency of INDOPY-1 was mostly unaffected by substitutions associated with resistance against NNRTIs or NRTIs, although NRTI substitutions at positions M184V and Y115F were shown to result in diminished susceptibility to INDOPY-1 (13, 15). A more potent novel benzo[4,5]furo[3,2,d]pyrimidin-2-one (BFPY) chemical series of NcRTIs, represented by Compound A, was also identified that retains potency against RT containing M184V but selects for a novel W153L substitution in RT (21). W153L mutant viruses were shown to have diminished replication capacity and were highly resistant to Compound A but sensitive to NVP and hyper-susceptible to each of abacavir (ABC), tenofovir (TFV), lamivudine (3TC) and emtricitabine (FTC) (17). This unique resistance profile makes it important to understand how the W153L substitution might impact on drug susceptibility,
Here, we have investigated the effect of W153L, alone or in combination with the clinically relevant RT substitutions K65R, M184I, K101E, K103N, E138K or Y181C on drug susceptibility and viral replication capacity. We found that W153L impairs viral replication but does not affect susceptibility to most NNRTIs or to 3TC, except, for the latter, when M184I was also present. All of the W153L-containing viruses that were tested were hyper-susceptible to TFV. We also studied relevant recombinant RT enzymes and found that the presence of W153L, alone or in combination with each of K65R, M184I, K101E, K103N, E138K and Y181C, diminished both enzyme processivity and the efficiency of DNA polymerization as assessed in gel-based assays. However, none of these mutant RT enzymes diminished RNase H activity. We also investigated the underlying mechanism of TFV hypersusceptibility by W153L.

MATERIALS AND METHODS

Chemicals, cells and nucleic acids

Etravirine (ETR) and rilpivirine (RPV) were gifts of Janssen Pharmaceuticals (Titusville, NJ). TFV was kindly provided by Gilead Sciences (Foster City, CA). 3TC was a gift of Glaxo-SmithKline (Greenford, UK). Efavirenz (EFV) and nevirapine (NVP) were obtained from the NIH AIDS Research and Reference Reagent Program. The NcRTI Compound A was obtained from Boehringer Ingelheim Canada Ltd (Laval, QC)(17).
The HEK293T cell line was obtained from the American Type Culture Collection (ATCC). The following reagents and cells were obtained through the NIH AIDS Research and Reference Reagent Program: the infectious molecular clone pNL4-3 from Dr. Malcolm Martin; the TZM-bl (JC53-bl) cell from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc.

The pNL4.3PFB plasmid DNA was a generous gift from Dr. Tomozumi Imamichi, National Institutes of Health, Bethesda, MD. The plasmid pRT6H-PROT was a generous gift from Dr. Stuart F. J. Le Grice, National Institutes of Health, Bethesda, Maryland, USA.

An HIV-1 RNA template, ~500 nucleotides (nt) in size, spanning the 5’ untranslated region (UTR) to the primer binding site, was transcribed in vitro from Accl-linearized pHIV-PBS DNA (18) by using an Ambion T7-MEGashortscript kit (Invitrogen, Burlington, ON, Canada) as described previously (19). The oligonucleotides used in this study were synthesized by Integrated DNA Technologies Inc. (Coralville, IA) and purified by polyacrylamide-urea gel electrophoresis. For 5’-end labeling of oligonucleotides with $\gamma^{32}P$ATP, the Ambion KinaseMax kit was used followed by purification through Ambion NucAway spin columns, according to protocols provided by the supplier (Invitrogen, Burlington, ON, Canada).

Site-directed mutagenesis and preparation of site-directed mutant (SDM) HIV-1\textsubscript{NL4.3} virus stocks

To construct HIV-1 RT expression plasmids and recombinant HIV-1\textsubscript{NL4.3} infectious clones harboring desired substitutions in the RT gene, site-directed mutagenesis reactions were carried out using a Stratagene QuickChange II XL site-directed mutagenesis kit (Agilent Technologies Canada Inc., Mississauga, ON). This work was performed with the HIV-1 RT expression plasmid DNA pbRT6H-PROT (19) and the pNL4.3PFB plasmid.
DNA (20) to generate recombinant RT enzymes and HIV-1$_{\text{NL4.3}}$ viruses containing the desired RT substitutions. DNA sequencing was performed to verify the absence of spurious substitutions and the presence of any desired substitution in the RT coding sequences. Recombinant HIV-1 wild-type (WT) and mutant viruses were generated by transfection of the corresponding proviral plasmid DNAs into HEK293T cells using Lipofectamine 2000 (Invitrogen, Burlington, ON) according to manufacturer's instructions. Viral supernatants were harvested at 48 hours post-transfection, centrifuged for 5 minutes at 800 $\times$ g to remove cellular debris, filtered through a 0.45-$\mu$M-pore size filter, aliquoted and stored at -80$^\circ$C. Levels of p24 antigen in viral supernatants were measured by a Perkin-Elmer HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's instructions (Perkin-Elmer Life Sciences, Boston, MA). BH10 WT or W153L viruses were generated as reported previously (17).

