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Short communication

Comparative analysis of *in vitro* processivity of HIV-1 reverse transcriptases containing mutations 65R, 74V, 184V and 65R + 74V

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ABSTRACT

While HIV-1 reverse transcriptase (RT) mutations of M to V at position 184 are commonly observed in the clinic, the double mutation of 65R + 74V is rarely seen. It has been demonstrated that rapid R → K reversion occurs at RT codon 65 during replication of HIV-1 in human peripheral blood mononuclear cells containing 65R + 74V mutations and that processivity of the RT is reduced relative to wild type. However, clinical studies show that M184V can be detected after several months of therapy interruption, suggesting more effective processivity. Herein, the *in vitro* RT processivity of genetically engineered M184V and double mutant 65R + 74V was compared. Virion-associated RTs of WT pNL4-3, K65R, L74V, M184V and 65R + 74V were used to perform RT processivity assays in the presence of trap, poly(rC)-oligo(dG). Both RTs with 184V and 65R + 74V mutations exhibited similar processivity when compared with each other and a significantly decreased processivity as compared to WT RT. Both mutant RTs synthesized shorter cDNA molecules (37–42 nt) relative to WT RT, which made longer (65–70 nt) cDNA molecules. Since these surprising biochemical results cannot explain the clinical phenotype, a hypothesis is presented to explain the discrepancy and suggest new approaches for future testing.

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During the past decade the effectiveness of highly active antiretroviral therapy in suppressing HIV load to undetectable levels has been demonstrated in several clinical trials. It has also become clear that most reverse transcriptase (RT) inhibitors are much more effective when given in combination than when given alone (Larder et al., 1995; Richman, 1996), although antagonism can be observed *in vitro* and in humans with nucleosides that use the same initial phosphorylation enzyme (Hernandez-Santiago et al., 2007) . It is widely believed that such synergistic drug interaction arises from the fact that certain combinations of drug resistance mutations are particularly detrimental for the enzyme, which leads to a less fit virus as shown by our group and others for RT variants containing mutations K65R, L74V and M184V (Back et al., 1996; Sharma and Crumpacker, 1997, 1999; White et al., 2002; Sharma et al., 2004; Deval et al., 2004a,b).

In addition, certain mutations are mutually exclusive. For example passaging of HIV_{LAI} in the presence of nucleoside reverse transcriptase inhibitors (NRTI), β-D-dioxolane guanine (DXG) or DAPD (Amdoxovir®), results in the selection of K65R or L74V, but both mutations were not selected together (Bazmi et al., 2000). Using human peripheral blood mononuclear (PBM) cell-based replication assays and genotype analysis, we demonstrated that the double mutant 65R + 74V in a pNL4-3 background is 'unstable' because a rapid 65R → K reversion occurs during replication of virus in PBM cells. We further showed that sub-optimal concentrations of human IL-2 slows down the replication of virus and 65R → K reversion. These studies provide evidence that 65R → K reversion is replication dependent and that RT mutations 65R and 74V are mutually exclusive (Sharma et al., 2004). Accordingly, the presence of 65R and 74V on the same viral genome mutations is rare in clinic (Wirden et al., 2005, and Stanford HIV drug resistance database, http://hivdb.stanford.edu). In contrast to the instability of 65R mutation in double mutant virus 65R + 74V, no reversion at codon 65 has been reported in double mutants 65R + 184V and 65R + 68S (Deval et al., 2004a; Røge et al., 2003). These observations indicate the significance of positioning of specific amino acid residues in 3D-RT structure in relation to the biological function of enzyme (Huang et al., 1998).

