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Amino Acid Substitutions Created in Reverse Transcriptase and their Influence on Hiv-1 Mutation Frequencies

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Amino acid substitutions created in Reverse Transcriptase and their influence on HIV-1 mutation frequencies

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Amani S. Alhejely ENTITLED Amino acid substitutions created in Reverse Transcriptase and their influence on HIV-1 mutation frequencies BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Amani Alhejely S. M.S., Program in Microbiology and Immunology, Wright State University, 2011. Amino acid substitutions created in RT and their influence on HIV-1 mutation frequencies.

As research has shown, a high mutation rate occurs during the reverse transcription RT process because HIV reverse transcriptase fails to correct erroneously incorporated nucleotides during the reverse transcription process. Based on various research articles, a series of amino acid substitutions created in RT were collected to find out their influence on virus mutation frequencies. Two tables were used; one for amino acid substitutions created in RT that increased the HIV-1 mutation rate and another for amino acid substitutions created in RT that decreased the HIV-1 mutation rate. The tables show which amino acid substitutions created in RT decreased or increased the mutation rate of HIV-1. With more amino acid substitutions showing a decrease in the mutation rate of HIV-1, the tables provide researchers with helpful information for future research. Additionally, the information resulted in three new findings. First, When viewing single amino acids identified with drug resistance, most showed a decrease in the frequency of the mutation rate the of HIV-1; however, if the specified amino acid is not identified with drug

resistance most showed an increase in the HIV-1 mutation rate. Second, when HIV-1 has an increased mutation rate multiple amino acids showed drug resistance. Finally, a structural rationale for the location of residues implicated in the resistance of nucleoside-analog inhibitors (Huang et al., 1998) showed that mutations that decrease and/or increase the mutation rate do not cluster together. Those specified mutations are all around the active site in the crystal structure. Also, these amino acids might be in the primer grip region or interact with the incoming dNTP, and form the dNTP binding site or residues in the fingers subdomain.

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INTRODUCTION:

Since the beginning of the AIDS epidemic researchers have made great efforts to understand the nature of the human immunodeficiency virus (HIV). There are two known species of HIV,HIV 1 and HIV 2, which belong to the L*entivirus* genus in the family *Retroviridae*. HIV virions like other retroviruses contain a viral capsid, which consists of the major capsid protein, p24; the nucleocapsid protein, p7/p9; the diploid single-stranded RNA genome; and the three viral enzymes, protease, reverse transcriptase, and integrase. The viral capsid is surrounded by a matrix protein and it is located underneath the virion envelope. The matrix protein is involved in the early stages of the viral replication cycle and plays an important role in the formation and transport of the preintegration DNA complex into the nucleus of the host cell. The envelope protein complex facilitates viral entry by binding to CD4, the main cellular receptor for all primate lentiviruses, via the outer envelope protein, which is gp120. The transmembrane protein, which is gp41, is involved in the fusion of the viral envelope with the cellular membrane (3).

With HIV-infected persons who are not on antiretroviral therapy, HIV replicates at an

extraordinarily high rate, typically with billions of virions produced daily (50). A key result in the HIV life cycle takes place when HIV RNA is reverse transcribed into HIV DNA (23). The HIV DNA formed in the reverse transcription process is comprised of nucleotides organized into codons (23). The HIV DNA is then transcribed into messenger RNA (mRNA), with each mRNA nucleotide triplet coding for an amino acid. The relationship between amino acids results in the synthesis of a polypeptide, and large polypeptides are subsequently modified to form proteins. A high mutation rate occurs during the reverse transcription process because HIV reverse transcriptase RT fails to correct erroneously incorporated nucleotides during the reverse transcription process (50, 30). The changed nucleotide sequences can result in amino acid substitutions during translation, with the potential formation of a mutated protein (30). In the setting of selective pressure from an antiretroviral medication, the overall effect is the generation of viruses that may have diminished preferences to the medication and these mutated HIV strains are referred to as resistant strains of HIV (30). If a mutation produces an HIV strain resistant to one or more antiretroviral agents currently used by an individual, the resistant strain typically has a selective advantage over

the wild type virus, and it may eventually become one of the dominant circulating strains of HIV (30).

The aim of antiretroviral therapy is to completely suppress viral replication and delay drug resistance. An increasing viral load is often the sign that HIV drug resistance has developed. Resistance in HIV therapy is defined as the virus' breakdown to respond following treatment and its subsequent active replication, or reproduction, of itself. It is possible that a person may have a virus that is resistant to a drug that the patient has never taken because the HIV in the patient spontaneously mutated and developed a resistance prior to treatment. Since each class of drug combats HIV in a different way, cross-resistance is not conveyed from one class to another; the three major classes of anti-HIV drugs are nucleoside reverse transcriptase inhibitors (NRTI), nonnucleoside reverse transcriptase inhibitors (NNRTI), and protease inhibitors (PI). Viral mutations may occur at a number of different places in the viral gename, and typically more than one mutation is needed for a virus to become drug resistant (1).

There are two types of antiretroviral resistance assays (drug-resistance assays) that are currently available to assist the clinician in assessing HIV resistance: genotypic

assays and phenotypic assays (22, 34, 30, and 24). Drug-resistance assays are unique laboratory tests used by HIV/AIDS specialists to determine whether the virus infecting a patient is likely to respond to specific antiretroviral medications (24). Recently, some investigators have employed newer techniques, such as allele-specific PCR, single-genome, and ultra-deep sequencing, to assess the role of minority HIV variants that harbor drug resistance, but are not detectable by current standard genotypic or phenotypic assays (34,30). Both genotypic assays and phenotypic assays complement each other in treating and monitoring HIV/AIDS patients (30).

