HIV-1 Fitness: Implications for Drug Resistance, Disease Progression, and Global Epidemic Evolution

Miguel E. Quiñones-Mateu[1] and Eric J. Arts[2]

[1] Department of Virology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH, USA
[2] Department of Medicine, Division of Infectious Diseases, Case Western Reserve University, Cleveland, OH, USA

[1] To whom correspondence should be addressed. Tel. (216) 444-2515, Fax (216) 444-2998, E-mail: quinonm@ccf.org

In the past five years, the HIV research field has shown renewed interest in the replicative capacity of human immunodeficiency virus type 1 (HIV-1) due to the potential impact of *ex vivo* viral fitness on population size (viral load), drug resistance, and disease progression. For example, studies on HIV-1 fitness of drug-resistant mutants under different selective pressures have led to a better understanding of emergence of specific drug resistance mutations during therapy, as well as risk/benefit of these mutations for HIV-infected individuals. In addition, other studies have associated HIV-1 fitness with the rate of transmission and disease progression. HIV-1 continually evolves and migrates through individual hosts, overcoming barriers to transmission, avoiding different immune responses, and resisting various antiretroviral regimens. The "fittest" HIV-1 isolate that survives in growth competition cultures may also lead to increased virulence within a host. However, rapid disease progression may be detrimental to fitness of the same HIV-1 isolate in the human population. Thus, the term "fitness" itself is an enigma requiring a multilayered definition. Rather than attempt to summarize all the studies on HIV-1 replication, this review concentrates on (i) the clinical implications of viral fitness within HIV-infected individuals in the presence or absence of antiretroviral therapy, (ii) changes in viral fitness upon emergence of drug resistance, and (iii) the role of HIV fitness in the AIDS epidemic, including virus transmission and viral/host evolution.

Concepts in viral fitness

Genetic variation is inherent to all RNA viruses but has been best characterized for HIV-1 [94;199]. The extensive heterogeneity observed in the worldwide epidemic originates from the rapid viral turnover (10^{10} viral particles/day) in an HIV-infected individual [102;226], high rate of incorrect nucleotide substitutions during HIV reverse transcription (10^{-4}/nt) in the absence of proof-reading mechanisms [182], and the pliant conformations/functions of many HIV-1 proteins. In addition to this rapid accumulation of minor genotypic changes, different HIV-1 strains can also recombine at a high rate, generating large genetic alterations [25;106;185;214;221;222]. HIV-1, like other RNA viruses, has a high mutation rate (3×10^{-5} mutations per base pair per cycle [146]), which coupled with selection and rapid turnover [225] results in the generation of swarms of mutants known as viral quasispecies [59;60;71]. Continuous production of viral mutants results in adaptation to most environmental changes [57;60]. It is important to highlight that the quasispecies, and not individual virus genomes, are the subject of selection and evolution [57;60;71]. Thus, HIV-1 quasispecies are evolutionary and clinically relevant since this genetic variability can in a sense respond to selective pressure (*e.g.*, host immune system and antiretroviral therapy).

Fitness is a parameter defining the replicative adaptation of an organism to its environment (reviewed in [57;60]). Survival of the fittest is the concept that drives evolution in a complex population. Within a given viral quasispecies each clone has a fitness, representative of those viral properties (*e.g.* activity and stability) undergoing selection in that particular environment. During viral replication within

a defined microenvironment, different genomes encode virus that replicate at high rates, continually mutate but generally remain under the same selective pressures [57]. Thus, positive (Darwinian) selection implies that one or more members of the quasispecies are better suited to a given environment while negative selection eliminates unfit variants [57;58;60]. In summary, the dynamics of the viral population is a continuous process of growth, competition, and selection that takes place in the sequence space (*i.e.*, all possible sequence permutations for an informational macromolecule). In the case of HIV-1, with a genome size of 10 Kb, the total possible sequence space is 410,000, although only a very small fraction corresponds to functional viruses [57]. The combination of sequence space and fitness (or replicative capacity) constitutes the "fitness landscape" [60;233]. This classical concept, first described seventy years ago [233], suggests that changes in viral fitness can be viewed as a movement of viral genomes in an irregular and adaptive landscape of peaks and valleys. As a result, RNA viruses can find multiple pathways to reach alternative high fitness peaks on the fitness landscape [72].

Multiple studies based on vesicular stomatitis virus (VSV), foot-and-mouth disease virus (FMDV), or HIV-1 quasispecies have assessed different fitness theories related to population size and quasispecies complexity. The Red Queen hypothesis states that viral quasispecies in competition tend to gain fitness with each viral passage [33;58;166]. However, the evolutionary interpretation of the statement implies that population size must continually increase to maintain or increase fitness gains. In contrast, the competitive exclusion principle asserts that in the absence of niche differentiation, one competing species will always eliminate or exclude the other [33;58]. Most experiments designed to test either fitness theory require the continual passage of an increasing virus population in cell culture. Failure to passage a significant proportion of the virus population and/or changes in the tissue culture environment results in bottlenecks and a decreased rate in fitness gain. Repeated bottlenecks will actually result in a net fitness loss [31;58;60;64;72;238]. The Muller's ratchet hypothesis suggests that limiting the population size will result in an accumulation of deleterious mutations which overwhelm the appearance of mutations improving fitness [31;58;64;72]. Extreme examples of Muller's ratchet have been well documented with plaque-to-plaque passages of VSV, FMDV, and bacteriophage f6 [58;64;72]. With the exception of few studies [237;238], HIV-1 has not been utilized as a tool to test fitness theories. This may be due in part to the slower replication rates and production yield of lentiviruses (*e.g.*, HIV-1) as compared to many single-strand RNA viruses (*e.g.*, VSV an FMDV).

Methods to determine HIV-1 fitness

Although differences in viral replicative capacities were described early in the HIV-1 epidemic [82], the role of HIV-1 fitness in drug resistance and pathogenesis have only recently been the focus of detailed investigations [34;164]. Hence, several key conceptual and technical shortcomings remain unresolved. For example, what is the proper assay to measure fitness of HIV-1 isolates or clones? Multiple methods have been employed to measure HIV-1 replication capacity *in vitro* [34;164] (Table 1). Although viral fitness is best defined by replicative capacity during growth competition experiments [103], many studies have extended the definition of relative fitness by comparing (i) the catalytic activity of HIV-1 enzymes [8;165], (ii) virus turnover on HIV-1 infected individuals [56;90;91], (iii) HIV-1 production in monoinfected cultures [40;62;151;204], (iv) the virion (infectious):virus particle ratios [19], and (v) an HIV-induced event in a single-cycle infection assay [52;143;239]. Use of these assays is generally justified or necessary to test a specific hypothesis. However, there is also little continuity between these studies creating specific ambiguities in the HIV-1 fitness field. Although each study was designed to address specific questions, the use of different assays and viral constructs to define the "fitness" of drug resistant HIV-1 can be very misleading for comparisons.

In vivo assays

Competitive ability of a virus is the result of many biological processes in its life cycle (*i.e.*, cell entry, genome replication, protein synthesis/processing, particle assembly and release from cells). A human host offers a variety of cell types and microenvironments to the infecting HIV-1 resulting in

Table 1. Methods used to estimate HIV-1 fitness

Assays	Methods	Detection techniques	References
In vivo	Viral kinetics in plasma	Sequencing	[45;56;90;91;142]
		Differential hybridization	[68;69;97]
		Primer-guided nucleotide incorporation assay	[85]
Ex vivo	Protease catalytic activity	Pr efficiency (Kcat / Km),	[8;40;62;165;220]
		Polyprotein processing/ maturation	[19;24;40;144;148;174;180;180;191; 201;239]
		Genetic complementation	[135;153]
	RT catalytic activity	RT polymerase, Rnase H activity	[2;8;26;79;88;120;205]
	Viral growth kinetics	p24 Antigen / RT activity	[2;8;16;19;20;26;40;43;62;67;87;88; 101;109;110;113;116;122;139;143; 144;148;151;177;180;195;197;204; 205;210;212;239]
	Single-cycle infection	β-galactosidase activity	[43;143-145;239]
		GHOST/CCR5-CXCR4 permissibility	[19]
		Luciferase activity	[52;92;149;180;234]
	Growth competition	Differential plaque assay	[155;191]
		Cloning/sequencing	[2;40;45;87;96;109;113;122;128; 150;152;165;180;204;205;212;236]
		Heteroduplex mobility assay	[162;188;192;238]
		Real-time NASBA	[45]
		Recombinant marker virus assay	[139]
Animal model	SCID mice	SCID-hu Thy/Liv	[210]
		SCID-hu PBL	[177]

various selective pressures. To date, most *in vivo* fitness studies have been performed on blood samples [68;90;91]. Although *in vivo* assays provide the best estimate of fitness, specific quasispecies (*e.g.*, drug-resistant variants) may be dominant in the blood but less fit in other compartments (*e.g.*, lung or CNS). Host-to-host comparisons of *in vivo* HIV-1 fitness is very difficult to evaluate due to differences in host genetics and immune response. Therefore, *in vivo* fitness studies are limited to the emergence of specific quasispecies or drug resistant mutants, and cannot determine the impact of specific substitutions on replicative capacity. In contrast, *ex vivo* fitness assays of HIV-1 primary isolates remove "variable" host constraints and focus on replication efficiency. Thus, *in vitro* fitness assays are still indispensable.

In vitro assays

In vitro studies using HIV-1 isolates or recombinant viruses cannot mimic the natural setting within human hosts. However, they are useful models for viral replication in a fixed environment and for the behavior of drug-resistant variants after the initiation of therapy. In the absence of a consensus method for quantifying viral replication capacity, many studies have used different techniques to assess *ex vivo* HIV-1 fitness. In general, methods to determine HIV-1 fitness *in vitro* could be grouped in two general

techniques: viral growth kinetic assays and growth competition experiments (Fig. 1).

a) Viral growth kinetic assays

Biochemical properties of the protease (PR) and reverse transcriptase (RT) enzymes, as well as the replication kinetics of HIV-1 with particular mutations, have been the subject of extensive investigations. Replicative capacity (viral fitness) of HIV-1 isolates or recombinant infectious clones is examined in monoinfections by determining the amount of virus production over time (*e.g.*, measuring p24 antigen, RT-activity, (β-galactosidase or luciferase activity in reporter cell lines, etc) [20;52;62;144;204] (Table 1). Differences in the replication kinetics of HIV-1 mutants can be compared in parallel infections (Fig. 1A). However, these assays can only discern gross changes in replicative capacity and cannot accurately define the impact of subtle genetic changes on the replication rates of HIV-1 isolates. In general, direct competition between two different viruses is a more accurate and sensitive assay to detect minute fitness differences [103;180] (Fig. 1B).

A novel recombinant virus technique, based on the PhenoSense assay (Virologic) used in drug susceptibility assays [176], has been recently adapted to measure HIV-1 replicative capacity [52;234] (Fig. 1C). Replicative capacity of the recombinant virus (generally PR-RT pseudotyped) is examined in a cell line transfected with a luciferase reporter gene replacing *env* to monitor a single round of virus replication. A single-cycle assay provides the best estimate of replication efficiency during a mono-infection. However, this assay is also limited by the use of recombinant viruses and assessment of only specific steps in the replication cycle (*i.e.*, only steps leading to viral transcription). This single-cycle infection assay cannot be used effectively in growth competition experiments.

It is important to note that a few studies have analyzed HIV-1 replication of drug-resistant variants using animal models, specifically SCID-hu mice [177;210]. This HIV-1 infection model has been successfully used to assess viral susceptibility to antiretroviral drugs. A recent study has compared the fitness and pathogenesis of protease inhibitor (PI)-resistant HIV-1 strains in SCID-hu mice (see below).

b) Growth competition experiments

Head on competitions in cell culture between two viral isolates of the same species provide the internal control lacking in monoinfections. However, an extreme limitation to growth competition experiments is developing an assay to accurate detect and quantify minute genotypic or phenotypic

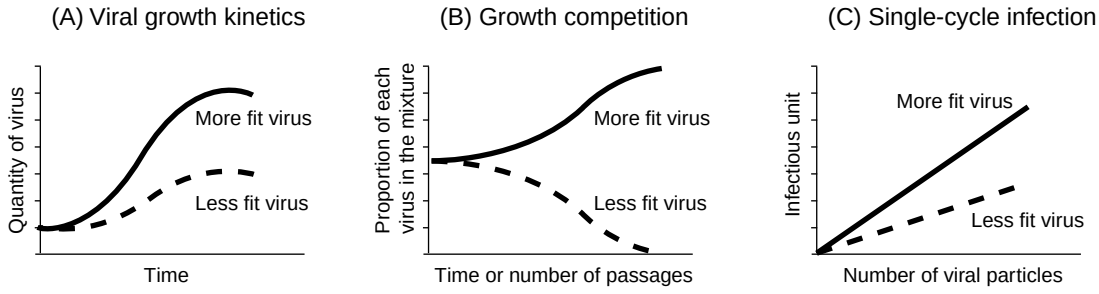


Figure 1. *Methods used to determine* in vitro *HIV-1 fitness. (A) Viral growth kinetics assays involves measuring virus production at various time points using diverse detection methods (*e.g.*, p24 antigen or RT-activity). (B) Growth competition experiments require dual infection with two different HIV-1 isolates (initial proportion of each may vary). Production and quantification of each specific HIV-1 isolate in the mixture can be determined through the use of different methods (Table 1). (C) Single-cycle infection assays. The number of cells infected after a single cycle of replication can be measured using various indicator systems (Table 1) (Figure adapted from [34]).*

differences between these viruses. Relative fitness can be directly compared in dual infections since virus populations in culture compete until one clone or quasispecies outgrows the other [60;103]. Following exposure of cells to a viral mixture, the proportion of viruses after several viral passages is compared with that in the initial mixture [58;103]. Despite being a laborious assay, growth competitions provide a more accurate measurement of relative viral fitness, and are generally reproducible in the same cell culture environment.

Although the concepts of viral competitions may be similar, different approaches have been developed to measure dual virus production. Most methods rely on laborious point mutation assays or on the sequencing of a large number of clones [40;96;109;150;151;204], whereas new studies utilize more rapid techniques to estimate the frequency of the two viruses in the competition [45;139;162;188;238] (Table 1). Martinez *et al.* [153] developed a bacteriophage lambda-based genetic screen to characterize the activity and phenotype of HIV proteases. In another study, a "fitness profile assay" was used to determine the relative fitness of drug-resistant variants and wild-type HIV-1 as a function of protease inhibitor concentration (by calculating the ratio of mutant:wt production with each drug concentration, in an assay similar to an IC_{90} determination) [145]. Heteroduplex tracking assay (HTA) has recently been used (i) to evaluate the production of HIV-1 variants in a competition [188;238] (ii) to detect specific V3-coding regions in *env* that are associated to NSI/R5 or SI/X4 phenotypes and (iii) to detect drug-resistant vs. wild-type codons in the PR-RT-coding region [162;192]. HTA can accurate detect drug-resistant variants comprising only 3% of the HIV-1 population, and can also be used to estimate *in vivo* viral fitness.

New technologies such as Real Time PCR have also been applied to dual virus detection. de Ronde [45] was the first to develop a real-time NASBA PCR and molecular beacons technique to quantify a specific AZT RT mutant within the quasispecies of treated patients (specifically, changes of codon 215 of the RT). Lu & Kuritzkes [139] developed a novel recombinant marker virus assay (RMVA) to perform growth competition assays and estimate RT fitness. The *nef* gene in an RT-deleted HIV-1 clone was replaced by the *Salmonella typhimurium* histidinol dehydrogenase (*his*D) or the human heat-stable placental alkaline phosphatase (PLAP) genes. Relative production of any two RT-pseudotyped HIV-1 isolates was measured by the corresponding marker (hisD or PLAP) using real-time PCR.

Finally, homologous recombination between two HIV-1 variants during competition can present a major obstacle in measuring relative HIV-1 fitness. Recombined HIV-1 isolates did emerge after four passages in a growth competition experiment [128]. This finding suggests that the recombinant was more fit than the two parental infectious clones. A recent study estimated a low frequency of HIV-1 recombination (approximately 3-5% / Kbp in a 5-day infection period) in a high-MOI (0.1 IU/ml) dual HIV-1 infection of PBMC [187]. Reducing the MOI of each isolate provided better estimates of HIV-1 fitness and reduced the frequency of co-infected cells leading to recombination (less than 0.1% for an MOI = 0.01). These results suggest that HIV-1 recombination is minimal in growth competition experiments with low MOI and limited passages.

c) HIV-1 isolates vs. recombinant viruses

To date, the vast majority of *in vitro* fitness studies are performed with cloned virus pseudotyped with various HIV-1 genes [19;98;119;151;195;206]. The entire gene or even segments of various coding regions (PR, RT, ENV, Gag-Pol protease cleavage sites) are often PCR-amplified directly from patient plasma or cells. One of the main advantages of these recombinant clones is the ease and flexibility of directly comparing fitness and drug sensitivity of various target genes. In HIV-1, protease and reverse transcriptase regions generally harbor the drug resistant mutations. In fact, recombinant viruses, as opposed to HIV-1 primary isolates, can determine the impact of specific sites or regions within a neutral HIV-1 backbone. This pseudotyping technique eliminates the possible fitness effects of polymorphisms or mutations found outside the target sequence in primary HIV-1 isolates [40;109;144;150;151;204;205]. However, comparison between HIV-1 fitness studies are difficult due to the use of a myriad of fitness assays and different HIV-1 clones for pseudotyping. Therefore, the *in vivo* relevance of fitness studies

on pseudotyped virus containing single drug resistance mutations or even the affected gene is still the subject of debate. Other genomic regions external to those containing the drug resistance mutations and not used for pseudotyping could have an equal or greater impact on fitness.