**Assays of virion-associated RT activity**

Virion-associated RT activity was measured by in vitro RT assay as described previously (21) with 50 $\mu$L of RT reaction mixtures containing 10 $\mu$L of culture supernatants (10 ng HIV-1 p24), 0.5 U/ml of poly(rA)/p(dT)$_{12-18}$ template/primer (T/P) (Midland Certified Reagent Company, Midland, Texas) in 50 mM Tris-HCl (pH 7.8), 75 mM KCl, 5 mM dithiothreitol (DTT), 5 mM MgCl$_2$, 0.05% Triton X-100, 2% ethylene glycol, 0.3 mM reduced glutathione, and 2.5 $\mu$Ci of $[^3\text{H}]$dTTP (70-80 Ci/mmol, 2.5 mCi/mL). Following a 240-min incubation at 37$^\circ$C, the reaction mixture was quenched by adding 0.2 mL of 10% ice-cold trichloracetic acid (TCA) containing 20 mM sodium pyrophosphate and...
incubated for at least 30 min on ice. The precipitated products were filtered onto Millipore 96-well Multi Screen HTS FC filter plates (MSFCN6B) and sequentially washed with 200 µL of 10% TCA and 150 µL of 95% ethanol. The radioactivity of incorporated products was analyzed by liquid scintillation spectrometry using a Perkin-Elmer 1450 MicroBetaTri Lux Microplate Scintillation and Luminescence Counter.

Measurements of HIV-1 replication capacity in TZM-bl cells

The relative replicative capacities of the following recombinant WT HIV-1<sub>NL4-3</sub> clones containing the substitutions W153L, W153L/K65R, W153L/M184I, W153L/K101E, W153L/K103N, W153L/E138K, and W153L/Y181C were evaluated in a non-competitive infectivity assay using TZM-bl cells as previously described (22, 23). Twenty thousand cells per well were added in triplicate into a 96-well culture plate in 100µl Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (Invitrogen, Burlington, ON), 1% penicillin-streptomycin, and 1% L-glutamine (Invitrogen, Burlington, ON). Viral stocks for both wild-type and mutant viruses were normalized by p24, and recombinant viruses were serially diluted from viral stock suspensions. After 4 hr, 50 µl of DMEM were removed from the wells and replaced by 50 µl of virus dilution; a control well did not contain virus. Viruses and cells were co-cultured for 48 hours, after which 100 µl of Promega Luciferase Assay Bright-Glo reagent (Fisher Scientific, Ottawa, ON)were added and luciferase activity was measured in a 1450 MicroBeta TriLux Microplate Scintillation and Luminescence Counter (Perkin Elmer, Waltham, MA) as described above. The viral
replication level of each viral variant was expressed as a percentage of relative light units (RLU) with reference to WT virus.

**Analysis of phenotypic drug susceptibility in TZM-bl cells**

Phenotypic susceptibility analyses of RT inhibitors were performed with recombinant HIV-1NL4.3 clones in a TZM-bl cell-based in vitro assay as described previously (22, 24, 25). Briefly, RT inhibitors at variable concentrations were added to TZM-bl cells (10⁴ cells/well) in 96-well plates grown in 100 μl supplemented medium. Immediately after drug addition, cells were infected with WT or mutant viruses. At 48 h post infection, cells were rinsed with 100 μl phosphate buffered saline and lysed with 50 μl/well Promega luciferase assay reagent (Fisher Scientific, Ottawa, ON). Cell lysates were then transferred to a white, opaque 96-well plate (Corning, Tewksbury, MA). Promega luciferase assay reagent (Fisher Scientific, Ottawa, ON) was added to each well, and relative luminescence units (RLU)/well were measured by a Perkin Elmer 1450 MicroBeta TriLux Microplate Scintillation and Luminescence Counter (Perkin Elmer, Waltham, MA). The EC₅₀ was calculated using the GraphPad Prism program (GraphPad Software, San Diego, CA).

**Recombinant reverse transcriptase expression and purification.**

Recombinant RTs in hetero dimeric form were expressed from plasmid pbRT6H-PROT (19) and purified as described (26, 27). In brief, RT expression in *Escherichia coli* M15 (pREP4) (Qiagen, Mississauga, ON) was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at room temperature. Pelleted bacteria were lysed under
native conditions with BugBuster protein extraction reagent containing Benzonase (Novagen, Madison, WI) according to the manufacturer’s instructions. After clarification by high speed centrifugation, the clear supernatant was subjected to the batch method of Ni-nitrilotriacetic acid (NTA) metal affinity chromatography (QIAexpressionist; Qiagen, Mississauga, ON, Canada). All buffers contained complete protease inhibitor cocktail (Roche, Mississauga, ON, Canada). Hexahistidine-tagged RT was eluted using an imidazole gradient. RT-containing fractions were pooled, passed through DEAE-Sepharose (GE Healthcare, Mississauga, ON, Canada), and further purified using SP-Sepharose (GE Healthcare, Mississauga, ON). Fractions containing purified RT were pooled, dialyzed against storage buffer (50 mM Tris-HCl [pH 7.8], 50 mM NaCl and 50% glycerol), and concentrated to 4-8 mg/ml with Centricon Plus-20 MWCO30 kDa (Millipore, Etobicoke, ON, Canada). Aliquots of proteins were stored at -80°C. Protein concentration was measured by a Bradford protein assay kit (Bio-Rad Laboratories, Saint-Laurent, QC, Canada), and the purity of the recombinant RT preparations was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The RNA-dependent DNA polymerase activity of each recombinant RT preparation was evaluated as described (28) using various concentrations of RT and a synthetic homopolymeric poly(rA)/p(dT)_{12-18} template/primer (T/P) (Midland Certified Reagent Company, Midland, TX). As a control, recombinant WT or W153L RT derived from HIV-1BH10 were generated as reported previously (17).

