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ScienceDirect
Antiviral Research 75 (2007) 93–103
Antiviral Research
www.elsevier.com/locate/antiviral

Review

Insertions in the β3–β4 loop of reverse transcriptase of human immunodeficiency virus type 1 and their mechanism of action, influence on drug susceptibility and viral replication capacity

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Received 6 December 2006; accepted 1 March 2007

Abstract

Introduction of antiretroviral therapy combining protease and reverse transcriptase (RT) inhibitors has dramatically improved the quality of life and survival of patients infected with the human immunodeficiency virus (HIV). However, effective long-term therapy of HIV-infection has been severely hampered by the development of drug resistance. Resistance to antiretroviral drugs is generally conferred by specific amino acid substitutions in the target gene of the drug. Yet, occasionally gene insertions are being observed. The most commonly observed insertion is seen during substrate analogue RT inhibitor therapy and is selected in the β3–β4 loop of the RT enzyme. This flexible loop is located in the fingers subdomain of the enzyme and plays an important role in substrate binding. The acquisition of drug resistance related mutations or insertions might come at a price, which is reduced performance of the enzyme resulting in a diminished replication capacity of the virus. Various types of insertions have been described, and, in this review, we have summarized these data and discussed the mechanism of action of the RT inserts and their impact on both drug susceptibility and replication capacity.

Keywords: Human immunodeficiency virus; Insertion; Reverse transcriptase; Drug resistance

Contents

Due to its central role in the life cycle of the human immunodeficiency virus type 1 (HIV-1), the virally encoded reverse transcriptase (RT) is a major target for drug discovery. Two classes of inhibitors that are directed against the polymerase domain of this enzyme are frequently administered in combination: nucleoside or nucleotide analogue RT inhibitors (NRTIs, NtRTIs) and non-nucleoside RT inhibitors (NNRTIs) (De Clercq, 1992). The NRTIs are intracellularly phosphorylated to their triphosphate forms and compete with natural dNTP substrates for incorporation. Once accepted and incorporated by the RT enzyme they block DNA synthesis through chain-termination, because they lack the 3′-OH group, which is required for the polymerization reaction (Squires, 2001). In contrast, NNRTIs bind in the vicin-

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doi:10.1016/j.antiviral.2007.03.001

ity, but not at the active site and cause a conformational change that affects the efficiency of nucleotide incorporation. Three different NNRTIs have been approved by the Food and Drug Administration (FDA): nevirapine (NVP), delavirdine (DLV) and efavirenz (EFV), and eight different NRTIs are currently in clinical use: 3′-azido-3′-deoxythymidine (zidovudine or AZT), 2′,3′-didehydro-2′,3′-dideoxythymidine (stavudine or d4T), 2′,3′-dideoxyinosine (didanosine or ddI), (−) -2′,3′-dideoxy-3′-thiacytidine (lamivudine or 3TC), 2′,3′-dideoxycytidine (zalcitabine or ddC), the carbocyclic nucleoside abacavir (ABC), the acyclic nucleoside phosphonate tenofovir (TFV), and, more recently, the fluorinated cytidine emtricitabine (FTC).

Unfortunately, despite considerable success in regard to treatment strategies that involve combinations of various RT inhibitors and also drugs that block the function of the viral protease (protease inhibitors), the emergence of resistant viruses is often difficult to prevent and remains a major reason for treatment failure (Geretti, 2006). The question is why has HIV-1 been so successful in adaptation to antiviral therapy? The answer presumably lies in its high mutation rate, the rapid turnover rate and the relatively large viral population size, resulting in a population of distinct, but closely related, genetic variants referred to a quasispecies. These quasispecies populations contain a huge reservoir of different viruses, some of which may be able to replicate in the presence of suboptimal antiviral pressure.

Reduced susceptibility to some clinically used RT inhibitors has been associated with specific genotypic changes in the RT gene (Johnson et al., 2006). For instance, high-level resistance to 3TC can be caused by a single mutation at codon 184, i.e. M184V/I (Boucher et al., 1993a; Gao et al., 1993; Gu et al., 1992; Keulen et al., 1997; Tisdale et al., 1993). In contrast, previous clinical studies and cell culture data have shown that the accumulation of mutations M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E correlates with a stepwise, incremental reduction in AZT susceptibility (Hooker et al., 1996; Kellam et al., 1992; Larder et al., 1989). In view of clinical data showing that these mutations may also reduce susceptibility to d4T, changes at positions 41, 67, 70, 210, 215, and 219 have been referred to as thymidine analogue mutations (TAMs), albeit the resistance conferring effects of TAMs is not restricted to thymidines. The accumulation of at least three TAMs can reduce susceptibility for TFV and ABC likewise. Another pattern of multidrug resistance (MDR) involves the Q151M substitution that, on its own, gives only low-level resistance to several NRTIs; however, Q151M in combination with A62V, V75I, F77L and F116Y comprises a cluster that confers high-level resistance to all NRTIs with the exception of TFV (Deval et al., 2002; Iversen et al., 1996; Johnson et al., 2006; Kaushik et al., 2000; Shirasaka et al., 1995).

A third pathway to NRTI multi-drug resistance involves the selection of amino acid insertions in the β3–β4 loop of RT (between amino acids 69 and 70). The crystal structure of wild type HIV-1 RT bound to a DNA/DNA primer/template and dNTP substrate shows that many residues that are linked to resistance to NRTIs are clustered around the nucleotide binding site (Huang et al., 1998). The flexible β3–β4 loop in the fingers subdomain plays an important role in this regard, since it traps the incoming nucleotide in a favorable position for catalysis. Various structural changes within this loop have been associated with resistance to multiple NRTIs. Mutations and deletions have been described, but dipeptide insertions are probably the most prominent examples in this regard. In this review we place emphasis on the effects of such fingers-insertions on structure–function relationships of the RT enzyme and related changes in drug susceptibility and viral replication.

1. Selection and generation of insertions in RT

Insertions in the loop between the β3-sheet (residues 56–63) and the β4-sheet (residues 73–77) were first observed in long-term treated patients in 1997 (De Antoni et al., 1997; de Jong et al., 1999; Ross et al., 1999; Winters et al., 1998). The prevalence of these rearrangements has been estimated to be less than 3% in therapy-failing patients. An overview of all described insertions, and if available the genotypic background, treatment regimen and phenotypic drug resistance analyses, has been made and is shown in Table 1 (Andreoletti et al., 2002; Balotta et al., 2000; Briones et al., 2001; Briones and Soriano, 1999; Bulgheroni et al., 2004; de Jong et al., 1999; Kaliki et al., 2000; Kim et al., 2001; Larder et al., 1999; Lobato et al., 2002; Mas et al., 2000; Masquelier et al., 2001; Matamoros et al., 2004; Miller and Larder, 2001; Prado et al., 2004; Quinones-Mateu et al., 2002; Rakik et al., 1999; Ross et al., 1999; Rousseau et al., 2001; Sato et al., 2001; Schneider et al., 2004; Sugiura et al., 1999; Tamalet et al., 1998, 2000; van der Hoek et al., 2005; Van Vaerenbergh et al., 2000; White et al., 2004; Winters et al., 1998; Yahi et al., 2000). A large variety of insertions is present in the β3–β4 loop between codons 68 and 69 or between codons 69 and 70. Because insertion variants usually have one or two additional substitutions just before or after the insertion, sequence alignment and numbering of the amino acids in this loop is ambiguous. In Table 1, all insertions are numbered as if they are located between residue 69 and 70. The majority of the insertions are neutral, hydrophobic, little polar dipeptides, mainly a double serine (SS), serine-glycine (SG) and serine-alanine (SA) (De Antoni et al., 1997; de Jong et al., 1999; Winters et al., 1998). Insertions of one amino acid have been described, as well as 3 to 11 amino acid insertions, but these are much less common (Huigen et al., 2005; Kaliki et al., 2000; Lobato et al., 2002; Masquelier et al., 2001; Sato et al., 2001).

