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HIV-1 Drug Resistance Mutations: an Updated Framework for the Second Decade of HAART

Robert W. Shafer[1] *and Jonathan M. Schapiro*[2]
[1]*Division of Infectious Diseases, Department of Medicine, Stanford University, USA;* [2]*National Hemophilia Center, Tel Hashomer, Israel*

Abstract

More than 200 mutations are associated with antiretroviral resistance to drugs belonging to six licensed antiretroviral classes. More than 50 reverse transcriptase mutations are associated with nucleoside reverse transcriptase inhibitor resistance including M184V, thymidine analog mutations, mutations associated with non-thymidine analog containing regimens, multi-nucleoside resistance mutations, and several recently identified accessory mutations. More than 40 reverse transcriptase mutations are associated with nonnucleoside reverse transcriptase inhibitor resistance including major primary and secondary mutations, non-polymorphic minor mutations, and polymorphic accessory mutations. More than 60 mutations are associated with protease inhibitor resistance including major protease, accessory protease, and protease cleavage site mutations. More than 30 integrase mutations are associated with the licensed integrase inhibitor raltegravir and the investigational inhibitor elvitegravir. More than 15 gp41 mutations are associated with the fusion inhibitor enfuvirtide. CCR5 inhibitor resistance results from mutations that promote gp120 binding to an inhibitor-bound CCR5 receptor or CXCR4 tropism; however, the genotypic correlates of these processes are not yet well characterized.
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Corresponding author: Robert W. Shafer, rshafer@stanford.edu

Key words

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Introduction

Nearly 25 antiretroviral drugs (ARV) have been licensed for the treatment of HIV-1: nine nucleoside reverse transcriptase inhibitors (NRTI), four nonnucleoside reverse transcriptase inhibitors (NNRTI), nine protease inhibitors (PI), one fusion inhibitor, one CCR5 inhibitor, and one integrase inhibitor. The first CCR5 and integrase inhibitors were approved in 2007, increasing the number of ARV classes from four to six. Commensurate with the increase in new ARV and ARV classes, there has been an increase in knowledge about drug resistance mutations. Entirely new vistas of mutations associated with integrase and CCR5 inhibitor resistance have also been opened, many new treatment-associated NRTI, NNRTI, and PI mutations have recently been described, and there has been a growing appreciation of the effects that different amino acid substitutions at the same position have on drug susceptibility.

Together with the expansion in the number of ARV classes and number of individual ARV, a consensus has emerged that ARV therapy can and should be used to completely suppress HIV-1 replication, even in patients in whom many previous ARV regimens have failed[1,2]. This unambiguous therapeutic endpoint (complete virologic suppression) necessitates a new framework in which the vast knowledge of drug resistance mutations should be cast. The identification of specific drug resistance mutations can increasingly be used to

Correspondence to:
Robert W. Shafer
Division of Infectious Diseases
Department of Medicine
Stanford University
Stanford, CA, USA
E-mail: rshafer@stanford.edu

avoid ARV that retain only minimal residual activity in favor of newer ARV that are likely to be either fully or nearly fully active. Therefore, as the breadth of knowledge about HIV-1 drug resistance continues to expand, many of the subtle distinctions among drug resistance mutations are becoming less clinically relevant.

Nucleoside reverse transcriptase inhibitors

The NRTI resistance mutations include M184V, thymidine analog mutations (TAM), mutations selected by regimens lacking thymidine analogs, multi-nucleoside resistance mutations, and many recently described non-polymorphic accessory mutations. There are two biochemical mechanisms of NRTI resistance: enhanced discrimination against and decreased incorporation of NRTI in favor of authentic nucleosides, and enhanced removal of incorporated NRTI by promoting a phosphorolytic reaction that leads to primer unblocking. Altogether, M184V, non thymidine analog-associated mutations such as K65R and L74V, and the multi-nucleoside resistance mutation Q151M act by decreasing NRTI incorporation[3,4]. Thymidine analog mutations, the T69 insertions associated with multi-nucleoside resistance, and many of the accessory mutations facilitate primer unblocking[5,7].

M184V

M184V is the most commonly occurring NRTI resistance mutation. *In vitro*, it causes high-level resistance to lamivudine (3TC) and emtricitabine (FTC), low-level resistance to didanosine (ddI) and abacavir, (ABC) and increased susceptibility to zidovudine (ZDV), stavudine (d4T), and tenofovir (TDF)[8]. The possibility that isolates with M184V are compromised was suggested by the initial 3TC monotherapy studies showing that plasma HIV-1 RNA levels remained about 0.5 log_{10} copies below baseline in patients receiving lamivudine for 6-12 months, despite the development of M184V and a high level of phenotypic resistance to 3TC[9-11]. Data from multiple 3TC-containing dual-NRTI regimens also suggest that 3TC continues to exert an antiviral effect even in patients whose virus isolates contain M184V[12-14].

M184V causes a median 1.5-fold and 3.0-fold reduction in susceptibility to ddI and ABC, respectively, in the PhenoSenseGT™ assay (Monogram Biosciences)[15,16]. These are levels of reduction that are above the wild-type range but below the level at which these NRTI are inactive[15]. Several clinical trials have also shown that ABC and ddI retain clinical activity in the presence of M184V[17-22]. For example, the addition of ddI or ABC to the regimen of a patient with virologic failure has been associated to plasma HIV-1 RNA reductions of 0.6 and 0.7 log_{10}, respectively, in patients harboring viruses with M184V and no other drug resistance mutations[19,21]. The phenotypic and clinical significance of M184V is influenced by the presence or absence of other NRTI resistance mutations. For example, the presence of K65R or L74V in combination with M184V is sufficient for high-level resistance to both ABC and ddI[16]. In contrast, three or more TAM plus M184V are required for high-level ABC and ddI resistance[8,16,19,20,23].

Thymidine analog mutations

Thymidine analog mutations are selected by the thymidine analogs ZDV and d4T. Thymidine analog mutations decrease susceptibility to these NRTI and to a lesser extent to ABC, ddI, and TDF[8]. Thymidine analog mutations are common in low-income countries in which fixed-dose combinations containing thymidine analogs are the mainstays of therapy. Thymidine analog mutations are also common in viruses from persons who began therapy in the pre-HAART era with incompletely suppressive thymidine analog-containing regimens, but are becoming less common in areas in which the fixed-dose combinations of TDF/FTC and ABC/3TC have become the most common NRTI backbones. However, even in these areas, TAM and in particular the partial T215 revertants remain the most common type of transmitted NRTI resistance mutation[24,25] (Table 1).

