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PREFACE

The year 1929, when Fleming published his seminal paper on the antibacterial action of a penicillium (Fleming, 1929), is generally considered as the year of birth of the antibiotic era. The birthday of the antivirals (De Clercq, 2009; Field & De Clercq, 2004) was 30 years later when Prusoff described the synthesis of 5-iodo-2′-deoxyuridine (IDU) (Prusoff, 1959). After Herrmann described its antiviral activity *in vitro* (Herrmann, 1961) and Kaufman its efficacy in the treatment of herpes simplex virus (HSV) keratitis (Kaufman, 1962), IDU became the first antiviral to be used topically for the treatment of herpetic eye infections. Two years later, in 1964, Kaufman and Heidelberger described the antiviral action of 5-trifluoro-2′-deoxythymidine (TFT) (Kaufman & Heidelberger, 1964). Like IDU, TFT would go on to be employed for the topical treatment of HSV keratitis.

In 1964, Privat de Garilhe and de Rudder described the antiviral activity of adenine arabinoside (ara-A) against HSV (Privat de Garilhe & de Rudder, 1964). This observation was corroborated by Schabel (1968) and, under the guidance of Whitley, ara-A (now also called vidarabine) became the first antiviral used systemically for the treatment of herpes virus infections, particularly herpes zoster (Whitley, Ch'ien, Dolin, Galasso, & Alford, 1976). While vidarabine is no longer prescribed, it paved the way for the use of acyclovir in the treatment of (herpes) virus infections.

Also in 1964, 1-adamantanamine (amantadine) was found to be an inhibitor of influenza A virus *in vitro* (cell culture) and *in vivo* (mice) (Davies et al., 1964). Although amantadine was approved for the treatment of influenza A virus infections, it has never been employed routinely for this condition because of the rapid development of resistance to this agent (for a review of influenza virus inhibitors, see De Clercq, 2006). Currently, influenza virus infections are treated with neuraminidase inhibitors, such as zanamivir, oseltamivir, peramivir, and laninamivir.

Ribavirin (Virazole) was first identified as a broad-spectrum antiviral agent by Sidwell and his colleagues in 1972 (Sidwell et al., 1972). Although at one time ribavirin was advocated for the therapy of arenavirus infections, such as Lassa fever (McCormick et al., 1986), for a long period it was a drug looking for a disease. Eventually, it found its "niche" as an adjunctive treatment for hepatitis C virus (HCV), and for the past decade, in combination with pegylated interferon it has been the standard of care. However, the days

of ribavirin and peg-IFN appear numbered, given the development of direct-acting antivirals (DAAs) that have proven highly effective as treatment options for hepatitis C (De Clercq, 2012).

In April 1978, the first selective antiviral agent, 9-(2-hydroxyethoxymethyl)guanine (acyclovir) (Schaeffer et al., 1978) was introduced. As a preview, Elion published a few months earlier (Elion et al., 1977) that the selectivity of acyclovir against herpesviruses, HSV in particular, is due to its phosphorylation by the HSV-encoded thymidine kinase. As of today, acyclovir is the gold standard for the treatment of HSV-1 and HSV-2 infections.

A few months after acyclovir was launched, another acyclic nucleoside analogue, (*S*)-9-(2,3-dihydroxypropyl)adenine (DHPA) was reported that, unlike the selective antiherpesvirus activity of acyclovir, displayed a broad range of antiviral effects (De Clercq, Descamps, De Somer, & Holý, 1978). Emerging from DHPA, and the known antiviral activity of phosphonoacetic acid, was the design of a new class of compounds, the prototype being (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine [(*S*)-HPMPA] (De Clercq et al., 1986). Starting from (*S*)-HPMPA, which was never commercialized, a new class of antiviral compounds emerged. Collectively, this class is referred to as the acyclic nucleoside phosphonates (ANPs) (De Clercq & Holý, 2005). Three of these compounds, cidofovir, adefovir, and tenofovir, are currently marketed, with tenofovir being included in different fixed-dose combinations.

In the 1980s, there were only a few antiviral compounds available in the physicians' armamentarium, including acyclovir. As of 2013, the number of antiviral drugs available for prescription has grown to around 50, half of which are employed for the treatment of AIDS, a condition that was not yet recognized in 1980. After the causative agent of AIDS was identified in 1983, an avalanche of drugs was developed for the treatment of AIDS, at a pace of almost one compound per year.

