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Virus population dynamics, fitness variations and the control of viral disease: an update

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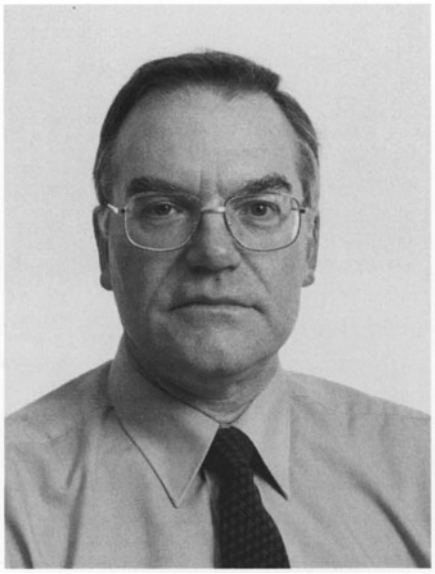

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Summary

Viral quasispecies dynamics and variations of viral fitness are reviewed in connection with viral disease control. Emphasis is put on resistance of human immunodeficiency virus and some human DNA viruses to antiviral inhibitors.

Future trends in multiple target antiviral therapy and new approaches based on virus entry into error catastrophe (extinction mutagenesis) are discussed.

Contents

Keywords

Virus, quasispecies, HIV, fitness, influenza, herpes, hepatitis B, inhibitor, resistance, extinction mutagenesis

Glossary of abbreviations

CMV, cytomegalovirus; CTL, cytotoxic T lymphocyte; EBV, Epstein-Barr virus; HAART, highly active anti-retroviral therapy; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; HSV, herpes simplex virus; PR, protease; RT, reverse transcriptase; TK, thymidine kinase; VSV, vesicular stomatitis virus; VZV, varicella zoster virus.

1 Introduction: overview of quasi-species complexity in relation to viral disease prevention and treatment

Previous versions of this article [1, 2] emphasized the need to design antiviral strategies taking into consideration that RNA viruses are highly heterogeneous and dynamic, rather than constrained genomes that would lend themselves to control by inhibitors and immune responses. It was proposed that a better control of viral replication should be achieved with combination antiviral therapy and multivalent vaccines [1, 2]. As deeper insight into the structure and dynamics of RNA viruses is gained, such proposals find increasing justification. The mutant spectra of many RNA viral quasi-species have been shown to hide mutants with altered biological properties (altered phenotypes). Of immediate relevance to disease control are inhibitor-resistant mutants [3–6], and antibody and cytotoxic T lymphocyte (CTL)-escape mutants [7–12]. The origin of the rich repertoire of genetic and phenotypic variants is a biochemical property of all known RNA-dependent DNA and RNA polymerases: low template-copying fidelity, partly associated with the absence (or very low efficiency) of proofreading-repair activities [13, 14]. By application of genetic and biochemical procedures, mutation rates averaging 10^{-3} to 10^{-5} misincorporations per nucleotide have been determined for several RNA viruses ([15] and references therein). This strong mutational pressure unavoidably yields mutants with different relative replication efficiency (fitness) and thus, different mutants are found at different frequencies in viral quasi-species. Mutation frequencies, measured as the number of mutations in a set of genomes relative to the number of nucleotides screened, range from 10^{-2} to 10^{-5} substitutions per nucleotide. This broad range reflects a complex set of parameters which influence the heterogeneity of a mutant spectrum: duration of an infection (either in cell culture or a natural host), number and extent of environmental perturbations affecting viral multiplication, and many others. Examples of very different mutation frequencies determined in our laboratory under disparate biological circumstances (clonal populations in cell culture *versus* prolongued infections *in vivo*) can be found in ([3, 16, 17]; as a recent general review see also [18]).

These well-documented mutation rates and mutation frequencies render it very likely that a selective pressure directed to a specific locus of a viral genome (or its protein product) will result in the selection of a viral variant capable of overcoming the selective constraint (the isolation of monoclonal

antibody-escape mutants is a classical example). Therefore, for statistical reasons, given the population size of replicating viruses – often higher than 10^{11} particles during human hepatitis C virus (HCV) or human immunodeficiency virus (HIV) infections [19–23] – an antiviral treatment must target three or more genetic loci of a virus. The evolution of therapeutic regimens implemented for the control of HIV-1 infections from the middle eighties until the present constitutes a revealing example. Monotherapy resulted in the systematic selection of inhibitor-resistant HIV-1 mutants in infected individuals [5, 24, 25, and many other studies]. In contrast, the so-called "highly active anti-retroviral therapy" (HAART) which involves administration of three or more anti-retroviral drugs targeting two different viral enzymes, has resulted in a generally effective suppression of HIV-1 replication and a reduction of AIDS cases [26–28]. Parallel arguments apply to vaccines [1, 2, 29, 30]. The main demonstration that multi-valent, multi-epitopic vaccines are required for protection against diseases associated with RNA viruses, is that no vaccines consisting of a single synthetic peptide capable of affording protection in a reproducible manner have been produced, despite much investment ([31, 32], among other reports].

Mutant swarms may hide additional types of mutants and their presence may affect viral pathogenesis. Individual components of vesicular stomatitis virus (VSV) quasi-species displayed either a higher or lower capacity to induce interferon than the average population in natural isolates of VSV [33]. For several RNA viruses, mutants with altered host cell tropism have been isolated from the quasi-species swarms [34–36]. In other cases, the presence of critical phenotypic variants may not be readily identified, but the number of different sequences in the mutant distribution may itself have a predictive value. Large quasi-species complexity correlated with a high pathogenic potential of the coronavirus mouse hepatitis virus for mice [37]. Evolution of the HCV quasi-species replicating in patients correlated with the transition from an acute to a chronic infection [38]. Also, despite early contradictory results, when the complexity of HCV quasi-species has been reliably measured by extensive nucleotide sequencing, a high mutant spectrum complexity predicts failure of elimination of the virus by the combined action of interferon α and the nucleoside analogue ribavirin ([39], reviewed in [40]).

Recently, a new feature of quasi-species has been revealed: the presence of memory genomes, as minority components of the mutant spectrum, which reflect those genomes which were dominant at an earlier stage in the evolu-

tionary history of the virus ([17, 41], review in [42]). Memory can be durable provided no genetic bottlenecks intervene during viral replication, showing that memory is a property of the quasi-species as a whole [further discussion in 42]. The presence of memory is a feature of complex adaptive systems, such as the immune system [43]. Its presence in RNA virus quasi-species suggests that adaptation to virology of concepts related to complexity in physics [44] may provide new grounds to study viral population dynamics.

The overview of long-established facts, together with recent observations, on the nature of RNA virus quasi-species suggests that the consensus nucleotide sequence (the one resulting from assigning to each genomic position the most abundant nucleotide found at the corresponding position, for the set of compared sequences) provides only a fragmentary information to guide decisions on antiviral treatment. Insights into complexity and composition of mutant spectra are required. Furthermore, RNA genome complexity has also inspired entirely new antiviral strategies, such as forcing virus replication into error catastrophe, an interesting possibility reviewed in section 9. HIV appears as a viral system where many of these issues have been thoroughly investigated, and here we summarize current knowledge on viral dynamics and molecular mechanisms leading to emergence of mutants escaping the immune response, as well as to the emergence of drug resistance. Finally, drug resistance in other RNA viruses, as well as in DNA viruses, will be discussed in the light of current treatment options.

2 HIV variation and strategies for HIV persistence

Rapid replication rates and high mutation rates are thought to be the engine of HIV-1 genetic diversity. The HIV-1 reverse transcriptase is error-prone, and its error rate has been estimated at between 10^{-4} to 10^{-5} mutations per nucleotide and cycle of replication [45–48]. Mutation rates may also show sequence- or structure-dependent variation; hence, all mutations may not occur with similar rates. If one assumes that 10^9–10^{10} virions are produced each day, then they must be the product of at least 10^7–10^8 replication cycles. Given the length of the HIV-1 genome (approximately 10^4 nucleotides), it is plausible that every single possible point-mutation (and probably many double mutations) will occur at least once each day, in an infected individual. Although specific combinations of multiple mutations may be rare, it is clear

that the degree of potential genetic change drives the diversification of HIV-1 in response to the selective pressure of host immune responses or anti-retroviral therapy.

