Quasispecies and Drug Resistance

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Contents

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Abstract

Mutant spectra of viral quasispecies are complex reservoirs of genetic and phenotypic variants, including drug-resistant mutants. Here we review basic features of RNA viral quasispecies such as internal interactions within mutant spectra and the effect of population size and bottleneck events as they affect the frequency of inhibitor-escape mutants. Genetic barriers to resistance and fitness cost of specific amino acid substitutions involved in resistance are discussed, with specific examples for human immunodeficiency virus type 1 (HIV-1) and hepatitis C virus (HCV). Prospects for new antiviral designs aimed at counteracting the adaptive potential of viral quasispecies are presented.

Introduction: Relevance of Quasispecies in Virus Biology

Viral quasispecies are mutant distributions (also termed mutant spectra, clouds, or swarms) that characterize genome populations of RNA viruses and at least some DNA viruses (Fig. 1). Both clonal analyses by classic nucleotide sequencing techniques and bulk population analyses by ultra-deep sequencing have documented that mutant distributions are extremely complex with many minority mutations occurring at low frequency (1 % which is the present standard cutoff value for reliable mutant frequency determination and probably lower according to studies that achieved lower cutoff values). From all evidence, mutant spectra originate from high mutation rates in RNA (and some DNA) viruses, which have been estimated in 10^{-3} to 10^{-5} mutations introduced per nucleotide copied, together with competition and intrapopulation interactions among genomes [reviewed in (Domingo et al. 2012)]. Viral quasispecies took its name from a theory of the origin of life developed by M. Eigen, P. Schuster, and their colleagues (Eigen and Schuster 1979). Theoretical studies on quasispecies have paralleled experimental investigations with RNA viruses, reaching a considerable degree of conceptual cross-fertilization (Eigen 2013; Holland 2006; Mas et al. 2010; Ojosnegros et al. 2011).

The biological behavior of viral quasispecies is not equivalent to that of sets of identical genomes undergoing only occasional mutations for two main reasons. One is that mutant spectra constitute vast reservoirs of genetic and phenotypic variants, including, notably, drug-, antibody-, or cytotoxic T-cell (CTL)-escape mutants. The second reason is that the variant genomes which dynamically arise, persist, increase, or decrease in frequency or are eliminated (transiently or irreversibly) do not act independently. Variants can complement each other to give rise to a new phenotype (Cao et al. 2014; Shirogane et al. 2012), to trigger large evolutionary transitions such as genome segmentation (Moreno et al. 2014), or to maintain a higher average fitness of the mutant ensemble relative to its individual components (Domingo et al. 1978, 2012;
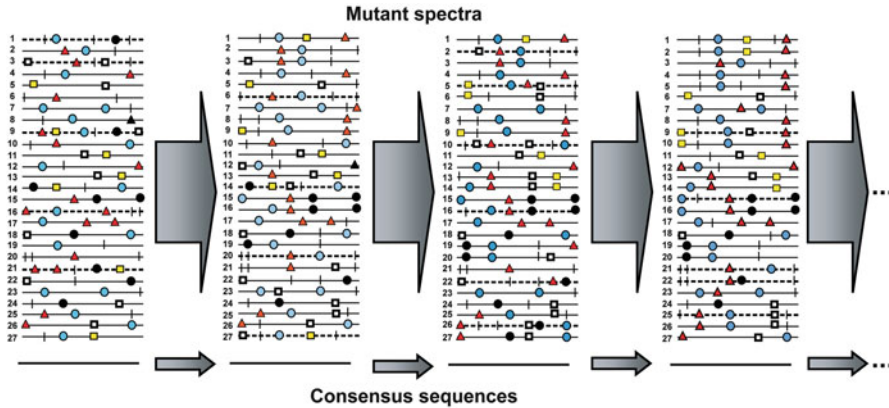
Mutant spectra

Consensus sequences

Fig. 1 Schematic representation of quasispecies evolution. The four successive populations (*lines* represent genomes and symbols on lines mutations) evolve by modification of the mutant spectrum while the consensus sequence remains invariant. In the simplified dynamics depicted here, genomes that acquire five or more mutations (genomes with discontinuous lines) do not survive. In reality, viral populations (even single replicative units in a replication complex) consist of hundreds or thousands genomes subjected to the dynamics of mutant generation, competition, and selection. (Figure reproduced from Domingo et al. (2012) with permission from ASM)

Duarte et al. 1994). A specific high-fidelity mutant of poliovirus that displayed limited mutant spectrum complexity was attenuated and could not adapt to complex environments or reach the central nervous system in a mouse model (Pfeiffer and Kirkegaard 2005a; Vignuzzi et al. 2006). However, an accompanying mutant spectrum allowed the mutant to reach its target organ *in vivo* (Vignuzzi and Andino 2010; Vignuzzi et al. 2006). Thus, mutant spectrum complexity is relevant to viral pathogenesis, and the control of replication fidelity may serve to engineer attenuated virus vaccines (Vignuzzi et al. 2008).

Quasispecies swarms can have an effect opposite to complementation: the suppression of individual viral mutant progeny which in isolation displays superior fitness than the parental quasispecies (de la Torre and Holland 1990). Theoretical quasispecies predicts that the behavior of any individual component may be modulated by the mutant spectrum that surrounds it. In one of the computer simulations, near an error threshold (preceding a second and final threshold where no genomes can be maintained), a slightly inferior mutant was strongly favored by virtue of its better mutant environment [(Swetina and Schuster 1982), reviewed in Eigen and Biebricher (1988)]. In the case of viruses, the suppressive effect of a mutant ensemble on particular variants is exerted through different biological mechanisms, derived from the biochemical reactions during genome replication and the effect of *trans*-acting proteins. Specifically, in poliovirus, four mechanisms of mutant-mediated interference were identified (Crowder and Kirkegaard 2005). Some capsid and polymerase mutants produced dominant negative phenotypes, attributed to the fact that these proteins function as oligomers. Mutations in *cis*-regulatory element (CRE) and VPg protein indicated that nonproductive priming of initiation of viral RNA replication is inhibitory. The authors confirmed that, as anticipated, a drug-

sensitive poliovirus inhibited the intracellular growth of a drug-resistant mutant (Crowder and Kirkegaard 2005). In line with these findings, a mutagenized, preextinction foot-and-mouth disease virus (FMDV) population interfered with replication of the standard virus (González-López et al. 2004). The accumulation of defective, mutated genomes in the heavily mutagenized FMDV population produced an interfering swarm, an event that was shown to participate in viral extinction by lethal mutagenesis (Grande-Pérez et al. 2005). A study with specific FMDV capsid and polymerase mutants confirmed their interfering activity on wild-type virus and showed that the mutants had to be competent in RNA replication to be inhibitory (Perales et al. 2007). This requirement is one of the factors that contribute to an advantage of sequential inhibitor-mutagen treatment over the corresponding combination, to prevent the selection of inhibitor-escape mutants and favor virus extinction (Iranzo et al. 2011; Moreno et al. 2012; Perales et al. 2009, 2012).