RT-catalyzed RNase H Activity
RNase H activity was assayed using a 41-mer 5’-[32P]-heteropolymeric RNA template termed kim40R that was annealed to a complementary 32-nucleotide DNA oligomer termed kim32D at a 1:4 molar ratio as described (29, 30). Reactions were conducted at 37°C in mixtures containing an RNA-DNA duplex substrate (20 nM) with equal amounts of RT enzymes in assay buffer, 50 mM Tris-HCl, pH 7.8, 60 mM KCl, and 5 mM MgCl2. Aliquots were removed at different time points after initiation of reactions and quenched by using an equal volume of formamide sample loading buffer (96% formamide, 0.1% each of bromophenol blue and xylene cyanol FF and 20 mM EDTA). The samples were heated to 90°C for 3 min, cooled on ice, and electrophoresed through 6% polyacrylamide-7M urea gels. The gels were analyzed by phosphorimaging.

**Processivity assays**

The processivity of recombinant RT proteins was analyzed as described using heteropolymeric RNA template in the presence of a heparin enzyme trap to ensure a single processive cycle, i.e., a single round of binding and of primer extension and dissociation (29). The template-primer (T/P) was prepared by annealing the 497-nt HIV PBS RNA with the [32P]-5’ end-labelled 25-nt DNA primer D25 at a molar ratio of 1:1, denatured at 85°C for 5 min, and then slowly cooled to 55°C for 8 min and 37°C for 5 min to allow for specific annealing of primer to the template. RT enzymes with equal amounts of activity and 40 nM T/P were pre incubated for 5 min at 37°C in a buffer containing 50 mM Tris-HCl (pH 7.8), 50 mM NaCl and 6 mM MgCl2. Reactions were initiated by the addition of dNTPs at 5 µM and heparin trap (final concentration 3.2 mg/mL) and incubated at 37°C for 30 min; then, 2 volumes of stop solution (90%
formamide, 10 mM EDTA, and 0.1% each of xylene cyanol and bromophenol blue) were added to stop the reaction. Reaction products were denatured by heating at 95°C and analyzed by 6% denaturing polyacrylamide gel electrophoresis and phosphorimaging. The effectiveness of the heparin trap was verified in control reactions in which the trap was preincubated with substrate before the addition of RT enzymes and dNTPs.

RNA-dependent DNA polymerase activity

The same 497-nt RNA and 5'-end $[^{32}P]$-labeled D25 primers described previously (29) were used to assess the polymerization efficiency of recombinant RT enzymes in time-course experiments. Final reaction mixtures contained 20 nM T/P, 400 nM RT enzyme, 50 mM Tris-HCl (pH 7.8), and 50 mM NaCl. Reactions were initiated by adding 6 mM MgCl$_2$ and dNTPs at 200 µM and sampled at 40 sec and 60 sec respectively, and mixed with 2 volumes of stop solution (90% formamide, 10 mM EDTA, and 0.1% each of xylene cyanol and bromophenol blue). Reaction products were separated by 6% denaturing polyacrylamide gel electrophoresis and analyzed as described (29).

Incorporation efficiency of tenofovir-diphosphate (TFV-DP) in gel-based primer extension assays

Incorporation of TFV-DP was monitored using DNA primer D25 and DNA template D42 (sequences are shown in Figure 6). The 5'-end $[^{32}P]$-labeled D25 primer was annealed to D42 at a molar ratio of 1:3. To monitor the inhibitory efficiency of TFV-DP, we performed a primer extension assay; final reaction mixtures contained 20 nM T/P, RT enzymes at similar activity, 1 µM dNTPs, 50 mM Tris-HCl (pH 7.8), 50 mM NaCl and
variable concentrations of TFV-DP. Reactions were initiated by adding 6mM MgCl₂ and incubated at 37°C for 20 min. Reactions were stopped by mixing with 2 volumes of stop solution (90% formamide, 10 mM EDTA, and 0.1% each of xylene cyanol and bromophenol blue). Reaction products were separated by 6% denaturing polyacrylamide gel electrophoresis and analyzed as described above.

Excision and rescue of chain-terminated DNA synthesis in the presence of ATP

The assay was performed as described previously (31). Briefly, the 5'-end [³²P]-labeled DNA primer 17D was annealed to DNA template 57D and subsequently extended with TFV-DP using WT RT. Complete primer termination with TFV monophosphate (TFV-MP) was verified by primer extension assay using WT RT and denaturing polyacrylamide gel electrophoresis. Excision and the ensuing rescue of chain-terminated DNA synthesis were monitored in time course experiments (0-120 min) in the presence of 3.5 mM ATP (pretreated with inorganic pyrophosphatase) and a dNTP cocktail consisting of 100 µM dATP, 100 µM dTTP, 10 µM dCTP and 100 µM ddGTP. All RT enzymes were normalized with equal amounts of activity as described previously (32). Samples were resolved by 6% denaturing polyacrylamide gel electrophoresis followed by phosphorimaging.