Use of HIV-1 clinical isolates in place of recombinant infectious clones to measure viral fitness will be dependent on the research question. Studies comparing viral fitness with HIV-1 pathogenesis (see below) [16;188] employed primary HIV-1 isolates since the entire genome may affect replicative capacity. Grant *et al.* [92] analyzed the fitness of PI-resistant and PI-susceptible viruses *in vivo* (ratio of wild-type:mutant in plasma) and *in vitro* using the PhenoSense HIV drug susceptibility single-cycle assay (see above). Both methods generated comparable fitness results suggesting that this recombinant/virus single-cycle infection may predict the prevalence of specific HIV-1 drug resistant mutations in the presence or absence of drug selective pressure. Viral fitness studies of amprenavir-resistant viruses also showed a strong correlation between the prevalence of resistant mutations and replication efficiency of PR-RT pseudotyped virus [180]. However, differences in replicative capacity were more apparent using growth competition experiments than in single-cycle mono-infection assays [19;180]. Recently, contributions of PR and/or RT to fitness has been compared in drug resistant HIV-1 primary isolates to fitness of recombinant HIV-1NL4-3 viruses pseudotyped with PR, RT, or PR-RT [19]. All of the HIV-1 clones pseudotyped with the drug-resistant PR and/or RT cassettes were less fit than the wild-type NL4-3 clones. In contrast, two of the three drug-resistant HIV-1 isolates had similar replication rates as the NL4-3 wild-type clone. These results suggest that other genomic regions can compensate the drug resistant substitutions in the PR and RT genes. Continual evolution of HIV-1 regions outside of drug-targeted genes will eventually compensate for defects in fitness. Thus, fitness results on specific viral clone or even primary isolates can only answer defined questions and should not be accepted as absolute *in vitro* or *in vivo* fitness.

Fitness of HIV-1 drug-resistant viruses

The ultimate goal of present therapy is to suppress HIV-1 replication for as long as possible. Maintaining low-to-undetectable plasma HIV-RNA levels prevents progression to AIDS and minimizes the possible emergence of drug resistant HIV-1 variants [100]. However, treatments with antiretroviral combinations do not eliminate or even completely suppress HIV-1 replication in all tissue compartments. Each of the sixteen antiretroviral drugs licensed in the United States falls into one of three classes: (i) nucleoside reverse transcriptase inhibitors (NRTI), nonnucleoside reverse transcriptase inhibitors (NNRTI), and protease inhibitors (PI). Aside from the initial pharmacokinetic and dynamic properties of these drugs, duration of treatment success is not measured by drug potency but rather the time required for the dominance of drug resistant isolates in the quasispecies [49;137;157]. In the absence of antiretroviral therapy, strains containing drug-resistance mutations have a reduced fitness compared to the wild-type (wt) quasispecies within the population [37]. Therefore, selective pressure in the form of drug therapy leads to dramatic shifts in the quasispecies distribution and fitness of those mutants with decreased sensitivity to the respective antiretrovirals [37;57;60;61]. During this *in vivo* selection, several drug-resistant variants will emerge and compete for dominance. These resistant isolates will pass through the drug-induced bottleneck and initiate a new quasispecies distribution that will be governed again by replication efficiency [37;138].

During antiretroviral therapy two types of mutations are associated with drug resistance and respective shifts in fitness (*i.e.*, *ex vivo* replication efficiency in the absence of drug). An initial decrease in fitness coincides with the appearance of *primary* substitutions conferring direct drug resistance. Continued drug pressure allows the quasispecies to re-explore the HIV-1 sequence space. *Secondary* or *compensatory* mutations are selected to restore the enzymatic activity of the drug-targeted resistant enzyme (PR or RT) leading to a rebound in fitness, similar if not greater than the fitness of the quasispecies

prior to treatment [13;34;100;164]. It is important to note that compensatory mutations are not limited to the target/resistant gene unless this resistant enzyme or protein is rendered inactive or severely impaired. In addition, appearance of specific mutations is often highly dependent on the baseline sequence and the sequential selection of "*de novo*" compensatory mutations that contribute to viral fitness [181;197;224]. These troughs and peaks in the HIV-1 fitness landscape during drug selection were first described in HIV-infected patients treated with lamivudine (3TC) [8]. Subsequently, multiple studies have reported impaired enzyme function and reduced viral fitness of HIV-1 isolates harboring PI and RTI resistant mutations [40;62;96;110;143;144;150;151]. Current treatment guidelines advocate a switch in antiretroviral treatment regimens following the emergence of primary drug resistant mutations and possibly prior to selection of compensatory changes. Several groups have described that an accumulation of drug-resistance mutations would have a debilitating effect on HIV-1 replication efficiency [10;20;62;69;155]. For example, high level resistance to protease inhibitors generally requires a combination of amino acid substitutions within or near the peptide binding/cleavage site [62]. Reduced recognition and cleavage of sites in the HIV-1 precursor proteins was expected but few had anticipated that mutations at the cleavage sites would compensate for changes in enzyme activity.

In the human host, the most fit HIV-1 sequence differs between patients due to variations in host genetics (*e.g.*, co-receptor polymorphisms), immune response, and several viral factors (*e.g.*, replication capacity, mutation rate, and host cell tropism) [34;164]. Several studies have reported that wild-type viruses from treatment-naïve patients have a broad range of replicative capacities (47% to 89%, median 73%) compared to laboratory strains [234]. In addition, pairwise competition experiments with HIV-1 isolates from untreated patients suggest that fitness differences do not necessarily follow a one-dimensional relationship (*e.g.*, since A > B, and B > C, A must be > C), but is much more complex and likely involves competition at multiple points in the replication cycle. Thus, the utility of a molecular HIV-1 clone as a fitness control decreases with the amount of genetic material introduced into that clone for fitness studies. Even the use of primary drug-resistant isolates requires a side-by-side (single-cycle) or head on (competition) comparison with several wild-type HIV-1 strains to approximate *ex vivo* fitness.

Protease inhibitor (PI)-resistant variants

HIV-1 protease is the enzyme responsible for cleavage of the viral Gag and Gag-Pol polyprotein into mature structural proteins and enzymes found in the infectious virion [157;173]. Six HIV-1 protease inhibitors have been approved to date in the United States, *i.e.*, amprenavir (APV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), and saquinavir (SQV). Numerous primary and/or secondary mutations have now been associated with HIV-1 resistance to these protease inhibitors [157;203]. The protease gene has shown great plasticity, with 49 natural polymorphisms and 20 drug resistance substitutions in the 99 amino acid protein, which is only active as a homodimer [157;203] (Fig. 2). Interestingly, primary drug resistant substitutions rarely dominate the quasispecies in PI-naïve HIV-infected individuals [133], suggesting they confer a selective disadvantage to the virus [129;203]. In fact, multiple mutations appear to be necessary for the development of a replication competent PI-resistant virus [11;228]. For example, viral infectivity was lost when two primary PI-resistance mutations (30N and 90M) were introduced into a HXB2 background, but restored when the entire PR-coding region from a clinical isolate, harboring the same two mutations, was cloned into HXB2 [159]. In patients displaying virological failure to a PI-based regimen, viral evolution leads to a gradual increase in both PI-resistance and replicative capacity (associated with the emergence of secondary mutations) [51;224].

For most of the protease inhibitors, primary PI resistant mutations cluster near the active site of the enzyme (Fig. 2B), reducing both catalytic activity and viral replicative capacity [20;40;144;157]. Secondary mutations within the protease gene (*e.g.*, 10,63,71,77) compensate for the impairment on HIV replication by helping the enzyme to adapt to the primary changes in the active site [20;68;101;144;163;165;197] (Fig. 2). In addition, increases in PI-resistance are often associated with substitutions in the protease cleavage sites (*gag* and *pol* genes) [62] (Fig. 3) [34;157;164;240]. Mutations in these regions provide better peptide substrates for the mutated protease and compensate for a potential

loss in viral fitness [34;62;144;164;239]. Another compensatory mechanism in RTV+SQV-treated patients may involve insertions in the proline rich region of the p6gag protein, the function of which is still not defined [117]. Although, some PI-resistant viruses display defects in the RT processing [43], mutations in the reverse transcriptase may increase packaging of the Gag-Pol precursor to effectively compensate for this defect and increase the amount of active RT in the virion. AZT-resistance mutations in RT can partially rescue the replicative defect of a PI-resistant virus, which could be relevant to the therapeutic control of HIV-1 infection [43].

During the last few years, multiple studies have described a significant reduction in HIV-1 replicative capacity as a consequence of PI resistance. A comprehensive list relating *ex vivo* fitness to each PI-resistant or secondary mutations is provided in Table 2. Most of these mutations reduce the replicative capacity but several amino acid substitutions (usually combinations of primary and secondary mutations) have been shown to restore or even increase fitness over a wild-type virus (Table 2). Initial studies reporting differences in the replicative capacity of viruses resistant to protease inhibitors introduced a relatively new field to HIV-1 biology and clinical therapy [20]. Transitions in *ex vivo* fitness associated with an accumulation of PI-resistant mutations selected during PI-based regimens (ritonavir, indinavir, saquinavir, nelfinavir and others) are now well documented in the literature (reviewed in

A

	10	20	24	30	32	33	36	46	47	48	50	54	63	71	73	77	82	84	88	90
	L	K	L	D	V	L	M	M	I	G	I	I	L	A	G	V	V	I	N	L
Amprenavir (APV)	FIRV	.	.	.	I	.	.	IL	V	.	V	VML	.	.	S	.	.	V	.	M
Indinavir (IDV)	IRV	MR	I	.	I	.	I	IL	.	V	.	V	APQ	TV	SA	I	AFTS	V	S	M
Lopinavir (LPV)	FIRV	MR	I	.	.	.	.	I	.	.	V	.	.	TV	.	.	AFTS	V	.	M
Nelfinavir (NFV)	FI	.	.	N	.	.	I	IL	.	.	.	.	I	TV	.	I	AFTS	V	DS	M
Ritonavir (RTV)	FIRV	MR	.	.	I	F	I	IL	.	.	.	VL	AP	TV	.	I	AFTS	V	.	M
Saquinavir (SQV)	IRV	.	.	.	.	.	.	.	.	V	.	VL	.	TV	S	I	A	V	.	M

B

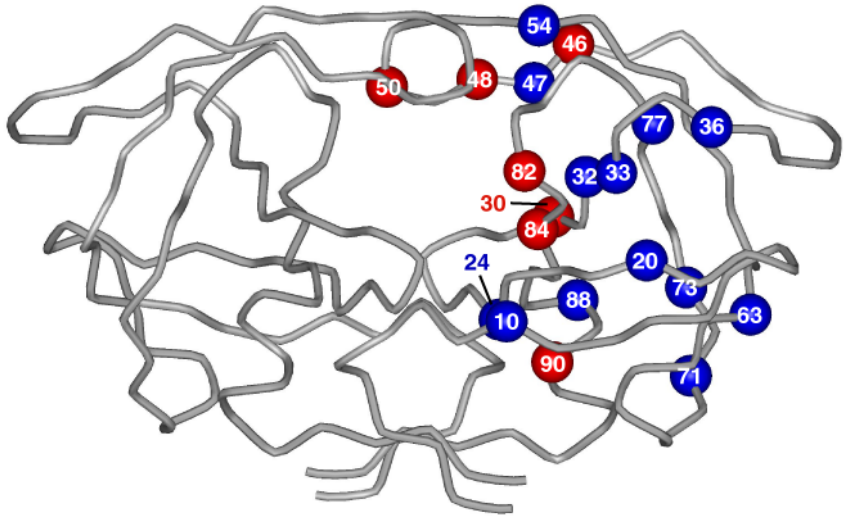
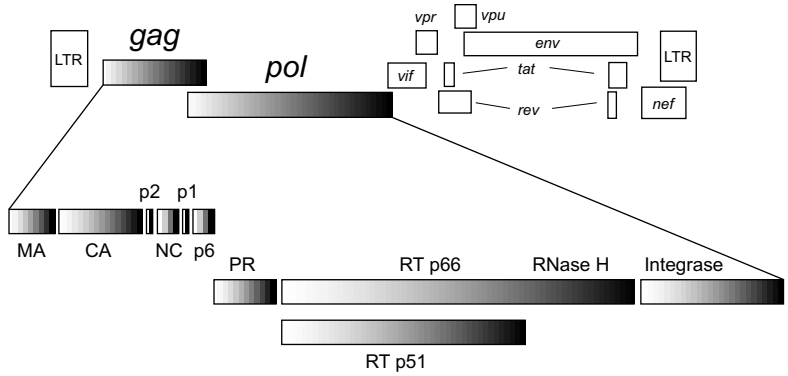


Figure 2. *(A) Summary of protease mutations associated with resistance to PR inhibitors. Amino acids in red and blue denote primary and secondary/compensatory mutations, respectively. Wild-type amino acids (HIV-1 subtype B) at the codons related to resistance to PI are indicated. Mutations related to loss of sensitivity to PI [99;203] were recently reviewed at http://www.iasusa.org. (B) Structure of the HIV-1 protease (pdb file: 1hxb) [130], indicating amino acid residues associated with PI-resistance. Each numbered circle indicates the codon position and nature of the resistant mutation (primary and secondary mutations in blue and red, respectively).*

[34;164]). Selection of mutant viruses with higher replication capacity than wild-type virus can be explained by the Wright's concept of adaptive landscape [233]; "natural selection drives a population to a local optimum, which is not necessarily the global optimum". In the absence of PI selection, it is unlikely that wild-type quasispecies would follow the same evolutionary pathway to achieve this high level of fitness. During suboptimal therapy, impaired replication of the intermediate HIV-1 isolate is compensated by the high level of drug resistance.

The latter studies on PI-resistant HIV-1 variants have focused on *ex vivo*/*in vitro* correlations of PI susceptibility and replication efficiency. Most fitness analyses were performed with recombinant HIV-1 clones pseudotyped with the PI-resistant protease or other Gag-Pol regions. Recent studies have compared *ex vivo* fitness of PI-resistant variants with an atypical response to antiretroviral therapy. Particular attention has been focused on discordant responses in HIV-infected patients treated with PI (*i.e.*, high viral loads and sustained $CD4^+$ T-cell counts). PI-resistant HIV-1 isolates and PR-pseudotyped HIV-1 clones from these patients were used to infect PBMC, human thymic organ cultures, and SCID-hu Thy/Liv mice [210]. Interestingly, replication of PI-resistant strains was highly impaired in the thymus, suggesting a possible preservation of $CD4^+$ T-cell counts in patients failing PI-based therapy [50].

A. Gag and Gag-Pol processing



B. HIV-1 protease cleavage sites

Site	Amino acid	Position	References
gag			
MA / CA	SQNY / PIV	1187 - 1188	[39;144]
CA / p2	ARVL / AEA	1880 - 1881	[39;144]
p2 / NC	ATIM / MQR	1920 -1 921	[39;144]
NC / p1	RQAN / FLG	2085 - 2086	[9;39;144;240]
p1 / p6	PGNF / LQS	2136 - 2137	[9;39;62;144]
pol			
TF / PR	SV / PQI	2257 - 2258	[39]
PR / RT	TLNF / PIS	2552 - 2553	[39]
RT (p51 / p66)	AETF / YVD	3869 - 3870	[39]
RT / IN	RKVL / FLD	4232 - 4233	[39]

Figure 3. *(A) Organization and processing of HIV-1 Gag and Pol proteins. The nine cleavage sites recognized by the HIV-1 protease in the Gag and Pol proteins are indicated. (B) HIV-1 protease cleavage sites. MA, matrix; CA, capsid; NC, nucleocapsid; TF, transframe; PR, protease; RT, reverse transcriptase; IN, integrase. Scissile bonds are presented as slashes in the amino sequence. Nucleic acid positions are based on the HIV-1HXB2 sequence (GenBank accession number K03455) (Figure adapted from [203]).*

Table 2. Effects of protease mutations on HIV–1 replication capacity

Amino acid substitutions	Fitness relative to wild–type[a]	Virus[b]	Method[c]	References
8K	.	Sel	Mono	[101]
8Q	–	Sel	Mono	[101]
10I	.	Sel/NL43	Mono/Enz	[145;174]
10I,F	–	Sel/NL43	Mono/Enz	[145;174]
17R*	–	HXB2	Mono/Comp	[122]
22V*	–	HXB2	Mono/Comp	[122]
25E	–	HXB2	Enz	[135]
25H*	–	HXB2	Mono/Comp	[122]
30N	–	NL43	Vivo/Mono/Comp	[56;151]
32I	–	Sel/NL43/HXB2	Mono/Comp/Enz	[20;40]
35TVLEE*	–	HXB2	Mono/Comp	[122]
35TD*	–	HXB2	Mono/Comp	[122]
35TN*	–	HXB2	Mono/Comp	[122]
36I	.	NL43	Mono	[145]
36NL*	–	HXB2	Mono/Comp	[122]
36GL*	–	HXB2	Mono/Comp	[122]
36DL*	–	HXB2	Mono/Comp	[122]
37D*	–	HXB2	Mono/Comp	[122]
37G*	–	HXB2	Mono/Comp	[122]
37N*	–	HXB2	Mono/Comp	[122]
46I	.	Sel/NL43/HXB2	Mono/Comp	[20;101;145;150]
46I,L	–	n.a.	Vivo	[56]
47V	–	Sel/NL43	Mono/Enz	[174]
48V	–	NL43	Mono	[145]
50V	–	Sel/NL43	Mono/Enz	[174]
54V	.	NL43	Mono	[145]
63P,A	.	Sel/NL43	Vivo/Mono/Comp	[68;147;150]
71V	.	Sel/NL43/HXB2	Mono	[20;145]
82A	.	Sel/NL43/HXB2	Mono	[20;145]
82A,T,F	–	Prim/Sel/NL43/HXB2	Vivo/Mono/ Comp/Enz/SCID	[24;68;147; 150;165;177]
84V	.	Sel	Mono	[147]
84A,V	–	Sel/NL43/HXB2	Mono/Comp/Enz	[40;165;191]
90M	.	NL43	Mono	[145]
90M	–	NL43	Mono/Comp	[151]
95TLNFPI*	–	HXB2	Mono/Comp	[122]
8K/46I	.	Sel	Mono	[101]
10I/48V	–	NL43	Mono	[145]
10F/50V	–	Sel/NL43	Mono/Enz	[174]
10F/84V	–	Sel/NL43	Mono/Comp/Enz	[180]
10I/90M	.	NL43	Mono	[145]
10F+449F**	–	Sel/NL43	Mono/Comp/Enz	[180]
30N/63P	–	NL43/HXB2	Mono/Comp	[151]
30N/88D	–	HXB2	Enz	[211]
30N/90M	–	HXB2	Enz	[211]
32I/71V	–	Sel/NL43/HXB2	Mono/Comp/Enz	[40]

Table 2. (cont.)