Thymidine analog mutations accumulate in two distinct but overlapping patterns[26-31]. The type I pattern includes the mutations M41L, L210W, and T215Y. The type II pattern includes D67N, K70R, T215F, and K219Q/E. Mutation D67N also occurs commonly with type I TAM[30,32]. However, K70R and L210W rarely occur together[33]. Type I TAM cause higher levels of phenotypic and clinical resistance to the thymidine analogs and cross-resistance to ABC, ddI, and TDF than do the type II TAM. Indeed, the presence of all three type I TAM markedly reduces the clinical response to ABC, ddI, and TDF[19,29,30,34,35]. The clinical significance of the type II TAM is not as well characterized.

Other mutations at several of the TAM positions are common. The most common of these are the partial T215 revertants T215C/D/E/I/S/V[36,37]. These mutations arise from the drug resistance mutations T215Y/F to

Table 1. Nucleoside reverse transcriptase inhibitor resistance mutations*

NRTI	184	Thymidine analog mutations (TAM)						Non thymidine analog regimen mutations					Multi-NRTI resistance mutations					
		41	67	70†	210	215	219	65	70†	74	75‡	115	69	151	62	75‡	77	116
	M	M	D	K	L	T	K	K	K	L	V	Y	T	Q	A	V	F	F
3TC	**VI**							RN	EG				Ins	M	V			
FTC	**VI**							RN	EG				Ins	M	V			
ABC	VI	L	N		W	FY		**RN**	EG	**VI**	TM	**F**	**Ins**	**M**	V	I	L	Y
DDI	VI	L	N		W	FY		**RN**	EG	**VI**	TM		**Ins**	**M**	V	I	L	Y
TDF		L	N		W	FY		**RN**	EG		M	F	**Ins**	M	V			
D4T		**L**	N	R	W	**FY**	QE	RN			**TM**		**Ins**	**M**	V	I	L	Y
ZDV		**L**	N	R	W	**FY**	QE						**Ins**	**M**	V	I	L	Y

*The first row of letters contains the consensus amino acid at the position indicated by the number in the preceding row. All amino acids are indicated by their one letter code with the exception of "Ins" which is an abbreviation for one or more amino acid insertions. Mutations in **bold** are associated with higher levels of phenotypic resistance or clinical evidence for reduced virologic response. Additional treatment-selected mutations at the positions in this table include D67G/E, T69DS/A/I/N/G, K70N, V75A/S, and K219NR. Additional accessory mutations include K43E/Q/N, E44D/A, V118I, H208Y, D218E, H221Y, and L228H/R. These accessory mutations generally occur with TAM and appear to be associated with a reduced level of susceptibility to multiple NRTI. Several mutations are associated with increased susceptibility: M184V/I increases susceptibility to ZDV, TDF, and d4T; L74V increases susceptibility to ZDV and TDF; K65R increases susceptibility to ZDV.
†K70R occurs in viruses from patients receiving thymidine analogs; K70E/G occur with non thymidine analog-containing regimens.
‡V75I occurs in combination with Q151M; V75TM occur in a variety of different treatment and mutational contexts.
NRTI: nucleoside reverse transcriptase inhibitor; 3TC: lamivudine; FTC: emtricitabine; ABC: abacavir; DDI: didanosine; TDF: tenofovir; D4T: stavudine; ZDV: zidovudine.

increase HIV-1 fitness in the absence of selective drug pressure. They occur more commonly than reversion to the wild-type T because most of the partial T215 revertants require only a single nucleotide mutation rather than the double nucleotide mutation required for Y or F to revert to T. The partial T215 revertants do not reduce drug susceptibility by themselves, but their presence in a previously untreated patient suggests that the patient may have been infected originally with a virus containing T215Y or F. Both K219N/R are two variants that unlike K219Q/E usually occur with type I rather than type II TAM[32]. Interestingly, two variants at position 70, K70E/G, are not selected by thymidine analogs and have phenotypic effects diametrically opposite to those of K70R, decreasing ABC, ddI, TDF, 3TC, and FTC susceptibility, and increasing ZDV susceptibility[38-40]. Both D67G and D67E are selected by NRTI therapy, but their phenotypic and clinical significance are not well characterized[41].

E44D/A and V118I are accessory mutations that generally occur with type I TAM. These mutations occur in about 1% of viruses from untreated patients and in a significantly higher proportion of viruses from patients receiving NRTI[27,42,43]. Although E44D plus V118I were first shown to cause low-level 3TC resistance when they occur in combination[44], subsequent studies have suggested that in combination with TAM, these mutations reduce the susceptibility and clinical activity of most NRTI[20,43,45-52]. F214L is a common polymorphism that is negatively associated with type I TAM, and as a consequence may raise the genetic barrier to resistance in viruses developing type I TAM[53,54].

Mutations occurring in the absence of thymidine analogs

The most common mutations in patients developing virologic failure while receiving a non thymidine analog-containing NRTI backbone include M184V alone or M184V in combination with K65R or L74V[55-57]. K65R causes intermediate resistance to TDF, ABC, ddI, 3TC, and FTC, low-level resistance to d4T, and increased susceptibility to ZDV[58-60]. L74V causes intermediate resistance to ddI and ABC, and a slight increase in susceptibility to ZDV and TDF[61]. L74I has similar phenotypic properties to L74V, but is found primarily in viruses with multiple TAM, possibly because it increases ZDV and TDF susceptibility less than L74V[62,63].

Mutations M184V plus K65R have been reported primarily in patients receiving the NRTI backbone TDF/3TC[64-65] and less commonly ABC/3TC[55,66] or TDF/FTC[56,67]. M184V plus L74V occurs primarily in persons

receiving ABC/3TC or ddI/3TC/FTC backbones[55,66,68,69]. K65R and L74V rarely occur in the same viruses; however, several patients developing virologic failure with L74V while receiving an ABC- or ddI-containing regimen have been found to have minor variants containing K65R[69,70].

There is a bidirectional antagonism between K65R and the TAM. K65R interferes with TAM-mediated primer unblocking and the TAM interfere with K65R-mediated NRTI discrimination[71,72]. As a result, viruses containing K65R in combination with TAM are uncommon[73]. The emergence of K65R is suppressed to a greater extent in regimens containing ZDV compared with d4T[59,74-81].

Less common mutations occurring during virologic failure with non thymidine analog regimens include K65N, K70E/G, and Y115F[38,40,55,82,83]. K65N and K70E/G have a resistance profile similar to K65R, but appear to cause less resistance than K65R to ABC, ddI, TDF, 3TC, and FTC[38-40,82,84,85]. Y115F reduces ABC susceptibility[86] and causes low-level cross-resistance to TDF[23,58,61,87]. Although T69D and V75T were originally identified as causing resistance to ddC[88] and d4T[89], respectively, a range of mutations at these positions (e.g. T69N/S/I/G and V75M/A) have been associated with reduced susceptibility to other NRTI, including ddI and d4T[23,89-92].

Two lines of evidence suggest that K65R may occur more commonly in non subtype B compared with subtype B viruses. K65R has emerged more rapidly during the *in vitro* passage of subtype C compared with subtype B isolates in the presence of increasing TDF concentrations[93]. Anecdotal reports have also suggested that K65R may occur more commonly in low-income countries when patients with non-B subtype viruses are treated with d4T/ddI and d4T/3TC[94,95], or TDF/3TC[96].

