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HIV-1 Fitness and Antiretroviral Drug Resistance

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Abstract

During the last few years, considerable new information has been obtained regarding HIV-1 replication capacity, often referred as viral fitness, and the potential effects on population size (viral load), drug resistance, and disease progression. Although viral fitness data originating from *in vitro* studies may not directly resemble *in vivo* clinical results, it offers a model to study and compare HIV-1 replication capacity and its relationship with drug resistance mutations. Treatment of HIV-1-infected individuals with antiretroviral drugs often results in selection of inhibitor-resistant variants with reduced replicative capacity. However, because of the remarkable plasticity of the HIV-1 genome, secondary/compensatory mutations are selected, which leads to the improving of viral fitness. Nevertheless, drug-resistant viruses with impaired fitness may pose a clinical benefit to the patient, by decreasing the levels of virus production and thereby delaying the emergence of highly resistant viruses. Characterization of the relative viral fitness of drug-resistant mutants under different selective pressures could lead to a better understanding of how specific drug resistance mutations emerge during therapy, and whether or not less fit viruses are beneficial for HIV-infected individuals.

Key words

HIV. Fitness. Drug resistance. Evolution. Genetic variability.

Introduction

RNA viruses, such as vesicular stomatitis virus (VSV) and foot-and-mouth disease virus (FMDV), offer a unique opportunity for the study of evolution at the molecular level, including viral fitness[1,2-7]. Mutation rates for these highly variable viruses are usually between 10^{-4} to 10^{-5} mutations per base incorporated per round of replication[8,9]. HIV-1 falls in the middle of these values at 3.4×10^{-5} mutations/bp/cycle[10]. As a consequence, RNA viruses replicate and evolve as complex mutant distributions termed viral quasispecies, which is powered by error-prone replication and high mutation frequency[1,11,12]. This continuous production of mutants favors adaptability of viruses in the event of environmental changes[1,13]. HIV-1, as other RNA viruses, is subject to a continuous process of mutation, competition, and selection of those genomes best adapted to a particular environment[1,9,13,14]. However, it is important to highlight that it is the quasispecies mutant swarm, and not individual virus genomes, which are the subject for selection and evolution[1,11,13]. Thus, HIV-1 quasispecies are evolutionary and clinically important since it provides the genetic variation needed to respond to selection pressure (e.g., host immune system and antiretroviral therapy).

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Fitness is a complex parameter aimed at describing the replicative adaptability or an organism to its environment (reviewed in[1,13]). Within a given viral quasispecies each sequence has a corresponding fitness, representing the combination of properties (e.g. activity and stability) undergoing selection in that particular environment. In the course of viral replication, different genomes are generated at high rates, and are subjected to a continuous process of competition and selection[13]. Newly arising, higher fitness variants often outcompete lower fitness viruses, and thus quasispecies can adapt rapidly to a changing environment. Thus, positive (Darwinian) selection implies that one or more members of the quasispecies are better suited, and consequently selected, to replicate in a new environment, whereas negative selection operates eliminating unfit variants[1,13,14]. In summary, the dynamics of the viral population can be visualized as a continuous process of growth, competition, and selection that takes place in the sequence space (i.e., all possible permutations of sequences for an informational macromolecule). In the case of HIV-1, having a genome size of 10 Kb, the total possible sequence space is an incredible $4^{10,000}$, although only a very small fraction corresponds to functional viruses[13]. The combination of sequence space and fitness description constitutes the "fitness landscape"[1,15]. This classical concept, first described seventy years ago[15], 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[16]. One of the consequences of the quasispecies structure of RNA viruses is an important effect of virus population size on fitness variation. Multiple studies, based in populations of VSV, FMDV, or HIV-1 quasispecies, have assessed different fitness theories related with this phenomenon. The Red Queen hypothesis states that populations of virus quasispecies in competition tend to gain fitness with each viral passage[17,3,14], whereas the competitive exclusion principle asserts that in the absence of niche differentiation, one competing species will always eliminate or exclude the other[14,17]. Massive passage of virus (i.e., large population size) in cell culture under defined environmental conditions tend to gain fitness in that environment[1,18]. In contrast, *in vitro* passage of reduced virus population size (e.g., plaque-to-plaque transfers) creates repeated bottlenecks and result in average fitness loses[4,5,14,16,19]. In this case, the Muller's ratchet hypothesis suggests that an irreversible gain of deleterious mutations in limited populations will overwhelm the appearance of mutations improving fitness[4,14,16,19]. All these previous studies, based on other RNA viruses, established many of the concepts and assays currently used to analyze HIV-1 fitness.

Methods to determine HIV-1 fitness

Although differences in replicative capacities of HIV-1 isolates were described early in the epidemic[20], the role of viral fitness in drug resistance and HIV-1 pathogenesis has been appreciated only recently[21,22]. Hence, many key conceptual and technical questions are still unsolved. For example, what is the proper system to determine viral fitness of HIV-1? Multiple methods have been used to measure HIV-1 replication capacity *in vitro*[21,22] (Table 1).

Table 1. *Methods used to estimate HIV-1 fitness*

Assays	Methods	Detection techniques	References
In vivo	Viral kinetics in plasma	Sequencing	25-27,39,132
		Differential hybridization	40-42
		Primer-guided nucleotide incorporation assay	110
In vitro	Protease catalytic activity	Pr efficiency (Kcat/Km),	23,24,28,29,155
		Polyprotein processing/maturation	29,32,33,44,45,45,95,100,156-158
		Genetic complementation	54,159
	RT catalytic activity	RT polymerase, Rnase H activity	23,64,105,107,109,114,115
	Viral growth kinetics	p24 Antigen/RT activity	23,28-34,36,43-45,48,49,53,63-65, 77,79,89,91,98,103-105, 111,114,115,118,149,156
	Single-cycle infection	β-galactosidase activity	33,34,44,55,91
		GHOST/CCR5-CXCR4 permissibility	32
		Luciferase activity	35,45,47,66,151
	Growth competition	Differential plaque assay	95,160
		Cloning/sequencing	24,29,30,36,39,45,50,51,58, 64,98,103,104,111,112,115,161
		Heteroduplex mobility assay	5,37,52,56
		Real-time NASBA	39
		Recombinant marker virus assay	53
Animal model	SCID mice	SCID-hu Thy/Liv	48
		SCID-hu PBL	49

However, while fitness of a virus is best defined by its replicative capacity in the course of growth competition experiments[7], most published literature extends the concept of HIV-1 fitness to various other measurements: (i) enzyme catalytic activities[23,24], (ii) kinetic analyses of virus production *in vivo*[25-27] or in cell cultures infected with one virus[28-31], (iii) infectivity/virion particle ratios[32], (iv) single-cycle assays[33-35], and the classical (v) growth competition between to different viral variants in a single mixed culture[29,31,36,37]. Most of these assays involve ex vivo (*in vitro* or animal model) experiments, with the limitations of not being assessed in the *in vivo*, natural human host environment (Fig. 1).

In vivo *assays*

In vitro studies using HIV-1 isolates or recombinant viruses could be very useful as models for defining the effects of know mutations on replication in a fixed environment and for the behavior of drug-resistant variants after the initiation of therapy. However, these methods cannot fully mimic the natural setting of the human host. As in the case of drug-resistant selection, the cell type used during viral fitness assays could influence the final result. For example, nonsyncytium-inducing/CCR5-tropic (NSI/R5) HIV-1 strains do not replicate efficiently in cell lines, but demonstrate efficient replication in primary cells (such as peripheral blood mononuclear cells, PBMC). Syncytium-inducing/CXCR4-tropic (SI/X4) isolates, can replicate efficiently in both, PBMC and cell lines. In addition, it has been described that the mutation rate could be increased by changes in deoxynucleotide triphosphate (dNTP) precursor pools[38], which may alter quasispecies distribution and viral fitness. Therefore, several studies, especially those focussed on viral dynamics during antiretroviral therapy, have evaluated HIV-1 fitness *in vivo*[25-27,39-42]. Goudsmit, et al.[25,26] analyzed the effect of AZT resistance mutations in viral replication fitness using nucleotide sequencing of several *in vivo* HIV-1 subpopulations. In other study, Eastman, et al.[40] applied a differential hybridization assay to determine relative amounts of wild-type and drug resistant viruses in the plasma HIV-RNA of infected individuals treated with ritonavir. They calculated the relative proportion of both populations using phosphatase-labeled probes, followed by chemiluminescent detection. However, despite of being an *in vivo* method to estimate viral fitness, the major disadvantage of this assay is that additional mutations responsible for increasing viral fitness, but not included in the probe used, could not be detected.

We should not forget that the competitive ability of a virus is the result of many biological processes in its life cycle (i.e., genome replication, protein synthesis/processing, particle assembly and release from cells). The entire individual offers a variety of cell types and microenvironments to the infecting HIV-1, with conflicting selective constrains. However, the main idea of a relative viral fitness is that it should allow extrapolation to other situations, and this can only be achieved by comparing viral replication fitness using *in vitro* assays.