In general, most insertions are selected during treatment with at least one thymidine analogue (AZT or d4T) (Lukashov et al., 2001; Moyle et al., 2000; Ross et al., 1999). This is most likely related to the selective advantage of the insertion in the background of several TAMs, especially the T215Y, M41L and L210W substitutions. In addition, a few other mutations seem to be associated with the presence of an insertion in RT. These mutations include the T69S, A62V and the D67E/G changes. Interestingly, the A62V substitution is part of the Q151M-complex. The Q151M- and the insertion-complex are two independent pathways leading to multi-NRTI-resistance and have never been observed in combination. A recent paper reported the coexistence of a one amino acid insertion in combination with the Q151M and F116Y mutations in a single patient,

Table 1
Overview of insertions in the fingers domain of HIV-1 RT with resistance-associated RT mutations and drug susceptibility results

	Insertion																			Resistance							
Reference/Sample	67	68	69	-	-	-	-	-	-	-	-	-	-	70	Resistance-associated RT mutations	Treatment (history)	AZT	3TC	d4T	ddI	ddC	ABC	PMEA	EFV	NVP
	D	S	T											K													
Winters 1998																											
Pt. 1	E	T	S	S	A										215Y		150,0	33,0	8,3	15,0	32,5		19,1		
Pt. 2			S	S	A										41L, 62V, 184V, 215Y	AZT, ddI, 3TC, d4T	270,0	>667	1,7	10,5	13,5		4,3		
Pt. 3			S	S	A										41L, 62V, 103N, 181C, 215Y	AZT, ddI, ddC, 3TC, d4T									
Pt. 4	E		S	S	G										41L, 215Y	AZT, ddC, ddI,	220,0	8,3	3,3	11,5	20,5		22,6		
Pt. 5			S	S	G									K/R	41M/L,184V, 215Y										
Pt. 6			S	S	G										62V, 181C, 215Y	AZT, ddC, 3TC, d4T, ddI									
Pt. 7	E		S	S	S									R	41L, 62V, 184M/V, 210W, 215Y	AZT, ddI									
Pt. 8			S	S	S										41L, 210W, 215Y	AZT, ddC	30,0	13,7	2,0	4,5	8,0		16,3		
Pt. 9			S	S	S										41L, 215Y	ddI, AZT									
Pt. 10			S	S	A/S										62V,184V, 210W, 215Y										
Pt. 11			S	S	S										41M/L, 62V, 184V, 210W, 215Y										
Pt. 12	G		S	E	A										41L, 210W, 215Y	AZT, ddI	100,0	34,7	7,0	15,5	23,0		30,6		
Pt. 13	E		S	T	S									R	210W, 215F	AZT, ddC, 3TC, ddI, d4T	80,0	11,3	3,3	7,0	10,0		32,0		
Pt. 14	E		A	S	G										41L, 62V, 74L/V, 103N, 184V, 210W, 215Y	AZT, ddC, ddI, d4T,									
Co.1			S	S	A											Site directed mutagenesis construct	6,0	4,7	0,7	6,0	10,0		2,0		
Co. 2			S	S	A										215Y	Site directed mutagenesis construct	14,0	9,7	4,0	9,5	19,0		20,8		
Co.3			S	S	G											Site directed mutagenesis construct	8,0	6,0	0,7	4,0	5,0		2,7		
Co.4			S	S	G										215Y	Site directed mutagenesis construct	18,0	17,3	4,0	8,5	45,5		11,4		
Co.5			S	S	G										41L, 215Y	Site directed mutagenesis construct	210,0	43,3	9,7	21,0	55,0		>42		
Co.6				S	G											Site directed mutagenesis construct	1,0	8,7	1,0	4,0	13,0		5,0		
Rakik 1999																											
Pt. 1 (D1)	E		S	S	S										41L, 62V, *184I*, 210W, 215Y	AZT, 3TC, SQV, NVP, RTV, ddI, ddC	113,0	52,0	4,3	1,4	3,8				0,7
Pt. 2 (D1)			S	S	G										62V, *181I*, 210W, 215Y	AZT, ddC, ddI, d4T, 3TC	1615,0	52,0	47,0	6,5	7,0	24,0		1,4	1250,0
Pt. 2 (Wk8)			S	S	G										62V, 103N, 181I/C/F/S, 188Y/F/L, 190G/A, 210W, 215Y										
Pt. 3 (D1)			S	S	G										62V, 181Y/C, 184M/V, 210W, 215Y	AZT, ddI, ddC, IDV, d4T, 3TC, NFV	1293,0	14,0	6,0	2,0	2,0	8,0		1,6	100,0
Pt. 3 (Wk8)			S	S	G										62V, 181 Y/C, 184M/V, 210W, 215Y										
Pt. 4 (D1)	G	Y		D										R	41L, *184V*, 215F, 219Q	d4T, AZT, IDV, ddC, 3TC, RTV,	1615,0	52,0	47,0	12,0	14,0	25,0			4,1
Pt. 4 (Wk8)	G	Y		D										R	41L, *103N*, 108I, 184V, 215Y, 219Q	SQV, NVP, NFV	1602,0	47,0	20,0	7,0	8,0	13,0		>10,000	>1200
De Jong 1999																											
Pt. 1	E			S	V	T									41L, 210W, 215Y	AZT, ddC, 3TC, SQV, IDV, d4T, ddI	>1493	>13	>32	6,0	17,0				3,0
Pt. 2	S			S	S	G								A	215Y	AZT, 3TC, ddI, K61V, SQV, d4T	1346,0	36,0	22,0	16,0	6,0				<1
Pt. 3	E			S	S	G									62V, 181C, 210W, 215Y	AZT, ddC, ddI, 3TC, d4T, NVP, RTV, SQV	1880,0	13,0	15,0	4,0	3,0				1046,0
Larder 1999																											
Co. 1				S	S	S										Site directed mutagenesis construct	2,2	6,2	2,3	1,7	2,1	2,6			
Co. 2				S	-	-									210W, 215Y	Site directed mutagenesis construct	19,0	3,8	1,1	1,2	1,2	1,5			
Co. 3				S	-	-									62V, 210W, 215Y	Site directed mutagenesis construct	8,0	1,3	0,7	0,5	0,8	0,7			
Co. 4				S	S	S									210W, 215Y	Site directed mutagenesis construct	220,0	20,0	4,8	2,1	4,2	4,6			
Co. 5				S	S	S									62V, 210W, 215Y	Site directed mutagenesis construct	>2500	15,0	5,2	1,8	2,7	5,4			
Sugiura 1999																											
Pt. JINST-1				S	S	A									41L, 215Y	AZT, ddI, 3TC, ddC, SQV, RTV, NFV	>50	4	1	6	8				
Pt. JINST-2	E			S	T	T									41L, 210W, 215Y	AZT, ddI, ddC, 3TC, IDV									
Pt. JINST-3				S	S	G									215Y	AZT, ddI, ddC, 3TC, d4T									
