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N348I in HIV-1 Reverse Transcriptase Decreases Susceptibility to Tenofovir and Etravirine in Combination with Other Resistance Mutations

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Summary

We previously demonstrated that N348I in HIV-1 reverse transcriptase confers zidovudine and nevirapine resistance. However, both of these inhibitors are currently infrequently used in developed countries and the impact of N348I on newer RT inhibitors, such as tenofovir and etravirine, is unknown. In this study, we demonstrate that N348I alone confers no resistance to tenofovir and low-level resistance to etravirine. However, N348I significantly decreases tenofovir susceptibility when combined with thymidine analogue mutations and etravirine susceptibility when combined with Y181C.

Keywords

HIV; N348I; reverse transcriptase; thymidine analogue mutations; etravirine; tenofovir

We recently identified the N348I mutation in the connection domain of the human immunodeficiency type I (HIV-1) reverse transcriptase (RT) that confers resistance to both zidovudine (AZT) and nevirapine [1]. N348I is highly prevalent in RT inhibitor (RTI)-experienced patients [1-5], occurs early in therapy usually prior to the appearance of recognized thymidine analogue mutations (TAMs) [1], and is associated with an increase in viremia [1]. In our study, N348I was selected by antiretroviral treatments that included AZT or the combination of AZT and nevirapine [1]. N348I has also been reported to confer resistance to didanosine and delavirdine and its emergence in a Japanese cohort was primarily associated with AZT and/or didanosine containing therapies [2].

The use of zidovudine, didanosine and nevirapine in antiretroviral therapies in the developed world has been largely replaced with more potent and less toxic RTIs [6]. For example, the International AIDS Society-USA panel recommends either tenofovir/emtricitabine (Truvada)

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or abacavir/lamivudine in combination with efavirenz or ritonavir boosted protease inhibitor for initial combination therapy [6]. Truvada is also used in the treatment of antiretroviral-experienced patients, as is the new nonnucleoside reverse transcriptase inhibitor (NNRTI), etravirine [6]. The genotypic determinants of tenofovir and etravirine have been established. Decreased susceptibility to tenofovir *in vitro* and *in vivo* is associated with the K65R mutation or the presence of three or more TAMs (e.g. M41L, L210W, T215Y) [7-10]. Decreased etravirine susceptibility requires at least 3 NNRTI-resistance mutations [11-14]. Surprisingly, etravirine activity is not compromised by the K103N mutation [11]. To date, it has not been established if N348I can reduce susceptibility to tenofovir or etravirine and compromise drug activity in treatment-experienced patients. Accordingly, in this study we determined whether N348I alone, or in combination with TAMs or Y181C, decreased susceptibility to tenofovir or etravirine.

N348I was introduced by site directed mutagenesis into the background of wild-type (WT), K103N, Y181C, M41L/L210Y and M41L/L210W/T215Y expressing RT genes of the pNL4.3 (NL) or HXB-2 (HX) infectious molecular clones [15,16]. HIV was recovered by transfection of 293T cells and drug susceptibility assays were performed in the TZM-bl indicator cell line, as described previously [1] with the exception that HIV replication was determined by measuring luciferase activity using the Steady-Glo Luciferase Assay System according to manufacturer's instructions (Promega). Statistically significant differences in the 50% effective dose (EC₅₀) were determined using the Wilcoxon Rank Sum Test [17].

Our data (Table 1) demonstrate that N348I (NL/348) alone conferred a 1.6-fold decrease (p=0.019, n=4) in etravirine susceptibility compared to the corresponding WT strain. By comparison, Y181C conferred 2.2-fold resistance to etravirine (p=0.02, n=4) while K103N did not confer a significant change in etravirine susceptibility compared to WT. When combined with K103N, no decrease in etravirine susceptibility was observed compared with K103N alone, while a small decrease in etravirine susceptibility was seen compared to WT (p=0.019, n=5)(Table 1). By contrast, when N348I was combined with Y181C, etravirine susceptibility was decreased 6.4-fold (p=0.02, n=4) relative to WT virus, and 2.9-fold (p=0.03, n=4) relative to Y181C HIV-1 (NL/181)(Table 1). Consistent with this finding, the Y181C/N348I double mutation also significantly decreased etravirine susceptibility at the enzyme level (data not shown). Taken together, these data demonstrate that N348I confers a small decrease in susceptibility to etravirine and significantly potentiates etravirine resistance in the context of Y181C but not K103N.

As reported previously [18], HIV-1 containing N348I conferred no significant increase in tenofovir EC₅₀ compared to the corresponding WT strain (Table 1). However, when combined with M41L and T215Y (NL/2AZT), N348I decreased tenofovir susceptibility by 1.7-fold (p=0.014, n=4) compared to WT. By contrast, the NL/2AZT strain was susceptible to tenofovir (Table 1). N348I also increased tenofovir resistance when combined with M41L, L210W and T215Y (HX/3AZT) by 6.0-fold compared to WT (p= 0.009, n=5) and 3-fold compared to the HX/3AZT strain (p=0.008, n=4). In this regard, A371V and Q509L in the connection and RNase H domains, respectively [19], and mutations located at residues that form part of the RNase H primer grip [20,21] potentiate resistance to tenofovir in cell culture based assays when combined with TAMs. Taken together, these data demonstrate that N348I decreases tenofovir susceptibility in the presence of TAMs, and notably this effect is observed with less than three TAMs.