Some of the compounds used for the treatment of HIV infection are also used for the management of chronic hepatitis B virus (HBV) infection, a condition that globally has a 10-fold higher incidence than HIV infection. For the treatment of HCV infection, a virus that until its identification in the 1989 was classified as non-A non-B, a host of new compounds was developed. These agents, termed DAAs, have appeared in rapid succession over the past few years. The DAAs (De Clercq, 2012) are poised to revolutionize the treatment of HCV which has for the last decade been dominated by the combined administration of pegylated interferon with ribavirin.

As detailed in this text, the strategies currently used, and/or considered, for the treatment of virus infections, are focused on

1. HIV [E. De Clercq (reverse transcriptase inhibitors and protease inhibitors), E. De Clercq (acyclic nucleoside phosphonates), and Y. Pommier (integrase inhibitors)],
2. HCV [J.M. Pawlotsky (standard of care) and M.J. Sofia (nucleo(s)tide polymerase inhibitors)],
3. HBV [S.J. Hadziyannis, D. Vassilopoulos, and E. Hadziyannis (management of HBV infection)],
4. HSV [A. Vere Hodge and H.J. Field (helicase/primase inhibitors)],
5. VZV [G. Andrei and R. Snoeck (VZV inhibitors)],
6. influenza virus [E. van der Vries, M. Schutten, P. Fraaij, C. Boucher, and Osterhaus (anti-influenza virus agents)].

Given the rapid advances made in the development of antiviral drugs, this is a particularly appropriate time for a review of this progress and its implications for the future of this uniquely productive field.

Erik De Clercq

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CHAPTER ONE

Antiviral Agents for Herpes Simplex Virus

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Contents

Abstract

This review starts with a brief description of herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), the clinical diseases they cause, and the continuing clinical need for antiviral

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chemotherapy. A historical overview describes the progress from the early, rather toxic antivirals to acyclovir (ACV) which led the way for its prodrug, valacyclovir, to penciclovir and its prodrug, famciclovir (FCV). These compounds have been the mainstay of HSV therapy for two decades and have established a remarkable safety record. This review focuses on these compounds, the preclinical studies which reveal potentially important differences, the clinical trials, and the clinical experience through two decades. Some possible areas for further investigation are suggested.

The focus shifts to new approaches and novel compounds, in particular, the combination of ACV with hydrocortisone, known as ME609 or zovirax duo, an HSV helicase–primase inhibitor, pritelivir (AIC316), and CMX001, the cidofovir prodrug for treating resistant HSV infection in immunocompromised patients. Letermovir has established that the human cytomegalovirus terminase enzyme is a valid target and that similar compounds could be sought for HSV. We discuss the difficulties facing the progression of new compounds.

In our concluding remarks, we summarize the present situation including a discussion on the reclassification of FCV from prescription-only to pharmacist-controlled for herpes labialis in New Zealand in 2010; should this be repeated more widely? We conclude that HSV research is emerging from a quiescent phase.

ABBREVIATIONS

ACV acyclovir or aciclovir
AD Alzheimer's disease
AIC316 AiCuris helicase–primase inhibitor (BAY 57-1293 or pritelivir)
BAY 57-1293 AiCuris helicase–primase inhibitor (AIC316 or pritelivir)
BVDU bromovinyldeoxyuridine, brivudin
CDC Centers for Disease Control and Prevention (USA)
CDV cidofovir
CMX001 hexadecyloxypropyl-cidofovir
DNA pol DNA polymerase
FCV famciclovir
FDA Food and Drug Administration (USA)
FOS foscarnet (phosphonoformic acid)
H2G omaciclovir
HK herpes keratitis
HPI helicase–primase inhibitor
HSV herpes simplex virus
IDU idoxuridine (5-iodo-2′-deoxyuridine)
i.v. intravenous
ME609 zovirax duo
PCV penciclovir
PK pharmacokinetics
$T_{1/2}$ half-life
TFT trifluorothymidine
TK thymidine kinase
VACV valacyclovir
VZV varicella-zoster virus
wt wild type

1. INTRODUCTION

1.1. The Herpesviridae

Herpes simplex virus (HSV) is a member of the alphaherpesvirus subgroup of the Herpesviridae. This is a family of large, double-stranded DNA viruses which are widely distributed among animals and man. The various members of this family have evolved complex relationships with their particular hosts over tens of millions of years (Davison, 2007). A notable feature of HSV is that infections are lifelong. Following primary infection which may, or may not, be accompanied by clinical signs of disease, the virus typically establishes a latent infection in the neurons of the peripheral nervous system. During periods of latency, no infectious virus is detected in the host and the quiescent virus provides a special challenge for antiviral chemotherapy. Antiviral compounds, which inhibit virus functions involved in the virus replication cycle, are inactive during the latent phase, and to date, no therapy has succeeded in eliminating latent HSV. Latent HSV may reactivate from time to time and recurrence of infectious virus, delivered via the sensory nerve supply to the skin, gives the possibility of transmission to a new susceptible host. Often (but not in all cases), recurrences of infectious virus are accompanied by the classic signs of recurrent herpes. These are typically lesions at or near the mucocutaneous junctions, for example, on or near the lip or genitalia.

