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Mechanisms of Resistance to Antiretroviral Drugs – Clinical Implications

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Abstract

HIV resistance to antiretroviral agents involves the selection of mutations within the reverse transcriptase (RT) and protease (PRO) genes, that result in structural changes causing in most instances a loss of affinity of RT and PRO inhibitors for their respective targets. Then, the inhibitory competition caused by these molecules in respect to the physiologic substrates of the RT and PRO enzymes is lost. For nucleoside analogs, a second mechanism of resistance involves the removal of the chain terminators (pyrophosphorolisis) and is caused by the classical AZT-resistance mutations. Complex interactions between drug resistance mutations make difficult how to interpret and predict the benefit of antiretroviral agents in the clinical arena. However, for most antiretroviral agents, resistance is not a dichotomic situation but rather a relatively continuous phenomenon, in which some partial activity of compounds is found even in the face of drug resistance mutations. Based in this fact, resistance to PRO inhibitors may be overcome when plasma levels of PRO inhibitors are boosted using low doses of ritonavir.

Key words

Drug resistance. NAMs. Antiretroviral drugs. HIV.

Introduction

Due to high mutation rates associated to RNA replication and retrotranscription, most RNA viruses replicate as complex and dynamic distributions of related, non-identical genomes termed viral quasispecies[1]. Naturally occurring polymorphisms at the reverse transcriptase (RT) and protease genes explain that resistant viruses may pre-exist in a given patient, even before being exposed to any antiretroviral therapy[2]. In this dynamic equilibrium of multiple viral variants, drug pressure leads to the selection of those minority viral populations harboring resistance genomes after a variable period of time (weeks to months), if residual active viral replication is allowed while on treatment[1]. This therapy-driven resistance is termed *secondary resistance*. In contrast, *primary resistance* refers to loss of drug susceptibility seen in *naïve* individuals mainly as a result of the transmission of HIV resistant viruses at the time of acute HIV infection[3].

HIV may evolve along more than one mutational pathway in developing resistance to a given drug. Each of these genotypic patterns may have different implications for cross-resistance and viral fitness. For example, resistance to nelfinavir may take either the 30N pathway, causing resistance to nelfinavir alone but paying a cost in virus replication capacity[4,5], or the 90M pathway, which confers broad cross-resistance among protease inhibitors, without compromising viral fitness[5,6]. Indeed, this information influences the preferences for sequencing antiretroviral agents[7].

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Resistance to nucleoside analogs

This family of compounds are prodrugs that are triphosphorylated by host cellular enzymes and, in

that form, compete with natural deoxynucleoside triphosphates (dNTP) for incorporation into newly synthesized viral DNA chains causing chain termination (Fig. 1). There are at least three biochemical mechanisms of resistance to NRTI. The first mechanism is mediated by mutations that increase the rate of hydrolytic removal (pyrophosphorolysis) of the chain terminating NRTI and enable continued DNA synthesis (Fig. 2)[8,9]. The second mechanism is mediated by mutations that allow the RT enzyme to discriminate against NRTI, thereby preventing their addition to the primer DNA chain (Fig. 3)[10]. The last mechanism has been described more recently[11] and is reminiscent of the inoculum effect described in bacteriology. Briefly, an increased packaging of RT molecules per virion could allow HIV to escape drug pressure to some extent (Fig. 4).

Nucleoside resistance mediated by pyrophosphorolysis

The most common mutations in HIV-1 obtained from patients receiving NRTI were originally identified through their involvement in causing AZT resistance. Various combinations of these mutations, which occur at six codons (41L, 67N, 70R, 210W, 215Y, and 219Q), have shown to mediate both ATP- and pyrophosphate (PP)-dependent removal of AZT-monophosphate from a terminated cDNA chain and cause a compensatory increase in RT processivity[8,9]. More recently, it has become clear that these mutations confer loss of susceptibility not only to AZT but also other NRTI, particularly d4T, abacavir (ABC), and didanosine (ddI)[12,13].

K70R causes low-level (4- to 8-fold) AZT resistance and is usually the first change to develop in

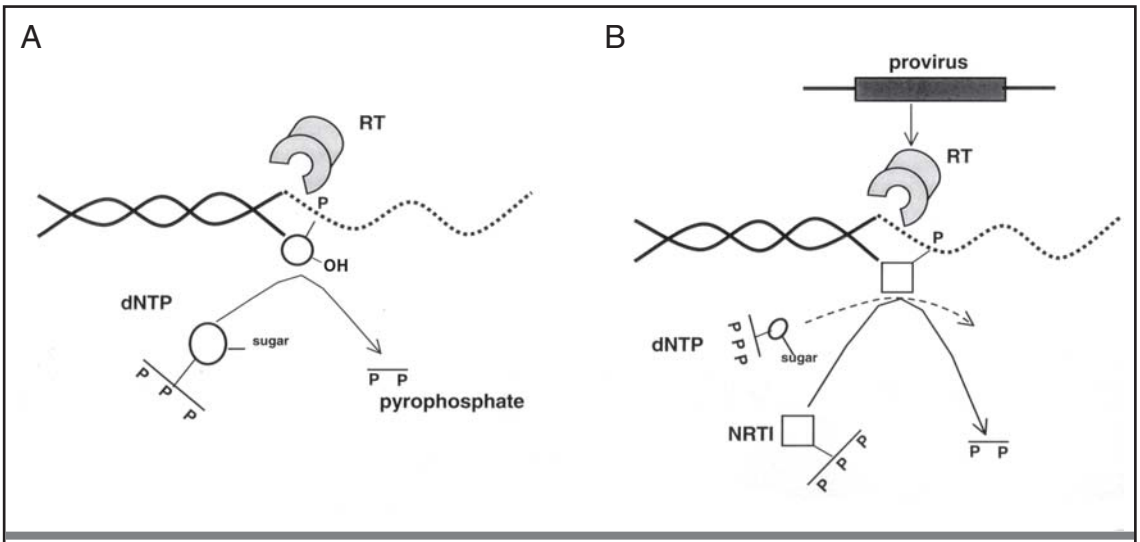

Figure 1. *Mechanism of action of nucleoside analogs acting as inhibitors of HIV replication. a) Natural extension. b) Blocking.*

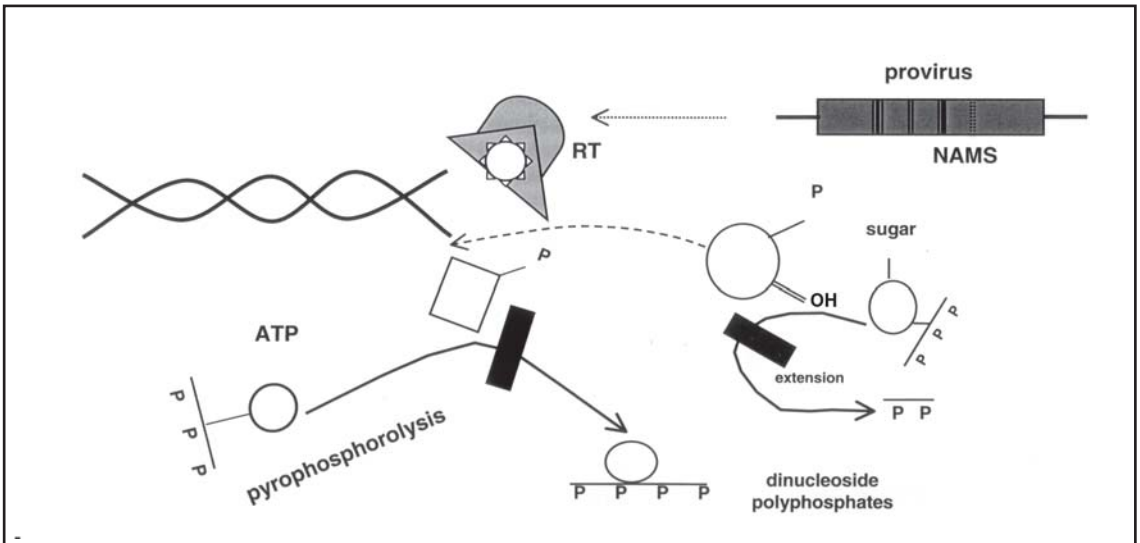

Figure 2. *Resistance to nucleoside analogs due to an increased pyrophosphorolysis.*