A biochemical mechanism to correlate attenuated replication capacity of a virus with altered RT function is the determination of *in vitro* RT processivity. However, controversial RT processivity results have been reported based upon the assay condition, choice of template/primer and the concentrations of dNTPs used in the reaction mixture (Arion et al., 1996; Back and Berkhout, 1997; Boyer et al., 1998; Sharma and Crumpacker, 1999; Deval et al., 2004b; Xu

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et al., 2009). Also, studies have demonstrated that replication defect by one mutation can be compensated by other distant mutation(s) leading to the generation of a virus which is replication competent (Olivares et al., 1999; Svarovskaia et al., 2008), and reduced enzyme activity due to single point mutation could be compensated by second-site mutation (Boyer et al., 1998; Harris et al., 1998). RT mutation M184V appears to be quite stable under therapy, however, upon discontinuation of lamivudine (3TC), 100% reversion to WT phenotype occurs after several months (Zaccarelli et al., 2003). The major goal of our study was to compare *in vitro* processivity of RTs containing a stable mutation M184V and an unstable double mutant 65R + 74V. We report herein a direct comparison of RTs containing 65R, 74V, 184V and 65R + 74V mutations in the same assay system.

Various point mutations were created in the background of proviral clone pNL4-3 (Adachi et al., 1986) by using pALTER^{-1} mutagenesis system of Promega (Madison, WI) according to manufacturer's guidelines and our previously described protocols (Sharma et al., 1996, 2004; Nurpeisov et al., 2003; Sharma and Crumpacker, 1999). To eliminate the possibility of reversion during reverse transcription in mutant K65R + L74V virus, we used primary human embryonic kidney cells (293), which are non-permissive for HIV-1 infection. Viruses were produced using SuperFect^{R} reagent of Quiagen (Valencia, CA) and manufacturer's guidelines. Cells were passed into 60 mm dishes 24 h prior to transfection in Dulbecco's Modified Eagles Medium (DMEM) containing 10% heat inactivated fetal bovine serum (FBS) and penicillin/streptomycin (complete medium). To generate virus, the complex containing 10 μg of DNA in 150 μL of serum-free medium and 30 μL of SuperFect reagent was incubated at room temperature for 10 min. One mL of complete DMEM was added dropwise onto 293 cells that were washed once with phosphate buffer saline (PBS). Cells were incubated at 37 °C in the presence of 5% CO_2 for 3 h. The remaining medium-complex was removed and the cells were washed with PBS (4 mL). Four mL of complete DMEM was added and dishes were incubated for 48–72 h. Culture supernatants were collected and centrifuged for 5 min at 833 × *g* (*g* = 1.2) to pellet any debris. The presence of specific mutation was confirmed by sequencing culture supernatant-derived HIV-1 RNA. One mL of each virus was centrifuged for 2 h at 15,000 rpm in a refrigerated centrifuge [Thermo Electron Corp., Marietta, OH; Model, MR 23i; Rotor, DRM6.M]. The pelleted virus was disrupted with 40 μL of lysis buffer containing NP40 and RT activity was determined using homopolymer template/primer [poly A-oligo d(T)] (Boehringer Mannheim, IN) and α-^{32}P dTTP (Morvaek Biochemicals Inc., Brea, CA) according to published protocols (Sharma and Crumpacker, 1999).

Previous, *in vitro* studies have shown that virion-associated protein Ncp7 (Li et al., 1996) and host proteins such as cellular topoisomerase I and p53 (Takahashi et al., 1995; Bakhanashvili, 2001) can interact with HIV RT and enhance RT activity and fidelity. Therefore, we used virion-associated RT lysates in all our assays. In order to compare *in vitro* processivity of various RTs, all assays were performed in the presence of 50-fold excess of trap [poly(rC).oligo(dG)]. The presence of trap ensures that processivity was measured during a single processive cycle.

Assays were performed as described elsewhere (Boyer and Hughes, 1995; Back et al., 1996; Sharma and Crumpacker, 1999). The reactions were terminated by placing the tubes in ice slurry and by adding an equal volume of buffered phenol. cDNA products were extracted by phenol:chloroform:isoamyl alcohol and the purified products were run on 6% polyacrylamide sequencing gel electrophoresis. The wet gels were exposed to autoradiography for 30 min to 2 h. The relative density of DNA bands was compared in autoradiograph using Intelligent Quantifier software (Bio Image Systems, Inc., Jackson, MI).