Table 1: Amino acid mutations, which result in a decrease in the mutation rate of nucleoside resistant of HIV-1 reverse transcriptase.

Mutant sequence	Region	Drug resistance	Fold change[1]	Type of assay	Reference
F61A	Fingers	ddTTP d4TTP	12	Gap filling, Gel based mispair extension assay	[14]
K65R	Fingers	ddc ddI 3TC Multi-NRTI[2]	3.3 8 0.3	Replicating Virus, Forward *lacZ* Gap filling	[37] [46]
L74V	Fingers	Abacavir & Didanosine (ddi)	3.5	*In vitro,* forward (gapped DNA), *lacZ(274 nt)* Replicating Virus, Forward *lacZ* Gap filling	[29] [37]
D76V	Fingers	None Reported	2.5 9.0-14.0 0.4 8.8	Replicating Virus, Forward *lacZ* *In vitro,*Forward *lacZa* M13mp2 lacZa forward mutation assay	[37] [32]
R78A	Fingers	AZT/ 3TC	3.3 9 8.9 0.3	Replicating Virus, Forward *lacZ* *In vitro,*Forward *lacZa*	[37] [32]

E89G	Palm	ddg 3TC LOOK reference 12 in #10	2	*In vitro* , Forward (gapped DNA), lacZ a(274 nt) Replicating Virus, Forward *lacZ*	[29] [13] [12] [37]
Y115F	dNTP Binding	abacavir	1.5-2.0 1.2= wt	*In vitro* , Forward (gapped DNA), lacZ a(174 nt)	[5] [7] [8]
Y115V	dNTP Binding	None Reported	3.0-4.0	*In vitro* , Forward (gapped DNA), lacZ a (174 nt)	[5] [7]
Q151N	dNTP Binding	Multi-NRTI (Part of 151 complex)3 AZT	5 13 0.2 5.7	Replicating Virus, Forward *lacZ* Misincorporation RT assay M13 lacZa forward mutation assay	[37] [51] [52]
K154A	dNTP Binding	None Reported	2.1	Replicating Virus, Forward *lacZ* Misincorporation RT assay	[51]
M184V	Palm	ddI/ddc/AZT Multi-NRTI[4]	2.4	*In vitro* , Forward (gapped DNA), lacZ a (274 nt) Mismatched primer extension assay	[39] [49] [13]
M184I	Palm	ddI/ddc Multi-NRTI[5]	4	Forward assay	[43]

W229A	Primer Grip	None Reported	2 0.5	Replicating Virus, Forward *lacZ*	[37]
Y318F	Thumb	Efavirenz & Nevirapine (NNRTIs)	1.4	Replicating virus, Reversion *luc*	[8]
T69I+AZT+Insertion	Fingers	Multi-NRTI[6]	1.4	Replicating virus, Reversion *luc*	[8]
T69s+Insertion+-AZT	Fingers	Multi-NRTI[6] AZT	4.0-16.0 9	M13 gapped duplex DNA, *lacZ* Virion-associated RT inhibition asay	[10] [33] [53]
D76V/R78A	dNTP Binding	None Reported	9	Replicating Virus, Forward *lacZ*	[37]
R78A/Q151N	Fingers & dNTP Binding	Multi-NRTI(part of 151 complex)[3]	14.3	Replicating Virus, Forward *lacZ*	[37]
K103N/Y318F	Palm&Thumb	Efavirenz & Nevirapine (NNRTIs)	2.2 2.69	Replicating virus, Reversion *luc*	[8]
R72A	finger	None Reported	3.3	Mismatch extension assay Running start fidelity assay Standing start fidelity assay lacz ? read it again	[35]
W229A AC:AG		None Reported	 2	Misamatch extension assay Running start fidelity assay	[54] [37]

			0.5	Standing start fidelity assay	
F227A		None Reported		Miamatch extension assay	[54]
GA:CA			5.5	Running start fidelity assay Standing start fidelity assay	[37]
V148I	?	None Reported	3.8	M13 lacZa forward mutation assay	[52]

[1]statistically significant with associated p-value < 0.05

Relative to wild type.

[2]Abacavir, Didanosine, Emtricitabine, Lamivudine, Tenofovir

[3]see references{26, 46}

[4]Abacavir, Emtricitabine, Lamivudine

[5]Emtricitabine, Lamivudine

[6]Zidovudine, Lamivudine, Stavudine, Didanosine, Zalcitabine

[7]Most frequent in patient treated with efavirenz (NNRTI)-containing therapies

Table 2: Increase in the mutation rate of nucleoside resistant of HIV-1 reverse transcriptase.