The emergence of mutant forms of HIV results in escape from neutralizing antibody responses and CTLs. Neutralizing antibodies found in sera of infected individuals are directed to the envelope glycoprotein complex. This complex is arranged in a trimeric configuration of heterodimers, each consisting of a gp120 surface subunit non-covalently associated with a gp41 transmembrane subunit. The infection progress is initiated by attachment of the virus to the target cell through the interaction of gp120 with the cellular receptor CD4. The subsequent interactions between gp120 and its coreceptors are complex and require conformational changes induced by binding to CD4 (for a review see [49]). The third variable (V3) loop of gp120 is the principal neutralizing domain, as evidenced by its dominant role in the neutralization of T cell line-adapted HIV-1 strains (i.e., IIIB, SF2, MN) by sera from HIV-1 infected individuals and gp120 vaccine recipients. Binding studies with monomeric gp120-CD4 complexes have demonstrated that monoclonal antibodies to the V3 loop inhibit the interaction of this complex with the coreceptor [50–52]. Neutralizing antibodies emerge only relatively late in the course of infection [53–55] and contribute to the control of viral replication, as shown by passive immunization in animal models, and also by the correlation between titers of neutralizing antibodies and lack of disease progression in long-term survivors of HIV-1 infection [56–58]. The emergence of neutralization escape mutants has been associated with viral persistence *in vivo*, and disease progression [12, 55, 59]. However, escape mutations are not always located in the V3 loop of gp120. It has been shown by many authors that the role of V3-specific antibodies in primary isolate neutralization is insignificant [60–62]. Recently, it has been suggested that neutralization resistance of primary isolates could be considered as an escape mechanism from humoral immune control [63]. This study revealed that the emergence of neutralization-resistant HIV correlated with increased variation in the env gene, and preceded disease development in an individual that was accidentally infected with the T cell line-adapted neutralization-sensitive IIIB isolate.

CTLs constitute an essential component of protective anti-retroviral immunity. HIV-1 can use mutational and nonmutational mechanisms to avoid the CTL response (for recent reviews see [8, 64]). Viral mutation can

lead to epitope deletion, failure of antigen processing, loss of major histocompatibility complex class I binding and impaired recognition by the T cell receptor. HIV-1 variants with impaired CTL recognition properties occur during the initial course of infection. Several examples illustrating how HIV can avoid CTL response through variations in T cell epitopes have been described. The first example was found following an attempt to treat an HIV-infected patient by adoptive transfer of a CTL clone, which had been grown to a very large number [65]. The CTL clone recognized an epitope presented by HLA A3. The clinical response was disappointing with no reduction in viral load or rise in $CD4^+$ T cells. The reason for the clinical failure was the emergence of a virus with a deletion in the nef gene, that was devoid of the T cell epitope spanning residues 69 to 78 of Nef. There are two examples of CTL escape occurring during the acute phase of infection. In one of them, the patient developed a strong monospecific response against an epitope (AENLWVTVY) including residues 30 to 38 of the Env protein, and presented by HLA B44 [66]. After a few weeks, variants appeared having Gly, Ala or Lys instead of Glu at the second position of the epitope, and several weeks later, they became predominant in the viral population. These variants escaped the initial CTL response of the patient. A similar case has been described by Price et al. [67], although in this case the T cell epitope includes residues 86 to 93 of Nef (sequence FLKEKGGL), and the CTL escape is associated with mutations at the fifth position of the epitope as well as with its deletion. Escape from CTL has also been observed late in HIV infection. Two patients who maintained a monospecific response to a single epitope in Gag, were found to select a virus with the same mutation as they progressed to AIDS [68]. The mutation changed an Arg to a Lys at position 2 of the epitope (KRWIILGLNK, residues 263–272 of the Gag precursor). The arginine residue was essential for binding of the peptide to HLA B27.

Strong support of the CTL "escape" hypothesis has been recently provided by a series of experiments showing the accumulation of amino acid replacements within CTL epitopes located on the proteins Env and Nef, and arising during the course of simian immunodeficiency virus disease progression in macaques [11]. Further studies revealed that Tat-specific CTLs select for SIV escape variants during the acute phase of infection [69]. The accumulation of mutations in Tat CTL epitopes suggests that responses against viral proteins that are expressed early during the viral life cycle are important to control viral replication.

3 Anti-retroviral therapy and development of drug resistance: population dynamics of HIV infection

Since the discovery of AZT (3'-azido-3'-deoxythymidine, zidovudine) as an effective anti-retroviral agent against HIV [70], a number of potent drugs that inhibit HIV-1 replication *in vivo* have been developed. As of March 2001, there are nine reverse transcriptase (RT) inhibitors and six protease (PR) inhibitors which have been approved for clinical use (Tab. 1). RT inhibitors prevent infection of new cells by blocking the synthesis of proviral DNA using the viral RNA as template, while PR inhibitors act on the late phase, interfering with the production of new infectious virions by infected cells. The obsolete monotherapy with AZT has become unacceptable and has been substituted by more powerful triple and quadruple combinations of drugs including RT and PR inhibitors [26–28]. Their use led to a significant reduction in the level of viral RNA in plasma, thereby indicating that the rate of virus production is impaired as a consequence of the treatment. Recent studies suggest that the half-life of free virus particles is extremely short, on the order of minutes to hours [23]. Analysis of dynamic changes in the level of plasma virus at different stages of disease and in response to anti-retroviral therapy has provided new insights into the mechanism of $CD4^+$ T cell depletion, the reasons for drug failure, the nature of viral reservoirs, and the intriguing possibility that prolonged therapy may lead to virus eradication (for reviews see [71, 72]). Following the initial infection, the immune response to HIV-1 develops, and plasma viral levels fall from a peak of viremia to lower steady-state values that vary in different individuals and are predictive of the rate of disease progression [20]. In untreated asymptomatic patients, the plasma HIV-1 RNA levels are typically in the range of 10^3–10^6 copies/ml in plasma. The viral burden is constant during the asymptomatic phase of infection and tends to be higher in patients showing more rapid declines in $CD4^+$ lymphocyte counts and more rapid progression to AIDS.

The introduction of HAART and sensitive molecular tools to measure HIV-1 RNA in plasma has permitted the kinetic analysis of HIV-1 infection. HIV-1 infection is characterized by rapid viral replication and rapid viral clearance [21–23]. The initiation of potent anti-retroviral therapy produced a very rapid exponential drop in the level of plasma virus. Typically, plasma viral levels decreased 100-fold in two weeks. The corresponding half-life ranges from 1.3 to 3.3 days [21, 22], and results from two different events: the clear-

Table 1.
Reverse transcriptase and protease inhibitors approved by the U.S. Food and Drug Administration for the treatment of HIV infection.[1]

Drugs	Commercial name	Company	Date of approval
Reverse transcriptase inhibitors			
Zidovudine (AZT)	Retrovir	Glaxo Wellcome	March 1987
Didanosine (ddI)	Videx	Bristol Myers-Squibb	October 1991
Zalcitabine (ddC)	Hivid	Hoffman-La Roche	June 1992
Stavudine (d4T)	Zerit	Bristol Myers-Squibb	June 1994
Lamivudine (3TC)	Epivir	Glaxo Wellcome	November 1995
Abacavir	Ziagen	Glaxo Wellcome	December 1998
Nevirapine	Viramune	Boehringer Ingelheim Pharmaceuticals, Inc.	June 1996
Delavirdine	Rescriptor	Pharmacia & Upjohn	April 1997
Efavirenz	Sustiva	DuPont Pharmaceuticals	September 1998
Protease inhibitors			
Saquinavir	Invirase	Hoffman-La Roche	December 1995
Ritonavir	Norvir	Abbott Laboratories	March 1996
Indinavir	Crixivan	Merck & Co. Inc.	March 1996
Nelfinavir	Viracept	Agouron Pharmaceuticals	March 1997
Amprenavir	Agenerase	Glaxo Wellcome	April 1999
Lopinavir	Kaletra[2]	Abbott Laboratories	September 2000

[1]Data shown have been taken from the WWW address: http://www.fda.gov/oashi/aids/virals.html, in March 2001.
[2]Kaletra is an approved oral capsule and solution, including lopinavir plus ritonavir. Other approved combinations of anti-retroviral drugs are Combivir (zidovudine and lamivudine) and Trizivir (zidovudine, lamivudine and abacavir), approved in September 1997 and November 2000, respectively.

ance of infectious virus (whose half-life was around 6 h), and the clearance of productively infected cells (whose half-lives were estimated to be 1.55 days) [23]. The short-lived cells that produce most of the plasma virus are $CD4^+$ T lymphoblasts.

