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Point Mutations and Antiviral Drug Resistance

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1. Introduction

Viruses are the most abundant biological entities on the planet and their life cycles include the infection of other organisms. Although the presence of viruses is obvious in host organisms that show signs of disease, many healthy organisms are also hosts of non-pathogenic virus infections, where some are active and others are quiescent. It is doubtless that the known viruses represent only a tiny fraction of the viruses on Earth. There is a strong correlation between how intensively a species is studied and the number of viruses found in that species. Our own species is the subject of the most attention, because we have the greatest interest in learning about agents and processes that affect our health. If other species received the same amount of attention, it is likely that many would be found to be hosts to similar numbers of viruses (Breitbart et al., 2005).

Viruses are important agents of many human diseases ranging from the trivial to the lethal, and they play roles in the development of several other types of disease. The history of viral pathogenesis is intertwined with the history of medicine. Humans were clearly aware of viral diseases in ancient times and, since recognizing viruses as disease agents, have taken

on the task of fighting them. This fight entails a need to understand the nature of viruses, how they replicate, and how they cause disease. Having such knowledge would facilitate the development of effective means for prevention, diagnosis, and treatment of viral diseases – medical applications that constitute the main aspects of the science of virology (John et al., 2007).

Currently, the principal strategy for treating viral infections is to use antiviral drugs. For a long time, very few antiviral drugs were available for clinical use compared with the number of anti-bacterial drugs. This was because it was difficult to find compounds that interfere specifically with viral activities without causing significant harm to host cells. However, drugs are now available to treat diseases caused by a variety of viruses and, in some circumstances, to prevent viral infections. The development of these drugs is closely connected with a better understanding of viral life cycles. Chemists are now applying their knowledge of the three-dimensional structure and the molecular function of viral proteins and other structures to the design of molecules that inhibit important functions of viral proteins (De Clercq, 2004; John et al., 2007).

In recent years, the demand for new antiviral strategies has increased markedly. Factors contributing to this growing demand include the ever-increasing prevalence of chronic viral infections and the emergence of new, more infectious viruses (De Clerq, 2004). One of the most dramatic aspects of virology is the emergence of new viral diseases or the re-emergence of viruses with increased pathogenicity (as in the outbreak of swine flu).

In some instances, the emergence of a viral disease represents the original identification of cause of this event (Geisbert et al., 2004). On occasion, a virus that is already widespread in a population can emerge as an endemic or epidemic disease because of an increase in the ratio of infectious cases. Such increases may result from either an increase in host susceptibility or an enhancement in the virulence of a virus. This can lead to large numbers of deaths among human populations, depending on the local social and environmental conditions (Weiss et al., 2004).

When a viral disease becomes pandemic, antiviral drugs are vital in the management of infections (Moscona et al., 2008). However, the emergence of drug-resistant viral strains is of great concern, because these can compromise the effectiveness of treatment, or even lead to its failure. Drug-resistant viruses began to appear soon after antiviral drugs were introduced into clinical practice. This should not have been surprising given that antibiotic-resistant bacteria and insecticide-resistant insects emerged as a result of natural selection. It is now known that viruses can mutate at high frequencies (RNA viruses have a mutation rate estimated at 10^{-4}, i.e., one mutation in 10,000 bases, while DNA viruses have a rate of 10^{-8}) and evolve rapidly, thereby allowing genotypes encoding for drug resistance to arise. Drug-resistant genotypes can be advantageous in hosts where the drug is present and they can become the dominant genotypes in such hosts (Nathanson et al., 2007).

The drug resistance of viruses is relative, rather than absolute. A measure of the degree of resistance can be obtained by determining the IC_{50}. A virus strain is considered to be 'resistant' to a drug if it is able to replicate in the body in the presence of a concentration of the drug that inhibits replication of 'sensitive' strains. Drug-resistant virus isolates are found to have one or more mutations in genes encoding for proteins that are drug targets (John et al., 2007).

Clinical problems arise when drug-resistant virus strains emerge in patients undergoing treatment and when resistant strains are transmitted to other individuals. In such cases, patients may be treated with alternative drugs or a strategy of multidrug therapy can be used, which is the current trend (John et al., 2007; l Nathanson et al., 2007). However, there are fewer therapeutic options when a new drug-resistant viral strain emerges (Tan et al., 2007).

Therefore, there is a great need to continue research programmes aimed at extending the range of drugs available. In particular, it is very important to know and understand the mechanisms by which viral strains can become resistant to existing drugs in order to design new drugs for which the development of resistance will prove more difficult. In this chapter, we focus on the molecular mechanisms involved in the emergence of drug resistance, using different types of virus to describe current knowledge.

2. Influenza virus

Influenza is one of the most prevalent viral diseases, affecting an estimated 10–20% of the world population annually, with 3–5 million cases of severe respiratory illness and up to 500 000 deaths. The aetiological agent is influenza virus (l Nathanson et al., 2007).

Influenza virus belongs to the Orthomyxoviridae family, which is subdivided into three serologically distinct types: A, B, and C. Only influenza viruses A and B appear to be of concern as human pathogens, because influenza C virus does not cause significant disease (Collier et al., 2006).

The virions are 100–200 nm in diameter with a spherical shape. The lipid envelope is covered with projections corresponding to different proteins on the virion surface (Itzstein et al., 2007). These proteins are: haemagglutinin (HA), which is a trimeric protein in the form of a spike; neuraminidase (NA), which is a tetrameric protein with a mushroom-like shape; and the M2 protein, which penetrates the lipid membrane of the virus during replication in the host cells and forms ion channels that allow the entry of protons from the interior of the virus (Figure. 1) (Horimoto et al., 2005).

HA is a glycoprotein with a triangular cross-section, which was first identified based on its ability to agglutinate erythrocytes (hence its name). It is now apparent that it also has important roles in the attachment and entry of the virus into host cells, thereby determining virulence. NA (also called sialidase) removes the neuraminic (sialic) acid from cellular glycoproteins to facilitate viral release and the spread of infection to new cells (Eun-Sun et al., 2011).

The genome of the influenza virus consists of eight discrete fragments of negative single-stranded RNA (approximately 13 kb in size). These fragments form a complex with various proteins (PA, PB1, and PB2) to form a ribonucleoprotein arranged in a helix. The M1 protein forms a shell that provides strength and rigidity to the lipid membrane and it is associated with the NS2 protein. RNA transcriptase, which is found inside the matrix shell, is essential for the transcription of viral RNA to mRNA during replication (Palese et al., 2004).

Influenza A viruses are designated based on the antigenic relationships of their external HA and NA proteins, i.e., they classified based on antibodies to H1–H16 and N1–N9. Only viruses with H1, H2, H3, N1, and N2 are known to infect humans or cause serious disease

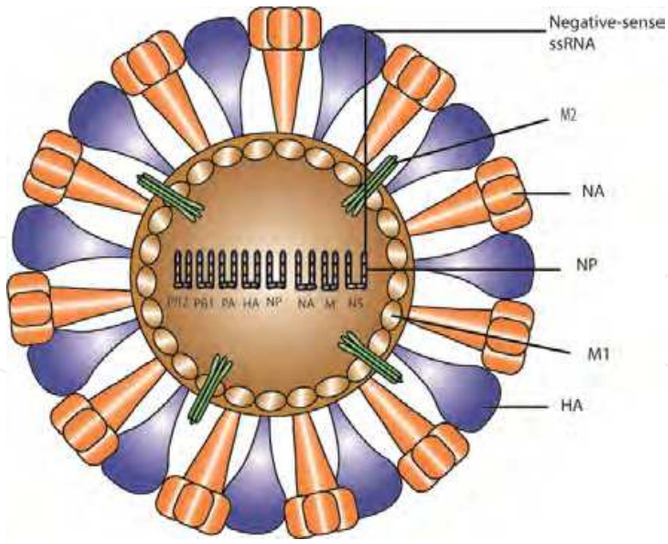

Fig. 1. Schematic diagram of influenza A virus. Two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), together with the M2 ion-channel protein, are embedded in the viral envelope, which is derived from the host plasma membrane. The ribonucleoprotein complex comprises a viral RNA segment associated with the nucleoprotein (NP) and three polymerase proteins (PA, PB1 and PB2). The matrix (M1) protein is associated with both ribonucleoprotein and the viral envelope. A small amount of non-structural protein 2 is also presen. (Modified from: Horimoto T, et. al. *Nature Rev. Microbiol.* 2005. 3: 591-600, reproduced with permission of the author)

outbreaks (Collier et al., 2006). Although influenza B viruses cause the same types of disease as influenza A, they do not cause pandemics, because the only hosts are humans and seals (Eun-Sun et al., 2011).

With at least 16 different HA and nine different NA subtypes, there is considerable antigenic variation among influenza viruses (Palese et al., 2004). This variation occurs because RNA viruses (e.g., influenza viruses) are extremely mutable and possess highly efficient strategies for generating viral diversity during evolution. RNA viruses have few or no proofreading mechanisms and many mutations are introduced during replication. Thus, RNA viruses exist as quasispecies, where viruses possess slightly different genetic compositions. Unfortunately, the evolution of viruses is not simply confined to point mutations inserted during replication. Viruses can also undergo leaps in evolution through the processes of recombination and reassortment. These processes produce population heterogeneity in viruses through the acquisition of large sections of genomic material from other viruses (Figure. 2) (Webby et al., 2004).

These same mechanisms of evolution allow viruses to develop resistance to drugs. This is the case with resistance to oseltamivir (Tamiflu®), where mutations confer resistance (mutations in NA) and there might be possible exchange of genetic information between resistant and susceptible viral strains (Janies et al., 2010).

As mentioned earlier, a pandemic can arise because of either an increase in host susceptibility or the enhancement of viral virulence, and antiviral drugs are vital in the

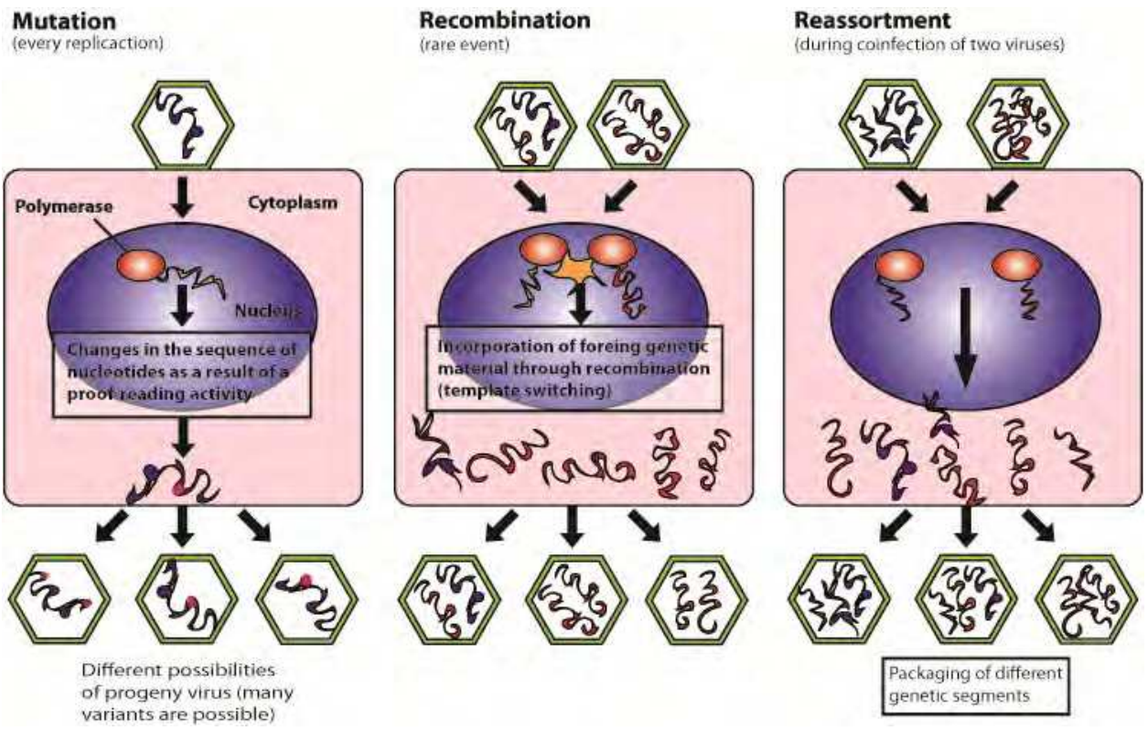

Fig. 2. Molecular mechanisms for generating viral diversity. The viruses have three main mechanisms for generating diversity on replication. (a) Mutation: during replication, single point mutations are incorporated into one or more genomic positions as a result of a lack of proof-reading activity of the viral polymerase. (b) Recombination: foreign genetic material is incorporated into the viral genome through mechanisms such as template switching during replication. (c) Reassortment: occurs on dual infection of a cell with segmented genome viruses, whole gene segments can be swapped. Any of the three mechanisms (which are not exclusive), may result in viruses that have new biological properties, such as new host range and pathogenic potential. (Modified from: Webby R., et. al. *Nature Med. Suppl*. 2005. 10: S77-S81, reproduced with permission of the author)

management of infection when a viral disease becomes pandemic. Therefore, it is important to combat the emergence of drug resistance in seasonal strains (normal) and pandemic strains (Janies et al., 2010).

Antiviral therapeutics can be divided into the following categories: drugs directed against the virus itself (either its genome or its proteins); drugs directed against host cell proteins that are critical for the replication of individual viruses; and therapeutics that mimic or enhance host defence mechanisms (Neal Nathanson et al., 2007). Two classes of antiviral agents are currently in use for the control of influenza infections: M2 ion channel blockers (directed against host cells) and NAIs (antivirus) (Fig. 3) (Palese et al., 2004; Okomo-Adhiambo et al., 2010).