The visual examination of autoradiograph clearly shows that the entire panel of mutant RTs had decreased processivity in comparison to WT RT (Fig. 1A). Similar RT processivities were noted in three to five independent assays. In order to determine the relative synthesis of cDNA bands generated during processive reverse transcription, a set of six bands were highlighted and the density of each set was calculated. The quantification of cDNA bands revealed that while there is not a significant difference between shorter cDNA products (bands 1–6) generated by WT, 65R and 74 RTs, a significant difference ($p = 0.05$) among larger cDNA products (>6 bands) was evident (Fig. 1B). In contrast, 184V RT and 65R + 74V RT made fewer and shorter cDNA products throughout, suggesting a major processivity defect in these two RTs as compared to WT RT (Fig. 1B and C, Table 1).

A nonparametric (Kruskal-Wallis) test was performed to compare the first 6 band values for the five viruses. While no significant difference was observed ($p = 0.11$) between WT and 65R and WT and 74V viruses, a significant difference between WT and 65R + 74V ($p = 0.05$) and WT and 184V ($p = 0.05$) was observed. The student *t*-test indicated that DNA density decreased at a significantly higher rate for RTs containing 65R + 74V and 184V mutations as compared to WT RT, 65R and 74V RTs. The relative processivities for different RTs were, WT > 65R ≥ 74V > 65R + 74V ≥ 184V. Under the assay conditions, the maximum lengths for cDNA synthesized by WT, 65R, 74V, 65R + 74V and M184V RTs were 66, 60, 62, 42 and 37 nucleotides, respectively. The decreased processivity observed for L74V and M184V RTs were similar to those seen previously (Back et al., 1996; Sharma and Crumpacker, 1999). In a recent study, investigators demonstrated that under low dNTP concentration K65R RT exhibits lower activity in single-cycle processivity assay (Xu et al., 2009) which is in agreement with our observation of a decreased processivity of K65R compared to WT RT.

X-ray crystal structure coordinates for wild type HIV RT complexed with thymidine analogs were downloaded from the international protein data bank at http://www.pdb.org (Huang et al., 1998; Sarafianos et al., 1999, 2002). File 1rtp.pdb (Huang et al., 1998), a covalently trapped catalytic complex of HIV RT and DNA including thymidine triphosphate (TTP), was modified by addition of 3′ hydroxyl to the primer residue. The entire system was protonated using Generalized Born/Volume Integral Formalism as implemented in MOE (v2007.09; CCG, Montreal). The protonated system including Mg^{2+} was typed for use in the Merck Molecular Force Field. Electrostatics were computed with a solvent dielectric of 80 and 15 Å cutoff. Steric interactions were calculated using a 12-6 Leonard-Jones potential. Systematic energy minimization and exploration of side chain rotomers associated with wild type and drug resistant mutants was performed as described previously (Hari et al., 2006). File 1n6q, wild type RT complexed with 3′-AZT-MP terminated DNA in the pre-translocation or "N" site, was fit to the 1rtd reference coordinates using "Matchmaker" in Chimera (Pettersen et al., 2004) to align functional activity space. Best-aligning pairs of chains between reference and match structure were computed using the Needleman-Wunsch alignment algorithm with a BLOSUM-62 Matrix and gap extension penalty of 1. Possible matches were pruned until no pair exceeded 2.0 Å. The 3′-azido group was modified to OH for comparisons with 1rtd.pdb.

The wild type complex containing TTP as prepared in methods is shown in Fig. 2. Energetic analysis of possible rotomer interactions associated with mutations at positions 65 and 74 within the catalytic TTP complex revealed significant shifting of H-bonding patterns and template alignments similar to those suggested previously for the A nucleotide (Deval et al., 2004b). Although the 74V mutation does not have direct contact with the TTP it does provide template positioning for the incoming nucleotide. Repositioning of finger region, β4–β3, in response to the changing L74V caused shifting of R72 and K65. This shifting became more pronounced in the