When potent anti-retroviral therapy is maintained, it is possible to observe a slower decay in plasma viral levels that follows the initial rapid clearance of infectious virus and productively infected T lymphocytes. This second phase of decay brings levels of plasma virus down to below the limit of detection of current assays (20–500 copies/ml), and corresponds to the elimination of latently infected cells or long-lived and chronically produc-

tive cells, for example, resting $CD4^+$ T cells with unintegrated HIV-1 DNA, infected macrophages or virions trapped on follicular dendritic cells [26, 73, 74]. From the half-lives of these viral reservoirs (8 to 15 days), it was estimated that 2–3 years of HAART would eliminate these viral pools.

However, despite the success of HAART in reducing viremia to undetectable levels in some patients, the recovery of replication-competent HIV-1 was reported for infected individuals who experienced complete suppression of new cycles of replication for the estimated 2–3 years required for the second phase of decay to be complete. Several recent studies suggest that latently infected $CD4^+$ CD45 RO^+ memory T cells with integrated provirus represent a extremely stable reservoir for HIV-1, that may be responsible for the third phase of decay in patients under potent anti-retroviral therapy [75–79]. Memory cells must survive for long periods of time in order to provide protection against a previously encountered pathogen. Sequence analysis of viruses isolated from memory T cells revealed little evidence for the evolution of drug resistance, suggesting that the viruses that persist in this compartment are derived from long-lived cells infected prior to initiation of HAART [78, 79]. Rough estimates of half-lives of these viral reservoirs are difficult to obtain due to the lack of longitudinal samples. The median lifespan of $CD4^+$ memory T cells is estimated to be approximately 200 days [80], but the range may extend for years. If this estimate is correct, then conventional treatment methods would not be effective in eradicating HIV-1.

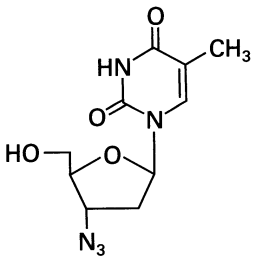
The contribution of the different pools of infected cells to the re-emergence of the virus after discontinuation of the anti-retroviral treatment is currently under investigation. Thus, using heteroduplex mobility and tracking assays, Chun et al. [81] have shown that the detectable pool of latently infected, resting $CD4^+$ T cells does not account entirely for the early rebounding plasma HIV in infected individuals in whom HAART was discontinued. In the majority of patients examined, the rebounding plasma virus was genetically distinct from both the cell-associated HIV-1 RNA and the replication-competent virus within the detectable pool of latently infected, resting $CD4^+$ T cells. These results point towards the existence of other persistent HIV reservoirs that could prompt rapid emergence of plasma viremia after cessation of HAART. Persistence of replication in lymphoid tissues, albeit at low levels, during seemingly effective treatment has been documented [82]. However, the precise anatomical location of the source of viral rebound remains undefined. Potential reservoirs are specific subsets of susceptible cells

(i.e., follicular dendritic cells) [83], or anatomical reservoirs such as the brain, gut-associated lymphoid tissue or the genital tract [84, 85].

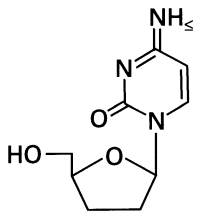
4 Selection of HIV variants resistant to anti-retroviral drugs

Current anti-retroviral drug regimens include RT and PR inhibitors (Figs. 1 and 2, respectively). A large number of mutations conferring partial or total resistance to those inhibitors has been observed *in vitro*, and subsequently detected *in vivo* after the examination of sequences obtained from patients failing monotherapy. The role of many of the resulting amino acid substitutions has been further verified by *in vitro* mutagenesis experiments, and large compilations of mutations have been presented in a previous review in this series [2] and elsewhere [86–89].

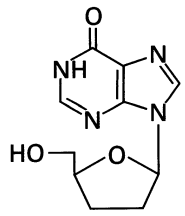
Development of high-level resistance to specific anti-retroviral drugs can be achieved either through the acquisition of specific mutations or through multiple amino acid substitutions. For example, a single mutation at codon 184 of HIV-1 RT (i.e., M184I or M184V) is sufficient to confer >1,000-fold resistance to lamivudine (3TC). On the other hand, high-level resistance to zidovudine (AZT) requires several amino acid substitutions (typically, M41L, D67N, K70R and T215F or T215Y), and one of them (T215Y) involves two nucleotide substitutions. The low genetic barrier to lamivudine resistance leads to the emergence of a resistant virus in just a few weeks [90], while in the case of zidovudine, its genetic barrier is higher, and the appearance of a resistant virus may take a few months [24]. Other examples of RT inhibitors having a low genetic barrier are nevirapine, delavirdine or efavirenz. In these cases, there are several single-nucleotide substitutions which can confer high-level resistance to the drug. For example, these include K103N, Y181C or G190A in the case of nevirapine, L100I, K103N, Y181C or P236L in the case of delavirdine, and L100I or K103N in the case of efavirenz. Interestingly, some of these mutations can confer cross-resistance to all inhibitors of this class, as occurs with K103N. In the case of PR inhibitors, the acquisition of high-level resistance is usually mediated by at least 3–4 changes. PR inhibitors are substrate analogues which contain a nonhydrolyzable peptidic bond. Resistance to PR inhibitors can be initially achieved through the acquisition of mutations affecting residues involved in substrate/inhibitor binding, such



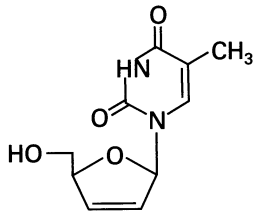
Zidovudine
(AZT)



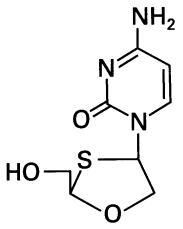
Zalcitabine
(ddC)



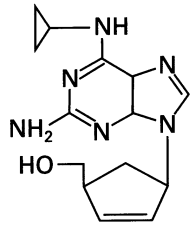
Didanosine
(ddI)



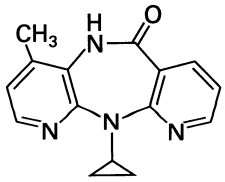
Stavudine
(d4T)



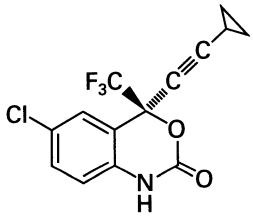
Lamivudine
(3TC)



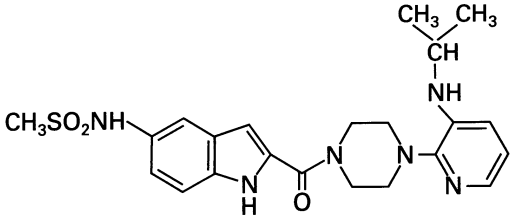
Abacavir



Nevirapine



Efavirenz



Delavirdine

Fig. 1
Structural formulae of approved drugs targeting HIV-1 reverse transcriptase.

as Asp-30, Val-82 or Ile-84. However, these amino acid changes reduce the proteolytic activity of the enzyme, compromising viral fitness, and further mutations appear to recover the viral replicative capacity.