36I/54V	–	HXB2	Comp/Enz	[165]
46I/63P	+	HXB2	Enz	[201]
46I/82A	.	NL43	Mono	[145]
48V/82A	–	NL43	Mono	[145]
48V/90M	–	NL43	Mono	[145]
54V/82A	–	Prim/NL43	Mono/SCID	[145;210]
62I/77I	.	HXB2	Comp/Enz	[165]
63P/90M	.	NL43	Mono/Comp	[151]
71V/82A	.	NL43	Mono	[145]
82T,F/84V	–	Sel/HXB2	Mono/Enz	[147;201]
82A/90M	–	NL43	Mono	[145]
10I/48V/82A	–	NL43	Mono	[145]
10I/48V90M	–	NL43	Mono	[145]
10I/82A/90M	–	NL43	Mono	[145]
10F/84V+p1/p6**	–	Sel/NL43	Mono/Comp/Enz	[150;180]
36I/50V/63P	–	HXB2	Mono	[195]
36I/54V/82T	–	HXB2	Comp/Enz	[165]
46I/47V/50V	–	Sel/NL43	Mono/Enz	[174]
46I/53L/82A	–	Prim/R8	Mono/Enz	[24]
46I/54V/82A	–	NL43	Mono	[145]
54V/71V/82A	.	NL43	Mono	[145]
63P/82F/84V	–	Sel	Mono	[147]
10I/23I/46I/84V	.	Sel/NL43/HXB2	Mono/Comp/Enz	[40]
10F/46I/50V+p1/p6**	–	Sel/NL43	Mono/Comp/Enz	[150;180]
10L/46I/82T/84V	.	NL43	Mono/Comp	[151]
20R/36I/54V/82A	–	NL43	Mono/Enz	[239]
20R/36I/63P/82S	–	NL43	Mono/Enz	[239]
20R/63P/82A/90M	–	NL43	Mono/Enz	[239]
36I/50V/63P+p1/p6**	–	HXB2	Mono	[195]
36I/54V/71V/82T	+	HXB2	Comp/Enz	[165]
46I/48V/63P/90M	–	NL43	Mono/Enz	[239]
46I/54V/71V/82A	.	NL43	Mono	[145]
46I/63P/82T/84V	.	NL43	Mono/Comp	[151]
54V/82A+ p2/NC+NC/p1	–	NL43	Mono/Enz	[144]
10I/23I/46I/84I+ p1/p6**	–	Sel/NL43/HXB2	Mono/Comp/Enz	[40]
10I/36I/48V/84V/90M	–	Prim/NL43	Mono/SCID	[210]
10F/46I/47V/50V+ p1/p6**	–	Sel/NL43	Mono/Comp/Enz	[150;180]
10L/46I/63P/82T/84V	.	NL43	Mono/Comp	[151]
20R/36I/54V/71V/82T	+	HXB2	Compt/Enz	[165]
32I/46I/71V/82A+ p1/p6**	–	Sel/NL43/HXB2	Mono/Comp/Enz	[40;135]
36I/46I/71V/84A+ p1/p6**	–	Sel/NL43/HXB2	Mono/Comp/Enz	[40;62]
10I/46I/63P/ 77I/84V/90M	–	Prim/NL43	Mono/SCID	[210]

Table 2. (cont.)

24I/46I/53L/ 63P/77I/82A	–	Prim/R8	Mono/Enz	[24]
54V/63P/71T/ 72E/82A/85V	–	HXB2	Mono	[195]
10I/36I/48V/84V/ 90M+MA/CA+p1/p6**	–	NL43	Mono/Enz	[144]
10I/54V/63P/71V/ 77I/82A/90M	–	Prim/NL43	Mono/SCID	[210]
14V/20R/32I/63P/ 64V/71V/82A	–	HXB2	Enz	[153]
10I/20R/36I/46L/ 48V/71V/82A/90M	–	Prim/NL43	Mono/SCID	[210]
10I/20R/36I/54V/ 63P/71V/82T/90M	–	Prim/NL43	Mono/SCID	[210]
10I/24I/46I/63P/ 71V/77I/82T/84V	–	Prim/NL43	Mono/SCID	[210]
10I/36I/46L/48V/ 63P/71V/82A/90M	–	Prim/NL43	Mono/SCID	[210]
54V/63P/71T/72E/82A/ 85V/85V/+p7/p1**	.	HXB2	Mono	[195]
10I/20R/36I/46I/53L/ 63P/71V/82A+ NCp1?**	–	NL43	Mono/Enz	[239]
23I/32I/46I/47V/54M/ 71V/84V+p1/p6+ p7/p1**	–	Sel/NL43/HXB2	Mono/Comp/Enz	[40;62;135]
10I/35D/37D/48V/ 54V/63P/71V/ 82A/90M/93L	–	HXB2	Enz	[153]
10I/14V/33F/36M/ 37C/54V/63P/67F/ 71V/72M/73S/77I/ 82A/84V/90M	–	HXB2	Enz	[153]

[a] Symbols: "–" decreased; "+" increased; "." comparable.

[b] HIV–1 isolates or pseudotyped viruses used to measure viral fitness (replicative capacity). Prim, HIV–[1] primary isolate; Sel, *in vitro* selection of drug–resistant strains in the presence of the corresponding antiretroviral drug; NL43, HXB2, and R8 correspond to the recombinant infectious clone used as a backbone to introduce drug–resistance associated mutations by site–directed mutagenesis or pseudotyping; n.a., not apply.

[c] Methods used to determine viral fitness. Vivo, *in vivo* determination of HIV–1 kinetics in plasma; Mono, *ex vivo* HIV–1 monoinfections (*i.e.*, viral growth kinetics and single–cycle infection assays); Comp, *ex vivo* HIV–1 growth competition experiments; Enz, catalytic activities of HIV–1 enzymes; SCID, SCID–hu Thy/Liv or hu–PBL–SCID mice as animal models.

* Insertion mutations at the corresponding codon position.

** Mutations in the protease gene accompanied for mutations in the Gag–processing sites.

Reverse transcriptase inhibitor (RTI)-resistant variants

Multiple studies have explored how RTI-resistant mutations affect HIV-1 replication capacity (Table 3, Fig. 4) (reviewed in [34;164]). Interestingly, amino acid changes conferring RTI-resistance do not appear to reduce viral fitness to the same extent as PI-resistance mutations [13;34]. This may be due in part to a restricted evolution in RT. However, there are other factors/attributes that differ between HIV-1 resistance to PI and to RTI. These include, (i) the appearance of discrete NRTI- or NNRTI-resistant mutations rather than a collection of PI-resistant substitutions during selection, (ii) minimal secondary/compensatory mutations selected during NRTI- or NNRTI-treatment as compared to PI-therapy, (iii) a decrease in relative fitness associated with increasing number of NRTI-resistant mutations (*e.g.*, AZT-resistant mutations) while the opposite is generally true with PI-resistance (*i.e.*, increase in viral fitness due to accumulation of compensatory mutations), and (iv) limited cross-resistance encoded by specific NRTI-resistant mutations as compared to a broad PI cross-resistance conferred by mutations selected during specific PI therapy. It is important to note that these are general observations and do not apply to HIV-1 resistance to all drugs in the NRTI, NNRTI, or PI classes. For example, almost all NNRTI-resistant mutations confer cross-resistance to all other drugs in this class. The next section will review studies on the fitness of RTI-drug resistant HIV-1.

a) Nucleoside reverse transcriptase inhibitors (NRTI)

Nucleoside analogue reverse transcriptase inhibitors (NRTI) were the first class of antiretrovirals to be approved for anti-HIV-1 therapy [137]. These drugs compete for binding to RT with the native deoxynucleoside triphosphates (dNTPs), can be incorporated into elongating HIV-1 DNA, and result in chain termination [6]. To date, six NRTI have been approved for therapy in the United States; *i.e.*, zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), and abacavir (ABC). Tenofovir (TNV) a nucleotide-RT-inhibitor has recently received approval from the FDA. In general, different pairs of NRTI remain the base component of all combination treatment regimens.

It is not surprising that some of the first studies showing the effect of drug-resistance mutations on viral replication fitness were related to AZT [90]. In AZT-experienced patients, the wild-type 215T HIV-1 isolate is more fit in the absence of AZT, can re-emerge from the diverse quasispecies and out-compete the AZT-resistant 215Y strain. This situation appears to be a replacement rather than a true genetic reversion but may be more representative of *ex vivo* fitness or replicative capacity. *In vivo* results showing that 215T, 215D, and 215S are equally fit [87] may be due to infection with a homogeneous HIV-1 population, specific mutational pathways in the absence of drug, and the 215T virus as competitor. A stepwise accumulation of AZT resistance mutations (70R, 215Y, and 41L) during *in vitro* selection was similar to that observed *in vivo* [96]. Interestingly, ordered accumulation of resistant variants was also predictive of relative fitness (*i.e.*, wt > 70R >> 215Y = 41L/215Y > 41L). Of course, this fitness-drug resistance relationship is inverse to that observed with the accumulation of primary and secondary PI-resistant mutations. However, each new AZT-resistant mutation confers a greater level of AZT-resistance (*i.e.*, 10-fold with 70R to >100-fold with 41L, 67N, 70R, 215Y, 219L) whereas the accumulation of compensatory mutations (*e.g.*, within the PR gene or at the protease cleavage sites) may improve fitness but have limited or no effect on susceptibility to PI.

Unlike other NRTI, AZT selects for mutations outside the RT domains involved in polymerase activity [6]. Residues M41, T215, and K219 reside adjacent but still external to the dNTP or primer binding sites. The role of the 41L mutation in resistance may be more related to enzyme stability [125]. In contrast, the 215Y and 219E substitutions appear to play a role in the reversal of pyrophosphorolysis responsible for removing AZT-MP from the primer terminus. Interestingly, the addition of low AZT concentrations to cell cultures resulted in increased replication of an AZT-resistant 41L/215Y HIV-1 clone, but did not affect growth of the 70R AZT-resistant HIV-1 virus and inhibited the wild-type virus [5]. Similar results were observed with AZT-resistant clinical isolates containing a combination of AZT-resistance mutations [5]. Although 70R is the first AZT-resistant mutation selected during AZT therapy, the less fit 215Y genotype eventually predominates the quasispecies. Thus, the AZT-mediated stimulation of the 215Y as opposed to 70R HIV-1 isolate may play a role in the *in vivo* selection of 215 mutants.

Table 3. Effects of RT mutations on HIV-1 replication capacity

Amino acid substitutions	Fitness relative to wild–type[a]	Virus[b]	Method[c]	References
41L	–	HXB2	Comp	[96]
62V	.	HXB2	Mono	[143]
67N	–	n.a.	Vivo	[56]
69SS*	–	HXB2	Vivo/Comp	[142;190]
70R	.	NL4	Mono/Comp	[204]
70T,R	–	NL43/HXB2	Mono/Comp	[96;204]
74V	.	HXB2	Mono	[143]
74V	–	NL43	Mono/Comp/Enz	[204;205]
75I	.	HXB2	Mono	[143]
77L	.	HXB2	Mono	[143]
89K	–	HXB2	Mono/Comp	[212]
92I	–	HXB2	Mono/Comp	[212]
98G	–	NL43	Mono	[110]
100I	–	NL43/HXB2	Mono/Comp	[110;191]
103N	.	NL4	Vivo/Mono	[56;110]
103N	–	NL43	Mono/Enz	[88]
106A	–	Sel/NL43	Mono/Comp/Enz	[2;108;110]
108I	.	NL43	Mono	[110]
115L,A,D,W	–	HXB2	Mono/Enz	[148]
116T	.	HXB2	Mono	[143]
151M	.	HXB2	Mono	[143]
151M	+	HXB2	Comp	[128]
151M,L	–	HXB2	Mono/Comp	[87]
156A	–	HXB2	Mono/Comp	[212]
163N	+	HXB2	Mono/Comp	[113]
179D	–	NL43	Mono/Comp/Enz	[2]
181C	.	NL43	Mono	[110]
181C	–	NL43	Mono/Comp/Enz	[2]
181C	+	Sel	Comp	[108]
184I,V	–	Sel/NL43/HXB2	Vivo/Mono/Comp/ Enz/SCID	[8;56;85;120;139; 149;177;205;236]
188C	.	NL43	Mono	[110]
190S,A	.	Sel/NL43	Mono/Comp	[108;110]
215Y,S,D	.	HXB2	Mono/Comp	[45;143]
215Y,F,N,S,D	–	HXB2	Vivo/Mono/Comp	[45;68;90;91; 96;128]
215S	+	HXB2	Comp	[128]
236L	–	NL43	Mono/Comp/Enz	[2;67;88]
501W,R	–	HXB2	Mono/Enz	[3]
41L/70R	–	HXB2	Mono/Comp	[113]
41L/215Y	–	HXB2	Comp	[96]
115W/230I	–	HXB2	Mono/Enz	[169]
151M/215Y	–	HXB2	Comp	[128]
Δ67/69G/74I	–	NL43	Mono	[110]
77L/116Y/151M	.	HXB2	Mono	[143]

Table 3 (cont.)

77L/116Y/151M	+	HXB2	Comp	[128]
67N/70R/ 215Y/219Q	+	NL43	Mono/Enz	[26]
75I/77L/ 116Y/151M	.	HXB2	Mono	[143]
75I/77L/ 116Y/151M	–	HXB2	Comp	[128]
62V/75I/77L/ 116T/151M	.	HXB2	Mono	[143]
62V/75I/77L/ 116Y/151M	+	HXB2	Comp	[128]
69G/70R/74I/ 103N/215F/219Q	–	NL43	Mono/Comp	[109]
Δ67/69G/70R/74I/ 103N/215F/219Q	.	NL43	Mono/Comp	[109]

[a] Symbols: "–" decreased; "+" increased; "." comparable.

[b] HIV–1 isolates or pseudotyped viruses used to measure viral fitness (replicative capacity). Prim, HIV–[1] primary isolate; Sel, *in vitro* selection of drug–resistant strains in the presence of the corresponding antiretroviral drug; NL43, HXB2, and R8 correspond to the recombinant infectious clone used as a backbone to introduce drug–resistance associated mutations by site–directed mutagenesis or pseudotyping; n.a., not apply.

[c] Methods used to determine viral fitness. Vivo, *in vivo* determination of HIV–1 kinetics in plasma; Mono, *ex vivo* HIV–1 monoinfections (*i.e.*, viral growth kinetics and single–cycle infection assays); Comp, *ex vivo* HIV–1 growth competition experiments; Enz, catalytic activities of HIV–1 enzymes; SCID, SCID–hu Thy/Liv or hu–PBL–SCID mice as animal models.

* Mutation at codon 69 (usually 69S), followed by an insertion of 2 or more amino acids (69 insertion complex).

Lamivudine-resistant viruses, harboring the 184V mutation, appear to have increased RT fidelity [79;171], diminished RT processivity and reduced replication capacity [8;132]. Back *et al.* [8] were among the first to report impaired replicative fitness of viruses carrying 3TC-resistance mutations. Viruses harboring the 184V mutation are more fit and show higher levels of resistance [8;85]. Thus, eventual outgrowth of the 184V 3TC-resistance variant is inevitable. Recent studies suggest that the combination of 184V and AZT-resistant mutations (specifically 215Y) may be incompatible for various RT activities [89]. In general, therapy with multiple nucleoside analogs has exhausted the possible sites for NRTI-resistant mutations in the RT-coding region, all of which reduce fitness. However, most NRTI-resistant mutations have a very modest effect on RT activity. In fact, there is a little evidence of any debilitating effect of AZT-resistant mutations on any HIV-1 RT activity *in vitro*. Thus, the minor decrease in fitness observed in HIV-1 clones containing NRTI-resistant mutations or pseudotyped with an NRTI-resistant RT-coding region may be overcome by secondary mutations increasing the fitness of other steps in the retroviral life cycle. Few studies have explored this possibility. Interestingly, reversion of most NRTI-resistant mutations is slower than most PI-resistant substitutions in the absence of therapy implying some compensation.

b) Nonnucleoside reverse transcriptase inhibitors (NNRTI)

Nonnucleoside reverse transcriptase inhibitors (NNRTI) are non- or un-competitive inhibitors that

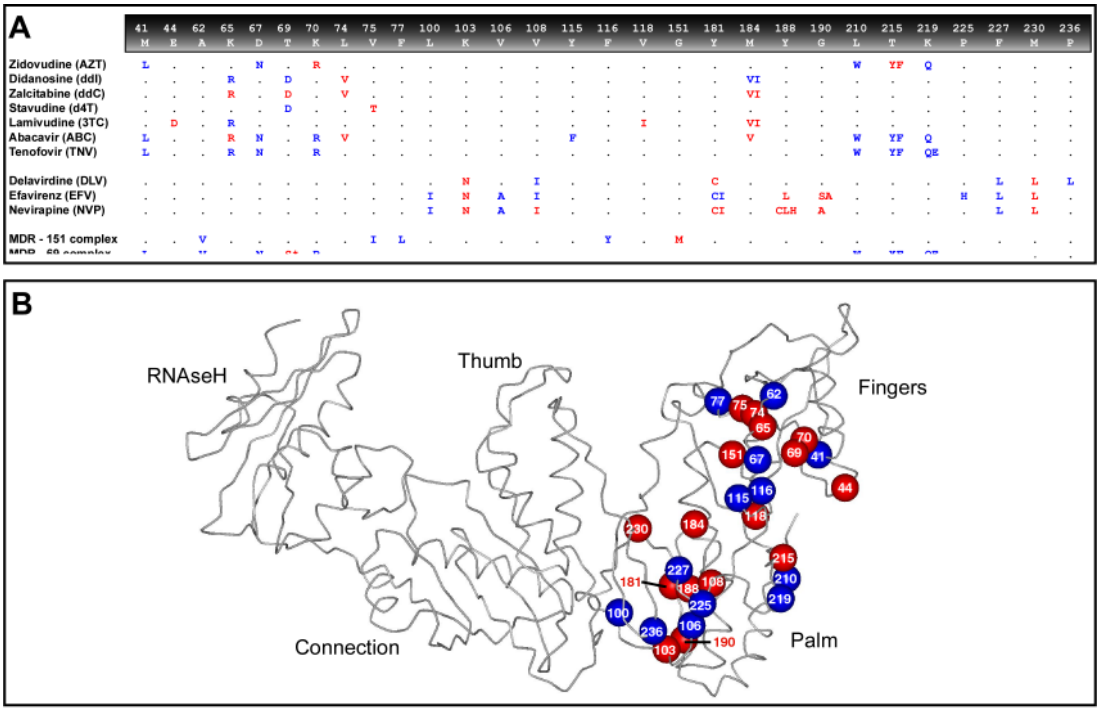


Figure 4. *(A) Summary of reverse transcriptase mutations associated with resistance to RT inhibitors. Amino acids in red and blue denote primary and secondary/compensatory mutations, respectively. Wild-type amino acids (HIV-1 subtype B) at the codons related to resistance to RTI are indicated. Mutations related to loss in sensitivity to RTI [99;203] were recently reviewed at http://www.iasusa.org. (B) Structure of the HIV-1 RT (pdb file: 3hvt) [223], and positions of each amino acid residue associated with resistance to RTI. Each numbered circle indicates codon position and the nature of the resistance mutation (primary and secondary mutations in blue and red, respectively).*

bind to a hydrophobic pocket adjacent to the polymerase active site of RT [49]. This binding inhibits DNA polymerization by an allosteric change of the polymerase active site [73]. Mutations in this hydrophobic binding pocket are rapidly selected during NNRTI-therapy [49;203] (Fig. 4). Three NNRTI are currently approved for antiretroviral therapy in the United States: delavirdine (DLV), efavirenz (EFV) and nevirapine (NVP). Presence of NNRTI resistance mutations results in a slight decrease in RT polymerase activities relative to RNase H activity of the enzyme [2;88]. However, the level of drug resistance *in vitro* does not always correlate with the likelihood that a drug-resistant variant will emerge *in vivo*. The viral replicative capacity of NNRTI-resistant viruses has not been extensively studied. Available data suggest that single-point mutations such as 103N or 181C, selected during NNRTI treatment, have limited effects on viral fitness but confer high level resistance and persist in the absence of drug pressure. Unlike other antiretroviral drugs, the genetic barrier to NNRTI resistance is minimal and is found at high frequencies in the HIV-1 quasispecies in the absence of therapy. The NNRTI binding pocket has only a structural role in the catalytic activity of RT in the absence of drug. Thus, changes that maintain the general hydrophobicity and architecture of the pocket will likely confer high level resistance without a negative impact on enzyme function. Interestingly, related lentiviral polymerases (*e.g.*, HIV-1 group O and HIV-2) sharing conserved structural RT domains are resistant to NNRTI [55;186;189]. Most HIV-1 group O isolates are intrinsically resistant to NNRTI due to the presence of three amino acid substitutions (*i.e.*, 98G, 179E, and 181C) in RT [186], which obviously do not affect the wild-type fitness.