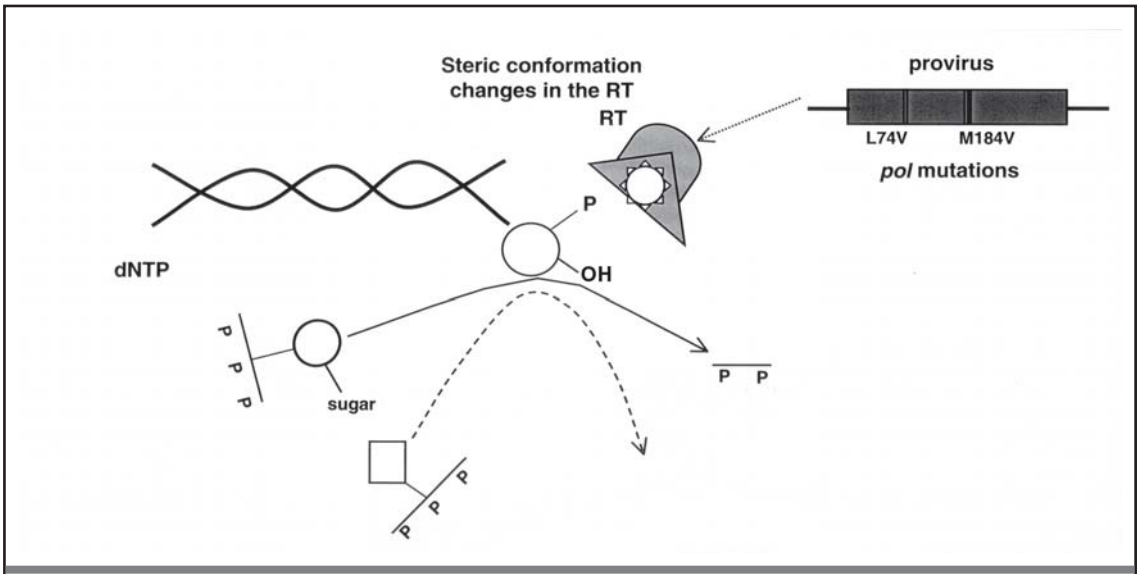

Figure 3. *Resistance to nucleoside analogs due to mutations enabling RT to discriminate against the binding of nucleoside analogs.*

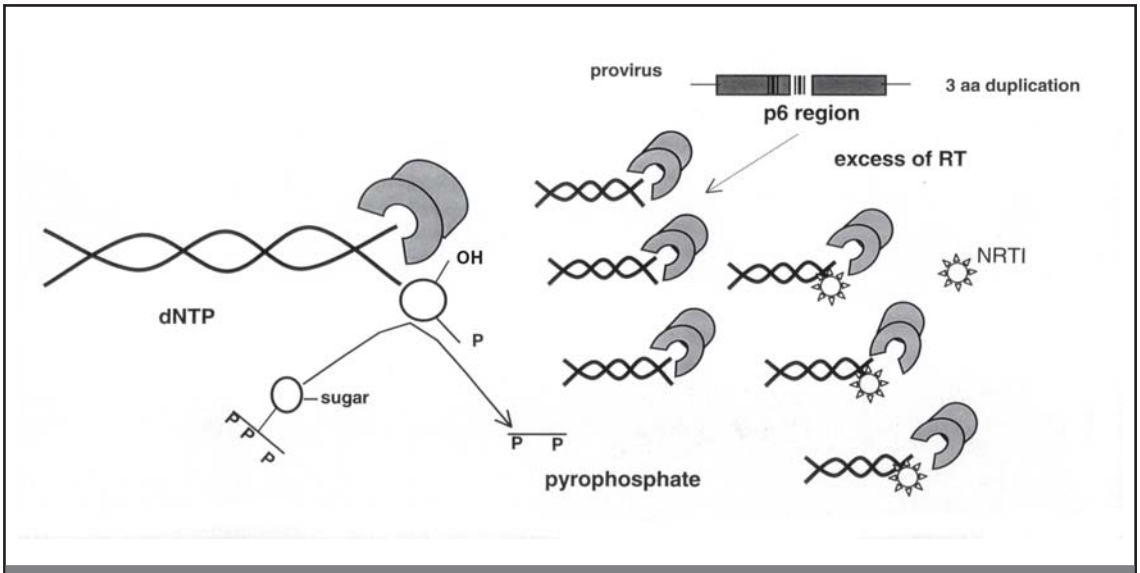

Figure 4. *Resistance to nucleoside analogs due to an increased packaging of RT molecules per virion.*

patients receiving AZT monotherapy[14]. T215Y/F result from a 2-base pair mutation and cause intermediate (10- to 20-fold) AZT resistance, and arise much later in subjects failing AZT. Mutations at positions 41 and 210 usually occur with mutations at position 215, and contribute to increasing the level of resistance. Mutations at positions 67 and 219 may be selected following mutations at codons 70 or 215. In patients having failed multiple nucleoside therapy it is common to see virus isolates harboring 4 or even all 6 of the classical AZT-resistance mutations.

Besides 215Y/F, other mutations have been described at this position, such as 215S/C/D, which are transitional mutations between wild-type (T) and resistant variants (Y or F) (Fig. 5). They do not cause reduced drug susceptibility but rather indicate a previous exposure to the drug[15,16]. They were firstly described in antiretroviral-*naïve* subjects who acquired a mutant virus at the time of HIV transmission. These persons might be more prone to select codon 215Y/F as soon as they start AZT or other nucleoside analogs, and therefore facilitate treatment failure[16]. The reason for that is that these changes differ from wild-type in only one nucleotide substitution at codon 215, whereas 215Y/F require two nucleotide changes (Fig. 6). In patients who stop nucleoside therapy, for example in the course of treatment interruptions, these intermediate reversal mutations do not arise since pre-existing wild-type viruses overgrow the mutant population more rapidly[17].

Most of the evidence linking AZT-resistant mutations with d4T and ddI resistance is based on clinical data: some patients fail on d4T and/or ddI without other

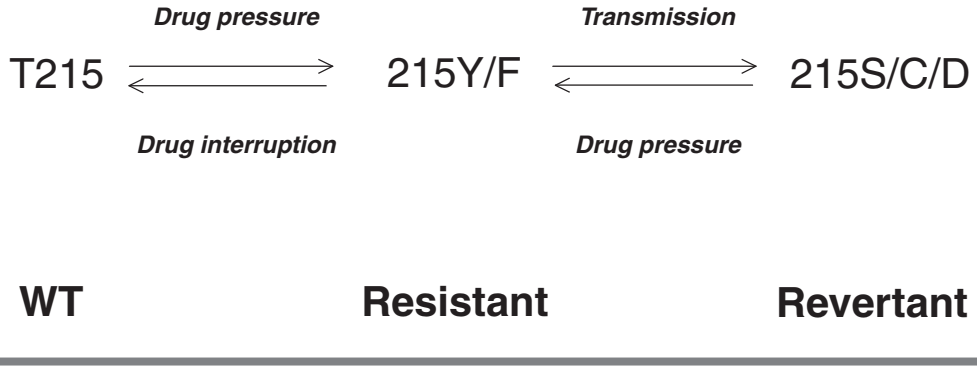

Figure 5. *Amino acid changes at codon 215 as consequence of drug pressure and/or transmission.*

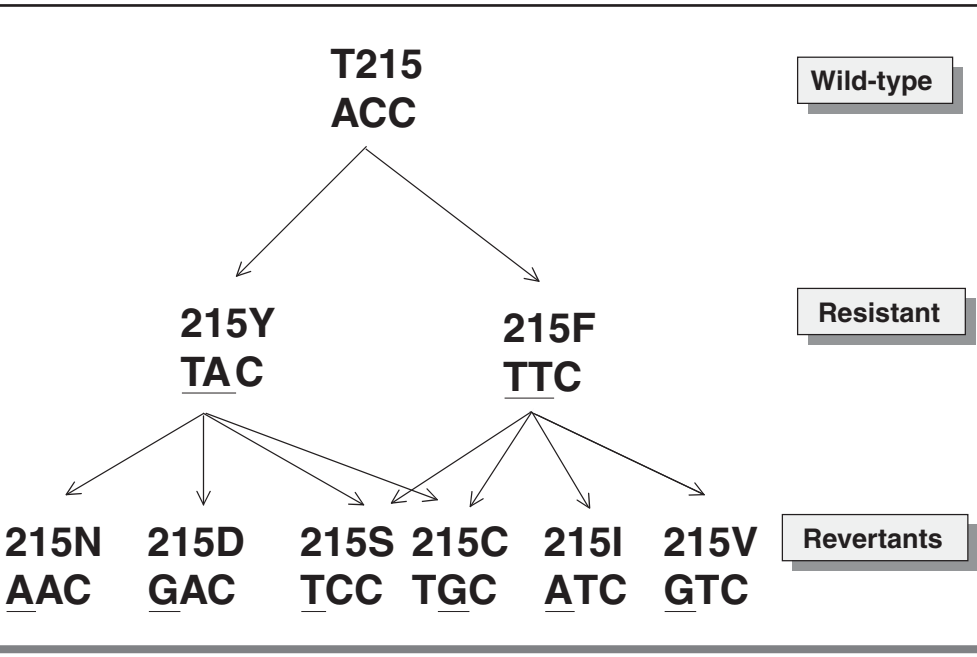

Figure 6. *Dynamics of nucleotide substitutions at codon 215 under drug pressure.*

apparent resistant genotypes[18-22]. In fact, these mutations arise in 10-35% of patients failing either d4T and/or ddI[22-26]. Finally, previous exposure to AZT and the presence of AZT-resistant mutations –particularly at position 215– leads to a diminished response to subsequent therapy including d4T[27,28].