Generally, there are two main processes leading to resistance-related treatment failure: pre-existing resistant strains may be selected by the drugs used, or resistant mutants are generated *de novo* by residual viral replication during treatment. Some genetic variability occurs in the PR and RT coding regions even in the absence of significant selective pressure, which result in sequence polymorphisms. Single mutations related to resistance to RT or PR inhibitors have been shown to pre-exist in the HIV-1 quasi-species of drug-naïve patients [3, 4]. However, the question is still open for a multidrug-resistant virus arising during potent combination therapies, since current methods are not sensitive enough to detect mutants at very low frequencies or are too laborious to be used in clinical practice. Models based on population genetics predict that treatment failure is most likely to be caused by the pre-existence of a multidrug-resistant virus, and they therefore propose that the key to drug resistance lies in the diversity of the viral population at the start of therapy [91].

5 Resistance mutations and their effect on viral fitness

Fitness is a complex parameter aimed at describing the replicative adaptability of an organism to its environment (for reviews see [92, 93]). For HIV (and other viruses), an experimental and useful approach to fitness is the relative ability of the virus to produce stable infectious progeny in a given environment (i.e., cell culture, blood stream, etc.). In general, drug-resistance mutations emerge at the expense of a loss in viral fitness. For example, the substitution of Met-184 by Val or Ile, which confers high-level resistance to lamivudine, leads to the loss of viral replication capacity [94-98]. The lower replication capacity of a virus harboring the substitutions M184V or M184I has been attributed to the diminished processivity of the viral RT [94], which is accentuated in peripheral blood mononuclear cells due to the low levels of dNTPs in the intracellular pools found in these cells [95]. The effects of the lamivudine resistance mutations on viral fitness have been observed not only in the sequence context of a wild-type subtype B HIV-1, but also in the presence of other drug-resistance mutations such as V75I, F77L, F116Y and

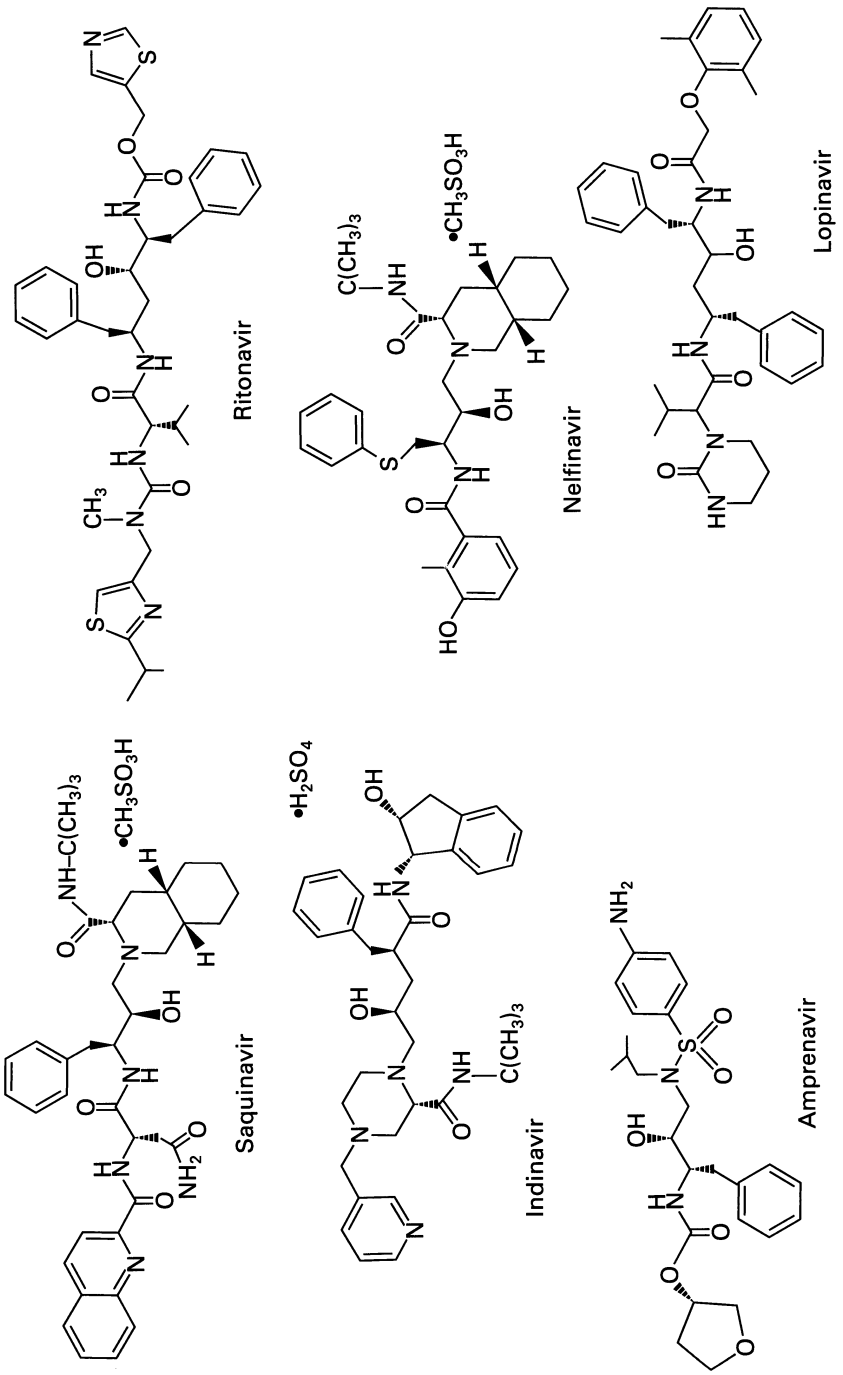


Fig. 2
Structural formulae of approved inhibitors of HIV-1 protease.

Q151M [99]. These results reported for cell cultures are also in good agreement with observations based on clinical experience. It has been shown that once the resistant virus becomes predominant in the viral quasi-species obtained after treatment with lamivudine, drug removal leads to the rapid replacement of the resistant virus by the wild-type. Estimates obtained from population dynamics *in vivo* revealed that in the presence of lamivudine, a virus having the mutation M184V showed only 10% of the fitness value calculated for the wild-type virus in the absence of the drug [100].

5.1 Complex genotypic pathways leading to resistance to multiple nucleoside analogue inhibitors of reverse transcriptase

In those cases in which two or more mutations are required to achieve high-level resistance, the sequential appearance of mutations in the viral population becomes critical. Thus, in the case of zidovudine, high-level resistance is acquired through the acquisition of several mutations including M41L, D67N, K70R, L210W, T215F/Y and K219Q/E. Different levels of resistance are observed depending on the particular combination of resistance-related mutations found in the viral clone. For example, IC_{50} values for zidovudine obtained with HIV-1 clones having the substitutions T215Y, M41L/T215Y, D67N/K70R/T215Y and M41L/D67N/K70R/T215Y are 3 to 16, 31, 33–70, and > 100 times higher, respectively, than the values obtained with wild-type virus (reviewed in [87]). Primary mutations for zidovudine resistance are K70R and T215F/Y. The K70R mutation appears frequently, since it requires only one nucleotide change, and does not have a major impact on viral fitness [101]. However, its effect on viral resistance is relatively small [102, 103]. The substitution of Thr-215 by Phe or Tyr implies two nucleotide changes and has a major impact on viral fitness [101, 104, 105], although it has a stronger effect on viral resistance.