Modulating effects of mutant spectra have been observed also *in vivo*. Virulent poliovirus can have its phenotype suppressed by attenuated virus in the population (Chumakov et al. 1991). A growth hormone deficiency syndrome induced by lymphocytic choriomeningitis virus can be suppressed by disease-negative variants (Teng et al. 1996) [reviewed in (Domingo et al. 2012)].

Major Factors in the Generation and Dominance of Drug Resistance in Viruses

Drug-resistant mutants are those present in quasispecies that can replicate more efficiently than other components of the mutant swarm in the presence of the drug. Their selection and maintenance in a viral population is conditional upon two events which are influenced by different parameters: (i) mutant generation and (ii) the efficiency of mutant replication relative to other components of the same population. Mutant generation depends on the genetic barrier to resistance, defined as the number and types of mutations required to reach the resistance phenotype. Telaprevir resistance in hepatitis C virus (HCV) can be achieved by amino acid substitution R155K in NS3. In HCV genotype 1a, these substitutions can be attained by a single nucleotide transition (AGA $\rightarrow$ AAA). In contrast, in genotype 1b, by virtue of the R codon being CGA, the same amino acid substitution requires two mutations: a transversion and a transition (CGA $\rightarrow$ AAA). Since the probability of occurrence of two independent mutations is the product of probabilities of occurrence of the individual mutations, and transversions are usually less frequent than transitions, the HCV genetic barrier to telaprevir resistance is higher for HCV genotype 1b than 1a. There is no molecular or evolutionary reason to exclude that genetic variations that modify the genetic barrier to a drug can occur among viruses of the same genotype or among components of a mutant spectrum. Obviously, the genetic barrier will be increased when two or more amino acid substitutions (each requiring at least one mutation) are needed to reach the drug-resistance phenotype. In general terms, requirements of multiple mutations (excessive number of steps in sequence space) are what preclude viruses of surviving in some environments. This is the main reason
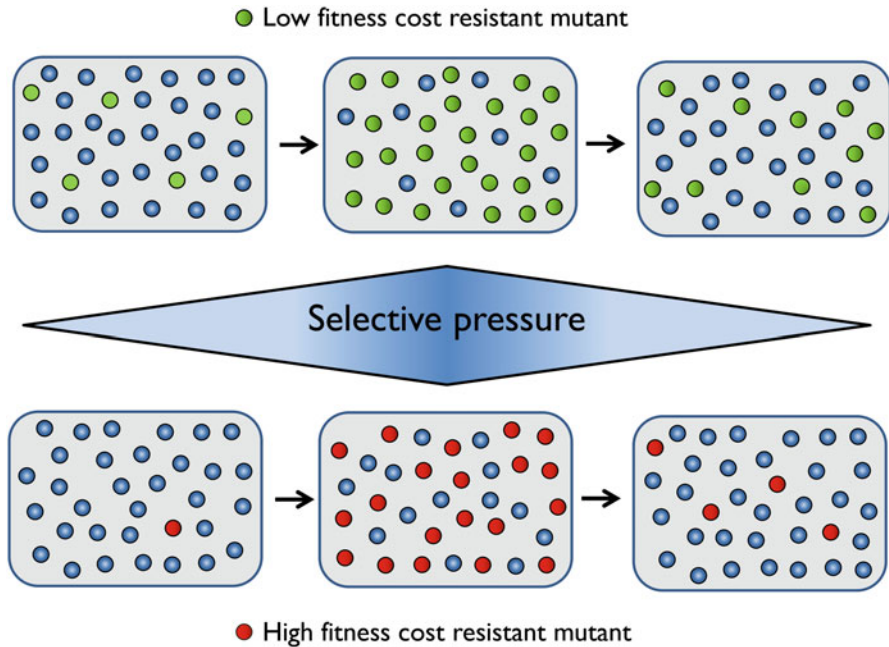
Fig. 2 Phenotypic barrier or fitness cost to overcome a selective pressure. The escape mutants that experience a low fitness cost (depicted as *green circles* in the upper three successive populations) may preexist with considerable frequency before the selective pressure is exerted; they can reach high proportions in the presence of the selective pressure and remain at elevated levels even when the selective pressure is removed (*upper right population*). The escape mutants that experience a high fitness cost (depicted as *red circles* in the bottom three successive populations) will be present at low frequency before the selective pressure is exerted; they can reach high proportions in the presence of the selective pressure and return to low levels when the selective pressure is removed (*bottom right population*)

of the advantages of combination therapies over monotherapy, with the exceptions discussed in section "Conclusions and New Prospects for Antiviral Therapy".

Once the genetic barrier has been overcome and the resistant mutant has been generated, a second barrier, termed phenotypic barrier or fitness cost, intervenes. If the relevant amino acid substitution, in addition to conferring drug resistance, impairs any step in the viral life cycle, the proportion of the mutant in the viral quasispecies will decrease. The higher the fitness cost, the lower the proportion of the mutant in the mutant spectrum. Two possible outcomes can be anticipated: either the fitness cost does not allow the mutant to become dominant or compensatory mutations (that counteract the fitness cost of the drug-resistance mutations without significantly altering the resistance level) occur that allow dominance of the resistant mutant. Fitness effects apply to viruses escaping any type of selective pressure (drugs, immune responses, tropism, host range changes, etc.). The consequence of fitness cost of a drug-resistance mutation has been schematically represented in Fig. 2, in which the frequency of the relevant escape mutant

(green circles in the upper population and red circles in the bottom population) is fitness-dependent. If the fitness cost is severe (even more than implied in the bottom panels of Fig. 2), the relevant escape mutant may not preexist in the population. With 10^{-3} to 10^{-5} mutations introduced per nucleotide copied (Domingo et al. 2012), a type of arms race is established between the occurrence of the relevant mutation and the opportunity of the genome harboring it to replicate sufficiently in the presence of the drug. These conflicting requirements may allow the virus to improve replication through compensatory mutations and evolve towards dominance or be irreversibly lost in the mutant spectrum. Studies of deep sequencing of viral populations that are confronted with a strong selective pressure [e.g., in human immunodeficiency virus type 1 (HIV-1)-infected patients treated with vicriviroc (Tsibris et al. 2009)] suggest that viral quasispecies screen multiple escape routes, and only a subset of those are successful. Drug-escape mutants are present at high frequencies in populations of many important pathogenic viruses such as HIV-1, hepatitis B virus, HCV, or influenza virus, and such mutants can dramatically lead to treatment failure. Yet, what the experimental studies on quasispecies dynamics suggest is that the observed drug-resistance mutations recorded are only a minor subset of all possible resistance mutations that would be found if fitness effects did not intervene.