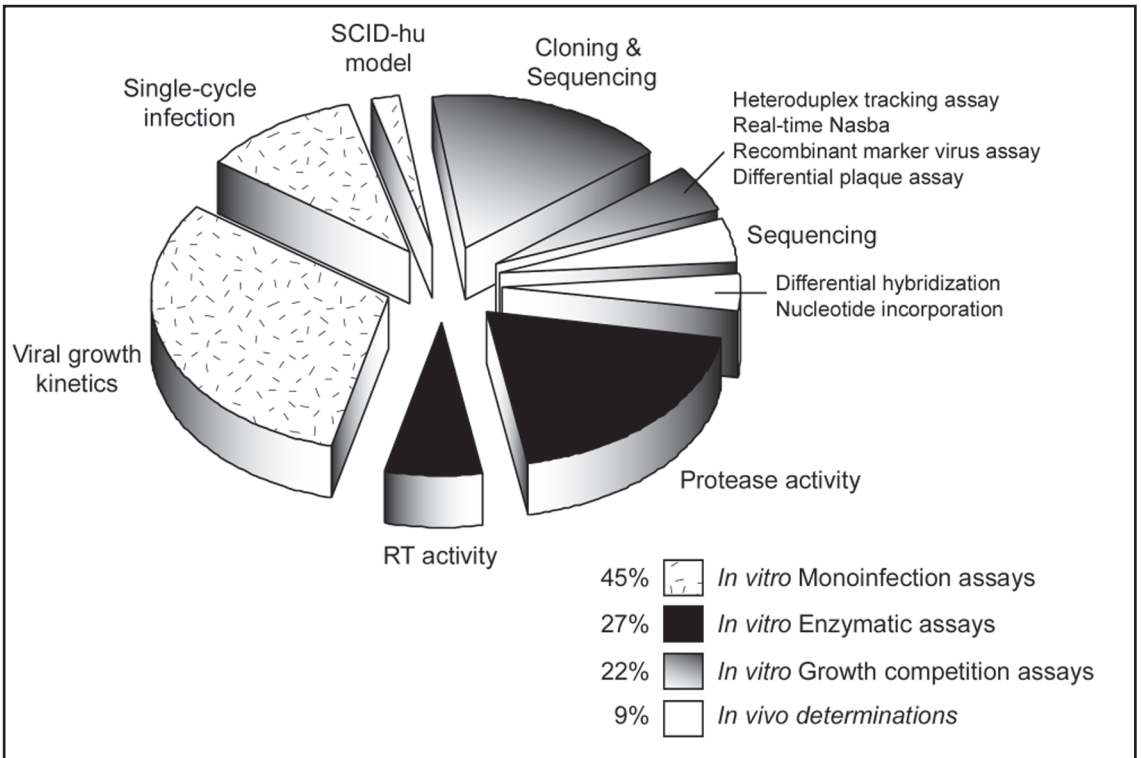

Figure 1. *Summary of HIV-1 fitness studies classified according to the method used to estimate viral replication capacity.*

In vitro *assays*

In the absence of a consensus method for quantifying viral replication capacity, many studies have used one or several different techniques to assess HIV-1 fitness, commonly in the clinical setting. 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. 2).

a) Viral growth kinetic assays

Biochemical properties of the protease (PR) and reverse transcriptase (RT) mutant enzymes, and the replication kinetics of HIV-1 have been broadly studied. In general, the replication capacity (viral fitness) of HIV-1 isolates or recombinant infectious clones is tested individually by determining the amount of virus production over time (e.g., measuring p24 antigen, RT activity, β-galactosidase or luciferase activity, etc)[28,30,35,43,44] (Table 1). Significant differences in replication kinetics of HIV-1 mutants can be observed in replicate parallel infections (Fig. 2A). However, although valuable for a broad estimation of viral fitness, conventional HIV-1 replication assays do not accurately define the impact of small differences (sometimes single base changes) in the replication rates among HIV-1 isolates. In general, direct competition between two different viruses is a more accurate and sensitive assay[7,45] (Fig. 2B).

A novel recombinant virus technique, based in the PhenoSense assay (Virologic) used in the evaluation of the phenotypic susceptibility of HIV-1 in plasma to antiretroviral drugs[46], has been recently adapted to measure HIV-1 replicative capacity[35,47] (Fig. 2C). The relative replication capacity of the virus tested is determined by measuring the amount of luciferase activity produced 72 hours after infection in the absence of drug. Then replication capacity is expressed as the percentage of the luciferase activity produced by the vectors containing mutant *gag-pol* sequences, compared to the luciferase activity from vectors containing the wild-type HIV-1_{NL4-3} *gag-pol* reference sequence. However, this single-infection assay cannot be used to perform growth competition assays. Finally, it is important to mention that few studies have analyzed HIV-1 replication capacity of drug-resistant variants using animal models, specifically SCID-hu mice[48,49]. This HIV-1 infection model have been successfully used to assess viral susceptibility to antiretroviral drugs, whereas have recently provided valuable information on the role of protease inhibitor-resistant strains with reduced viral fitness on HIV-1 pathogenesis (see below).

b) Growth competition experiments

These assays involve mixed infections, at similar of different proportions, in which the initial viruses are genetically and/or phenotypically distinguishable, and where the outgrowth of one of the populations is measured[7]. The relative fitness of the two viruses may be then directly compared since two virus populations in culture compete with each other until one clone outgrows the other[1,7]. In general,

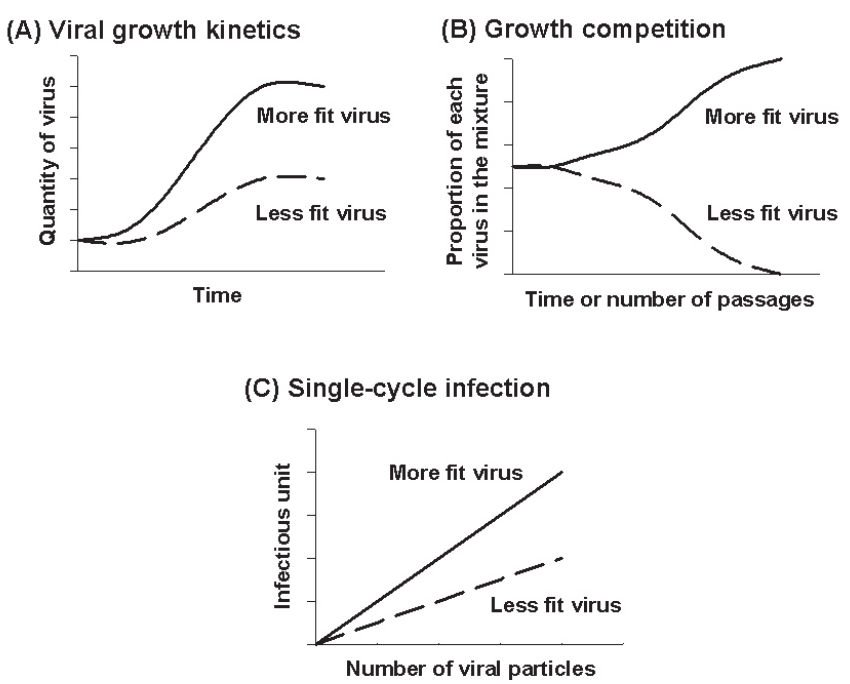

Figure 2. *Methods used to determine in vitro HIV-1 fitness. (A) Viral growth kinetics assays correspond to quantification of virus production at various time points using suitable detection methods (e.g., p24 antigen or RT activity). (B) Growth competition experiments involve dual infection with two different HIV-1 isolates (initial proportion may vary). Production and quantification of each specific HIV-1 in the mixture can be determined using 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[21]).*

cells are infected with the viral mixture, and after several passages, the proportion of both viruses is determined and compared with their proportion in the initial mixture[7,14]. Thus, despite being more laborious, growth competition assays provide a more accurate measure of viral fitness by allowing direct comparison of each mutant against the wild-type reference strain or other mutants. Nevertheless, although the concept of viral competition may be similar, different approaches have been developed in order to quantify the final proportion of both viruses in the mixture and further estimation of relative viral fitness.

Most of these methods rely on point mutation assays or depend on the sequencing of a large number of clones[29-31,36,50,51], whereas new studies use more rapid techniques to estimate the frequency of the two viruses in the population[5,37,39,52,53] (Table 1). Martinez, et al.[54] developed a bacteriophage lambda-based genetic screen to characterize the activity and phenotype of HIV proteases. A competitive phage replication assay, in the presence or absence of drug, was used to measure the relative fitness phages carrying different drug-resistant HIV-1 proteases. On the other hand, a "fitness profile assay" was used to determine the replicative fitness capacity of drug-resistant variants as a function of protease inhibitor concentration (by calculating the ratio of mutant:wt infectivity for each drug concentration, in an assay similar to an IC_{90} determination)[55].

Different versions of the heteroduplex tracking assay (HTA) have been used to evaluate the production of both HIV-1 variants in a competition. Quiñones-Mateu, et al.[37] utilized growth competition experiments of HIV-1 isolates and a rapid and sensitive HTA to analyze the correlation between viral fitness and disease progression. Similarly, a modified V3-specific HTA was used to determine the relative abundance of different *in vivo* V3 populations in HIV late-stage infections[52]. Resch, et al.[56] developed a multiple-site-specific HTA capable of detecting the presence of single, specific mutations or set of linked mutations (i.e., HIV-1 protease gene at positions 46, 48, 54, 82, 84, and 90, HIV-1 RT at positions 181 and 184). This assay allowed the accurate quantification of variants comprising 3% or more of the population, and could also be used to estimate viral fitness *in vitro*.