According to the International AIDS Society-USA drug resistance mutations update, the presence of 3 or more TAMs inclusive of M41L and L210W is expected to give a reduced *in vivo* response to tenofovir [22]. Therefore, in current genotyping algorithms tenofovir could be prescribed in the presence of 2 TAMs (e.g. M41L and T215Y) and N348I, which may result

in reduced *in vivo* drug efficacy. Similarly, we have shown that N348I enhances resistance to etravirine in the context of Y181C, a mutation that is associated with reduced virological response *in vivo* [13,14]. Since the presence of three or more NNRTI mutations V90I, A98G, L100I, K101E/P, V106I, V179D/F, Y181C/I/V and G190A/S results in no response to etravirine treatment [13,14], the presence of two of these NNRTI mutations and N348I at baseline may also reduce etravirine efficacy *in vivo*.

N348I is not a polymorphism. It is found in treatment experienced, but rarely in treatment naïve individuals infected with HIV-1 clades A, B, AE, AG, C, D, F and G (<http://hivdb.stanford.edu/cgi-bin/AgMutPrev.cgi>). Furthermore, the prevalence of Y181C and K103N is 11% and 22%, respectively. Accordingly, at least 1 in 10 HIV infected individuals will have Y181C prior to etravirine exposure, particularly in patients failing first line antiretroviral therapies in resource poor settings due to the continued use of nevirapine [23]. Accordingly, the acquisition of N348I in HIV-1 RT may significantly impact both first and second line antiretroviral therapies in resource poor settings.

Taken together, our *in vitro* data warrant studies to determine the clinical significance of the appearance of a pre-existing N348I mutation in regimens containing tenofovir or etravirine.

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Table 1

Effect of N348I on Etravirine and Tenofovir Resistance

Strain	Amino acid at Indicated RT Codon ^d								Etravirine		Tenofovir	
	41	103	181	210	215	348	348	Mean EC ₅₀ ± SE (μM) ^b	Resistance (fold) ^c	Mean EC ₅₀ ± SE (μM) ^d	Resistance (fold) ^c	
NL	M	K	Y	L	T	N		0.0056 ± 0.0003	I	6.1 ± 0.6	I	
NL/348	-	-	-	-	-	I		0.0090 ± 0.0014	1.6	4.0 ± 0.9	0.7	
NL/103	-	N	-	-	-	-		0.0084 ± 0.0025	1.5	ND	-	
NL/103+348	-	N	-	-	-	I		0.0086 ± 0.0014	1.5	ND	-	
NL/181	-	-	C	-	-	-		0.0125 ± 0.0024	2.2	ND	-	
NL/181+348	-	-	C	-	-	I		0.0360 ± 0.0084	6.4	ND	-	
NL/2AZT	L	-	-	-	Y	-		ND	-	7.5 ± 0.8	1.2	
NL/2AZT+348	L	-	-	-	Y	I		ND	-	10.6 ± 0.6	1.7	
HX	-	-	-	-	-	-		ND	-	3.0 ± 0.3	I	
HX/348	-	-	-	-	-	I		ND	-	4.1 ± 0.9	1.4	
HX/3AZT	L	-	-	W	Y	-		ND	-	5.8 ± 1.2	1.9	
HX/3AZT+348	L	-	-	W	Y	I		ND	-	18.0 ± 4.1	6.0	

^aRT amino acid residues shown are numbered as for NL4.3 (NL) and HXB-2 (HX) and are those that differ from WT sequences.

^bEffective concentration (EC₅₀) and standard error (SE) values were determined in drug susceptibility assays performed in the TZM-bl indicator cell line from at least four independent assays. The differences between the etravirine EC₅₀ values for NL compared to NL/348 (p=0.019, n=4), NL/103+348 (p=0.019, n=5), NL/181 (p=0.02, n=4) and NL/181+348 (p=0.02, n=4) were statistically significant as was NL/181+348 compared to NL/181 (p=0.03, n=4). The difference in the etravirine EC₅₀ values for NL/103 (p=0.54, n=5) compared to NL was not statistically significant. Statistical analyses were performed using the Wilcoxon Rank Sum test. ND denotes not done.

^cThe differences between the etravirine or tenofovir EC₅₀ values for mutant strain divided by the EC₅₀ for corresponding WT strain. Values >1 indicate resistance. Statistically significant differences compared to WT are denoted in bold-type.

^dThe differences between the tenofovir EC₅₀ for NL and NL2AZT+348 (p=0.014, n=4), and HX compared to HX/3AZT (p=0.05, n=4) and HX/3AZT+348 (p=0.009, n=5) were statistically significant as was HX/3AZT+348 compared to HX/3AZT (p=0.008, n=4). The difference in the tenofovir EC₅₀ values for NL/2AZT and HX/348 compared to the corresponding WT strain was not statistically significant. ND denotes not done.