Pt. JINST-4				S	S	S									41L, 184V, 210W, 215Y	AZT, ddI, 3TC, NFV									
Tamalet 2000																											
Pt. AR 11197	G				T									R	184I, 215F, 219Q	various combinations of	9								
Pt. BP 07/96				S	S	S									41L, 62V, 74L/V, 108V/I, 184V, 210W, 215Y	AZT, 3TC, ddI, d4T, NVP and Indinavir	3745								
Pt. BR 03/99				S	S	T									41L, 181C, 190A, 210W, 215Y	for all these patients	189								
Pt. GB 02/99	E			S	E	S									62A/V, 181C, 190A, 210W, 215Y		1999								
Pt. MP 03/99				S	S	S								R	62V, 184V, 215Y		36								
Mas 2000/Melamorox 2004																											
Co. T69S				S	-	-										Site directed mutagenesis construct	0,4	0,4	2,2	0,4	0,5				
Co. T69SSS				S	S	S										Site directed mutagenesis construct	0,3	3,3	1,1	1,5	0,6				
Co. T69SW				S	S	W										Site directed mutagenesis construct									
Pt. SS				S	S	S								R	41L, 62V, 108I, 181C, 184I, 210W, 215Y	a.o. AZT, ddI, NEV, SQV, NFV	786,0	>35	9,1	10,6	4,7				
Co. 2S3S				S	-	-								R	41L, 62V, 108I, 181C, 184I, 210W, 215Y	Site directed mutagenesis construct	143,0	>35	24,2	8,9	12,2				
Co. S24S				S	S	S	S	S						R	41L, 62V, 74I, 108I, 181C, 184I, 210W, 215Y	Site directed mutagenesis construct	3,6	>35	1,9	5,0	1,6				
Vaerenbergh 2000																											
Pt. SW3				S	S	S									41L, 62V, *184I*, 210W, 215Y	AZT, ddI, 3TC	>1873	>14	16,0	1,0	9,0	3,0	1,2	1,0	
Pt. CHC				S	S	S								R	215Y	AZT, ddI, ddC									
Masquelier 2001																											
Pt. 1	E		S	S	G										215Y	d4T, 3TC, RTV, SQV	183	15	9,0	2,8		10,1		0,6	1,0
Pt. 2	G		S	T	T									R	184I, 215F, 219Q	d4T, 3TC, RTV	9	>339	5,3	4,4		18,3		0,7	0,7
Pt. 3	G		Y	S	D									R	70R, 181C. 184I, 219Q	AZT, 3TC, NVP									
Pt. 4	A/D		S	S	S	G									41L, 215Y	d4T, ddI, IDV	>1805	>245	55,1	3,4		>9		0,5	1,7
Pt. 5	C		G	S	G										41L, *103N*, 181C, 215Y	AZT, ddI, NFV	>1805	>245	66,7	0,0		>9		6,8	>463
Pt. 6	E	N/S	S	S	C										210W, 215Y	ABC, ddI, NFV, SQV	>1805	>245	67,8	3,7		>9		3,9	9,0
Pt. 7	S		S	S	C	T									41L, 184I, 215Y	d4T, 3TC, IDV	297	>245	68,1	3,0		>9		0,7	0,8
Pt. 8	S		S	S	C	T									41L, 184I, 210W, 215Y	d4T, 3TC, IDV	131	>245	27,4	5,6		6,3		0,2	1,0
Pt. 9	D/N		S	S	C	A									41L, 74V, 210L, 215Y, 219Q	d4T, NFV	>301	>25	>28	6,2		21,1		>68	>947
Pt. 10	E		S	V	G										41L, *181C*, 215I	d4T, ddI, ABC, IDV	>301	>25	>28	7,4		>24		23,9	>947
Pt. 11	D		S	S	S	G									41L, *181C*, 210W, 215Y, 219K/E	treatment interruption	>301	>25	6,9	5,1		>24		9,1	20,4
Pt. 12	E		S	S	S	G									41L, 210W, 215Y	d4T, 3TC, NFV	>301	>25	20,6	5,4		>24		1,5	5,8
Pt. 13	D		S	S	S	G									41L, *184I*, 210W, 215Y	d4T, ddI	>301	>25	>28	4,7		15,2		0,2	0,4
Pt. 14	S		L	A	V	G									41L, 74V, *184V*, 210W, 215Y	d4T, ddI, hydroxyurea	>30,1	>25	>28	10,7		>24		1,2	2,5
Pt. 15	D		S	S	T	T									41L, 210W, 215Y	AZT, 3TC, RTV									
Pt. 16	D		S	S	S	G									210W, 215Y	d4T, ddI, NFV, SQV									
Pt. 17	D		S	S	S	A									41L, *103N*, 181C, 210W, 215Y	d4T, ddI, NVP, IDV	>1805	>245	67,7	2,5		>9		91	>1,463
Pt. 18	Q		S	S	S	A									108I, 184I, 210W, 215Y	3TC, ABC									
Pt. 19	D		S	S	S	T									41L, 74V, *181C*, *103N*, *190A*, 210W, 215Y	d4T, ABC, EFV, APV	169	4	1,8	2,1		7,2		>209	>2,894
Pt. 20	D		S	S	S	S								R	70R, 184V, 215F	d4T, 3TC IDV	36	>339	1,3	1,3		12,2		0,3	0,4
Pt. 21	E		S	S	E	S									190A, 210W, 215Y	ABC, NVP, NFV	1999	>339	32,3	11,7		32,4		43,5	>2,894
Pt. 22	D		S	S	S	K									41L, 74V, *103N*, 108I, 184V, 190A, 210W, 215Y	ddI, EFV, APV	3745	>339	9,7	2,7		>48		1,2	19
Pt. 23	D		S	S	S	G									41L, *103N*, 184V, 190A, 210W, 215Y	AZT, 3TC, NVP									
Pt. 24	E		S	S	S	T									41L, 184I, 210W, 215Y	treatment interruption									
Pt. 25	E		S	S	M	T									210W, 215F	AZT, ddI, DLV									
Pt. 26	D		S	S	S	T									41L, 210W, 215Y	AZT, ddC	>1805	46	7,3	2,5		7,5		0,4	0,4
Pt. 27	E		S	S	S	A									41L, *103N*, 210W, 215Y	AZT, RTV, SQV	>1805	>245	20,3	3,1		>9		0,8	1,6
Pt. 28	E		S	S	S	G									215Y	d4T, 3TC, NFV	>1805	>245	17,1	5,1		>9		0,7	0,9
Pt. 29	D		S	S	S	S									184V, 215Y	d4T, 3TC, RTV	188	>245	11,9	2,3		7,2		0,5	1,1
Pt. 30	G		S	S	S	G									None	ABC, SQV, RTV									
Pt. 31	V/A		S	S	S	G								R	T215Y	AZT, 3TC	>1805	>245	26,8	4,3		5,6		0,6	1,4
Pt. 32	K		G	S	T	T								R	41L, 74V, *103N*, 184V, 215F, 219Q	AZT, 3TC, ddI	>1805	>245	32,4	5,1		8,1		>137	>1,463
Pt. 33	G		S	S	V	G									184I	d4T, ABC									
Pt. 34	C		T	S	V	T									41L, *108I*, 103N, 215Y	ABC, EFV, RTV, SQV	>3397	>339	53,9	10,8		>48		>209	>2,894
Pt. 35	E		S	S	S	G									62V, 74V, 75I, 103N, 181C, 215F, 219E	ABC, EFV, NFV	>1805	>245	35,3	2,6		5,7		>137	>1,463
Pt. 36	E		S	S	V	T									41L, 62V, *184I*, 210W, 215Y	AZT, 3TC, NVP, IDV	758	>245	203,2	5,9		7,7		1,0	0,1
Pt. 37	D		S/T	S	S	A									41L, 62V, 215Y	d4T, 3TC, RTV									