De Ronde[39] used a real-time NASBA and molecular beacons technique to quantify individual mutant viruses in a mixture (specifically, changes in the codon 215 of the RT) in order to determined viral fitness. This assay allowed a reliable quantification of either mutant at a level as low as 4%. Lu & Kuritzkes[53] developed a novel recombinant marker virus assay (RMVA) to perform growth competition assays to estimate fitness of HIV-1. They used RT-deleted proviral clones of HIV-1 in which the *nef* gene was replaced by the *Salmonella typhimurium* histidinol dehydrogenase (*his*D) or the human heat-stable placental alkaline phosphatase *(PLAP)* genes. Replication-competent viruses were generated by homologous recombination of PCR-derived RT-coding sequences from patients and one of these clones. Following growth competition experiments, the proportion of any RT sequence in the mixture was determined by quantifying the corresponding marker (*his*D or *PLAP*) using real-time PCR. This technique could be used to analyze the fitness of viruses resistant to any other HIV-1 inhibitor (e.g., protease, integrase, *env* inhibitors).

Finally, since homologous recombination represents one mechanism for HIV-1 to acquire drug resistance[57], it could be argued that growth competition experiments may produce HIV-1 recombinant variants, which could modify the measurements of relative fitness. In fact, Kosalaraksa, et al.[58] described the emergence of a new HIV-1 recombinant variant after the fourth passage in a growth competition experiment, which was apparently more fit than the two parental infectious clones. However, in a recent study, we have calculated that the percentage of viral recombination in competition experiments is approximately 3-5%/1 Kbp in a 15-days infection period[59]. Altogether, these results suggest that growth competition experiments with reduced number of passages may exclude the occurrence of this potential artefact.

c) HIV-1 isolates *vs.* recombinant viruses

Multiples groups have constructed different plasmids and designed methods for rapid cloning of HIV-1 PCR products from patient specimens, which can be applied to generate infectious recombinant virus clones for antiretroviral drug resistance testing and to determine replicative viral fitness[31,32,60-63]. Novel cloning vectors are constructed to carry patient-derived sequences encoding HIV-1 protease, reverse transcriptase, and Gag-Pol cleavage sites. One of the main advantages of these recombinant clones is the flexibility for studying clinical specimen-derived clones of the reading frames selected by current protease (PI) and reverse transcriptase (RTI) inhibitors in the same genetic background. In fact, using recombinant viruses, as opposed to HIV-1 primary isolates, eliminates possible effects of polymorphisms or mutations outside the targeted coding region on relative fitness. Most of the studies analyzing the effects of drug resistance mutations on HIV-1 replication capacity (viral fitness) have used recombinant viruses based in the same genetic backbone (usually, HIV-1_{NL4-3} or HIV-1_{HXB2}) [29-31,36,44,51,64]. However, although useful in correlating amino acid substitutions with alterations in replicative capacity, the great diversity of methods to estimate viral fitness makes difficult an adequate comparison among different studies. Furthermore, we cannot circumvent the fact that although no single viral background or cell type can be considered an ideal system to analyzed HIV-1 fitness *in vitro*, other genomic regions (external to those used in the recombinant virus) could have an equal or higher impact on the fitness of the virus.

Should we use HIV-1 clinical isolates instead of recombinant infectious clones to measure viral fitness? It is obvious that it will depend on the topic to be investigated. Studies correlating viral fitness with HIV-1 pathogenesis[37,65] used viral isolates since the

whole genome is likely to be responsible for the viral replicative capacity of the virus. In the case of viral fitness of HIV-1 drug-resistant variants, multiple studies have shown that recombinant viruses carrying PCR-amplified products from patient samples can provide useful information[29-31,36,44,51,64]. However, direct comparison of different methods to estimate viral fitness has produced contradictory results. Grant, et al.[66] analyzed fitness differences between PI-resistant and PI-susceptible viruses using *in vivo* measures of the wild-type:mutant ratio in the plasma and an adaptation of the PhenoSense HIV drug susceptibility assay (see above). They found that both methods compared well, indicating that this rapid recombinant virus assay may provide virologically relevant information. In addition, Prado, et al.[45], analyzing viral fitness of amprenavir-resistant viruses, showed a concordance of replication capacity measurements generated using this single-cycle replication assay and a growth competition assay. However, as described above, the authors acknowledged that small relative fitness differences were more apparent using growth competition experiments. Moreover, Bleiber, et al.[32] have recently described the individual contributions of mutant PR and RT to viral fitness of drug-resistant HIV-1. They analyzed the fitness of HIV-1 clinical isolates and whether or not recombinant viruses carrying PR, RT, or PR-RT cassettes in a NL4-3 background, reproduce the behavior of these parental isolates. Interestingly, despite the reduced viral fitness of recombinant clones, fitness of two of the HIV-1 isolates was comparable to that of the wild-type, suggesting an extensive compensation by genomic regions away from PR and RT. They showed discrepancies in the results, depending on the cellular system used for testing (e.g., CEM T-cell line accentuated defects linked to PR, but improved the fitness of RT recombinants). Thus, it is evident that fitness of drug-resistant HIV-1 variants is a complex interplay of cellular and virological factors, and *in vitro* results must be carefully interpreted, since *in vivo* and *in vitro* assays imposes different environmental constraints for viral fitness.

Fitness of HIV-1 drug-resistant viruses

The ultimate goal of the present therapy is to suppress HIV-1 replication as much and as long as possible. Maintaining plasma low-to-undetectable HIV-RNA would prevent progression to AIDS and minimize the risk of emergence of HIV variants resistant to the drugs used[67]. However, treatments with combination of antiretrovirals do not completely inhibit HIV-1 replication, eventually leading to treatment failure. Each of the sixteen antiretroviral drugs licensed in the United States belongs to one of three classes: (i) nucleoside reverse transcriptase inhibitors (NRTI), nonnucleoside reverse transcriptase inhibitors (NNRTI), and protease inhibitors (PI). However, the effectiveness of all these drugs is limited by the emergence of drug-resistant variants, frequently showing extensive cross-resistance within each drug class[68-70].

Mutations related to resistance to reverse transcriptase or protease inhibitors preexist at low levels in the HIV-1 quasispecies of patients undergoing no therapy with the relevant inhibitors[42,71,72]. In consequence, plasma HIV-RNA rebound during therapy is largely due to replication of drug-resistant mutant viruses selected from the viral quasispecies population by antiretroviral therapy[73,74]. Coffin[73] suggested that, in the absence of antiretroviral therapy, viruses containing drug resistance mutations have a reduced fitness compared to wild-type (wt) viruses and as a result, wt variants are prevalent within the population in the absence of therapy. Clearly, drug-resistance mutant viruses are highly fit in the presence of antiretroviral drugs, as it has been demonstrated by a number of studies over the past few years (see below). Therefore, selective pressure introduced through drug therapy can result in rapid shifts in the relative replicative fitness of these mutants, leading to dramatic changes in the relative prevalence of different genotypes within a patient's HIV-1 quasispecies[1,13,73,75]. During this *in vivo* selection, several drug-resistant variants will emerge in parallel and competition will result in outgrowth of the most fit variant.

Two types of mutations associated with drug resistance have been described: *primary* mutations, which allow the wild-type virus to escape drug inhibition, and *secondary* or *compensatory* mutations, which increase the fitness of the drug resistant virus[67]. Accordingly, two phases in the evolution of viral fitness during antiretroviral therapy have been proposed: (i) selection of drug resistance variants, usually accompanied by a decrease in viral fitness, and (ii) selection of compensatory mutations, which although do not increase drug resistance, gave rise to variants with increased replication capacity [21,22,76]. A decreased in viral fitness was first reported for HIV-1 variants with the mutation M184V within the catalytic core of the RT, associated with resistance to the nucleoside analog lamivudine (3TC)[23]. Subsequently, multiple studies have reported impaired enzyme function and reduced viral fitness for HIV-1 isolates harboring amino acid substitutions related to reverse transcriptase and protease inhibitors[28,29,31,34,44,50,51,77].

As described below, the reduced sensitivity to antiretroviral therapy may not only be associated with mutations at PR and RT sites, but also with genotype alterations outside these regions, for example at Gag-Pol cleavage sites, increasing viral fitness by improving the cleavage and processing of precursor proteins[28,32,33,44,78]. Therefore, both the specific sequence background within which a resistance mutation develops (baseline sequence) and the selection of *de novo* compensatory mutations, contribute to the evolution of viral fitness[79,80]. Myint, et al.[81] showed that the presence of two primary PI-resistance mutations 30N and 90M, in an HXB2 background, abolished the infectivity completely. However, a recombinant virus carrying a protease PCR fragment from a clinical isolate which harbored these two mutations, was replicative competent. Deeks, et al.[82] analyzed the evolution of viral fitness

in patients experiencing virological failure of a PI-based regimen, showing a gradual increase in both PI resistance and replicative capacity (associated with the emergence of secondary mutations). In a similar study, Weber, et al.[83] demonstrated that the viral genetic background previous the initiation of PI-based therapy influences HIV-1 fitness evolution. Altogether these results suggest that the context of sequence (genetic background) may compensate drug resistance mutations and is necessary to partially recover the impaired viral fitness.