To complicate matters even further, as noted in the Introduction, fitness levels are not only a property of individual viral genomes. Rather, the frequency of a given mutant can be influenced by the surrounding quasispecies. Specifically, the presence of a complex mutant spectrum can suppress a drug-resistant mutant to avoid or delay its dominance (see Introduction for references).

Effect of Variations in Population Size and Viral Load

The virus population size often varies during the course of the natural infectious cycle of viruses. It generally increases from the initial infecting dose to a viremic state, and it may decrease again if persistence or chronicity is established. During an acute infection, subsets of viruses may invade new cells, tissues, or organs, and such invasions may involve reductions of population size (bottleneck events). Since viral populations consist of mutant swarms, the viral population size that is transmitted (from host to host, organ to organ, or cell to cell) will determine the numbers and types of mutants that can continue replicating (Fig. 3). Drug-resistant mutants may be generated in an infected host subjected to therapy with the drug (e.g., the mutants depicted as yellow stars in Fig. 3). This mutant will not contribute directly to drug resistance in a recipient virus-naïve individual unless the transmitted population reaches a critical size. The term primary resistance was coined during the AIDS epidemics to denote infections by HIV-1 which harbored an antiretroviral resistance mutation selected prior to transmission.

Not only fluctuations of population size and bottleneck events are important to understand quasispecies evolution, the total population size (viral load) is also relevant. We have previously emphasized the connections between four parameters in virus survival: viral load, replication rate, genetic heterogeneity, and viral
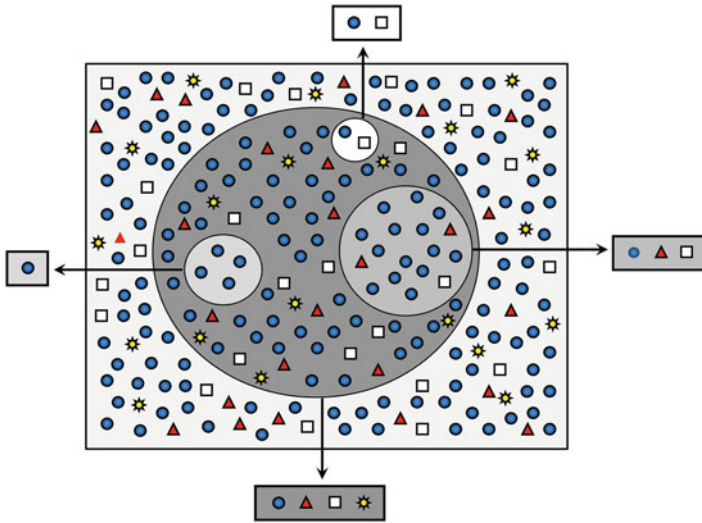
Fig. 3 The effect of population size in a mutant repertoire. The *large square* represents a viral quasispecies, in which four types of genomes are present. Small sample sizes will result in detection of only the highest frequency genomes (*small gray circle*) but may randomly fluctuate based on chance detection (*small white circle*). Greater diversity will be detected in larger sample sizes, represented by larger *gray circles* (Figure modified from Domingo et al. (2012) with permission from ASM)

fitness. As explained elsewhere (Domingo et al. 2012), potent replication which is a key component of fitness values tends to produce elevated viral loads. When endowed with the adequate diversity (mutant spectrum amplitude), high viral loads will contribute to adaptedness and survival. Several lines of evidence suggest that these four interconnected parameters are linked to disease progression, again emphasizing the relevance of quasispecies for viral pathogenesis (Domingo et al. 2012).

Clinical Impact of Drug-Resistant Viral Mutants

Escape mutants have been reported ever since the first controlled studies with viral populations subjected to antiviral inhibitors were performed (Eggers and Tamm 1965; Melnick et al. 1961). Many examples, both historical and current, have been periodically reviewed [Domingo (1989), Domingo et al. (2012), Richman (1996) and references therein]. Several data banks offer updated information on drug resistance of important viral pathogens.

Here we review, as specific examples, drug-resistance mutations of some clinically relevant viruses such as HIV-1 and HCV.

HIV Variability and Antiretroviral Therapy

HIV is a retrovirus that causes the acquired immunodeficiency syndrome (AIDS) in humans. Currently, there are around 34 million people worldwide infected with HIV. Although HIV-1 strains are responsible for most of the global AIDS pandemic, there are about 1–2 million people infected with HIV type 2 (HIV-2). Genetic variability is one of the hallmarks of HIV. These viruses have high mutation rates (around 10^{-4} to 10^{-5} mutations per nucleotide and replication cycle) and high recombination frequencies (reviewed in (Menéndez-Arias 2009)). This is partly due to the relatively low fidelity of the viral reverse transcriptase that like other polymerases found in RNA viruses is devoid of proofreading activity. In addition, it has been estimated that the minimum duration of the HIV life cycle *in vivo* is only 1.2 days, while the average number of virions produced per day in an infected individual is 1.03×10^{10} (Perelson et al. 1996). These characteristics of the HIV infection are responsible for the generation of complex quasispecies that facilitate the selection of strains resistant to antiretroviral drugs, as discussed in sections "Introduction: Relevance of Quasispecies in Virus Biology" to "Effect of Variations in Population Size and Viral Load".

Six years after the first clinical observation of AIDS in the United States (Centers for Disease Control [CDC] 1981), AZT (3′-azido-3′-deoxythymidine; zidovudine) became the first drug approved for treatment of HIV-1 infection (Fig. 4). AZT is a prodrug that in its triphosphate form is incorporated into the viral genome by the reverse transcriptase, while blocking DNA synthesis due to the absence of a 3′-OH in its ribose ring. For several years, AZT was administered to patients in monotherapy, leading to the selection of resistant HIV-1 strains with amino acid substitutions such as M41L, D67N, K70R, L210W, T215F or T215Y, and K219E or K219Q in the reverse transcriptase [(Larder et al. 1989); reviewed in Menéndez-Arias (2008)]. Other nucleoside analogs (e.g., didanosine, zalcitabine, stavudine, and lamivudine) were approved in the following years, and very often these drugs were prescribed alone after failure of AZT monotherapy due to the emergence of drug-resistant HIV-1. Sequential treatments facilitated the selection of multidrug-resistant viral strains due to accumulation of resistance mutations specific for each drug. On the other hand, the combined use of AZT and didanosine or zalcitabine in untreated patients facilitated the selection of multidrug-resistant HIV-1 variants containing a different set of mutations including A62V, V75I, F77L, F116Y, and Q151M (Shirasaka et al. 1995).