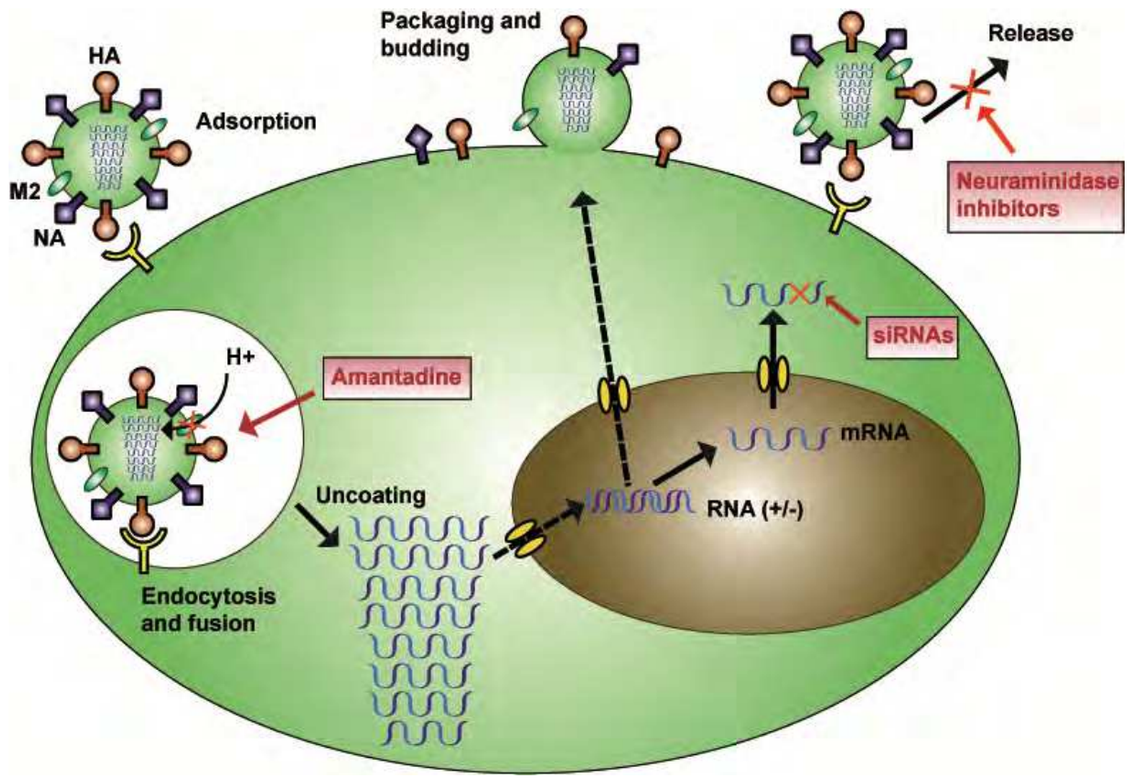

Fig. 3. Inhibition of influenza virus replication cycle by antivirals. After binding to sialic acid receptors, the virus is internalized by receptor-mediated endocytosis. The low pH in the endosome triggers the fusion of viral and endosomal membranes and the influx of H+ ions through the M2 channel releases the viral genes into the cytoplasm. Amantadine blocks this uncoating step. RNA replication and transcription occur in the nucleus. siRNA inhibition may affect the stability of mRNA, preventing translation of viral protein. Packaging and budding of virions occurs at the cytoplasmic membrane. Neuraminidase inhibitors block the release of the virus from the infected cell. Because sialic acid receptors are not removed by the neuraminidase, aggregates of virus stick to the cytoplasmic membrane of the infected cell and cannot move on to infect other cells. (Modified from: Peter P. et al. *Nature Med*. 2004. Supp. 10: S82-S87)

The M2 blockers (amantadine and rimantadine) are effective against influenza A viruses, but not influenza B viruses. However, their effectiveness has been compromised by drug-resistant mutants that are frequently isolated from patients within a few days of therapy (Okomo-Adhiambo et al., 2010).

2.1 Antivirals used to the treatment of influenza

Oseltamivir and zanamivir are neuraminidase inhibitors (NAIs) that are commonly used against type A and type B influenza infections (Fig. 4). Oseltamivir is administered orally, whereas zanamivir is inhaled. Another drug of this type is peramivir, which is currently

being studied. NAIs competitively bind to the highly conserved NA active site by mimicking sialic acid (N-acetylneuraminic acid), which is the natural substrate of NA. This inhibits the enzyme's key function by destroying neuraminic acid-containing receptors, which prevents the release of progeny virions from infected cells and any possible dissemination to neighbouring cells (Neal Nathanson et al., 2007).

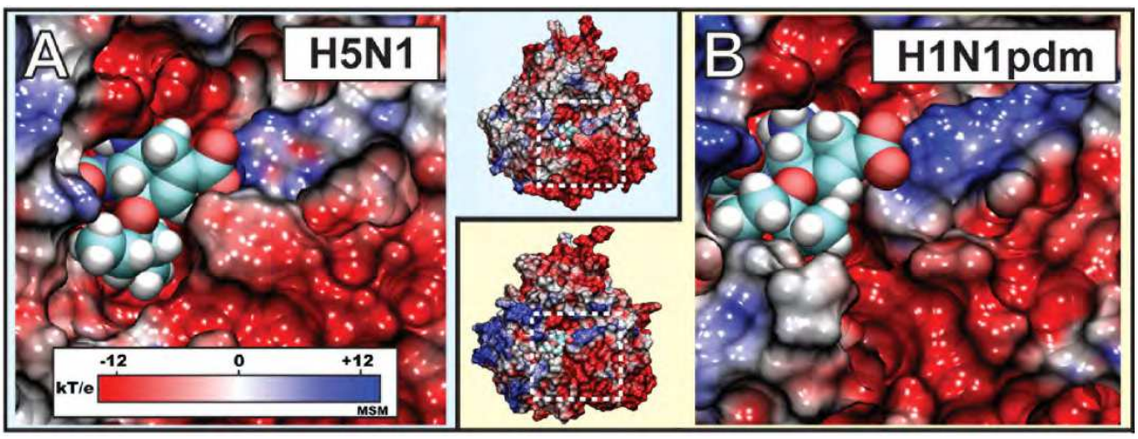

Fig. 4. Electrostatic surface potential of the sialic acid (SA) binding pocket of H1N1pdm and oseltamivir. Shown in A) and B) are closeup views of the SA binding pocket with drug bound H1N1pdm and avian H5N1 neuraminidase, respectively. The region of the binding pocket, where the drug binds, exhibits a negative potential (colored red), whereas the opening of the pocket is surrounded by a highly positive potential ring (colored blue). (Reproduced with permission of the author).

The highly conserved NA enzyme active site is comprised of catalytic amino acid residues that directly interact with the substrate (R118, D151, R152, R224, E276, R292, R371, and Y406) and framework (E119, R156, W178, S179, D198N, I222, E227, H274, E277, N294, and E425) residues that support the catalytic residues (Ferraris et al., 2008; Le et al., 2010).

The influenza A H1N1 virus can develop resistance to oseltamivir because of a point mutation at one of several sites in the NA protein (e.g., D79G, S247G, N294S, or H274Y). Resistance to zanamivir (Relenza®) by the influenza A H1N1 virus can occur because of the NA point mutations H126N or Q136K (Janies et al., 2010).

2.2 Antiviral resistance

Resistance to oseltamivir in pandemic influenza strains can appear in various forms: 1) sporadic evolution in an infected patient in response to treatment; 2) evolution of resistance to oseltamivir in an infected patient and transfer of the strain among personal contacts; 3) maintenance of a genotype that confers resistance to oseltamivir in a viral lineage due to selection pressure; and 4) a reassortment event between oseltamivir-resistant seasonal H1N1 and a pandemic strain (Janies et al., 2010).

The frequency of resistance to oseltamivir was previously low, except in hospitalized children and immunocompromised patients. However, during the 2007–2008 and 2008–2009 influenza seasons, the emergence and transmission of oseltamivir-resistant seasonal

influenza A (H1N1) viruses with an H274Y mutation were detected globally in untreated individuals, which emphasized the need for close monitoring of oseltamivir resistance. Mutations at catalytic (R292K) and framework (E119V and N294S) NA residues have been detected in the N2 viral subtype in oseltamivir-treated patients. A four-amino-acid deletion mutation (deletion of residues 245 to 248) was reported to confer oseltamivir resistance in an influenza A (H3N2) virus isolated from an immunocompromised patient treated with the drug (Okomo-Adhiambo et al., 2010). This shows that the clinical use of oseltamivir is associated with the emergence of drug resistance resulting from subtype-specific NA mutations (Janies et al., 2010; Okomo-Adhiambo et al., 2010).

Zanamivir-resistant mutants are less common than oseltamivir-resistant mutants, partly because of differences between how the two drugs bind to the NA active site and possibly because of lower frequency of the prescription and use of zanamivir (Okomo-Adhiambo et al., 2010).

Variant strains with an advantage are quickly amplified when selective conditions are present, such as the conditions after jumping to a new host, and they become the dominant strain by selective pressure, e.g., when a host is treated with a drug that the strain can resist (Webby et al., 2004).

Several mutations in seasonal or pandemic strains confer resistance to oseltamivir, which is currently the most widely used drug. These mutations are found mainly in the framework of the enzyme and they appear to destabilize drug binding to the target enzyme, thereby reducing viral susceptibility to the treatment (Itzstein et al., 2007; Janies et al., 2010; Okomo-Adhiambo et al., 2010).

Mutants in E119 (influenza A H3N2) are known to emerge after oseltamivir treatment. The E119V variant was resistant to oseltamivir, but it did not exhibit reduced susceptibility to peramivir. The E119I variant was resistant to oseltamivir, but it also showed decreased susceptibility to zanamivir, peramivir, and A-315675 (another NAI under study) (Okomo-Adhiambo et al., 2010). Mutants in E119 (influenza A H3N2) were also reported to disrupt E276-R224 salt bridges that accommodate the hydrophobic pentyl group of oseltamivir, although further studies are required to confirm this (Wang et al., 2009). Another very important mutation is H274Y (influenza H5N1 and H1N1), which is a mutation in the framework of the NA.

Despite advances in our understanding of some viruses, we generally know very little about the specific molecular changes that allow many viruses to overcome known barriers (Webby et al., 2004). Therefore, new studies are investigating the molecular mechanisms that allow a virus to become drug resistant. One example is a computer simulation study of the union and disunion of oseltamivir with NA, and the effects that might be caused by known mutations (Le et al., 2010).

These studies rely on the recent elucidation of crystal structures of both wild-type and mutant H5N1 NAs, which have opened the way for the investigation of drug resistance mechanisms and structure-based drug design at the atomic level. These crystal structures represent a frozen-in-time snapshot of a possible conformation during drug–protein interaction. However, drug binding is a dynamic process and computational studies using

crystal structures as starting points can shed light on how protein flexibility and point mutations influence drug–protein endpoint interactions (Le et al., 2010).

The major finding with this approach is the discovery that the union of oseltamivir to the NA occurs through charged groups on the protein surface. These interactions through a charged pathway have proved to be very important in the process of interaction between oseltamivir and the NA, because they facilitate binding or stabilize it. It is now known that many mutations prevent or weaken key interactions that allow the union or stabilization between oseltamivir and the NA enzyme (Fig. 5) (Itstein, 2007; Le et al., 2010; Eun-Sun et al., 2011).

This finding opens up a whole new landscape for drug design. Chance and virological screening will continue to play a part in the discovery of new antivirals, but knowledge of viral gene structure and protein functioning is leading to a new generation of drugs (Collier et al., 2006). The insights gained so far should assist in the rational design of NAIs and other types of drugs, while avoiding drug resistance (Le et al., 2010).

Given the results of simulations of the interaction between oseltamivir and the NA enzyme, the next generation of drugs for the treatment of influenza will probably consist of inhibitors with positively charged groups, because these compounds have potential as antiviral therapeutics, although the strain specificity of these inhibitors must be resolved (Eun-Sun et al., 2011).

Alternative drug discovery targets, such as RNA polymerase, HA, or the M2 ion channel protein, which are essential components of the viral life cycle, are also under investigation. This research may lead to a combination therapy approach or the use of sialidase inhibitors alone, to provide new classes of anti-influenza drugs. Combination therapy might also reduce the potential of resistance development (Izstein, 2007).

It is also very important to limit the prescription of antivirals in order to reduce the possibility of the emergence of drug-resistant strains and thus maintain the ability of antivirals to treat high-risk patients (Le et al., 2010).

Finally, a precise diagnosis of influenza A is very important, including the identification of resistant strains and point-of-care pathogen genotyping, because this information can help to identify the appropriate antiviral in each situation. Some studies have indicated that genomics technology, bioinformatics, and geographical information systems can be immediately applicable to help in the treatment of infectious diseases. In addition to data collection, analyses are useful for turning raw data into prospective public health intelligence on drug resistance in a specific region, and into a form that provides easy visualization (Janies et al., 2010).

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3. HIV drug-resistance related point mutations

3.1 Introduction

The advent of highly active antiretroviral therapy [HAART] has changed the natural history of human immunodeficiency virus infection [HIV], extending the survival of carrier subjects and reducing progression to human immunodeficiency syndrome. The success in the combination of antiretroviral treatments to suppress viral replication depends on several factors, including the host, the virus and medications. Nowadays, therapeutic options against HIV-1 include more than 20 drugs, classified according to their action mechanism and targeted to four different points of the viral replication cycle: the entry of the virus into the cell, inverse transcription, the integration of viral genetic material into the cell nucleus, and maturation of virions [Fig. 5] (Altman et al., 2007). Since its introduction, ARV therapy showed marginal and short-duration benefits when drug combinations did not achieve a satisfactory control of viral replication. This phenomenon has been associated with the high replicative capacity of the virus and the high error rate in the transcription of its genetic material, but also with a Darwinian phenomenon of quasispecies selection and accumulation exhibiting resistance due to the presence of specific mutations resulting from pharmacological pressure and suboptimal viral suppression under a treatment scheme (Johnson et al, 2010). That is to say, both the preexistence and selection of resistance mutations are important failure predictors for an ARV therapy (Hatano, 2006; Perno, 2002; Poveda, 2010; Shafer, 2006).