In the natural host, the best-fit HIV sequence differs strongly between patients due to individual variation in multiple host (e.g., immune response, genetic background, and target cell availability) and viral (e.g., replication capacity, mutation rate, and host cell tropism) factors[21,22]. Recently, Wrin, et al.[47] reported that wild-type viruses from treatment-naïve patients have a broad range of replicative capacities (47 to 89%, median 73%) compared to the HIV-1_{NL4-3} virus used as control. This study emphasizes the necessity of a careful interpretation of viral fitness measurements, especially on the significance of reduced replication capacity of HIV-1 drug-resistant variants. For instance, what is the contribution of protease and RT drug resistance mutations to viral fitness?

Viral fitness of PI-resistant variants

The HIV-1 protease is the enzyme responsible for the cleavage of the viral Gag and Gag-Pol polyprotein precursors during virion maturation, which yields the structural proteins and the enzymes of the viral particle[68,84]. 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). Many mutations associated with HIV-1 resistance to these protease inhibitors have now been identified[68,85]. The protease gene has shown great plasticity, with polymorphisms detect in 49 of the 99 amino acids of the HIV-1 protease monomer and substitutions at more than 20 amino acids being associated with resistance to PI[68,85] (Fig. 3). Although they can be sporadically witnessed in viral quasispecies[72], most of these primary mutations are rarely found in PI-naïve HIV-infected individuals, suggesting that they confer a selective disadvantage to the virus[85,86]. In fact, multiple mutations appear to be necessary for the development of a PI-resistant virus that is able to replicate, where most of these residues are highly conserved within the different subtypes of HIV-1[87,88].

For most of the protease inhibitors, primary PI resistance mutations cluster near the active site of the enzyme (Fig. 3), reducing both protease catalytic activity and viral replicative capacity[29,43,44,68]. Secondary mutations (e.g, 10, 63, 71, 77) which partially compensate the impairment on HIV replication, are usually located outside of the substrate-binding region of the enzyme suggesting conformational adaptation to the primary changes in the active site[24,40,43,44,79,89,90] (Fig. 3). In addition, changes located

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	.	.	I	V	.	V	VM	.	.	.	.	.	V	.	M
Indinavir (IDV)	IRV	MR	I	.	I	.	IV	IL	.	V	.	V	APQ	TV	STA	I	AFTS	V	S	M
Lopinavir (LPV)	FIRV	MR	I	.	.	.	.	I	.	.	V	.	.	TV	.	.	AFTS	V	.	M
Nelfinavir (NFV)	FI	.	.	N	.	.	IV	IL	.	.	.	.	I	TV	.	I	AFTS	V	D	M
Ritonavir (RTV)	I	MR	.	.	I	FI	I	IL	.	.	.	VL	AP	TV	.	I	AFTS	V	.	M
Saquinavir (SQV)	IRV	.	.	.	.	.	.	.	.	V	.	V	.	TV	S	I	A	V	.	M

B

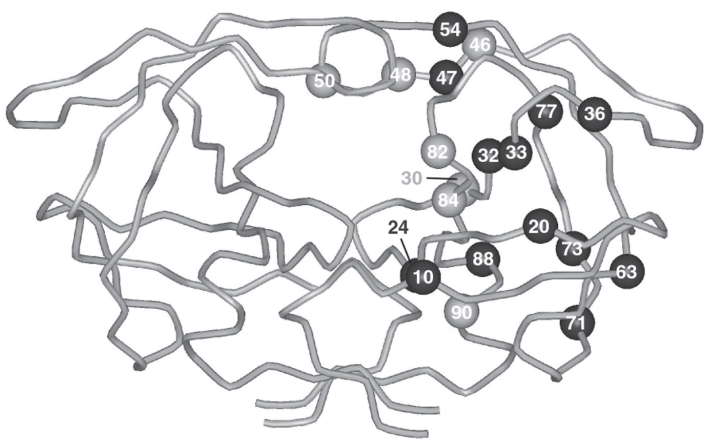

Figure 3. *(A) Summary of protease mutations associated with resistance to Protease inhibitors. Amino acids in grey and black denote primary and secondary/compensatory mutations, respectively. Wild-type amino acids (HIV-1 subtype B) at the codons related to resistance to PI are indicated. Mutations related to loss of sensitivity to PI[85,127] were recently reviewed at http://www.iasusa.org. (B) Structure of the HIV-1 protease (pdb file1hxb)[165], indicating amino acid residues associated with resistance to PI. Each numbered circle indicates codon position and the nature of the resistance mutation (primary and secondary mutations in black and grey, respectively).*

within eight major protease cleavage sites (*gag* and *pol* genes), have been associated with resistance to protease inhibitors[21,22,28,68,78]. Mutations in these regions provide better peptide substrates for the mutated protease, which partially compensate for the resistance associated loss of viral fitness[21,22,28,33,44]. Moreover, it has been reported that some PI-resistant viruses display defects in the processing of the RT enzyme[91], reducing the levels of RT in the virions and perhaps contributing to a reduction in viral fitness. Interestingly, AZT resistance mutations in the RT can partially rescue the replicative defect of a PI-resistant virus, which could be relevant to the therapeutic control of HIV-1 infection[91].

In summary, after the introduction of PI-based antiretroviral treatment, the virus follows a "step-by-step" general pathway to overcome the drug selection: (i) acquisition of primary resistance mutations in the protease gene, (ii) selection of secondary/compensatory protease mutations to repair the enzyme function, which directly rescue the viral fitness, and (iii) selection of mutations in the major cleavage sites of the Gag and Gag-Pol polyprotein precursors, to restore protein processing and to increase production of HIV-1 protease enzyme[22,76,92-94]. However, the exact evolutionary pathway will depend on the type of protease inhibitor, the viral genetic background, and stochastic mutations.

During the last few years, multiple studies have described a significant reduction in viral replicative capacity as a consequence of the development of PI resistance, generating a comprehensive list of mutations related to drug resistance and impairment/rescue of viral fitness (Table 2). Most of these mutations reduce the replicative capacity of the virus. However, several amino acid substitutions (usually combinations of primary and secondary mutations) have been shown to restore the impaired viral fitness to similar, or even higher, levels than the wild-type virus (Table 2). *In vitro* selected highly resistant HIV-1 variants to the PI BILA-1906-BS and BILA-2185-BS contained mutations in the protease gene and also in one or two Gag cleavage sites, with slower growth kinetics than wild-type viruses. Mutations in cleavage sites (i.e., p7/p1 and p1/p6) compensated for the impaired ability of mutant viruses to replicate, but do not contributed to viral resistance to protease inhibitors[28,29]. A similar result was obtained using a different PI (DMP 450 or mozenavir): drug-resistant viruses carrying the 84V mutation were less fit than the wild-type strain, in the absence of drug[95].

Borman, et al.[43] were one of the first to report differences on replicative capacity in viruses resistant to the protease inhibitor ABT-77003 (which harbored the 32I mutation). Further accumulation of mutations at secondary sites (46I, 71V, and 82A) led

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

Amino acid substitutions	Relative fitness to wild-type	References
8K	Comparable	89
8Q	Decreased	89
10I	Comparable	55,158
10I,F	Decreased	55,158
17R*	Decreased	98
22V*	Decreased	98
25E	Decreased	159
25H*	Decreased	98
30N	Decreased	27,31
32I	Decreased	29,43
35TVLEE*	Decreased	98
35TD*	Decreased	98
35TN*	Decreased	98
36I	Comparable	55
36NL*	Decreased	98
36GL*	Decreased	98
36DL*	Decreased	98
37D*	Decreased	98
37G*	Decreased	98
37N*	Decreased	98
46I	Comparable	43,51,55,89
46I,L	Decreased	27
47V	Decreased	158
48V	Decreased	55
50V	Decreased	158
54V	Comparable	55
63P,A	Comparable	40,51,162
71V	Comparable	43,55
82A	Comparable	43,55
82A,T,F	Decreased	24,40,49,51,100,162
84V	Comparable	162
84A,V	Decreased	24,29,95
90M	Comparable	55
90M	Decreased	31