A remarkable breakthrough in the antiretroviral treatment was achieved in 1995 when the first HIV-1 protease inhibitor (i.e., saquinavir) was approved. Highly active antiretroviral therapy (HAART) was then introduced as a combination of two nucleoside analogs (e.g., AZT, lamivudine, etc.) and a protease inhibitor (Gulick et al. 1997; Hammer et al. 1997). By targeting two different steps in the HIV life cycle (i.e., viral genome replication and maturation), it was possible to decrease viral loads below the limits of detection and minimize the impact and emergence of drug resistance. In the following years, the approval of nonnucleoside analog inhibitors of
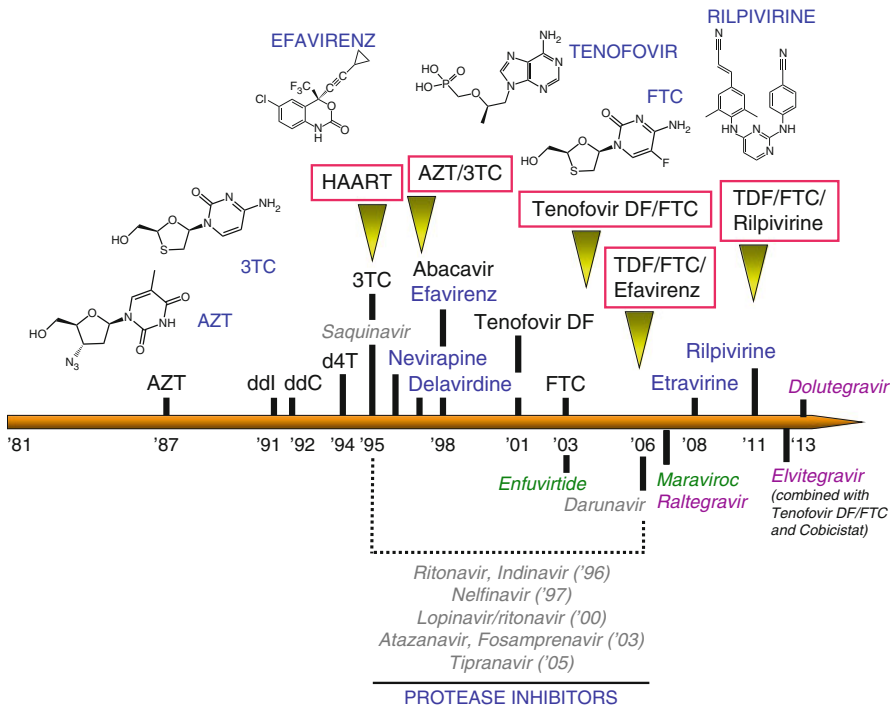
Fig. 4 Timeline for approval of antiretroviral drugs. Nucleoside reverse transcriptase inhibitors are shown in *black*, nonnucleoside reverse transcriptase inhibitors in *blue*, protease inhibitors in *gray*, integrase inhibitors in *purple*, and entry inhibitors in *green*. Abbreviations: 3TC (lamivudine), β-L-(−)-2′,3′-dideoxy-3′-thiacytidine; AZT (zidovudine), β-D-(+)-3′-azido-3′-deoxythymidine; d4T (stavudine), β-D-(+)-2′,3′-didehydro-2′,3′-dideoxythymidine; ddC (zalcitabine), β-D-(+)-2′,3-′-dideoxycytidine; ddI (didanosine), β-D-(+)-2′,3′-dideoxyinosine; FTC (emtricitabine), β-L-(−)-2′,3′-dideoxy-5-fluoro-3′-thiacytidine; HAART, highly active antiretroviral therapy; tenofovir DF, tenofovir disoproxil fumarate

HIV-1 reverse transcriptase increased the number of available HAART regimens by allowing novel drug combinations acting on different HIV targets.

Effective Combination Therapies for HIV-1 Infection

Despite their impressive success in reducing AIDS mortality, combination therapies developed in the late 1990s were still problematic due to the poor pharmacokinetic properties of HIV protease inhibitors (very high doses and a large number of pills were needed) and the low genetic barrier to resistance of nevirapine and other nonnucleoside reverse transcriptase inhibitors. In 2006, the introduction of Atripla®, a combination of two nucleoside analogs (tenofovir disoproxil fumarate and emtricitabine) and the nonnucleoside reverse transcriptase inhibitor efavirenz,

constituted a significant accomplishment as it became the standard of care for therapy-naïve patients. With one pill a day, its dosage is optimal and facilitates adherence to antiretroviral therapy. Based on the same principles, other recently approved combinations include a single tablet tenofovir disoproxil fumarate and emtricitabine either with rilpivirine (a nonnucleoside reverse transcriptase inhibitor) or with elvitegravir (an integrase inhibitor which is administered together with cobicistat). Cobicistat is an inhibitor of cytochrome P450 3A enzymes that boosts blood levels of elvitegravir.

The Molecular Basis of Drug Resistance in HIV-1

In addition to the simplification of dosing regiments, drugs used today are more potent and have longer half-lives than compounds used 15 years ago. Furthermore, current regimens have less toxicity and are more tolerable over time. At present, viral suppression using combination therapies is effective and emergence of resistance has been significantly reduced in the clinical setting. However, there are still patients infected with drug-resistant strains that were selected after successive treatments with different antiretroviral drugs or individuals that were infected with drug-resistant HIV-1 (i.e., transmitted drug resistance). In addition, natural resistance to various antiretroviral drugs has been observed in several HIV-1 clades, as well as in HIV-2 (Menéndez-Arias and Álvarez 2014). Therefore, in this scenario, compounds targeting different steps of the virus life cycle are still needed. Currently licensed drugs target (i) viral entry (e.g., maraviroc and enfuvirtide), (ii) reverse transcription (nucleoside and nonnucleoside reverse transcriptase inhibitors), (iii) integration (integrase inhibitors, such as raltegravir, elvitegravir, and dolutegravir), and (iv) viral maturation (protease inhibitors) [reviewed in (Menéndez-Arias 2013)]. A list of amino acid substitutions associated with resistance to antiretroviral drugs is given in Table 1.

Nucleoside reverse transcriptase inhibitors are the backbone of current antiretroviral therapies. Some of those drugs have a relatively low genetic barrier (section "Major Factors in the Generation and Dominance of Drug Resistance in Viruses"). For example, high-level resistance to lamivudine and emtricitabine is conferred by single mutations generating the amino acid substitutions M184I or M184V. These amino acid changes reduce the ability of the reverse transcriptase to incorporate the inhibitor relative to its natural substrates (i.e., dNTPs) [reviewed in Menéndez-Arias (2008)]. On the other hand, at least 2–3 mutations are needed to produce an AZT-resistant HIV-1 strain. The relevant thymidine analog resistance mutations (e.g., M41L, D67N, T215Y, etc.) facilitate the excision of AZT-monophosphate, stavudine-monophosphate, or tenofovir from the 3′ end of blocked DNA primers, in a reaction mediated by ATP and other pyrophosphate donors (Meyer et al. 1999; Tu et al. 2010). The same molecular mechanism operates for HIV variants having reverse transcriptases that contain a dipeptide insertion between codons 69 and 70 and thymidine analog resistance mutations such as M41L or T215Y [reviewed