Mutant Sequence	RT region	Drug Resistance(s)	Fold change 1	Type of assay	Reference
Y115A	dNTP Binding palm	AZT	4.0 2.3	*In vitro* , Forward (gapped DNA), *lacZ* α(274 nt) Replicating Virus, Forward *lacz*	[29] [37]
Y183F	palm	efavirenz	1.6	*In vitro* , Forward (gapped DNA), *lacZ*α (274 nt) Mismatch extension assay Running start fidelity assay (1.2)	[29] [54] [8]
F227A A:A	Primer Grip	None Reported	5.5	*In vitro* , Forward (gapped DNA), *lacZ* α (274 nt) Mismatch extension assay Running start fidelity assay	[29] [54]

W229A A:A	Primer Grip	None Reported	27	Mismatch extension assay Running start fidelity assay 3 assay	[54] [37]
Q258A	Thumb	None Reported	2.3	*In vitro,* framshift	[4]
G262A	Thumb	None Reported	4.4	*In vitro,* Forward *lacz a*	[2] [3] [37]
W266A	Thumb	None Reported	3.2	*In vitro* , Forward (gapped DNA), *lacZ* α (274 nt)	[2] [3], [37]
Q269A	Thumb	None Reported	2.6 3.5	*In vitro* , Forward (gapped DNA), *lacZ* α (274 nt) *In vitro,* framshift (4nt)	[2] [4]
Y501W	RNaseH Primer Grip	None Reported	2.7*	Replicating Virus, Forward *lacz*	[37]
M41L/T215Y	Fingers & palm	AZT (NRTI) 3TC	2.0* 3.3	Replicating Virus, Forward *lacz*	[36]
M41L/D67N/K70R/T215Y	Fingers & palm	AZT (NRTI) 3TC	3.0* 4.3	Replicating Virus, Forward *lacz*	[36]
L74V/Y115F/M184V	Fingers & palm	Abacavir and Zalcitadine (NRTIs)	1.91 3.39	Replicating Virus, Reversion *luc*	[8] [29]

Y115WW/M230I	Primer grip & dNTP Binding	None Reported	3-.35	Misinsertion/mispair extension assay *In vitro*, Forward *lacz* α	[17]
R72A	Finger	None Reported	3.3	Misamatch extension assay Running start fidelity assay Standing start fidelity assay	[35]
Q151N/ R78A		AZT 3TC Hu Thy TG	13/9	Replication a lacz α	[37]
W229A A:A		None Reported	27	Misamatch extension assay Running start fidelity assay Standing start fidelity assay	[54]
F227A A:A		NNRTI	5.5	Misamatch extension assay Running start fidelity assay Standing start fidelity assay	[54]

As shown in tables 1 and 2 when viewing single amino acids identified with drug resistance, most showed a decrease in the frequency of the mutation rate the of HIV-1 genome ; however, if the specified amino acid is not identified with drug resistance most showed an increase in the HIV-1 mutation rate:

In untreated patients, HIV undergoes mutations at every position in the genome numerous times a day, allowing the emergence of viruses that can evade the immune response (10). Studies of *in vivo* viral dynamics have estimated the viral turnover to be 10^7 to 10^9 in a single patient, with the *in vivo* half-life of a virion estimated at 2 days (41, 42). This large, short-lived population of virus is sustained by a rapid rate of viral replication. Calculations of probable viral growth rates and mutation frequencies suggest that the replication rate is sufficient to create a population of viruses of sufficient diversity to respond to any immunological or pharmacological challenge.

Drug-resistant viruses have a greater ability to replicate under conditions of drug pressure when compared to the wild type as determined via viral fitness measurements (10). Conversely, the overall rate of

replication for most drug resistant viruses is lower than that of the wild type virus in the absence of treatment (34). This lowered replication rate, referred to as decreased viral fitness, can affect the rate of appearance of new variants (33). Therefore, nucleoside-analog resistance mutations in RT can contribute to the rate of emergence of new viral variants. Thus, a good understanding of the influence of drug resistance mutations on viral mutation rate and viral variation remains an important goal of HIV research.

HIV-1 forward mutation frequencies have been analyzed in a single round of replication in culture using HIV-1 vectors containing reporter genes *lacZ and tk*, or in vitro using gapped DNA repair and other types of assays (see Tables 1&2). A series of amino acid substitutions were created in RT to find out their influence on virus mutation frequencies. The focus of the current study is on amino acid residues that were targets for site directed mutagenesis, generally chosen based on their involvement in drug resistance. These data indicate that single amino acids associated with drug resistance mostly showed a decrease in the frequency of the mutation rate of the HIV-1 genome; however, if the specified amino acid is not associated with drug resistance most showed an increase in

the HIV-1 mutation rate (Tables1 and 2).

Some studies tested these mutations to determine what influence they would have on virus mutation frequencies by using an assay for measuring the HIV-1 mutation frequency in one round of replication. In some cases, researchers chose residues located in the finger and palm subdomains such as F61, K65R, L74V, D76V, R78A, R72A, which confere resistance to drugs such as ddTTP, d4TTP, ddc, ddI, 3TC, and combinations likeNRTI andAZT.

In Tables 1 all single-amino-acid substitutions in RT that significantly decreased HIV-1 mutation frequencies confered drug resistance, except D76V and R72A, for which drug resistance has not yet been reported. Also, mutations E89G, M184V, and M184I, located in the palm subdomain confer drug resistence to ddI, ddc, AZT, and combinations of NRTI5, ddg and 3tc.

In other cases, researchers chose amino acid residues that interact with the incoming dNTP and form the dNTP binding site identified in structural studies. For example, a mutation in the dNTP binding site of HIV-1 RT, Q151N, has been reported to increase fidelity by a factor of 13, using *lacZ* or a factor of 5.7 using M13 *lacZ* a forward mutation assay (51). The Q151 residue in RT is implicated in drug resistance to multiple drugs. Also, there is Y115F has been

reported to increase fidelity by factors of 1.5 to 2.0 by using forward gapped DNA repair (7).