The influence of these mutations is not limited to AZT and d4T, which are thymidine analogs, since to a different extent they are selected and contribute in causing resistance to ABC[29,30] and ddI[21,23] as well. About 10-15% of individuals failing therapy with ddI develop AZT-resistant mutations instead of the classical L74V[21,23], and the presence of these mutations reduces the chances of response to ddI[31]. Only lamivudine (3TC) escapes, but not absolutely, its action[12,13]. On the other hand, it is interesting that 41L and 210W are particularly relevant in conferring resistance to tenofovir[32,33], the last FDA-approved antiretroviral drug. Taking into account all these data, the term NAM (nucleoside-associated mutations) has been applied to this set of classically AZT mutations[9].

Although NAMs arise using almost all nucleoside analogs (except 3TC), the rate in which they appear and the loss of sensitivity they produce varies widely for each drug. Three NAMs plus 184V and/or 74V result in resistance to ABC, whereas high-level of resistance to AZT appears with only two NAMs. Resistance to ddI or d4T requires three or more NAMs plus other mutations (Table 1)[34]. Therefore, the presence of NAM alone should not preclude the use of d4T, ABC or ddI, since some of the antiviral activity of these compounds can be expected to be retained in this context. Therefore, the degree of loss of antiviral activity as a consequence of NAM varies among NRTI. Resistance is not a yes/no property in this setting. It means that residual antiviral activity is retained in most instances as long as the number of NAM is not high enough. This information is particularly useful when salvage regimens are designed based on the results of drug-resistance testing. Moreover, this knowledge may favor the use of some NRTI rather than others in first-line

Table 1. *Resistance to nucleoside analogues caused by NAMs plus other RT mutations*

Genotype	Abacavir (>4.5-fold)	ddI (>3.5-fold)	d4T (>3.0-fold)
3 NAMs	No	No	No
+44/118	No	No	Yes
+44/118 + 184	Yes	Yes	Yes
+44/118 + 69	Yes	No	Yes
+184	Yes	Yes	No
+74	Yes	Yes	No

therapies[7,35]. Figure 7 emphasizes the relative compromise in antiviral activity provided by NAMs in respect to the different NRTI.

Nucleoside resistance mediated by discriminatory mechanisms

The presence of NAMs results in loss of sensitivity to nucleoside analogues by increasing its removal (pyrophosphorolysis) from the nascent DNA chain in which these artificial compounds have been incorporated, blocking its further extension[8,9]. Once removed, natural nucleosides can again be incorporated by the RT. This mechanism is markedly different from the competitive inhibition operated by mutations such as 184V or 74V, which modify the steric conformation of the RT, nearby the catalytic site, complicating the binding of artificial nucleotides 3TC and ddI, respectively (Fig. 3).

Mutation M184V. Codon 184 is in a conserved part of the RT, close to the active site (two of the catalytic aspartates are at positions 185 and 186). M184V sterically hinders certain NRTI, particularly 3TC, while still allowing the enzyme to function[36]. The possibility that isolates containing M184V are compromised in their replicative capacity was suggested by the initial 3TC monotherapy studies, which showed that HIV-RNA levels remained about 0.5 logs below their starting value in patients continuing 3TC for 6 to 12 months, despite the presence of 3TC-resistant viruses harboring M184V. Experimental studies have shown that RT enzymes with M184V display increased fidelity[36] and/or decreased processivity[37]. The clinical relevance of these biochemical findings is not well known, since the increased fidelity does not appear to limit the ability of HIV to develop new mutations under any drug pressure[38,39].

M184V causes high-level (>100-fold) 3TC resistance and emerges rapidly in patients receiving 3TC monotherapy[40]. It is also the first mutation to develop in isolates from patients receiving incomplete suppressive triple combinations including 3TC[41-44]. M184V is also selected during failures with ABC[29,30] and only rarely with ddI[20], and causes about 2-fold resistance to these drugs. M184V alone renders 3TC ineffective but does not significantly compromise the response to ABC[45] or ddI. However, M184V in combination with multiple AZT mutations or with changes at positions 65, 74 or 115 leads to significant ABC and ddI resistance[29,45-47].

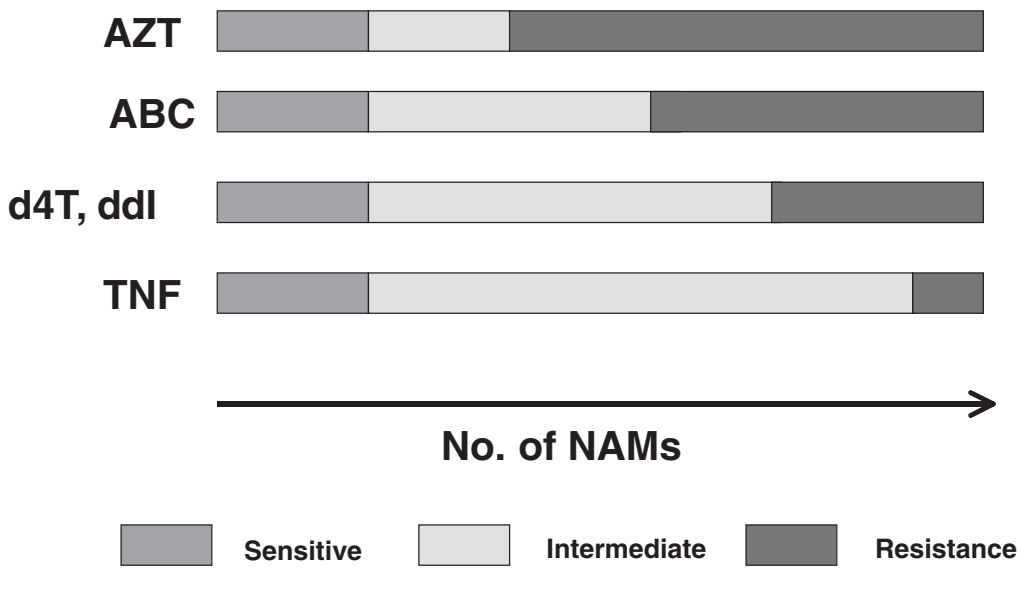

Figure 7. *Level of resistance to different nucleoside/nucleotide analogs as a function of the number of NAM.*

Mutation L74V. It is selected failing ddI[20,48] and abacavir[29] monotherapy. It confers 2- to 5-fold resistance to ddI[48] and ddC and 2- to 3-fold resistance to ABC[49]. L74V is sufficient to cause virological failure in patients receiving ddI monotherapy[48,50], but additional mutations are required to cause failure to ABC.

Nucleoside resistance mediated by p6 mutations

Genetic changes at the p1-p6^{gag}-p6^{pol} region, localized immediately upstream of the *pol* gene, seem to be involved in drug resistance[11,51]. The p1 region carries structures regulating gag-pol frameshift activities. The p6^{gag} region encodes a protein involved in the late viral cycle, including pol packaging, particle size determination, and budding[52-55]. The transframe protein encoded by the p6^{pol} region acts as a regulator of protease activation[56,57]. Thus, the p1-p6^{gag}-p6^{pol} regions have the potential to affect anti-HIV drug activities by several mechanisms, including greater pol production through frameshift regulation, enhanced packaging of viral enzymes, and control of viral protease. In other words, the introduction of these downstream changes may lead to resistance to antiretroviral agents through a mechanism of gene or protein dosing or titration (Fig. 4).

Insertions in p6^{gag} are seen in a significant proportion of viruses from antiretroviral-experienced patients. More precisely, duplications of the initial 11 amino acids of p6, including the motif PTAP, were identified as the first mutation selected under NRTI pressure, or emerging during the stepwise process of accumulation of resistance mutations, leading to high-level NRTI resistance[11]. Theoretically, the duplication of polyproline motifs could improve cellular protein recruitment at membrane locations, modulating viral assembly and enhancing pol incorporation into the budding virion (Fig. 4). In previous reports[11], these insertions were identified in plasma viruses collected from 21% of patients under nucleoside analogs, whereas they were seen in only 5% of drug-*naïve* individuals. However, recent studies have demonstrated similar rates of insertions in the PTAP motif comparing drug-*naïve* and pretreated individuals (Table 2)[58,59]. Therefore, p6 duplications may be just natural polymorphisms[58-60], although they may affect the susceptibility to antiretroviral drugs and may be clinically relevant. In fact, there is a trend towards earlier treatment failure for individuals harboring HIV with PTAP insertions, suggesting that these insertions may be clinically relevant[58].

Multi-nucleoside resistant genotypes

Q151M complex. Mutation Q151M is a 2-base-pair change in a conserved RT region that is close to the first nucleotide of the single-stranded nucleotide template[49,61,62]. This mutation develops in 3 to 5% of patients who fail dual NRTI therapy with ddI in combination with AZT or d4T[23,24,61-65]. Q151M alone causes intermediate levels of resistance to AZT, ddI, ddC, d4T and ABC[62,66,67]. The selection of Q151M is generally followed by mutations at positions 62, 75, 77 and 116. Isolates with V75I, F77L, F116Y and Q151M show high-level resistance to all NRTI, although they affect 3TC and tenofovir to a lesser extent[49,62]. The mechanism why Q151M reduces NRTI susceptibility seems to involve a discriminatory pathway against nucleoside analogs favoring physiologic nucleosides.