RESULTS

The W153L substitution in HIV-1 RT impairs virion-associated RT activity
To validate the impact of the W153L substitution on RT activity in two HIV-1 variants, HIV-1\textsubscript{NL4-3} and HIV-1\textsubscript{BH10}, equivalent amounts (10 ng p24) of virion-containing cell-free supernatants of transfected 293T cells were assessed. The W153L viral mutants from both HIV-1\textsubscript{NL4-3} and HIV-1\textsubscript{BH10} showed significantly decreased RT activity, ie 7.7% and 6.9% of the corresponding WT activities, respectively (Figure 1). The W153L mutant viruses from HIV-1\textsubscript{NL4-3} and HIV-1\textsubscript{BH10} exhibited similar levels of RT activity to each other. Levels of RT activity associated with the two WT viruses were also similar. Unless specified, viruses derived from HIV-1\textsubscript{NL4-3} were used in this study.


The W153L substitution alone has been shown to impair viral replication capacity in Jurkat cells (21). Now, we wished to investigate the impact of interactions between W153L and each of substitutions at positions K65R, M184I, K101E, K103N, E138K and Y181C on viral replication. For this purpose, we used a well-established non-competitive high throughput infectivity assay with TZM-bl cells that were infected with recombinant HIV-1 viruses that were normalized in terms of inoculum on the basis of p24 antigen (22, 23). The infectiousness of the WT and mutant viruses was determined by measuring luciferase activity at 48 hr post-infection. The results show that the replication of all the mutant viruses containing W153L was diminished compared to WT (Figure 2). At a p24 input of $2.5 \times 10^7$ pg/ml, the relative replication capacity (RC) of virus containing W153L alone was decreased by ~7-fold compared to WT, consistent with previous
W153L enhances susceptibility to TFV

It was previously shown that HIV-1 containing W153L displays high level resistance to the novel NcRTI Compound A (160-fold) without affecting susceptibility to NVP (17). In contrast, W153L conferred hypersusceptibility (5- to 10-fold) to the N(t)RTIs ABC, TFV, 3TC and FTC. Now, we determined the impact of W153L, alone or in combination with each of K65R, M184I, K101E, K103N, E138K and Y181C, on susceptibility to each of the NRTIs TFV and 3TC, the NNRTIs ETR, RPV, EFV and NVP, and to the NcRTI Compound A in TZM-bl cells. Table 1 shows that all of the W153L-containing mutant viruses, including W153L, W153L/K65R, W153L/M184I, W153L/K101E, W153L/K103N, W153L/E138K, and W153L/Y181C, displayed high-level (>133-fold) resistance to the novel NcRTI Compound A, confirming that W153L plays a crucial role in conferring resistance to this compound. Moreover, the presence of W153L together with other common RT-resistance substitutions did not reverse resistance to Compound A. K65R on its own conferred hypersusceptibility to Compound A (0.3-fold), in agreement with a previous report (17). In contrast, M184I alone displayed slight resistance to Compound A (2.5-fold). This indicates that M184I, like M184V as demonstrated in a previous report (17), confers modest resistance to Compound A.
In regard to NNRTIs, the W153L-containing viruses displayed little or no resistance to ETR, RPV, NVP, and EFV. Moreover, W153L did not enhance the resistance of common NNRTI substitutions to these compounds. The double mutants W153L/K101E, W153L/K103N and W153L/Y181C conferred high level resistance (>60-fold) to NVP, while all the other mutant viruses were susceptible to NVP. The roles of each of the K101E, K103N E138K, and Y181C substitutions in regard to NNRTI resistance have been well characterized previously (33-36).

W153L alone displayed hypersusceptibility to 3TC as demonstrated by fold-changes in EC50 (0.3-fold), consistent with previous observations (17). However, the W153L/M184I combination conferred high level resistance to 3TC as did as M184I alone, indicating that W153L could not reverse the 3TC resistance phenotype of M184I. None of the other W153L double mutants showed resistance to 3TC.

For TFV, W153L alone displayed hypersusceptibility as demonstrated by a fold-change in EC50 of 0.3-fold. Interestingly, the double W153L/K65R mutant reversed the resistance associated with K65R to a phenotype of hyper susceptibility (fold change in EC50 from 2.7 to 0.3). Hypersusceptibility to TFV was also observed with all the double mutants with fold changes in EC50 from 0.3-0.5. These data show that W153L can enhance TFV susceptibility and can reverse the TFV resistance that is associated with K65R. In agreement with previous reports (17), K65R alone displayed hypersusceptibility to Compound A as demonstrated by a fold change in EC50 of 0.2-fold.