Several studies have described a decreased replicative capacity in viruses carrying mutations associated with nevirapine (*i.e.*, 103N and 181C) [97] and efavirenz (*i.e.*, 100I, 103N, and 190S) [215] resistance. However, the high level of delavirdine resistance conferred by the 236L mutation is insufficient to compensate for its decreased fitness compared to other NNRTI resistance mutants (*e.g.*, 103N or 181C). It may also explain why 236L is selected infrequently during DLV therapy [53;65;67;88]. In most cases, the major NNRTI-resistant mutations 103N and 181C have a minimal impact on viral fitness, which could explain for the rapid failure of NNRTI when used in suboptimal treatment regimens. A recent analysis of *in vitro* selected nevirapine-resistant variants showed that, in the absence of drug, the 181C mutant was more fit than the wild-type virus (fitness gradient: 181C > wt > 106A > 190A) [108]. It is difficult to explain why a drug-resistant substitution that confers increased fitness is not found as the wild-type sequence. Detailed studies on RT activity suggest that several of these mutations may actually increase fidelity thereby slowing the mutation rates. Although a small decrease in mutation frequency will have little impact on intrapatient population size, avoidance of specific selective pressures (*e.g.*, immune response) requires constant evolution (*i.e.*, accumulation of specific mutations).

Multiple dideoxynucleoside resistance (MDR) variants

It is well known that current antiretroviral agents have achieved a remarkable reduction in HIV-related morbidity and mortality. However, a recent study showed that at least 50% of HIV-positive individuals in the United States are infected with drug-resistant variants [194]. Thus, a "second epidemic" of AIDS may involve patients who harbor drug-resistant HIV-1 strains. Prevalence of drug resistant mutations in patients with primary HIV-1 infection has been assessed in several studies conducted in Europe [183] and in the United States [134]. Clinical and virological consequences of primary HIV-1 infection with drug resistant viruses may include suboptimal treatment responses, reduced viral fitness, and the potential for transmission of drug-resistant virus [134]. Moreover, MDR strains acquired during primary HIV infection can persist for more than nine months, despite decreased viral fitness [22].

Accumulation of multiple mutations associated with MDR does not occur at random but follows a sequential order [118]. The 151M mutation is thought to be the first MDR mutation selected in RT, followed by 75I, 77L, 116Y, and then 62V (also know as the MDR-151 complex) [203;207]. As discussed before, HIV-1 can evolve under drug pressure by selecting drug-resistance mutations at the expense of viral fitness, but generally compensate for this defect with secondary mutations. Few studies have documented a replicative advantage of drug-resistant variants over wild-type viruses in the absence of drugs (Tables 2 and 3). Several studies have assessed the *in vitro* fitness of viruses containing the MDR-151 complex [128;143], but specific combinations of these mutations actually appear more fit than the wt in the absence of drug [128;143]. Garcia-Lerma *et al.* [87] evaluated the replication capacity of different MDR recombinant HIV-1 clones carrying the 151M mutation along with two intermediate mutations (151L or 151K). They showed that a virus harboring the 151M mutation was more fit than the 151L. Thus, the 151L mutants may be an intermediate of these MDR variants.

Another set of mutations associated with high-level resistance to multiple RT inhibitors centers around the 69G substitution (MDR-69 complex) [99] (Figure 4). An amino acid deletion at codon 67 (Δ67) improves the replication efficiency of a highly AZT-resistant virus (Δ67/69G/70R/74I/103N/215F/219Q) to wild-type levels [109]. Recent studies have also reported a rare nucleotide insertion in the reverse transcriptase gene between codons 69 and 70 (*e.g.*, 69SS) that results in high-level resistance to multiple NRTI, including AZT, 3TC, d4T, ddI, and ddC [23;154;229]. Decreased viral fitness in the absence of NRTI is quite evident in HIV-1 clones harboring a dipeptide insertion (69SS) in RT [190]. Finally, Lukashov *et al.* [142] studied the *in vivo* evolution of a MDR HIV-1, which contained an insertion of 2 amino acids between positions 68 and 69, and several other mutations within RT. These mutants were replaced by wild-type variants following cessation of therapy, indicating a competitive advantage of the wild-type over the insertion mutant in the absence of selective drug pressure (16% less fit than the wt virus). However, these MDR mutants were able to maintain high viral loads in the presence of antiretroviral therapy.

Resistance to other HIV-1 inhibitors

Within each drug class targeting PR or RT, there is extensive cross-resistance. For example, 103N in RT confers high-level resistance to the other three NNRTI, 90M in PR results in some degree of resistance to all four available PI, and 151M in RT confers resistance to every NRTI except 3TC. New drugs in development target different steps in the virus life cycle, including host cell entry and viral integration. There are three classes of drugs that interfere with HIV-1 entry into host cells, and are classified as inhibiting: (i) HIV-1 envelope glycoprotein (gp120) interaction with its CD4 receptor [112], (ii) gp120 binding to the chemokine receptors CCR5 or CXCR4 [35;208], or (iii) the fusion of the viral and cellular membranes [121].

Identification of the major HIV-1 co-receptors was facilitated by the initial finding that some (β-chemokines, *i.e.* RANTES, macrophage inflammatory protein-1α (MIP-1α), and MIP-1β, natural ligands of CCR5 could block infection of nonsyncytium-inducing/CCR5-tropic (NSI/R5) HIV-1 [35]. In contrast, T-cell line tropic isolates, forming cell syncytia during active replication in tumor T cell lines, utilize the CXCR4 co-receptor for entry (SI/X4) [1;54;63;80]. This discovery in the mid 1990 led to rapid development of several drugs that blocked HIV-1 binding or utilization of these co-receptors. The heterogeneous HIV-1 envelope glycoproteins and these RANTES analogs compete for the same CCR5 receptors on the cell surface. Torre *et al*. examined the sensitivity of 18 primary NSI/R5 HIV-1 isolates to AOP-RANTES inhibition and discovered a 30-fold variation in IC_{50} values [217]. Although all of these viruses are considered "wild type" quasispecies, there were also significant variations in *ex vivo* fitness [188]. Considering that the rate of host cell entry appears to control replicative capacity in wild-type viruses [4] (see below), it is quite conceivable that increased viral fitness is also linked to decreased susceptibility to these CCR5 agonists or antagonists.

Several X4 entry inhibitors are now in development (Met-SDF-1β, an α-chemokine analog) or already in clinical trials (AMD3100, a bicyclam compound) [75;235]. Met-SDF-1β inhibits X4 viral entry by a mechanism similar to R5 HIV-1 inhibition by RANTES analogs [75;235]. However, IC_{50} values required for Met-SDF-1β inhibition of X4 HIV-1 isolates are at least 1,000-fold greater than the concentration required for R5 inhibition by the RANTES analogs [235]. AMD3100 is a low molecular weight bicyclam compound that has an IC_{50} value in the low nanomolar concentrations (~1.4 nM) and does not induce receptor signaling [42;75]. Resistance to this drug is conferred by a collection of single amino acid substitutions in the *env* gene or a reduction in the overall positive charge of HIV-1 gp120 V3 loop [42;76]. AMD3100-resistance was not associated with a co-receptor switch (*e.g.* CXCR4 to CCR5 usage) but did have a negative impact on HIV-1 replication [77].

Another class of entry inhibitors was designed to block virus-cell fusion and target conserved fusion domains in gp41. Included in this class of inhibitors are T-20, T-1249, C34, 5helix, and IQN-17 [30;32;70;84;93;111;161]. T-20 and T-1249 are synthetic peptide analogs that bind to the alpha helix bundle region in the gp41 transmembrane domain and prevent formation of a hairpin structure necessary for membrane fusion. Both drugs are currently in clinical trials [30] and appear to act synergistically with co-receptor antagonists and agonists to inhibit HIV-1 *in vitro*. Lu & Kuritzkes [140] demonstrated that the introduction of known T-20-resistant mutations (37T, 38M or 36S/38M) into the gp41 coding region of the $HIV\text{-}1_{NL4\text{-}3}$ clone reduced replication to levels less than the wild-type control (relative fitness order: wt > 37T > 38M > 36S/38M). T-20-resistant HIV-1 isolates are currently being isolated from patients in ongoing clinical trials and utilized in various fitness assays.

Integrase inhibitors (*i.e.*, diketo acids analogs) comprise the most recent class of drugs shown to effectively inhibit HIV-1 replication [230]. Resistance to these compounds is related to specific mutations in the integrase active site (*i.e.*, 153Y, 66I, and 155S), which also impair enzymatic function *in vitro*. Increasing levels of resistance to integrase inhibitors are associated with a significant loss of viral fitness [230]. However, the actual appearance or net effect of any resistant mutation on HIV-1 fitness can only be assessed in HIV-infected patients treated with this drug.

HIV-1 fitness and AIDS

A topic that is often overlooked in HIV-1 research and in scientific literature is the impact of fitness on HIV-1 transmission, disease progression, evolution, and prevalence in the human population. This review has thus far focused on the effect of drug resistance on HIV-1 fitness. However, the vast majority of HIV-1 infections worldwide are not being treated with conventional antiretroviral therapies. The rate of new infections continues to increase, people progress to AIDS, and die and yet little is still known about the phenotypic differences between the heterogeneous etiological agent. Recent studies suggest that the nature of the virus itself, and not solely manifestations of host factors and the immune response is contributing to HIV disease progression [188]. Numerous studies have also shown that there are possible differences in interpatient and intersubtype HIV-1 fitness [4;115;188;209]. However, these fitness studies are plagued by the same difficulties and shortcomings described for research on the viral fitness of drug resistant HIV-1 strains. What is appropriate assay to measure *ex vivo* fitness? Are primary isolates or pseudotyped HIV-1 clones most suitable for accessing differences in fitness? How do we compare *ex vivo* HIV-1 replicative capacity to viral fitness within a patient or even within the human population? Although this field remains controversial, recent studies aided by new technologies are now making progress in answering some of the questions related to HIV-1 fitness and AIDS [4;115;188;209].

Fitness of the transmitted HIV-1 isolate

A significant genetic bottleneck is quite apparent during transmission by any route of HIV-1 infection (see above). However, individuals may be exposed to varying amounts of virus depending on the mode of transmission. Sexual and vertical transmission generally involves small amounts of virus and infected cells, whereas a bolus dose of virus may be transmitted during direct blood-to-blood contact, *e.g.* contaminated needles or blood transfusions [170;232;241]. Regardless, the virus population recovered from primary HIV-1 infections is more homogeneous with a narrow genetic distribution of quasispecies as compared to the donor HIV-1 quasispecies. The transmitted variants are likely less fit than the donor quasispecies due to the genetic bottleneck. The selective factors imposing the bottleneck are diverse and likely include: (i) host factors such as innate immune response, (ii) density of target cells at the site of infection, (iii) number of transmitted virions, and (iv) the structure of transmitted viral quasispecies. Quasispecies distribution and size of the transmitted pool may have a significant effect on fitness of the infecting isolate and subsequent disease progression [60;167]. The viral properties selected during initial infection may not be the same attributes necessary for efficient dissemination and rapid turnover during acute disease. A combination of these viral characteristics may only be found in exposures with a significant load of diverse HIV-1 quasispecies. Finally, environmental differences (pH, target cells, mucosal composition) at the site of exposure may not only affect the efficiency of transmission but also fitness of the infecting isolates [18;170]. For example, there appear to be phenotypic and genotypic differences between HIV-1 variants infecting men and women following heterosexual contact [170].

Although there may be an element of chance in the expansion of a particular HIV-1 clone, phenotypic selection does occur in nearly every HIV-1 infection. SI/X4 HIV-1 isolates often dominate the quasispecies late in disease and yet the NSI/R5 variant is typically transmitted to a recipient (reviewed in [83]). Preferential transmission of NSI/R5 over SI/X4 HIV-1 isolates is contradictory to increased turnover of SI/X4 HIV-1 over NSI/R5 isolates in culture (see below) [15;215]. Thus, efficiency or fitness of transmission is likely not related to the HIV-1 replicative capacity measured in normal cell culture. As described below, this fitness dichotomy is not restricted to co-receptor usage but may also be observed in transmission of different NSI/R5 HIV-1 isolates in the human population [17]. Although *in vivo* findings suggest that NSI/R5 HIV-1 isolates may out-compete the SI/X4 variants at the site of primary infection, one report suggests that the NSI/R5 isolates only predominate after a temporary expansion of SI/X4 HIV-1 isolates is quenched by an activated immune response [38]. However, this observation is difficult to reconcile with the finding that humans who are homozygous for a deletion in the CCR5 gene (*i.e.* lack CCR5 on any cell surface) are typically resistant to HIV-1 infection [48;168]. To date, the

universal factors (*i.e.* found in almost every human host) involved in the selection of NSI/R5 HIV-1 isolates during transmission and asymptomatic disease are not well defined. Langerhans' cells (LC) are found embedded in mucosa (*e.g.* vaginal mucosa) and may be the first cell targets for primary heterosexual transmission [18;209]. LC may play a role in NSI/R5 HIV-1 selection since CCR5 and not CXCR4 is preferentially expressed *in situ* and in the absence of external stimuli [18]. A recent report describes increased replication of an NSI/R5 ($HIV\text{-}1_{Bal}$) over an SI/X4 ($HIV\text{-}1_{III\text{-}B}$) isolate in LC embedded in skin-derived explants even though the opposite is true in PBMC cultures or other permissive cell lines [18;209]. This discrepancy only reinforces the definition of fitness, *i.e.* a measure of an organism's replicative capacity in a given environment.

Impact of HIV-1 fitness on disease progression

Although HIV-1 is the etiological agent of AIDS, it is often assumed that phenotypic characteristics and replication efficiency (*ex vivo* fitness) of the infecting, wild type HIV-1 isolates has little impact on the rate of disease progression. In general, acute HIV-1 infection is followed by a chronic and progressing disease resulting in immunodeficiency and acquisition of various life-threatening opportunistic infections. Multiple studies have tried to compare both, host and viral factors, with HIV-1 pathogenesis and progression to AIDS. The strongest correlates of HIV-1 disease progression include various host immunological and genetic factors: (i) a strong HIV-specific CD8+ cytotoxic lymphocyte throughout disease, and (ii) retention and proliferation of HIV-specific $CD4^+$ lymphocyte response following acute infection [27;28;66;158;172;179;198], (ii) some polymorphisms in the CCR5, RANTES, and SDF-1 promoter regions or altered expression of the chemokines ([47], reviewed by [12]), and (iii) specific HLA class I genes [44]. However, there are several general observations suggesting that HIV fitness may play an important role in disease progression, *i.e.*, (i) HIV-1 load is the best marker to date for disease progression [156], (ii) treatment with highly active antiretroviral therapy (HAART) may reduce viral loads to undetectable levels and delays progression to AIDS indefinitely, and (iii) emergence of drug resistant isolates and rebound in viral load results in resumption of normal disease progression.

In addition to these general observations, there are also three clear examples that HIV-1 phenotype can affect disease progression. First, the faster replicating SI/X4 HIV-1 isolates are generally isolated during AIDS or in late HIV disease, whereas the slower replicating, NSI/R5 strains generally predominate during asymptomatic stages [7;14;215]. Several studies have now shown that appearance of SI/X4 isolates does coincide with rapid decline in CD4 cells, a burst in viral load, and the onset of AIDS. However, SI/X4 isolates are inconsistently isolated in late stages of disease and are not a prerequisite for progression or AIDS [32;83;202]. Using a mathematical model, Wodarz & Nowak [231] suggested that the evolution of SI/X4 strains depends on the fitness landscape of the HIV-1 quasispecies. Variation in intrapatient HIV-1 evolution and the fitness landscape would explain why SI/X4 viruses only appear in half of HIV-infected individuals. Recently, the dogma that all SI/X4 isolates are more fit in cell culture than NSI/R5 isolates have been challenged by competing several NSI/R5 - SI/X4 pairs in PBMC cultures [188]. Although most SI/X4 were more fit in cell culture, NSI/R5 isolates from rapid progressors could out-compete SI/X4 isolates from long-term survivors or even SI/X4 isolates from patients displaying typical HIV-1 progression [188].