In other studies, the focus was on amino acids in the primer grip. The αH helix motif of the HIV-1 RT thumb subdomain binds to the minor groove of the template-primer complex and is connected with alterations in RT processivity and fidelity. The 3‘ end of the primer is positioned close to the RT active site by conserved residues 224 to 335 on the B11b loop and the β12 and β13 hairpins (37). The region bound by residues 227 to 235 is considered the primer grip (37). The W229A mutation in the αH helix has been reported to modify misincorporation and mispair extension frequencies, presumeably through an increase in the rate of RT dissociation from the template DNA or through an increase in strand slippage resulting in lower enzyme fidelity (54). These mutations in RT were tested to determine what influence they would have on virus mutation frequencies by using an assay for measuring the HIV-1 mutant frequency in one round of replication. The W229A RT variant influenced virus mutant frequencies. The W266 and G262A residues are located in the thumb domain and have been found to increase HIV-1 RT cell-free fidelity in the *lacZ* reporter assay (37). The W266A and G262A variants have decreased fidelity for template-primer slippage

errors, such as frameshift and deletion mutations; drug resistance has not yet been reported for these. Another point of interest is amino acid residues in the RNase H domain. The RNase H domain is responsible for the RNase H activity essential for the degradation of the viral RNA present in the RNA-DNA replication intermediate, allowing the DNA to act as a template for the DNA-dependent DNA polymerase activity of RT to complete synthesis of the double-stranded viral DNA. The RNase H activity of RT is necessary for the strand transfered that occur during reverse transcription. Amino acid residues in the RNase H domain that are involved in the RNase H primer grip contain residues 473 to 476, 501, and 505. Some researchers tested whether residues in the RNase H primer grip could influence virus mutant frequencies. In particular mutation at Y501 was analyzed. Analysis of these RT variants on virus mutant frequencies indicated that Y501W significantly influenced virus mutation frequencies compared to wild-type RT; drug resistance for this location has not yet been reported (37).

In summary, several RT variants with single amino-acid changes have been characterized in both the polymerase and RNase H domains that influence virus mutation frequencies and have been implicated in drug resistance to multiple

drugs. Many mutations that increase mutation rate have not yet been implicated in drug resistance.

When HIV-1 has an increased mutation rate multiple amino acids showed drug resistance:

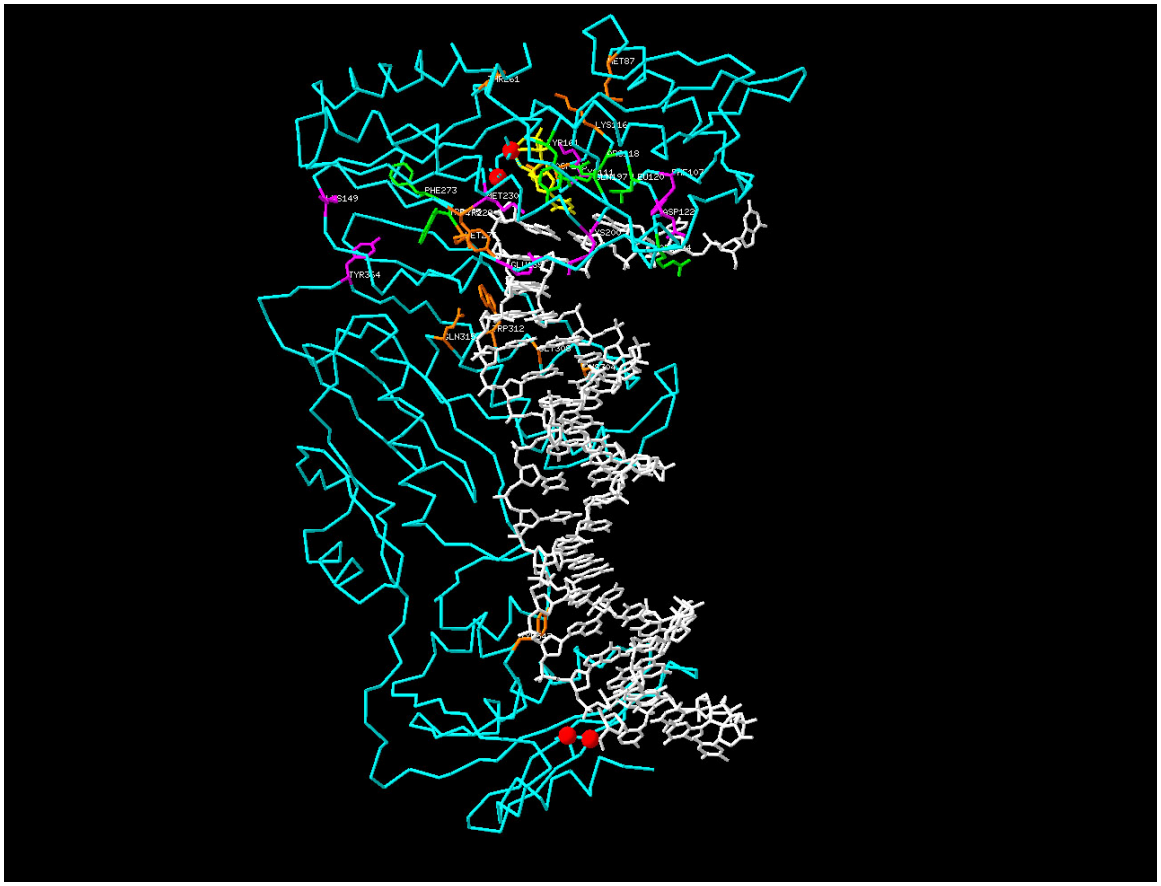
Most drug-resistant RT enzymes that increase the viral mutation rate have multiple amino acid substitutions. Some researchers have been identified several amino acid residues in RT that, when mutated, can significantly alter virus mutant frequencies. Researchers tested whether the mutated residues could act together to alter virus mutant frequencies by analyzing a series of RT variants with combined mutations. The variants tested integrated the D76V/R78A, R78A/Q151N mutations, which corresponded to mutations in the finger subdomain. The HIV-1 RT variants included either the D76V/R78A(-) or the R78A/Q151N(-,+) double mutation. These substitutions led to mutation frequencies that were significantly lower than the frequencies of wt HIV-1 (Tables 1 and 2). It was shown that the mutation frequency of R78A/Q151N was appreciably lower than the variants with each mutation alone, while the mutation frequency of D76V/R78A was significantly lower than that of the R76V RT variant but not that of the R78A RT variant alone (37). As shown in Table 2 the drug resistance mutation that identified an increase in HIV