Table 2. *Effects of protease mutations on HIV-1 replication capacity (cont.)*

Amino acid substitutions	Relative fitness to wild-type			References
95TLNFPI*	Decreased			98
8K/46I		Comparable		89
10I/48V	Decreased			55
10F/50V	Decreased			158
10F/84V	Decreased			45
10I/90M		Comparable		55
10F+449F**	Decreased			45
30N/63P	Decreased			31
30N/88D	Decreased			163
30N/90M	Decreased			163
32I/71V	Decreased			29
36I/54V	Decreased			24
46I/63P		Increased		157
46I/82A		Comparable		55
48V/82A	Decreased			55
48V/90M	Decreased			55
54V/82A	Decreased			48,55
62I/77I		Comparable		24
63P/90M		Comparable		31
71V/82A		Comparable		55
82T,F/84V	Decreased			157,162
82A/90M	Decreased			55
10I/48V/82A	Decreased			55
10I/48V90M	Decreased			55
10I/82A/90M	Decreased			55
10F/84V+p1/p6**	Decreased			45,51
36I/50V/63P	Decreased			63
36I/54V/82T	Decreased			24
46I/47V/50V	Decreased			158
46I/53L/82A	Decreased			100
46I/54V/82A	Decreased			55
54V/71V/82A		Comparable		55
63P/82F/84V	Decreased			162
10I/23I/46I/84V		Comparable		29
10F/46I/50V+p1/p6**	Decreased			45,51
10L/46I/82T/84V		Comparable		31
20R/36I/54V/82A	Decreased			33
20R/36I/63P/82S	Decreased			33
20R/63P/82A/90M	Decreased			33
36I/50V/63P+p1/p6**	Decreased			63
36I/54V/71V/82T			Increased	24
46I/48V/63P/90M	Decreased			33
46I/54V/71V/82A		Comparable		55
46I/63P/82T/84V		Comparable		31
54V/82A+p2/NC+NC/p1	Decreased			44
10I/23I/46I/84I+p1/p6**	Decreased			29
10I/36I/48V/84V/90M	Decreased			48
10F/46I/47V/50V+p1/p6**	Decreased			45,51
10L/46I/63P/82T/84V		Comparable		31
20R/36I/54V/71V/82T			Increased	24
32I/46I/71V/82A+p1/p6**	Decreased			29,159
36I/46I/71V/84A+p1/p6**	Decreased			28,29
10I/46I/63P/77I/84V/90M	Decreased			48
24I/46I/53L/63P/77I/82A	Decreased			100
54V/63P/71T/72E/82A/85V	Decreased			63
10I/36I/48V/84V/90M+MA/CA+p1/p6**	Decreased			44
10I/54V/63P/71V/77I/82A/90M	Decreased			48
14V/20R/32I/63P/64V/71V/82A	Decreased			54
10I/20R/36I/46L/48V/71V/82A/90M	Decreased			48
10I/20R/36I/54V/63P/71V/82T/90M	Decreased			48
10I/24I/46I/63P/71V/77I/82T/84V	Decreased			48
10I/36I/46L/48V/63P/71V/82A/90M	Decreased			48
54V/63P/71T/72E/82A/85V/85V/+p7/p1**		Comparable		63
10I/20R/36I/46I/53L/63P/71V/82A+NCp1?**	Decreased			33
23I/32I/46I/47V/54M/71V/84V+p1/p6+p7/p1**	Decreased			28,29,159
10I/35D/37D/48V/54V/63P/71V/82A/90M/93L	Decreased			54
10I/14V/33F/36M/37C/54V/63P/67F/71V/72M /73S/77I/82A/84V/90M	Decreased			54

* Insertion mutations at the corresponding codon position.

** Mutations in the protease gene accompanied by mutations at Gag-processing sites.

to an improvement in fitness. A further study showed the role of the 63P/A mutation in improving the fitness of the 82A/F ritonavir resistance mutation, perhaps by compensating the change around the active site of the enzyme[40]. Nijhuis, et al.[24] described how ritonavir-resistant variants increased their viral fitness by acquiring compensatory mutations during sub-optimal therapy. Drug-resistant viruses selected *in vivo* evolved to novel variants with new compensatory mutations, which increased protease activity and replicative capacity when compared to the wild-type virus (viral fitness order: 36I/54V/71V/82T > 20R/36I/54V/71V/82T > wt > 82T > 84V > 36I/54V = 36I/54V/82T). They explained the selection of viruses with increased replication capacity by the Wright's concept of adaptive landscape[15]: "natural selection drives a population to a local optimum, which is not necessarily the global optimum". Generation of these viral variants in the absence of drug could be not viable since the intermediate protease variants have an inferior viral fitness and thus, are exposed to negative selection in the absence of antiretroviral therapy. More recently, Mammano, et al.[55] analyzed the effect of single and multiple mutations in the HIV-1 protease gene on viral fitness in the absence and presence of RTV, establishing a "fitness profile" that correlates with the order of accumulation of resistance mutations selected in treated patients. The 82A mutation conferred a replicative advantage in the presence of 20 to 400 nM of RTV, whereas a marked increased replicative capacity was observed for a mutant harboring 71V/82A in the presence of 1,000 nM of RTV.

Several studies have analyzed the effects of both ritonavir and saquinavir based therapy on viral replicative capacity and protease-mediated processing of Gag and Gag-Pol precursors. Zennou, et al.[33] found that all recombinant resistant mutant viruses harboring protease sequences from patients were less fit that recombinant clones carrying parental pre-therapy proteases. This replicative defect involved mutations in the protease gene (46I, 48V, and 90M) and in at least one cleavage site (NC/p1). A similar study from the same group showed that mutations in the Gag cleavage sites (MA/CA, CA/p2, p1/p6) in patient-derived HIV-1 resistant variants corrected only partially the loss of viral fitness due to selection of RTV/SQV-resistance mutations[44]. Recently, Kaufmann, et al.[96] reported that insertions in the proline rich of the p6*gag* protein may affect the virological response to RTV+SQV therapy. Thus, mutations in the Gag cleavage sites (p7/p1 and p1/6) or C-terminal p6* residues serve as compensatory mutations to increase HIV-1 replicative capacity.

Martinez-Picado, et al.[31] determined the relative replicative fitness of HIV-1 mutants selected by nelfinavir, saquinavir, and indinavir. Using different methods to measure viral fitness, they compared each PI-resistant virus to the HIV-1_{NL4-3} wild-type strain. Interestingly, the 63P substitution (considered a major natural polymorphism) compensated the fitness loss of the 90M SQV-resistance mutation, but only slightly improved the fitness of the 30N NFV-resistance substitution. Overall, the fitness order for the different mutants assayed was: wt = 46I/63P/82T/84V = 10R/46I/63P/82T/84V > 90M > 30N. The relative fitness of viruses harboring the 90M or 30N mutations was calculated as 90% and 63% of the wild-type, respectively. A similar study analyzed the viral replicative capacity of recombinant clones harboring indinavir-selected mutations (46I and 82T) and the polymorphism 63P. Only viruses carrying the 82T mutation showed a decrease in viral fitness (wt = 46I = 63P > 82T)[51].

Few studies have analyzed the replication capacity of amprenavir-resistant viruses. Using both drug-resistant viruses selected *in vitro* and recombinant infectious clones, Prado, et al.[45] showed that APV-resistant variants accumulated mutations at codons 10, 46, 47, 50, and 84 in the protease gene and in the Gag p1/p6 cleavage site (codon 449). When the viral fitness was assayed, all the APV-mutant variants had an impaired replication capacity compared to the wild-type virus (wt > 10F > 10F/84V > 10F/46I/50V > 10F/46I/47V/50V). Interestingly, in contrast to other studies in which, compensatory mutations increased viral replication capacity[21,22], in this study the progressive accumulation of PR mutations did not re-establish viral fitness.

Several years ago it was inconceivable to find amino acid insertions on "conserved" HIV-1 enzymes as the protease and RT. However, as the pool of HIV sequences increase, we have found that the HIV genome is extremely variable and that *pol* sequences are only two-to-three-fold less variable that the *env* gene[97]. Recently, an analysis of over 24,000 HIV-infected individuals reported that 0.09% of these patients possess HIV-1 isolates with an insert in the protease gene (1, 2, 5, or 6 amino acids mapping between codons 35-38, 17-18, 21-25, or 95-96)[98]. Most of the inserts (79%) mapped between codons 35 and 38. These isolates, although less fit that the wild-type NL4-3 strain, showed an increased replicative capacity in respect to their counterparts insertion/lacking viruses. Although rarely, insertion in the PR gene can occur, do not increase resistance to PR, but may provide an advantage in replication capacity. In addition, Grant, et al.[99] found that an HIV-1 isolate with an insertion in the protease gene at codon 35, had normal replication capacity, was susceptible to PI and, even more important, proved to be transmissible (it was found in both partners in an HIV-infected couple), suggesting that it confers an advantage in terms of viral fitness. Recently, Buhler[100] analyzed the HIV response to the broad-based retroviral protease inhibitor TL-3, which is effective against HIV, SIV, FIV, and is able to inhibit several isolates with PI-resistance mutations. They showed that an HIV-1 variant with six amino acid changes in the protease (i.e., 24I/46I/53L/63P/77I/82A) was found to be TL-3 resistant (17-fold), with a replicative capacity comparable to the wild-type virus. Taken together, these results demonstrate that the high plasticity of the HIV genome allows the virus to explore and find different ways to escape diverse selection pressures.

Finally, recent studies have correlated the impairment on viral fitness of PI-resistant variants with an

atypical response to antiretroviral therapy, perhaps with consequences on HIV-1 control and disease progression. In order to understand "discordant responses" in HIV-1-infected patients (high viral loads and sustained CD4+ T-cell counts in PI-treated individuals), Stoddart, et al.[48] used HIV-1 isolates and recombinant HIV-1 clones containing wild-type or PI-resistant clones to infect (i) PBMC, (ii) human thymic organ cultures, and (iii) SCID-hu Thy/Liv mice. Interestingly, viral replication capacity of PI-resistant strains in the thymus was highly impaired, perhaps contributing to the preservation of CD4+ T-cell counts in patients failing PI-based therapy. Further studies are necessary to determine why PI-resistant viruses have a more severe impairment in replicative capacity in thymocytes.