The situation becomes more complex in a virus displaying multidrug resistance. Combined therapies with two or more nucleoside analogue inhibitors of RT leads to the emergence of drug resistance through mutational pathways which are different from those obtained with monotherapeutic regimens. Resistance to multiple nucleoside inhibitors of RT has been associated with a substitution at the nucleotide binding site of the enzyme (i.e., Q151M) and

with insertions or deletions around positions 67–70 of the RT. The acquisition of resistance through the Q151M pathway was initially observed in the virus isolated from patients receiving zidovudine and didanosine [106]. In this situation, the first amino acid change that appears in the viral population is Q151M, which involves two nucleotide changes. Viral clones harboring this amino acid substitution display moderate resistance to zidovudine and zalcitabine, and low resistance to other nucleoside analogues [106, 107]. However, further acquisition of additional mutations, such as A62V, V75I, F77L, or F116Y led to the appearance of highly resistant virus. Fitness assays involving the determination of replication kinetics or growth competition experiments have shown that the mutations at codons 62, 75, 77 and 116 improve the replication capacity of the resistant virus [108, 109]. The frequency of emergence of Q151M and its related mutations has been estimated as ranging from 3.5% to more than 19% in patients receiving combination chemotherapy with multiple dideoxynucleosides for more than a year [106, 107, 110–112]. Its low frequency is probably related to the requirement of two nucleotide changes to obtain the initial Q151M mutation (from CAG to AUG). In addition, any intermediate step, involving either the substitution of Q151K (CAG to AAG) or Q151L (CAG to CUG), results in a virus whose replication efficiency is very low, as demonstrated by introducing these mutations in proviral HIV clones [109]. The conservative change to Q151L appears to be lethal for viral replication and has been rarely observed *in vivo*. However, a recent study suggests that in the presence of Gly instead of Ser at position 68, this mutant retains some replicative capacity [113], and therefore could facilitate the emergence of multidrug resistance through the Q151M pathway.

An insertion of two amino acids (often Ser-Ser, Ser-Gly or Ser-Ala) between residues 69 and 70 of HIV-1 RT has recently been described in patients subjected to prolonged therapy with AZT, often together with (or followed by) administration of other nucleoside inhibitors [114–120]. The insertion appears to be associated with multiple amino acid substitutions, including AZT-resistance mutations, such as T215Y. The insertion is critical for the acquisition of zidovudine resistance, since it improves the enzyme's ability to unblock and further extend AZT-terminated primers in the presence of ATP [121]. Interestingly, this effect occurs only in the background of a virus having many resistance mutations, where it probably increases its replication capacity. Although the molecular mechanism leading to the emergence of the

insertion is not known, it could result from the duplication of a six-nucleotide sequence, after the replacement of Thr-69 by Ser. In any case, this event would be rare, in agreement with frequency estimates of insert-containing strains in patients undergoing potent anti-retroviral therapy, which are usually between 1 and 3% [116, 117, 120, 122].

A deletion at codon 67 (Δ67 mutation) has been recently reported to be associated with multidrug resistance either in the context of mutations of the Q151M pathway [123] or in the presence of known zidovudine resistance mutations (i.e., K70R, T215F or K219Q) and non-nucleoside RT inhibitor resistance mutations (i.e., K103N) [124]. The deletion appears to be associated with a substitution at codon 69 (T69G). An extensive analysis of the fitness of a virus carrying the deletion and/or the T69G mutation in different sequence contexts revealed that the substitution of T69G is critical for the acquisition of high-level resistance to zidovudine, while the Δ67 mutation is important to restore viral fitness in the context of a heavily mutated multidrug-resistant virus [125, 126].

5.2 Resistance to protease inhibitors

Currently approved PR inhibitors share relatively similar chemical structures (Fig. 2), and therefore, cross-reactivity is frequently observed in the clinical setting [25]. Primary resistance mutations involved substitutions at positions of the substrate/inhibitor binding site. Examples are V82A or I84V, among others. A multidrug-resistant virus appearing during prolonged therapy with indinavir bore the substitutions M46I, L63P, V82T and I84V in the PR [25]. Crystallographic studies of the mutant enzyme revealed that the substitutions at codons 82 and 84 were critical for the acquisition of resistance, while the amino acid changes at codons 46 and 63, which are away from the inhibitor binding site, appear as compensatory mutations [127, 128]. In a similar way, saquinavir resistance implies the acquisition of substitutions G48V and L90M [129], where G48V exerts the major influence on resistance, and L90M, which is located away from the substrate/inhibitor binding site, contributes to the stability of the HIV PR.

The analysis of viral fitness of clones bearing PR inhibitor resistance mutations is broadly consistent with the hypothesis suggesting that the acquisition of resistance implies a significant cost in terms of viral replicative capac-

ity. Primary mutations, such as D30N, M46I or M46L, G48V and V82A or V82T, which arise after treatment with nelfinavir, indinavir, saquinavir or ritonavir, respectively, have been shown to have a severe impact on viral fitness [130–134]. In all cases, these substitutions affect residues which are important to stabilize the substrate in its binding pocket.

The acquisition of drug resistance in the case of PR inhibitors usually involves several mutations in the protease gene, as well as in secondary loci of the genome. One example that illustrates this concept comes from a clinical study in which the emergence of indinavir resistance was monitored in patients treated for over a year [135]. Sequential analysis of the genotype of viral isolates obtained at different times after initiation of therapy revealed that the substitution V82A appeared first, but additional mutations in the PR gene emerged shortly afterwards or simultaneously in some patients. These mutations were L10I, M46L and I54V, and are commonly observed as secondary mutations during treatment with other PR inhibitors. It was also observed that several isolates bore an additional change at the cleavage site between the gag-encoded proteins p1 and p6. Further treatment implied additional mutations in the PR coding region (i.e., K20M/R, L24I, G48V, A71V/T, G73S, L89M/P and L90M), and another substitution in one of the amino acids of the cleavage site between the nucleocapsid protein, p7 (NC), and the peptide p1. These results revealed that the virus optimizes its viral fitness through the accumulation of changes in PR, but also through the acquisition of mutations at cleavage sites in precursor polyproteins (in HIV-1, termed as Gag and Gag-Pol), which need to be hydrolyzed by the PR in order to complete viral maturation successfully. Mutations at Gag cleavage sites may increase proteolytic processing and compensate for the functional impairment shown by the PR bearing drug-resistance mutations [135, 136]. An interesting effect of the substitution found at the p1/p6 cleavage site is that it produces a 3- to 11-fold increase in the expression of pol, as a consequence of an increased level of frameshifting [137]. The consequence is that the same mutation improves Gag cleavage and stimulates PR synthesis.

6 Multidrug-resistant virus: an obstacle to eradication

In addition to the enormous variability of HIV derived from the inaccuracy of its RT and the high viral loads involved in the infection, recombination

events can also contribute to substantial genetic variation. Recombination between strains resistant to RT inhibitors and to PR inhibitors has been demonstrated *in vitro* as a source of dual resistance [138]. In this scenario, suboptimal therapies, such as the sequential treatment with multiple drugs, may accelerate the appearance of a multidrug-resistant virus through the accumulation of inhibitor-specific drug resistance mutations [139], or through mutational pathways discussed above.

7 Resistance to antiviral drugs specific for other RNA viruses

Influenza is the paradigm of a viral disease in which continued evolution of the virus has an enormous influence in annual epidemics and occasional pandemics of disease in humans. Point mutations (antigenic drift), gene assortment (genetic shift), defective-interfering particles, and RNA recombination are significant contributors to antigenic variation which ultimately leads to viral escape from immune control (reviewed in [140]). Approved drugs for treatment of influenza are amantadine, rimantadine, zanamivir and oseltamivir (Fig. 3) (reviewed in [141]). Amantadine and rimantadine act at an early stage of the viral life cycle, impairing the functionality of the proton channel formed by the viral protein M2. These compounds block the flow of hydrogen ions that goes into the viral particle in the initial steps of viral infection. Inside the virion, the low pH promotes dissociation of the M1 protein from the ribonucleoprotein and triggers initiation of viral replication [142, 143]. Resistant mutants have been isolated *in vitro* and have been recognized in about 25–35% of the treated patients. Escape mutants were usually resistant to both drugs and most of them contained the amino acid substitution S31D at the transmembrane domain of the M2 protein [144].