Table 1 Amino acid substitutions associated with HIV-1 resistance to antiretroviral drugs

Drugs	Amino acid substitutions associated with drug resistance
Nucleoside analog reverse transcriptase inhibitors	
Zidovudine (AZT)	M41L, D67N, K70R, V118I, L210W, **T215F/Y**, K219E/Q
Didanosine (ddI)	K65R, L74V, M184V
Lamivudine (3TC)	(E44D/V118I), K65R, **M184I/V**
Stavudine (d4T)	M41L, D67N, K70R, V118I, L210W, T215F/Y, K219E/Q
Abacavir	K65R, L74V, Y115F, M184V (M41L, D67N, K70R, L210W, T215F/Y, K219E/Q)
Emtricitabine (FTC)	(K65R/Q151M), **M184I/V**
Tenofovir	**K65R**, K70E
Combinations of mutations that confer resistance to various nucleoside analogs	
	(i) M41L, D67N, K70R, L210W, T215F/Y, K219E/Q; (ii) A62V, V75I, F77L, F116Y, Q151M; (iii) insertions between codons 69–70 (i.e., T69SSS or T69SSG or T69SSA), M41L, A62V, K70R, L210W, T215F/Y
Nonnucleoside reverse transcriptase inhibitors	
Nevirapine	L100I, K101P, K103N/S, V106A/M, V108I, Y181C/I, Y188C/L/H, G190A/C/E/Q/S/T
Delavirdine	K103H/N/T, V106M, Y181C, Y188L, G190E, P236L
Efavirenz	L100I, K101P, K103H/N, V106M, V108I, Y188L, G190A/S/T, P225H, M230L
Etravirine	V90I, A98G, L100I, K101E/H/P/Q, V106I, **E138**A/G/K/**Q/R**/S, **V179**D/**F/I/L**, Y181C/I/V, G190A/S, F227C, M230L, T386A, E399D
Rilpivirine	V90I, K101E/P, **E138**A/G/**K**/Q/**R** (±M184I/V), V179F/I/L, Y181C/I/V, Y188L, V189I, H221Y, F227C, M230I/L
Combinations of mutations that confer cross-resistance to nevirapine, delavirdine and efavirenz	
	(i) K103N alone; (ii) V106M alone; (iii) Y188L alone; (iv) two or more amino acid changes of the group: L100I, V106A, Y181C/I, G190A/S, M230L, and Y318F
Protease inhibitors	
Saquinavir	L10I/R/V, **G48V**, I54L/V, A71T/V, G73S, V77I, V82A, I84V, **L90M**, and A431V [in the Gag polyprotein cleavage site p7(NC)/p1)]
Ritonavir	L10I/R/V, K20M/R, V32I, L33F, M36I, M46I/L, I54L/V, A71T/V, V77I, **V82A/F/S/T**, **I84V**, L90M, and A431V [in the Gag polyprotein cleavage site p7(NC)/p1)]
Indinavir	L10I/R/V, K20M/R, L24I, V32I, M36I, **M46I/L**, I54V, A71T/V, G73A/S, V77I, **V82A/F/S/T**, **I84V**, L90M, and in the Gag cleavage sites: A431V [in p7(NC)/p1)] and L449F [in p1/p6]
Nelfinavir	L10F/I, **D30N**, M36I, M46I/L, A71T/V, V77I, V82A/F/S/T, I84V, N88D/S, **L90M**, and in the Gag cleavage sites L449F and P453L [in p1/p6]
Amprenavir (fosamprenavir)	L10F/I/R/V, V32I, M46I/L, I47V, **I50V**, I54V/M, **I84V**, L90M, and Gag cleavage sites L449F and P453L [in p1/p6]

Table 1 (continued)

Drugs	Amino acid substitutions associated with drug resistance
Lopinavir	L10F/I/R/V, G16E, K20I/M/R, L24I, **V32I**, L33F, E34Q, K43T, M36I/L, **M46I**/L, **I47A**/V, G48M/V, I50V, **I54L**/**V**/A/M/S/T, Q58E, I62V, L63T, A71T, G73T, T74S, **L76V**, **V82A**/F/S/T, I84V, L89I/M, L90M, and A431V [in the Gag polyprotein cleavage site p7(NC)/p1]
Atazanavir	L10F/I/V, K20I/M/R, L24I, L33F/I/V, M36I/L/V, M46I/L, G48V, **I50L**, I54L/V, L63P, A71I/T/V, G73A/C/S/T, V82A/F/S/T, **I84V**, **N88S**, L90M
Tipranavir	L10I/S/V, I13V, K20M/R, **L33F**/**I**/V, E35G, M36I/L/V, K43T, M46L, I47V, I54A/M/V, Q58E, H69K, T74P, **V82L**/**T**, N83D, **I84V**, L89I/M/V, L90M
Darunavir	V11I, V32I, L33F, I47V, **I50V**, I54L/M, T74P, L76V, V82F, I84V, L89V, and in the Gag cleavage sites A431V [in p7(NC)/p1] and S451T and R452S [in p1/p6]
Combinations that confer resistance to multiple protease inhibitors	
	L10F/I/R/V, **M46I/L**, I54L/M/V, **V82A/F/T/S**, **I84V**, **L90M**[a]
Fusion inhibitors	
Enfuvirtide	G36D/E/S, I37T/N/V, V38A/E/M, Q40H, N42T, N43D/K/S (all in gp41)
Integrase inhibitors	
Raltegravir	G140S, **Y143C/R**, **Q148H/K/R**, **N155H**
Elvitegravir	T66A/I/K, L74M, E92Q/V, **Q148H/K/R**, V151L, **N155H**
Dolutegravir	F121Y, E138A/K, G140A/S, Q148H, R263K
CCR5 antagonists	
Maraviroc	Resistance usually develops through the selection of viruses that use the CXCR4 (X4) coreceptor. In addition, maraviroc resistance mutations have been selected in vitro in the V2, V3, and V4 loops of gp120 (Westby et al. 2007)

For additional information, see Clotet et al. (2014), Wensing et al. (2014), and the websites of the International Antiviral Society–USA (http://www.iasusa.org) and the Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu)

Major resistance mutations are shown in *bold*. Most protease inhibitors are usually prescribed in combination with a low dose of ritonavir that has a boosting effect on the protease inhibitor concentration in plasma

[a]Multiple protease inhibitor resistance can be achieved through the accumulation of four or five mutations of those indicated in the list

in (Menéndez-Arias et al. 2006)]. The substitution of Gln[151] by Met, considered as the initial step in the Q151M pathway, requires two nucleotide changes. Q151M and accompanying mutations confer resistance by reducing the viral polymerase ability to incorporate nucleoside analogs in the DNA chain.