Aside from these changes in co-receptor usage/cell tropism, evidence that HIV-1 genetic alterations could affect disease progression was clearly demonstrated in a few long term nonprogressor patients (LTNP) shown to harbor HIV-1 strains with *nef* deletions [46;124]. Independently of these observations, Daniel *et al.* [41] had generated several HIV-1 clones with similar *nef* deletions, all of which were replication defective in PBMC cultures. Interestingly, several of the LTNP infected with *nef*-deleted viruses have eventually progressed to AIDS (after >10 years of asymptomatic disease) [21]. Changes in HIV-1 fitness during this time period have not been published but would provide valuable information on the possible accumulation of compensatory mutations. It is important to note that a similar rebound in HIV-1 fitness does occur during the emergence of drug-resistant mutations, albeit on a shorter time scale. In these LTNP now progressing to AIDS, the Red Queen Hypothesis would support the notion that slow but continual replication of HIV-1 would expand the quasispecies diversity and lead to increased

fitness. Only genetic bottlenecks due to changes in selective pressure could reverse this trend (*i.e.* Muller's Ratchet).

As described above, several viral parameters such as infectious dose, route of infection, and viral fitness may be contributing to the clinical course of HIV-1 infection [36;141;216;241]. Subtle differences in viral fitness may also contribute to HIV-1 pathogenesis and even overwhelm an HIV-specific immune response. In an attempt to correlate *ex vivo* HIV-1 replicative capacity and disease progression, several studies have compared the replication differences of primary HIV-1 isolates using mono-infections of PBMC or a $CD4^+$ tumor cell line [16;82;202;215]. Variations in the micro-environment of tissue culture systems, the lack of an internal control, and the possibility of a high virus titer saturating the target cells all lead to inconsistent results with mono-infections. Moreover, a moderate fitness cannot be accurately measured in mono-infections because of the inherent variability between cultures. This may explain why this assay has only characterized drastic defects in the replication kinetics of HIV-1 isolates from long-term nonprogressor (LTNP). Several studies have examined the replication kinetics of HIV-1 isolated from patients with atypical progression [16;82;202]. Using a mono-infection assay with primary HIV-1 isolates, Blaak *et al*. [16] showed that some LTNP harbored NSI isolates with slow replication kinetics. A recent study competed HIV-1 isolates from long-term survivors (LTS) and typical progressors (PRO) with four reference HIV-1 strains (*i.e.* primary isolates) [188]. PRO HIV-1 isolates had out-competed the reference strains in growth competition experiments while the opposite was observed for HIV-1 isolated from long-term survivors. Interestingly, all patient isolates and reference HIV-1 strains had similar replication kinetics in PBMC monoinfections. These results suggest that regardless of the viral phenotype (NSI or SI), HIV-1 isolates from long-term survivors were less fit than strains obtained from progressors [188].

The relative fitness values of each LTS and PRO isolate, derived from competitions with four reference strains, showed strong correlations with viral RNA load. Comparisons of viral load vs. *ex vivo* fitness indicated that the linear regressions derived from LTS and PRO analyses had similar slopes but did not intersect [188]. This data support earlier findings that the fitness of the infecting HIV-1 isolate may predict the clinical course of disease [141]. In a current model, decreased genetic diversity in donor quasispecies and low infectious dose during transmission result in infection with an HIV-1 clone of reduced fitness. Rosenberg *et al*. [198] have suggested that rapid depletion of $CD4^+$ cells during acute/early infection results in an irreplaceable loss of HIV-specific T helper cells. In contrast, proliferation and retention of HIV-specific T helper cells during early disease is associated with slower disease progression [198]. Thus, it is quite conceivable that infection with an isolate of poor replicative capacity may lead to limited depletion of $CD4^+$ cells and retention of HIV-specific T helper cells. Slower disease progression may be due to a combination of an enhanced HIV-specific immune response and reduced HIV-1 fitness. However, the model also predicts that continual HIV-1 replication and evolution will eventually increase genetic diversity and the fitness of the quasispecies. This may explain why even LTNP fail to eradicate virus and eventually progress to AIDS. It is important to note that infection with an HIV-1 clone outside of the normal distribution of quasispecies would be rare which could explain the low frequency of atypical progression (*e.g.* LTS or rapid progressors).

Although the impact of HIV-1 fitness on disease progression is still not well understood, studies on SIV pathogenesis have provided valuable information about the disease process that leads to simian AIDS (reviewed in [227]). Using the SIV model, Kimata *et al* [123] showed that antigenic and cytopathic properties of the SIV strain and not phenotype (*e.g.* SI vs. NSI) predict fitness in the host. Emerging SIV variants have increased replicative capacity over the SIV strain used in the initial infection. Furthermore, infectious dose and virulence ("viral fitness") of the initial inoculum did influence viral load during disease progression in SIV-infected macaques [104].

Are there intersubtype HIV-1 variations in viral fitness?

Divergent interpatient HIV-1 evolution coupled with new introductions into susceptible human populations has lead to the current HIV-1 diversification from original zoonotic jumps in central Africa

[95;127]. Although a founder effect results in further HIV-1 spread and divergent evolution in different regions in the world, the phylogenies of most non-African HIV-1 strains can be traced to central African isolates [95;127]. At least two to three separate zoonotic jumps from chimpanzees into humans led to the disproportionate spread of HIV-1 groups M (main), O (outlier), and N (non-M/non-O) [86;95;131]. Increased fitness has likely played a key role in the predominance and extreme variation of HIV-1 group M over group N or O isolates. Phylogenetic and recombination analyses further subdivided the HIV-1 group M into nine subtypes (A, B, C, D, F, G, H, J, and K) and fourteen circulating recombinant forms (CRF) (reviewed in [175;185]). These subtypes and CRFs are unequally distributed across the globe, *e.g.* subtype B in the Americas and Europe whereas A, C, and CRF02-AG are the most prevalent clades in Africa [175;185]. To date, CRF have been identified in nearly every region of the world where two or more subtypes co-circulate and may account for over 10% of new HIV-1 infections [126;175;185].

The proportions of subtypes in defined populations are not stable but are in constant flux due to new introductions of HIV-1 subtypes, changes in human behavior, therapeutic intervention, mode of transmission, and possibly, subtype fitness. Over the past decade, there has been a considerable shift in the epicenter of the HIV-1 epidemic from Sub-Saharan Africa to Southern Africa, India, and Southeast Asia [74;114;136;200]. Subtype C has now emerged as the predominant clade in the world due to these regional pandemics and accounts for at least half of all infections worldwide [105;196]. Although subtype B likely preceded subtype C as a founder clade in India and China, most of the new infections in these countries are attributable to subtype C isolates or intersubtype B/C recombinants [178;196]. Similar trends have been observed in Kenya, Tanzania, and South America (*e.g.* Brazil and Argentina) [74;105;136;184;219]. This rapid insurgence of subtype C may be due to a founder effect or to intersubtype fitness differences.

Any difference in *ex vivo* fitness likely reflects genotypic or phenotypic variations between subtypes. For example, most subtype C isolates appear to have an extra or third NFkB element in the LTR as compared to the two sites found in most subtype, which augment transcription in the presence or absence of HIV-1 Tat protein [107;196;218]. A subsequent study on HIV-1 protease activity showed increased cleavage of peptide substrates by HIV-1 subtype C versus subtype B protease [220]. This phenotypic data suggests that dominance of subtype C in the HIV-1 epidemic could be due to increased replicative capacity of subtype C isolates over other HIV-1 subtype isolates. In addition to these phenotypic differences, subtype C isolates rarely switch from a NSI/R5 phenotype to an SI/CXCR4 phenotype during late disease [29]. However, a recent study has compared the fitness of nine subtype B and six subtype C HIV-1 isolates in a pair-wise competition experiment in PBMC culture [4]. The relative fitness values were not statistically different in pair-wise competitions involving isolates of the same subtype (intrasubtype B or C competitions). In contrast, almost all of the subtype C isolates were out-competed by the subtype B isolates in the pair-wise competitions. In contrast to previous reports with recombinant HIV-1 clones containing fragments of the subtype C genome, this data suggests that subtype C isolates are likely less fit than subtype B isolates [4]. The poor relative fitness of subtype C isolates can also fit into model for subtype C dominance in the epidemic. Decreased fitness is linked to slower disease progression, which could result in increased in transmission time [188].

Is the fitness and pathogenicity of HIV-1 decreasing?

It is apparent that some simian immunodeficiency viruses (SIV) have become highly adapted to their host species over an extended period of time, resulting in asymptomatic, non-pathogenic infections [227]. Based on the subtype C fitness results, is it possible that HIV-1 is evolving to an attenuated state? This hypothesis was proposed by Temin more than ten years ago [213]. Rapid attenuation of virus was first observed in the myxoma virus infection of Australian rabbits [78]. Several introductions of this highly lethal virus resulted in a decrease of nearly half of the rabbit population followed by rapid evolution of a less virulent (fit) virus [81]. When a virus "jumps" and infects a new species, an increase in the initial virulence can only be supported in minor pandemics (*e.g.* Ebola pandemics). It is evident that to survive, viruses must continue to propagate in a living host and that a low/attenuated level of pathogenesis

represents a tradeoff between virulence and transmissibility. Ewald [78] suggested that more virulent viruses would rapidly kill their hosts reducing the time for transmission and permitting the expansion of less virulent viruses that were still capable of establishing new infections. In the case of HIV-1, the continual spread of this lethal virus is a consequence of long asymptomatic but transmissible periods following initial infection. Individuals infected with a more pathogenic (high replication) strain will progress faster to HIV-1 disease, decreasing the probability of viral transmission. In contrast, attenuated HIV-1 strains (*i.e.* lower replicative capacity) would in theory delay disease progression and increase the likelihood of transmission. Based on this theory, changes in HIV-1 pathogenesis in the population may not be easily identified by common correlates of disease progression (*e.g.* viral load and CD4 cell counts). For example, the nonpathogenic infection of Sooty mangabeys monkeys with SIV [193] results in extremely high viral load while maintaining transmissibility in the absence of symptomatic disease. Thus, the higher viral loads observed in patients infected with subtypes B or A as compared to subtypes C or D (respectively) may be unrelated to the effect of subtype on disease progression.

Acknowledgments

We would like to thanks Drs. Esteban Domingo, Daniel R. Kuritzkes, and Richard D'Aquila for their critical reading and valuable comments to this review. M.E.Q-M was supported by research grants from the National Heart, Lung, and Blood Institute, NIH (5-KO1-HL67610-02) and the NIH Center for AIDS Research (AI36219) at Case Western Reserve University. E.J.A. was supported by research grants from the National Institute of Child Health and Development, NIH (NO1-HD-0-3310-502-02) and from the National Institute of Allergy and Infectious Diseases, NIH (AI49170)

References

1 Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* 1996; **272**:1955–1958.

2 Archer RH, Dykes C, Gerondelis P, Lloyd A, Fay P, Reichman RC, Bambara RA, Demeter LM. Mutants of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase resistant to nonnucleoside reverse transcriptase inhibitors demonstrate altered rates of RNase H cleavage that correlate with HIV-1 replication fitness in cell culture. *J Virol* 2000; **74**:8390–8401.

3 Arion D, Sluis-Cremer N, Min KL, Abram ME, Fletcher RS, Parniak MA. Mutational Analysis of Tyr-501 of HIV-1 Reverse Transcriptase. Effects on ribonuclease H activity and inhibition of this activity by n- acylhydrazones. *J Biol Chem* 2002; **277**:1370–1374.

4 Arts EJ, Ball SC, Quinones-Mateu ME, Marozsan A. Competition assays clearly indicates that subtype C HIV-1 isolates are less fit than isolates of other subtypes. 1st IAS Conference on HIV Pathogenesis and Treatment, Buenos Aires, Argentina, July 2001.

5 Arts EJ, Quinones-Mateu ME, Albright JL, Marois JP, Hough C, Gu Z, Wainberg MA. 3′-Azido-3'-deoxythymidine (AZT) mediates cross-resistance to nucleoside analogs in the case of AZT-resistant human immunodeficiency virus type 1 variants. *J Virol* 1998; **72**:4858–4865.

6 Arts EJ, Wainberg MA. Mechanisms of nucleoside analog antiviral activity and resistance during human immunodeficiency virus reverse transcription. *Antimicrobial Agents and Chemotherapy* 1996; **40**:527–540.

7 Asjö B, Morfeldt-Manson L, Albert J, Biberfeld G, Karlsson A, Lidman K, Fenyˆ EM. Replicative capacity of human immunodeficiency virus from patients with varying severity of HIV infection. *The Lancet* 1986; ii:660–662.

8 Back NK, Nijhuis M, Keulen W, Boucher CA , Oude Essink BO, van Kuilenburg AB, van Gennip AH, Berkhout B . Reduced replication of 3TC-resistant HIV-1 variants in primary cells

due to a processivity defect of the reverse transcriptase enzyme. *EMBO J* 1996; **15**:4040–4049.
9 Bally F, Martinez R, Peters S, Sudre P, Telenti A. Polymorphism of HIV type 1 gag p7/p1 and p1/p6 cleavage sites: clinical significance and implications for resistance to protease inhibitors. *AIDS Res Hum Retroviruses* 2000; **16**:1209–1213.
10 Barbas CF, III, Hu D, Dunlop N, Sawyer L, Cababa D, Hendry RM, Nara PL, Burton DR. *In vitro* evolution of a neutralizing human antibody to human immunodeficiency virus type 1 to enhance affinity and broaden strain cross-reactivity. *Proc Natl Acad Sci U S A* 1994; **91**:3809–3813.
11 Barrie KA, Perez EE, Lamers SL, Farmerie WG, Dunn BM, Sleasman JW, Goodenow MM. Natural variation in HIV-1 protease, Gag p7 and p6, and protease cleavage sites within gag/pol polyproteins: amino acid substitutions in the absence of protease inhibitors in mothers and children infected by human immunodeficiency virus type 1. *Virology* 1996; **219**:407–416.
12 Berger EA, Murphy PM, Farber JM. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu Rev Immunol* 1999; **17**:657–700.
13 Berkhout B. HIV-1 evolution under pressure of protease inhibitors: climbing the stairs of viral fitness. *J Biomed Sci* 1999; **6**:298–305.
14 Bjorndal A, Deng H, Jansson M, Fiore JR , Colognesi C, Karlsson A, Albert J, Scarlatti G, Littman DR, Fenyo EM. Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. *J Virol* 1997; **71**:7478–7487.
15 Bjorndal A, Deng H, Jansson M, Fiore JR , Colognesi C, Karlsson A, Albert J, Scarlatti G, Littman DR, Fenyo EM. Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. *J Virol* 1997; **71**:7478–7487.
16 Blaak H, Brouwer M, Ran LJ, de Wolf F, Schuitemaker H. *In vitro* replication kinetics of human immunodeficiency virus type 1 (HIV-1) variants in relation to virus load in long-term survivors of HIV-1 infection. *J Infect Dis* 1998; **177**:600–610.
17 Blackard JT, Renjifo B, Fawzi W, Hertzmark E, Msamanga G, Mwakagile D, Hunter D, Spiegelman D, Sharghi N, Kagoma C, Essex M. HIV-1 LTR subtype and perinatal transmission. *Virology* 2001; **287**:261–265.
18 Blauvelt A, Glushakova S, Margolis LB. HIV-infected human Langerhans cells transmit infection to human lymphoid tissue ex vivo. *AIDS* 2000; **14**:647–651.
19 Bleiber G, Munoz M, Ciuffi A, Meylan P, Telenti A. Individual contributions of mutant protease and reverse transcriptase to viral infectivity, replication, and protein maturation of antiretroviral drug-resistant human immunodeficiency virus type 1. *J Virol* 2001; **75**:3291–3300.
20 Borman AM, Paulous S, Clavel F. Resistance of human immunodeficiency virus type 1 to protease inhibitors: selection of resistance mutations in the presence and absence of the drug. *Journal of General Virology* 1996; **77**:419–426.
21 Brambilla A, Turchetto L, Gatti A, Bovolenta C, Veglia F, Santagostino E, Gringeri A, Clementi M, Poli G, Bagnarelli P, Vicenzi E. Defective nef alleles in a cohort of hemophiliacs with progressing and nonprogressing HIV-1 infection. *Virology* 1999; **259**:349–368.
22 Brenner B, Petrella M, Spira B, Routy J , Wainberg MA. Persistence of multidrug resistant HIV-1 with diminished fitness acquired in primary infection. 1st IAS Conference on HIV Pathogenesis and Treatment, Buenos Aires, Argentina, July 2001.
23 Briones C, Mas A, Gomez-Mariano G, Altisent C, Menendez-Arias L, Soriano V, Domingo E. Dynamics of dominance of a dipeptide insertion in reverse transcriptase of HIV-1 from patients subjected to prolonged therapy. *Virus Res* 2000; **66**:13–26.
24 Buhler B, Lin YC, Morris G, Olson AJ, Wong CH, Richman DD, Elder JH, Torbett BE. Viral evolution in response to the broad-based retroviral protease inhibitor TL-3. *J Virol* 2001; **75**:9502–9508.
25 Burke DS. Recombination in HIV: an important viral evolutionary strategy. *Emerging Infectious Diseases* 1997; **3**:1–9.
26 Caliendo AM, Savara A, An D, DeVore K, Kaplan JC, D'aquila RT. Effects of zidovudine-selected human immunodeficiency virus type 1 reverse transcriptase amino acid substitutions on