Multi-nucleoside resistance mutations

Amino acid insertions at codon 69 generally occur in the presence of multiple TAM, and in this setting are associated with intermediate resistance to 3TC and FTC and high-level resistance to each of the remaining NRTI[97-101]. Q151M is a 2-bp mutation (CAG→ATG) that is usually accompanied by two or more of the following mutations: A62V, V75I, F77L, and F116Y. The Q151M complex causes high-level resistance to ZDV, d4T, ddI, and ABC, and intermediate resistance to TDF, 3TC, and FTC[61,102,103]. This complex developed in 5% of patients who received ddI in combination with ZDV or d4T[98,104], but is rarely selected by 3TC- or FTC-containing regimens. Q151M may be uncommon because the two intermediate amino acids Q151L (CAG→CTG) and Q151K (CAG→AAG) are poorly replicating and rarely observed[105-107]. Q151M is a common genetic mechanism of NRTI resistance in HIV-2-infected persons[108,109]. The optimal NRTI combination to use in patients with codon 69 insertions or Q151M is not known[110,111].

Miscellaneous mutations

Mutations K43E/Q/N, E203D/K, H208Y, D218E, H221Y, K223Q, and L228H/R are non-polymorphic NRTI-selected mutations which generally follow TAM and which have subtle effects on HIV-1 NRTI susceptibility and replication[27,53,61,112]. Q145M is a rare mutation that has been reported by one group to reduce susceptibility to multiple NRTI and NNRTI[113,114]. P157S, which is homologous to the mutation causing 3TC resistance in FIV, has been reported once in an HIV-1 isolate[115,116].

Several mutations in the connection and RNaseH domains of HIV-1 RT play an accessory role in reducing HIV-1 susceptibility in combination with TAM, most likely by slowing the activity of RNaseH and thereby allowing more time for TAM-mediated primer unblocking[117]. The single most important of these mutations may be N348I, a non-polymorphic mutation that occurs in about 10% of NRTI-treated patients[118]. N348I causes a twofold reduction in ZDV susceptibility when it occurs in combination with multiple TAM[118]. G333E/D, A360T, and A371V, mutations with similar phenotypic effects, occur in about 5% of NRTI-naive and 10% of NRTI-treated patients[119-122]. Although several RNaseH mutations may potentially reduce ZDV susceptibility in combination with TAM[123], few have been observed in clinical isolates[124,125].

Nonnucleoside reverse transcriptase inhibitors

The NNRTI inhibit HIV-1 RT allosterically by binding to a hydrophobic pocket close to but not contiguous with the RT active site. Nearly all of the NNRTI resistance mutations are within the NNRTI binding pocket or adjacent to residues in the pocket[126,127]. There is a low genetic barrier to NNRTI resistance, with only one or two mutations required for high-level resistance. High levels of clinical cross-resistance exist among the NNRTI because many of the NNRTI resistance mutations reduce susceptibility to multiple NNRTI and because the low genetic barrier to resistance allows a single NNRTI to select for multiple NNRTI resistance

mutations in different viruses, even if only a single mutation is detected by standard population-based sequencing[128,129].

The NNRTI resistance mutations can be classified into the following categories: (i) primary NNRTI resistance mutations that cause high-level resistance to one or more NNRTI and that are among the first to develop during NNRTI therapy; (ii) secondary NNRTI resistance mutations that usually occur in combination with primary NNRTI resistance mutations, but that also have clinically significant implications for choosing an NNRTI, particularly etravirine; (iii) minor non-polymorphic mutations that may occur alone or in combination with other NNRTI resistance mutations and that cause consistent but low-level reductions in NNRTI susceptibility; and (iv) polymorphic accessory mutations that modulate the effects of other NNRTI resistance mutations. Table 2 summarizes effect of the major primary, major secondary, and minor NNRTI resistance mutations on delavirdine, efavirenz, etravirine, and nevirapine.

Because delavirdine is rarely used, it is not discussed in the sections that follow. The resistance profile of delavirdine is distinguished from that of the other NNRTI by the fact that G190A/S increase delavirdine susceptibility, providing perhaps the only virologic rationale for its use[130]. Although there have been case reports of virologic responses to delavirdine-containing salvage treatment regimens in treating viruses with G190A/S, the extent to which delavirdine contributed to these successes is not known[131].

Primary nonnucleoside reverse transcriptase inhibitor resistance mutations

Each of the primary NNRTI resistance mutations – K103N/S, V106A/M, Y181C/I/V, Y188L/C/H, and G190A/S/E – cause high-level resistance to nevirapine and variable resistance to efavirenz, ranging from about twofold for V106A and Y181C, sixfold for G190A, 20-fold for K103N, and more than 50-fold for Y188L and G190S[61,132,133]. Although transient virologic responses to an efavirenz-based salvage therapy regimen occur in some NNRTI-experienced patients, a sustained response has been uncommon[128,134-136]. In contrast, patients with any single one of the primary NNRTI resistance mutations may benefit from etravirine salvage therapy, although the mutations at position 181 and to a lesser extent 190 compromise etravirine response and may provide the foundation for the development of high-level etravirine resistance[137-139].

Major secondary nonnucleoside reverse transcriptase inhibitor resistance mutations

L100I, K101P, P225H, F227L, M230L, and K238T are secondary mutations that usually occur in combination with one of the primary NNRTI resistance mutations. L100I and K101P, which occur in combination with K103N, further decrease nevirapine and efavirenz susceptibility from 20-fold with K103N alone to more than 100-fold[61]. Although viruses with K103N are fully susceptible to etravirine, viruses with L100I plus K103N display about 10-fold decreased susceptibility[133]. P225H and K238T/N usually occur in combination with K103N and synergistically reduce nevirapine and efavirenz susceptibility[132,140,141]. F227L nearly always occurs in combination with V106A, leading to synergistic reductions in nevirapine susceptibility[142]. M230L, which may occur alone, decreases the susceptibility of all NNRTI including etravirine by 20-fold or more[133,143].

V179F, F227C, L234I, and L318F are rare mutations that are of increased importance now that etravirine is licensed. V179F occurs solely in combination with Y181C/I/V and acts synergistically to increase etravirine resistance from fivefold to 10-fold with Y181C/I/V alone to more than 100-fold[133]. F227C, an exceedingly rare mutation, reduces etravirine susceptibility 10-fold to 20-fold[144,145]. L234I, which has been selected *in vitro* by etravirine, acts synergistically with Y181C to reduce etravirine susceptibility[133]. L318F, which was first reported to reduce delavirdine and nevirapine susceptibility by 15-fold and threefold, respectively[146], has also been selected *in vitro* by etravirine and found to reduce etravirine susceptibility synergistically with Y181C[133].