1.1.1 HSV-1 and HSV-2 are closely related viruses

Genetic analysis reveals that HSV comprises two closely related viruses: HSV-1 and HSV-2. The former is most frequently associated with recurrent oral lesions (herpes labialis known as cold sores) and HSV-2 with recurrent genital lesions (herpes genitalis or genital herpes). Recent surveys suggest that seropositivity for HSV in Europe varies widely by country but approximately 50–80% of adults are infected with HSV-1 and a lesser proportion (4–24%) with HSV-2 (Pebody et al., 2004). It was of interest that this survey showed no protective effect of prior infection with HSV-1 in susceptibility to HSV-2 although the clinical signs may be less in some cases. In recent years, there appears to have been an increase in the seroprevalence of HSV-2. Furthermore, there appears to be an increasing prevalence of isolates of HSV-1 from genital lesions suggesting a change in sexual practices (Gilbert et al., 2011). The frequency of recurrent lesions varies among seropositive individuals for both types of virus from never or very rare to

many times per year with consequent frequent suffering in such patients. However, HSV-1 genital infections appear to produce recurrent lesions less frequently than HSV-2 genital infections (Laferty, Coombs, Benedetti, Critchlow, & Corey, 1987; Wald, Zeh, Selke, Ashley, & Corey, 1995). Similarly, recurrent oral lesions due to HSV-2 are less frequent than HSV-1. In any case, the treatment or suppression of HSV lesions in patients prone to suffer recurrences accounts for the majority of HSV antiviral chemotherapy.

1.2. Rarer forms of HSV disease

As well as causing the familiar labial and genital herpes, other forms of disease occur, albeit at a much lower frequency. Herpes keratitis (HK) and conjunctivitis are recurrent infections of the eye usually caused by HSV-1. In some cases, the virus may infect the deeper tissues of the eye (stromal tissue) and together with the concomitant scarring may produce permanent damage to eyesight (Farooq & Shukla, 2012). At the advent of antiviral chemotherapy in the 1960s, this was one of the first manifestations of herpes disease to be treated successfully using nucleoside analogue inhibitors applied topically (see below). HSV-1 infection may also result in life-threatening encephalitis. Although this condition is relatively rare (about 1 per million adults each year), it remains the most common cause of encephalitis and prompt antiviral therapy for this condition is essential to prevent disease progression (Whitley, 2006). Infection with HSV at or immediately after birth can result in generalized neonatal herpes. Again, this type of infection can also result in death or permanent damage to the neonate if untreated (Whitley, 2004).

Bell's palsy (face drop) is another less common clinical manifestation long thought to be due to HSV-1 although definitive proof is still lacking. Gronseth and Paduga (2012) reviewed the results of trials including antiviral therapy over the previous decade. Steroid therapy was found to be helpful but little or no benefit was reported for specific antiviral therapy although the duration of therapy based on acyclovir (ACV) was relatively short (2–5 days). To date, there has been no report to our knowledge on the possible benefit of famciclovir (FCV) or extended periods of antiviral therapy.

HSV can produce extensive lesions after gaining entry through damaged skin causing, for example, eczema herpeticum (Studdiford, Valko, Belin, & Stonehouse, 2011; Wollenberg, Zoch, Wetzel, Plewig, & Przybilla, 2003)

or herpes gladiatorum (Anderson, 2006). It should be noted that antiviral therapy is very effective in both these cases.

Finally, HSV is a particular problem in immunocompromised individuals (Fatahzadeh & Schwartz, 2007), for example, undergoing bone marrow transplant. In these cases, lesions caused by HSV-1 or -2 may become extensive and prolonged. Furthermore, prolonged HSV therapy in immunocompromised patient is likely to be associated with the development of antiviral resistance (see below).

2. HISTORICAL OVERVIEW: THERAPIES FOR HSV INFECTIONS

2.1. Early progress toward highly selective and effective antivirals

The story of antiviral therapies for herpes started at Yale, USA, in 1959. A team, led by the late William (Bill) Prusoff, synthesized idoxuridine (IDU) which was to be the first Food and Drug Administration (FDA)-approved antiviral drug, used to treat HK. For this work and for his long career in antiviral therapy, Bill was affectionately known as the "father of antiviral chemotherapy." Trifluorothymidine (TFT) followed, also to treat HK. Although still in use today, both IDU and TFT are limited by their toxicities to topical treatment of eyes infected with HSV. Although the introduction of these compounds represented a big step forward, their limitations seemed to reinforce the widely held view that truly selective and effective antiviral therapies were an impossible dream.