Pt. 38	S		S	S	S	G									62V, 184V	d4T, 3TC, NFV	1,214	>245	11,2	1,6		5		1,1	2,1
Pt. 39	E		S	S	V	T									41L, 62V, *184I*, 210W, 215Y	d4T, 3TC, SQV	1,129	>245	37,8	3,2		6,5		0,6	0,7
Pt. 40	G		S	S	S	G									103N, 190A, 210W, 215Y	AZT, 3TC, EFV, RTV, APV	>3397	>339	19,6	4,4		>48		76,1	262,5
Pt. 41	D		S	S	S	G									188C, 215Y/F	ddI, ABC, NVP									
Pt. 42	G		S	S	I	G									215Y	d4T, ddI	>3397	>339	10,5	2,4		17,9		0,3	0,3
Pt. 43	D		S	S	S	G									41L, *103N*, 210W, 215Y	d4T, ddI, EFV, RTV	>586	>619	153,8	10,4		>19		1,1	2,5
Pt. 44	E		S	S	S	G									62V, 184I, 215Y	d4T, 3TC, NFV, SQV	>586	>619	>382	22,6		>19		1,0	0,8
Pt. 45	D		S	S	V	S								R	62V, 70R, 184V, 215F	d4T, 3TC, NFV	>586	>619	321,7	40,2		>19		0,4	0,7
Pt. 46	D		S	S	V	A								R	41L, 62V, 70R	d4T, ddI	>3688	>346	14,8	6,1		9,2		1,0	1,0
Pt. 47	N		N	I	T	T	R	V	M				G	41L, 67N, 215Y, 219E	d4T, 3TC, RTV	>301	>25	24,7	5,8		18,3		1,0	3,0
Pt. 48	D		S	T	T	E	G	K	K	D	S	T	R	W R K	41L, *103N*, 181C, 190A, 210W, 215Y	d4T, ddI, NVP, NFV	188	2	2,8	1,3		4,1		38,3	>947
Sato 2001																											
Pt. 89JP-NH3-4-vm	N		N	I	H	G	G	R	D	Q	G	P	A	S I	41L, 210W, 215Y	AZT, ddI, 3TC, PI	400	200	>50	50	90				<1
Co. ERT-m11															41L, 210W, 215Y	Site directed mutagenesis construct	36	1,4	0,7	1,1	0,4				0,3
Co. ERT-m12	N		N	I	H	G	G	R	D	Q	G	P	A	S T		Site directed mutagenesis construct	1,1	5,2	1,4	1,5	1,0				0,4
Co. ERT-m13	N		N	I	H	G	G	R	D	Q	G	P	A	S I		Site directed mutagenesis construct	8,8	2,4	2	4,3	0,5				<0,1
Co. ERT-m14	N		N	I	H	G	G	R	D	Q	G	P	A	S T	210W, 215Y	Site directed mutagenesis construct	>400	30	12	8,7	0,4				0,2
Co. ERT-m15	N		N	I	H	G	G	R	D	Q	G	P	A	S T	41L, 210W, 215Y	Site directed mutagenesis construct	>400	16	4,3	9,3	2,3				0,1
Co. ERT-m16	N		N	I	H	G	G	R	D	Q	G	P	A	S I	41L, 210W, 215Y	Site directed mutagenesis construct	>400	192	25	30	3,6				0,2
Andreoletti 2002																											
Co. 1				S	E	S								R	62V, 181C, 190A, 215Y	N/A	>301	>619	>550	4,0		>400		20	>947
Co. 2				S	V	A								R	41L, 62V, *184V*	d4T, ddI	>3688	>346	14,8	6,0		9,0		1	1
Co. 3				S	V	S								R	62V, 184I, 215F	3TC, d4T, NFV	>586	>619	322,0	40,0		>19		<1	<1
Lobeto 2002																											
Pt. 15 bp ins	N		G	T	T	R	V	M	G						41L, 215Y, 219Q	N/A	371	84	15	12	4	32			
Quinones-Mateu 2002																											
Co. BH10 T69S				S	-	-										Site directed mutagenesis construct	0,4								
Co. BH10 T69SS				S	S	S										Site directed mutagenesis construct	0,3								
Pt. SS				S	S	S								R	41L, 62V, *108I*, 181C, 184I, 210W, 215Y	(a.o AZT, ddI, d4T, NEV, SQV, NFV)	786								
Co. 2S3S				S	-	-								R	41L, 62V, *108I*, 181C, 184I, 210W, 215Y	Site directed mutagenesis construct	143								
Co. 2S4S				S	S	S	S	S						R	41L, 62V, *108I*, 181C, 184I, 210W, 215Y	Site directed mutagenesis construct	3,6								
Balzarini 2004																											
Pt. 001	E			S	S	G								R	184M/V, 215Y	AZT, ddC, 3TC, ddI, d4T, RTV, SQV	103	7,4	41	<1		9,2			
Pt. 002	G			S	T	G									184V	AZT, ddC, SQV, d4T, 3TC, IDV	>500	>50	>50	18		>10			
Pt. 003				S	V	G									210L	AZT, ddC, SQV, ddI, d4T, 3TC	>500	3,8	<1	>3					
White 2004																											
Pt. FS-SSS				S	S	S									41L, 74V, *108I*, 206W, 215Y	AZT, ddI, d4T, ddC, 3TC, PIs, NNRTIs	>1,180	6,2	7,7	3,0	3,5	13,0			
Co. FS					-	-									41L, 74V, *108I*, 206W, 215Y	Site directed mutagenesis construct	25	3,2	1,8	1,6	1,1	2,6			
Malamoros 2004																											
SS_D67N	N			S	S	S								R	41L, 62V, *108I*, 181C, 184I, 210W, 215Y	Site directed mutagenesis construct	536	>35	10,9	7,6	11,5				
SS_Y215T				S	S	S								R	41L, 62V, *108I*, 181C, 184I, 210W	Site directed mutagenesis construct	1	>35	2,1	9,2	10,5				
SS_Y215S				S	S	S								R	41L, 62V, *108I*, 181C, 184I, 210W, 215S	Site directed mutagenesis construct	2,9	>35	1,3	5,0	6,5				
SS_Y215N				S	S	S								R	41L, 62V, *108I*, 181C, 184I, 210W, 215N	Site directed mutagenesis construct	0,9	>35	1,2	3,9	5,6				
Prado 2004																											
Co. SSSY				S	S	S									215Y		4	5,3	5,5	1	3,7				
Pt. SS				S	S	S								R	41L, 62V, *108I*, 181C, 184I, 210W, 215Y	a.o. AZT, d4T ddI, NVP, SQV, NFV	>1,600	>34	13,9	8,7	12,8				
Co. SS/215S				S	S	S								R	41L, 62V, *108I*, 181C, 184I, 210W, 215S	Site directed mutagenesis construct	708	>34	14	4,7	6,3				
Co. SS/215N				S	S	S								R	41L, 62V, *108I*, 181C, 184I, 210W, 215N	Site directed mutagenesis construct	1,4	>34	2,6	5,7	5,7				
Co. SS/215T				S	S	S								R	41L, 62V, *108I*, 181C, 184I, 210W	Site directed mutagenesis construct	4,1	>34	1,7	3,1	3,5				
Co. SS/67N	N			S	S	S								R	41L, 62V, *108I*, 181C, 184I, 210W, 215Y	Site directed mutagenesis construct	1,2	>34	1,6	2,4	3,1				
Van der Hoek 2005																											
Pt. 1				S	T	G	K	K	D	S	T		R		41L, 210W, 215Y	AZT, ddC	>2,000	8,8	5,1	2,3	2,4	7,1		0,4	0,4