Minor nonnucleoside reverse transcriptase inhibitor resistance mutations

A98G, K101E, V108I, and V179D/E are common NNRTI resistance mutations that reduce susceptibility to nevirapine and efavirenz about twofold to fivefold[147]. Although K103R alone, which occurs in about 1% of untreated persons, has no effect on NNRTI susceptibility, the combination of K103R plus V179D reduces nevirapine and efavirenz susceptibility by 15-fold[141]. Data are not available on the effect of these mutations on etravirine susceptibility. V179D, and rarely A98G and V108I, are observed in patients who have never been

Table 2. Nonnucleoside reverse transcriptase inhibitor resistance mutations*

	98	100	101	103	106	108	179	181	188	190	225	227	230	236	238
	A	**L**	**K**	**K**	**V**	**V**	**V**	**Y**	**Y**	**G**	**P**	**F**	**M**	**P**	**K**
NVP	G	**I**	**EP**	**NS**	**AM**	I	DEF	**CIV**	**LHC**	**ASE**		LC	**L**		**NT**
DLV	G	**I**	**EP**	**NS**	**AM**	I	DEF	**CIV**	**LHC**	**E**		C	**L**	**L**	**NT**
EFV	G	**I**	E**P**	**NS**	A**M**	I	DEF	CIV	**L**HC	**ASE**	H	C	**L**		NT
ETR†	G	**I**	E**P**				DE**F**	**CIV**	LHC	A**SE**		**C**	**L**		

*The first row of letters contains the consensus amino acid at the position indicated by the number in the preceding row. All amino acids are indicated by their one letter code. Mutations in **bold** are associated with higher levels of phenotypic resistance or clinical evidence for reduced virologic response. Several additional uncommon mutations at the positions in this table are also associated with NNRTI therapy or phenotypic resistance including: K101N/H, K103T/H, G190Q/C/T/V. Additional NNRTI resistance mutations that are not in the table include E138K, L234I, and L318F. E138K has been selected *in vitro* by ETR and been shown to cause low-level reductions in susceptibility to each of the NNRTI[133,145]. L234I has been selected *in vitro* by ETR, acts synergistically with Y181C to reduce ETR susceptibility[133]. L318F is a non-polymorphic NNRTI-selected mutation that decreases susceptibility to DLV and to a lesser extent to nevirapine and possibly ETR[133,146]. Several polymorphic mutations such as K101Q, I135T/M, V179I, and L283I and NRTI selected mutations such as L74V, H221Y and N348I may cause subtle reductions in NNRTI susceptibility[118,152]. A98S, K101R/Q, K103R, V106I, E138A, V179I, and K238R are polymorphic substitutions with little if any effect on drug resistance on their own. However, the combination of K103R + V179D (each of which occurs in 1-2% of untreated persons) reduces susceptibility to NVP, DLV, and EFV about 15-fold[141].
†In the DUET study, a univariate analysis showed that persons with viruses with three or more of the following mutations responded similarly to placebo and ETR: V90I, A98G, L100I, K101E/P, V106I, V179D/F, Y181C/I/V, and G190A/S[139].
NNRTI: nonnucleoside reverse transcriptase inhibitor; NVP: nevirapine; DLV: delavirdine; EFV: efavirenz; ETR: etravirine.

treated with NNRTI[148]. The optimal management of patients with viruses containing these mutations is not known. Although low-level baseline resistance has not been shown to decrease the virologic responses to first-line NNRTI-containing regimens[149], efavirenz and etravirine may be preferable to nevirapine because these NNRTI have generally been more active than nevirapine against these and other NNRTI-resistant variants[127,150].

Miscellaneous nonnucleoside reverse transcriptase inhibitor resistance mutations

Several highly polymorphic RT mutations, such as K101Q, I135T/M, V179I, and L283I, reduce susceptibility to nevirapine and efavirenz by about twofold and may act synergistically with primary NNRTI resistance mutations[151,152]. Other mutations such as L74V, H221Y, K223E/Q, L228H/R, and N348I are selected primarily by NRTI, yet also cause subtle reductions in NNRTI susceptibility[41,107,112,118,152-154]. V90I and V106I are highly polymorphic mutations that were associated with decreased virologic response to etravirine in the DUET clinical trial, but may owe this association to their correlation with other NNRTI resistance mutations[139]. Mutations at positions 31, 135, and 245 have been reported to cause low-level NNRTI resistance in a non subtype B context[155,156]. Conversely, there is a large body of evidence showing that type I TAM increase NNRTI susceptibility[157,158].

Protease inhibitors

As the PI class has expanded to nine licensed ARV, the individual PI have evolved increasingly specific roles. Ritonavir is used solely for pharmacokinetic boosting (indicated by/r). Lopinavir/r, atazanavir/r, fosamprenavir/r, and less commonly saquinavir/r are used for first-line therapy, whereas lopinavir/r, tipranavir/r, and darunavir/r are used for salvage therapy[1,2]. Nelfinavir, which cannot be boosted by ritonavir, and unboosted atazanavir and fosamprenavir are alternative but suboptimal choices for first-line therapy because of their higher risk of virologic failure with drug resistance compared with boosted PI. Although indinavir/r may be effective for first-line or salvage therapy, it is not recommended because of its high risk of nephrolithiasis.

More mutations are selected by the PI than by any other ARV class. The effect of PI resistance mutations on individual PI may be difficult to quantify when many mutations are present in the same virus isolate or when mutations occur in unusual patterns. The effect of PI resistance mutations on drug susceptibility can also be modulated by *gag* cleavage site mutations and possibly other parts of *gag* that influence Gag-Pol processing. Although multiple protease mutations are often required for HIV-1 to develop clinically significant resistance to a ritonavir-boosted PI[159-161], some mutations indicate that a particular PI, even when boosted, may not be effective. Many protease mutations are accessory, compensating for the replication impairment

Table 3. Protease inhibitor resistance mutations*

	23	24	30	32	33	46	47	48	50†	53	54	73	76†	82	84	88†	90
	L	**L**	**D**	**V**	**L**	**M**	**I**	**G**	**I**	**F**	**I**	**G**	**L**	**V**	**I**	**N**	**L**
ATVr		I			F	IL	V	**VM**	**<u>L</u>**	L	VTALM	**ST**		ATFS	**<u>VAC</u>**	D**<u>S</u>**	**M**
DRVr‡				**I**	F		VA		V		**LM**	ST	**V**		VAC		M
FPVr				**<u>I</u>**	F	**IL**	**<u>VA</u>**		**<u>V</u>**		VTA**<u>LM</u>**	ST	**V**	ATFS	**<u>VAC</u>**		**M**
IDVr		**I**		V		**IL**	V			L	**VTA**LM	ST	V	**<u>AFTS</u>**	**<u>VAC</u>**	S	**M**
LPVr		I		I	F	IL	V**<u>A</u>**	VM	**V**		**VTALM**		**V**	**AFTS**	VAC		M
NFV	**<u>I</u>**	**I**	**<u>N</u>**		F	**<u>IL</u>**	V	**<u>VM</u>**		L	**VTALM**	**ST**		**AFTS**	**<u>VAC</u>**	**<u>DS</u>**	**<u>M</u>**
SQVr		I						**<u>VM</u>**		L	**VTA**LM	**ST**		AT	**<u>VAC</u>**	S	**<u>M</u>**
TPVr§				I	F	IL	**V**				**VAM**			A**<u>T</u>**FS**<u>L</u>**	**VAC**		M