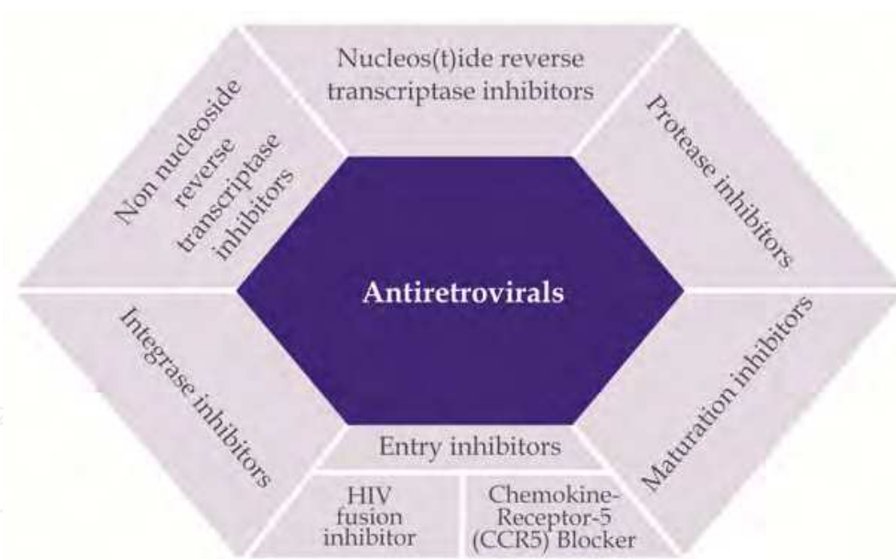


Fig. 5. Antirretroviral drug classes

Retroviruses use the reverse transcription of viral RNA into linear double-stranded DNA, with subsequent integration into the host genome. The characteristic enzyme used for this process is known as reverse transcriptase (Arnold & Sarafianos, 2008). This enzyme is error-prone; with the massive turnover of virions in the infected host, these errors accumulate in the viral DNA, accounting for the relatively high mutability of HIV-1. Retroviruses have the survival advantage of great genetic diversity, and latency, because the DNA provirus is integrated into the chromosomal DNA of the infected cell (Reitz & Gallo, 2009).

3.2 HIV biology and resistance point mutations

Pathogenic human retroviruses include lentiviruses [HIV-1 and -2] and oncoviruses [HTLV-I and –II]. Current knowledge places retroviral infection of humans as zoonoses that originated in primate-to-human species-jumping events. For HIV-1 and HIV-2, these events occurred in Central and West Africa. All retroviral genomes consist of at least 4 *genes: gag, pro, pol and env*. The *gag* gene encodes the major structural polyprotein Gag. The viral protease is encoded by the *pro* gene and is responsible for facilitating the maturation of viral particles. Products of the *pol* gene include reverse transcriptase, RNase H and integrase, while *env* is responsible for the viral surface glycoprotein and transmembrane proteins that mediate cellular receptor binding and membrane fusion. In addition, complex retroviruses such as HIV-1 encode accessory proteins that enhance replication and infectivity (Reitz & Gallo, 2009).

Resistance to ARV therapy implies the selection and accumulation of mutations in one or more HIV genes [mainly *gag* and *pol*], which reduce the antiviral activity of one or more ARV drugs. It can be "primary" when occurring in individuals not previously and directly exposed to these drugs and whose transmission of resistant strains is presumed or "secondary" when there is history of previous exposure. Interestingly, the frequency in the selection and transmission of mutations conferring resistance can also vary according to the adaptive and reproductive capacity preserved by the mutant virus in a given environment [viral fitness], and to the greater or lower ability of the drug to retain its antiviral activity despite the presence of specific resistance mutations [genetic barrier] (De Luca, 2006).Table 1 shows the mutations suggested for the monitoring of resistance transmitted to the HIV by the World Health Organization [WHO] for the three primary classes of antiretroviral treatment (Bennett et al, 2009).

NRTI	**M41L, K65R, D67N/G/E, T69D/Ins, K70R/E, L74V/I, V75M/T/A/S, F77L, Y115F, F116Y, Q151M, M184V/I, L210W, T215Y/F/I/S/C/D/V/E, K219Q/E/N/R**
NNRTI	L100I, K101E/P, K103N/S, V106M/A, V179F, Y181C/I/V, Y188L/H/C, G190A/S/E, P225H, M230L
PI	L23I, L24I, D30N, V32I, M46I/L, I47V/A, G48V/M, I50V/L, F53L/Y, I54V/L/M/A/T/S, G73S/T/C/A, L76V, V82A/T/F/S/C/M/L, N83D, I84V/A/C, I85V, N88D/S, L90M

Table 1. List of mutations for surveillance of transmitted drug resistant HIV, World Health Organization 2009.NRTI: nucleotide reverse transcriptase inhibitors, NNRTI: non-nucleotide reverse transcriptase inhibitors, PI: protease inhibitors

3.3 Impact of resistance on clinical response to antiretroviral treatment

The main purpose of antiretroviral therapy is to achieve a sustained control of HIV replication. Virological failure is defined as the inability to achieve or maintain the viral replication suppression at levels below 200 copies/mL.

An incomplete virological response refers to the presence of two consecutive readings of HIV RNA higher than 200 copies/mL in plasma, after 24 weeks on continuous ARV therapy; and viral rebound is a detectable viral load [VL] higher than 200 copies/mL after achieving viral suppression (Department of Health and Human Services [DHHS], 2011). Usually, once the ARV therapy is initiated and constantly administered, the HIV VL in

plasma is quantitatively reduced in two phases, a fast initial phase during the first weeks, and a slower late phase that can extend up to 6 months. There are multiple factors involved in the viral response to the ARV therapy, such as evolutionary issues of the virus (Esté & Telenti, 2009; Parkin et al, 2005; Rivas et al, 2006), host immunology (Gragsted et al, 2004; Telenti & Goldstein, 2006), compliance of the individual with the treatment (Muller et al, 2011; Protopopescu et al, 2009), pharmacogenetics (Clifford et al, 2009; Telenti et al, 2002), pharmacologic interactions (Zhu et al, 2001), and the occurrence of comorbidities. Table 2 shows examples for the abovementioned issues.

Item	Example
Virus	- In some non-B strains, the accumulation of certain polymorphisms and secondary mutations in the enzyme protease has been associated with a reduction in susceptibility in vitro to protease inhibitors, even in absence of primary mutations. - Primary mutations associated with resistance are the same regardless the viral group and subtype. However, some clearly appear more frequently in some groups or subtypes than in others.
Host immunity	- The adequate response to antiretroviral treatment is inversely proportional to the number of basal CD4+ cells.
Adherence to treatment	- The satisfactory virological suppression depends on the adherence to the antiretroviral treatment. On the other hand, social factors such as stigmatization, the fear of knowing the diagnosis and the attitude of health providers to the patient can also affect response to treatment.
Pharmacogenetics	- Study ACTG 5097s found that clearance of efavirenz from the body was increased by 32% in non-hispanic whites compared to blacks and Hispanics. There was a slight association between higher blood levels of efavirenz and study discontinuations. In a subsequent study, the authors found that a single nucleotide polymorphism (SNP) that changed the DNA code from a “G” to a “T” at position 516 of the gene for the CYP2B6 metabolic enzyme was associated with slower clearance of efavirenz, higher blood levels of the drug and more CNS-related side effects.
Pharmacologic interactions	- The pharmacologic interaction between antiretroviral drugs and other drugs can cause a reduction in the minimum and maximum levels, as well as the area under the curve of one or more antiretroviral agents. This can be associated with the exposure to suboptimal doses of these agents and treatment failures (atazanavir-omeprazole, protease inhibitors-rifamycins). - Additionally, the pharmacologic interaction between certain drugs can also increase the level of co-administered agents generating adverse effects to such drugs (protease inhibitors-phosphodiesterase inhibitors, protease inhibitors-statins).
Comorbidity	- The association between the HIV and other pathologies is capable to deteriorate the response to treatment (e.g. absorption issues at intestinal level), or to increase adverse effects of the antiretroviral agent. This is the case of co-infection with the Hepatitis B and C viruses.

Table 2. Determinants of response to antiretroviral treatment.

Blips are transient episodes of low-level viremia [from 51 a 1,000 copies/mL]. Some of their causes are: sample processing artefacts due to the use of collection tubes with PPT™ versus ethylenediaminetetraacetic acid [EDTA], frequency of 70% and 5.4%, respectively (Lee et al, 2006); immunizations, acute infections or viral release from cell compartments known as "reservoirs". A recent report determined that annual frequency of a viral rebound was 85%, two events in 13% and three or more in 1.9%, defined as a VL detectable between 50 and 400 copies/mL in previously undetectable in individuals. The persistent rebounds (identified in two or more readings) behaved as an independent risk factor increasing from 1.4 to 2.18 times the relative risk of viral failure compared to individuals with transient viremia [blips] (Geretti et al, 2008).

On the other hand, it has been found that the incidence of persistent low-level viremia is more frequent than high-level viremia when evaluating the virological response in subjects with HIV infection and HAART, reporting incidences of 29% and 6.7% for readings > 50 copies/mL and > 1,000 copies/mL, respectively (Van Sighem et al, 2008). Likewise, the UK-CHIC study reported that the cumulative risk for resistance mutation detection for two of the three primary classes of ARV therapy within a population increases in time, passing from 6% to 14% and 20% after a follow-up period of 2, 4, and 6 years for each period (Phillips et al, 2005).

3.4 Resistance assessment

There are, at least, four types of assays to identify the resistance or viral susceptibility to drugs available in clinical practice: genotype, actual phenotype, virtual phenotype, and viral tropism tests.

3.4.1 Genotype

Standard genotypic assays are primarily based on the amplification of the inverse transcriptase and protease genes in order to identify resistance mutations already known for their ability to reduce viral susceptibility to certain drugs. Generally, these studies only provide data on the substitutions associated with resistance to NRTI, NNRTI and protease inhibitors [PI]. Usually, these substitutions reduce the susceptibility to the drug compared with that of the wild-type viral strain through changes in the molecular target of the therapy or in other viral proteins that indirectly interfere with the drug activity. There are also resistance detection tests for fusion inhibitors [FI] and INI, but their access is more limited and they are not performed routinely (Fransenet al, 2009; Long et al, 2009; Perno & Mertoli, 2006).

Genotype is indicated in the following situations:

- Confirmed viral failure and VL > 1,000 copies/mL [considered in individuals with VL > 500 but < 1,000 copies/mL if there are available special techniques to easy ultrasensitive amplification].
- Suboptimal response to treatment [e.g. fall of HIV VL < 1 $\log^{10}$ during the first four weeks of therapy].
- ARV therapy naive subjects in whom transmitted resistance is suspected.

- In populations with prevalence of transmitted resistance > 5% in chronically ill individuals, its execution is recommended on all the cases at diagnosis, and its repetition before initiating the HAART when the decision is to change the treatment (Hirsch et al, 2003).

The search of resistance mutations for FI and INI must be performed in subjects with virological failure to these drugs. However, in the absence of an adequate compliance, the lack of pharmacologic pressure reduces the possibility of identifying situations associated with resistance [due to the predominance of the wild-type strain]. This is more frequent in individuals with increased HIV VLs [more than 100,000 copies/mL], notwithstanding the type of ARV scheme administered (Geretti et al, 2008).

On the other hand, ultrasensitive genotypic tests have a very high probability to identify resistance mutations [even in individuals with VL < 500 copies/mL], although their clinical utility has not been reliably demonstrated. This is explained because at present it is considered that the resistant mutant viral strain must represent at least 2-3% of the existing population in order to have an impact on the response to a given drug, and the diagnostic sensitivity of such tests is higher than this cut-off [up to 0.1%] (Mackie et al, 2004; Villahermosa et al, 2000). There are two commercial methods approved by the Food and Drug Administration [FDA] and the European CE Notified Bodies: the TruGene HIV-1 genotypic assay and the ViroSeq assay. For both, the sequence is determined by amplifying the interest genes using the reverse transcriptase-polymerase chain reaction [RT-PCR] with primers in conserved regions capable to align most M strains of HIV-1. The mutations that have been associated with a reduction in the antiretroviral susceptibility as of today are included in Table 3.

3.4.2 Phenotype

This test measures the ability of the virus to multiply under different ARV concentrations in comparison with a wild-type reference strain. It is based in the HIV-1 isolation in plasma or serum mononuclear cells, and the measurement of its susceptibility to ARV drugs *in vitro*. This is performed through the insertion of genetic sequences of reverse transcriptase and protease obtained from the HIV under study [recently also from integrase and viral envelope], which are inserted into a laboratory viral clone. Thus, the recombinant HIV carries the genetic characteristics of the test virus, regarding the genes involved in resistance expression. The viral strain is effectively replicated *in vitro* and exposed to different ARV drug concentrations. That is how the mean inhibitory concentration [IC50] is calculated, which represents the drug concentration capable of limiting the viral replication by 50%. The ratio difference between IC50 of the HIV under study and the reference strain is reported as the change in the IC50 ratio [fold-change] and the significance is determined according to clinical or biological cut-off values (Perno & Mertoli, 2006).

