

Does 3'-Azidothymidine Select Mutations in the RNase H Domain of HIV-1 Reverse Transcriptase?

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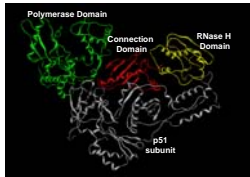
Background

HIV-1 reverse transcriptase (RT) is a heterodimer composed of a 560 amino acid, 66 kDa subunit (p66), and a 440 amino acid 51 kDa subunit (p51), see Figure 1. Both the DNA polymerase and ribonuclease H (RNase H) catalytic active sites of HIV-1 RT reside in the p66 subunit, which are spatially separated by the connection domain (see Fig.1).

Nucleoside RT inhibitors (NRTI), such as 3'-azidothymidine (AZT), inhibit HIV-1 RT DNA polymerization activity. Mutations associated with NRTI resistance have been primarily identified in the DNA polymerase domain of RT. In fact, most commercial resistance genotyping algorithms do not consider mutations beyond codon 350 of RT.

Figure 1: Structural Representation of HIV-1 RT.

The DNA polymerase domain (residues 1-318), connection domain (residues 319-426) and RNase H domain (residues 427-565) are colored green, red and yellow, respectively. The catalytically inert p51 subunit is colored grey.



Structure drawn using MOE, based on coordinates from Hsiao et al., 1996. Structure 1S:853. Pdb access number: 1DLO.

Nikolenko et al. (PNAS 2005; 102:2093) have reported that mutations introduced into the RNase H domain of HIV-1 reverse transcriptase (RT) that decrease RNase H activity also cause resistance to 3'-azidothymidine (AZT). Hypothetically, these mutations in the RNase H domain reduce RNA template degradation, thereby prolonging the window of opportunity for AZT-monophosphate to be excised from the terminated primer.

It is not known, however, whether mutations in the RNase H domain are selected by AZT. Therefore, we carried out in vitro selections of AZT-resistant HIV-1, and sequenced the entire coding region of RT to identify all AZT-associated resistance mutations.

Methods

1. Selection of AZT-Resistant HIV-1

- Two independent selections were performed in MT-2 cells in increasing AZT concentrations:
 - One starting with wild-type (WT) HIV-1_{LAI}
 - The second starting with HIV-1_{LAI} encoding 41L, 210W, 215Y site resistance mutations
- AZT susceptibility was monitored in P4/R5 cells that express β-galactosidase under the control of the HIV-1 LTR.
- RT PCR was used to amplify the entire RT sequence from viral RNA.
- Four primers located throughout RT were separately added to ~40 ng of purified PCR DNA and sequenced with an ABI3100.

2. Clonal Analysis of Mutation Linkage

- The amplified RT of HIV_{LAI} was cloned into the TOPO[®] vector (Invitrogen) and transformed into TOPI0 competent *E. coli*.
- DNA from isolated colonies was purified and sequenced as described above.

3. Site-Directed Mutagenesis and AZT Susceptibility (IC₅₀)

- Mutations identified in selections were introduced into p6HRT by QuickChange[®] site-directed mutagenesis (Stratagene) and subcloned into xHIV-1_{LAI}.
- AZT susceptibility (IC₅₀) was determined in a single-cycle replication assay in P4/R5 cells.

Results

Selection of AZT-Resistant HIV-1

Figure 2: Progressive Increase in AZT Resistance of WT HIV-1_{LAI}

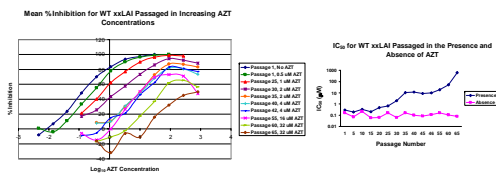
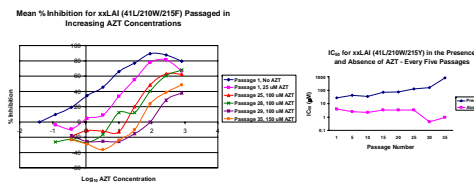


Figure 3: Progressive Increase in AZT Resistance of HIV-1_{LAI} with 41L/210W/215Y



Sequence Analysis of Passaged Viruses

Table 1: Mutations Selected by AZT in WT xxLAI

Passage Number	AZT Concentration	IC ₅₀ (µM)	Fold-R	Mutations
15	1 µM	0.16	1.1	None
15	1 µM	0.2	1.1	None
16	1 µM	0.28	2.1	67N mutation
17	1 µM	ND	ND	67N mutation
18	1 µM	ND	ND	67N and 70R mutations
19	1 µM	ND	ND	67N and 70R mutations
20	2 µM	2.4	20	67N
35	2 µM	154	93	67N, A371V, Q509L
40	4 µM	9.0	113	67N, A371V, Q509L
42	4 µM	13	69	70R, A371V, Q509L
55	16 µM	31	205	67N, T215I, A371V, Q509L
60	32 µM	109	1099	67N, T215I, A371V, Q509L
65	32 µM	608	7508	67N, T215I, A371V, Q509L