*The first row of letters contains the consensus amino acid at the position indicated by the number in the preceding row. All amino acids are indicated by their one letter code. Mutations in **bold** have been shown to reduce *in vitro* susceptibility or *in vivo* virologic response. Mutations in **<u>bold underline</u>** are relative contraindications to the use of specific PI. Several additional uncommon mutations at the 17 positions in this table are also selected by PI, but have not been evaluated phenotypically including L24F, L33I, M46V, F53Y, I54S, G73C/A, V82M/C, and N88T/G. In contrast, V82I and L33V are polymorphisms that are not associated with PI therapy. Accessory protease mutations that are not in the table include the polymorphic mutations L10I/V, I13V, K20R/M/I, M36I, D60E, I62V, L63P, A71V/T, V77I, and I93L and the non-polymorphic mutations L10F/R, V11I, E34Q, E35G, K43T, K45I, K55R, Q58E, A71I/L, T74P/A/S, V75I, N83D, P79A/S, I85V, L89V, T91S, Q92K and C95F.

†I50L increases susceptibility to all PI except ATV; I50V and I54L increase TPV susceptibility; N88S increases FPV susceptibility; L76V increases ATV, SQV and TPV susceptibility.

‡A genotypic susceptibility score (GSS) for DRV based on the POWER clinical trials includes the number of the following 11 mutations: V11I, V32I, L33F, I47V, I50V, I54L/M, G73S, L76V, I84V, and L89V[201]. In a subsequent update the substitution of T74P for G73S led to an improved model[202].

§A GSS for TPV/r based on the RESIST studies identified 21 mutations at 11 positions: L10V, I13V, K20M/R/V, L33F, E35G, M36I, K43T, M46L, I47V, I54A/M/V, Q58E, H69K, T74P, V82L/T, N83D, and I84V[184]. An updated TPV/r GSS excluded I13V, K20M/R/V, E35G, and H69K; reclassified I47V, I54A/M/V, Q58E, T74P, V82L/T, and N83D as major mutations; reclassified L10V, M36I, K43T, M46L, and I84V as minor mutations; and included L24I, I50L/V, I54L, and L76V as mutations likely to improve TPV susceptibility and virological response[200]. A complete listing of studies of genotypic PI response predictors can be found at: http://hivdb.stanford.edu/pages/geno_clinical_review/PI.html

ATVr: atazanavir/ritonavir(r); DRVr: darunavir/r; FPVr: fosamprenavir/r; IDVr: indinavir/r; LPVr: lopinavir/r; NFV: nelfinavir; SQVr: saquinavir/r; TPVr: tipranavir/r.

of other PI resistance mutations or reducing PI susceptibility only in combination with other PI resistance mutations.

Major protease inhibitor resistance mutations

Table 3 lists mutations at 17 largely non-polymorphic positions that are of the most clinical significance. Mutations at 13 of these 17 positions have been shown to reduce susceptibility to one or more PI, including mutations at the substrate cleft positions 23, 30, 32, 47, 48, 50, 82, and 84, the flap positions 46 and 54, and interior enzyme positions 76, 88, and 90. Mutations at four of these 17 positions (24, 33, 53, and 73) are included because they are non-polymorphic, occur commonly, and have disparate effects on different PI[61].

Whereas many mutations reduce nelfinavir susceptibility, L23I, D30N, M46I/L, G48V/M, I84V, N88D/S, and L90M are relative contraindications to the use of nelfinavir in that an inferior virologic response to therapy relative to that obtainable with most other PI would be expected[14,162-167]. I50L and N88S and possibly I84V, are relative contraindications for the use of atazanavir/r[23,61,168-174]. G48V/M, I84V, and L90M are relative contraindications to the use of saquinavir/r[175-177]. V32I, I47V/A, I54L/M, and I84V are relative contraindications to the use of fosamprenavir/r[174,178-181]. Mutations at position 82 as well as I84V may be relative contraindications to the use of indinavir/r. There are few known contraindications to the salvage therapy PI (lopinavir/r, tipranavir/r, darunavir/r), except V47A for lopinavir/r[178,182,183] and V82L/T for tipranavir/r[184].

At six of the 17 PI resistance mutations in table 3, only a single mutation has been shown to be associated with PI resistance – L23I, L24I, D30N, V32I, L76V, and L90M. At 11 positions, different mutations are associated with PI resistance, and at positions 50, 54, 82, and 88 these differences can be responsible for dramatically different effects on PI susceptibility. Additional, uncommon, PI-selected mutations not shown in table 3 include L33I, M46V, F53Y, I54S, G73C/A, V82M/C, and N88T/G[23,41,185]. V82I, which does not contribute to PI resistance, is a polymorphism that is the consensus residue for subtype G isolates. L33V is another polymorphism that is not associated with PI therapy or resistance. L33F and M46I/L, although non-polymorphic in most subtypes, occur at a prevalence of about 0.5-1%

in subtype A and CRF01_AE isolates (http://hivdb.stanford.edu/cgi-bin/MutPrevBySubtypeRx.cgi)[148].

Several resistance mutations are associated with increased susceptibility to one or more PI, including I50L which increases susceptibility to all PI other than atazanavir[168], I50V and I54L which increase tipranavir susceptibility[186], N88S which increases fosamprenavir susceptibility[187], and L76V which increases susceptibility to atazanavir, saquinavir, and tipranavir[23,188].

Accessory protease inhibitor resistance mutations

Mutations at positions 10, 20, 36, 63, and 71 upregulate protease processivity to compensate for the decreased fitness associated with the major PI resistance mutations[189-193]. Positions 20, 36, and 63 are highly polymorphic. In contrast, L10I/V and A71V/T occur in 5 and 10%, respectively, of PI-naive patients, and in a much higher proportion of PI-treated patients, while L10F/R and A71I/L do not occur in the absence of PI therapy[147]. In one retrospective study, baseline mutations at positions 10 and 36 were associated with an increased risk of virologic failure in patients receiving older PI-based regimens containing nelfinavir or an unboosted PI[194,195].