Viral fitness of RTI-resistant variants

Multiple studies have explored how RTI resistance mutations affect HIV-1 replication capacity (Table 3, Fig. 4) (reviewed in[21,22]). However, RTI-associated amino acid changes do not appear to reduce viral fitness at the same levels that PI do[21,76], perhaps due to a restricted evolution of RT inhibitor resistance (no compensatory changes have been observed in other loci of the HIV-1 genome). Here we discuss some of the most relevant studies involving RTI drug resistance and viral fitness.

a) NRTI

Nucleoside analogue reverse transcriptase inhibitors (NRTI) were the first class of antiretrovirals to be developed and to provide effective antiviral therapy in the setting of HIV-1 infection[70]. These drugs compete with deoxynucleoside triphosphates (dNTPs) during polymerization and act as premature chain terminators upon incorporation[101]. Up to date, six NRTI have been approved to be used on the United States; i.e., zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), and abacavir (ABC). In addition, tenofovir (TNV) is the leading drug among nucleotide reverse transcriptase inhibitors (NtRTI) and the only approved to be used in patients. Altogether, these drugs remain the backbone of nearly all anti-HIV treatment strategies.

It is not surprising that some of the first studies showing the effect of drug resistance mutations in viral replication fitness were related to AZT. Analyses of *in vivo* HIV fitness[25], using a single AZT resistance mutation at codon 215, concluded that the mutation 215S (susceptible to AZT) was 0.4 to 2.3% more fit than the resistant mutation 215Y. Afterward, the same group used blood samples from an untreated individual infected with an AZT-resistant strain to quantify the relative fitness *in vivo* of three subpopulations of HIV-1 marked by mutations at codons 41 and 215 of the RT. They found that 1 to 2% of fitness difference was sufficient for significant replicative advantage (fitness order wt > 215S > 215D >215T)[26]. Yerly, et al.[102] analyzed successive HIV-1 RNA plasma samples from seroconverting individuals infected with virus harboring the AZT-associated 215Y/F mutation. Only one patient

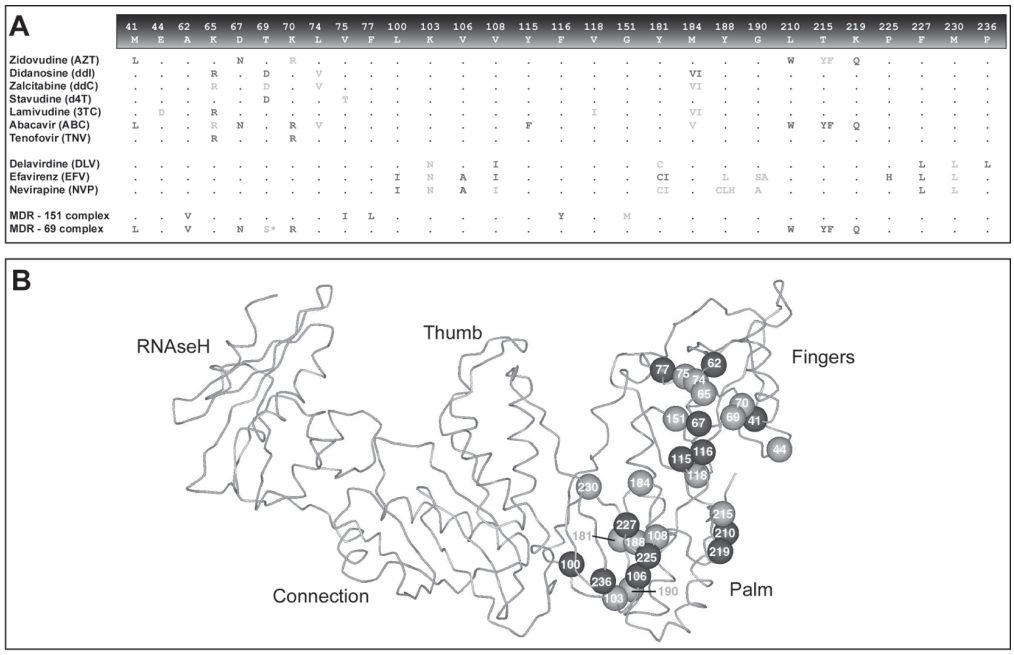

Figure 4. *(A) Summary of reverse transcriptase mutations associated with resistance to RT inhibitors. Amino acids in grey and black denote primary and secondary/compensatory mutations, respectively. Wild-type amino acids (HIV-1 subtype B) at the codons related to resistance to RTI are indicated. Mutations related to loss of sensitivity to RTI 85,127 were recently reviewed at http://www.iasusa.org. (B) Structure of the HIV-1 RT (pdb file1hxb) 166, indicating amino acid residues associated with resistance to RTI. Each numbered circle indicates codon position and the nature of the resistance mutation (primary and secondary mutations in black and grey, respectively).*

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

Amino acid substitutions	Relative fitness to wild-type			References
41L	Decreased			50
62V		Comparable		34
67N	Decreased			27
69SS*	Decreased			131,132
70R		Comparable		30
70T,R	Decreased			30,50
74V		Comparable		34
74V	Decreased			30,64
75I		Comparable		34
77L		Comparable		34
89K	Decreased			111
92I	Decreased			111
98G	Decreased			77
100I	Decreased			77,95
103N		Comparable		27,77
103N	Decreased			114
106A	Decreased			77,115,119
108I		Comparable		77
115L,A,D,W	Decreased			156
116T		Comparable		34
151M		Comparable		34
151M			Increased	58
151M,L				103
156A	Decreased			111
163N			Increased	104
179D	Decreased			115
181C		Comparable		77
181C	Decreased			115
181C			Increased	119
184I,V	Decreased			23,27,49,53,64,109, 110,112,151
188C		Comparable		77
190S,A		Comparable		77,119
215Y,S,D		Comparable		34,39
215Y,F,N,S,D	Decreased			25,26,39,40,50,58
215S			Increased	58
236L	Decreased			114,115,118
41L/70R	Decreased			104
41L/215Y	Decreased			50
115W/230I	Decreased			164
151M/215Y	Decreased			58
r67/69G/74I	Decreased			77
77L/116Y/151M		Comparable		34
77L/116Y/151M			Increased	58
67N/70R/215Y/219Q			Increased	105
75I/77L/116Y/151M		Comparable		34
75I/77L/116Y/151M	Decreased			58
62V/75I/77L/116T/151M		Comparable		34
62V/75I/77L/116Y/151M			Increased	58
69G/70R/74I/103N/215F/219Q	Decreased			36
r67/69G/70R/74I/103N/215F/219Q		Comparable		36

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

reverted to the wild-type 215T, whereas unusual 215D/C mutations appeared in the revertant viruses. These viruses were more fit than the original 215Y drug-resistant variant (*in vivo*). Their data suggest that most patients will revert from 215Y to other amino acids within a year in the absence of drug pressure. A further study confirmed these results, showing that unusual variants at codon 215 of the RT in treatment naïve patients (i.e., 215C/D/S) are more fit that the AZT-resistant 215Y mutant[103]. Moreover, it has been shown that after primary infection with HIV-resistant AZT variants, some viruses are readily replaced by drug sensitive strains (e.g., 215Y to 215D,S,N) indicating the establishment of new wild-type populations[39]. In this study, the authors used infectious molecular clones to analyze the contribution of these mutations to viral fitness, obtaining results according to the *in vivo* observations (i.e., fitness order: 215T = 215S = 215D > 215Y). These new wild-type viruses are AZT sensitive but the presence of a single mutation allow them to develop resistance to AZT (215Y), and thus,

expected to arise rapidly if treatment with AZT is initiated in these patients.

Harrigan, et al.[50] analyzed the relative viral fitness of AZT-resistant HIV-1 isolates *in vitro*. They demonstrated a stepwise accumulation of AZT resistance mutations (41L, 70R, and 215Y) in a manner similar to that observed *in vivo*, which may be related to the initial proportion of the given variant as is determined for its intrinsic fitness (i.e., wt > 70R >> 215Y = 41L/215Y > 41L). Although the 41L mutation appears to be relatively stable *in vivo* in the absence of selection[26], its low fitness in a zidovudine-free environment may explain the fact that this mutation is rarely observed in untreated patients[26,71]. In a different *in vitro* study, Jeeninga, et al.[104] showed that the AZT resistance 41L/70R double mutant virus has a poor replication capacity, whereas introduction of the 215Y mutation restores the viral fitness. These results would explain the absence of the 41L/70R mutations in clinical samples. In addition, *in vitro* replication of this double mutant lead to the selection of compensatory mutations (e.g. 163N), which restored the function and increased the fitness of the 41L/70R HIV-1 mutant. Interestingly, an early study showed the combinatorial effects of AZTresistance mutations (67N/70R/215Y/219Q), which increased viral replication capacity in PHA-stimulated PBMC, perhaps by increasing DNA synthesis[105].