Classical nonnucleoside reverse transcriptase inhibitors (e.g., nevirapine, delavirdine, and efavirenz) have a very low genetic barrier. Single nucleotide changes occurring at several codons in the reverse transcriptase-coding region can individually confer high-level resistance to those drugs. Interestingly, some of them (notably, K103N) confer cross-resistance to all three drugs. Next-generation inhibitors such as etravirine and rilpivirine are more potent and show a higher genetic barrier to resistance. However, E138K and other substitutions at this position are

sufficient to confer partial resistance to these drugs (Asahchop et al. 2013). Nevertheless, unlike in the case of nevirapine, delavirdine, or efavirenz, two amino acid substitutions are needed to attain high-level resistance in vitro (Azijn et al. 2010; Javanbakht et al. 2010). Rilpivirine is now substituting efavirenz in the most effective antiretroviral drug combinations.

Resistance to HIV protease inhibitors is relatively complex, since for most drugs in this class, high-level resistance involves a relatively large number of amino acid substitutions [for a recent review, see Menéndez-Arias (2013)]. Major mutations associated with resistance map within the substrate/inhibitor binding site (e.g., D30N, G48V, V82A, I84V, etc.). These amino acid changes usually have a significant impact on the viral replication capacity. Secondary mutations that are selected later during treatment increase viral fitness and usually locate out of the substrate binding site. In some cases, these amino acid substitutions have an impact on protease stability (e.g., L10I or A71V) (Chang and Torbett 2011). Further viral fitness recovery during treatment can be facilitated by mutations occurring at the viral polypeptide substrates cleaved by the HIV protease (e.g., at Gag cleavage sites NC/p1 and p1/p6). These mutations facilitate viral polyprotein processing by improving Gag susceptibility to protease cleavage.

Approved integrase inhibitors bind to the catalytic domain of the enzyme blocking its strand transfer activity. Resistance to raltegravir and elvitegravir is associated with single amino acid substitutions (usually Q148K/R/H, but also N155H). Integrase inhibitors have been recently combined with nucleoside analogs in HAART regimens. Interestingly, the latest integrase inhibitor approved for treatment (i.e., dolutegravir) shows a surprisingly high genetic barrier. In phase III clinical trials, approximately 88 % of the patients treated with dolutegravir and two nucleoside reverse transcriptase inhibitors attained viral load suppression to <50 copies of RNA/ml, without developing drug-resistance-associated mutations after 48 weeks of treatment (Raffi et al. 2013; Wainberg et al. 2013). It is possible that development of dolutegravir resistance mutations may result in viruses with greatly diminished replicative capacity, thereby constituting a major barrier towards the development of resistance.

Other drugs used in antiretroviral rescue therapy include entry inhibitors targeting either the step involving the recognition of the viral coreceptor (CCR5 antagonists) or fusion inhibitors (enfuvirtide). Enfuvirtide is a largely helical polypeptide that interferes with the packaging of HIV-1 gp41 α-helical segments required for the fusion of the viral envelope and the cell membrane. Resistance is achieved by mutations in gp41 that encode amino acid changes that disrupt interactions between α-helices in the transmembrane protein and enfuvirtide (Greenberg and Cammack 2004). On the other hand, maraviroc is a CCR5 antagonist. This drug binds to a pocket in the chemokine receptor and makes it unavailable for the HIV-1 surface glycoprotein gp120. Viral strains resistant to maraviroc may still infect the host by using other chemokine receptors (e.g., CXCR4) (Westby et al. 2006). In addition, resistance to maraviroc mediated by specific amino acid substitutions in the V3 loop of gp120 allows HIV-1 to continue using CCR5 coreceptors, even in the presence of bound maraviroc (Westby et al. 2007). This use of a drug-bound coreceptor

illustrates that viruses have multiple resources to overcome a selective pressure intended to limit their replication and that even drugs that target a cellular function are not free of the problem of selection of virus-escape mutants (see section "Conclusions and New Prospects for Antiviral Therapy").

HCV Variability and Current Therapy

HCV is a member of the *Flaviviridae* family affecting approximately 170 million individuals worldwide. HCV shows a very high variability which is mainly due to the absence of proofreading activity of the RNA polymerase and very high rate of virion production, approximately 10^{12} per infected individual per day (Neumann et al. 1998). For over ten years, the standard of care treatment for HCV infection was a combination of pegylated interferon-α (IFN-α) and ribavirin, which achieved viral eradication in 40–50 % of patients infected with HCV genotype 1 and 80 % in those infected with genotypes 2 and 3 (Quer et al. 2008; Shiffman 2008). Unfortunately, the use of IFN-α and ribavirin results in moderate to severe side effects in many patients. The approval of telaprevir and boceprevir in 2011 for the treatment of chronic HCV infection was a major breakthrough in the field of anti-HCV therapy. Therapy consisting of a protease inhibitor combined with IFN-α and ribavirin for HCV genotype 1 patients significantly increased sustained virological response (SVR) rates compared with IFN-α and ribavirin treatment alone and reduced the rate of selection of resistant variants (Bacon et al. 2011; Jacobson et al. 2011; Poordad et al. 2011; Zeuzem et al. 2011). There are currently several new compounds targeting various HCV proteins that have been or will soon be added to the arsenal of drugs available for new combination therapies, that might render possible the implementation of IFN-free regimens (deLemos and Chung 2014; Lange and Zeuzem 2013). The newest direct-acting antiviral agents (DAAs) are candidates to be included in these regimens, such as second-generation NS3, NS5A, and viral polymerase (NS5B) inhibitors. However, appropriate combinations of these inhibitors must be selected to avoid cross-resistance and overcome problems associated with low barrier of resistance to individual drugs.

Resistance to Interferon-α and to Ribavirin

The administration of exogenous IFN-α exerts antiviral effects via activation of innate immunity. It is not clear why some patients respond differently to IFN–αbased treatments, though several host factors (gender, age, ethnicity, obesity, etc.) have been implicated. Sequence polymorphisms within the IL28B locus (IFN-λ3) have been linked to variations in the virological response to IFN-α-based therapy (Ge et al. 2009). Particularly difficult cases are null responders to previous treatment with IFN-α and ribavirin, those infected by certain HCV genotypes, patients coinfected with HIV-1, or those with advanced liver fibrosis (Lange and Zeuzem 2013). The number of escape routes that a virus may use to avoid suppression by

antivirals depends on the complexity of the response exerted by the drugs. For DAAs that target specific viral proteins, resistance often depends on one or a few key amino acid substitutions. In contrast, the pluricomponent antiviral response exerted by IFN-α affects multiple cell signaling pathways, which may explain why IFN-α resistance has been linked to several HCV genes (Kozuka et al. 2012; Perales et al. 2013, 2014; Serre et al. 2013). Among other examples, the viral protease NS3/4A cleaves mitochondrial antiviral signaling (MAVS) and TIR-domain-containing adaptor-inducing interferon-β (TRIF), interrupting signal transduction via retinoic acid-inducible gene-I (RIG-I), toll-like receptor 3 (TLR3), and protein kinase R (PKR) response pathways. Additionally, HCV core protein has been linked to decreased signaling via Jak-STAT, resulting in decreased expression of interferon-stimulated genes (ISGs) (Horner and Gale 2013).