Table 1 (*Continued*)

Reference/Sample	67	68	69	Insertion										70	Resistance-associated RT mutations	Treatment (history)
	D	S	T											K		
Tamalet 1998																
Pt. 1 month 0			S	S	S										41L, 62V, 184V, 210W, 215Y	(a.o. AZT, 3TC, IDV)
Pt. 1 month 10			S	S	G										41L, 62V, 184V, 210W, 215Y	(a.o. AZT, 3TC, IDV)
Pt. 1 month 12			S	S	G										41L, 62V, 103N, 184V, 210W, 215Y	(a.o. AZT, 3TC, IDV)
Pt. 2 month 0	Q		S	S	S										184V, 215Y	(a.o. AZT, 3TC, RTV)
Pt. 2 month 9	Q		S	S	S										184I, 215Y	(a.o. AZT, 3TC, RTV)
Pt. 2 month 10	Q		S	S	S										ND	(a.o. AZT, 3TC, RTV, d4T, SQV)
Pt. 3 month 0	G			T										R	184I, 215F, 219Q	(a.o. d4T, 3TC, RTV)
Pt. 4 month 0	E		S	S	A										41L, 215Y	(a.o AZT, ddI, RTV, SQV)
Pt. 5 month 0			S	S	T										41L, 210W, 215F	(a.o. AZT, ddC)
Pt. 6 month 0	E		S	M	T										103N, 210L/W, 215Y/F	(a.o. d4T, 3TC, IDV)
Pt. 7 month 0			S	S	S										215Y	(a.o d4T, 3TC, NFV)
Pt. 8 month 0	E		S	S	G										215Y	(a.o d4T, 3TC, NFV)
Briones 1999																
Pt. 1 05/97	N		S	S	S									R	41L, 215Y	AZT, ddI
Pt. 2 02/94			S	S	S										41L, 215Y	ATZ, ddC
Pt. 2 01/95			S	S	S										41L, 215Y	AZT, ddC, ddI,
Pt. 2 08/95		S/N	S	S	G										41L, 215Y	ATZ, ddC, ddI, 3TC,
Pt. 2 05/96			S	S	G										41L, 215Y	ATZ, ddC, ddI, 3TC,
Ross 1999																
Pt. 1			S	S	G									R	62V, 184V, 215Y	AZT, d4T, ddC, 3TC, SQV, RTV, IDV
Pt. 2			S	S	A										41L, 184V, 215Y	ddI, 3TC, AZT, IDV
Pt. 3			S	S	A										ND	d4T, 3TC
Pt. 4			S	S	A										ND	d4T, 3TC
Kalliki 2000																
Pt. 1	H			K	D	S	I								41L, 210W, 215Y	
Pt. 2	G	T		D										R	74V, 103N, 181C, 184V, 215V, 219Q	
Pt. 3	G			T										R	106M, 190A, 219Q	
Balotta 2000																
Pt. 3			S	S	G										41L, 62A/V, 184M/V/I, 210L/W, 215Y	AZT, ddI, SQV, d4T, 3TC, IDV
Pt. 4	E		S	S	G										210W, 215Y	AZT, ddC, d4T, 3TC, SQV, RTV, IDV
Pt. 5			S	S	S									K/R	41L,184I, 210W, 215Y	AZT, ddC, d4T, 3TC, RTV, IDV
Pt. 6			S	V	G										210W	AZT, ddI, ddC, SQV
Tamalet 2000																
Pt. BS 02/97	E		S	S	S										184V, 210W, 215Y	various combinations of
Pt. BS 12/97	E		S	S	S										108V/I, 181Y/C, 184I, 210W, 215Y	AZT, 3TC, ddI, d4T, NVP and Indinavir
Pt. JY 01/97			S	S	S										41L, 62V, 184V, 210W, 215Y	for all these patients
Pt. JY 01/98			S	S	G										41L, 62V, 103N, 184V, 190A, 210W, 215Y	
Pt. JY 08/99	E	G	S	S	G										41L, 62V, 98G, 108I, 184V, 190A, 210W, 215Y	
Pt. AR 01/96	G			T										R	184I, 215F, 219Q	
Pt. AR 04/99	G			T										R	184M/V, 215T/I/F/S, 219Q	
Pt. GM 10/98	E		S	V	T										41L, 210W, 215Y/F	
Pt. GM 06/99	E		S	V	T										41L, 100L/I, 188L, 210W, 215Y	
Pt. GM 11/99	E		S	V	T										41L, 188L, 210W, 215Y/F	
Pt. BP 07/99			S	S	S										41L, 62V, 74V, 103K/N, 108I, 184V, 190G/A, 210W, 215Y	
Pt. BP 08/99															41L, 210W, 215Y	
Pt. BP 10/99															41L, 210W, 215Y	
Pt. BP 11/99			S	S	S										41L, 62V, 74V, 108I, 181C, 184V, 190A, 210W, 215Y	
Pt. AC 01/95			S	T	G									R		
Pt. AC 04/98			S	V	G											
Pt. AC 06/99			S	V	G										103N, 181C, 190G/A	
Pt. DD 08/98	G		S	S	G										184V, 215Y	
Pt. DD 02/99	D/G/E		S	S	G										184V, 215Y	
Pt. DD 09/99	G		S	V	G									Q	184V, 215Y	
Pt. GR 09/97			S	S	A										41M/L, 62A/V, 210L/W, 215Y	
Pt. GR 08/98			S	S	T										41L, 62V, 210W, 215Y	
Pt. NR 04/97			A	S	G										41L, 210W, 215Y	
Pt. SR 02/99	G	Y	S	D										R	181C, 184I, 219Q	
Pt. NO 10/99	E		S	V	T										41L, 210W, 215Y	
Pt. JS 12/99	E		S	S	S										62V, 100I, 103N, 116F/Y, 184I, 210W, 215Y	
Yahi 2000																
Pt. D 07/98			S	S	S										41L, 62A/V, 74L/V, 108V/I, 184V, 210W, 215Y	AZT, d4T, ABC, NFV, EFV, IND, SQV, RTV
Pt. D 07/99			S	S	S										41L, 62V, 74V, 103K/N, 108I, 184V, 210W, 215Y	AZT, d4T, ABC, NFV, EFV, IND, SQV, RTV
Pt. D 08/99				-	-										210W, 215Y	AZT, d4T, ABC, NFV, EFV, IND, SQV, RTV (after therapy stop)
Pt. D 10/99				-	-										210W, 215Y	AZT, d4T, ABC, NFV, EFV, IND, SQV, RTV
Pt. D 11/99			S	S	S										41L, 62V, 74V, 108I, 181C, 184V, 210W, 215Y	AZT, d4T, ABC, NFV, EFV, IND, SQV, RTV
Briones 2001																
Pt. 1			S	S	S									R	41L, 215Y	AZT, ddI
Pt. 2			S	S	S									R	41L, 62V, 184I, 210W, 215Y	AZT, ddC
Pt. 3			S	S	G										41L, 215Y	AZT, ddC
Pt. 4			S	S	G									R	215Y	AZT, ddC
Rousseau 2001																
Pt. 13	N	G	Q	C										V	41L, 62V, 181C, 190A, 210W, 215Y	d4T, NVP. SQV, NFV, 3TC
Pt. 15															41L, 103N, 181C, 190A, 215Y	ABC, NFV, EFV, RTV, IDV
Pt. 16															41L, 62V, 188L, 215Y	ABC, EFV, NFV,
Kim 2001																
Pt. W176	G		S	E	A										41L, 210W, 215Y	N/A
Pt. QGC	E		S	S	A										215Y	N/A
Miller 2001																
Pt. 14 wk0			S	S	S										41L, 74V, 184V, 210W	Several ART drugs
Pt.14 wk24			S	S	S										41L, 74V, 210W, 215Y	Several ART drug s+ PMEA
Schneider 2004																
Pt.1 Month 0			S	S	T										41L, 62V, 184V, 210W, 215Y	AZT, ddI, 3TC, d4T, IDV
Pt.1 Month 31	E		S	S	T										41L, 62V, 74V, 103N, 184V, 190A, 210W, 215Y	AZT, ddI, 3TC, d4T, IDV, ddC, SQV, NFV, EFV, RTV
Pt. 2 Month 5			S	S	A										41L, 184V, 210W, 215Y	AZT, ddI, 3TC, d4T, IDV
Pt. 2 Month 10			S	S	A/G										41L, 62V, 184V, 210W, 215Y	AZT, ddI, 3TC, d4T, IDV
Pt. 2 Month 17	E		A	S	G/S										41L, 62V, 210W, 215Y	AZT, ddI, 3TC, d4T, IDV, RTV
Pt. 3 Month 2	E		S	S	T										41L, 215Y	AZT, ddI, 3TC
Pt. 3 Month 26	E		S	S	S										41L, 62V, 184I, 215Y	AZT, ddI, 3TC, IDV, d4T, NFV
Pt. 4 Month 7			A	S	A										41L, 62V, 210W, 215Y	AZT, ddC, 3TC
Pt. 4 Month 12			A	V	A										41L, 62V, 210W, 215Y	AZT, ddC, 3TC, d4T, IDV
Pt. 5 Month 5			S	S	S										41L, 215Y	AZT,ddI
Pt. 5 Month 17			S	S	G										41L, 184V, 215Y	AZT, ddI, ddC, 3TC
Pt. 5 Month 36	E		S	S	G										41L, 184I, 215Y	AZT, ddI, ddC, 3TC, d4T, IDV
Pt. 6 Month 37			A	S	A										41L, 184V, 215Y	AZT, ddI, ddC, 3TC
Pt. 7 Month 16			S	Q	S										41L, 181C, 210W, 215F	AZT, ddC, 3TC, IDV, d4T, ddI, RTV
Pt. 8 Month 0			S	S	A										41L, 210W, 215Y	AZT, ddC, 3TC
Pt. 8 Month 35			S	C	A										41L, 181C, 190A, 210W, 215Y	AZT, ddC, 3TC, IDV, RTV, d4T, SQV
Pt. 9 Month 9			S	S	A										62V, 210W, 215Y	AZT, ddI
Pt. 9 Month 14			S	S	G										62V, 210W, 215Y	AZT, ddI, d4T, 3TC
Pt. 9 Month 19	E		S	S	G										62V, 210W, 215Y	AZT, ddI, d4T, 3TC
Pt. 10 Month 36			A	S	G										41L, 184V, 210W, 215Y	AZT, ddI, 3TC
Pt. 10 Month 51		G	A	V	G										41L, 210W, 215Y	AZT, ddI, 3TC, d4T, IDV
Pt. 11 Month 0	G	Y	S	D										R	215F, 219Q	ND
Tamalet 2004																
	N		S	T										R	219Q	AZT, 3TC, ddI, d4T
	N		T	T										R	F116Y, Q151M, 219Q	AZT, 3TC, ddI, d4T

Fold increases in IC_{50} compared to a wild type reference strain. Shown are the resistance-associated mutations as indicated by the International AIDS Society (Johnson et al., 2006). *Abbreviations*: AZT, zidovudine; ddI, didanosine; ddC, zalcitabine; 3TC, lamivudine; d4T, stavudine; ABC, abacavir; NVP, nevirapine; DLV, delavirdine; EFV, efavirenz; SQV, saquinavir; IDV, indinavir; RTV, ritonavir; NFV, nelfinavir; PMEA, 9-[2-phosphonyl-methoxy-ethyl] adenine; PI, protease inhibitors; ART, anti-retroviral therapy.