Lamivudine-resistance viruses, especially viruses harboring the 184V mutation, have been shown to increase RT fidelity[106,107], to diminish RT processivity and further decrease viral replication fitness[23,108]. Back, et al.[23] were among the first to report impaired replicative fitness of viruses carrying 3TC resistance mutations (fitness, wt > 184V >184I). *In vitro* selection with 3TC showed that the mutation bias of the RT enzyme allows a higher frequency of the 184I (56%) over the 184V (12.5%), explaining the initial appearance of this mutation in patients under 3TC treatment[109]. However, viruses harboring the 184V mutation are more fit than those carrying the 184I substitution [23], explaining the eventual outgrowth of the most common 184V drug resistance variant. Frost, et al. [110] estimated the viral fitness of the 184V mutation, in the presence of 3TC, as 10% of that of the wild-type prior of therapy, whereas 184V has a replicative advantage over 184I of approximately 23%. In summary, increased RT fidelity and decreased fitness of the M184V HIV-1 virus, perhaps due to decreased polymerase activity[23], may be factors contributing to the strong antiviral effect of AZT/3TC combination therapy.

Few studies have addressed the association of other NRTI with HIV-1 replicative capacity. Didanosine (ddI)-related 74V mutation has been shown to confer a viral replication disadvantage (11% loss of fitness) in PBMC[30], whereas viruses containing ddI-resistance 74V and 3TC resistance 184V mutations have shown a decreased RT processivity[64]. These results suggest that the reduced replication capacity of viruses harboring these mutations (wt > 184V > 74V) is due in part to this biochemical mechanism. Viral replication analyses of HIV-resistant mutants to less conventional nucleoside analogs have demonstrated a similar pattern: wild-type viruses are generally more fit than drug-resistant mutants. Tachedjian, et al.[111] showed that foscarnet-resistant HIV strains (carrying the 89K, 92I, 156A RT mutations) have impaired fitness. Mutant viruses with resistance to the NRTI QYL-685 and QYL-609 NRTI, which selected the 184I/V RT mutation, have a reduced replication competence in the absence of the drugs[112].

b) NNRTI

Nonnucleoside reverse transcriptase inhibitors (NNRTI) are noncompetitive inhibitors that bind to a hydrophobic pocket adjacent to the polymerase active site of RT[69]. This binding causes an allosteric change of the polymerase active site which inhibits DNA polymerization[113]. NNRTI-associated mutations are usually in the hydrophobic pocket and a single mutation may result in high-level resistance to one or more NNRTI[69,85] (Fig. 4). Three NNRTI are currently approved for antiretroviral therapy in the United States: delavirdine (DLV), efavirenz (EFV) and nevirapine (NVP). The most commonly observed effect of NNRTI resistance mutations is a change in the ratio of RNase H to polymerase activities[114,115]. However, the level of drug resistance *in vitro* does not always correlate with the likelihood of a drug-resistant variant emerging *in vivo*. Although the viral replicative capacity of NNRTI-resistant viruses has not been extensively studied, available data suggest that NNRTI-selected single-point mutations, such as 103N or 181C. These mutations confer little damage to viral fitness, creating highly resistant viruses without compromising viral replication, thus persisting during long-term virologic failure.

The most common nevirapine resistance mutations are 103N and 181C, which confer cross-resistance to other NNRTIs[85]. Havlir, et al.[42] analyzed the *in vivo* viral replicative capacity of nevirapine-resistant HIV variants, based on quantitation of wild-type and 181C-mutant strains (e.g., different kinetics of viral turnover in plasma and PBMC). The gradual turnover of the population to the 181C mutant suggested that this is the most fit population under the selective pressure of nevirapine monotherapy. *In vitro* resistance to efavirenz has been associated with 100I, 190S and/or 103N mutations[69]. Rayner, et al.[95] showed that viruses carrying the EFV-resistance 100I mutation impair viral replication in the absence of efavirenz. The 190S mutation confers higher levels of resistance than 103N[69,85], but this last mutation occurs more frequently *in vivo*. In the absence of drug, the replicative capacity order of these variants was wt > 103N > 190S, while viruses containing the 190S mutation outcompeted 103N variants only at high concentration of efavirenz[69].

In vitro passage of HIV-1 in the presence of delavirdine (DLV) selects for a unique 236L mutation[116] which confers higher resistance that 103N and 181C. However, only 6% of DLV resistant HIV isolates from patients receiving DLV monotherapy contained this mutation[117]. Interestingly, Gerondelis, et al.[114] demonstrated that the NNRTI resistance mutation 236L, in a HIV-1_{NL4-3} backbone, is replica-

tion-defective relative to the wild-type and 103N viruses. This suggests that the high level of DLV resistance conferred by the 236L mutation is insufficient to compensate for its decreased fitness relative to other NNRTI resistance mutants (e.g., 103N or 181C), and it may explain why 236L is not commonly selected during therapy with DLV. This was the first description of clinical drug-resistant mutants of HIV-1 with abnormalities in RNase H cleavage[114]. A further study showed that the 106A mutation confers resistance to NNRTI and affects the rates of both DNA 3'-end- and RNA 5'-end-directed RNase H cleavage, whereas is associated with a significant reduction in the replication fitness of HIV-1 (relative order for replication fitness was wt > 179D ~ 181C > 106A ~ 236L)[115]. In addition, viruses harboring NNRTI-resistance 98G or 106A mutations have a decreased virus replication[77]. However, this impaired viral fitness can be compensated by the addition of compensatory mutations (such as 69S/74I), perhaps influencing the RNase H cleavage[77].

Dykes, et al.[118] confirmed that the mutation 236L in the background of the HIV-1 laboratory strain NL4-3 reduce the replication capacity of the virus, whereas other NNRTI-resistance mutations, such as 103N and 181C, do not. They postulated that HIV-1 isolates from patients with the 236L mutation must carry compensatory mutations. To test this hypothesis, they constructed HIV-1_{NL4-3} recombinant viruses with delavirdine-resistant RT sequences derived from patients' isolates. Although recombinant clones containing 236L replicate slower than clones with either 103N or 181C, most of the patients who acquire 236L during delavirdine therapy do not have compensatory mutations within the RT sequence. Altogether, these results suggest that (i) mutations outside RT may compensate for the replication defect conferred by 236L or (ii) HIV-1 variants with highly impaired fitness can be selected during treatment with NNRTI.

As described above, in most cases, the 103N and 181C mutations have minimal impact on viral fitness, which could explain the failure of NNRTI when used in suboptimal regimens. A recent analysis of *in vitro* selected nevirapine-resistant variants showed that, in the absence of drug, the Y181C mutant was more fit than the wild-type virus (fitness gradient: 181C > wt > 106A > 190A)[119]. Furthermore, 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 the RT[120], which obviously do not affect the wild-type fitness. However, no mutations at position 236 have been identified in any group O RT sequences[120].

Viral fitness of multiple dideoxynucleoside resistance (MDR) variants

It is well known that currently available 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[121]. Thus, a "second epidemic" of AIDS may be under way among patients who harbor HIV-1 strains that are resistant to several available drugs. The prevalence of drug resistant mutations in patients with primary HIV-1 infection has been assessed in several studies conducted in Europe[122] and in the United States[123]. 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[123]. Moreover, MDR strains acquired during primary HIV infection can persist for more that nine months, despite a decreased viral fitness[124].

Accumulation of multiple mutations associated with antiretroviral drug resistance does not occur at random but follows a certain order in most cases[125]. Thus, as discussed before, HIV-1 can evolve under drug selection pressure by selecting few drug resistance mutations (at expense of viral fitness), but later improving viral replication competence through further mutations. Very few studies have documented drug resistance mutant variants with a replicative advantage over wild-type viruses when examined in absence of drugs (Table 3). A set of five mutations in the RT (62V/75I/77L/116Y/151M), associated with MDR[34,126], comprise MDR-151 complex[127] (Fig. 4). Several studies have assessed the *in vitro* fitness of viruses containing these mutations[34,58], where some combinations have been reported to be more fit than the wt in the absence of drug (fitness order: 62/75/77/116/151/ > 77/116/151 > 151 > wt > 75/77/116/151 > 151/215 > 215)[34,58]. The 151M mutation is thought to be the first mutation to develop, followed by 75I, 77L, 116Y, and then 62V[85,126]. Garcia-Lerma, et al.[103] evaluated the replication capacity of different MDR recombinant viruses carrying the 151M mutation and two different intermediates (151L or 151K) in different RT genetic backgrounds. They showed that a virus harboring the 151M mutation was more fit than the 151L. Thus, 151L mutants could be a potential intermediate of these MDR variants.