Table 2: Mutations Selected by AZT in xxLAI with 41L/210W/215Y

Passage Number	AZT Concentration	IC ₅₀ (µM)	Fold-R	Mutations
1	No AZT	3.8	---	210W, 215Y
25	100 µM	123	36	41L, 210W, 214F, 215Y
28	100 µM	161	112	41L, 67N, 210W, 214F, 215Y
29	100 µM	~810	365	41L, 67N, 210W, 214F, 215Y
35	150 µM	~810	863	41L, 67N, 210W, 214F, 215Y

*ND = Not determined

Clonal Analysis of Passage 65

Table 3: 67N, 70R, 215V/F, 371V, and 509L are Linked on the Same Genomes
Yellow indicates mutations detected by population sequencing

Mutations/Polymorphisms	Frequency (Percent)	clone 1	clone 2	clone 3	clone 4	clone 5	clone 6	clone 7	clone 8	clone 9	clone 10	clone 11
Q180	9											
S385	9											
T399	9											
D37N	100	*	*	*	*	*	*	*	*	*	*	*
Q509	100	*	*	*	*	*	*	*	*	*	*	*
T335	9											
Y371	9											
Q38E	9											
E346F	9											
K104N	9											
E174L	9											
K118	9											
G133V	9											
S190Q	9											
E174L	9											
Q227	18											
P11P	64	*	*	*	*	*	*	*	*	*	*	*
E150	9											
Q242R	9											
P11P	9											
R137R	9											
A371V	73	*	*	*	*	*	*	*	*	*	*	*
Q509L	9											
P11P	9											
P11P	9											
A371V	9											
Q509L	9											
K118	9											
N185Y	9											
Q187R	9											
Q188R	9											
Q189R	9											
Q190R	9											
K118	10	*	*	*	*	*	*	*	*	*	*	*
K118	9											
E174L	9											
Q188R	9											
Q189R	9											
Q190R	9											
Q191R	9											

Mutagenesis Results

Table 4: Impact of Mutations Selected by AZT on AZT Susceptibility
Mutation combinations in BOLD were selected by AZT

Mutation	Mean IC ₅₀ (µM)	Fold - Resistance
WT xxLAI	0.21 ± 0.07	---
A371V	0.15 ± 0.04	0.73
Q509L	0.29 ± 0.16	1.4
67N/70R	1.1 ± 0.74	5.1
67N/70R/371V	1.4 ± 0.47	6.6
67N/70R/509L	3.0 ± 0.98	14
67N/70R/371V/509L	9.9 ± 5.9	46
67N/70R/215I	0.29 ± 0.18	1.4
67N/70R/215I/371V	0.57 ± 0.30	2.7
67N/70R/215I/509L	3.0 ± 2.2	14
67N/70R/215I/371V/509L	9.3 ± 4.6	44
67N/70R/215F	4.8 ± 0.50	23
67N/70R/215F/371V	4.9 ± 2.9	23
67N/70R/215F/509L	28 ± 17	131
67N/70R/215F/371V/509L	203 ± 40	956

* Mean ± SD from at least three experiments

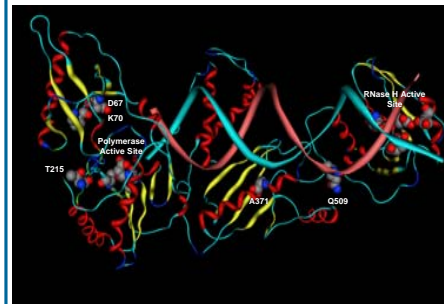
Summary of Results

AZT selections:

- >1000-fold AZT-resistant virus was selected starting with WT xxLAI
 - A371V in the connection domain and Q509L in the RNase H domain of RT were selected on the same genome with "classic" TAMs (67N, 70R, 215V/F).
- >1000-fold AZT-resistant virus was selected from xxLAI with 41L, 210W, 215Y
 - 67N and 214L were selected but no mutations beyond codon 350 were detected.

Figure 4: Structural Representation of AZT Selected Mutations in the p66 Subunit of RT. A371V is located in the connection domain and Q509L is in the RNase H domain of RT. DNA Primer: turquoise ribbon; RNA Template: pink ribbon.

Structure drawn using MOE, based on coordinates from Sarafianos et al., 2001. EMBO J 20:1449. Pdb access number: 1HY5.



A371V and Q509L Mutants:

- A371V and Q509L in combination with "classic" TAMs in the polymerase domain of RT (67N, 70R, 215F) significantly increased HIV-1 resistance to AZT.
- The 67N/70R/215F/371V/509L mutant exhibited ~1000-fold AZT resistance compared with:
 - ~130-fold resistance for 67N/70R/215F/509L, and
 - ~20-fold resistance for 67N/70R/215F
- A371V (0.7 Fold-R) and Q509L (1.35 Fold-R) alone did not alter AZT susceptibility without "classic" TAMs.

Conclusions

- This work provides the first clear evidence that mutations beyond codon 350 of RT are selected by AZT and increase resistance conferred by "classic TAMs" in the polymerase domain.
- Studies to assess the biochemical mechanisms involved and the clinical significance of these findings are in progress.