processive DNA synthesis and viral replication. *J Virol* 1996; **70**:2146–2153.
27 Cao Y, Qin L, Zhang L, Safrit J, Ho DD. Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. *N Eng J Med* 1995; **332**:201–208.
28 Carotenuto P, Looij D, Keldermans L, de Wolf F, Goudsmit J. Neutralizing antibodies are positively associated with CD4+ T-cell counts and T-cell function in long-term AIDS-free infection. *AIDS* 1998; **12**:1591–1600.
29 Cecilia D, Kulkarni SS, Tripathy SP, Gangakhedkar RR, Paranjape RS, Gadkari DA. Absence of coreceptor switch with disease progression in human immunodeficiency virus infections in India. *Virology* 2000; **271**:253–258.
30 Chan DC, Fass D, Berger JM, Kim PS. Core structure of gp41 from the HIV envelope glycoprotein. *Cell* 1997; **89**:263–273.
31 Chao L. Fitness of RNA virus decreased by Muller's ratchet [see comments]. Nature (London) 1990; **348**:454–455.
32 Cheng-Mayer C, Seto D, Tateno M, Levy JA. Biologic features of HIV-1 that correlate with virulence in the host. *Science* 1988; **240**:80–82.
33 Clarke DK, Duarte EA, Elena SF, Moya A, Domingo E, Holland J. The red queen reigns in the kingdom of RNA viruses. *Proc Natl Acad Sci U S A* 1994; **91**:4821–4824.
34 Clavel F, Race E, Mammano F. HIV drug resistance and viral fitness. *Adv Pharmacol* 2000; **49**:41–66.
35 Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV- suppressive factors produced by CD8+ T cells [see comments]. *Science* 1995; **270**:1811–1815.
36 Coffin JM. Genetic variation in AIDS viruses. *Cell* 1986; **46**:1–14.
37 Coffin JM. HIV population dynamics *in vivo*: implications for genetic variation,pathogenesis, and therapy. *Science* 1995; **267**:483–489.
38 Cornelissen M, Mulder-Kampinga G, Veenstra J, Zorgdrager F, Kuiken C, Hartman S, Dekker J, van Der HL, Sol C, Coutinho R, . Syncytium-inducing (SI) phenotype suppression at seroconversion after intramuscular inoculation of a non-syncytium-inducing/SI phenotypically mixed human immunodeficiency virus population. *J Virol* 1995; **69**:1810–1818.
39 Cote HC, Brumme ZL, Harrigan PR. Human immunodeficiency virus type 1 protease cleavage site mutations associated with protease inhibitor cross-resistance selected by indinavir, ritonavir, and/or saquinavir. *J Virol* 2001; **75**:589–594.
40 Croteau G, Doyon L, Thibeault D, McKercher G, Pilote L, Lamarre D. Impaired fitness of human immunodeficiency virus type 1 variants with high-level resistance to protease inhibitors. *J Virol* 1997; **71**:1089–1096.
41 Daniel MD, Kirchhoff F, Czajak SC, Sehgal PK, Desrosiers RC. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* 1992; **258**:1938–1941.
42 De Clercq E. Inhibition of HIV infection by bicyclams, highly potent and specific CXCR4 antagonists. *Mol Pharmacol* 2000; **57**:833–839.
43 de la Carriere LC, Paulous S, Clavel F, Mammano F. Effects of human immunodeficiency virus type 1 resistance to protease inhibitors on reverse transcriptase processing, activity, and drug sensitivity. *J Virol* 1999; **73**:3455–3459.
44 De Maria A, Moretta L. HLA-class I-specific inhibitory receptors in HIV-1 infection. *Hum Immunol* 2000; **61**:74–81.
45 De Ronde A, van Dooren M, van Der HL, Bouwhuis D, de Rooij E, van Gemen B, de Boer R, Goudsmit J. Establishment of new transmissible and drug-sensitive human immunodeficiency virus type 1 wild types due to transmission of nucleoside analogue-resistant virus. *J Virol* 2001; **75**:595–602.
46 Deacon NJ, Tsykin A, Solomon A, Smith K , Ludford-Menting M, Hooker DJ, McPhee DA, Greenway AL, Ellet A, Chatfield C, Lawson VA, Crowe S, Maerz A, Sonza S, Learmont J, Sullivan JS, Cunningham A, Dwyer D, Dowton D, Mills J. Genomic structure of an attenuated

quasispecies of HIV-1 from a blood transfusion donor and recipients. *Science* 1995; **270**:988–991.

47 Dean M, Carrington M, Winkler C, Huttley GA, Smith MW, Allikmets R, Goedert JJ, Buchbinder SP, Vittinghoff E, Gomperts E, Donfield S, Vlahov D, Kaslow R, Saah A, Rinaldo C, Detels R, Hemophilia growth and development study, Multicenter AIDS cohort study, Multicenter hemophilia cohort study, San Francisco city cohort, ALIVE study, O'Brien SJ. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. *Science* 1996; **273**:1856–1862.

48 Dean M, Carrington M, Winkler C, Huttley GA, Smith MW, Allikmets R, Goedert JJ, Buchbinder SP, Vittinghoff E, Gomperts E, Donfield S, Vlahov D, Kaslow R, Saah A, Rinaldo C, Detels R, O'Brien SJ. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science* 1996; **273**:1856–1862.

49 Deeks SG. International perspectives on antiretroviral resistance. Nonnucleoside reverse transcriptase inhibitor resistance. *J Acquir Immune Defic Syndr* 2001; 26:S25-S33.

50 Deeks SG, Barbour JD, Martin JN, Swanson MS, Grant RM. Sustained CD4+ T cell response after virologic failure of protease inhibitor-based regimens in patients with human immunodeficiency virus infection. *J Infect Dis* 2000; **181**:946–953.

51 Deeks SG, Wrin T, Barbour JD, Martin JN , Hellmann NS, Grant RM, Petropoulos CJ. Evolution of phenotypic drug susceptibility and viral replication capacity during stable protease inhibitor-based therapy despite incomplete viral suppression. 5th International Workshop on HIV Drug Resistance & Treatment Strategies, Scottsdale, Arizona June 2001.

52 Deeks SG, Wrin T, Liegler T, Hoh R, Hayden M, Barbour JD, Hellmann NS, Petropoulos CJ, McCune JM, Hellerstein MK, Grant RM. Virologic and immunologic consequences of discontinuing combination antiretroviral-drug therapy in HIV-infected patients with detectable viremia. *N Engl J Med* 2001; **344**:472–480.

53 Demeter LM, Shafer RW, Meehan PM, Holden-Wiltse J, Fischl MA, Freimuth WW, Para MF, Reichman RC. Delavirdine susceptibilities and associated reverse transcriptase mutations in human immunodeficiency virus type 1 isolates from patients in a phase I/II trial of delavirdine monotherapy (ACTG 260). *Antimicrob Agents Chemother* 2000; **44**:794–797.

54 Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 1996; **381**:661–666.

55 Descamps D, Collin G, Letourneur F, Apetrei C, Damond F, Loussert-Ajaka I, Simon F, Saragosti S, Brun-Vezinet F. Susceptibility of human immunodeficiency virus type 1 group O isolates to antiretroviral agents: *in vitro* phenotypic and genotypic analyses. *J Virol* 1997; **71**:8893–8898.

56 Devereux HL, Emery VC, Johnson MA, Loveday C. Replicative fitness *in vivo* of HIV-1 variants with multiple drug resistance-associated mutations. *J Med Virol* 2001; **65**:218–224.

57 Domingo E, Escarmis C, Menendez-Arias L, Holland J. Viral quasispecies and fitness variations. In: Domingo E, Webster R, Holland J, editors. Origin and evolution of viruses. San Diego: Academic Press, **1999**:141–161.

58 Domingo E, EscarmÌs C, Sevilla N, Moya A, Elena SF, Quer J, Novella IS, Holland JJ. Basic concepts in RNA virus evolution. *The FASEB Journal* 1996; **10**:859–864.

59 Domingo E, Holland JJ. High error rates, population equilibrium, and evolution of RNA replication systems. In: Domingo E, Holland JJ, Ahiquist P, editors. RNA genetics. Boca Raton : CRC Press, **1988**:3–36.

60 Domingo E, Holland JJ. RNA virus mutations and fitness for survival. Annu. Rev. Microbiol. 1997; **51**:151–178.

61 Domingo E, Menendez-Arias L, Quinones-Mateu ME, Holguin A, Gutierrez-Rivas M, Martinez MA, Quer J, Novella IS, Holland JJ. Viral quasispecies and the problem of vaccine-escape and

drug-resistant mutants. *Prog Drug Res* 1997; **48**:99–128.
62 Doyon L, Croteau G, Thibeault D, Poulin F, Pilote L, Lamarre D. Second locus involved in human immunodeficiency virus type 1 resistance to protease inhibitors. *J Virol* 1996; **70**:3763–3769.
63 Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC- CKR-5. Nature (London) 1996; **381**:667–673.
64 Duarte EA, Novella IS, Weaver SC, Domingo E, Wain-Hobson S, Clarke DK, Moya A, Elena SF, de la Torre JC, Holland JJ. RNA virus quasispecies: significance for viral disease and epidemiology. *Infectious Agents and Disease* 1994; **3**:201–214.
65 Dueweke TJ, Pushkarskaya T, Poppe SM, Swaney SM, Zhao JQ, Chen IS, Stevenson M, Tarpley WG. A mutation in reverse transcriptase of bis(heteroaryl)piperazine- resistant human immunodeficiency virus type 1 that confers increased sensitivity to other nonnucleoside inhibitors. *Proc Natl Acad Sci U S A* 1993; **90**:4713–4717.
66 Dyer WB, Ogg GS, Demoitie MA, Jin X, Geczy AF, Rowland-Jones SL, McMichael AJ, Nixon DF, Sullivan JS. Strong human immunodeficiency virus (HIV)-specific cytotoxic T- lymphocyte activity in Sydney Blood Bank Cohort patients infected with nef-defective HIV type 1. *J Virol* 1999; **73**:436–443.
67 Dykes C, Fox K, Lloyd A, Chiulli M, Morse E, Demeter LM. Impact of clinical reverse transcriptase sequences on the replication capacity of HIV-1 drug-resistant mutants. *Virology* 2001; **285**:193–203.
68 Eastman PS, Mittler J, Kelso R, Gee C, Boyer E, Kolberg J, Urdea M, Leonard JM, Norbeck DW, Mo H, Markowitz M. Genotypic changes in human immunodeficiency virus type 1 associated with loss of suppression of plasma viral RNA levels in subjects treated with ritonavir (Norvir) monotherapy. *J Virol* 1998; **72**:5154–5164.
69 Eastman PS, Urdea M, Besemer D, Stempien M, Kolberg J. Comparison of selective polymerase chain reaction primers and differential probe hybridization of polymerase chain reaction products for determination of relative amounts of codon 215 mutant and wild-type HIV-1 populations. *J Acquir Immune Defic Syndr Hum Retrovirol* 1995; **9**:264–273.
70 Eckert DM, Malashkevich VN, Hong LH, Carr PA, Kim PS. Inhibiting HIV-1 entry: discovery of D-peptide inhibitors that target the gp41 coiled-coil pocket . *Cell* 1999; **99**:103–115.
71 Eigen M. Viral quasispecies. *Sci Am* 1993; **269**:42–49.
72 Escarmis C, Carrillo EC, Ferrer M, Arriaza JF, Lopez N, Tami C, Verdaguer N, Domingo E, Franze-Fernandez MT. Rapid selection in modified BHK-21 cells of a foot-and-mouth disease virus variant showing alterations in cell tropism. *J Virol* 1998; **72**:10171–10179.
73 Esnouf R, Ren J, Ross C, Jones Y, Stammers D, Stuart D. Mechanism of inhibition of HIV-1 reverse transcriptase by non- nucleoside inhibitors. *Nat Struct Biol* 1995; **2**:303–308.
74 Essex M. Human immunodeficiency viruses in the developing world. *Adv Virus Res* 1999; **53**:71–88.
75 Este JA. HIV resistance to entry inhibitors. *AIDS Reviews* 2001; **3**:121–132.
76 Este JA, Cabrera C, Blanco J, Gutierrez A, Bridger G, Henson G, Clotet B, Schols D, De Clercq E. Shift of clinical human immunodeficiency virus type 1 isolates from X4 to R5 and prevention of emergence of the syncytium-inducing phenotype by blockade of CXCR4. *J Virol* 1999; **73**:5577–5585.
77 Este JA, Quinones-Mateu ME, Barretina J, Armand-Ugon M, Schols D, Blanco J, Gutierrez A, Clotet B, De Clercq E. Reduced fitness of HIV-1 that are resistant to CXCR4 antagonists due to alterations in gp120 function . 5th International Workshop on HIV Drug Resistance & Treatment Strategies, Scottsdale, Arizona June 2001
78 Ewald PW. The evolution of virulence. *Sci Am* 1993; **268**:86–93.
79 Feng JY, Anderson KS. Mechanistic studies examining the efficiency and fidelity of DNA synthesis by the 3TC-resistant mutant (184V) of HIV-1 reverse transcriptase. *Biochemistry* 1999; **38**:9440–9448.

80 Feng Y, Broder CC, Kennedy PE, Berger EA. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 1996; **272**:872–877.
81 Fenner F, Kessler H. Evolution of the poxviruses, including the coevolution of virus and host myxomatosis. In: Morse SS , editor. The evolutionary biology of viruses. New York: Raven Press, **1994**:273–292.
82 Fenyo EM, Albert J, Asjö B. Replicative capacity, cytopathic effect and cell tropism of HIV. *AIDS* 1989; 3:S5–12.
83 Fenyo E, Schuitemaker H, Asjˆ B, McKeating J, Sattentau Q, EC Concerted ActionHIV Variability. The History of HIV-1 Biological Phenotypes. Past, Present and Future. Review in 1997 Human Retroviruses and AIDS Compendium .
84 Fischl MA, Richman DD, Griego MH, Gottlieb MS, Volberding PA, Laskin OL, Leedom JM, Groopman JE, Mildvan D, Schooley RT, Jackson GG, Durack DT, Phil D, King D, AZT Collaborative Working Group. The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. *N Eng J Med* 1987; **317**:185–191.
85 Frost SD, Nijhuis M, Schuurman R, Boucher CA, Brown AJ. Evolution of lamivudine resistance in human immunodeficiency virus type 1-infected individuals: the relative roles of drift and selection. *J Virol* 2000; **74**:6262–6268.
86 Gao F, Bailes E, Robertson DL, Chen Y, Rodenburg CM, Michael SF, Cummins LB, Arthur LO, Peeters M, Shaw GM, Sharp PM, Hahn BH. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature* 1999; **397**:436–441.
87 Garcia-Lerma JG, Gerrish PJ, Wright AC, Qari SH, Heneine W. Evidence of a role for the Q151L mutation and the viral background in development of multiple dideoxynucleoside-resistant human immunodeficiency virus type 1. *J Virol* 2000; **74**:9339–9346.
88 Gerondelis P, Archer RH, Palaniappan C, Reichman RC, Fay PJ, Bambara RA , Demeter LM. The P236L delavirdine-resistant human immunodeficiency virus type 1 mutant is replication defective and demonstrates alterations in both RNA 5'-end- and DNA 3'-end-directed RNase H activities. *J Virol* 1999; **73**:5803–5813.
89 Gotte M, Arion D, Parniak MA, Wainberg MA. The M184V mutation in the reverse transcriptase of human immunodeficiency virus type 1 impairs rescue of chain-terminated DNA synthesis. *J Virol* 2000; **74**:3579–3585.
90 Goudsmit J, De Ronde A, de Rooij E, de Boer R. Broad spectrum of *in vivo* fitness of human immunodeficiency virus type 1 subpopulations differing at reverse transcriptase codons 41 and 215. *J Virol* 1997; **71**:4479–4484.
91 Goudsmit J, De Ronde A, Ho DD, Perelson AS. Human immunodeficiency virus fitness *in vivo*: calculations based on a single zidovudine resistance mutation at codon 215 of reverse transcriptase. *J Virol* 1996; **70**:5662–5664.
92 Grant RM, Liegler T, Bonhoeffer S, Elkin C, Nijjar S, Wrin T, Ho R, Kolberg J, Hellerstein MK, Hellmann NS, Petropoulos CJ, Deeks SG. Large fitness differences between protease inhibitor susceptible and resistant HIV-1 *in vivo*. 8th Annual HIV Dynamics and Evolution Meeting. 2001.
93 Greenberg M, McDanal C, Stanfield-Oakley S, Jin L, Trembaly C, Sista P, Hirsch M, Matthews T. Virus sensitivity to T-20 and T-1249 is independent of coreceptor usage. 8th Conference on Retroviruses and Opportunistic Infections. 2001.
94 Hahn BH, Gonda MA, Shaw GM, Popovic M, Hoxie JA, Gallo RC, Wong-Staal F. Genomic diversity of the acquired immune deficiency syndrome virus HTLV-III: Differnt viruses exhibit greatest divergence in their envelope genes. *Proc Natl Acad Sci USA* 1985; **82**:4813–4817.
95 Hahn BH, Shaw GM, De Cock KM, Sharp PM. AIDS as a zoonosis: scientific and public health implications. *Science* 2000; **287**:607–614.
96 Harrigan PR, Bloor S, Larder BA. Relative replicative fitness of zidovudine-resistant human immunodeficiency virus type 1 isolates *in vitro*. *J Virol* 1998; **72**:3773–3778.
97 Havlir DV, Eastman S, Gamst A, Richman DD. Nevirapine-resistant human immunodeficiency virus: kinetics of replication and estimated prevalence in untreated patients. *J Virol* 1996;