Vidarabine had activity against both HSV-1 and HSV-2. It was sufficiently selective to allow systemic administration to seriously ill patients, mainly those with herpes encephalitis although low solubility required relatively large volumes to be administered. Vidarabine has now been superseded by much safer drugs, and it is no longer commercially available.

In 1977, the Wellcome group published their discovery of acyclovir (ACV), spelt aciclovir in some countries. In one "quantum leap," this discovery moved antiviral chemotherapy from poorly selective agents to a highly active and selective therapy which has been taken by individual patients for years, even decades, with remarkably few side effects. A true antiviral therapy was no longer a dream (Crumpacker, Schnipper, Zaia, & Levin, 1979; Elion et al., 1977).

The Wellcome group investigated the mechanism of action that offered an explanation for the exceptional antiviral selectivity of ACV. ACV can pass easily into and out of cells but remains inactive. In cells infected with HSV (or varicella-zoster virus, VZV), the viral thymidine kinase (TK) is able to phosphorylate ACV to its monophosphate (ACV-MP), a surprising observation as ACV is a purine analogue. Having been converted into a charged molecule, ACV-MP is trapped within the virus-infected cell. Cellular kinases convert ACV-MP to the di- and triphosphates (ACV-DP and ACV-TP), the latter being the active form of the drug. In this way, the virus provides a "safety gate" by which the antiviral compound is activated. The viral DNA polymerase (DNA pol) can use ACV-TP as a substitute for GTP and attaches ACV to the end of the growing viral DNA chain. As ACV lacks a second hydroxyl group, there is no possibility of adding another residue and so ACV has been referred to as an "obligate DNA chain terminator."

The discovery of ACV prompted other research groups to search for new nucleoside analogues which could make use of this "safety gate." Within a short time, publications reported on brivudin (BVDU), bromovinylarauridine (BV-araU), ganciclovir (GCV), penciclovir (PCV), and omaciclovir (H2G). BVDU has been approved for therapy of HSV eye infections and herpes zoster, first in Germany and then in several other European countries but not in United Kingdom or United States due to safety concerns. GCV is not used to treat HSV but it and its prodrug valganciclovir have become the drugs of choice for the management of human cytomegalovirus (HCMV) in immunocompromised patients. The prodrug of H2G, valomaciclovir stearate, is being evaluated in clinical trials for the treatment of herpes zoster (shingles) (Tyring et al., 2012) and infectious mononucleosis (glandular fever) (Balfour et al., 2009). Of these compounds following on from ACV, only PCV has good activity against HSV-1 and -2 and VZV together with a good safety profile in preclinical toxicology. Now, some two decades later, ACV and PCV, mostly in the form of their prodrugs (valacyclovir (VACV) and famciclovir (FCV), respectively), are the only therapies for HSV-1 and -2 widely available worldwide. These compounds have also become the mainstay of therapy for VZV (Chapter 4).

Although the oral availability of ACV (Fig. 1.1A) was poor (about 20% in human subjects), it was sufficient to allow good clinical benefit, especially when high doses were taken frequently. Because PCV had such poor (<10%) oral bioavailability in animal models, the search for a prodrug was started early during the research phase. FCV (Fig. 1.1B) was the