Neuraminidase is an influenza virus enzyme which allows the virus to penetrate the host cell. The neuraminidase inhibitors, zanamivir and oseltamivir are analogues of N-acetylneuraminic acid (the cell-surface receptor for influenza viruses) [145, 146]. Zanamivir-resistant viruses have been selected *in vitro* [147, 148], although resistance mutations have not been described so far in clinical trials with this drug [149]. In the case of oseltamivir, resistance has been shown to occur in 1.5% of the treated patients [149].

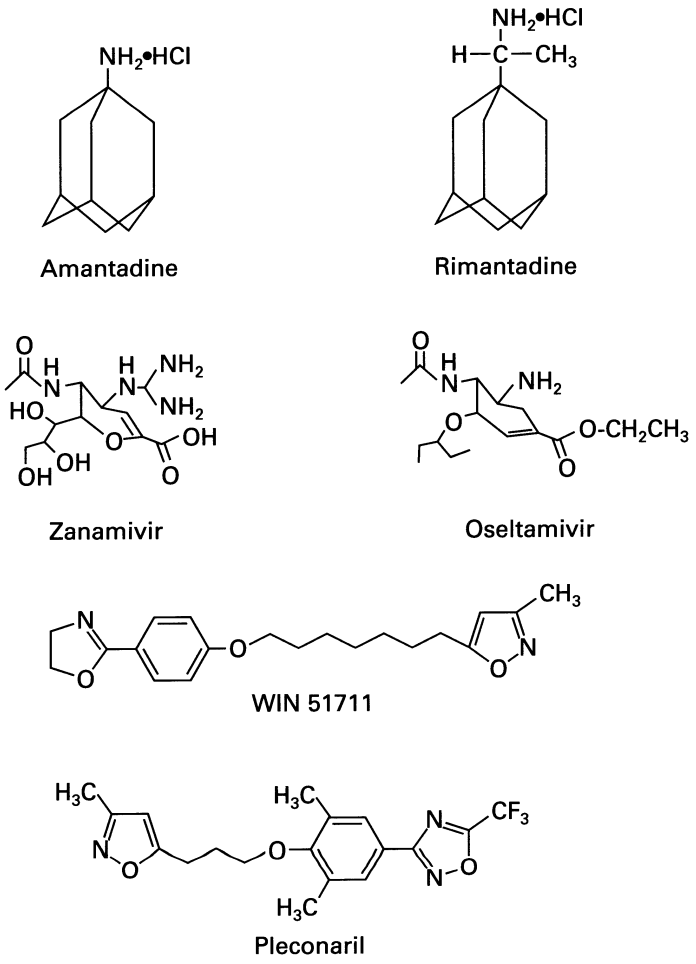


Fig. 3
Structural formulae of antiviral drugs acting on influenza viruses (amantadine, rimantadine, zanamivir and oseltamivir) and other RNA viruses (WIN 54954 and pleconaril).

Some members of the *Picornaviridae* family are important human or animal pathogens. Although effective vaccines have been developed for some of these viruses, such as the polio virus or hepatitis A virus, for other members of this group, the development of antiviral agents has become a priority (for a review see [150]). Structural analysis of the surface of the rhinovirus and enterovirus has revealed the presence of a hydrophobic pocket (usually

referred as a 'canyon') which constitutes a target of antiviral compounds blocking viral uncoating and/or attachment. These compounds are collectively referred to as WIN compounds [151]; several drugs have been extensively studied, and resistance mutations to these drugs have been characterized (reviewed in [152]). One of the most promising agents acting on the capsid function is pleconaril, which is a metabolically stable WIN derivative (Fig. 3). This compound showed broad-spectrum and potent antiviral activity against the enterovirus and rhinovirus [153]. As previously noted for various anti-retroviral drugs, the development of resistance to pleconaril and other WIN derivatives leads to severely reduced infectivity and virulence or attenuated viral growth characteristics in cell culture ([154, 155]; reviewed in 152]).

Other antiviral agents targeting other viral functions include guanidine, a viral replication inhibitor effective against members of the *Picornaviridae* family (polio virus, rhinovirus and foot-and-mouth disease virus, which has not been used in the clinical setting due to its adverse side-effects and the rapid selection of resistant mutants [156]. Guanidine acts at the RNA synthesis step and resistance mutations map to the protein 2C, an ATPase involved in viral replication and encapsidation [157]. Despite significant efforts, efficient inhibitors of viral RNA polymerases, such as inhibitors of the polio virus or hepatitis C polymerase, have not been described, although the recently published structures of these two enzymes [158–161] may pave the way towards its design. A recent report described a new compound (VP32947) which inhibits the RNA-dependent RNA polymerase (NS5B) of bovine viral diarrhoea virus (a pestivirus of the *Flaviviridae* family) [162]. Although mutants resistant to this drug have been generated in the laboratory, VP32947 and other related compounds could represent potentially useful antiviral agents for treating and controlling pestivirus infections, and could also be helpful to design new drugs targeting the elusive viral RNA polymerases.

8 Resistance to antiviral drugs specific for DNA viruses

An important, still largely unanswered question is whether DNA viruses may participate in the high mutation frequencies and quasi-species dynamics which are typical of RNA viruses. The hepadnaviruses, which replicate *via* a

RT step catalyzed by a DNA polymerase devoid of a proofreading-repair activity, share high mutation frequencies with RNA viruses [163]. A strong case for quasi-species dynamics has been made for the geminivirus, a family of plant viruses with a circular, single-stranded DNA genome [164]. Other viruses with single-stranded DNA genomes of small size, such as the parvovirus, also display considerable heterogeneity and variations in host range [165]. For large DNA viruses, it has been suggested that loci-specific variations, for example, tandemly repeated sequences, will be far more likely than generalized high mutation frequencies at many loci (reviewed in [18]). Nevertheless, population heterogeneity and selection of inhibitor-resistant mutants may complicate the control of diseases associated with complex DNA viruses, as documented in the next section.

8.1 Inhibitors of viral replication of *Herpesviridae*

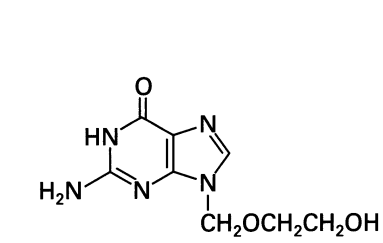
Approximately 90% of the human population is infected by at least one member of the herpes viruses. These viruses are responsible for genital and labial herpetic disease, chickenpox, retinitis, several tumors (lymphomas, sarcomas), etc. The most relevant infectious agents of this family are the herpes simplex virus types 1 and 2 (HSV-1 and -2), varicella zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV) and human herpes virus type 8 (HHV-8). The treatments currently in clinical use include nucleoside derivatives (acyclovir, vidarabine, famciclovir, valacyclovir, ganciclovir, trifluridine and cidofovir), pyrophosphate analogues (foscarnet), interferon α and an oligonucleotide of 21 nucleotides in length (fomivirsen) (for reviews see [166–169]).

Herpes virus isolates, whether from patients or adapted to cell culture, are heterogeneous populations. Data from a study of the molecular evolution of HSV-1 showed that its evolution *in vivo* is slower than for RNA viruses, with an estimated rate of evolution of 3.5×10^{-8} substitutions per site per year [170]. Despite the relatively low level of replication errors found in these viruses, drug-resistance mutations appear in the absence of antiviral drugs [171]. A drug-resistant mutant frequency of approximately one in 1500 plaque-forming viruses has been determined for HSV-1 populations that have never been exposed to selective pressure [172–174], and this rate could be 9- to 16-fold higher in the case of HSV-2 [175]. Once the treatment begins,

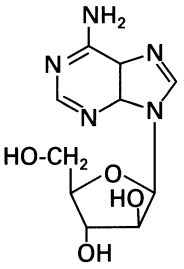
the proportion of pre-existing treatment-resistant viruses would increase as a result of Darwinian positive selection. Therefore, prolonged antiviral treatments, often required for clinical management of herpes virus infections in immuno-compromised patients, favor the emergence of drug-resistant strains ([176, 177] and reviewed in [178]).