Codon 67-69 inserts. Positions 65 to 72 form a loop between the β2 and β3 strands in the fingers region of the RT and this loop makes important contacts with the incoming dNTP during polymerisation[68]. In addition to AZT-resistant mutations at codons 67 and 70, this region contains several other NRTI-resistant mutations, the most common of which occur at position 69 and include T69D/N/S/A, as well as single and double amino acid insertions[69-71]. T69D was initially identified as causing resistance to ddC, but substitutions at this position have since been reported with each of the available NRTI. In fact, mutations at this position contribute to resistance to each NRTI when they occur in the presence of other classical AZT-resistant mutations. By themselves, insertions at position 69 cause low-level resistance to each of the NRTI, but isolates containing insertions together with T215Y/F and other AZT-resistant mutations show high-level resistance to each of the NRTI[72-74] and tenofovir[75].

Interactions between nucleoside resistance mutations

NAMs with M184V or L74V. M184V reverses T215Y-mediated AZT resistance[76]. For example,

Table 2. *Prevalence (%) of p1-p6 changes in viruses from* naïve *and pre-treated patients*

p6^{gag} genotypes	Gallego, et al.[59]	Peters, et al.[11]	Dong, et al.[58]		
	Naïve **(n = 74)**	**Pretreated (n = 82)**	***Naïve*** **(n = 74)**	**Pretreated (n = 222)**	***Naïve*** **(n = 510)**
Insertions at the PTAPP motif	17.6	12.2	5.4	21.2	16
Insertions at the KQE motif	0	12.2	4.1	4.0	–
Other changes in p6^{gag} (P5 I/L/T[a], E20 A/G/K[b], I31 K/R/T/V[c])	10.8[a,b]	42.7[a,b]	5.4[c]	14.4[c]	–

HIV-1 isolates harboring M41L/T215Y display 64-fold resistance, while isolates containing this set of mutations together with M184V are just 4-fold resistant. This effect is clinically significant and explains the slow evolution of phenotypic AZT resistance in patients receiving AZT plus 3TC[77]. However, it can be overcome by the presence of 3 or more AZT-resistant mutations. The favorable effect of M184V on AZT in the setting of AZT resistance seems to be caused by the ability of M184V to impair the rescue of chain-terminated DNA synthesis[78] and does not apply to AZT resistance caused by other mechanisms, such as Q151M. Presumably M184V also reverses the effect of the classical AZT mutations on d4T and tenofovir, which explains the *in vivo* synergy observed when using these drugs in combination[79,80].

By evaluating paired genotypic and phenotypic susceptibilities to NRTI in a large number of samples, investigators from Virologic have concluded that the degree of cross-resistance to NRTI caused by NAMs is modulated by the M184V[12]. This mutation generally restores the sensitivity to AZT, d4T and tenofovir, whereas it impairs much more that of ABC, ddI, ddC and 3TC (Fig. 8).

As with M184V, the presence of L74V restores, at least in part, the sensitivity to AZT when a few AZT-resistant mutations are present[45]. A similar effect most likely occurs with d4T[49]. This circumstance explains why L74V is rarely seen in patients failing dual nucleoside therapy with ddI plus either AZT[61] or d4T[23].

Mutation G333E. A polymorphism recognized in around 10% of *naïve* subjects, G333E, has been reported to facilitate AZT resistance in isolates from patients failing AZT plus 3TC and already harboring multiple AZT-resistant mutations[81,82]. This substitution by itself does not affect the susceptibility to AZT or 3TC. However, it avoids the reversal in AZT susceptibility caused by M184V in the presence of AZT-resistant mutations.

E44D and V118I. Each of these mutations occurs in about 1% of untreated individuals. The prevalence of these genotypes is much higher in isolates obtained from patients failing dual NRTI combinations, particularly in viruses from subjects containing multiple AZT-resistant mutations. When present in combination, E44D and V118I cause intermediate 3TC resistance[83-85], and contribute to enhancing the loss of sensitivity to other NRTI, including ddI, d4T and ABC[62,86,87] (Table 1). Taking into account all these data, a significant loss of susceptibility to 3TC may result from a set of genotypes and not just M184V (Table 3).

In summary, the mechanisms of resistance to nucleoside analogues can be grouped into three pathways. The first causes an enhanced excision/removal of the chain terminator. The second reduces the affinity of the mutated enzyme for the inhibitors. The third mechanism allows evading drug pressure by packaging a high number of drug targets into the virions, and therefore "distracting" their action. Other properties distinguishing these mechanisms are recorded in table 4. Finally, table 5 records the list of mutations known so far to confer resistance to NRTI.

Resistance to non-nucleosides

The non-nucleoside reverse transcriptase inhibitors (NNRTI) bind to a hydrophobic pocket in the RT enzyme close to, but not continuous with, the catalytic site. These compounds inhibit HIV-1 replication allosterically by displacing the catalytic

Table 3. *Genotypes conferring clinically significant reduced susceptibility to lamivudine*

1. M184V
2. E44D plus/or V118I
3. 67/69 inserts

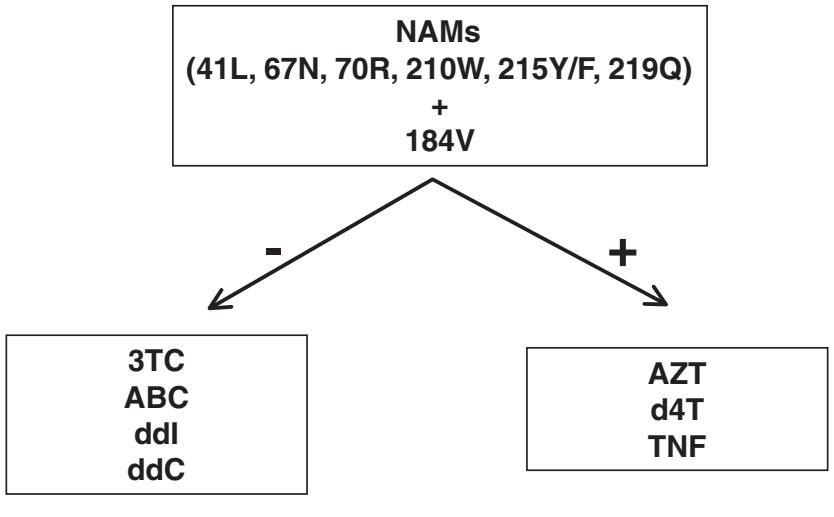

Figure 8. *Interaction between NAMs and M184V results in an opposite effect on the susceptibility to distinct nucleoside analogs.*

Table 4. *Mechanisms of resistance to nucleoside analogs*

	↓ binding	↑ removal (NAMs)	↑ number of RT molecules
Mechanism	Inhibitory competition	Pyrophosphorolysis	Inoculum effect
Specificity	Single drugs	Broad spectrum	Broad spectrum
Fitness	Reduced	Unchanged	Reduced
Codons	74, 184	41, 67, 70, 210, 215, 219	p6 region
Drugs	ddI, 3TC	AZT > d4T > ABC > ddI > 3TC	All NRTIs

aspartic residues relative to the polymerase-binding site[49,88]. The mutations responsible for NNRTI resistance are in the hydrophobic pocket to which they bind. A single mutation in this pocket may result in high-level resistance to one or more NNRTI. Resistance usually emerges rapidly when NNRTI are administered as monotherapy or in the presence of incomplete virus suppression, suggesting that resistance may be caused by the selection of a pre-existing population of mutant viruses[2,89].

HIV-2 and HIV-1 group O are intrinsically resistant to most NNRTI[90-94]. In addition, wild-type HIV-1 group M isolates tend to have greater inter-isolate variability in their susceptibility to NNRTI than to NRTI and PI[95]. In fact, moderate decreases in NNRTI susceptibility (<10-fold) in the absence of previous NNRTI exposure or known NNRTI-resistant mutations does not compromise the virological response to NNRTI-containing regimens[96,97].

Nucleotide substitutions affecting the susceptibility to NNRTI are found in three main regions: between codons 98 and 108, 179 to 190, and 225 to 236[49]. Table 5 summarizes the codon substitutions reported to be associated with NNRTI resistance so far, and the loss of susceptibility they confer to each of the commercially available compounds.

Mutations at codons 98-108

K103N is the most clinically important NNRTI-resistant mutation[98,99]. It causes 20 to 50-fold resistance to each of the available NNRTI, which is sufficient to cause virological failure with each of them[100,101]. A different mutation at this position, K103R, is seen in 3% of NNRTI-*naïve* subjects and does not confer NNRTI resistance[49].