In a few words, the fold-change represents the similarity or loss of partial or total susceptibility of the test strain versus the reference strain. It is important, however, to point out that there are multiple technical difficulties for these procedures and that the integrated proviruses are not necessarily representative of the circulating virus, but they can constitute stored viruses or residual, free virions in plasma.

	Antiretroviral	Mutations potentially associated with high-level resistance	Other significant mutations
Nucleotide reverse transcriptase inhibitors	Abacavir	K65R, L74V	Y115F, M184V, TAMs, Q151M complex
	Didanosine	K65R, L74V	TAMs, Q151M complex
	Emtricitabine, Lamivudine	M184V	K65R, Q151M complex
	Stavudine	M41L, K65R, D67N, L210W, T215 Y/F, K219Q/E	Q151M complex
	Tenofovir	K65R	K70E, TAMs
	Zidovudine	M41L, D67N, K70R, L210W, T215Y/F, K219Q/E	Q151M complex
Non-nucleotide reverse transcriptase inhibitors	Efavirenz	L100I, K103N/S/T, V106A/M, Y181C/I/V/S, Y188L, G190A/C/E/Q/S/V/T, P225H, M230L	K103I/P, V108I, V179F, Y181S, Y188C/H, F227C
	Nevirapine	L100I, K103N/S/T, V106A/M, V179F, Y181C/I/V/S, Y188C/L, G190A/C/E/Q/S/V/T, F227C, M230L, K238T/N, Y318F	K101E/P, V108I, Y188H
	Etravirine	Y181I/V, E138K	V90I, A98G, L100I, K101E/H/P, V106I, E138A, V179D/F/T, Y181C/I/V, G190S/A, M230L
Protease inhibitors	Atazanavir	I50L, G73S/T, I84A/C/V, N88S, L90M	G48V/M, F53L, I54A/L/M/T/V, V82A/F/S/T, N88D
	Darunavir	I50V/I	V11I, V32I, L33F, I47A/V, I54L/M, G73S/T, L76V, V82A/F/S/T,I84A/C/V, L90M
	Fosamprenavir	V32I, I47A/V, I50V, I54L/M, L76V, I84A/C/V, L90M	L24I, L33F, M46I/L, I54L/T/V, G73ST, V82A/F/S/T
	Indinavir	V82A/F/S/T, I84A/C/V	L24I, V32I, M46I/L, I47V, F53L, I54A/L/M/T/V, G73S/T, L76V, N88S, L90M
	Lopinavir	I47A	L10F/I/R/V, K20M/N/R,L24I, V32I, L33F, M36I, M46I/L, I47V,G48M/V, I50V, I54A/L/M/T/V, L76V, V82A/F/S/T, I84A/C/V, L90M
	Nelfinavir	L23I, D30N, M46I/L, G48M/V, I84A/C/V,	L24I, L33F, I47/V, F53L, I54A/L/M/T/V, G73S/T,

	Antiretroviral	Mutations potentially associated with high-level resistance	Other significant mutations
		N88D/S, L90M	V82A/F/S/T
	Saquinavir	G48M/V, L90M	L24I, F53L, I54A/L/M/T/V, G73S/T, V82A/T, I84A/C/V, N88S
	Tipranavir	V82L/T, N83D	L10V,V32I, L33F, M36I/L/V,K43T,M46I/L, I47V, I54A/M/V, Q58E, T74P, V82A/F/S, I84A/C/V, L89I/M/V, L90M
Fusion inhibitors	Enfuvirtide	G36D/S, I37V, V38A/M/E, Q39R, Q40H, N42T, N43D	
Integrase inhibitors	Raltegravir	Q148R	E92Q, E138A/K,G140A/S,Y143R/H/C, Q148H/K, N155H

Table 3. Overview of drug resistance mutations in HIV-1. TAMs: Thymidine analog mutations (M41L, D67N, K70R, L210W, T215Y/F and K219Q/E). Q151M complex: A62V, V75I, F77L, F116Y and Q151M.

Phenotype is considered as supplementary to genotype, and it is reserved for cases where a complex mutation pattern is suspected, in which resistance-associated mutations and hypersusceptibility have been selected, and that specially involves multiple PI o more than two classes of ARV drugs. Virco and Monogram laboratories currently offer the technology to develop commercial phenotypes.

3.4.3 Virtual phenotype

Virtual phenotype combines the knowledge generated by both resistance assays: genotype and phenotype. In the virtual phenotype, the investigational virus is sequenced and the identified changes are compared with the information of a great database that contains genotype-phenotype paired data for viruses previously sequenced. That is how the fold-change of a HIV under study is inferred, calculated based on the mean of all the results of the actual phenotype obtained for virus existing in the database and with genotypic characteristics similar to those of the test virus. Generally, concordance of results obtained for NNRTIs and PIs is very good, although that observed with NRTIs is very lower. This is laid to a poor representation of certain genotypes in the system and it is expected that this issue be corrected (Mazzotta et al, 2003; Torti et al, 2003; Loutfy et al, 2004). Virtual phenotype shares the same advantages and disadvantages as that the genotype. The indications, advantages and disadvantages of the viral genotype and phenotype are included in Table 4.

3.4.4 Tropism test

The assay for the identification of the type of tropism to co-receptors that the HIV exhibits [CCR5 or CXCR4] must be performed when considering the initiation of a co-receptor antagonist and there is viral replication. The purpose of these tests is to identify the receptor

molecules CCR5 and CXCR4 in the host cell membranes. Phenotypic and, to a lesser extent, genotypic studies have been developed to determine co-receptors of the dominant viral population in each case.

Viruses can be monotropic [CCR5 or CXCR4], or can exhibit a mixed tropism or D/M dual tropism. Prevalence of tropic CCR5 viruses in heavily treated individuals or with CD4 < 100 cells/mm^3 has been reported of 50% (Hunt et al, 2006; Wilkin, 2007). Currently, Trofile [Monogram Biosciences, Inc.], Phenoscript assay, [VIRalliance], Xtrack C/Phen X-R [inPheno] and Virco, provide phenotypic tropism assays.

This study is expensive and requires an average time of 3-4 weeks to have the results. Detection of minority variants is the primary limitation and varies from 1% to 10%, depending on the assay used. Likewise, it is difficult to establish the cut-off points for the minority species X4, and this is affected by multiple variables such as sample quality, collected volume, HIV-1 VL, detection limits and and PCR variations (Braun & Wiesmann, 2007).

3.4.5 Genotropism test

Several bioinformatic tools have been proposed to predict co-receptor usage by interpretation of genotypic data—mainly through the use of V3 loop sequencing. Nevertheless, there are important differences between algorithms in detection sensitivity of X4 isolates. In one report, the most sensitive bioinformatic tools were PSSM and Geno2pheno, with sensitivities of about 60%. Moreover, more studies will be needed to further the study on the prediction of co-receptor use in HIV-1 non-B subtypes and throughout different stages of the HIV infection (Recordon-Pinson et al, 2010; Coakley et al, 2011).

Changes on the treatment supported in resistance assays have a greater potential impact on the virological response, especially when an expert in the interpretation of the results is available (Palella et al, 2009; Sax et al, 2005; Tural et al, 2002).

3.4.6 Integration of the rescue scheme

There is a high risk that every new rescue may offer less possibilities to maintain a long-term virological control as a result of stored mutations underestimated when implementing the rescue treatment. Currently, there are interpretation algorithms facilitating the physician to select the ARV therapy, to predict the activity of a drug or its potential virological response. There are computing platforms that function through different methodological rules and which are populated from the results of clinical studies or *in vitro* assays concentrated in big databases. Some of the best known are the ANRS HIV-1 genotypic drug resistance interpretation algorithms and the Stanford HIV Drug Resistance database [both with on-line free access].

The selection of point mutations in HIV infection is a complex event, frequency and causes of which vary notoriously depending on different factors. Some of such factors are associated to the socioeconomic characteristics of the studied population, the antiretroviral drugs used, prevalent viral groups and subtypes, etc. These mutations have a great relevance in HIV infection because of their negative impact on the response to antiretroviral treatment, which induces a suboptimal or transient viral suppression, immune deterioration and clinical progression to the acquired immune deficiency syndrome.

	Genotype	Phenotype
Advantages	- It is the most inexpensive resistance assay. - Detects changes in the viral genome associated with resistance faster than the phenotypic assay. - Technically less complex. - Results are obtained in 1-2 weeks. - Preferred assay to guide rescue strategies in naïve individuals, with suboptimal response, or after the first and second HAART failure. - The occurrence of mutations may precede to the development of phenotypic resistance.	- Higher sensitivity to identify viral susceptibility to PIs. - Direct and quantitative measurement of viral susceptibility. - It is capable to measure the accumulated impact of different viral mutations (resistance and hypersusceptibility mutations).
Disadvantages	- Qualitative result. - Low sensitivity to detect resistance mutations in patients with viral replication lower than 1000 copies/mL. - Indirect measurement of resistance. - Incapable to quantify the interaction between mutations. - It cannot be used to detect the residual activity of some drugs. - Its capacity to detect resistance mutations falls drastically in absence of pharmacologic pressure; it has to be performed preferably before discontinuing the HAART and not beyond the firsts 4 weeks after its discontinuation. - Low sensitivity to identify resistance in viral strains with representativeness lower than 20% of the circulating population (minorities). - Requires specific training for its interpretation. - It cannot be used to identify resistance mutations that have not been previously associated with loss of viral susceptibility.	- Supplementary to genotype. - Very limited availability. - Expensive. - *In vitro* assay. - The results are available in 3-4 weeks when performed through automated assays. - Interpretation is complex and not completely standardized. - Lack of information clinically correlating to fold-change of some available ARV with suboptimal response or viral failure. - Its capacity to detect resistance mutations falls drastically in absence of pharmacologic pressure; it has to be performed preferably before discontinuing the HAART and not beyond the firsts 4 weeks after its discontinuation. - Low sensitivity to identify resistance in viral strains with representativeness lower than 10% or 20% of the circulating population (minorities). - Mutant viral strains with higher viral fitness may reduce the identification of strains with superior resistance profile but lower replicative capacity. - Requires specific training for its interpretation.

Table 4. Resistance tests, genotype and phenotype: advantages and disadvantages.

The prescription of new agents with better pharmacologic profiles and a higher genetic barrier does not replace the need of a comprehensive approach, a narrow clinical follow-up and an appropriate diagnosis of the viral failure. There are still multiple subjects for basic and clinical investigation, such as to specify the role of minority populations who express resistance to antiretroviral drugs in the clinical settings, to evaluate the importance of resistance assays and genotropism tests in proviral DNA, to implement strategies optimizing the pharmacokinetics and pharmacodynamics of the current antiretroviral agents, to identify new agents with a higher genetic barrier and with different action mechanisms. All of the above with the main objective of extending the productive life of subjects infected with the HIV.

4. Point mutations and herpes virus resistance to antivirals

4.1 Introduction

Herpesviruses comprise a large group of enveloped DNA-containing viruses infecting a wide range of vertebrate hosts, such as humans, horses, cattle, mice, pigs, chickens, turtles, lizards, fish, and even in some invertebrates, such as oyster. According to the International Committee on Taxonomy of Viruses, these viruses are classified in the Order *Herpesvirales,* with three families: *Alloherpesviridae*, *Herpesviridae* and *Malacoherpesviridae*. The human herpesviruses are included in the *Herpesviridae* family (Fauquet et al., 2005). All of them share four biological properties: 1) they express a large number of enzymes involved in metabolism of nucleic acid (e.g. thymidine kinase), DNA synthesis (e.g. DNA helicase/primase) and processing of proteins (e.g. protein kinase). 2) The synthesis of viral genomes and assembly of capsids occurs in the nucleus. 3) The success of herpes virus infection depends upon viral inhibition of several cell functions, such as turning off host protein synthesis, inhibition of mRNA splicing, blocking presentation of antigenic peptides on the cell surface and apoptosis. 4) They have the ability to hide their bare, circularized genome in the nucleus of lymphocytes and neuron cells and return to productive infection months, even years later. These latent herpes virus infections are often benign, but can be devastating especially to newborns and immuno-suppressed individuals (Roizman et al., 2007).

Human herpeviruses infections are a leading cause of human viral disease, second only to influenza and cold viruses (Murray et al., 2009). There are eight known human herpesviruses (Table 5): herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7), and Kaposi's sarcoma-associated herpesvirus (KSHV) (Pellett & Roizman, 2007). Herpesvirus infections are endemic and sexual contact is a significant method of transmission for HSV-1, HSV-2, also HCMV and likely KSHV. The increasing prevalence of genital herpes and corresponding rise of neonatal infection and the implication of EBV and KSHV as cofactors in human cancers are of great public health concern (Anzivino et al, 2009, Shiley & Blumberg, 2011). But also, the association between herpes genital and susceptibility to HIV infection has clearly emerged in the last years (Van de Perre et al., 20008).

Table 6 shows a summary of the most common anti-herpes drugs and their inhibition mechanism. It is important to mention that antiviral therapy is available for HSV, VZV and HMCV infections. Therefore the other herpesviruses infections will not be discussed further.