Additional PI-selected accessory mutations include the highly polymorphic mutations I13V, D60E, I62V, V77I and I93L, and many uncommon non-polymorphic mutations including V11I, E34Q, E35G, K43T, K45I, K55R, Q58E, T74P/A/S, V75I, N83D, P79A/S, I85V, L89V, T91S, Q92K and C95F[23,41,196-199]. Several of the non-polymorphic mutations have become part of the genotypic susceptibility scores for tipranavir/r (E35G, K43T, Q58E, T74P, and N83D) and darunavir (V11I, T74P, and L89V), based on analyses of the RESIST[184,200] and POWER and DUET[201,202] clinical trials. These mutations, however, have not been evaluated for their effects on other PI, but their presence at baseline in these two clinical trials for heavily PI-experienced patients suggests that they are also associated with decreased susceptibility to the older PI.

Gag cleavage site mutations

The *gag* gene codes for the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins, a protein of uncertain function, p6, and two spacer peptides: p2 (between CA and NC) and p1 (between NC and p6). The gag polypeptide is cleaved at the MA/CA, CA/p2, p2/NC, NC/p1, and p1/6 junctions. A stem-loop structure between p1 and p6 stimulates the frame shifting necessary to create the Gag-Pol polypeptide. The residues surrounding each protease cleavage site are designated 5'-P4, P3, P2, P1/P1', P2', P3', P4'-3'.

Mutations that improve the kinetics of PI-resistant proteases emerge at several protease cleavage sites during PI treatment[203-205]. Most *gag* cleavage site mutations occur at NC/p1 and p1/p6[203,206] – sites at which cleavage may be rate limiting for gag and Gag-Pol polyprotein processing[207]. A431V, at the P2 position of NC/p1, is associated with mutations at protease positions 24, 46, and 82[208,209]. L449F, at the P1' position of p1/p6, is associated with the protease mutation pair D30N/N88D and with I84V[209,210]. P453L, at the P5' position of the p1/p6 site, is associated with protease mutations at positions 32[211], 47[211], 50[212], 84, and 90[209,213]. A set of three NC/p1 mutations (A431V, K436E, and I437T/V) developing during *in vitro* selection with the investigational PI RO033-4649 was found to cause a twofold reduction in susceptibility to multiple PI, even in the absence of mutations in protease[214]. Several p6 mutations, including insertions in a proline-rich region containing a conserved PTAP motif, occur more frequently in viruses with PI resistance mutations than in wild-type viruses[215-219].

Subtype-specific mechanisms of protease inhibitor resistance

Naturally occurring polymorphisms in the different protease subtypes often occur at sites of accessory PI resistance mutations in subtype B isolates[220]. For example, the accessory PI resistance mutations I13V, K20I, M36I, and I93L represent the consensus variant in one or more non-B subtypes[221]. Although these mutations may result in subtle structural and biochemical differences among subtypes[222-224], the vast majority of *in vitro* and *in vivo* studies suggest that the licensed PI are as active against wild-type non-B viruses as they are against wild-type subtype B viruses[220,225].

With several notable exceptions, the genetic mechanisms of PI resistance are also highly similar among the different subtypes[226]. Although both D30N and L90M occur in non-B viruses during nelfinavir therapy, D30N occurs more commonly in subtype B viruses and L90M occurs more commonly in subtype C, F, G, and CRF01_AE viruses[227-231]. The increased predilection for certain subtypes to develop L90M may relate to the presence of variants other than L (the subtype B consensus) at position 89[230-232]. Similarly, T74S, a polymorphism that occurs in 8% of subtype C

sequences, but rarely in other subtypes, is associated with reduced susceptibility to nelfinavir[61,233].

The fact that V82I is the consensus amino acid for subtype G affects the spectrum of mutations observed at this position in PI-resistant subtype G isolates: V82T and the rare mutation V82M occur more frequently than V82A in subtype G isolates because T and M require a single base pair change, whereas A requires two base pair changes[234]. However, for nearly all other subtypes and protease mutations, a similar number of nucleotide changes is required to convert a wild-type residue into one associated with drug resistance[235].

Integrase inhibitors

The HIV-1 integrase contains 288 amino acids encoded by the 3' end of the HIV-1 *pol* gene. It has three functional domains: the N-terminal domain (NTD), which encompasses amino acids 1-50 and contains an HHCC motif that coordinates zinc binding[236], the catalytic core domain (CCD) which encompasses amino acids 51-212 and contains the catalytic triad D64, D116, and E152, known as the DDE motif, and the C-terminal domain (CTD), which encompasses amino acids 213-288 and is involved in host DNA binding through an as yet poorly defined mechanism.

A multimeric form of integrase catalyzes the cleavage of the conserved 3' dinucleotide CA (3' processing) and the ligation of the viral 3'-OH ends to the 5'-DNA of host chromosomal DNA (strand transfer)[237]. Crystal structures of the CCD plus CTD domains[238] and the CCD plus NTD[239] have been solved, but the relative conformation of the three sub domains and of the active multimeric form of the enzyme are not known. There has been one published crystal structure of the CCD bound to an early prototype inhibitor (5CITEP)[240] but no structures of the CCD bound to one of the integrase inhibitors (INI) in clinical use or to a DNA template.

The current generation of clinically relevant INI (the FDA-licensed inhibitor raltegravir and the investigational inhibitor elvitegravir) preferentially inhibit strand transfer by binding to the target DNA site of the enzyme. These INI as well as the initial series of strand-transfer diketo acid inhibitors including S-1360[241] and L870,810[242] select for mutations in the part of the integrase bound to 5CITEP[240,243,244]. *In vitro* drug susceptibility data and surveys of integrase sequences from HIV-1-infected patients previously treated with other ARV classes or who were treatment-naive suggest that there is no cross-resistance between the INI and the other HIV-1 enzyme inhibitors[245-248].

Most INI resistance mutations are in the vicinity of the putative INI binding pocket. Some of the INI resistance mutations decrease susceptibility by themselves, whereas others compensate for the decreased fitness associated with other INI resistance mutations[249]. There is a high level of cross-resistance between raltegravir and elvitegravir, as well as between these INI and the first generation of strand-transfer inhibitors, suggesting that the development of non cross-resistant INI will be challenging[245,250-255].

Among 38 patients with virologic failure in Merck Protocol 005, nearly all developed INI resistance mutations including N155H or Q148H/R/K, each of which reduces raltegravir susceptibility by 10-fold to 25-fold[251]. Higher levels of raltegravir resistance occurred with the accumulation of additional mutations. E92Q and the two polymorphic mutations L74M and G163R generally occurred with N155H, whereas G140A/S generally occurred with Q148H/R/K[251]. Additional mutations reported to the FDA as being selected either *in vitro* or *in vivo* by raltegravir include the non-polymorphic mutations L74R, E138A/K, Y143R/C/H, N155S, H183P, Y226D/F/H, S230R, and D232N and the polymorphic mutations T97A and V151I[256,257].