Diminished HIV-1 RT processivity is a determinant of impaired viral replication capacity (37-40). For example, the M184I/V substitution is associated with a deficit in enzyme processivity, which, in turn, can be correlated with lower replication fitness, especially in cell types that have low dNTP pools (37, 41). To investigate whether W153L alone, or in the presence of other common resistance substitutions, i.e., K65R, M184I, K101E, K103N, E138K and Y181C, might impact enzyme processivity, we performed gel-based single-cycle processivity assays using recombinant WT RT or RT containing each of the substitutions mentioned above. Initial experiments were performed with 0.5 µM of dNTPs, and we found that the mutant RT enzymes were barely able to extend primer (not shown). In order to enhance extension and better validate relative processivity among RT variants, we next used 5 µM of dNTP in the processivity assays. Figure 3 shows that only WT RT was able to synthesize substantial amounts of full-length DNA product, i.e. 471 nt in length in this circumstance. In contrast, all of the mutant W153L-containing RT enzymes were diminished in processivity as demonstrated by shorter DNA products compared with the WT RT control (Figure 3). Similar results were obtained with the BH10 WT and W153L RTs as with the NL4.3WT and W153L RTs (not shown). These findings show that the W153L substitution in RT, alone or in combination with each of the K65R, M184I, K101E, K103N, E138K and Y181C substitutions impairs enzyme processivity. Therefore, the lower replication capacity of W153L-containing viruses is correlated with a defect in RT enzyme processivity.
The W153L substitution alone or in tandem with common resistance substitutions diminishes the efficiency of DNA synthesis.

In addition to enzyme processivity, RT polymerization efficiency is an important determinant of viral replication capacity that can also be assessed by primer extension efficiency in gel-based assays. For example, E138K RT is known to have processivity similar to WT RT but causes a decrease in the efficiency of RT polymerization at high dNTP concentrations (29). In contrast, M184I does not result in a defect in the efficiency of polymerization at high dNTP concentration (29, 42), but the simultaneous presence of M184I and E138K can restore the efficiency of processive DNA synthesis and viral RC (29). Another example is the N348I substitution in the connection subdomain of HIV-1 RT, which significantly decreases catalytic efficiency (43), and diminishes polymerization efficiency as demonstrated in gel-based assays without impairing enzyme processivity (30). To determine whether W153L might affect the efficiency of DNA polymerization, we ran gel-based RNA-dependent DNA polymerase assays at high dNTP concentrations (200 μM) in time-course experiments using the recombinant RT enzymes WT, W153L, W153L/K65R, W153L/M184I, W153L/K101E, W153L/K103N, W153L/E138K, and W153L/Y181C. RT molecules were used at ~20-fold excess over substrate, so that any RTs that dissociated from the primer terminus during synthesis would be rapidly replaced and the rate-limiting step would be the addition of nucleotides (nts) (29, 42). The efficiency of polymerization among RT variants was compared by studying the longest extension products at 60 sec (indicated by arrows in Figure 4). The results of Figure 4 reveal that W153L RT enzyme showed a decreased efficiency of primer extension compared to WT RT, as seen by the longest extension products after 60 sec of
polymerization. The double variants W153L/M184I, W153L/K103N and W153L/Y181C showed similar impairments inefficiency of polymerization as W153L alone. However the double mutants W153L/K65R, W153L/K101E and W153L/E138K all showed further impairment in efficiency of primer extension. Similar results were obtained with the BH10 RTs WT and W153L as with the NL4.3 RTs WT and W153L (data not shown). These data confirm that W153L-containing RTs are impaired in efficiency of processive DNA synthesis, and this contributes to the lower replication capacity of W153L-containing viruses.

W153L does not diminish RNase H activity either alone or in a background of RT containing K65R, M184I, K101E, K103N, E138K or Y181C.

Substitutions at certain residues in HIV-1 RT can impair RNase H activity and contribute to reductions in HIV-1 replication fitness (44, 45). In addition, some NNRTI resistance substitutions are associated with impaired RNase H activity (44, 46-50) and reduced RNase H activity may also be associated with enhanced resistance to both NRTIs and NNRTIs (51, 52). To investigate the impact of W153L alone, or in a background of K65R, M184I, K101E, K103N, E138K and Y181C, on RNase H activity, we monitored multi-cycle RNase H-mediated RNA cleavage in time-course experiments using WT RT and the RT variants W153L, W153L/K65R, W153L/M184I, W153L/K101E, W153L/K103N, W153L/E138K, and W153L/Y181C. The results showed that all the mutant enzymes yielded the same cleavage profiles and at the same efficiencies as did WT enzyme, as demonstrated by the relative band densities of the uncleaved RNA substrates and the...
cleaved products (Figure 5). These data demonstrate that the W153L substitution, either alone or in combination with other resistance substitutions, did not diminish RNase H activity. Thus, RNase H does not likely contribute to the diminished viral replication capacity associated with viruses containing these various substitutions.