Virus	Main disease(s)
Herpes simplex virus type 1 or human herpesvirus 1 (HSV-1 or HHV-1) Herpes simplex virus type 2or human herpesvirus 2 (HSV-2 or HHV-2)	The causal agents of oral and genital herpes, respectively (Roizman et al., 2007).
Varicella-zoster virus or human herpesvirus 3 (VZV or HHV-3)	The causal agent of chickenpox and shingles (Cohen et al., 2007).
Human cytomegalovirus or human herpesvirus 5 (HCMV or HHV-5)	The major cause of infectious morbidity and mortality in immunocompromised individuals and developing fetuses, and HCMV-caused disease is called cytomegalovirus inclusion disease (CID) (Mocarski et al., 2007).
Human herpes virus 6A (HHV6A) Human herpesvirus 6B (HHV-6B)	Associated with roseola (Yamanishi et al., 2007).
Human herpesvirus (HHV-7)	HHV-7 has not been definitively documented to cause a specific disease and has been associated to pityriasis rosea (Blauvelt, 2001)
Epstein-Barr virus or human herpesvirus 4 (EBV or HHV-4)	Associated with several diseases, most notably infectious mononucleosis (colloquially known as *mono* or *kissing disease*) and Burkitt's lymphoma (Rickinson & Kief, 2007).
Kaposi's sarcoma herpesvirus or Human herpesvirus 8 (KSHV or HHV-8)	Associated with Kaposi's sarcoma , but KSHV can also cause B-cell lymphoma (Ganem 2007)

Table 5. Human herpesviruses and their main diseases

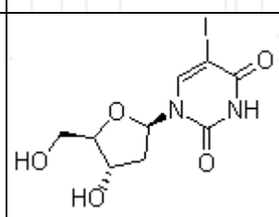
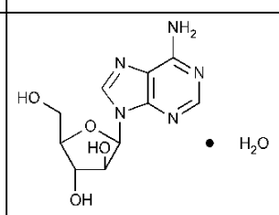
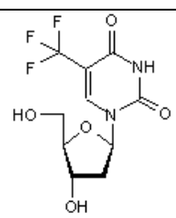
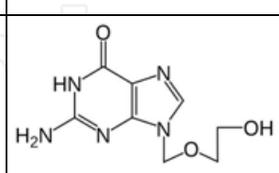
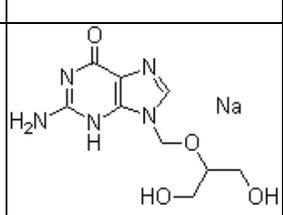
EBV is a gammaherpes virus responsible for several clinical entities, is the causative agent of infectious mononucleosis, Burkitt lymphoma, and nasopharyngeal carcinoma, and accounts for 90% of the cases of posttransplant lymphoproliferative disorder (PTLD). The cornerstone of therapy is decreasing the level of immunosuppression whenever is possible. Some antiviral drugs as acyclovir and ganciclovir have been used as a prophylactic measure but their efficacy has not showed clear results. HHV-6, the causative agent of the common childhood disease roseola infantum (exanthema sabitum), has received attention in the past several years as an opportunistic infection in the posttransplant population.

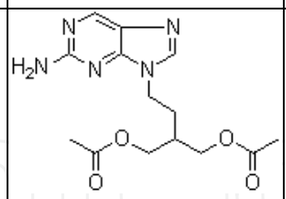
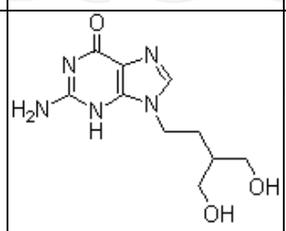
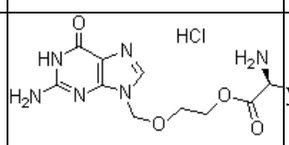
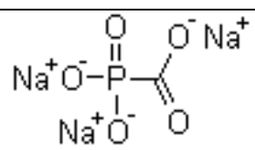
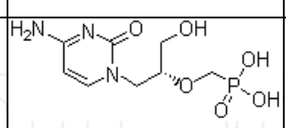
4.2 Antiviral agents for herpesvirus infections

In vitro data has shown that HHV-6 is inhibited by ganciclovir, foscarnet, and cidofovir, but no prospective clinical trials have evaluated the use of antiviral-drugs in the treatment of HHV-6 associated disease.

KSHV is responsible for the malignant entities of Kaposi sarcoma (KS) and primary effusion lymphoma (PEL), as well as some forms of multicentric Castleman disease (MCD). As the case with EBV-associated PTLD, reduction in immunosuppression is the first-line therapy

for KS and is often curative (Shiley & Blumberg, 2011). Herpesvirus primary infections are followed by latency and subsequent periodic reactivations in most patients. Both primary and recurrent infections may require therapeutic or prophylactic interventions. Individuals at particularly high risk of developing severe consequences from HSV infection include immunocompromised hosts, such as transplant recipients, patients who receive cytotoxic drugs, HIV-infected individuals, and pregnant women and their newborns.

Drug	Chemical formula[A]	Name[A]	Antiviral mechanism	Active against	Uses
Idoxuridine		2'-Deoxy-5-iodouridine CAS # 54-42-2	Thymidine analog	HSV-1, HSV-2, VZV, and CMV	Limited to topical ophthalmic treatment of herpes simplex keratoco-njunctivitis.
Vidarabine (adenine arabinoside, ara-A)		9-beta-D-Arabinosyla-denine CAS # 5536-17-4	Adenine arabinoside	HSV-1, HSV-2	Ophthalmic preparations: Effective for acute keratoco-njunctivitis and recurrent superficial keratitis.
Trifluridine (trifluorothymidine)		2'-Deoxy-5-(trifluoromethyl) uridine CAS # 70-00-8	Thymidine analog	HSV-1, HSV-2	Topical: Ophthalmic treatment of primary keratoco-njunctivitis and recurrent keratitis or ulceration caused by herpes simplex 1 and 2.
Acyclovir		9-[(2-Hydroxyethoxy)methyl]guanine CAS # 59277-89-3	Guanosine analog	HSV-1, HSV-2, VZV, and EBV Minimal activity against CMV	Oral or IV (IV indicated when a higher serum drug level is required, as for herpes simplex encephalitis).
Ganciclovir		2-Amino-1,9-dihydro-9-[[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl]-6H-purin-6-one sodium salt CAS #107910-75-8	Guanosine analog	A homologue of acyclovir,	Effective in the treatment of HCMV. It inhibits all the herpesviruses and transformation of normal cord-blood

Drug	Chemical formula[A]	Name[A]	Antiviral mechanism	Active against	Uses
					lymphocytes by EBV.
Famciclovir		2-[(acetyloxy) methyl]-4-(2-amino-9H-purin-9-yl)butyl acetate CAS # 104227-87-4	Guanosine analog	Antiviral spectrum similar to acyclovir	As effective as acyclovir for genital herpes and herpes zoster and more bioavailable after oral administration.
Penciclovir		2-amino-9-[4-hydroxy-3-(hydroxymethyl)butyl]-6,9-dihydro-3*H*-purin-6-one CAS # 39809-25-1	Guanosine analog	Potent activity against CMV (inhibits CMV protein synthesis)	Intravitreal injection: For patients with HIV infection and CMV retinitis that is resistant to other therapies
Valacyclovir		L-Valine 2-(guanin-9-ylmethoxy)ethyl ester CAS # 124832-26-4	Guanosine analog	HSV, VZV	Management of herpes simplex and herpes zoster (shingles). It is a prodrug, being converted *in vivo* to acyclovir.
Foscarnet		Trisodium phosphonoformate CAS # 63585-09-1	Pyrophosphate analog	EBV, HHV-8, HHV-6, HSV and VZV, CMV	Efficacy similar to that of ganciclovir for treating and delaying progression of CMV retinitis.
Cidofovir		[1-(4-Amino-2-oxo-pyrimidin-1-yl)-3-hydroxy-propan-2-yl]oxymethylphosphonic acid CAS # 113852-37-2	Cytosine analog	HSV-1, HSV-2, VZV, CMV, EBV, KSHV,	Generally used for CMV, but use limited by renal toxicity.
Fomivirsen	5'-GCG TTT GCT CTT CTT CTT GCG-3'	Oligonucleotide with phosphorothioate linkages	The first antisense antiviral approved by the FDA	CMV	CMVretinitis in immunocompromised patients.

*Adapted from Porter & Kaplan, 2004; Shors, 2009; [A]Online Database of Chemicals from Around the World

Table 6. FDA-Approved antiherpesvirus drugs*.

4.2.1 Acyclovir and analogs

The first report detailing the selective antiviral activity of acyclovir against herpesviruses was published in 1977 (Elion et al., 1977). Penciclovir, a structurally related compound identified in the 1980s (Boyd et al., 1993), is also a potent and selective inhibitor of many human herpesviruses. Both compounds are analogues of the natural nucleoside deoxyguanosine. Oral prodrugs of penciclovir (famciclovir) and acyclovir (valaciclovir) were subsequently developed to improve their oral bioavailability (Beauchamp et al., 1992).

Acyclovir (ACV), valacyclovir and famciclovir have a similar mechanism of antiviral action against HSV (Corey et al., 2004; Mertz et al., 1997; Sacks, 2004). Acyclovir is a purine nucleoside analogue with inhibitory activity against HSV-1, HSV-2 and VZV. It has a highly selective inhibitory activity due to its affinity for the thymidine kinase enzyme (TK) encoded by HSV and VZV. The TK converts ACV into ACV-monophasphate, a nucleotide analogue. This monophosphate is further converted into diphosphate by cellular guanylate kinase and into triphosphate by a number of cellular enzymes. The ACV-triphosphate stops viral replication by competitive inhibition of viral DNA polymerase, incorporation and termination of the growing viral DNA chain, and inactivation of the viral DNA polymerase (GlaxoSmithKline).

4.2.2 Foscarnet

Foscarnet is a pyrophosphate analogue with activity against herpesviruses, human immunodeficiency virus (HIV), and other RNA and DNA viruses. Foscarnet and its analogues achieve their antiviral effects via inhibition of viral polymerases. Current evidence indicates that foscarnet interferes with exchange of pyrophosphate from deoxynucleoside triphosphate during viral replication by binding to a site on the herpesvirus DNA polymerase or HIV reverse transcriptase (Crumpacker, 1992).

4.2.3 Cidofovir

Cidofovir is a monophosphate nucleotide analogue of deoxycytidine (dCTP), which does not require a viral activation. After undergoing cellular phosphorylation, it competitively inhibits the incorporation of dCTP into viral DNA by viral DNA polymerase, disrupting further chain elongation (Lea & Bryson, 1996, De Clercq, 2004).

4.3 Herpesvirus-resistance to antiviral drugs

Treatment of HSV-infections with nucleoside analogs has been used for more than 20 years, and the isolation of drug-resistant virus from immunocompetent patients has been an infrequent event, from 0.1 to 0.7% (James et al., 2009; Kriesel et al., 2005; Reyes et al., 2003; Shin et al., 2003; Bacon et al., 2002; Blower et al., 1998; Christophers et al., 1998; Crumpacker et al., 1982). However, herpes-resistance to antiviral drugs is well established (7–14%) in imunocompromised patients (Griffiths, 2009; Chen et al., 2000; Levin et al., 2004; Safrin et al., 1994) and neonates (Levin et al., 2001; Nyquist et al., 1994; Oram et al., 2000). It has been found that the most significant risk factors to developing antiviral-resistance are the degree of immunosuppression and prolonged exposure to the antiviral agent (Levin et al., 2004). Nowadays, the drug-resistant viruses have become a very important issue, since the

immunocompromised population has been increasing around the world, either due to viral infections such as AIDS, or immunosuppression for cancer treatment, organ transplant, and chronic diseases treatment, among others. It's estimated that about 10 million people in the United States (3.6 percent of the population) are immunocompromised. But that's likely an underestimate because it only includes those with HIV/AIDS, organ transplant recipients, and cancer patients; but there's a sizable population that takes immunosuppressive drugs for other disorders such as rheumatoid arthritis and inflammatory bowel disease (Kahn, 2008).

4.3.1 Resistance to acyclovir

It is known that HSV has a low inherent propensity to develop mutations within its genome because its polymerase has a proof reading mechanism, therefore many replication cycles are required statistically to generate a virus that has resistance to ACV or similar drugs, and also due to the potency of these drugs to inhibit viral replication, the chance that this may occur in practice is decreased. Nevertheless, mutations of the viral Thymidine kinase (TK) and DNA polymerase (DNApol) can occur and both are intimately involved in mechanisms of resistance to acyclovir and penciclovir (Bacon et al., 2003; Boyd et al., 1993; Coen and Schaffer 1980; Schnipper & Crumpacker, 1980; Schmit & Boivin, 1999). Among ACV-resistant HSV, 95% are due to mutations in the TK gene and 5% in the DNA pol gene (Frobert et al., 2008), and resistance mutations are located mainly in catalytic or conserved domains of TK and DNA polymerase (Chibo et al., 2002; Gibs et al., 1988; Schmit & Boivin, 1999; Stránská et al., 2004; Sauerbrei et al., 2010).

4.3.1.1 Mutations in the TK gene

HSV TK is a 376-amino-acid protein, encoded by the UL23 gene. Known primarily as a TK, this enzyme is a wide spectrum nucleoside kinase capable of phosphorylating both purine and pyrimidine nucleosides and their analogues (Roizman et al., 2007). It contains an ATP binding site (codons 51 to 63), a nucleoside binding site (codons 168 to 176 for HSV-1 and 169 to 177 for HSV-2), and a highly conserved cysteine residue at position 336 for HSV-1 and 337 for HSV-2 (Balasubramaniam et al., 1990; Kit et al., 1983). TK utilizes ATP to phosphorylate deoxythymidine (dT) in the formation pathway of deoxythymidine triphosphate for DNA synthesis.