V106A causes > 30-fold resistance to nevirapine (NVP), intermediate resistance to delavirdine (DLV), but low-level resistance to efavirenz (EFZ)[49,102]. L100I causes intermediate resistance to EFZ and DLV, but low-level resistance to NVP[49,102]. L100I usually occurs with K103N in patients receiving EFZ and significantly enhances the resistance to this drug. Other mutations causing low-level resistance to NNRTI are A98G, K101E, and V108I.

Mutations at codons 179-190

Y181C/I cause > 30-fold resistance to NVP and DLV, but only 3-fold resistance to EFZ[49,102]. Nevertheless, NVP-treated patients with isolates containing Y181C generally have only transient virological responses to EFZ-containing regimens[103]. Recently, a trial has investigated whether subjects experiencing an early virological failure on NVP could be rescued with EFZ[104]. Overall, only subjects lacking NNRTI-resistant mutations were able to regain sustained virological response. Therefore, genotyping at the time of early failure with NVP-containing regimens may prove to be useful is some circumstances[7].

Y188C/L/H and G190A/S cause high-level resistance to NVP and EFZ, but not to DLV[49,102]. Moreover, G190A/S increase the level of resistance to NVP and EFZ in the presence of Y181C/I and/or K103N.

Mutations at codons 225-236

P225H causes low-level resistance to EFZ and NVP. By itself, P225H seem to result in DLV hypersusceptibility. However, it usually occurs with K103N in patients receiving EFZ[105,106].

M230L is a recently identified, rare mutation that causes 20-fold resistance to EFZ, and 40-fold resistance to NVP and 60-fold resistance to DLV[107]. P236L is a rare mutation that causes high-level resistance to DLV and hyper-susceptibility to NVP[49,102].

Other NNRTI resistance mutations

A mutation Y to F at codon 318 is associated with resistance to NNRTI[108]. It mainly causes resistance to DLV and only slightly contributes to enhancing the resistance to EFZ and NVP, in the presence of the classical K103N and/or Y181C.

Mutational interactions between NNRTI- and NRTI-resistant mutations exist, and may be clinically relevant. For example, Y181C and L100I hypersensitize HIV-1 to AZT[109]. Likewise, some NRTI-resistant mutations appear to hyper-sensitize HIV-1 to certain NNRTI[49]. These interactions could explain the success of dual NRTI-NNRTI regimens in certain salvage situations[110]. Moreover, they suggest that the number of ways in which HIV-1 can develop simultaneous high-level resistance to both NRTI and NNRTI is limited.

Resistance to protease inhibitors

Following entry of HIV into the host cell, viral RNA is reverse transcribed to cDNA, which is further integrated into chromosomes as proviral DNA. The

Table 5. *List of mutations known to cause resistance to antiretroviral drugs*

Nucleoside analogs (NRTI)

Mutation		Loss of susceptibility (increase in IC_{50})						
		AZT	**DdI**	**ddC**	**d4T**	**3TC**	**ABC**	**TNF**
M41L	ATG→**T**TG	4	<2	–	<2	–	–	–
K65R	AAA→A**G**A	–	4-10	4-10	–	–	3	3-5
D67N	GAC→**A**AC	X	<2	–	<2	–	–	–
T69D	ACT→**GA**T	–	3	5	–	–	–	–
K70R	AAA→A**G**A	X	<2	–	–	–	–	–
K70E	AAA→**G**AA	–	–	–	–	–	–	–
L74V **	TTA→**G**TA	–	5-10	5-10	–	–	4	–
V75T	GTA→**AC**A	–	<2	5	7	–	–	–
Y115F	TAT→T**T**T	–	–	–	–	–	2	–
Q151M	CAG→**AT**G	10	5	5	–	2	–	–
P157S	CCG→**T**CG	↑3	–	–	–	5	–	–
I178M	ATA→AT**G**	–	–	–	4	–	–	–
M184V **	ATG→**G**TG	↑2	2-5	2-5	–	>100	4	–
M184I **	ATG→AT**A**	–	–	–	–	X	–	–
T215Y	ACC→**TA**C	–*	<2	–	<2	–	–	–
T215F	ACC→**TT**C	–*	–	–	–	–	2	–
K219Q	AAA→**C**AA	X	<2	–	<2	–	–	–
M41L + T215Y		60-70	–	–	–	–	–	–
M41L + D67N + K70R + T215Y		180	–	–	–	–	–	–
D67N + K70R + T215Y + K219Q		120	–	–	–	–	–	–
E44D/A ± V118I + M41L + T215Y		30-50	–	–	–	8-50	–	–
K65R + M184V		–	–	–	–	–	8	–
K65R + L74V±Y115F + M184V		–	–	–	–	–	10	–
L74V + M184V		–	–	–	–	–	9	–
L74V + Y115F + M184V		–	–	–	–	–	>12	–
A62V + V75I + F77L + F116Y + Q151M		190	50	20	>10	6	–	–
K67N + K70R + T215Y + K219Q		120	–	–	–	–	–	–
T69SSX + T215Y 140			11	17	3	20	–	–
M184V + R211K, con F214L		X	–	–	–	X	–	–
M184V + M41L + T215Y		4	–	–	–	>100	5	–
M184V + M41L + T215Y + T69D		60-70	–	–	–	>100	10	–
G333E + other mutations		30-600	–	–	–	>100	–	–

Non-nucleosides **(NNRTI)**

Mutation		NVP	EFV	DLV
A98G	GCA→G**G**A	2-10	–	3
L100I **	TTA→**A**TA	8.5-14	33->100	50->90
K101E	AAA→**G**AA	7-15	<8-16	5
K103N	AAA→AA**C**	>100	33-67	20-78
K103T	AAA→A**C**A	–	–	35
V106A	GTA→G**C**A	>100	–	–
V106I	GTA→**A**TA	>100	–	–
V108I	GTA→**A**TA	3-30	2	–
V179D	GTT→G**A**T	–	2	1-21
Y181C **	TAT→T**G**T	>100	2-4	>100
Y181I	TAT→**AT**T	100	–	–
Y188C	TAT→T**G**T	>100	–	–
Y188H	TAT→**C**AT	>100	–	–
Y188L	TAT→T**TA**	–	1,000	9
G190A	GGA→G**C**A	>63-75	–	–
G190S	GGA→**A**G**C**	–	>100	–
P225H	CCT→C**A**T	2.8	–	–
M230L	ATG→**C**TG	39	23	58
P236L	CCT→C**T**T	0.2-2	–	>100
Y318F	TAT→T**T**T	–	–	17.3
Y318W	TAT→T**GG**	53.7	–	–
L100I + K103N		X	4,000	X
L100I + V108I		X	1,000	X
L100I + V179D + Y181C		>100	1,000	X
K103N + V108I		>100	100	45
K103N + M230L		>780	270	>250
I135T/V ± L283V (Polimorphisms)		2,5-7	–	–
Y181C + M230L		>780	25	>250

Table 5. *List of mutations known to cause resistance to antiretroviral drugs (cont.)*

Protease inhibitors (PI)

Mutation		Loss of susceptibility (increase in IC_{50})						
		SQV	**RTV**	**IDV**	**NFV**	**LPV**	**TPV**	**APV**
R8Q	CGA→C**A**A	4	–	–	–	–	–	–
D30N	GAT→**A**AT	–	–	–	9*	–	–	–
M46I	ATG→AT**A**	–	–	–*	–	–	–	–
M46L	ATG→**T**TG	–	–	–*	–	–	–	–
G48V	GGG→G**T**G	3-8*	–	–	3	–	–	–
I50V	ATT→**G**TT	–	–	–	–	–	–	3
V82F	GTC→**T**TC	–	5*	3*	2	–	–	2
V82A	GTC→G**C**C	–	2*	–*	2	–	–	–
V82S	GTC→**TC**C	–	6*	–	–	–	–	–
V82T	GTC→**AC**C	–	3*	–*	3	–	–	–
V82I	GTC→**A**TC	–	–	–*	–	–	–	2.4
I84V	ATA→**G**TA	–	10	5	5	–	–	–
N88D	AAT→**G**AT	–	–	–	–*	–	–	–
L90M	TTG→**A**TG	3*	–	–	5	–	–	–
L10I + M46I + I54V + L63P + A71V + V82A + I84V		3	294	34	–	X	X	X
L10R + M46I + L63P + V82T + I84V		8	80	47	>100	X	6	X
K20R + M36I + I54V + V82A		–	41	–	–	X	X	X
K20R + M36I + I54V + A71V + V82T		–	28	–	–	X	X	X
L10I + K20R + M36I + I54V + I62V + L63A + A71V + V82A + L90M		X	67	20	X	X	3	X
D30N + A71V		–	–	–	7	–	–	–
V32I + E34K + M36I + A71V + I82V		9	260	76	–	X	X	X
V32I + M46L + A71V + V82A		–	–	14	–	X	X	X
L33F + I54V + L63P + V82F		–	56	19	–	X	X	X
E35D + M36I + I54V + A71V + V82T		–	17	8	3	X	X	X
M46L + I54V + V82A		–	–	10	–	X	X	X
M46I + L63P + A71V + I84V		–	–	–	30	X	X	X
M46I + L63P + A71V + V82F + I84V		–	27	–	–	X	X	X
L10F + V32I + M46I + I47V + I84V + T91S		X	X	X	X	25-100	X	X
L10F + G16E + M46I + I47A + H69Y + I89V + T91S		X	X	X	X	>100	X	X
L10F + M46I + I84V + T91S		X	X	X	X	9-14	X	X
M46I + I50V		–	X	X	–	–	–	7
M46I + I84V		–	9	–	5	6-8	X	X
M46I + I47V + I50V		–	X	X	–	–	–	14
G48V + L90M		>100	–	–	–	X	X	X
G48V + I54V + L90M		>50	–	–	–	X	X	X
G48V + I84V + L90M		>30	–	30	–	X	X	X
G48V + I54V + A71T + V82A		18	–	13	–	X	X	X
G48V + A71T + V82A		9	–	12	–	X	X	X
I54V + M46I		–	9	–	–	X	X	X
I54V + V82T		–	9	5	3	X	X	X
V82T + I84V / I84V + L90M		X	X	X	X	X	X	X
V82F + I84V		–	10	–	–	X	X	12.3