**W153L enhances incorporation of TFV-DP in gel-based primer-extension assays**

The two major mechanisms that account for resistance to NRTIs are discrimination and excision (for reviews see (11, 12)). In discrimination, the mutant RT enzyme has decreased incorporation efficiency of NRTIs relative to that of the natural dNTP substrate. The consequence is diminished incorporation of drugs into the DNA chain. In order to determine the biochemical mechanism of the hypersusceptibility phenomenon of HIV-1\(^{W153L}\) to TFV in cell-based assays, we performed primer extension assays at a fixed concentration of dNTPs (1 µM) to compare the incorporation efficiency of TFV-DP in DNA-dependent DNA polymerization by WT, W153L and K65R RT enzymes (Figure 6). Use of the W153L RT mutant resulted in a 3-fold increase in susceptibility to TFV-DP, as demonstrated by a FC in IC50 of 0.3 (Table 2). In contrast, the K65R mutant RT yielded a decrease in susceptibility to TFV-DP as demonstrated by a FC of 3.3 in IC50. These data are consistent with the results of studies performed using cell-based assays. The 3-fold decrease in IC50 for TFV-DP as a result of W153L within RT suggests that the latter substitution can enhance the incorporation efficiency of TFV-DP.

**Efficiency of ATP-dependent excision of TFV-MP and rescue of DNA synthesis**
The excision of incorporated NRTIs is a second mechanism whereby resistance to NRTIs by mutant RTs can occur. Using the recombinant RT enzymes WT, W153L, and K65R, we determined the excision efficiency of TFV-MP in gel-based ATP-dependent excision/rescue experiments. The results of Figure 7A and 7B show that the W153L RT only displayed a modestly diminished efficiency of excision of incorporated TFV-MP while the K65R RT severely compromised this activity. These data indicate that the slightly decreased excision efficiency of the W153L RT cannot account for the hypersusceptibility of TFV that is associated with the W153L substitution.

**DISCUSSION**

Due to the pivotal role of HIV-1 RT in viral replication and the fact that drug resistance occurs against all RTIs, efforts are required toward the development of new antiretroviral compounds with distinct mechanisms and resistance profiles. The recent identification of a novel BFPY NcRTI compound termed Compound A is exciting. In spite of its improved antiviral potency, however, Compound A selects for a substitution at position W153L in RT and does not possess an advantageous pharmacokinetic profile. Nonetheless, compound A possess a superior profile compared with the NcRTI known as INDOPY-1 that displays overlapping resistance with certain NRTIs such as 3TC and FTC. Furthermore, most substitutions associated with decreased susceptibility to INDOPY-1 are clustered around the dNTP binding site (13-15).

The W153 residue in HIV-1 RT is highly conserved but is not located at the dNTP binding site. Until recently, this substitution had not been associated with resistance to NNRTIs or NRTIs (17). The W153L substitution appears to not only confer drug...
resistance but also to play a critical role in RT structure and function. Ours is the first
study to characterize W153L by both cell-based and biochemical methods. We show that
W153L, alone or in a background of clinically relevant substitutions for NRTIs and
NNRTIs, severely diminished viral replicative fitness by impairing enzyme processivity
and polymerization efficiency. We have also provided phenotyping data that demonstrate
that W153L can reverse the resistance to TFV conferred by K65R and that W153L
together with K65R can yield hypersusceptibility to TFV.

K65R confers reduced susceptibility not only to TFV but also to most NRTIs except
zidovudine (53). An increasing number of reports now show that levels of K65R are
increasing worldwide, especially in subtype C infected patients who have failed
antiretroviral therapy (54-56). It was previously shown that K65R confers
hypersusceptibility to Compound A, while W153L confers hypersusceptibility to TFV
(17). Our phenotyping data in TZM-bl cells now confirm this mutual hypersusceptibility
and, more importantly, we also demonstrate that W153L together with K65R can
hypersensitize to TFV. This unique phenotype may make it advantageous to co-
administer an NcRTI with a resistance profile similar to Compound A together with TFV
to suppress viral replication as well as to suppress viruses resistant to either of these drugs.

Previously, K65R variants were shown to be hypersusceptible to earlier NcRTI
compounds such as INDOPY-1 and to another investigational RT inhibitor i.e. 4'-
ethynyl-2-fluoro-2'-deoxyadenosine (EFdA) (57). Resistance to INDOPY-1 was
conferred by M184V (5-fold)(58). It was also previously shown that combining TFV
with INDOPY-1 in cell culture can prevent the selection of resistance (59). However, the
simultaneous presence of K65R with M184V did not result in hypersusceptibility to INDOPY-1 as did the combination of K65R with W153L in regard to Compound A. We are currently studying whether combining TFV with Compound A may affect the selection of resistance to both of these compounds. The mechanism of K65R resistance to TFV has been demonstrated to be a combination of decreased drug incorporation and decreased excision, that both occur in a balanced fashion (60, 61). Here, we have shown that the mechanism of TFV hypersusceptibility that is conferred by the W153L substitution is mainly due to increased efficiency of TFV-DP incorporation by RT.

The emergence of HIV-1 drug resistance is often associated with reductions in viral fitness, but the subsequent selection of additional compensatory substitutions can often restore fitness while increasing levels of resistance(62). Here, we have demonstrated that W153L-harboring viruses display diminished replicative fitness compared to WT virus. No compensatory effects were observed between W153L and other clinically relevant substitutions that confer resistance against NRTIs or NNRTIs.