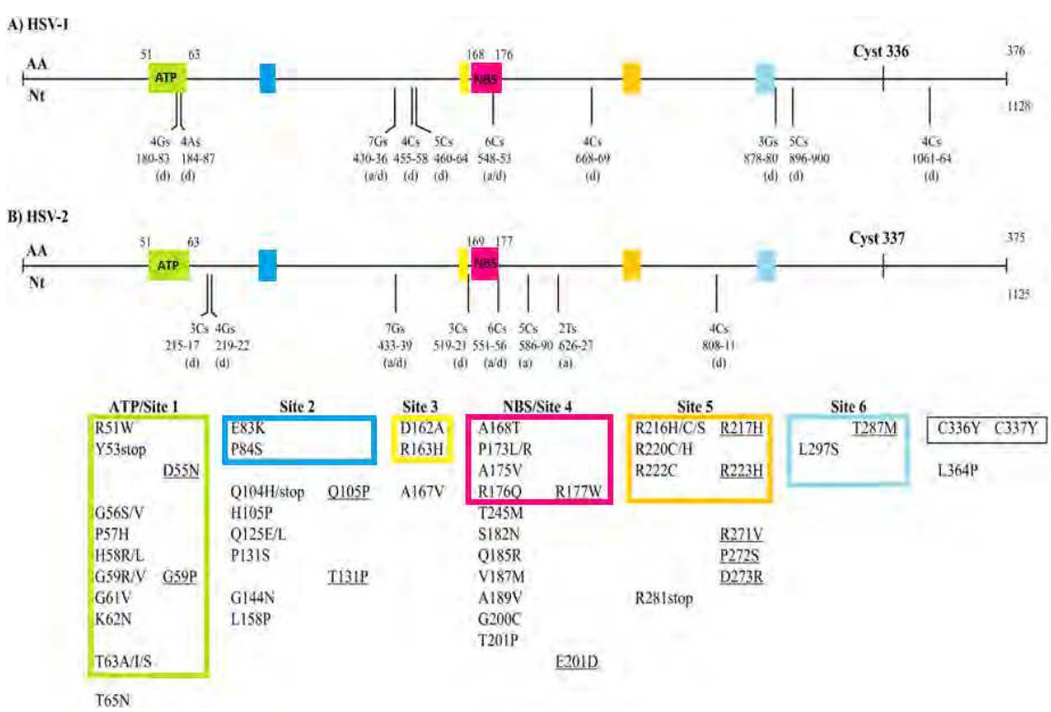
Three distinct classes of acyclovir resistant TK mutants have been identified:

1. TK-negative (TKN) mutants. They lack of TK activity. These phenotypes are the consequence of either single-base insertions/deletions occurring in guanosine (G) or cytidine (C) homopolymer repeats, leading to the shift of the translational reading frame of UL23 TK, or missense point mutations (Gaudreau et al., 1998; Morfin et al., 2000; Sarisky et al., 2001). Therefore, a deletion, insertion or point mutation, often within a hotspot, of the viral UL23 gene, originates a premature stop codon at one of several different places within the gene (Sasadeusz et al., 1997), resulting in a truncated TK protein, which does not have enzymatic activity (Gilbert et al., 2002; Summers et al., 1975). The TKN strains replicate very slowly because they lack the activity of the viral TK gene, which is required to synthetize deoxythymidine triphosphate for DNA synthesis, and these negative mutants have shown to be impaired in their pathogenicity, establishment of latency and low reactivation efficiency (Piret et al.,

2011). In immunocompetent individuals the immune response can rapidly dealt with the viral mutants before they can become clinically apparent (Coen, 1994). However, it is not always the case, it has been published that acyclovir-resistant HSV-2 mutants can be developed rapidly in neonatal infection and cause clinically significant disease, in spite of the *in vitro* decreased replication and attenuated virulence in an animal model showed by the viral strain (Oram, 2000).

2. TK-partial (TKP), express reduced levels of TK activity. Rare mutations can produce a virus which maintains the ability to reactivate as well as some virulence for an immunocompetent animal host, and some residual TK activity is present (Collins & Darby, 1991; Bacon et al., 2003; Coen, 1994).
3. TK-altered (TKA) mutants are substrate specificity mutants, which phosphorylate thymidine but not acyclovir and/or penciclovir, due to a specific mutation in the TK gene such, that it recognizes acyclovir/penciclovir poorly, but can still phosphorylate the natural nucleosides required by the virus for replication (Darby et al., 1981).

Approximately 95 to 96% of acyclovir-resistant HSV isolates are TK deficient (TKN or TKP), and the remaining isolates are usually TKA. It has been generally shown that acyclovir-resistant mutants do not appear to be capable of initiating a latent infection that can subsequently be reactivated (Pottage & Kessler, 1995). Gaudreu et al., 1998 analyzed 30 acyclovir-resistant HSV isolates from immunocopromissed patients, finding 17 TKN, 12TKP and 1TK undefined. Half of them had an insertion or deletion of one or two nucleotides, within homopolymers of G or C, which are considered resistance hot spots. The two longest homopolymers, one composed of 7 Gs and one of 6 Cs, are the sites of the most frequently reported mutations in ACV-resistant clinical isolates; other resistance cases are due to nucleotide substitutions usually in the conserved sites of the UL23 gene (Piret et al., 2011) (Figure 6)



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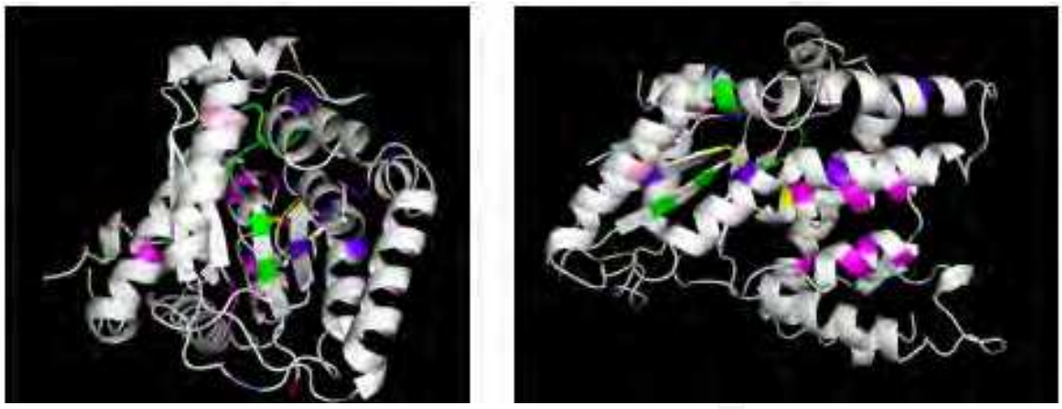


Fig. 6. Mutations identified in the UL23 gene of HSV isolates resistant to ACV. A) and B) maps of mutations of HSV-1 and HSV-2 isolates resistant to ACV, respectively. The ATP-binding site (ATP), the nucleoside-binding site (NBS), and the six regions of the *UL23* gene that are conserved among *Herpesviridae* are shown by the color boxes. The six highly conserved regions are located at amino acids (AA) 56 to 62 (site 1), 83 to 88 (site 2), 162 to 164 (site 3), 171 to 173 (site 4), 216 to 222 (site 5), and 284 to 289 (site 6) for HSV-1 and 56 to 62, 83 to 88, 163 to 165, 172 to 174, 217 to 223, and 285 to 290 for HSV-2. The additions (a), deletions (d), or both additions and deletions (a/d) reported in homopolymer runs, as well as the nucleotides (Nt) involved, are indicated below vertical bars. Substitutions of amino acids reported in the *UL23* gene that are included in the boxes correspond to those identified in conserved regions, and those outside the boxes are located in nonconserved regions. Underlined mutations correspond to the HSV-2 mutations (Adapted from Piret & Boivin, 2011; reproduced with permission of the American Society for Microbiology). **C**) Two views of the 3D structure of the TK protein (PDB:1KI2) of HSV-1 in ribbon diagram. The TK protein is in white, the NH2 end in red, the carboxyl end in blue, and the HSV-1 mutations published by Piret & Boivin 2011 are shown in different colors: Site 1 mutations are in green (R51W, Y53Stop, G56S/V, P57H, H58R/L, G59R/V, G61V, K62N, T63A/I/S, T65N; Site 2 mutations in pale blue (E83K, P84S, Q104H, H105P, Q125E/L, P131S, G144N, L158P); Site 3 mutations in yellow (D162A, R163H, A167V); Site 4 mutations in magenta (A168T, P173L/R, A175V, R176Q, T245M, S182N, Q185R, V187M, A189V, G200C, T201P); Site 5 mutations in orange (R216H/C/S, R220C/H, R222C, R281STOP); Site 6 mutations in cyan (L297S); mutations in the carboxyl end are in pink (C336Y, C337Y, L364P).

No consistent differences have been identified in the mutations associated to acyclovir-resistant isolates from immunocompetent and immunocompromised patients (Levin et al., 2004). Genotyping of the UL23 TK gene of HSV-1 has confirmed its uncommonly high polymorphism, in comparison to the TK gene of HSV-2 isolates, which was considerably less polymorphic. These findings are in agreement with the fact that the variability of the HSV-1 genome is about fourfold higher than that of HSV-2 (Sauerbrei et al., 2010; Chiba et al., 1998).

Recent work by Burrel et al., 2010 analyzing the natural polymorphism of UL23 TK and UL30 DNA pol among HSV-1 and HSV-2 strains to identify the amino acids changes potentially associated to antivirals HSV-resistance, identified 15 and 51 new natural polymorphisms within the TK and DNA pol, respectively (Fig. 7). Several amino acid changes among drug-resistant HSV were identified in the TK (S29A for HSV-2) or in the

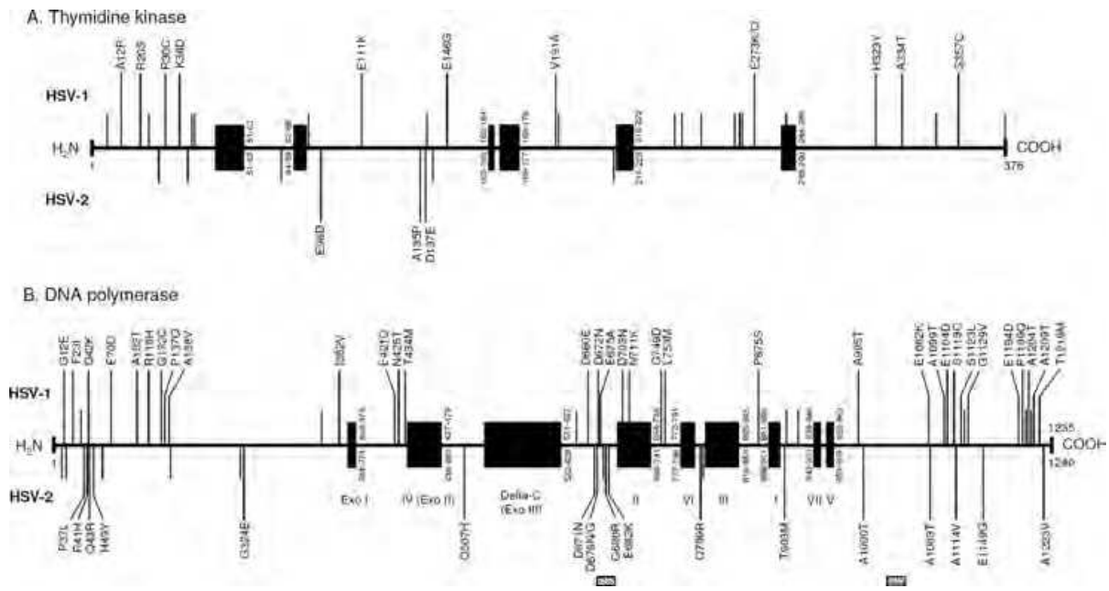


Fig. 7. Natural polymorphism map (to scale) of thymidine kinase (TK) (A) and DNA polymerase (B) among HSV-1 and HSV-2 strains. For each viral enzyme, conserved regions and functional domains are indicated by the black boxes, and natural polymorphisms are represented separately for HSV-1 (top) and HSV-2 (bottom). Amino acid changes related to natural polymorphism are indicated by vertical bars: short vertical bars correspond to changes previously reported, long vertical bars labeled with amino acid change correspond to changes newly described in this study. Regarding HSV-2 DNA polymerase, hatched boxes indicate amino acid insertions or deletions related to natural polymorphism (Burrel and Boutolleau, 2010; reproduced with permission of the American Society for Microbiology).

DNA pol (H98Y, V117L, L267M, A870G, L1188F, and R1229I for HSV-1; I291V, V544A, Y823C, and H837R for HSV-2) proteins. Moreover, the genotypic characterization of 25 drug-resistant HSV isolates revealed 8 new amino acid changes located in TK, potentially accounting for acyclovir-resistance (Y53D, I101S, L170P, and A207P for HSV-1; and S66P, A72S, R176W, and M183I for HSV-2). Wang et al., 2011 analyzed 68 ACV-resistant HSV-1 isolated from children, and identified 21 mutations in the TK gene, 11 of them have not been previously reported (D14H, Q15L, A28V, Q67R, .E95A, I203L, A207S, D215N, Q275P, A365T, Q342K). It is known that viruses with TK mutations are normally-resistant to other drugs which require the viral TK for activation, such as penciclovir; but generally remain susceptible to antiviral agents that act directly on DNA polymerase, such as foscarnet and cidofovir (Bacon et al., 2003).

In summary there is not a unique mutation pattern to explain HSV ACV-resistance, and further work is necessary to construct a more complete mutations data base of the TK gene.