Another set of mutations associated with high-level resistance to multiple RT inhibitors involves the 69G amino acid substitution. This complex has been denominated MDR-69[127] (Fig. 4). Whereas the 69G mutation appeared to be critical for high-level resistance, the combination with an amino acid deletion at codon 67 (?67), improved the fitness replication of a highly AZT-resistant virus containing the 69G/70R/74I/103N/215F/219Q in the RT, to wild-type levels[36]. Recent studies have also reported that nucleotide insertions in the reverse transcriptase gene with a 69S mutation lead to high-level resistance to multiple NRTI, including AZT, 3TC, d4T, ddI, and ddC[128-130]. HIV-1 encoding a dipeptide insertion between codons 69 and 70 does not show a clear selective *in vivo* advantage over other genomes lacking the insertion (i.e., low frequency in cohort studies and a fluctuating nature of the genomes harboring the insertion)[128,129]. Moreover, a decrease in viral fitness in the absence of drugs has

been observed in viruses harboring a dipeptide insertion (SS) between codons 69 and 70 of the RT[131]. Finally, Lukashov, et al.[132] 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 the RT. After termination of therapy, these mutants were replaced by wild-type variants, indicating a competitive disadvantage of the insertion mutant in the absence of therapy (less than 84% viral fitness compared to the wt virus). However, these MDR mutants were able to maintain high viral loads in the presence of antiretroviral therapy.

Viral fitness and resistance to other HIV-1 inhibitors

Maintaining plasma low-to-undetectable HIV-RNA would prevent progression to AIDS and minimize the risk of emergence of HIV variants resistant to the drugs used. However, treatments with combination of antiretrovirals do not completely inhibit HIV replication, eventually leading to treatment failure. Furthermore, within each drug class, there is extensive cross-resistance (e.g. 103N causes high-level resistance to the three available NNRTI, 90M produces some degree of resistance to the four available PI, and 151M confers resistance to each NRTI except 3TC). Thus, there is a substantial need for the availability of new and novel agents, which target different sites involved in the virus life cycle. Currently, the area of most interest is in drugs that interfere with attachment, fusion or entry of HIV into susceptible cells.

Several novel antiretrovirals focus in the interactions between HIV-1 and the cell surface, which lead to viral entry. Based on this process, entry inhibitors can be grouped into different classes: (i) those blocking the binding of HIV-1 envelope glycoprotein (gp120) to the primary receptor (CD4)[133], (ii) agents inhibiting the binding to the chemokine receptors CCR5 or CXCR4[134,135], and (iii) drugs blocking the fusion of the viral and cellular membranes[136].

A number of inhibitors of the chemokine receptors, CCR5 and CXCR4, have been developed (e.g., analogues of RANTES, the natural ligand of CCR5, and AMD-3100, a positive charged bicyclam with *in vitro* activity against HIV-1 strains that use CXCR4) (reviewed in[137]). Although, there has been concern that CCR5 inhibitors may select for syncytium-inducing, CXCR4-using HIV variants, which could lead to more rapid disease progression[138], resistance to CCR5[139] or CXCR4[140] can occur without a switch in coreceptor usage. In addition, selected AMD3100-resistant viruses without a change in co-receptor use, have been shown to have significantly diminished fitness compared to wild-type virus (HIV-1_{NL4-3})[140].

Among the fusion inhibitors, T-20 (a 36-amino-acid peptide matching the HR-2 sequence of the viral envelope protein, gp41) is currently in clinical trials. Although of importance in highly treatment-experienced patients, resistance to T-20 has been already described[136]. A second and more potent fusion inhibitor (T-1249) is in development, which is active against T20-resistant HIV variants[141]. Lu & Kuritzkes[142] showed that recombinant viruses in which mutations known to confer resistance to T-20 (37T, 38M or 36S/38M in the extracellular portion of the *env* gp41) were introduced by site-directed mutagenesis in a HIV-1_{NL4-3} background, were significantly less fit than the wild-type control (relative fitness order: wt > 37T > 38M > 36S/38M). Further studies using data from HIV-infected patients in ongoing T-20-based clinical trials would add more information about the *in vivo* fitness of these variants.

Finally, recent studies on integrase inhibitors (i.e., diketo acids analogs) have shown to be effective on inhibiting HIV-1 replication[143]. Resistance to these compounds has been related with specific mutations in the integrase active site (i.e., 153Y, 66I, and 155S), which impair enzymatic function *in vitro*. Furthermore, as with other antiretroviral drugs, accumulation of high-level resistance to integrase inhibitors is associated with a significant loss of viral replicative capacity[143]. More studies are necessary to study the fitness of these drug-resistant variants, as well as to assess their clinical significance.

Viral fitness and structured treatment interruptions (STI)

Overall, virological failure has been defined as the inability to achieve complete suppression of viral replication[144]. However, although complete viral suppression may be impossible to achieve with the current antiretroviral therapies[145], this may not be a requisite for durable treatment benefit. As previously described, drug resistance mutations in the *pol* gene are generally accompanied by a reduction in the viral fitness in the absence of antiretroviral drugs. Nevertheless, when antiretroviral treatment is interrupted, drug-resistant members of the quasispecies are often replaced by the most fit wild-type virus[35,73], which raise the question of the value to continue therapy once drug-resistant virus has been detected.

Several studies have suggested that continuation of antiretroviral therapy in patients with persistently detectable viral load and presence of multidrug-resistant variants could still has some benefits[35,146,147]. In fact, the intentional generation of drug resistance virus variants (with reduced viral fitness) using a sub-optimal therapy has been proposed as an alternative antiretroviral approach[146]. From the viral standpoint, continue therapy would maintain the selective pressure over a less fit resistant virus, although further viral evolution could generate more fit viral strains due the accumulation of compensatory mutations. Conversely, discontinuing therapy will allow the switch to a more fit wild-type strain and the potential rebound of viral load[147,148]. In addition, the fitness difference between drug-resistant and wild-type viruses may be quite small (the range of wt fitness is quite large[47]).

Does STI affect HIV-1 fitness evolution? Verhofstede, et al.[147] studied the effect of the interruption of RTI therapy or a switch from RTI to PI, on the genotypic resistance pattern of plasma HIV-1. Rapid reappearance of more fit wild-type viruses was evident after the interruption of therapy (14 to 60 days). In another study, repopulation of the quasispecies with wild-type drug sensitive viruses, following cessation of therapy, was consistent with drug resistant viruses being less fit than the wt counterparts[148]. Deeks, et al.[144] analyzed the effect of cessation of therapy in HIV-infected individuals with detectable viremia (HIV-RNA plasma level > 2500 copies/ml), showing a change from PI-resistant to PI-susceptible phenotype with increased viral replicative capacity after the discontinuation of therapy. They concluded that antiviral activity against drug-resistant viruses with decreased replication capacity contribute to partial suppression of viral replication and still provides some immunologic benefits. In a similar study, Kaufmann, et al.[149] reported that continued drug pressure may contribute to immunological benefit, but could result in increasing drug resistance and improved viral fitness. Interestingly, after cessation of antiretroviral therapy, primary PI-associated mutations reverted more rapid to wt than secondary PI- and primary RT mutations[150]. Over 90% of the primary PI-mutations reverted to wild-type within a month after treatment discontinuation, suggesting that primary PI mutations cause a more impaired viral fitness than primary RT mutations. Finally, Martinez-Picado, et al.[151] analyzed the selection of drug resistance HIV-1 mutants (mainly 184V) in response to repeated STI. After three consecutive STIs, drug-resistant viruses (specifically the 184V RT mutation) increased in frequencies in virus populations, despite the reduced viral fitness of these variants[23,64]. These results suggest that the positive selection of the 184V during therapy is higher that the negative selection against this mutation during the treatment interruptions (perhaps because the length of interruption is relatively short, 30 days), resulting in the emergence of 184V to high levels.

The switch from drug resistance mutant to wild-type virus, can be the result of reversion of the mutant codons, or can be due to a replication rebound of the original wild-type strains (although reversion to the original wt amino acid occur infrequently[102]). It is possible that a latent cellular reservoir of wild-type virus, capable of high replication after removing the drug pressure, is preserved for a long period of time[152]. However, wt HIV-1 variants could continue to replicate at a very low level within the quasispecies population, and is ready to take over as soon as the pressure is interrupted. This concept called "memory of *quasispecies*" has been described for other RNA viruses[153]. Recently, Hance, et al.[154] detected a small proportion of residual drug resistance variants more than 5 months after the discontinuation of antiretroviral treatment. These mutants persisted within the viral population even after re-emergence of wild-type virus (actively replicating as minority species within the *quasispecies* memory, or due to the activation of long-lived cells acting as reservoirs). These results suggest that the usual duration of 3 months for STI might be too short for washout of resistant viruses in most patients. Furthermore, in patients carrying drug resistance variants with high viral fitness, re-emergence of wild-type virus could be slow or may even not occur after STI.

Summary

Relative viral fitness, defined as the overall replicative capacity of the virus in a given environment can be better quantitated in growth competition experiments. However, *in vitro* fitness differences cannot easily be extrapolated to *in vivo* situations, since they depend on the actual replication rate under the *in vitro* tissue culture conditions. More studies are necessary to establish a validated protocol and measurement tool for evaluating viral fitness in both the presence and absence of drugs.

What is the clinical significance of reduced viral fitness? The fact that mutant viral strains have a lower replication capacity than wild-type viruses have been an argument in favor of continuing a partially suppressive regimen, which selectively maintains a poorly fit virus, despite the emergence of viral resistance. However, the virus is continuously looking for to improve its fitness, which is even more marked during selection with antiretroviral drugs. Understanding the impact that drug resistance mutations have on HIV-1 replication fitness could lead to the design of drug combinations that are not only effective in reducing the viral load to below detection limits, but that additionally result in maximum decrease of viral fitness once drug resistance has developed.

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