mutation frequency represents a combination of mutations in HIV-1 RT. For example, M42L/T215Y, M41L/D67N/K70R/T215Y, Y115W/M230I and Q151N/R78A have been implicated in drug resistance to AZT, 3TC, abacavir and NRTI. The data support the conclusion that most of the drug resistance mutations that increase virus mutation frequencie functions as residues in RT that act together.

Figure 1: Images from the Swiss-PdbViewer software-assisted visualization of the amino acid residues in RT that lead to decrease or increase mutation rate.

Figure 1-a: The image shows the residues for which mutation leads to a decrease in mutation rate are colored in purple, the residues mutation leads to increase in mutation rate are colored in orange, the residues who mutation leads to increase and decrease in mutation rate are colored in green, Mg cations (red) and the protein backbone color in white:(left side)

a



1-b (right side)

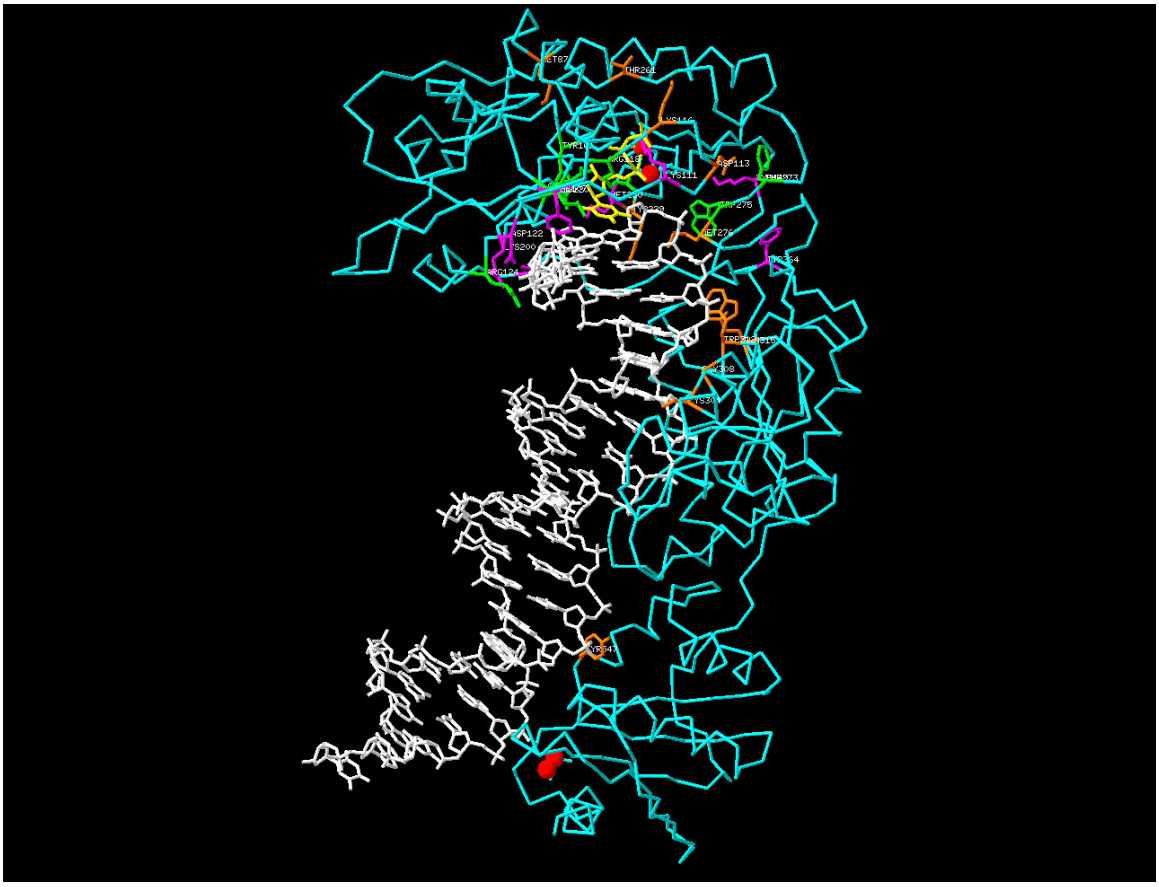
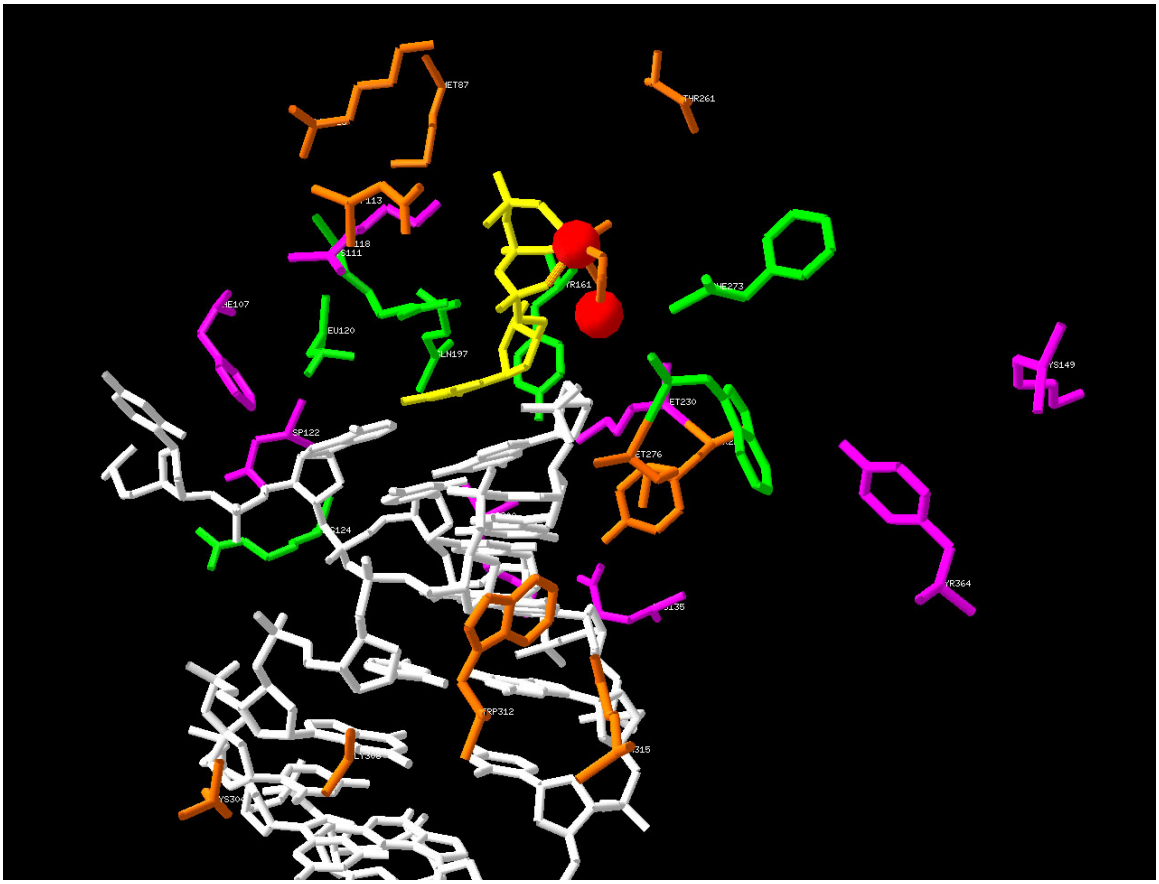


Figure 2: Close-up of each of the grouped amino acid residues:
a



b

Mutations that decrease and/or increase the mutation rate do not cluster together. They are all around the active site:

The structure of a ternary complex with dNTP bound at the active site gave a more detailed explanation of the dNTP-binding pocket, and provided a structural rationale for the location of residues implicated in resistance to nucleoside-analog inhibitors (25). The ternary structure suggests that upon binding of the dNTP substrate, HIV-1 RT forms a closed structure, whereby residues in the fingers subdomain become a component of the binding pocket (25). A number of residues in this region are mutated in nucleoside-analog resistant variants, and have also been shown to affect the fidelity of HIV-1 RT (25). As shown in Table 1 and 2 and Fig 1 and 2, mutations that decrease and/or increase the mutation rate do not cluster together. They are all around the active site. These amino acids are residues in the fingers subdomain or primer grip region of HIV-RT or represent residues that interact with the incoming dNTP and form the dNTP binding site. As previously mentioned, the primer grip region (residues 227–235) correctly positions the 3‘ ends of primers. Point mutations were formed by alanine substitution at positions 224–235 and error frequencies were measured by extension of a dG:dA