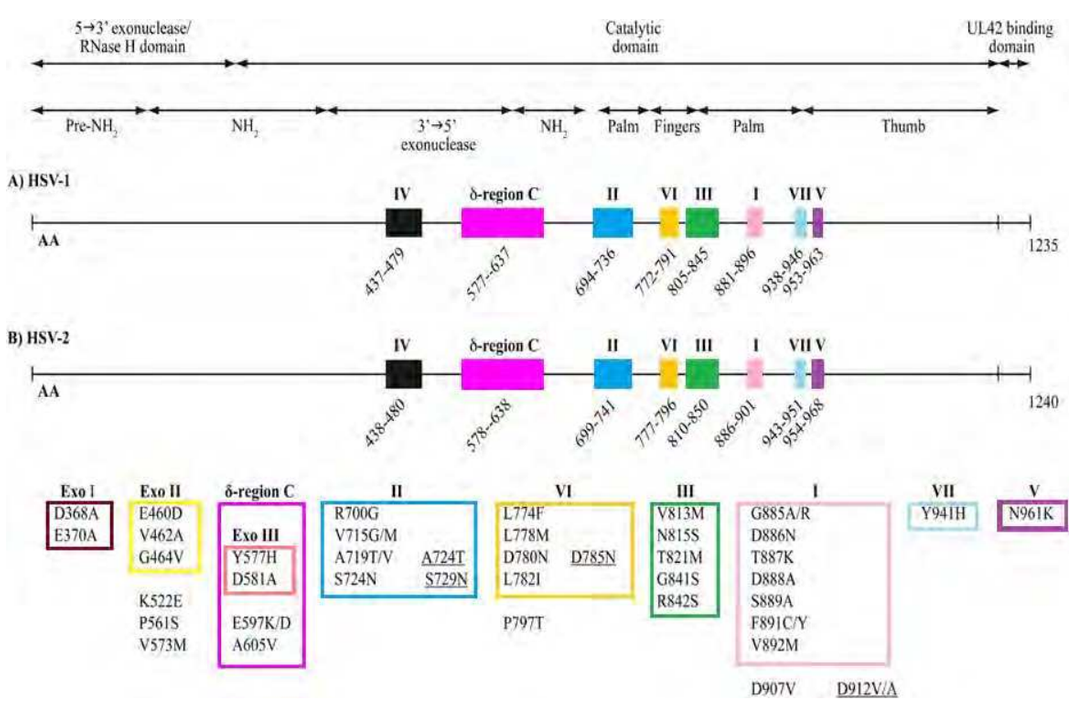
4.3.1.2 Mutations in the DNA pol gene

The herpes simplex virus-1 DNA polymerase is a heterodimer, which consists of the products of the *UL30 (Pol)* and *UL42* genes. The *UL30* gene encodes the catalytic subunit, while the *UL42* gene encodes a phosphoprotein that possesses double-stranded DNA-

binding activity. DNA pol is a multifunctional enzyme which possesses a polymerase activity for the extension of DNA primer chains, an intrinsic 3-5´ exonuclease proofreading activity, and an RNase H activity that could be removed the RNA primers to initiate the synthesis of Okazaki fragments at a replication fork during herpes DNA replication (Crute & Lehman, 1989). HSV DNA pol belongs to the family of α-like DNA polymerases. It is formed by 6 structural domains; **1**) A pre-NH2 domain, from NH2-terminal to aa 140; **2**) An NH2-terminal domain, from aa 141–362 and 594–639; **3**) A 3´-5´ exonuclease domain, from aa 363–593, contains three highly conserved sequence motifs: Exo I, Exo II, and Exo III; **4**) polymerase palm domain (catalytic site), from aa 701–766 and 826–956, contains the conserved regions I, II, and VII; **5**) Fingers domain, from aa 767–825, contains the conserved regions III and VI**,** and the base subdomain may play a role in positioning the template and primer strands ; **6**)Thumb domain, from aa 957–1197, contains the conserved region V (Liu et al., 2006). In clinical isolates, mutants with altered DNA polymerase conferring resistance to nucleoside analogues are less frequent detected (Sacks et al., 1989). The mutations are single amino acid substitutions located in regions which are directly or indirectly involved in the recognition and binding of nucleotides or pyrophosphate, as well as in catalysis. Fig 8 shows several identified mutations in the UL30 gene of HSV-1 and HSV-2 (Piret, et al., 2011).

4.3.2 Foscarnet-resistance

Foscarnet (FOS) is a pyrophosphate analogue that inhibits the viral DNA pol by mimicking the structure of pyrophosphate produced during the elongation of DNA, and it acts as a noncompetitive inhibitor of DNA pol activity. FOS does not require phosphorylation by viral and cellular kinases and binds to the pyrophosphate binding site on the viral DNA pol and blocks the release of pyrophosphate. It is only available as an intravenous formulation, and is indicated as a second-line therapy for HSV infections.



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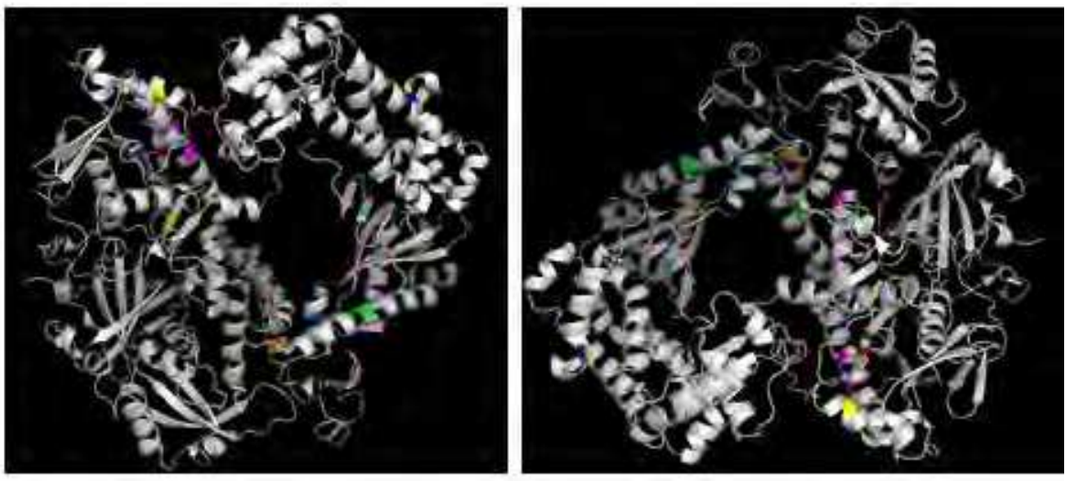

Fig. 8. Mutations identified in the UL30 gene of HSV isolates resistant to ACV. A) and B) maps of mutations of HSV-1 and HSV-2 isolates resistant to ACV, respectively. Regions conserved among Herpesviridae genes are shown by the color boxes. The roman numbers (I to VII and δ-region C) corresponding to each of these regions are indicated above the boxes. Amino acid (AA) locations are noted below each of these regions for HSV-1 and HSV-2. Substitutions reported in the UL30 gene that are included in the boxes correspond to those identified in conserved regions, and those outside the boxes are located in nonconserved regions. Underlined mutations correspond to the HSV-2 mutations. Mutations E460D, G464V, K522E, and P561S in and outside Exo II are lethal to the virus; mutations Y577H and D581A in the Exo III motif in δ-region C are associated with hypersusceptibility to ACV; and none of the mutations in region I are spontaneously induced (Adapted from Piret & Boivin 2011; reproduced with permission of the American Society for Microbiology). **C**) Two views of 3D structure of the DNA pol protein (PDB 2GV9) of HSV-1 in ribbon diagram. The DNA pol is in white, the NH2 end in red, the carboxyl end in blue, and the HSV-1 mutations from the conserved regions published by Piret & Boivin 2011 are shown in different color: in the region 1 are in pink (G885A/R, D886N, T887K, D888A, S889A, F891C/Y and V892M); in the region II in pale blue (R700G, V715G/M, A719T/V and S724N); in the region III in green (V813M, N815S, T821M, G841S and R842S); in the region V in purple (N961K); in the region VI in orange (L774F, L778M, D780N and L782I); in the region VII en cyan (Y941H); in the Exo I motif in dark red (D368A and E370A); in the Exo II in yellow (E460D, V462A and G464V); in the Exo III in salmon pink (Y577H and D581A); in the δ-region C, in magenta (E597K/D ad A605V)

Most FOS-resistant clinical HSV isolates contain single base substitutions in conserved regions and in a non-conserved region of the DNA pol gene. Some of these isolates retained their susceptibility or, at the most, borderline levels of susceptibility to ACV and cidofovir. However, some mutations conferring resistant to both ACV and FOS have been found in clinical isolates: V715G, S724N/S729N and Y941H (HSV-1/HSV-2). Mutants with alterations in both HSV TK and DNA pol can also occur, resulting in double resistance to both ACV and FOS (Piret et al., 2011).

4.3.3 Cidofovir and adefovir

These drugs are acyclic nucleoside phosphonates derivatives of cytosine (cidofovir) and adenine (adefovir), which are converted into active ANP-diphosphates by cellular kinases. Thus, do not require the activation by the viral TK enzyme, and act as competitive inhibitors of the viral DNA pol, and chain terminators. Cidofovir is indicated for HCMV infections, including ganciclovir-resistant strains, and also against HSV and VZV strains. Several mutations linked to cidofovir resistance have mapped in HSV DNA pol gene: R700M, G841C and G850I, L773M, Y941H and V573M, and in laboratory-derived HSV strains resistant to cidofovir, L1007M and I1028T (Piret et al., 2011).

4.4 VZV-resistant to antiviral drugs

The current drug of choice for the antiviral treatment of VZV infections in patients at risk is ACV (De Clercq, 2004). VZV-resistant to ACV or foscarnet has only been reported in rare cases of immunocompromised patients, and the resistance to ACV is associated to alterations in the TK activity as it happens with HSV. One of the problems to study the antiviral-resistance of VZV, is the low rate of VZV isolations in cell culture, usually between 20% and 43% from vesicle samples; and even more, there is a restricted spectrum of permissive cell cultures and a long time (up to a few weeks) is required to developing cytopathic effect (Cohen et al., 2007). On the other hand, there is not enough information about the natural polymorphism or resistance associated to the TK and DNA pol genes. Sauerbrei et al., 2011 analyzed genotypically 16 VZV strains with clinical diagnosis of ACV-resistance, finding seven strains with alterations in the TK gene: three were associated to amino acid substitutions (L73I, W225R, T256M), three with stop codon generation (A163-, Q303-, N334-) and one frameshift due to a deletion of nucleotides 19–223. All of them were not previously reported, except the Q303- mutation, which has been reported in two patients with AIDS treated with ACV to control persistent zoster (Fillet et al., 1998; Saint-Léger et al., 2001).

Even though herpes viruses have a low rate of mutations, it is clear that apparition of viral strains resistant to the current antivirals drugs is a serious problem, especially for the immunocompromised patients. The resistance is associated with a wide range of mutations located mainly in catalytic or conserved domains of the viral TK and DNA pol enzymes, enzymes that play a critical role to achieve herpes viruses inhibition by the current antiviral drugs. Therefore it is necessary to monitor the efficacy of the antiviral therapy administered to the patients, but also to develop new antiviral drugs against different viral targets.

5. Point mutations and antiviral resistance in cytomegalovirus: Molecular basis and clinical implications

Despite progress in the diagnosis and management of cytomegalovirus (CMV) infections in different groups of immunosuppressed patients, this virus continues to be an important pathogen that can establish acute and chronic infections (Snydman et al., 2011) with high morbidity and mortality in immunosuppressed individuals and/or the immunologically immature. The main risk groups are: a) newborns; b) individuals receiving organ transplants; c) patients with cancer; and d) AIDS patients, although the incidence in this group has declined with the advent of HAART (Emery et al., 2001; Snydman et al., 2011).

Drugs approved by the Food Drug Administration FDA for treating CMV infection include ganciclovir (GCV), foscarnet (FOS), cidofovir (CDV), and valganciclovir (VGV). The characteristics of these drugs are described in Table 6

5.1 Viral proteins targeted by the antivirals

5.1.1 UL97 protein

A protein encoded by the UL97 gene. The natural role of this protein in viral replication is not fully known, but recent studies suggest it is involved in the regulation of viral replication (Wolf et al., 2001) and other studies highlight the involvement of UL97 in viral envelope assembly (Goldberg et al., 2011). This protein has a kinase activity, so it may be involved in the monophosphorylation of GCV. This feature has been widely studied and is exploited in the pharmacokinetics of GCV.

5.1.2 UL54 protein

The viral polymerase UL54 is encoded by the UL54 gene. Several functional regions have enzyme activity. They are divided into I to VII with polymerization activity and EXO I to EXO III with exonuclease activity. The first function allows polymerization activity, while the second provides an editing function that significantly reduces the rate of mutation and leads to high fidelity (Picard-Jean, 2007).

5.2 Drugs and their mechanism of action

5.2.1 Ganciclovir

This drug is an analogue of guanosine. It was the first drug approved by the FDA for the treatment of CMV infection. GCV is a pro-drug that is inactive until phosphorylated. This process is carried out by a viral protein kinase encoded by the UL97 gene, which phosphorylates GCV. The product from this biochemical reaction is ganciclovir monophosphate, which is further phosphorylated twice more by cellular kinases to produce ganciclovir triphosphate. This form is recognized by the polymerase and it competes with deoxyguanosine triphosphate (dGTP) to block chain elongation (Figure. 9) (Faulds et al., 1990; Sullivan et al., 1992).

5.2.2 Valganciclovir

Although GCV has proved to be an efficient CMV induction therapy in various groups of patients, such patients also require maintenance therapy with oral GCV. This therapy is limited because oral GCV has low bioavailability (Markham et al., 1994; Anderson et al., 1995). By contrast, the pro-drug VGV has the advantage of a high oral bioavailability. VGV is hydrolysed by esterases in the gut and liver in a first step; the product is GCV, which can be phosphorylated to block viral replication (Sugawara et al., 2000).

5.2.3 Foscarnet

This drug is a conjugate base of phosphonoformic acid that has the advantage that it does not need to be phosphorylated. It is capable of inhibiting the activity of the viral polymerase,

because it binds to a site that recognizes pyrophosphates on bases. It interferes with the exchange of pyrophosphates and phosphates by dideoxynucleosides and inhibits viral replication (Crumpacker et al., 1992) (Fig. 9).