The mechanisms underlying the diminished viral replicative fitness of W153L-containing viruses have been addressed here using purified recombinant RT enzymes. We have shown by gel-based assays that W153L is associated with decreased RT processivity and polymerization efficiency. The fact that the W153L substitution, alone or in combination with other common resistance substitutions, has a negative impact on RT polymerase function may be encouraging in regard to future development of antiretroviral drugs that
select for this substitution. Further structural and biochemical studies of the role of the W153 residue are in progress in our lab.

Previous findings from our laboratory and by others have shown that RT polymerase functions can be affected by interactions between HIV-1 drug-resistance substitutions. For example, a mutual compensatory effect exists between E138K and M184I in regard to enzyme processivity and polymerization efficiency (29, 63, 64). In contrast, an antagonistic interaction exists between Y181C and E138K that results in more severe impairment in enzyme processivity and polymerization efficiency (34), which explains why Y181C may prevent the emergence of E138K in cell culture under conditions of selections with ETR. More recently, the N348I substitution in the connection domain of RT was also shown to restrict the emergence of E138K in cell culture as a result of a negative impact on RT polymerization efficiency (30). Future studies should document how W153L affects the transit kinetics of reverse transcription and further detail the mechanisms of resistance and hypersusceptibility associated with this substitution.

In conclusion, we have provided virological data demonstrating that W153L, either alone or in a background of K65R or other clinically relevant RAMs such as M184I, K101E, K103N, E138K and Y181C, can enhance susceptibility to TFV. Moreover, K65R conferred hypersusceptibility to the NeRTI termed Compound A and W153L, alone or in tandem with other resistance substitutions, impaired enzyme processivity and efficiency of polymerization, albeit without diminishing RNase H activity. We have also shown that the mechanism of TFV hypersusceptibility by the W153L substitution in RT is due to
increased efficiency of TFV-DP incorporation which occurs without compromising the efficiency of excision.

These data demonstrate that combining a NcRTI such as Compound A with TFV or a new pro-drug of TFV termed TAF could suppress K65R resistance and that the emergence of W153L may also be restricted in this circumstance. In the likelihood that the pharmacology of Compound A will make it unsuitable for clinical development, the synthesis and evaluation of derivatives of Compound A that possess excellent tolerability and pharmacokinetics, while sharing the resistance profile of Compound A, should be pursued.

Acknowledgments

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REFERENCES


30.


### TABLE 1

Drug susceptibilities for recombinant HIV-1<sub>NL4-3</sub>wild-type and site-directed mutant viruses containing W153L alone or together with other substitutions in reverse transcriptase as assessed in TZM-bl cultures<sup>a</sup>

<table>
<thead>
<tr>
<th>Virus</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (fold change in resistance) and drug concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TFV(µM)</td>
</tr>
<tr>
<td>WT</td>
<td>4.7±0.9</td>
</tr>
<tr>
<td>W153L</td>
<td>1.4±0.3 (0.3)</td>
</tr>
<tr>
<td>W153L/K65R</td>
<td>1.3±0.5 (0.3)</td>
</tr>
<tr>
<td>K65R</td>
<td>12.7±3.2(7)</td>
</tr>
<tr>
<td>W153L/M184I</td>
<td>1.9±0.6(0.4)</td>
</tr>
<tr>
<td>M184I</td>
<td>ND</td>
</tr>
<tr>
<td>W153L/K101E</td>
<td>2.5±0.4 (0.5)</td>
</tr>
<tr>
<td>W153L/K103N</td>
<td>1.2±0.10(3)</td>
</tr>
<tr>
<td>W153L/E138K</td>
<td>1.8±0.2(0.4)</td>
</tr>
<tr>
<td>W153L/Y181C</td>
<td>2.5±0.4 (0.5)</td>
</tr>
</tbody>
</table>

**TABLE 2** TFV-DP susceptibilities for recombinant HIV-1RT enzymes as assessed in gel-based primer extension assays

<table>
<thead>
<tr>
<th></th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Fold change in resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3.4±0.6</td>
<td>1</td>
</tr>
<tr>
<td>W153L</td>
<td>0.9±0.1</td>
<td>0.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> EC<sub>50</sub>: 50% drug effective concentrations.

ND: not determined.

Data are the means and standard deviations (SD) of 3 independent experiments.
aIC50: 50% inhibitory concentration.

Data are the means and standard deviations (SD) of results of 3 independent experiments.

**Figure Legends**

**FIGURE 1.** Virus particle-associated RT activities. Clarified virus-containing culture supernatants (10 ng viral p24) were assayed for RNA-dependent DNA polymerase activity by the incorporation of [3H]dTTP into poly(rA)/p(dT)12-18. Reaction mixtures were prepared as described in Materials and Methods, incubated at 37°C for 4 h, and quenched with 10% trichloroacetic acid-sodium pyrophosphate. Values represent incorporated radioactivity measured as counts per minute (cpm). Each bar represents the means of three separate measurements. Error bars represent standard deviations.