Among 30 patients developing virologic failure while receiving elvitegravir in GS-US-1830105, 28 developed INI resistance mutations including E92Q, E138K, Q148H/R/K, or N155H in 11 patients, and S147G or T66I/A/K in nine and five patients, respectively[252]. Additional mutations selected *in vitro* by elvitegravir include the non-polymorphic mutations H51Y, Q95K, F121Y, Q146P, S153Y, and R263K, and the slightly polymorphic mutation E157Q[245,250]. For both raltegravir and elvitegravir, virologic failure has generally been accompanied by 100-fold or greater decreases in susceptibility and the development of two or more INI resistance mutations.

Table 4 lists the non-polymorphic INI resistance mutations that have been selected in patients receiving raltegravir or elvitegravir, or that have been characterized *in vitro* for susceptibility to both drugs. Mutations at positions 92, 121, 140, 148, and 155 are associated with more than fivefold to 10-fold decreased susceptibility to both INI, whereas mutations at positions 66 and 147 are associated with marked decreases in susceptibility only to elvitegravir.

Fusion inhibitors

Enfuvirtide, the only licensed fusion inhibitor, inhibits the interaction of the heptad repeat (HR) 1 and 2 domains

Table 4. Integrase inhibitor resistance mutations*

	66	92	121	138	140	143	147	148	153	155	157	263
	T	**E**	**F**	**E**	**G**	**Y**	**S**	**Q**	**S**	**N**	**E**	**R**
Raltegravir†		**Q**	**Y**	AK	**AS**	CHR	G	**HRK**		**H**S	Q	
Elvitegravir‡	**I**	**Q**	**Y**	AK	**AS**	n/a	**G**	**HRK**	Y	**H**S	Q	K

*The first row of letters contains the consensus amino acid at the position indicated by the number in the preceding row. All amino acids are indicated by their one letter code. INI-resistance mutations selected in patients receiving raltegravir[251,257] or elvitegravir[252] and characterized *in vitro* for susceptibility[250,252,253,255]. Mutations in **bold** are associated with > 5-10 fold decreased susceptibility[256].
†Other mutations selected *in vitro* or *in vivo* by raltegravir include the non-polymorphic mutations H183P, Y226DFH, S230R, and D232N, and the polymorphic mutations L74M, T97A, V151I, G163R, I203M, and S230N[256].
‡Other mutations selected *in vitro* or *in vivo* by elvitegravir include the non-polymorphic mutation H51Y, Q95K, and Q146P. Additional integrase mutations selected by other investigational integrase inhibitors include the non-polymorphic mutations T125K, A128T, Q146K, N155S, K160D and the polymorphic mutations V72I, M154I, V165I and V201I[249].

of gp41 by mimicking a part of HR2 (amino acids 127-162) that binds to a conserved part of HR1. It has antiviral activity approaching that of the most active ARV such as efavirenz, lopinavir/r, and raltegravir. However, resistance may develop rapidly in patients receiving enfuvirtide for salvage therapy who do not receive a sufficient number of additional active drugs. Indeed, the emergence of resistance strains followed by virologic rebound has been observed in some patients within two to four weeks[258,259].

The extra-viral portion of gp41 is the most conserved region in the HIV-1 envelope glycoprotein and there is little naturally occurring variation in the HR1 binding site among the different group M subtypes[260-265]. Nonetheless, there is about 10-fold variation in enfuvirtide susceptibility among isolates from enfuvirtide-naive persons, possibly resulting from gp41 polymorphisms outside of the HR1 binding site[266-269]. However, despite the wider range in baseline enfuvirtide susceptibility than for other ARV, there is no evidence that enfuvirtide-naive patients infected with viruses at the lower ranges of enfuvirtide susceptibility respond less well to enfuvirtide[267,269].

Mutations in gp41 codons 36 to 45, the region to which enfuvirtide binds, are primarily responsible for enfuvirtide resistance[269-274]. Table 5 lists the most commonly observed enfuvirtide resistance mutations in this region. A single mutation is generally associated with about 10-fold decreased susceptibility, whereas double mutations can decrease susceptibility more than 100-fold. Several accessory mutations in the HR2 region corresponding to the peptide sequence of enfuvirtide including N126K, N137K, and S138A appear to improve fitness in combination with specific mutations at positions 36-45[274-277]. Similar enfuvirtide resistance mutations appear to emerge in subtype B and non-B isolates[278,279].

Enfuvirtide-resistant HIV-1 isolates replicate less well than enfuvirtide-susceptible isolates, as evidenced by *in vitro* competition studies[280] and by the rapid reversion to wild-type that occurs in patients who discontinue enfuvirtide[281]. There are some conflicting data on the clinical benefit of continued therapy in the presence of incomplete virologic suppression. One study showed that interruption of therapy was associated with a mean increase in plasma HIV-1 RNA levels of just 0.2 $\log_{10}$ and no decrease in CD4 count[281]. However, other studies have suggested that some enfuvirtide resistance mutations, particularly those at position 38, may be associated with CD4 count increases[282], possibly because mutations at this position may decrease virus replication or render the virus more susceptible to neutralizing antibodies that target fusion intermediates[283].

CCR5 inhibitors

The licensed small molecule inhibitor maraviroc and the investigational small molecule inhibitor vicriviroc (formerly SCH-D) allosterically inhibit HIV-1 gp120 binding to the seven-transmembrane G protein-coupled CCR5 coreceptor. Whereas HIV-1 gp120 binds to the N-terminus and second extracellular loop region of CCR5[284], site-directed mutagenesis and molecular modeling studies suggest that most small molecule inhibitors bind to a pocket formed by the transmembrane helices[285-290].

The HIV-1 gp120 has a highly variable sequence, and different HIV-1 isolates display variable susceptibility to inhibition by different ligands and small molecule inhibitors[291,292]. Nonetheless, there appear to be minimal differences in the susceptibility of wild-type viruses (even those belonging to different subtypes), to maraviroc[293] and vicriviroc[294], suggesting that these

Table 5. Fusion inhibitor resistance mutations*

	G36	I37	V38	Q40	N42	N43	L44	L45
Enfuvirtide	**DE**VS	V	**EA**MG	**H**	T	**DKS**	M	M

*Mutations in bold reduce enfuvirtide susceptibility > 10-fold in site-directed mutants and most clinical isolates. N42S is the only common polymorphism between codons 36 to 45. It occurs in about 15% of untreated isolates and does not decrease enfuvirtide susceptibility[269]. Most other mutations at these positions are likely to have been selected by enfuvirtide, although their effect on enfuvirtide susceptibility may not have been reported. Several accessory mutations in the HR2 region corresponding to the peptide sequence of enfuvirtide including N126K, N137K, and S138A have been shown to emerge to improve fitness in combination with specific mutations at positions 36 to 45[274-277].

inhibitors disrupt a highly conserved protein-protein interaction. Moreover, *in vitro* passage experiments have generally demonstrated that high-level resistance emerges only after several months of passage, suggesting that the genetic barrier to resistance to CCR5 inhibitors is not low[295-298]. Nonetheless, HIV-1 may escape from CCR5 inhibition by developing CCR5 inhibitor resistance or by utilizing the CXCR4 coreceptor.