Table 2
Several examples of dipeptide insertions to illustrate possibilities of duplications on nucleotide level

	Reference	Amino acid in HIV-1 reverse transcriptase					
		68	–	69	–	–	70
	Wild type (consensus B)	AGT (S)	–	ACT (T)	–	–	AAA (K)
Example 1	De Antoni et al. (1997)	AGT (S)	–	AGT (S)	AGT (S)	AGT (S)	AAA (K)
Example 2	Winters et al. (1998)	AGT (S)	–	TCT (S)	AGT (S)	TCT (S)	AAA (K)
Example 3	Sugiura et al. (1999)	AGC (S)	AGC (S)	ACT (T)	ACT (T)	–	AAA (K)

but did not show a mechanistic interplay between these changes (Tamalet et al., 2004).

Looking at the nucleotide level, insertions seem to be duplications of prior codons. For example the frequently occurring double serine insertion is likely to be a duplication of six nucleotides on a row (codons 68 and 69), which is illustrated by the first two examples in Table 2 (De Antoni et al., 1997; Winters et al., 1998). That a dipeptide insertion can also be generated by two duplications of three nucleotides (codons 68 and 69) is also shown (example 3, Table 2) (Sugiura et al., 1999). Furthermore it has been demonstrated that a dipeptide insertion is generated by an insertion of 9 nucleotides (three amino acids) between codons 68 and 69 and a deletion of three nucleotides (codon 70) (Larder et al., 1999). Longer inserts could be explained by (long-distance) duplications as well, such as a duplication of amino acids 66–70 or 15 nucleotides of the *env* gene, although not all large insertions can be justified by a doubling of viral and/or cellular genes (Lobato et al., 2002; van der Hoek et al., 2005).

Duplication of preceding nucleotides could be explained by primer/template slippage. Strand-transfer reactions during reverse transcription seem to occur at so-called pausing sites (Harrison et al., 1998). The likelihood of slippage of the primer/template at these sites could be increased by the T69S mutation. If insertions are indeed generated by a duplication of codon 68 and/or codon 69 after a T69S substitution, the majority of the insertions should initially be a double serine. In fact, clinical samples have shown the initial generation of a double serine insertion in a patient, which can evolve towards a serine-glycine (SG) insertion variant. This single nucleotide change probably confers a selective advantage when compared to its predecessor, since the SG-insertion completely replaces the SS-variant and remains stably present. Other evolutionary pathways of insertions have been described as well, e.g. TG to VG, SG to VG, SA to ST and 69SSS to 69SSG or 69EGS.

Considering the high-level of recombination during reverse transcription of the HIV-1 genome, it is suggested that recombination might also play a role in the generation of insertions (Temin, 1993).

2. Influence of insertions in RT on drug susceptibility

As shown in Table 1, most of the patient-derived insertion variants show high-level resistance (average of >500 fold increase in IC_{50}) to AZT and moderate to high-level resistance to 3TC, d4T, ddI, ddC and ABC (an average increase in IC_{50} of >50, >15, >5, >5 and >10, respectively). In contrast to the Q151M-complex (Deval et al., 2002; Johnson et al., 2006; Shirasaka et al., 1995), insertions in the fingers domain confer resistance against TFV as well (Johnson et al., 2006).

Variants in which the insert was deleted while the background remained unchanged, revealed that the susceptibility of the virus for several NRTIs was increased. However, phenotypic drug resistance analyses of insertion mutants in a wild type background (i.e. no accompanying TAMs) have shown only a minor to no decrease in susceptibility to most NRTIs (Larder et al., 1999; Mas et al., 2000; Meyer et al., 2003; Sato et al., 2001; Winters et al., 1998). This indicates that an insertion on its own does not have a large influence on the reduction of drug susceptibility. High-level multi-drug resistance requires the presence of additional mutations, especially the classical TAMs. Remarkably is the low-level resistance against 3TC that has been found in the "insertion-only" constructs. Apparently, no additional mutations are required to confer a very weak resistance towards 3TC (Larder et al., 1999; Mas et al., 2000; Sato et al., 2001; Winters et al., 1998).

In a recent paper by van der Hoek et al. a total of 134 insertion variants in the Monogram Biosciences database have been analyzed and it was demonstrated that a larger insert correlates with increased AZT resistance (van der Hoek et al., 2005). Also, it was shown that the sequence of the insert has a modest effect on the resistance phenotype. However, these genotype–phenotype correlations did not take the presence of background mutations into account (van der Hoek et al., 2005).

To investigate the impact of different TAMs in insertion variants on drug susceptibility, these changes were added to the inserts in a wild type background. A large decrease in susceptibility to NRTIs was observed when the T215Y amino acid substitution was added to these constructs (Table 1) (Winters et al., 1998). The importance of this mutation has also been emphasized by replacing the 215Y by a 215T, 215S or 215N in an insert-containing clinical isolate. Reversion of the T215Y towards wild type, S or N resulted in a decrease in resistance to all tested NRTIs (Table 1) (Matamoros et al., 2004; Prado et al., 2004). A reduced susceptibility for several NRTIs was also observed for the M41L or A62V changes in combination with an insert. The importance of the A62V substitution was confirmed by comparing clinical isolates with and without 62V (see Table 1), which indicates that the A62V is able to increase resistance in the presence of L210W and T215Y (Larder et al., 1999; Tamalet et al., 2000). Together with the M41L, the A62V change seems to play a role in positioning the β3–β4 loop and is probably affecting the ATP-dependent excision rate of primers that are terminated by thymidine analogues (Cases-Gonzalez et al., 2007). The role of the flanking amino acid substitutions

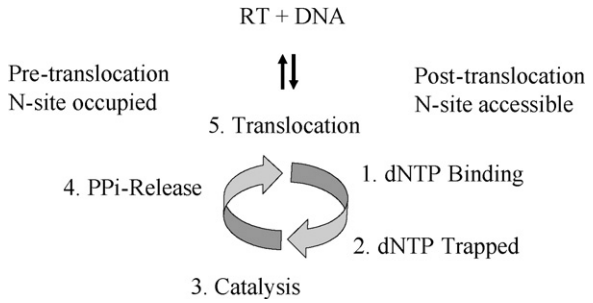

Fig. 1. Single cycle of nucleotide incorporation. The incorporation of a novel nucleoside monophosphate by HIV-1 RT involves dNTP, a conformational change that traps the incoming nucleotide, catalysis, release of pyrophosphate and ultimately polymerase translocation to clear the nucleotide binding site (N-site). The enzyme remains bound to its nucleic acid substrate (processive mode of DNA synthesis), or dissociates and rebinds in the post-translocational state before the incorporation of the next nucleotide (distributive mode).

D67E, T69S and K70R in drug resistance is less clear. Several experiments show that the D67E substitution confers only little resistance to most drugs and also the T69S seems to give only small differences in susceptibility (Winters et al., 1998). These flanking amino acid substitutions, which may facilitate the initial generation of the insertion, could have a compensatory function to maintain enzyme activity or compensate for loss of fitness due to the insert (Boyer et al., 1999).