70:7894–7899.
98 Hertogs K, de Bethune MP, Miller V, Ivens T, Schel P, Van Cauwenberge A, Van Den EC, Van G, V, Azijn H, Van Houtte M, Peeters F, Staszewski S, Conant M, Bloor S, Kemp S, Larder B, Pauwels R. A rapid method for simultaneous detection of phenotypic resistance to inhibitors of protease and reverse transcriptase in recombinant human immunodeficiency virus type 1 isolates from patients treated with antiretroviral drugs. *Antimicrob Agents Chemother* 1998; **42**:269–276.
99 Hirsch MS, Brun-Vezinet F, D'aquila RT, Hammer SM, Johnson VA, Kuritzkes DR, Loveday C, Mellors JW, Clotet B, Conway B, Demeter LM, Vella S, Jacobsen DM, Richman DD. Antiretroviral drug resistance testing in adult HIV-1 infection: recommendations of an International AIDS Society-USA Panel. *JAMA* 2000; **283**:2417–2426.
100 Hirsch MS, Conway B, D'aquila RT, Johnson VA, Brun-Vezinet F, Clotet B, Demeter LM, Hammer SM, Jacobsen DM, Kuritzkes DR, Loveday C, Mellors JW, Vella S, Richman DD. Antiretroviral drug resistance testing in adults with HIV infection: implications for clinical management. International AIDS Society—USA Panel. *JAMA* 1998; **279**:1984–1991.
101 Ho DD, Toyoshima T, Mo H, Kempf DJ, Norbeck D, Chen CM, Wideburg NE, Burt SK, Erickson JW, Singh MK. Characterization of human immunodeficiency virus type 1 variants with increased resistance to a C2-symmetric protease inhibitor. *J Virol* 1994; **68**:2016–2020.
102 Ho DD, Neumann AU, Perelson AS, Chen W , Leonard JM, Markowitz M. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* 1995; **373**:123–126.
103 Holland JJ, de la Torre JC, Clarke DK, Duarte E. Quantitation of relative fitness and great adaptability of clonal populations of RNA viruses. *J Virol* 1991; **65**:2960–2967.
104 Holterman L, Niphuis H, Koornstra W, Dubbes R, ten Haaft P, Heeney JL. The rate of progression to AIDS is independent of virus dose in simian immunodeficiency virus-infected macaques. *J Gen Virol* 2000; **81**:1719–1726.
105 Hu DJ, Dondero TJ, Rayfield MA, George R, Schochetman G, Jaffe HW, Luo C, Kalish ML, Weniger BG, Pau C, Schable CA, Curran JW. The emerging genetic diversity of HIV. The importance of global surveillance for diagsnostics, research, and prevention. *JAMA* 1996; **275**:210–216.
106 Hu W-S, Temin HM. Genetic consequences of packaging two RNA genomes in one retroviral particle: pseudodiploidy and high rate of genetic recombination. *Proc Natl Acad Sci USA* 1990; **87**:1556–1560.
107 Hunt G, Tiemessen CT. Occurrence of additional NF-kappaB-binding motifs in the long terminal repeat region of South African HIV type 1 subtype C isolates. *AIDS Res Hum Retroviruses* 2000; **16**:305–306.
108 Iglesias-Ussel MD, Casado C, Yuste E, Olivares I, Lopez-Galindez C. *In vitro* analysis of HIV-1 resistance to nevirapine and fitness determination of resistant variants. *J Gen Virol* 2002; **83**:93–101.
109 Imamichi T, Berg SC, Imamichi H, Lopez JC, Metcalf JA, Falloon J, Lane HC. Relative replication fitness of a high-level 3'-azido-3'-deoxythymidine- resistant variant of human immunodeficiency virus type 1 possessing an amino acid deletion at codon 67 and a novel substitution (Thr—>Gly) at codon 69. *J Virol* 2000; **74**:10958–10964.
110 Imamichi T, Murphy MA, Imamichi H, Lane HC. Amino acid deletion at codon 67 and Thr-to-Gly change at codon 69 of human immunodeficiency virus type 1 reverse transcriptase confer novel drug resistance profiles. *J Virol* 2001; **75**:3988–3992.
111 Ivanoff LA, Dubay JW, Morris JF, Roberts SJ, Gutshall L, Sternberg EJ, Hunter E, Matthews TJ, Petteway SR Jr. Ivanoff LA. V3 loop region of the HIV-1 gp120 envelope protein is essential for virus infectivity Virology 1992; **187**:423–32.
112 Jacobson JM, Lowy I, Fletcher CV, O'Neill TJ, Tran DN, Ketas TJ, Trkola A, Klotman ME, Maddon PJ, Olson WC, Israel RJ. Single-dose safety, pharmacology, and antiviral activity of the human immunodeficiency virus (HIV) type 1 entry inhibitor PRO 542 in HIV- infected adults. *J Infect Dis* 2000; **182**:326–329.
113 Jeeninga RE, Keulen W, Boucher C, Sanders RW, Berkhout B. Evolution of AZT resistance in

HIV-1: the 41–70 intermediate that is not observed *in vivo* has a replication defect. *Virology* 2001; **283**:294–305.
114 Kalish ML, Baldwin A, Raktham S, Wasi C, Luo C-C, Schochetman G, Mastro TD, Young N, Vanichseni S, R¸bsamen-Waigmann H, von Briesen H, Mullins JI, Delwart E, Herring B, Esparza J, Heyward WL, Osmanov S. The evolving molecular epidemiology of HIV-1 envelope subtypes in injecting drug users in Bangkok, Thailand: implications for HIV vaccine trials. *AIDS* 1995; **9**:851–857.
115 Kanki PJ, Hamel DJ, Sankale JL, Hsieh C, Thior I, Barin F, Woodcock SA, Gueye-Ndiaye A, Zhang E, Montano M, Siby T, Marlink R, NDoye I, Essex ME, Mboup S. Human immunodeficiency virus type 1 subtypes differ in disease progression. *J Infect Dis* 1999; **179**:68–73.
116 Kaufmann GR, Bloch M, Zaunders JJ, Smith D, Cooper DA. Long-term immunological response in HIV-1-infected subjects receiving potent antiretroviral therapy. *AIDS* 2000; **14**:959–969.
117 Kaufmann GR, Suzuki K, Cunningham P, Mukaide M, Kondo M, Imai M, Zaunders J, Cooper DA. Impact of HIV type 1 protease, reverse transcriptase, cleavage site, and p6 mutations on the virological response to quadruple therapy with saquinavir, ritonavir, and two nucleoside analogs. *AIDS Res Hum Retroviruses* 2001; **17**:487–497.
118 Kellam P, Boucher CA, Tijnagel JM, Larder BA. Zidovudine treatment results in the selection of human immunodeficiency virus type 1 variants whose genotypes confer increasing levels of drug resistance. *J Gen Virol* 1994; **75**:341–351.
119 Kellam P, Larder BA. Recombinant virus assay: a rapid, phenotypic assay for assessment of drug susceptibility of human immunodeficiency virus type 1 isolates. *Antimicrob Agents Chemother* 1994; **38**:23–30.
120 Keulen W, Nijhuis M, Schuurman R, Berkhout B, Boucher C. Reverse transcriptase fidelity and HIV-1 variation. *Science* 1997; **275**:229–1.
121 Kilby JM, Hopkins S, Venetta TM, DiMassimo B, Cloud GA, Lee JY, Alldredge L, Hunter E, Lambert D, Bolognesi D, Matthews T, Johnson MR, Nowak MA, Shaw GM, Saag MS. Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. *Nat Med* 1998; **4**:1302–1307.
122 Kim EY, Winters MA, Kagan RM, Merigan TC. Functional correlates of insertion mutations in the protease gene of human immunodeficiency virus type 1 isolates from patients. *J Virol* 2001; **75**:11227–11233.
123 Kimata JT, Kuller L, Anderson DB, Dailey P, Overbaugh J. Emerging cytopathic and antigenic simian immunodeficiency virus variants influence AIDS progression. *Nat Med* 1999; **5**:535–541.
124 Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, Desrosiers RC. Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. *N Eng J Med* 1995; **332**:228–232
125 Kohlstaedt LA, Wang J, Rice PA, Friedman JM, Steitz TA. The structure of HIV-1 reverse transcriptase. In: Skalka AM, Goff SP, editors. Reverse Transcriptase. Cold Spring Harbor, N. Y.: Cold Spring Harbor Laboratory Press, **1993**:223–249.
126 Korber B, Hahn B, Foley B, Mellors J, Leitner T, Myers G, McCutchan F, Kuiken C. Human Retroviruses and AIDS 1997. Los Alamos, NM, Theoretical Biology and Biophysics Group, Los Alamos National Laboratory.
127 Korber B, Muldoon M, Theiler J, Gao F, Gupta R, Lapedes A, Hahn BH, Wolinsky S, Bhattacharya T. Timing the ancestor of the HIV-1 pandemic strains. *Science* 2000; **288**:1789–1796.
128 Kosalaraksa P, Kavlick MF, Maroun V, Le R, Mitsuya H. Comparative fitness of multi-dideoxynucleoside-resistant human immunodeficiency virus type 1 (HIV-1) in an *In vitro* competitive HIV-1 replication assay. *J Virol* 1999; **73**:5356–5363.
129 Kozal MJ, Shah N, Shen N, Yang R, Fucini R, Merigan TC, Richman DD, Morris D, Hubbell E, Chee M, Gingeras TR. Extensive polymorphisms observed in HIV-1 clade B protease gene using high-density oligonucleotide arrays. *Nat Med* 1996; **2**:753–759.
130 Krohn A, Redshaw S, Ritchie JC, Graves BJ, Hatada MH. Novel binding mode of highly potent

HIV-proteinase inhibitors incorporating the (R)-hydroxyethylamine isostere. *J Med Chem* 1991; **34**:3340–3342.
131 Kuiken CL, Foley B, Hahn B, Korber B, McCutchan F, Marx P, Mellors J, Mullins JI, Sodroski J, Wolinsky S. Human Retroviruses and AIDS 1999: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences. Los Alamos, NM: Theoretical Biology and Biophysics Group, Los Alamos National Laboratory.
132 Larder BA, Kemp SD, Harrigan PR. Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. *Science* 1995; **269**:696–699.
133 Lech WJ, Wang G, Li Yang Y, Chee Y, Dorman K, McCrae D, Lazzeroni LC, Erickson JW, Sinsheimer JS, Kaplan AH. *In vivo* sequence diversity of the protease of human immunodeficiency virus type 1: presence of protease inhibitor-resistant variants in untreated subjects. *J Virol* 1996; **70**:2038–2043.
134 Little SJ, Daar ES, D'aquila RT, Keiser PH, Connick E, Whitcomb JM, Hellmann NS, Petropoulos CJ, Sutton L, Pitt JA, Rosenberg ES, Koup RA, Walker BD, Richman DD. Reduced antiretroviral drug susceptibility among patients with primary HIV infection. *JAMA* 1999; **282**:1142–1149.
135 Loeb DD, Swanstrom R, Everitt L, Manchester M, Stamper SE, Hutchison CA, III. Complete mutagenesis of the HIV-1 protease. Nature (London) 1989; **340**:397–400.
136 Lole KS, Bollinger RC, Paranjape RS, Gadkari D, Kulkarni SS, Novak NG , Ingersoll R, Sheppard HW, Ray SC. Full-length human immunodeficiency virus type 1 genomes from subtype C- infected seroconverters in India, with evidence of intersubtype recombination. *J Virol* 1999; **73**:152–160.
137 Loveday C. International perspectives on antiretroviral resistance. Nucleoside reverse transcriptase inhibitor resistance. *J Acquir Immune Defic Syndr* 2001; 26:S10-S24.
138 Loveday C, Hill A. Prediction of progression to AIDS with serum HIV-1 RNA and CD4 count. *Lancet* 1995; **345**:790–791.
139 Lu J, Kuritzkes DR. A novel recombinant marker virus assay for comparing the relative fitness of hiv-1 reverse transcriptase variants. *J Acquir Immune Defic Syndr* 2001; **27**:7–13.
140 Lu J, Kuritzkes DR. Impaired fitness of HIV-1 site-directed mutants resistant to T-20. 5th International Workshop on HIV Drug Resistance & Treatment Strategies, Scottsdale, Arizona June 2001
141 Lukashov V, Goudsmit J. Founder virus population related to route of virus transmission: a determinant of intrahost human immunodeficiency virus type 1 evolution? J Virol 1997; **71**:2023–2030.
142 Lukashov VV, Huismans R, Jebbink MF, Danner SA, De Boer RJ, Goudsmit J. Selection by AZT and rapid replacement in the absence of drugs of HIV type 1 resistant to multiple nucleoside analogs. *AIDS Res Hum Retroviruses* 2001; **17**:807–818.
143 Maeda Y, Venzon DJ, Mitsuya H. Altered drug sensitivity, fitness, and evolution of human immunodeficiency virus type 1 with pol gene mutations conferring multi- dideoxynucleoside resistance. *J Infect Dis* 1998; **177**:1207–1213.
144 Mammano F, Petit C, Clavel F. Resistance-associated loss of viral fitness in human immunodeficiency virus type 1: phenotypic analysis of protease and gag coevolution in protease inhibitor-treated patients. *J Virol* 1998; **72**:7632–7637.
145 Mammano F, Trouplin V, Zennou V, Clavel F. Retracing the evolutionary pathways of human immunodeficiency virus type 1 resistance to protease inhibitors: virus fitness in the absence and in the presence of drug. *J Virol* 2000; **74**:8524–8531.
146 Mansky LM, Temin HM. Lower *in vivo* mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. *J Virol* 1995; **69**:5087–5094.
147 Markowitz M, Mo H, Kempf DJ, Norbeck DW, Bhat TN, Erickson JW, Ho DD. Selection and analysis of human immunodeficiency virus type 1 variants with increased resistance to ABT-538, a novel protease inhibitor. *J Virol* 1995; **69**:701–706.

148 Martin-Hern·ndez AM, Domingo E, MenÈndez-Arias L. Human immunodeficiency virus type 1 reverse transcriptase: role of Tyr115 in deoxynucleotide binding and misinsertion fidelity of DNA synthesis. *EMBO Journal* 1996; **15**:4434–4442.
149 Martinez-Picado J, Morales-Lopetegui K, Wrin T, Garcia-Prado J, Frost SD, Petropoulos CJ, Clotet B, Ruiz L. Selection of the M184V mutation during repetitive cycles of structured antiretroviral treatment interruptions. 5th International Workshop on HIV Drug Resistance & Treatment Strategies, Scottsdale, Arizona June 2001
150 Martinez-Picado J, Savara AV, Shi L, Sutton L, D'aquila RT. Fitness of human immunodeficiency virus type 1 protease inhibitor- selected single mutants. *Virology* 2000; **275**:318–322.
151 Martinez-Picado J, Savara AV, Sutton L, D'aquila RT. Replicative fitness of protease inhibitor-resistant mutants of human immunodeficiency virus type 1. *J Virol* 1999; **73**:3744–3752.
152 Martinez-Picado J, Sutton L, De Pasquale MP, Savara AV, D'aquila RT. Human immunodeficiency virus type 1 cloning vectors for antiretroviral resistance testing. *J Clin Microbiol* 1999; **37**:2943–2951.
153 Martinez MA, Cabana M, Parera M, Gutierrez A, Este JA, Clotet B. A bacteriophage lambda-based genetic screen for characterization of the activity and phenotype of the human immunodeficiency virus type 1 protease. *Antimicrob Agents Chemother* 2000; **44**:1132–1139.
154 Mas A, Parera M, Briones C, Soriano V, Martinez MA, Domingo E, Menendez-Arias L. Role of a dipeptide insertion between codons 69 and 70 of HIV-1 reverse transcriptase in the mechanism of AZT resistance. *EMBO J* 2000; **19**:5752–5761.
155 Masuda T, Harada S. Interaction between two distinct plaque-cloned human immunodeficiency viruses (HIVs): the possible existence of heterozygous virus. *Microbiol Immunol* 1990; **34**:1055–1063.
156 Mellors JW, Rinaldo CR, Jr., Gupta P, White RM, Todd JA, Kingsley LA. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma Science 1996; **272**:1167–1170.
157 Miller V. International perspectives on antiretroviral resistance. Resistance to protease inhibitors. *J Acquir Immune Defic Syndr* 2001; 26:S34-S50.
158 Montefiori DC, Pantaleo G, Fink LM, Zhou JT, Zhou JY, Bilska M, Miralles GD, Fauci AS. Neutralizing and infection-enhancing antibody responses to human immunodeficiency virus type 1 in long-term nonprogressors. *J Infect Dis* 1996; **173**:60–67.
159 Myint L, Matsuda Z, Yokomaku Y, Matsuo K, Iwasaki T, Yamada K, Sugiura W. Contribution of accumulated Gag and protease mutations towards the recovery of the fitness and the virus particle formation in the protease inhibitor-resistant HIV-1 with D30N and L90M . 5th International Workshop on HIV Drug Resistance and Treatment Strategies, Scottsdale, Arizona June 2001
160 Najera I, Holguin A, Quinones-Mateu ME, Munoz-Fernandez MA, Najera R, Lopez-Galindez C, Domingo E. Pol gene quasispecies of human immunodeficiency virus: mutations associated with drug resistance in virus from patients undergoing no drug therapy. *J Virol* 1995; **69**:23–31.
161 Najera I, Richman DD, Olivares I, Rojas JM, Peinado MA, Perucho M , Najera R, Lopez-Galindez C. Natural ocurrence of drug resistance mutations in the reverse transcriptase of human immunodeficiency virus type 1 isolates. *AIDS Research and Human Retroviruses* 1994; **10**:1475–1484.
162 Nelson JA, Baribaud F, Edwards T, Swanstrom R. Patterns of changes in human immunodeficiency virus type 1 V3 sequence populations late in infection. *J Virol* 2000; **74**:8494–8501.
163 Nijhuis M, Boucher CA, Schipper P, Leitner T, Schuurman R, Albert J . Stochastic processes strongly influence HIV-1 evolution during suboptimal protease-inhibitor therapy. *Proc Natl Acad Sci U S A* 1998; **95**:14441–14446.
164 Nijhuis M, Deeks S, Boucher C. Implications of antiretroviral resistance on viral fitness. *Curr Opin Infect Dis* 2001; **14**:23–28.
165 Nijhuis M, Schuurman R, de Jong D, Erickson J, Gustchina E, Albert J , Schipper P, Gulnik S, Boucher CA. Increased fitness of drug resistant HIV-1 protease as a result of acquisition of compensatory mutations during suboptimal therapy. *AIDS* 1999; **13**:2349–2359.