– Mutation not involved with drug resistance so far
X Unknown degree of resistance
–* Primary mutations usually appearing in combination
** Changes that could reverse AZT resistance
↑ Increase in susceptibility

viral capsid proteins and replicative enzymes are initially expressed as two large, nonfunctional polyproteins called p55 and p160. These polyproteins are assembled and packaged at the cell surface from where immature virions are released into the plasma. In order to assemble complete mature virus particles, these polyproteins must undergo post-translational processing to be cleaved into their functional constituents by the protease (Fig. 9).

HIV-1 protease is an aspartic protease composed of two non-covalently associated, structurally identical monomers, 99 amino acids in length (Fig. 10). The protease has a substrate-binding **cleft** that recognizes and cleaves 9 different sequences on viral precursor polyproteins. The top of the cleft is covered with a mobile **flap** that forms a turn over the cleft, but can move away to let substrates enter and products leave[49]. Drug resistance

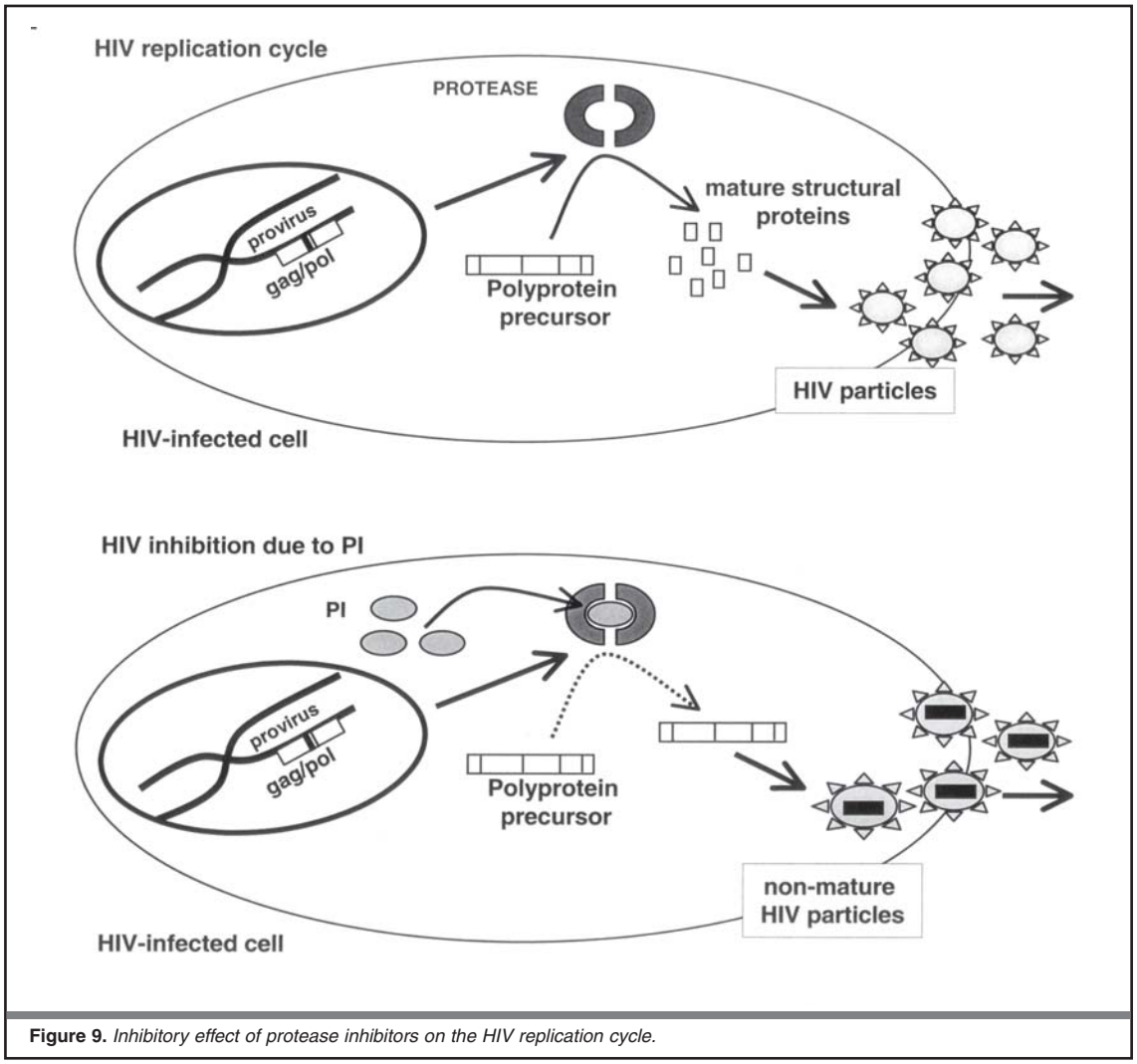

Figure 9. *Inhibitory effect of protease inhibitors on the HIV replication cycle.*

is mediated by structural changes in the substrate cleft that result in a reduction in drug-binding affinity to the mutant target molecule[111-113]. The effects of non-active site mutations are less obvious and appear to involve other mechanisms such as alterations in enzyme catalysis, effects on dimer stability, alterations in inhibitor binding kinetics, or active site re-shaping through long-range structural perturbations[114,115].

Sequence analysis of drug resistance clones has shown mutations not only within the protease but also at several of the protease cleavage sites[116-118]. In some circumstances, mutations at these positions improve the kinetics of protease enzymes containing drug resistance mutations, suggesting that they are compensatory rather than primary. There are no reports so far showing that changes at cleavage sites alone can cause PI resistance. Therefore, they do not appear in the absence of protease mutations, and genetic sequencing of these sites does not seem to be necessary for detecting PI resistance in the clinical setting. Figure 11 summarizes the mechanisms of resistance to PI described so far.

Mutations at more than 20 positions have been associated with PI resistance, including mutations in the substrate cleft, the flap, other conserved sites of the enzyme, and polymorphic sites (Table 5). The spectrum of mutations selected during therapy with indinavir (IDV), nelfinavir (NFV), saquinavir (SQV), and ritonavir (RTV) has been well characterized[49,86,102,114-116,120]. Less data are available for amprenavir (APV)[121,122] and lopinavir (LPV)[123-125].

Mutations at the protease catalytic site

V82A/T/F/S occur predominantly in HIV-1 isolates from patients receiving treatment with IDV and RTV[44,119,126,127]. V82A also occurs in isolates from patients failing SQV for a long time, following the development of the G48V mutation[128]. By themselves, mutations at codon 82 cause resistance to IDV, RTV but not to NFV, SQV or APV. However, when present with other PI mutations, V82A/T/F/S contribute to resistance to each of the available PI[119,126,129]. V82I can be recognized in about 1% of untreated subjects with subtype B and in 10% of *naïve* subjects with non-B isolates[130,131]. This mutation does not confer resistance to IDV[132].