primer-template mismatch. Wisniewski et al. (1999) found that mutants F227A and W229A extended the dG:dA mismatch 40% and 66% less efficiently, respectively, as compared with the wild type. These researchers also examined the misinsertion rates of dG, dC, or dA across from a DNA template dA using RT mutants F227A and W229A. They used running start misinsertion assay on a template that allows misalignment Synthesis. They also performed a running start experiment using substrate A. In this experiment the template has two T residues and one G after the 3′ end of the annealed primer and it is similar to substrate E. After the G, the substrate A template has two additional T residues that possibly promote a misalignment. By this mechanism, once the first two T residues in the template are extended by RT, the last A of the primer can bind to the next set of T residues in the template, and it causes the G to loop out. By that the third or fourth A could be added. Then the enzyme can continue synthesis with the G looped out to create a deletion. On the other hand, the third A added to the primer could mispair with the G and the fourth could correctly pair with a T, allowing further extension. They observed F227A and wild type RTs synthesizing a misinsertion of an A across from a G on this template. Mutant F227A did not extend past the misinsertion

as efficiently as the wild type RT, because the mutant required a several fold higher concentration of dATP in order to sustain comparable synthesis past the mismatch. Also, mutant F227A was not able to make a detectable misincorporation product with the same mismatch on substrate E, which did not contain the two T residues after the G on the template. It suggests that the two T residues in substrate A promote extension past the G in the template and that the F227A mutant uses the misalignment mechanism for this process. It is possible that F227A misinserted an A across from a G. Misinsertion cauld have been favored by the particular sequence context of substrate A. They found W229A was not able to catalyze an A misinsertion across from G, similar to its behavior on substrate E, and this mutant was not able to catalyze extension of a misalignment like F227A or wild type RT. Especially, the F227A mutant catalyzed the dA:dG misincorporation and mismatch extension with a lower efficiency than the wild type RT. Also, extension past the 32-nucleotidelong product by F227A to create longer products having a mismatch required a 40-fold higher concentration of dATP in order to generate an equal amount of extension products as compared with wild type RT. In contrast, F227A extended the DNA primer to create a 38-nucleotide product at 0.5 mM dATP. This product is hardly

visible with the wild type RT at all concentrations of dATP added. To make this extension product, the mutant had to misinsert three A nucleotides across from three A residues in the template. They found the F227A mutant was more efficient at creating and extending dA:dA mismatches than the wild type RT. They conclude that the F227A mutant displays higher fidelity for dA:dG mismatches and lower fidelity for dA:dA mismatches than wild type. So their result shows that a single amino acid substitution can increase or decrease fidelity, depending on the base pair being formed in the reaction.

They found that mutant W229A exhibited high fidelity and did not produce a dG:dA or dC:dA mismatch. Also, mutant F227A displayed high fidelity for dG:dA and dC:dA mismatches but low fidelity for dA:dA misinsertions. This indicated that F227A discriminates in opposition to particular base substitutions. Also, a primer extension assay with three dNTPs showed that F227A generally displays higher fidelity than the wild type RT. Noticeably, primer grip mutations can improve or worsen either the overall or base-specific fidelity of HIV RT. They hypothesized that wild type RT has evolved to a fidelity that allows genetic variation without compromising yield of viable viruses.

After HIV-RT has secured onto the DNA (or RNA)

template, it faces the job of adding base specific nucleotides (dNTPs) to produce a new daughter strand (38). With the stabilizing features of the polymerase site, there are also RT-template or RT-metal interactions that stabilize the dNTP binding site and promote the polymerization reaction. A majority of these stabilizing interactions are found within the palm domain, contacting the minor groove of DNA. Tyr 183 (+) which hydrogen bonds to the minor groove, increased HIV-RT mutation rate. There stands a multitude of amino acids that coordinate the triphosphate moiety. For instance, the sidechain of Arg 72 which was found to both increase and decrease the mutation rate, lies flat against the dNTP base, donates hydrogen bonds to the alpha-phosphate and esterified oygen bridging the alpha and beta phosphates. *The NH group of Lys 65* that decreased HIV-RT mutation rate also donates hydrogen bonds to the gamma phosphate at both the esterified, and non-esterified oxygens. The main chains of Asp 113 and Ala 114 (not shown in the table), give hydrogen bonds to the beta and gamma phophates, respectively, while the main chain-NH of Tyr 115 which both increases and decreases the mutation rate donates a hydrogen bond to the 3'OH of the incoming dNTP (38).

It can be concluded that the mutations that decrease

and/or increase the mutation rate do not cluster together. They are all around the active site in the crystal structure. Also, these amino acids might be in the primer grip region or interact with the incoming dNTP and form the dNTP binding site or residues in the fingers subdomain.

Discussion:

Nucleoside-analog inhibitors of HIV-1 RT were the first drugs used against the virus. It is known that monotherapy with these and other drugs leads to the rapid development of viral resistance. Also, it is being increasingly appreciated that a significant percentage of individuals receiving highly active antiretroviral therapy (HAART) also develop resistance. RT is responsible both for optimal rate of replication and an accurate copying of the viral genome, the consequence of drug resistance mutations in RT to the biochemistry of this enzyme and to the biology of the virus are critically important. In this review, we describe a number of studies investigation the polymerase fidelity of nucleoside-analog resistant HIV-1 reverse transcriptase as well as the mutation rate of HIV-1 harboring these mutations (44).

As shown in Tables (1 and 2), several RT variants with single amino-acid changes have been characterized in both

the polymerase and RNase H domains that decrease virus mutation frequencies have been implicated in drug resistance to multiple drugs. Many single amino-acid changes in the polymerase and RNase H domains that increase mutation rate have not yet been implicated in drug resistance. Also, as shown in Tables (1 and 2) drug resistant mutations that increase HIV mutation are found in combinations.