3. Structural basis for drug action and drug resistance

The crystal structures of HIV-1 RT in the presence of a bound nucleoside triphosphate shed light on molecular mechanisms involved in nucleotide incorporation and resistance to NRTIs (Huang et al., 1998). The fingers subdomain plays a dual role in substrate binding, as it affects both binding of the nucleic acid and binding of the incoming nucleotide. Crystallographic data show that fingers and thumb are wrapped around the bound primer/template duplex (Ding et al., 1998; Huang et al., 1998; Jacobo-Molina et al., 1991) The distance between the tips of the two subdomains is relatively large as compared to other polymerases, which helps to explain the facile dissociation of the complex and the relatively poor processivity of HIV-1 RT (Patel et al., 1995). Following binding of the primer/template substrate, the incoming dNTP binds to the nucleotide binding site (N-site), while the 3′-end of the primer occludes the so-called priming site (P-site) (Fig. 1) (Boyer et al., 2001; Gotte, 2004). The fingers close down, which traps the dNTP substrate, and aligns its α-phosphate with the 3′-hydroxyl group of the primer terminus for catalysis. Thus, changes in the fingers subdomain can conceivably affect both nucleotide binding and primer/template binding.

Resistance to NRTIs has been associated with two major biochemical mechanisms (Goldschmidt and Marquet, 2004; Gotte, 2004; Selmi et al., 2003). The first mechanism is based on an improved discrimination between the nucleotide analogue and its natural dNTP counterpart. The mutant enzyme disfavours binding and/or incorporation of the drug over the natural dNTP to a greater extent as seen with wild type RT. A single mutation can already influence the affinity and/or proper positioning of NRTI-TPs to the N-site, which may lead to decreases in rates of polymerization. The M184V or M184I mutations that confer high-level resistance to 3TC are prominent examples in this regard (Krebs et al., 1997). Crystallographic data suggest a steric conflict between the β-branched amino acid and the sugar moiety of a modelled 3TC-TP (Sarafianos et al., 1999). The second major mechanism is based on increased rates of excision of the incorporated inhibitor. In this case, the mutant enzyme may incorporate the inhibitor with efficiencies that are comparable with wild type RT; however, chain-termination is not irreversible, and the incorporated nucleotide analogue can be excised in the presence of pyrophosphate, i.e. the back reaction (pyrophosphorolysis), or in the presence of a pyrophosphate donor such as ATP or other NTPs (Arion et al., 1998; Meyer et al., 1998, 1999). Enzymes containing classical TAMs can dramatically increase rates of excision of incorporated AZT-MP. The difference between the mutant enzyme is far more pronounced in the presence of ATP, which suggests that ATP is likely to be the physiologically relevant substrate (Meyer et al., 1999).

Given that the fingers mutations in the β3–β4 loop are located in close proximity to the incoming nucleotide, and in view of the fact that the insertions are almost exclusively seen together with TAMs, it is conceivable that changes in substrate discrimination as well as changes in rates of excision could play a role in resistance associated with these genotypes. Slight increases in the ability to discriminate between the NRTI and natural dNTP pools have been reported for 3TC (Boyer et al., 2002; Mas et al., 2002) and TFV (White et al., 2004), while the selectivity for AZT remains largely unchanged. However, rates of ATP-dependent removal of AZT-MP and many other NRTIs are dramatically increased with enzymes containing fingers insertions in a background of TAMs, which suggests that excision is probably the major mechanism involved in resistance to multiple NRTIs. This was first demonstrated by Mas et al. (2000). The authors generated a recombinant mutant enzyme that was derived from a clinical isolate containing numerous known resistance conferring mutations, including T215Y and a double serine insertion between residues 69 and 70. The ATP-dependent excision was highly effective with this enzyme, while the rate of the reaction dropped significantly when the double serine insertion was selectively deleted. Rates of excision of incorporated AZT-MP follow the order M41L/T215Y/69S-SS RT > M41L/T215Y RT > T69S-SS RT ≈ wild type RT (Mas et al., 2000; Meyer et al., 2003). This trend correlates well with the different levels of phenotypic drug resistance measured with corresponding mutant viruses. Similar effects have been described with other dipeptide insertions that were either derived from clinical isolates or generated by site-directed mutagenesis. Taken together, the aforementioned biochemical studies suggest that an aromatic side chain at position 215 (Y or F) is a prerequisite for the observed high rates of ATP-dependent primer unblocking, and the fingers insertion further increases the efficiency of the reaction. Kinetic data suggest that the T215Y mutation improves the alignment between the pyrophosphate donor ATP and the terminal phosphodiester bond of the primer (Matamoros et al., 2004).

There are subtle differences among the various insertion mutations in rates of excision of incorporated NRTIs. It appears that the SG insertion in combination with TAMs is more efficient as compared to SS, AG or SA insertion in the same background (Boyer et al., 1999; Meyer et al., 2003).

Although crystal structures of HIV-1 RT with primer/template and ATP are not available, it is highly likely that the β- and γ-phosphates of the pyrophosphate donor occupy the pyrophosphate binding site (Boyer et al., 2001). Based on this assumption it has been proposed that excision may only occur pre-translocation, i.e. in the same configuration that would exist immediately after catalysis before the release of PPi when the chain-terminator still occupies the N-site (Boyer et al., 2001). This model is supported by crosslinking studies showing that excision can occur in a trapped pre-translocation complex, while the reaction cannot occur post-translocation when the 3′-end of the primer occludes the P-site (Sarafianos et al., 2003). Site-specific footprint data suggest that access to the N-site is controlled by a dynamic equilibrium between pre and post-translocational configurations. The enzyme can rapidly shuttle between both positions, which has been described as translocational equilibrium (Fig. 2). This equilibrium ensures that the 3′-end of the primer can occupy the N-site even after incorporation of the chain-terminator and the release of PPi (Marchand and Gotte, 2003, 2004).

The footprints show that the translocational equilibrium depends on various factors including the concentration of the next templated nucleotide, the chemical nature of the chain-terminator, and the specific mutational background, notably the finger insertions. High concentrations of the next nucleotide can trap the post-translocated complex. This configuration allows binding of the nucleotide immediately downstream of the chain-terminator; hence, the formation of a stable, ternary complex. This is essentially a "dead-end-complex" (DEC) (Tong et al., 1997), because the chain-terminator blocks the incorporation of the bound dNTP, and excision of the chain-terminator is prevented by the fact that the primer terminus resides in the P-site (Meyer et al., 1999). Relatively high concentrations of dNTPs are required to block excision of incorporated AZT-MP as compared to d4T-MP, ddAMP, or ddTMP that all lack a bulky azido group. This effect appears to be amplified with enzymes containing fingers insertions (Meyer et al., 2002, 2003). Footprinting and band shift experiments have shown that the formation of the ternary DEC complex is severely compromised with these mutant enzymes (Boyer et al., 2002; Marchand and Gotte, 2003; Matamoros et al., 2004). This will result in an equilibrium shift to the pre-translocation configuration, thereby enhancement of excision and thus resistance.

Together these findings suggest that resistance to AZT, d4T, ddI, and ddC by insertion variants is probably mediated through increased rates of ATP-dependent primer unblocking. Mechanisms based on substrate discrimination at the level of NRTI binding and/or incorporation are of minor importance in this context; however, such mechanisms might play a role for 3TC and TFV resistance. Rates of incorporation of TFV appear to be slightly diminished; however, TFV undergoes excision with wild type RT and this effect is significantly increased with insertion-containing mutant enzymes (White et al., 2004).

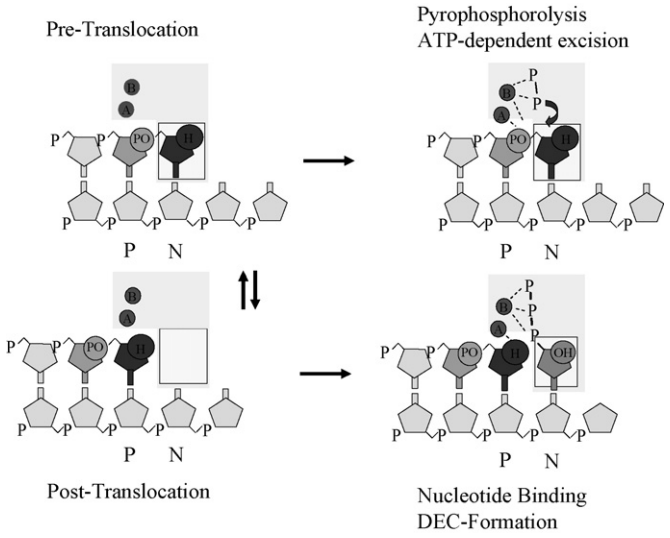

Fig. 2. Translocation state and enzymatic function. Specific binding sites for two divalent specific binding sites for two divalent metal ions (circles with A and B) are shown according to the general two-metal ion mechanism for polymerases proposed by Steitz (1999). Excision of a newly incorporated chain-terminator (darkest grey) can only occur in the pre-translocation configuration when the metal ions are positioned in the vicinity of the ultimate phosphodiester bond (top). The nucleophilic attack is indicated by the dark grey arrow. In contrast, nucleotide binding and the formation of a dead-end-complex (DEC) can only occur in the post-translocation configuration after the 3′-end of the primer cleared the N-site and moved to the P-site (bottom).