Another unsettled issue is whether resistant variants with IFN-α-specific mutations are directly responsible for treatment failure. Though sequence analysis of HCV from patients failing treatment with IFN-α and ribavirin has been performed, no consensus amino acid changes have been associated with genotype-specific IFN-α response. However, variations detected in broad regions of core, E2, and NS5A have been correlated with treatment outcome (Chayama and Hayes 2011; Enomoto et al. 1996; Pawlotsky et al. 1998). This is consistent with the results of *in vitro* studies, where HCV passaged in the presence of IFN-α has selected multiple substitutions throughout the viral genome (Perales et al. 2013, 2014; Serre et al. 2013). Interestingly, the mutations seen in patients failing therapy are not the same as those observed after *in vitro* selection in the presence of IFN-α (Kozuka et al. 2012; Perales et al. 2013, 2014).

There seems to be a strong link between enhanced fitness and IFN-α resistance *in vitro*, making it more difficult to distinguish *bona fide* IFN resistance from cell culture adaptation. Thus, the need of HCV to cope with multiple ISG proteins renders IFN-α resistance a far more complex issue than resistance to standard antiviral inhibitors that target a specific viral protein (Perales et al. 2014). In the serial passages of HCV to select IFN-α-resistant mutants, it was observed that virus that had been passaged in human hepatoma Huh-7.5 cells in the absence of IFN-α also acquired partial resistance to IFN-α (Perales et al. 2013). Further studies with the multiply passaged populations documented that the partial resistance extended to several DAAs and ribavirin, despite the virus not having been exposed to the drugs. Mutant spectrum analyses and the kinetics of progeny production by serially diluted populations and by individual clones excluded that drug resistance was associated with the presence of drug-escape mutants in the multiply passaged populations. The results established viral fitness as a multidrug-resistance factor in HCV (Sheldon et al. 2014).

The inclusion of ribavirin in combination therapies increased the rates of SVR compared with treatment using IFN-α alone, though the mechanism is not fully understood (Sostegni et al. 1998). Several antiviral mechanisms of ribavirin have been described: (i) immunomodulation and enhancement of the Th1 antiviral immune response, (ii) upregulation of genes involved in IFN signaling, (iii) inhibition of viral RNA-dependent RNA polymerases, (iv) depletion of intracellular GTP

levels, (v) inhibition of mRNA cap formation, and (vi) lethal mutagenesis. Although the precise mechanism (or combination of mechanisms) of ribavirin-mediated viral inhibition during anti-HCV therapy has not been elucidated, several lines of evidence suggest that lethal mutagenesis is involved (Asahina et al. 2005; Cuevas et al. 2009; Dietz et al. 2013; Dixit et al. 2004; Lutchman et al. 2007). The ribavirin-induced bias in the mutant spectrum (an excess of G-A and C-U transitions), which reflects the mutagenic activity of ribavirin, has been observed both *in vivo* (Dietz et al. 2013) and in cell culture (Ortega-Prieto et al. 2013). In general, resistance mutations against a mutagen are less frequent than for classical inhibitors. The first identification of a ribavirin-resistance mutation (F415Y in NS5B) in HCV was during ribavirin monotherapy in patients (Young et al. 2003). Experiments with HCV replicon containing cell lines showed that ribavirin resistance occurred by changes in the cell lines (the resistant cell lines were defective in ribavirin import) or from mutations in NS5A (G404S and E442G) (Ibarra and Pfeiffer 2009; Pfeiffer and Kirkegaard 2005b). Additionally, serial passage of a genotype 2a replicon in the presence of ribavirin resulted in reduced sensitivity to the drug, and NS5B mutation Y33H was determined to be responsible, presumably due to a decrease in replicative fitness (Hmwe et al. 2010). Passage of infectious J6/JFH1 of HCV in the presence of ribavirin yielded a resistant virus with many mutations, but the responsible mutation was not identified (Feigelstock et al. 2011).

Resistance to Directly Acting Antiviral Agents (DAAs)

The number of HCV antiviral drugs under development has increased greatly over the past few years, with many drugs now approved by the US Food and Drug Administration and more in late-phase clinical trials. These DAAs are taking a more central role in therapy, with the aim of shortening treatment duration and avoiding IFN in standard of care therapy.

Telaprevir and boceprevir were the first two DAAs to be approved for use in anti-HCV therapy. However, despite their exceptionally potent antiviral activity, use of these first-generation inhibitors of the NS3/4A protease results in the rapid selection of resistance mutations and viral "breakthrough" of monotherapy (Sarrazin et al. 2007; Susser et al. 2009). *In vitro* studies have identified many single amino acid changes associated with reduced sensitivity to protease inhibitors, indicating a low barrier to resistance that has also been evidenced in clinical trials (Lange and Zeuzem 2013; Thompson et al. 2011). The ease of crossing the resistance barrier can be partly explained by the structural characteristics of the NS3/4A protease active site. Only a few side-chain interactions are needed for the binding of inhibitors to the greatly exposed protease active site (Romano et al. 2010). There are mutations at key positions in NS3 (Arg[155], Ala[156], and Asp[168]) that make HCV resistant to nearly all protease inhibitors (Sarrazin and Zeuzem 2010; Thompson et al. 2011; Wyles 2013). Some newer protease inhibitors, such as MK-5172, have shown increased potency against variants containing Arg[155] mutations (Summa et al. 2012). Most of the protease inhibitors currently in use were developed to target the NS3 protease

domain of genotype 1 HCV. Due to differences in NS3 amino acid sequence, the efficacy of protease inhibitors in genotypes 2–6 is decreased (Lange et al. 2010). As mentioned in section "Major Factors in the Generation and Dominance of Drug Resistance in Viruses" above, there is also a difference in susceptibility to NS3/4A inhibitors based on HCV subtype, due to differences in nucleotide sequence at key amino acid-coding positions (McCown et al. 2009).

Daclatasvir was the first NS5A inhibitor to be used in clinical trials. NS5A inhibitors show a very potent antiviral activity across all genotypes due to conservation of targeted domains, but the barrier to resistance is relatively low, as for protease inhibitors. Substitutions at positions Met^{28}, Gln^{30}, Leu^{31}, Pro^{32}, and Tyr^{93} are frequently selected by this class of inhibitor (Gao 2013; Halfon and Sarrazin 2012; Nakamoto et al. 2014; Nettles et al. 2011). Similar to protease inhibitors, the viral genotype was found to influence the rate of resistant mutant selection, with virus from patients infected with genotype 1b being less likely to acquire resistance than virus from genotype 1a-infected patients (Fridell et al. 2011; Nettles et al. 2011).