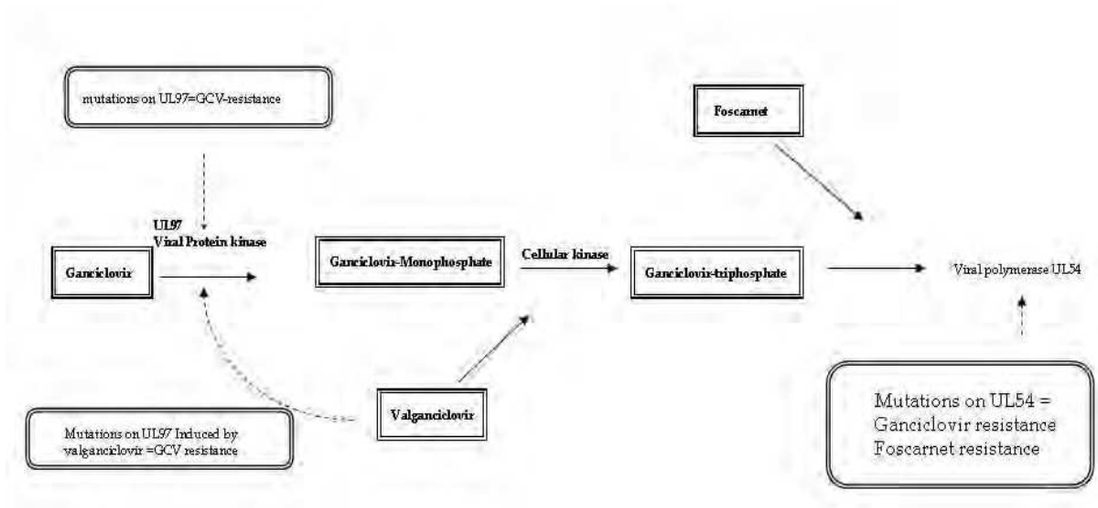

Fig. 9. Mechanism of action to ganciclovir, valganciclovir and foscarnet. GCV and valganciclovir, need phosphorilation while foscarnet recognize the UL54 without previous phosphorilation. Ganciclovir is initially phoshorilated by a viral protein kinase UL97. Mutations on UL97 confer them ganciclovir resistance, valganciclovir can induce ganciclovir resistance. Mutations on UL54 gene can confer ganciclovir and/or foscarnet resistance.

5.2.4 Cidofovir

This drug is the acyclic nucleotide analogue 1-(3-hydroxy-2-phosphonomethoxyethyl) cytosine and it competes with cytosine. Several cellular enzymes are involved in the phosphorylation of this compound. These include pyrimidine nucleoside monophosphate kinase, pyruvate kinase, creatine kinase, and nucleoside diphosphate kinase (Cihlar et al., 1996). This antiviral is not dependent on UL97 phosphorylation and it inhibits viral polymerase activity through a similar mechanism to GCV (Fig. 9) (De Clercq, 2003).

5.2.5 Fomivirsen

This drug is an antisense nucleotide phosphorothioate that contains 21 nucleotides. It is resistant to cellular nucleases and binds to transcripts of the immediate early viral proteins (6) (Azad et al., 1993). Studies show that this oligonucleotide can specifically block viral genes without interfering with cellular translation.

5.3 Point mutations and antiviral drug resistance

Strains resistant to antivirals emerged following the treatment of active infections, with this emergence linked to the prolonged and intermittent administration of drugs to immunocompromised patients. The most studied drug in this context is GCV. Several point mutations in viral phosphotransferase sequences (UL97) or DNA polymerase sequences

(UL54) are involved. The role of UL97 in the CMV life cycle is not clear, but its participation in the phosphorylation of GCV is well known. The mechanism occurs as follows. The GCV domain is recognized by the UL97 protein kinase. The deoxyguanosine analogue (9-[1,3-dyhydroxy-2-propoxymethyl]guanine) or monophosphate deoxyguanosine are the products of GCV phosphorylation. Two subsequent phosphorylations are mediated by cellular phosphate kinases. Triphosphorylated GCV is the active molecule for the inhibition of viral replication, because it competes with the natural substrate deoxyguanosine triphosphate for the viral DNA polymerase.

5.3.1 Resistance to ganciclovir

GCV resistance is most frequently a result of amino acid substitutions due to mutations in the codons 460, 594, and 595, which are in the coding sequence of the protein UL97. These mutational changes reduce the affinity for GCV without affecting polymerase activity (Chou et al., 1995). GCV resistance was first reported in isolates obtained from blood samples of immunosuppressed hospitalized patients. Two had been diagnosed with AIDS, while another had lymphoblastic leukaemia. These strains showed poor response to GCV with *in vitro* cell cultures (Erice et al., 1989). Resistance to GCV was previously obtained *in vitro* by the selective pressure of low doses of GCV on the strain AD169 (Biron et al., 1986). Subsequently, Lurain et al. reported the isolation of three mutants resistant to GCV with a reduced ability to phosphorylate GCV. Resistance was associated with the emergence of a mutation in the exonuclease function of polymerase (Lurain et al., 1992). The same authors later reported isolates with a point mutation at position 460 in region IV of the UL97 gene, where a change of methionine for isoleucine (M460I) was involved (Lurain et al., 1994).

Several mutations that are known to change the active site of UL97 and prevent the phosphorylation of GCV have been described and characterized. More than 25 mutations are associated with this process. The most common are at codons 460 (methionine to valine), 594 (methionine to isoleucine), and 595 (aspartic acid to serine). In some cases, there is more than one mutation.

UL54 mutations are also involved with resistance to GCV. These mutations produce changes in the amino acid sequence of the viral polymerase, which affects the affinity for a guanosine triphosphate analogue (GCV triphosphate). More than 20 mutations have been described, and combinations of mutations are also found in UL97 and UL54. Strains with mutations in UL97 alone are resistant to GCV but susceptible to FOS and CDV, strains with mutations in UL54 show a pattern of resistance to GCV and CDV, and strains with double mutations in UL97 and UL54 are highly resistant to GCV.

5.3.2 Induction of resistance with valganciclovir

This antiviral is a GCV pro-drug, so its prolonged administration may be a factor in developing resistance to GCV. This effect has a low incidence, but greater use of this antiviral is now encouraged. Reports have described mutations in UL97 as V466G, V466M, and V466G, and these were obtained after treatment with VGV (Boivin et al., 2001; Foulongne et al., 2004; Martin et al., 2010).

5.3.3 Foscarnet

Mutations involved with FOS resistance are located on the UL54 gene. A great diversity of point mutations can change the amino acid sequence of the viral polymerase and lead to FOS resistance. These mutations are located in regions II and III (codons 696–845). The presence of these mutations changes the reading frame of the sequence leading to blocking of antiviral recognition by the polymerase (Lurain et al., 2010).

5.3.4 Cidofovir

As with GCV, most mutations that confer resistance to CDV are concentrated in the exonuclease domain of the polymerase (codons 301, 408–413, and 501–545) and region V (codons 981–987) (Bowen et al., 1999).

5.3.5 Fomivirsen

An antisense nucleotide inhibits viral replication by two different mechanisms that are either dependent on or independent of the sequence. The antiviral mechanism was analysed *in vitro* using fibroblasts, and it was shown that the oligonucleotide recognizes the mRNA sequence of the viral Immediate Early Protein IE2 following internalization. This results in reduced expression levels of the IE proteins. Mulabampa et al. obtained an *in vitro* mutant with fomivirsen resistance. It was expected that the mutation leading to resistance would be located on the complementary region to fomivirsen. However, no mutation was found when the region was sequenced. Despite this result, the induction of fomivirsen resistance induced antiviral and the clinical impact is not clear.(Mulamba et al., 1998, Anderson et al., 2004).

5.4 Multidrug resistance

Mutations can provide different levels of resistance or cross-resistance to antivirals. Occasionally, mutations in both UL97 and UL54 occur, resulting in multidrug resistance. For example, Eckle et al. reported a paediatric patient undergoing bone marrow transplantation with a disseminated infection during the conditioning procedure and encephalitis on day +100. FOS was started, which was followed by combination therapy and subsequently GCV, FOS, and CDV on day +167. Multidrug resistance with multiple mutations was observed seven months later (Eckle et al., 2000). Rodriguez et al. reported on a 58-year-old patient undergoing a liver transplant. On day +86, they isolated a possible mutation at A594V in UL97 and six point mutations that potentially affected the amino acid sequence of the UL54 protein. The resistance to GCV and FOS in this patient was apparently associated with multiple factors, but prolonged therapy with GCV and co-infection may have produced at least two strains (Rodríguez et al., 2007).

5.5 Laboratory methods for the detection of antiviral drugs

Based on *in vitro* analysis, antiviral resistance can be classified as phenotypic or genotypic.

Phenotypic resistance is tested using a cell culture assay to determine an antiviral's ability to reduce viral replication. This technique is considered the gold standard. The AD169 strain is used as a reference where the profile drug susceptibility is already known. The reference

values for the drugs are 6 μM for GCV, 2 μM for CDV, and 400 μM for FOS. The *in vitro* assay determines the antiviral's ability to reduce the number of lytic plaques, which quantifies the cytopathic effect induced by the virus. This can be determined as a 50% inhibitory concentration (IC_{50}) (Chou et al., 1999; Landry et al., 2000). This is a difficult method that requires trained personnel and standard antiviral concentrations. Simplified techniques have been developed that can be applied to a large number of samples, which significantly reduces the workload (Prix et al., 1998). However, this is still a laborious technique and it is difficult to apply it routinely. Nevertheless, these are necessary tests that are required to confirm potential drug resistance to a new drug or new mutations found by genotypic assays (Lurain et al., 2010).

Phenotypic resistance tests are perhaps the most widely used, because a number of mutations associated with resistance are already known. Thus, the presence of a mutation can be inferred if the strain is resistant. Several molecular methods have been proposed for the detection of mutations that are known to be associated with resistance to antiviral drugs. These methods are described below.

5.5.1 Polymerase chain reaction coupled to restriction fragment length polymorphism (PCR–RFLP)

This method has been used for several years, because it is easy to perform and it requires no sophisticated equipment. However, it cannot identify new mutations associated with resistance. This method is more applicable to routine detection of the most common mutations, particularly those in codons 520, 460, 594, 595, 591, and 592 that confer GCV resistance (Hanson et al., 1995; Prix, 1999).

5.5.2 Real-time PCR

Several studies report the use of real-time PCR as a tool for the detection of mutations associated with resistance. Yeo et al. (2005) used a strategy of molecular beacons for the detection of mutations in codon 460 in UL97. Liu et al. developed a method based on SYBR green for the detection of mutations at position 460 in UL97. Liu et al. (2008) and Göhring et al. (2008) developed a method based on real-time PCR for the detection of mutations in codons 594, 595, 603, and 607.

5.5.3 PCR sequencing

This is the gold standard method for detecting the presence of mutations known to be involved in resistance. It also allows the genotypic detection of new mutations that are correlated with phenotypic tests to determine whether they are associated with resistance.

5.6 Clinical impact of cytomegalovirus drug resistance

Published evidence indicates the emergence of resistance and severe complications in patients. For example, Jabs et al. (2010) observed that the emergence of resistance associated with mutations was closely linked to high mortality in a study of 266 patients with AIDS who were treated with GCV or FOS for CMV retinitis. We observed a fatal outcome with

viral pneumonitis and graft rejection in a paediatric patient undergoing bone marrow transplant, where two resistance mutations (M460V and M460I) appeared following non-response to treatment with GCV. We attributed these complications to the lack of response to the treatment, which apparently allowed high viral replication coupled with strong resistance while toxic damage induced by the antiviral therapy led to rejection of the graft. (Arellano-Galindo et al., 2011).

6. Conclusions

At present, it is clear that mutations conferring resistance to the currently approved antiviral drugs is a growing problem , so it is very important to continue research in various areas that would allow better resolution of the problem:

First, the knowledge of mutation's type that allows virus avoid the action of drugs, will allow an understanding as to how these mutations arise and how we must avoid them. Also, this knowledge, will allow a better design of new drugs.

It is also, very important to establish definitive criteria for the diagnosis of resistance and specific treatment, mainly in immunocompromised population, where several factors are involved.

Also, there are still multiple subjects for basic and clinical investigation, such as specify the role of minority populations who express resistance to the antiviral drugs in the clinical settings, implement strategies optimizing the pharmacokinetics and pharmacodynamics either of the current or of the new antiviral agents. All with the main objective to improve the action of drugs, reduce the likelihood of emergence of resistant strains and have under control those which are already presented.

On the other hand, we must not forget the monitoring of the efficacy of the antiviral therapy which is administered to the patients, to avoid the appearance of point mutations conferring drug resistance. Finally, it is also important to bear in mind that clinical centres attending immunosuppressed patients should implement special techniques for the detection of genotypic viral resistance.

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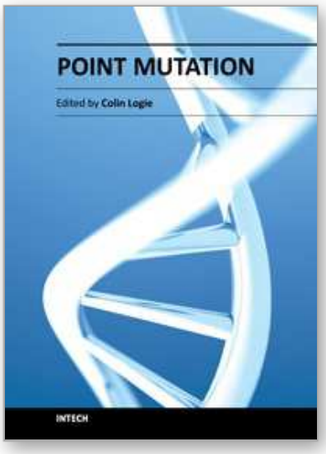

Point Mutation

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This book concerns the signatures left behind in chromosomes by the forces that drive DNA code evolution in the form of DNA nucleotide substitutions. Since the genetic code predetermines the molecular basis of life, it could have been about any aspect of biology. As it happens, it is largely about recent adaptation of pathogens and their human host. Nine chapters are medically oriented, two are bioinformatics-oriented and one is technological, describing the state of the art in synthetic point mutagenesis. What stands out in this book is the increasing rate at which DNA data has been amassed in the course of the past decade and how knowledge in this vibrant research field is currently being translated in the medical world.

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