I84V is selected in patients failing almost all PI except NFV[133]. G48V occurs primarily in patients

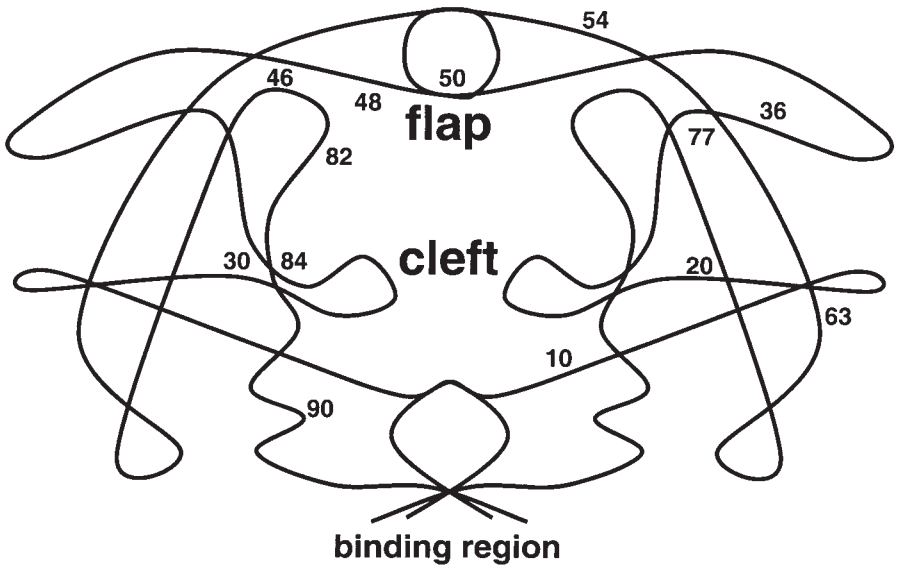

Figure 10. *HIV protease. Residues more frequently involved in producing resistance to protease inhibitors. Primary (left side) and secondary (right side) mutations.*

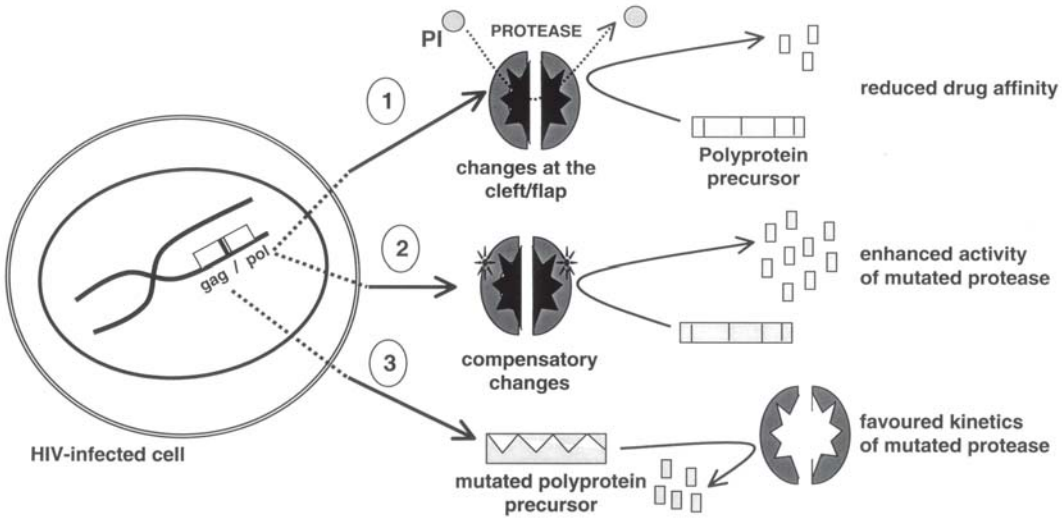

Figure 11. *Resistance may emerge as result of three different mechanisms: (1) mutations at the protease active site; (2) mutations at non-active sites; and (3) mutations at gag cleavage sites.*

receiving SQV[128,134] and rarely in patients receiving IDV[44]. This mutation causes 10-fold resistance to SQV and about 3-fold resistance to IDV and RTV[126,128,133,134].

D30N occurs solely in patients exposed and failing NFV[135,136]. Although preliminary reports[6] highlighted that resistance to NFV may develop using two pathways (Fig. 12), in two thirds of instances involving D30N and the rest through selection of L90M, more recent data have noted that early failures on NFV almost always occur with D30N[136]. However, in subjects carrying HIV-1 subtype G the selection of L90M seems to be the rule[137]. This observation is of interest in two ways: first, D30N compromises the replicative capacity of HIV to a greater extent than other PI-resistant mutations[4,138]; second, D30N does not confer cross-resistance to other PI; therefore, failures on NFV may be rescued more successfully with other PI[7,139]. These features have favored the preference of NFV as the initial PI, at least in subjects with subtype B.

I50V has been reported only in patients receiving APV as their first PI[121,122]. In addition to reducing APV susceptibility, it causes low-level RTV resistance of uncertain clinical significance. V32I is selected in

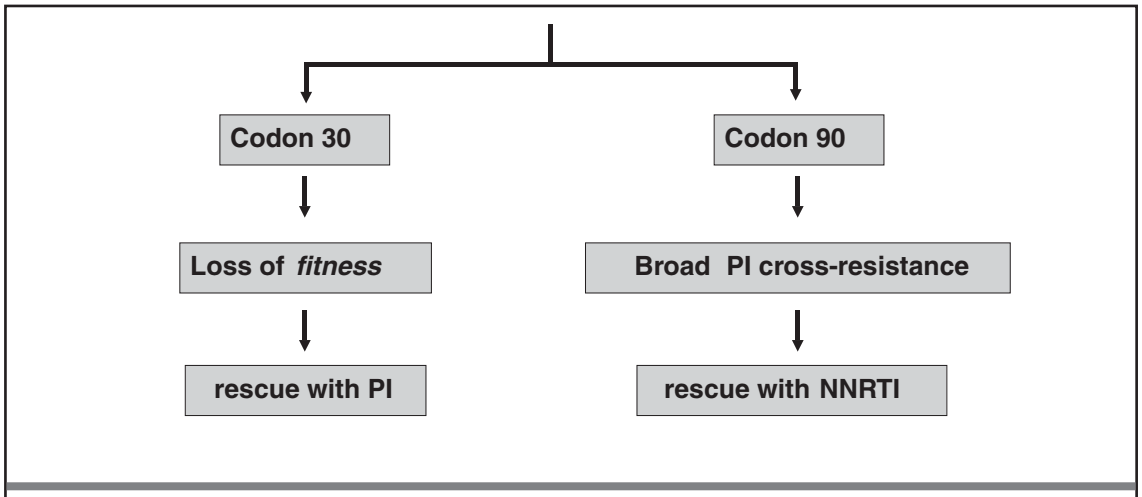

Figure 12. *Two different pathways lead to nelfinavir resistance.*

patients failing IDV, RTV and APV. It often appears in association with other PI-resistant mutations in the substrate cleft or flap.

The protease flap region (positions 46-56) extends over the substrate binding cleft and must be flexible to allow the entry and exit of the polypeptide substrates and products[49,62]. In addition to G48V and I50V, which are also in the substrate cleft, mutations at positions 46, 47, 53 and 54 make important contributions to drug resistance. Mutations at position 54 (generally I54V, less commonly I54T/L/M) contribute resistance to all currently available PI and have frequently been reported in subjects failing IDV, RTV, SQV and APV[140,141]. Mutations at codon 47 have been reported in patients failing APV, IDV or RTV, and often occur in conjunction with the nearby substrate cleft V32I mutation. F53L has rarely been reported in patients failing any PI monotherapy, but is selected in more than 10% of patients under multiple PI combinations[142].

Mutations at position 46 contribute resistance to any PI except SQV, and have frequently been reported in failures under IDV, RTV, APV and NFV[126,133,139].

L90M has been recognized in isolates from patients treated with SQV, NFV, IDV and RTV. L90M either contributes to, or directly confers resistance to, each of the currently available PI and plays a role in causing clinical cross-resistance to each of the PI[140]. In a recent study[129], it was concluded that G48V, V82A/F/T, I84V, and L90M are the major determinant genotypes producing multiple PI resistance.

All previously described mutations associated with PI resistance were single codon substitutions that resulted from 1- or 2-base point mutations in the protease gene. Recently, inserts of 1 to 5 amino acids between codons 35 and 38 have been described in PI-experienced subjects[143]. They are located at the flap region, and cause conformational changes over the catalytic site, influencing PI access to their binding site. However, the inserts described so far do not seem to increase the level of resistance due to other protease mutations but provide an advantage in replication capacity[143].

Non-active site protease mutations

Distinct amino acids at seven polymorphic positions, including codons 10, 20, 36, 63, 71, 77 and 93, also make major contributions to drug resistance. While these mutations do not cause PI resistance by themselves, some of them contribute to resistance when present together with other protease mutations, whereas others compensate for the decrease in catalytic efficiency caused by protease mutations affecting the catalytic site[138,144-147].

Mutations at codon 10, 20, 36 and 71 occur in up to 5-10% of untreated persons. However, in heavily-treated patients harboring isolates with multiple mutations in the substrate cleft, flap, or at codon 90, the prevalence of mutations at these positions increases dramatically. Mutations at codon 10 and 71 increase to 60-80%, whereas mutations at codons 20 and 36 increase to 30-40%[49,129,140].

Codon 63 is the most polymorphic protease position. In untreated persons, about 45% of isolates have 63L, which is considered the subtype B consensus. However, nearly 45% have 63P, and about 10% have other residues at this position. When only subjects heavily treated with PI are examined, the prevalence of amino acids other than L at position 63 increases to 90%[149]. Mutations at codons 77 and 93 double in prevalence from 15-20% in untreated persons to 30-40% in heavily-treated patients[149].