166 Novella IS, Duarte EA, Elena SF, Moya A, Domingo E, Holland JJ. Exponential increases of RNA virus fitness during large population transmissions. *Proc Natl Acad Sci USA* 1995; **92**:5841–5844.
167 Novella IS, Quer J, Domingo E, Holland JJ. Exponential fitness gains of RNA virus populations are limited by bottleneck effects. J Virol 1999 Feb;**73**:1668–71
168 O'Brien SJ, Moore JP. The effect of genetic variation in chemokines and their receptors on HIV transmission and progression to AIDS. *Immunol Rev* 2000; **177**:99–111.
169 Olivares I, Sanchez-Merino V, Martinez MA, Domingo E, Lopez-Galindez C, Menendez-Arias L. Second-site reversion of a human immunodeficiency virus type 1 reverse transcriptase mutant that restores enzyme function and replication capacity. *J Virol* 1999; **73**:6293–6298.
170 Overbaugh J, Kreiss J, Poss M, Lewis P , Mostad S, John G, Nduati R, Mbori-Ngacha D, Martin H, Jr., Richardson B, Jackson S, Neilson J, Long EM , Panteleeff D, Welch M, Rakwar J, Jackson D, Chohan B, Lavreys L, Mandaliya K, Ndinya-Achola J, Bwayo J. Studies of human immunodeficiency virus type 1 mucosal viral shedding and transmission in Kenya. *J Infect Dis* 1999; 179:S401-S404.
171 Pandey VN, Kaushik N, Rege N, Sarafianos SG, Yadav PN, Modak MJ. Role of methionine 184 of human immunodeficiency virus type-1 reverse transcriptase in the polymerase function and fidelity of DNA synthesis. *Biochemistry* 1996; **35**:2168–2179.
172 Pantaleo G, Menzo S, Vaccarezza M, Graziosi C, Cohen OJ, Demarest JF , Montefiori D, Orenstein JM, Fox C, Schrager LK, Margolick JB, Buchbinder S, Giorgi JV, Fauci AS. Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. *N Eng J Med* 1995; **332**:209–216.
173 Park J, Morrow CD. Mutations in the protease gene of human immunodeficiency virus type 1 affect release and stability of virus particles. *Virology* 1993; **194**:843–850.
174 Pazhanisamy S, Stuver CM, Cullinan AB, Margolin N, Rao BG, Livingston DJ. Kinetic characterization of human immunodeficiency virus type-1 protease-resistant variants. *J Biol Chem* 1996; **271**:17979–17985.
175 Peeters M. Recombinant HIV sequences: Their role in the global epidemic. In: Kuiken C, Foley B, Hahn B, Korber B, McCutchan F, Marx P, Mellors J, Mullins JI, Sodroski J, Wolinsky S, editors. Human Retroviruses and AIDS 2000. Los Alamos, NM: Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, 2000: I-39-I-54.
176 Petropoulos CJ, Parkin NT, Limoli KL, Lie YS, Wrin T, Huang W, Tian H, Smith D, Winslow GA, Capon DJ, Whitcomb JM. A novel phenotypic drug susceptibility assay for human immunodeficiency virus type 1. *Antimicrob Agents Chemother* 2000; **44**:920–928.
177 Picchio GR, Valdez H, Sabbe R, Landay AL, Kuritzkes DR, Lederman MM , Mosier DE. Altered viral fitness of HIV-1 following failure of protease inhibitor- based therapy. *J Acquir Immune Defic Syndr* 2000; **25**:289–295.
178 Piyasirisilp S, McCutchan FE, Carr JK, Sanders-Buell E, Liu W, Chen J, Wagner R, Wolf H, Shao Y, Lai S, Beyrer C, Yu XF. A recent outbreak of human immunodeficiency virus type 1 infection in southern China was initiated by two highly homogeneous, geographically separated strains, circulating recombinant form AE and a novel BC recombinant. *J Virol* 2000; **74**:11286–11295.
179 Pontesilli O, Klein MR, Kerkhof-Garde SR, Pakker NG, de Wolf F, Schuitemaker H, Miedema F. Longitudinal analysis of human immunodeficiency virus type 1-specific cytotoxic T lymphocyte responses: a predominant gag-specific response is associated with nonprogressive infection. *J Infect Dis* 1998; **178**:1008–1018.
180 Prado JG, Wrin T, Beauchaine J, Ruiz L , Petropoulos CJ, Frost SD, Clotet B, D'aquila RT, Martinez-Picado J. Amprenavir resistant HIV-1 exhibits lopinavir cross-resistance and reduced replication capacity. AIDS 2002 (In press).
181 Precious HM, Gunthard HF, Wong JK, D'aquila RT, Johnson VA, Kuritzkes DR, Richman DD, Leigh Brown AJ. Multiple sites in HIV-1 reverse transcriptase associated with virological response to combination therapy. *AIDS* 2000; **14**:31–36.

182 Preston BD, Dougherty JP. Mechanisms of retroviral mutation. *Trends in Microbiology* 1996; **4**:16–21.
183 Puig T, Perez-Olmeda M, Rubio A, Ruiz L, Briones C, Franco JM, Gomez-Cano M, Stuyver L, Zamora L, Alvarez C, Leal M, Clotet B, Soriano V. Prevalence of genotypic resistance to nucleoside analogues and protease inhibitors in Spain. The ERASE-2 Study Group. *AIDS* 2000; **14**:727–732.
184 Quinn TC. Global burden of the HIV pandemic. *The Lancet* 1996; **348**:99–106.
185 Quinones-Mateu ME, Arts EJ. Recombination in HIV-1: Update and implications. *AIDS Reviews* 1999; **1**:89–100.
186 Quinones-Mateu ME, Ball SC, Arts EJ. Role of human immunodeficiency virus type 1 group O in the AIDS pandemic. *AIDS Reviews* 2001; **2**:190–202.
187 Quinones-Mateu ME, Ball SC, Marozsan AJ, Arts EJ. Analyses of *in vitro* and *in vivo* intersubtype recombination in the HIV-1 env gene. Meeting on Retroviruses. Cold Spring Harbor, NY 2001.
188 Quinones-Mateu ME, Ball SC, Marozsan AJ, Torre VS, Albright JL, Vanham G, van der GG, Colebunders RL, Arts EJ. A dual infection/competition assay shows a correlation between ex vivo human immunodeficiency virus type 1 fitness and disease progression. *J Virol* 2000; **74**:9222–9233.
189 Quinones-Mateu ME, Soriano V, Domingo E, Menendez-Arias L. Characterization of the reverse transcriptase of a human immunodeficiency virus type 1 group O isolate. *Virology* 1997; **236**:364–373.
190 Quinones-Mateu ME, Tadele M, Parera M, Mas A, Clotet B, Domingo E, Menendez-Arias L, Martinez MA. Decreased viral fitness conferred by a multidrug-resistant HIV-1 reverse transcriptase harbouring a dipeptide insertion between codons 69 and 70. 5th International Workshop on HIV Drug Resistance & Treatment Strategies, Scottsdale, Arizona June 2001
191 Rayner MM, Cordova B, Jackson DA. Population dynamics studies of wild-type and drug-resistant mutant HIV in mixed infections. *Virology* 1997; **236**:85–94.
192 Resch W, Parkin N, Stuelke EL, Watkins T, Swanstrom R. A multiple-site-specific heteroduplex tracking assay as a tool for the study of viral population dynamics. *Proc Natl Acad Sci U S A* 2001; **98**:176–181.
193 Rey-Cuille MA, Berthier JL, Bomsel-Demontoy MC, Chaduc Y, Montagnier L, Hovanessian AG, Chakrabarti LA. Simian immunodeficiency virus replicates to high levels in sooty mangabeys without inducing disease. *J Virol* 1998; **72**:3872–3886.
194 Richman DD. Two unrelated studies from 1 lab: HIV drug resistance in the US and decay of latency. 2nd HIV DRP Symposium Antiviral Drug Resistance, Chantilly, Virginia December 2001 .
195 Robinson LH, Myers RE, Snowden BW, Tisdale M, Blair ED. HIV type 1 protease cleavage site mutations and viral fitness: implications for drug susceptibility phenotyping assays. *AIDS Res Hum Retroviruses* 2000; **16**:1149–1156.
196 Rodenburg CM, Li Y, Trask SA, Chen Y, Decker J, Robertson DL, Kalish ML, Shaw GM, Allen S, Hahn BH, Gao F. Near full-length clones and reference sequences for subtype C isolates of HIV type 1 from three different continents. *AIDS Res Hum Retroviruses* 2001; **17**:161–168.
197 Rose RE, Gong YF, Greytok JA, Bechtold CM, Terry BJ, Robinson BS, Alam M, Colonno RJ, Lin PF. Human immunodeficiency virus type 1 viral background plays a major role in development of resistance to protease inhibitors. *Proc Natl Acad Sci U S A* 1996; **93**:1648–1653.
198 Rosenberg ES, Billingsley JM, Caliendo AM, Boswell SL, Sax PE, Kalams SA, Walker BD. Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia [see comments]. *Science* 1997; **278**:1447–1450.
199 Saag MS, Hahn BH, Gibbons J, Li Y, Parks ES, Parks WP, Shaw GM. Extensive variation of human immunodeficiency virus type-1 *in vivo*. *Nature* 1988; **334**:440–444.
200 Santiago ML, Santiago EG, Hafalla JC, Manalo MA, Orantia L, Cajimat MN, Martin C, Cuaresma C, Dominguez CE, Borromeo ME, De Groot AS, Flanigan TP, Carpenter CC, Mayer

KH, Ramirez BL. Molecular epidemiology of HIV-1 infection in the Philippines, 1985 to 1997: transmission of subtypes B and E and potential emergence of subtypes C and F. *J Acquir Immune Defic Syndr Hum Retrovirol* 1998; **18**:260–269.

201 Schock HB, Garsky VM, Kuo LC. Mutational anatomy of an HIV-1 protease variant conferring cross- resistance to protease inhibitors in clinical trials. Compensatory modulations of binding and activity . *J Biol Chem* 1996; **271**:31957–31963.

202 Schuitemaker H, Koot M, Kootstra NA, Dercksen MW, de Goede RE, van Steenwijk RP, Lange JM, Schattenkerk JK, Miedema F, Tersmette M. Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population. *J Virol* 1992; **66**:1354–1360.

203 Shafer RW, Dupnik K, Winters MA, Eshleman SH. A guide to HIV-1 reverse transcriptase and protease sequencing for drug resistance studies. HIV Sequence Compendium 2000.

204 Sharma PL, Crumpacker CS. Attenuated replication of human immunodeficiency virus type 1 with a didanosine-selected reverse transcriptase mutation. *J Virol* 1997; **71**:8846–8851.

205 Sharma PL, Crumpacker CS. Decreased processivity of human immunodeficiency virus type 1 reverse transcriptase (RT) containing didanosine-selected mutation Leu74Val: a comparative analysis of RT variants Leu74Val and lamivudine-selected Met184Val. *J Virol* 1999; **73**:8448–8456.

206 Shi C, Mellors JW. A recombinant retroviral system for rapid *in vivo* analysis of human immunodeficiency virus type 1 susceptibility to reverse transcriptase inhibitors. *Antimicrob Agents Chemother* 1997; **41**:2781–2785.

207 Shirasaka T, Kavlick MF, Ueno T, Gao WY, Kojima E, Alcaide ML, Chokekijchai S, Roy BM, Arnold E, Yarchoan R, . Emergence of human immunodeficiency virus type 1 variants with resistance to multiple dideoxynucleosides in patients receiving therapy with dideoxynucleosides. *Proc Natl Acad Sci U S A* 1995; **92**:2398–2402.

208 Simmons G, Clapham PR, Picard L, Offord RE, Rosenkilde MM, Schwartz TW, Buser R, Wells TN, Proudfoot AE. Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. *Science* 1997; **276**:276–279.

209 Soto-Ramirez LE, Renjifo B, McLane MF, Marlink R, O'Hara C, Sutthent R , Wasi C, Vithayasai P, Vithayasai V, Apichartpiyakul C, Auewarakul P, Pena C, V, Chui DS, Osathanondh R, Mayer K, Lee TH, Essex M. HIV-1 Langerhans' cell tropism associated with heterosexual transmission of HIV. *Science* 1996; **271**:1291–1293.

210 Stoddart CA, Liegler TJ, Mammano F, Linquist-Stepps VD, Hayden MS, Deeks SG, Grant RM, Clavel F, McCune JM. Impaired replication of protease inhibitor-resistant HIV-1 in human thymus. *Nat Med* 2001; **7**:712–718.

211 Sugiura W, Matsuda Z, Yokomaku Y, *et al*. Evidence of a mutually exclusive relationship between the HIV-1 protease inhibitor resistance mutations D30N and L90M. *Antiviral Therapy* 2001; **5**:33.

212 Tachedjian G, Mellors JW, Bazmi H, Mills J. Impaired fitness of foscarnet-resistant strains of human immunodeficiency virus type 1. *AIDS Res Hum Retroviruses* 1998; **14**:1059–1064.

213 Temin HM. Is HIV unique or merely different? J Acquir Immune Defic Syndr 1989; **2**:1–9.

214 Temin HM. Retrovirus variation and reverse transcription: Abnormal strand transfers result in retrovirus genetic variation. *Proc Natl Acad Sci USA* 1993; **90**:6900–6903.

215 Tersmette M, de Goede REY, Al BJM, Winkel IN, Coutinho RA, Cuypers HThM, Huisman JG, Miedema F. Differential syncytium-inducing capacity of HIV isolates. Frequent detection of syncityum-inducing isolates in patients with AIDS and ARC. *J Virol* 1988; **62**:2026–2032.

216 Tersmette M, Miedema F. Interactions between HIV and the host immune system in the pathogenesis of AIDS. *AIDS* 1990; 4:S57-S66.

217 Torre VS, Marozsan AJ, Albright JL, Collins KR, Hartley O, Offord RE, Quinones-Mateu ME, Arts EJ. Variable sensitivity of CCR5-tropic human immunodeficiency virus type 1 isolates to inhibition by RANTES analogs. *J Virol* 2000; **74**:4868–4876.

218 van Harmelen J, Williamson C, Kim B, Morris L, Carr J, Karim SS, McCutchan F. Characteriza-

tion of full-length HIV type 1 subtype C sequences from South Africa. *AIDS Res Hum Retroviruses* 2001; **17**:1527–1531.
219 Van Harmelen JH, van der RE, Loubser AS, York D, Madurai S, Lyons S, Wood R, Williamson C. A predominantly HIV type 1 subtype C-restricted epidemic in South African urban populations. *AIDS Res Hum Retroviruses* 1999; **15**:395–398.
220 Velazquez-Campoy A, Todd MJ, Vega S, Freire E. Catalytic efficiency and vitality of HIV-1 proteases from African viral subtypes. *Proc Natl Acad Sci U S A* 2001; **98**:6062–6067.
221 Wain-Hobson S. Multiply infected cells and rampant recombination. 6th Annual International Discussion Meetingf on HIV Dynamics and Evolution. Atlanta, 1999.
222 Wain-Hobson S. Quasispecies *In vivo* and Ex vivo. *Current Topics in Microbiology and Immunology* 1992; **176**:181–193.
223 Wang J, Smerdon SJ, Jager J, Kohlstaedt LA, Rice PA, Friedman JM, Steitz TA. Structural basis of asymmetry in the human immunodeficiency virus type 1 reverse transcriptase heterodimer. *Proc Natl Acad Sci U S A* 1994; **91**:7242–7246.
224 Weber J, Valdez H, Rangel HR, Chakraborty B, Connick E, Smith K, Landay A, Kuritzkes DR, Lederman MM, Quinones-Mateu ME. A pilot study to analyze HIV-1 fitness evolution under a protease inhibitor-based therapy shows a diverse response depending of the basal genotypic context of the virus. 9th Conference on Retroviruses and Opportunistic Infections, Seattle. 2002.
225 Wei X, Ghosh SK, Taylor ME, Johnson VA , Emini EA, Deutsch P, Lifson JD, Bonhoeffer S, Nowak MA, Hahn BH, . Viral dynamics in human immunodeficiency virus type 1 infection. Nature (London) 1995; **373**:117–122.
226 Wei X, Ghosh SK, Taylor ME, Johnson VA , Emini EA, Deutsch P, Lifson JD, Bonhoeffer S, Nowak MA, Hahn BH, Saag MS, Shaw GM. Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* 1995; **373**:117–122.
227 Whetter LE, Ojukwu IC, Novembre FJ, Dewhurst S. Pathogenesis of simian immunodeficiency virus infection. *J Gen Virol* 1999; **80**:1557–1568.
228 Winslow DL, Stack S, King R, Scarnati H, Bincsik A, Otto MJ. Limited sequence diversity of the HIV type 1 protease gene from clinical isolates and *in vitro* susceptibility to HIV protease inhibitors. *AIDS Res Hum Retroviruses* 1995; **11**:107–113.
229 Winters MA, Coolley KL, Girard YA, Levee DJ, Hamdan H, Shafer RW, Katzenstein DA, Merigan TC. A 6-basepair insert in the reverse transcriptase gene of human immunodeficiency virus type 1 confers resistance to multiple nucleoside inhibitors. *J Clin Invest* 1998; **102**:1769–1775.
230 Witmer M, Schleif W, Gabryelski L, grobler J, Felock P, Stillmock K , Espeseth A, Danzeisen R, Blau C, Danovich R, Miller M, Hazuda D. Evolution of resistance to integration inhibitors: the selection of integrase active site mutations results in mutliple affects on viral replication that are partially restored by truncating Vpu. 2nd HIV DRP Symposium Antiviral Drug Resistance, Chantilly, Virginia December 2001.
231 Wodarz D, Nowak MA. The effect of different immune responses on the evolution of virulent CXCR4-tropic HIV. *Proc R Soc Lond B Biol Sci* 1998; **265**:2149–2158.
232 Wolinsky SM, Wike CM, Korber BT, Hutto C, Parks WP, Rosenblum LL, Kunstman KJ, Furtado MR, Munoz JL. Selective transmission of human immunodeficiency virus type-1 variants from mothers to infants. *Science* 1992; **255**:1134–1137.
233 Wright S. Evolution in Mendelian populations. *Genetics* 1931; **16**:97–159.
234 Wrin T, Gamarnik A, Whitehurst N, Beauchaine J, Whitcomb JM, Hellmann NS, Petropoulos CJ. Natural variation of replication capacity measurements in drug-naive/susceptible HIV-1. 5th International Workshop on HIV Drug Resistance & Treatment Strategies, Scottsdale, Arizona June 2001
235 Yang OO, Swanberg SL, Lu Z, Dziejman M , McCoy J, Luster AD, Walker BD, Herrmann SH. Enhanced inhibition of human immunodeficiency virus type 1 by Met- stromal-derived factor 1beta correlates with down-modulation of CXCR4. *J Virol* 1999; **73**:4582–4589.
236 Yoshimura K, Feldman R, Kodama E, Kavlick MF, Qiu YL, Zemlicka J, Mitsuya H. *In vitro*

induction of human immunodeficiency virus type 1 variants resistant to phosphoralaninate prodrugs of Z-methylenecyclopropane nucleoside analogues. *Antimicrob Agents Chemother* 1999; **43**:2479–2483.

237 Yuste E, Lopez-Galindez C, Domingo E. Unusual distribution of mutations associated with serial bottleneck passages of human immunodeficiency virus type 1. *J Virol* 2000; **74**:9546–9552.

238 Yuste E, Sanchez-Palomino S, Casado C, Domingo E, Lopez-Galindez C. Drastic fitness loss in human immunodeficiency virus type 1 upon serial bottleneck events. *J Virol* 1999; **73**:2745–2751.

239 Zennou V, Mammano F, Paulous S, Mathez D, Clavel F. Loss of viral fitness associated with multiple Gag and Gag-Pol processing defects in human immunodeficiency virus type 1 variants selected for resistance to protease inhibitors *in vivo*. *J Virol* 1998; **72**:3300–3306.

240 Zhang YM, Imamichi H, Imamichi T, Lane HC, Falloon J, Vasudevachari MB, Salzman NP. Drug resistance during indinavir therapy is caused by mutations in the protease gene and in its Gag substrate cleavage sites. *J Virol* 1997; **71**:6662–6670.

241 Zhu T, Mo H, Wang N, Nam DS, Cao Y, Koup RA, Ho DD. Genotypic and phenotypic characterization of HIV-1 patients with primary infection. *Science* 1993; **261**:1179–1181.