In contrast, nucleoside analog NS5B inhibitors display high antiviral activity, broad genotype coverage, and relatively high barrier to resistance. Nucleoside inhibitors of NS5B are analogs of the polymerase substrates and bind directly to the NS5B active site. Importantly, because the active site of NS5B is highly conserved, nucleoside analogs have similar efficacy across all HCV genotypes. While amino acid substitutions resulting in weak resistance to nucleoside analog are readily selected, the resulting loss of replicative fitness limits breakthrough (McCown et al. 2008; Sarrazin and Zeuzem 2010). This contrasts with substitutions selected by NS3/4A protease and nonnucleoside inhibitors, which have greater resistance and do not profoundly affect replication capacity (Pawlotsky 2009). Sofosbuvir is an approved pyrimidine-derived nucleoside analog NS5B inhibitor that may be of great importance in future IFN-free treatment regimens. The barrier to resistance is relatively high, as only a few NS5B mutations have been confirmed to confer resistance [S282T (Sofia et al. 2010); L159F/L320F (Tong et al. 2014; Donaldson et al. 2014)].

Ongoing clinical trials with newly approved DAAs aim at finding effective, IFN-free combinations applicable to all HCV genotypes, which is challenging due to the continuing diversification of HCV in nature and the many escape routes that viruses find to combat drugs. To avoid rapid selection of cross-resistant mutant populations of HCV, combinations of inhibitors should be directed against several viral genes simultaneously (Lange and Zeuzem 2013). This concept is supported by the known sensitivity of viruses with protease inhibitor-resistance mutations to other classes of DAAs (such as NS5A, NS5B, and cyclophilin inhibitors) (Thompson et al. 2011) and the accumulated experience with treatments against HIV-1 infections (section "Effective Combination Therapies for HIV-1 Infection"). In a study where treatment-naïve HCV genotype 1a and 1b patients were treated with mericitabine, danoprevir (NS3/4A inhibitor), and ribavirin, viral breakthrough was mainly associated with NS3/4A resistance mutants while specific resistance mutations in NS5B were obtained in a single patient (Lange and Zeuzem 2013). In clinical trials with patients infected by genotype 1 HCV treated with a combination of the NS5A

inhibitor ledipasvir and sofosbuvir, high rates of SVR were achieved (>94 %), regardless of prior treatment history (Afdhal et al. 2014a, b). Combination therapy using the NS5A inhibitor daclatasvir with sofosbuvir resulted in SVR rates of 98 %, 92 %, and 89 % in patients infected by genotypes 1, 2, and 3, respectively (Sulkowski et al. 2014). Resistance mutations present in patients were limited to known NS5A resistant variants, and no sofosbuvir-specific mutations were observed. Thus, the combination of inhibitors used in IFN-free therapies must be chosen carefully in order to minimize risk of breakthrough resistance. It is difficult to anticipate to what extent a wider use of IFN-free treatments will affect HCV evolution and select for new drug-resistance mutations that will acquire epidemiological relevance (as was the case with HIV-1). It will largely depend on the administration of the new combinations to poorly responding patients, who provide environments that are prone to select for escape mutants, and also on the fitness and frequency of transmission of the newly generated mutants.

Due to the high basal mutation frequency in natural HCV populations, it may be important to evaluate the presence of preexisting resistance mutations within patient HCV quasispecies. Presence of a naturally occurring variant of genotype 1a (Q80K) has been associated with decreased SVR after simeprevir-based triple therapy (Forns et al. 2014). When resistant mutants emerge after treatment failure, it is not well known how long they remain in the population and whether they can impact future therapy. Studies using population sequencing techniques have revealed a rapid loss of detection of resistant variants (Mauss et al. 2014) although in other studies, sequencing detected resistant variants even several years after treatment with telaprevir or boceprevir (Susser et al. 2011). Furthermore, increased failure of simeprevir-based triple therapy was observed after re-treatment of patients who had developed simeprevir resistance previously during monotherapy, likely an effect of persistent resistance variants within the viral population (Lenz et al. 2012). The analysis of resistance mutations within the quasispecies both at baseline and after failure of IFN-free regimens will guide future selection of inhibitor combinations to be used.

Conclusions and New Prospects for Antiviral Therapy

High mutation rates and quasispecies dynamics confer to RNA viruses an adaptive potential that may be counteracted using five main strategies: (i) combination therapy (i.e., HAART for AIDS, as has been described in section "HIV Variability and Antiretroviral Therapy"), (ii) splitting of the treatment into a first induction regimen (to decrease the number of viral mutants and viral load) and a second maintenance regimen (to maintain a viral load sufficiently low) (von Kleist et al. 2011), (iii) targeting of cellular proteins (taking advantage of viral reliance on host cell functions) (Geller et al. 2007; Hopkins et al. 2010; Kumar et al. 2011), (iv) combined use of immunotherapy and chemotherapy (in order to stimulate a broad adaptive immune response) (Li et al. 2005; Seiler et al. 2000; Webster et al. 1986), and (v) lethal mutagenesis [Domingo et al. (2012) and references therein].

It has been proposed that the targeting of cellular functions should limit selection of viral escape mutants. This is not necessarily the case, as evidenced by selection of mutations in NS5A that were associated with resistance to cyclosporine A (Chatterji et al. 2010; Delang et al. 2011) and maraviroc-resistant HIV-1 mutants (section "The Molecular Basis of Drug Resistance in HIV-1"). Moreover, such agents may produce side effects derived from the perturbation of cellular functions. However, the ability of these compounds to simultaneously inhibit the replication of multiple virus types may increase their therapeutic potential (Pawlotsky 2014).

Lethal mutagenesis aims at extinguishing viruses by increasing mutation rates via administration of mutagenic agents. Population behavior of RNA viruses is strongly influenced by interactions among viral genomes within the mutant spectra. Thus, the generation of defective viral genomes (as a consequence of increased mutagenesis) and the collapse of the whole ensemble due to interfering interactions with the replication of the standard virus are consistent with the features of viral quasispecies (Grande-Pérez et al. 2005; Perales et al. 2007). A first clinical trial using a mutagenic nucleoside analog was conducted against HIV-1 in AIDS patients, showing that lethal mutagenesis could be effective *in vivo* (Mullins et al. 2011). Therapies involving inhibitors and mutagenic agents should consider the mechanism of action of both drugs due mainly to two reasons: (i) defective mutants (generated by the mutagen) should be replication-competent to exert their interfering activity and this is impeded in the presence of an inhibitor and (ii) due to the mutagen-induced error rate, the selection of inhibitor-escape mutants could be favored when both drugs are administered simultaneously, and this probability will increase with the viral load (Iranzo et al. 2011; Perales et al. 2009, 2012). In light of this, sequential therapies with a first phase of viral load reduction (via a combination of inhibitors) followed by a second phase of increased mutagenesis deserve further investigation. From a general perspective, such explorations of new treatment designs will become even more justified if, as can be anticipated, the new combinations fail to eradicate current and emerging pathogenic viruses worldwide.

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