CCR5 inhibitor resistance

The genotypic and phenotypic mechanisms of CCR5 inhibitor resistance and cross-resistance are complex and poorly understood. Dose-response curves suggest that there are at least two phenotypic mechanisms of resistance: (i) enhanced binding to unbound CCR5, manifested by a shift in the typical sigmoid dose-response curves resulting from the requirement of a several-fold increase in the concentration of inhibitor required to suppress virus in a manner similar to wild-type[292,297-300]; and (ii) enhanced binding to a CCR5-inhibitor complex, manifested by a plateau in the maximal percent inhibition (MPI) regardless of inhibitor concentration. Such plateaus have been observed when testing both individual virus clones as well as virus populations within a clinical isolate. For a virus clone, the level of the MPI plateau is expected to be inversely proportional to the relative affinity of the clone for the bound compared with the unbound form of the receptor[298]. For a virus isolate, the level of the MPI plateau is expected to be inversely proportional to the number of viruses within the isolate with affinity for the bound compared with the unbound form of the receptor[292].

Viruses with high levels of CCR5 inhibitor resistance (> 1,000-fold reductions in IC_{50} as well as an MPI plateau) have been identified during *in vitro* passage experiments with most CCR5 inhibitors[297-299,301,302]. The amino acid changes responsible for resistance may be entirely within the V3 loop[298,299], entirely outside of the V3 loop[297], or may result from synergistic interactions between substitutions in the V3 loop and other parts of *env*[302]. These amino acid changes may include known polymorphisms as well as novel substitutions, insertions, and deletions. Further complicating the genetic basis of CCR5 inhibitor resistance is the observation that the same inhibitor may select for different mutations in different virus isolates[297,298,302].

The mechanisms of CCR5 inhibitor resistance *in vivo* may be even more complicated than those that have been observed to emerge *in vitro*. First, virus isolates from the majority of patients developing virologic failure while receiving maraviroc[303] or vicriviroc[304] have not demonstrated phenotypic resistance. Second, the few viruses with phenotypic resistance (four of 37 for maraviroc and one of seven for vicriviroc) have demonstrated only subtle MPI reductions rather than the MPI reductions and large increases in IC_{50} that have been observed during the emergence of resistance *in vitro*. Finally, the mutations that have been observed *in vivo* have been highly variable, differing for each virus isolate[303,304].

CXCR4 tropism

At the time of initial HIV-1 infection, at least 80-90% of patients have viruses that exclusively use CCR5 as their coreceptor (R5 tropic). During the course of infection, about 50% of patients with subtype B infections are eventually found to harbor viruses that use the CXCR4 coreceptor (X4 tropic)[305-308]. The emergence of X4 tropism usually occurs in later disease stages and, in the absence of ARV therapy, is followed by accelerated CD4 cell depletion. When X4-tropic viruses emerge, they usually co-circulate with R5-tropic viruses as minor variants[306,309-311]. Some X4-tropic viruses are also R5 tropic, although most such dual-tropic HIV-1 clones usually infect only one of the two coreceptors efficiently[311,312].

The main determinants for coreceptor tropism are in the V3 loop, although changes outside of the V3 loop may also influence tropism, either in combination or independently of V3 changes[284,313-319]. The presence

of positively charged amino acids at positions 11 and 25 in the V3 loop, combined with several other V3 sequence characteristics, have a specificity of about 90% and sensitivity of 70-80% for predicting X4 tropism of individual virus clones belonging to subtype B[284,316-318]. However, the number and type of mutations by which an R5-tropic virus becomes X4 tropic is complex and depends on the sequence of the baseline R5 virus[284,313-315,320]. Preliminary data also suggests that the frequency and genetic basis for tropism switches may be different for different subtypes[284,312].

A phenotypic assay has recently been developed to assess the tropism of complete *env* genes (gp120 plus gp41) amplified from patient samples (Trofile™, Monogram Biosciences)[311]. Amplified *env* genes are ligated into *env* expression test vectors (eETVs), which following co-transfection with *env*-deleted genomic vectors are used to create a population of pseudovirions. CD4+/U87 cells expressing CCR5 or CXCR4 are inoculated with these pseudovirions, and infection of each cell type is measured using a luciferase-based reporter system. In reconstruction experiments, X4-tropic variants can also be detected even when they constitute 1-5% of a mixed virus population[311,321]. However, because the amplification sensitivity of the assay is reliable only when plasma HIV-1 RNA levels are > 1,000 copies/ml, the full sensitivity of the assay will be achievable only in patients with plasma HIV-1 RNA levels > 10,000 copies/ml.

The factors responsible for the emergence of X4 tropism and for the proportion of X4 variants relative to R5 variants in those patients in whom X4-tropic viruses do emerge are not known. Yet, these factors have implications for detecting X4-tropic viruses to determine whether a CCR5 inhibitor will be effective. In a 10-day monotherapy study of maraviroc in 62 patients with CD4 counts > 250 cells/ul and the absence of X4 variants by Trofile™ testing, X4 emergence and virologic rebound occurred in two patients[322,323]. Phylogenetic analysis of *env* clones from pre- and posttreatment time points indicated that the X4 variants probably emerged by outgrowth from a pretreatment X4 reservoir[323]. Considering that seven patients had been excluded from this study owing to the presence of X4 variants at screening, X4 variants were detected successfully at baseline in only seven of nine cases[322]. Similar findings were reported in patients receiving maraviroc in the MOTIVATE I and II trials[324], as well as in clinical trials of vicriviroc[325] and aplaviroc[326].

Improved sensitivity for detecting X4 variants is required to ensure that CCR5 inhibitors are optimally used. The Trofile™ test is more sensitive than genotypic methods for detecting X4-tropic viruses in clinical samples for at least two reasons. First, because the assay uses complete patient-derived *env* genes, it can detect X4 tropism even when the changes responsible are outside of the V3 loop[327]. Second, the assay is more sensitive at detecting X4-tropic variants that are below the 20-30% limit of detection of standard population-based genotypic assays. Indeed, this factor alone appears to be responsible for the drop in sensitivity for V3 genotyping from 70-80% on individual clones to 30% on clinical samples[318,327]. Novel genotypic approaches such as ultra-deep pyrosequencing methods that simultaneously sequence multiple individual clones in a patient sample[328,329] and novel bioinformatic approaches for analyzing these sets of sequences will be required to attain sensitivities approaching that of phenotypic assays. Although the complex genetic basis for coreceptor tropism poses a hurdle for genotypic relative to phenotypic approaches, this drawback may be offset if genotypic methods are capable of identifying transitional R5 to X4 variants that may be surrogates for the presence of low-level X4 emergence.

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