**FIGURE 2.** The HIV-1 RT substitution W153L impairs viral replication capacity alone or in the background of each of the RT substitutions K65R, M184I, K101E, K103N, E138K and Y181C. Viral stocks of a wild-type HIV-1NL4.3 clone, W153L, W153L/K65R, W153L/M184I, W153L/K101E, W153L/K103N, W153L/E138K, and W153L/Y181C-containing virus were normalized for p24 and used to infect TZM-bl cells. Luciferase activity was measured at 48 hours postinfection as an indication of viral replication. The relative infectivity of WT compared to mutant viruses is shown on the Y-axis while the X-axis denotes the input of p24. The Figure is representative of two independent experiments. Error bars represent the standard deviations (SD).
FIGURE 3. Comparative analysis of enzyme processivity of WT RT and RT enzymes containing the W153L substitution, alone or in combination with other mutations. The processivity of purified recombinant RT enzymes was analyzed using 5'-end labelled DNA primer (D25) annealed to a 471-nt HIV-1 PBS RNA template as substrate; the resulting full-length DNA (FL DNA) is 471 nt in length. Processivities were determined by the size distribution of DNA products in fixed-time experiments at 5 μM dNTP in the presence of heparin trap. The sizes of some fragments of the 32P–labeled 25bp DNA ladder in nucleotide (nt) bases are indicated on the left side of the panel. All reaction products were resolved by denaturing 6% polyacrylamide gel electrophoresis and visualized by phosphor imaging. The position of the 32P-labeled D25 primer (32P-D25) is indicated on the right. A representative image is shown from three independent experiments, in all of which similar results were obtained.

FIGURE 4. Time course experiments showing the effect of the W153L, W153L/K65R, W153L/M184I, W153L/K101E, W153L/K103N, W153L/E138K, and W153L/Y181C substitutions in HIV-1 RT on the efficiency of processive DNA polymerization. The [32P]-labelled D25 primer (32P-D25) was annealed to the 497-nt RNA template, and extension assays were performed at an excess of recombinant RT enzymes at dNTP concentrations of 200 μM. Reactions were stopped at 40 sec and 60 sec, respectively. All reaction products were resolved by denaturing 6% polyacrylamide gel electrophoresis and visualized by phosphorimaging. Sizes of some fragments of the [32P]–labelled 25-bp DNA ladder in nucleotide (nt) bases are indicated on the left side of the panel. The positions of [32P]–labelled D25 primer (32P–D25) are indicated on the right.
extension products generated at 60 sec are identified by arrows and indicate differences in the efficiency of polymerization. The Figure shows a gel from a representative experiment.

**FIGURE 5.** RNase H activity of WT and mutant recombinant RT enzymes. (A) Graphic representation of the RNA/DNA (kim40R/kim32D) substrate duplex used to monitor the cleavage efficiency of mutant and WT RTs. Positions of cleavage sites relative to the 3'-end of the primer are shown at the top. The 40-mer RNA kim40R was labelled at its 5'-terminus by $^{32}$P and annealed to a 32-mer DNA oligo (kim32D). (B) RNase H activity was analyzed by monitoring substrate cleavage in time-course experiments at 0.2, 0.5, 1, 3, 5, and 10 min, respectively. The time point 0 shows the uncleaved substrate in a control reaction without RT enzyme. The uncleaved substrate and cleaved products relative to the 3'-terminus of the DNA primer are indicated on the left. All reactions were resolved by denaturing 6% polyacrylamide gel electrophoresis. Experiments were repeated at least twice with similar results being obtained each time. The Figure shows a gel from a representative experiment.

**FIGURE 6.** Inhibitory efficiency of TFV-DP of recombinant WT and mutant RT enzymes. (A) Graphic representation of the DNA template/DNA primer (D42/D25) substrate duplex used to monitor the inhibitory efficiency of TFV-DP of WT and mutant RTs. Positions of incorporation sites of TFV-DP are shown at the bottom. The 25-mer DNA primer was labelled at its 5'-terminus by $^{32}$P and annealed to a 42-mer DNA oligo (D42). (B) Inhibitory efficiency of TFV-DP was analyzed by monitoring full-length DNA
(FL DNA) products in fixed-time primer extension experiments with RT enzymes at variable concentrations of TFV-DP. Lanes 0 to 8 represent concentrations of TFV-DP at 0, 0.8, 1.6, 3.1, 6.3, 12.5, 25, 50, and 100 µM, respectively. The lane P shows the position of labeled D25 primer in a control reaction without RT enzymes. Positions of incorporation sites of TFV-DP and FL DNA products are indicated on the left. All reactions were resolved by denaturing 6% polyacrylamide gel electrophoresis. Experiments were repeated at least twice with similar results being obtained each time. The Figure shows a gel from a representative experiment.

**FIGURE 7.** Efficiency of ATP-dependent unblocking of TFV-MP terminated primer and rescue of DNA synthesis by WT and mutant RT enzymes. (A) The primer 17D was initially 5’-end labeled with [$\gamma^{32}$P] ATP, annealed to DNA template 57D, and chain terminated with TFV-DP. Combined excision/rescue reactions were compared in time course experiments. Lanes 1 to 8 represent time points at 0, 5, 10, 20, 30, 45, 60, 90, and 120 min, respectively. Experiments were repeated at least twice with similar results being obtained each time. The Figure shows a gel from a representative experiment. (B) Graphic representation of efficiency of rescued DNA synthesis from the representative gel-based experiment.