A protease mutation N88S is selected in most patients failing atazanavir[150] and occasionally in subjects showing virological failure on NFV and/or IDV, in which another change at codon 88 (N→D) is more frequently seen[44,151]. Interestingly, it seems to result in an enhanced sensitivity (hyper-susceptibility) to APV[152], which might be of clinical relevance[153]. Moreover, viruses harboring the N88S substitution have low fitness compared to wild-type[152]. More recently, hyper-susceptibility to ritonavir has been claimed for viruses harboring the nelfinavir-associated D30N mutation[154], although the clinical relevance of this finding is unknown.

Resistance using boosted PI combinations

Lopinavir (LPV) is the latest PI introduced in the market. Its resistance profile is not well characterized so far. Preliminary evidence suggested that 6 to 8 of a set of 11 mutations conferred a greater than 10-fold increase in LPV IC_{50}, which was arbitrarily defined as a clinically relevant cut-off[155]. At the 2001 Drug Resistance Workshop, data on over 1,300 clinical samples collected from PI-treated patients and on nearly 1,000 samples from drug-*naïve* individuals were presented[156]. In untreated patients, 98% of samples showed a reduced susceptibility to LPV below 2.5-fold compared to wild-type, which they subsequently defined as the biological cut-off for this drug. In treated patients, there was considerable cross-resistance between LPV (n = 400 samples) and other PI, especially RTV and IDV. More than 30 mutations were identified as being associated with decreased susceptibility to LPV, but mainly 11 of them (i.e. 10I/F/R/V, 20M/R, 24I, 46I/L, 53L, 54VIL, 63P, 71V/T, 82A/F/T/S, 84V, 90M) were relevant for the definition of the biological cut-off. In agreement, the presence of mutations at positions 82, 54 and 10, plus a median of 4 additional PI mutations, predicts treatment failure in NNRTI-*naïve* patients receiving NRTI together with EFZ and Kaletra (LPV/RTV)[155]. However, similar mutational patterns may be detected in patients having good treatment response. These viruses often had significantly lower phenotypic resistance levels at baseline, arguing in favor of the use of phenotypic tests in addition to DNA sequencing.

The usefulness of a simple mutation score for LPV has been questioned by some authors[158], who found up to 31-fold LPV resistance among *in vitro* APV-selected mutants with less than 6 PI-mutations.

Table 6. *Predictive value of genotyping on virologic response (HIV-RNA < 500 copies/ml at 24 weeks) to ritonavir-boosted PI regimens in salvage therapy[161]*

	SQVsg	IDV	LPV	APV
No.	60	47	76	16
≤5 PI mutations	95%	90%	88%	100%
>5 PI mutations	21%	23%	47%	25%

SQVsg: saquinavir soft gel. IDV: indinavir. LPV: lopinavir. APV: amprenavir

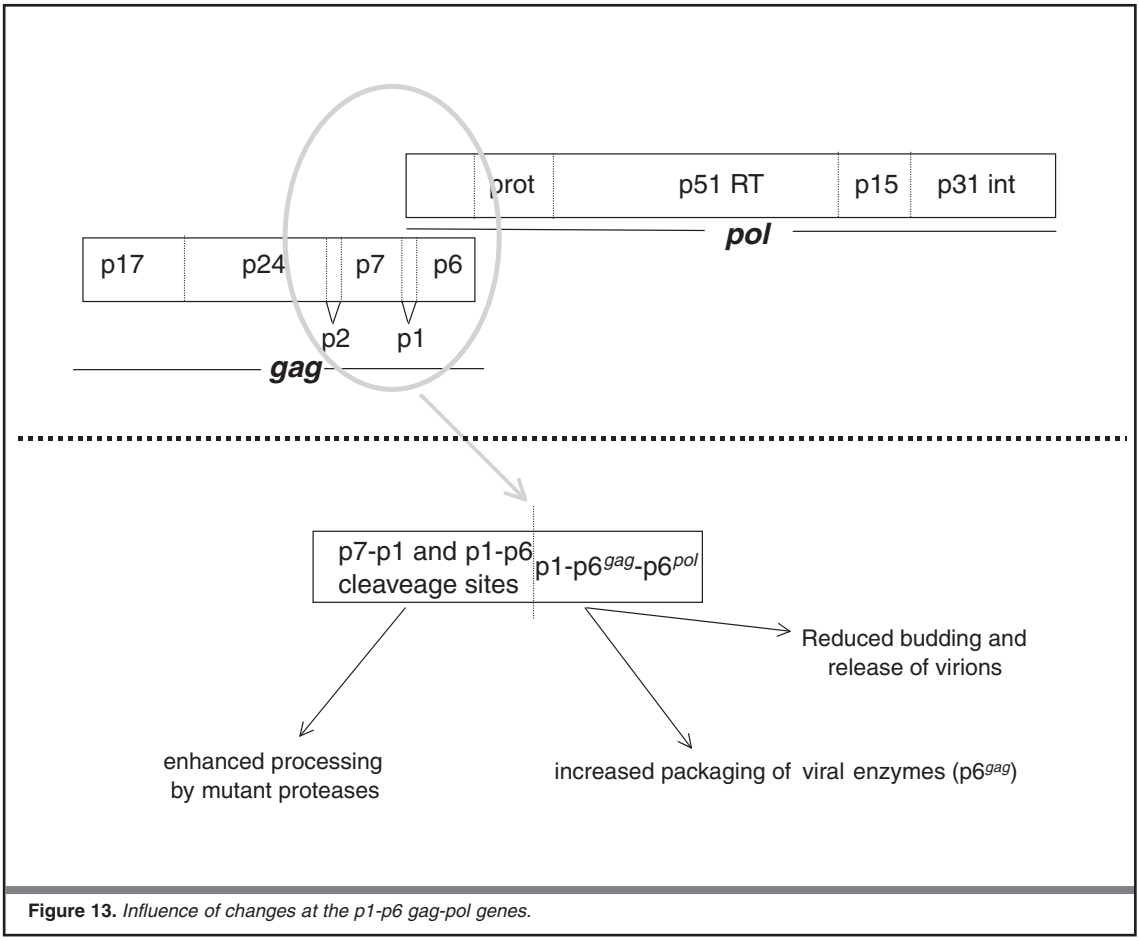

Figure 13. *Influence of changes at the p1-p6 gag-pol genes.*

These mutants had a significant reduction (>90%) of replicative capacity, too. Moreover, other groups of investigators have shown that specific key mutations are associated either with resistance to LPV (i.e. V82A/T, I54V) or APV (I84V)[159]. However, multiple data are emerging supporting the notion that resistance using ritonavir-boosting PI combinations is mainly dependent of the number of PI-resistant mutations, with the impact of specific key mutations being less important in the face of high PI levels. This is explained, at least in part, by the fact that PI-resistant mutations only cause slight reductions in PI susceptibility, which is often overcome when using ritonavir-boosted PI combinations. This has been shown clearly with saquinavir[160], and in a preliminary trial[161] a threshold of 4-5 PI resistance mutations has been shown to predict significantly the response to salvage therapy using almost any PI boosted by ritonavir (Table 6).

Mutations at Gag cleavage sites and the p6* transframe

In addition to mutations at the protease gene, PI resistance may develop as a consequence of amino acid substitutions in protease cleavage sites (Fig. 13). Some of them involve positions related to protease scission sites whereas others (i.e., L75R, H219Q) render the polyprotein cleavage sites more accessible to mutant proteases or improve gag functions, such as polymerization of viral proteins and/or assembly[116-118].

HIV-1 p6, a protein involved in virus budding, has recently been investigated by several groups with regard to its potential role in HIV drug resistance and viral fitness. Due to a shift in the reading frame, two variants of p6 (i.e. p6 gag and p6 pol, also called p6*) are synthesized by HIV[162]. A hypothetical role for p6 pol as a competitive inhibitor of the protease activity has been postulated[163], raising the question of a possible interaction of this protein with PI resistance mechanisms.

In a recent report[51] in which the impact of HIV-1 protease, RT, cleavage sites and p6 mutations in the response to salvage therapy was examined, genotype alterations outside the protease gene were found to be responsible for treatment failure. Although a high prevalence of mutations at cleavage sites p6/p1 and p1/p7 was found, no significant relationship between these changes and the virological outcome was evident. However, the number of mutations at p1/p7 cleavage sites was associated with a greater number of protease mutations, which supports the concept that these alterations may act as compensatory mutations, increasing viral fitness[116,117].

Another interesting finding in this study was a trend towards a larger prevalence of mutations of the last two residues in the C-terminal p6* domain in subjects with virological failure. It has been suggested that C-terminal residues of the p6* protein regulate HIV-1 protease function[163,164]. It can be speculated that, *in vivo*, mutations in this region might increase protease activity, compensating for reduced viral fitness in individuals with primary protease mutations.

Taking together all these considerations, it may be concluded that p1/p7 cleavage sites and C-terminal p6* mutations are associated with protease mutations. Changes in these regions most likely act as compensatory mutations improving the activity of mutated HIV proteases.

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