The mutation rate of HIV-1 is influenced by a multiplicity of viral and host factors (44). The contribution of the virus to this variation includes three things. First, the high error rate of HIV-1 reverse transcriptase is estimated to be 3×10^{-5} per base per replication cycle (20). Second, the diploid nature of the genome facilitates recombination between the two RNA genomes (44). Finally, the high rate of viral replication (10). The host contributes to viral variation by the use of the cellular machinery for transcription, physiological fluctuations in dNTP pools, and asymmetric error repair. With the generation of errors, variation is further affected by a variety of selective forces. Examples of selective forces involve the relative fitness of a given mutant virus, the balance between the abundance of specific cell types, the cell tropism of a given variant of HIV, and

the action of the immune system. Some studies show that the host and virus act together to produce G-to-A hypermutations (44). Host cells produce a protein known as APOBEC3G, a DNA deaminase, which is packaged in the virion leading to the deamination of deoxycytosine residues in the first strand of viral DNA synthesized during reverse transcription to deoxyuracil. Therefore, APOBEC3G serves a host-encoded antiviral function by producing a large number of G-to-A mutations in the HIV-1 genome, thus rendering it nonfunctional. The virally encoded protein Vif allows HIV to overcome the activity of APOBEC3G, resulting in a non-lethal level of G-to-A mutations, and contributing to the overall viral variation. Although all of these factors contribute to the overall mutation rate, a principal consideration is the viral RT, which is itself subject to alteration by mutations and selection during the course of disease and treatment. The majority of drug combination protocols used currently include nucleoside analogs that target RT. Resistance to this class of drugs develops through mutations that affect (44).

A dose-dependent association between increased drug concentration and increased virus mutation frequency has been reported for zidovudine, lamivudine, and dideoxyinosine (36, 37). The maximum increase in virus

mutation frequency in the presence of dideoxyinosine was six fold higher than the virus mutation frequency observed during replication in the absence of drug. Chen et al. (2005) indicate that increased virus mutation frequencies occur when drug concentrations increase.

Chen et al (2005) theorized that both RT variants and drugs together could increase virus mutation frequencies (36). Chen et al (2005) tested this ideaby using selected NRTIs and drug-resistant RT variants and analyzing them for their influence on virus mutation frequencies. They found mutant viruses containing either the Y115F or the L74V/Y115F/M184V RT mutations could grow in the presence of abacavir at its IC50 concentration. Both mutations were associated with a statistically significant increase in virus mutation frequency compared to those observed through virus replication with the wild-type RT in the presence of abacavir. Chen et al (2005) also observed that HIV-1 replication with the L74V/Y115F/M184V RT variant in the presence of zalcitabine significantly influenced HIV-1 mutation frequencies compared to that observed during virus replication with wild-type RT in the presence of zalcitabine (3.39-fold versus 1.36-fold). Also, in the presence of stavudine, the V75T RT variant affected virus mutation frequency as compared to wild-type RT (3.46-fold

versus 1.65-fold) (9).

It is important to understand the influence of mutations on viral properties such as replicative fitness, fidelity, and mutation rates. It is considered that the increased fidelity of K65R RT is due to an altered interaction with the dNTP substrate.

Insight into residues that constitute the dNTP-binding pocket as well as mechanisms by which mutations at different sites may confer drug resistance or increased fidelity was provided by the shah crystal structure of a covalently trapped catalytic complex of HIV-1 RT with template-primer and dTTP (46). In some studies the researchers found the presence of dNTP substrate in the binding pocket led to a conformational change involving an inward movement of the finger β3– β4 hairpin loop, bringing it closer to the active site. In that conformation, the residues in the β3– β4 hairpin loop, a lot of which are implicated in nucleoside analog resistance, directly contact the incoming dNTP, as well as indirectly influence its binding through interactions with the templating base. For example, the ammonium group of Lys65 contacts the γ-phosphate of the dNTP, whereas its aliphatic portion interacts with Arg72. Also, Leu74 locks the templating base tightly in place and contacts the side chain of Arg72 and

Gln151, which in turn stack on the base of the dNTP. All of these residues except Arg72 are changed in variant viruses displaying resistance to nucleoside analogs. A Lys-to-Arg substitution at codon 65 associated with resistance to ddC and cross-resistance to 3TC and ddI was first reported by Gu et al. This mutation was shown to confer cross-resistance to (9R)-2-phosphonylmethoxyethyladenine (16) and to bisisopropyloxymethylcarbonyl (48). A Leu-to-Val substitu- tion at codon 74 is the most frequently described mutation in patients taking ddI (53) and confers a 5–10-fold decrease in sensitivity to ddI. Also, The L74V substitution has been shown to confer cross-resistance to ddC and 3TC, although both mutations can be found in a single patient, they usually exist as separate viral quasi-species. When wild-type RT is compared with K65R RT, it is clear that K65R RT tended to generate very few products beyond the template site that served as a barrier. The wild-type residues at positions 65 and 74 create key contacts with the incoming dNTP and stabilize the templating base, respectively. In addition, the differential effect on mutation rate by substitutions at both residues that confer resistance to nucleoside analogs is intriguing. It appears that the alteration at Lys65 leads to an ability to discriminate against several 2^{-}

deoxy-NTP analogs as against non-Watson-Crick base-paired incoming dNTPs. On the other hand, the effects of the L74V substitution appear to be limited to discrimination against 2^{-} deoxy analogs(46).

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