4. Polymerase activity, processivity, and replication capacity

The combined data showing that fingers insertions can diminish the stability of ternary complexes are in agreement with previous studies showing certain defects on DNA synthesis in the absence of RT inhibitors. A comparison of polymerase activities of insertion-containing constructs showed that mutant enzymes display lower levels of DNA synthesis compared to wild type activity (Boyer et al., 1999, 2002; Kew et al., 1998; Meyer et al., 2003). Steady state kinetic measurements suggest that fingers insertions can diminish the efficiency of nucleotide incorporation, which is largely attributable to increases in Km values. Moreover, insertion-containing enzymes show reduced levels of processive DNA synthesis, presumably as a direct consequence of the diminished stability of ternary complexes. Defects in regard to the efficiency of nucleotide incorporation or processivity of DNA synthesis correlate with deficiencies in viral replication capacity.

These findings are consistent with previous clinical data showing the disappearance of insertions and other resistance conferring mutations in the absence of drug pressure; however, viruses with these alterations reappear after restart of therapy. In one patient an insertion (SG) was selected during the first period of therapy (AZT monotherapy) and the insertion variants were the only viruses found during ongoing therapy. About 4 months after the first treatment interruption the insertion mutants were

Table 3
Replication capacity of several insertion variants

Reference	Insertion	Resistance-associated RT mutations	Replication capacity relative to WT	Method[a]
Quinones-Mateu et al. (2002)	69S + SS	41L, 70R, 62V, 108I, 181C, 184I, 210W, 215Y	Decreased	Comp
			Increased	Comp (in presence of inhibitors)
			Increased	Comp[b]
	69S + SSSS	41L, 70R, 62V, 108I, 181C, 184I, 210W, 215Y	Decreased	Comp
			Increased	Comp (in presence of inhibitors)
Lobato et al. (2002)	T69 + TRVM G	41L, 67N, 215Y, 219Q	Decreased	Comp
			Decreased	Mono
			Increased	Comp (in presence of inhibitors)
			Increased	Mono (in presence of inhibitors)
Sato et al. (2001)	67N + NIHGGRDQGPA	41L, 210W, 215Y	Decreased	Enz[c]
			Comparable	Mono[c]
Kew et al. (1998)	K66 + SSSSSSGGGSSSSSS	None	Comparable	Enz
	K66 + SIHPRLEAVPPSSSS	None	Comparable	Enz
Lukashov et al. (2001)	69S + SG/SD	215Y	Decreased	*In vivo*
			Increased	*In vivo* (in presence of inhibitors)
Prado et al. (2004)	69S + SS	215Y	Decreased	Comp
	69S + SS	41L, 62V, 70R, 108I, 181C, 184I, 210W, 215Y	Decreased	Comp
	69S + SS	41L, 62V, 70R, 108I, 181C, 184I, 210W, 215S	Decreased	Comp
	69S + SS	41L, 62V, 70R, 108I, 181C, 184I, 210W, 215N	Decreased	Comp
	69S + SS	41L, 62V, 70R, 108I, 181C, 184I, 210W	Decreased	Comp
	69S + SS	41L, 62V, 67N, 70R, 108I, 181C, 184I, 210W, 215Y	Decreased	Comp

Shown are the resistance-associated mutations as indicated by the International AIDS Society (Johnson et al., 2006).
[a] Method—vivo: *in vivo* determination of HIV-1 kinetics in plasma; mono: *ex vivo* HIV-1 monoinfections (viral growth kinetics and single cycle infection assays); comp: *ex vivo* HIV-1 growth competition experiments; enz: catalytic activities of HIV-1 enzymes (Quinones-Mateu et al., 2002).
[b] Compared to wild type with 69S + SS.
[c] Compared to wild type of patient before treatment.

completely replaced by wild type viruses in plasma. The wild type viruses were again completely replaced by the insertion variant about 1 month after initiation of triple therapy (d4T, 3TC, SQV) and more heterogeneity was found in the insertion region (SG/SD/RG). Two months after termination of this triple therapy the insertion variants were already completely replaced in plasma (Boyer et al., 1999).

Phylogenetic analysis (two parameter distances and synonymous distances) indicates that the insertion variants originate from the patient wild type virus and that the reappearance of the insertion variants is due to persistence in the proviral population.

The fast replacement of the wild type virus by the insertion mutant during therapy and the fast outgrowth of wild type after termination of therapy suggest a large reduction in replication capacity in the absence of drugs. It takes far more time to replace AZT resistance associated mutations after termination of therapy (Albert et al., 1992; Boucher et al., 1993b).

Also in vitro, in replication competition experiments (RCE) in the absence of drugs, recombinant viruses with a wild type RT out competed the recombinant viruses with a SS-insertion-containing RT derived from clinical isolates. On the other hand, in the presence of inhibitor (AZT) the RCEs revealed a replicative fitness advantage for the insertion-containing viruses (Table 3) (Kew et al., 1998; Lobato et al., 2002; Lukashov et al., 2001; Prado et al., 2004; Quinones-Mateu et al., 2002; Sato et al., 2001). In general in the absence of inhibitor, all insertion variants, demonstrated a highly reduced fitness level when compared to wild type. And clinically observed isolates (insertion with background mutations) were only slightly more fit than a double serine insertion in a wild type backbone.

Thus, viruses with fingers insertions in the RT enzyme replicate less efficiently as compared to viruses that lack the insertion (Quinones-Mateu et al., 2002). This could explain the low prevalence of insertion variants and the fact that these variants quickly disappear after therapy interruption (Quinones-Mateu et al., 2000). Mutations R211K and L214F have been associated with such a phenotype (Meyer et al., 2003).

5. Conclusion

The prevalence of insertions in the β3–β4 loop of RT is relatively low as compared to amino acid substitutions conferring RT drug resistance. However, once an insertion in this region in RT has been selected, the virus becomes moderate to high-level resistant to all substrate analogue RT inhibitors.

Inserts in the β3–β4 loop of RT are selected in the background of thymidine therapy associated substitutions, especially in the presence of T215Y/F, M41L and L210W changes. These amino acid substitutions cause drug resistance by increasing the level of excision of incorporated nucleotide analogue RT inhibitors. Rates of excision are further enhanced by selection of inserts in the fingers domain of RT. A few other substitutions (A62V, D67E/G and T69S) seem to be associated with a β3–β4-loop insertion in RT. The exact role of these mutations is not known, although studies demonstrate that selection of A62V is directly related to reduced NRTI susceptibility. The flanking amino acid changes at codons 67 and 69 may facilitate the initial generation of the insert. Further studies are warranted to investigate mechanisms associated with the selection of such insertions in RT.

Selection of drug resistance mutations normally comes at a price. Enzymatic analysis demonstrates that selection of insertions in the fingers subdomain of RT results in a diminished efficiency of nucleotide incorporation and DNA synthesis. This relates to a reduced replication capacity, which, in turn, is in line with the observation that drug therapy interruption results in the rapid replacement of these mutants with a wild type HIV variant. However, continuous replication in the presence of inhibitors may result in the selection of compensatory mutations. There are several candidates in this regard. Changes at codons 67, 69, 211 and 214 may compensate for the reduced processivity and replication capacity.

Taken together, selection of inserts in the β3–β4 loop of RT, although low in frequency, compromises the usefulness of a complete class of HIV RT inhibitors and therefore poses a serious threat for subsequent treatment. Especially if continuous replication in the presence of antiretroviral therapy results in the selection of amino acid changes compensating for the initially reduced processivity and replication capacity.

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