Nucleoside analogues (Fig. 4) must be converted into their tri-phosphorylated form in order to be biologically active. For acyclovir, famciclovir, valacyclovir and ganciclovir, the first phosphate is added by viral thymidine kinase (TK) in HSV or VZV, or by UL97 phosphotransferase in the case of CMV [179, 180], to obtain the monophosphate form of the inhibitor. Further phosphorylations are mediated by cellular kinases. The active triphosphate forms of these nucleosides competitively inhibit viral DNA polymerase and act as chain terminators. In general, the acquisition of resistance to nucleoside analogues in HSV and VZV appears to be mediated by mutations in the *tk* gene, usually rendering truncated or inactivated proteins [181–184], or alternatively through mutations leading to decreased TK activity. Examples of resistance associated with alterations in the viral TK have been reported for acyclovir [176, 178, 185, 186], famciclovir [187] and ganciclovir [178, 188, 189]. In addition, acyclovir, penciclovir (the oral prodrug of famciclovir), and cidofovir resistance-associated mutations have been found in the viral DNA polymerase-coding region, albeit less frequently than in the *tk* gene (for a review see [190]). For example, approximately 95 % of the clinical isolates resistant to acyclovir are TK mutants rather than DNA polymerase mutants [191, 192]. Polymerase mutations involve amino acid substitutions that either diminish inhibitor binding or its incorporation into the growing DNA chain [193-196].

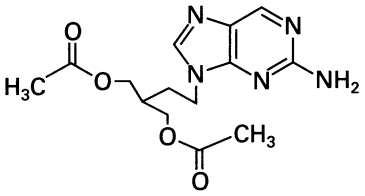
Foscarnet is a pyrophosphate analogue (Fig. 4) and therefore is a prototype of a different class of inhibitors. It has a broad spectrum, being active against many DNA polymerases, including the ones from HSV-1 and -2, VZV, CMV, EBV, and HHV-6, apart from being also an inhibitor of HIV-1 RT and the hepatitis B virus DNA polymerase [197]. Foscarnet does not require intracellular metabolism for activation [198]. It is a non-competitive (or mixed-type) inhibitor that is not incorporated into the growing DNA [199]. It binds to the polymerase pyrophosphate-binding site blocking the pyrophosphate exchange during the catalytic cycle. As expected, the resistant phenotype has been related exclusively to mutations at the DNA polymerase level [178, 199, 200]. Foscarnet-resistant strains may remain susceptible to acyclovir and



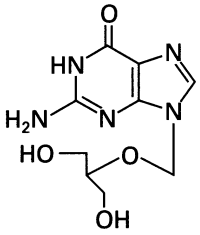
Acyclovir



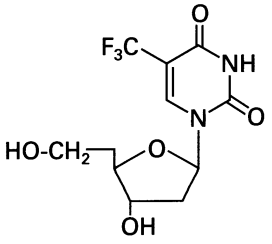
Vidarabine



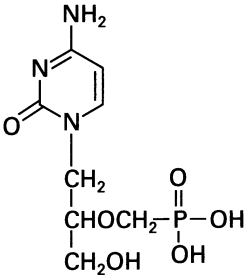
Famciclovir



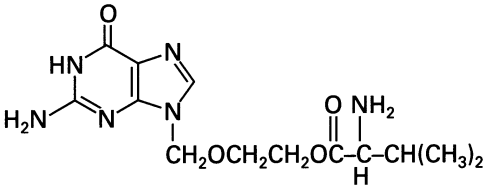
Ganciclovir



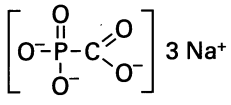
Trifluridine



Cidofovir



Valacyclovir



Foscarnet

Fig. 4
Structural formulae of antiviral drugs targeting herpes virus replication.

ganciclovir [178], although dually resistant clinical isolates have been described [201].

There are other drugs in clinical development, such as herpes virus PR inhibitors (for a review see [202]) or compounds interfering with encapsidation [203], among others. Although development of resistance will probably be unavoidable, these new drugs will certainly be needed to design new alternative treatments based on drug combinations, and to inhibit strains resistant to acyclovir or other nucleoside drugs widely used in the clinical setting.

8.2 Inhibitors of reverse transcription as antiviral drugs for chronic hepatitis B

The hepatitis B virus (HBV) chronically infects 350 million people worldwide, and approximately 10% of these carriers will die as a direct consequence of persistent viral infection. HBV is a hepatotropic DNA virus with a 3.2-kb partial double-stranded circular genome with four overlapping reading frames encoding the viral polymerase, capsid, and surface proteins and a putative transcriptional transactivator protein termed "X". The HBV polymerase converts pre-genomic RNA into double-stranded DNA by reverse transcription. The viral RT is a multifunctional enzyme displaying protein priming activity, as well as DNA-dependent and RNA-dependent DNA polymerase, and RNase H activities. Retro-transcription involves minus-strand DNA synthesis using pre-genomic viral RNA as the template. Then, the RNase H degrades the pre-genomic RNA of the DNA/RNA hybrid, and then the viral polymerase synthesizes plus-strand DNA from minus-strand DNA templates (for a review see [204]). The HBV polymerase shares significant structural similarity with HIV-1 RT (Fig. 5), and nucleoside inhibitors of the HIV polymerase, such as lamivudine or adefovir, have turned into effective inhibitors of HBV replication both *in vitro* and *in vivo* (for a recent review see [206]). Lamivudine (Fig. 1) has been licensed for clinical treatment of hepatitis B (for a recent review of its therapeutic potential in chronic hepatitis B, see [207]). However, as also occurs in the case of HIV infection, the development of resistance constitutes an important limitation on its therapeutic success.

Resistance to lamivudine usually develops after 6 months of treatment and is associated with mutations in the highly conserved motif YMDD (Fig. 5). As in the case of HIV-1, the methionine residue found in this motif

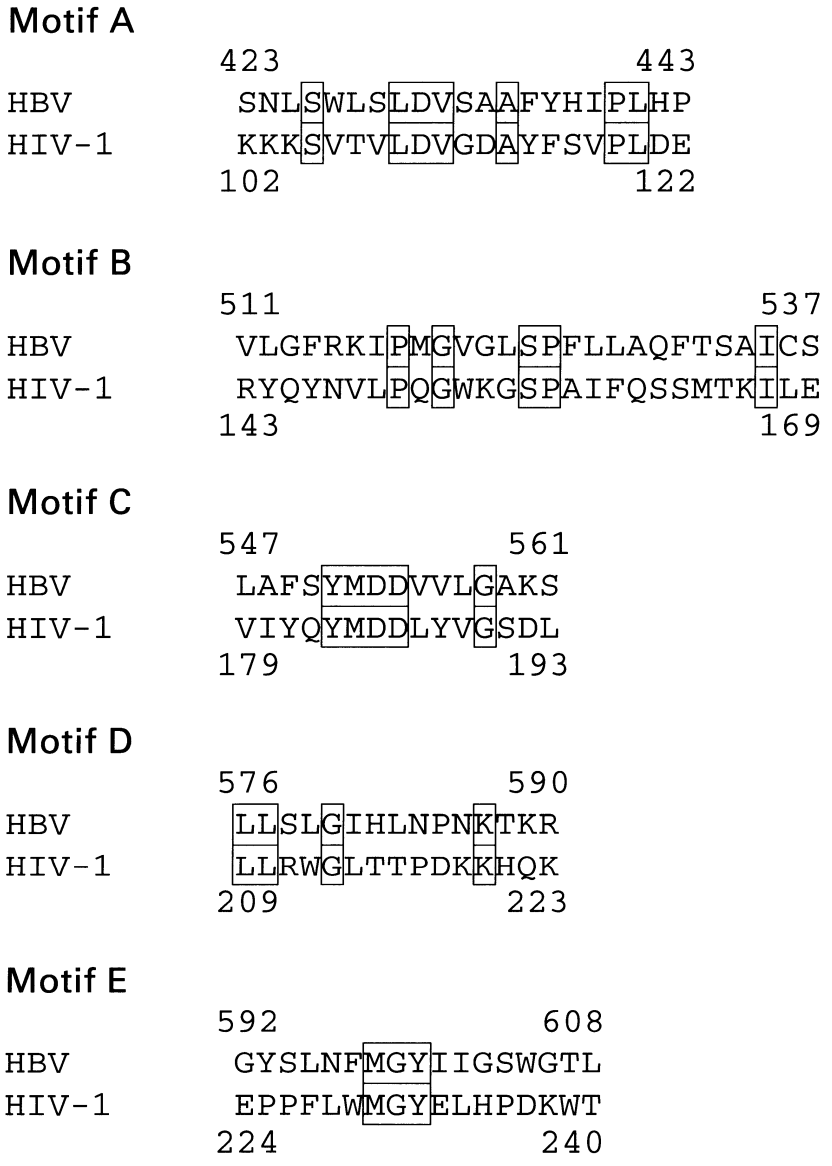


Fig. 5
Alignments of conserved motifs within HBV polymerase and HIV-1 RT. Conserved residues are boxed. Sequences were taken from EMBL/GenBank accession files J02205 (HBV subtype adw2) and M15654 (isolate BH10), and the alignments were based on those given by Poch et al. [205].

is replaced by valine (M552V) or isoleucine (M552I) in lamivudine-resistant clinical isolates [208–211]. The resistance levels shown by isolates carrying the substitution M552I were significantly higher than those observed when the mutation M552V was present [209]. As in the case of HIV-1, the M552V polymerase variant showed reduced ability to synthesize viral DNA, indicating that the viral fitness would be compromised in the lamivudine-resistant mutants [212]. The M552V mutation is found *in vivo* always associated with an additional mutation, L528M, which is located in the conserved region B of HBV polymerase and HIV-1 RT. It has been shown that L528M in combination with M552V confers high-level resistance to lamivudine, although L528M alone has only a moderate impact on resistance [209]. The acquisition of lamivudine resistance appears to be associated with loss of viral fitness [213, 214]. In agreement with this proposal, it has been shown that in patients failing therapy (which occurred after 8 months of the initiation of lamivudine monotherapy), the resistant virus was quickly substituted by the wild-type HBV species after lamivudine withdrawal [215]. However, in some of these patients viral resistance reappeared sooner than with the initial course of treatment [215], suggesting that the resistant virus would persist, either inaccessible to the inhibitor or in the form of minority components of the viral quasi-species, constituting a sort of "molecular memory" ([41]; see also Section 1), that would result in a more efficient response to a selective constraint, such as the introduction of a previously administered drug.

No crystal structure is available for the HBV RT. However, amino acid sequence alignments indicate that the lamivudine-resistance mutations in HBV polymerase are the same as those observed in HIV-1 RT (i.e., M184V or M184I). In HIV-1 RT, the equivalent position of Leu-528 is occupied by Phe-160. This residue is highly conserved in HIV isolates, and non-conservative substitutions at this position lead to the loss of polymerase activity [216]. Phe-160 interacts with Tyr-115, and close to Met-184 [217]. If we assume a similar conformation at the nucleotide binding site of HBV polymerase, Phe-436, Leu-528, and Met-552 would be forming a cluster below the sugar moiety of the incoming nucleotide that would be critical for dNTP binding. Single mutants, such as M552I or M552V, and the double mutant L528M/M552V have shown to confer low-level or no resistance to other nucleoside inhibitors, such as lobucavir or adefovir [218, 219], which are under development to treat HBV infection. On the other hand, resistance to famciclovir (the oral form of penciclovir; Fig. 3) is acquired through the amino acid sub-

stitution L528M, in addition to other mutations whose relevance for drug resistance is still under investigation ([220–222] and references therein).

Mutations in the catalytic domain of the polymerase can also affect the amino acid sequence of the envelope protein (HBsAg) and *vice versa*. In particular, the genetic sequence for the neutralization domain of HBV known as the a determinant, which is located between residues 99 and 169 of HBsAg, overlaps with the major catalytic regions A and B of the polymerase. Although substitutions at motif C are likely to occur in the HbsAg region which is embedded in the lipid envelope [209], the lamivudine- and famciclovir-resistance mutation L528M is a potential neutralization escape mutant (for reviews see [223, 224]).

The estimated rate of evolution for HBV is approximately $< 2 \times 10^{-4}$ base substitutions/site/year [225], which is one to two orders of magnitude lower than the rate of evolution calculated for the positive- and negative-strand RNA viruses, including HIV. However, its half-life (24 h), the total daily production of virus (10^{11} virions), and its high viral load (2×10^{11}), together with the rate of cell death, with a half-life of infected hepatocytes of 10–100 days and a daily turnover of infected hepatocytes of 1–7%, reflect a highly dynamic process with a large production of the virus [226]. This situation parallels what we observed in the case of HIV infection, and suggests that many lessons learnt from drug-resistance management in HIV should also apply to HBV infection. However, emergence of resistance to lamivudine in HBV infection is slower and rarer than in HIV infection. The 10- to 100-day life of HBV-producing cells suggests that the generation time is 5 to 50 times longer in HBV than in HIV, and this could explain the slower adaptive response [227]. In the HIV field it is clear that therapeutic success implies prolonged effective therapy in order to achieve complete suppression of viral replication and, therefore, avoid the emergence of drug resistance. In order to improve the results of lamivudine monotherapy, studies on long-term therapy of combinations of lamivudine with interferon α or with other nucleoside analogues are certainly warranted.

9 Future directions: multiple-target antiviral therapy

Even though drug resistance seems inevitable, the development of new antiviral drugs fighting a resistant virus, as well as exploiting new targets,

appears to be important to design more successful combination therapies, or to introduce treatments that would cope successfully with the genetic variation found in many viruses. For example, in the case of HIV-1, promising new drugs targeting viral entry or fusion and integration are in preclinical or clinical trials (for recent reviews see [228, 229]). The use of additional inhibitors could prolong suppression of viral replication, and should aim at treatments with diminished side-effects as compared with the combination therapies presently available.

One possibility for antiviral intervention that exploits the intrinsic nature of the viral quasi-species is the use of mutagens to increase viral mutation frequency. The mutation rate of many RNA viruses may approach the error threshold for viral population viability. Classical studies showed that chemical mutagenesis can cause a significant increase in mutation frequency in RNA viruses [230]. It has also been reported that chemical mutagenesis or X-ray irradiation of polio-virus-VSV-infected cells yielded a two-fold increase in viral mutation frequency together with a much larger decrease in viral replicative capacity [231]. More recently, Loeb and colleagues [232] showed that the sequential passage of HIV in cells grown in the presence of a mutagenic nucleoside (5-hydroxydeoxycytidine) resulted in the loss of viral replication. Viral extinction with a concomitant increase in the mutation frequency has been also reported for foot-and-mouth disease virus and polio virus, using 5-fluorouracil and ribavirin, respectively, as mutagenic nucleosides [233, 234]. Although the emergence of drug resistance could also be the Achilles's heel for therapies based on lethal mutagenesis, resistance to mutagenic analogues seems less likely to occur. Thus, the number of mutations required for drug resistance is expected to be relatively small in comparison with the number of nucleotide changes that would cause lethality, and in addition, it is unlikely that an immediate selective advantage for resistance to mutagenic nucleotides would arise, since the situation is different from what occurs with chain terminators, where resistance mutations afford an immediate selective growth advantage. Extinction (or lethal) mutagenesis appears as a promising new antiviral strategy, and research to develop this new approach is currently under way in several laboratories.

Acknowledgements

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