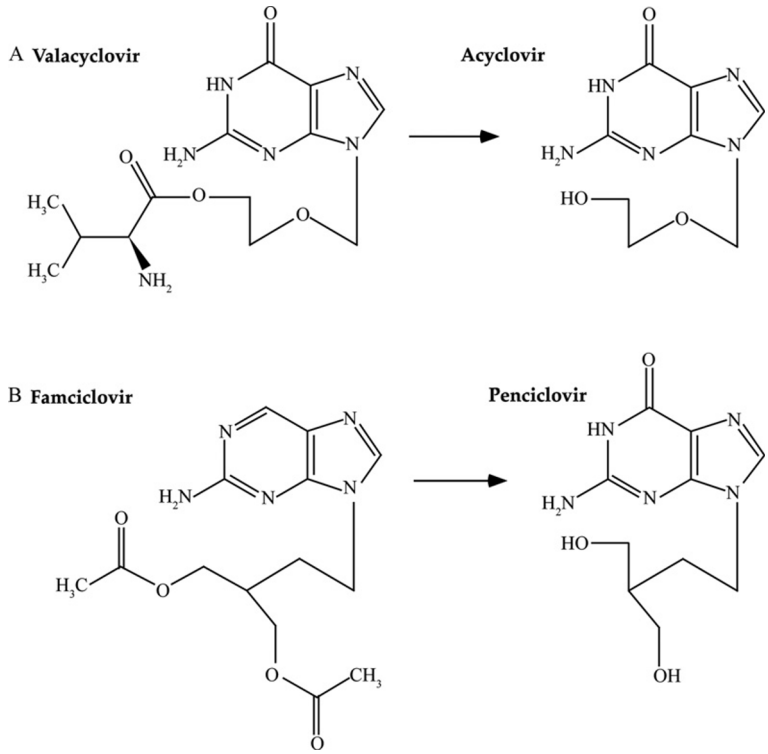


Figure 1.1 The structural formulae of (A) valacyclovir, acyclovir, (B) famciclovir, penciclovir. These guanosine nucleoside analogues and their orally bioavailable prodrugs are the current mainstay for treatment or prevention of HSV.

compound selected for development (Jarvest, Sutton, & Vere Hodge, 1998; Vere Hodge, Sutton, Boyd, Harnden, & Jarvest, 1989), and FCV became the first licensed oral prodrug for an antiviral agent. The good bioavailability (77%) of PCV from FCV prompted the search for an oral prodrug for ACV; VACV (Fig. 1.1A) was selected. All three compounds for oral therapy, ACV, VACV, and FCV are now available generically. As FCV and VACV have shown either equivalence or superiority compared with ACV in clinical trials and as their dosing regimens are certainly more patient-friendly than ACV, there seems to be little reason for using ACV orally. Of course, ACV and PCV are the compounds of choice for the intravenous (i.v.) treatment of seriously ill patients, although it should be noted that i.v. PCV is not licensed in the United States. FCV and VACV are often regarded as having

similar clinical efficacies but their preclinical evaluation revealed some marked differences which may not, as yet, have been fully evaluated in clinical trials. Below, we suggest some novel designs for clinical studies.

2.2. ACV and PCV: Preclinical evaluations

ACV and PCV share a similar mode of action in that both are selectively activated in HSV-infected cells. There are, however, some potentially important differences. PCV makes good use of the "safety gate" because its affinity for viral TK is about 100-fold higher than that for ACV, leading to 100-fold higher concentrations of PCV-TP than ACV-TP. The position is reversed when comparing the affinities of their triphosphates for HSV DNA pol. The affinity of ACV-TP for the viral DNA pol is about 100-fold that of PCV-TP. Both compounds act as competitive inhibitors of the viral DNA pol and also are substrates, being into viral DNA. Because ACV has only a single hydroxyl group, there is no possibility of a further nucleotide being added to the ACV residue and therefore ACV has been called an "obligate" DNA chain terminator. Although DNA chain extension was seen following incorporation of PCV when the template strand had a single deoxycytidine base, there was no extension when the template strand had two deoxycytidine bases (Earnshaw et al., 1992). Under conditions designed to represent HSV-2-infected cells, with the four natural nucleotide triphosphates, ACV-TP and PCV-TP at their expected concentrations, PCV-TP was more effective than ACV-TP in inhibiting DNA chain extension by HSV-2 DNA pol (Earnshaw & Vere Hodge, 1992). In five replicates, the DNA extended by <20 nucleotides with PCV-TP but extended by 40–70 nucleotides with ACV-TP.

In standard cell culture antiviral assays in which the compounds are present continuously, ACV and PCV have similar activities (Vere Hodge & Perkins, 1989). It seems that the differing enzyme affinities compensate each other for antiviral activity in this assay. Nevertheless, with ACV establishing a remarkable safety record in the clinic, it was a comfort to know that PCV was making such good use of the same "safety gate." However, standard cell culture assays are not a good model for human pharmacokinetics (PK) as these assays do not reflect the varying concentrations of antiviral compound in plasma following oral dosing to patients, especially for ACV and PCV with relatively short plasma half-lives ($T_{1/2}$) of 2–3 h. Therefore, the stability of the active triphosphate becomes an important factor; ACV and PCV differ markedly in the stabilities of their triphosphates and in the durations of their

antiviral effects. ACV-TP has short half-lives ($T_{1/2}$) in HSV-1- and HSV-2-infected cells, 0.7 and 1.0 h, respectively. In contrast, PCV-TP has much greater stability ($T_{1/2} = 10$ and 20 h) in HSV-1- and HSV-2-infected cells, respectively. In a simple adaptation of conventional HSV assay, once-daily dosing in patients was modeled by incubating infected cells with either PCV or ACV for just 2 h and then measuring infectious virus at 19 h (Vere Hodge & Perkins, 1989). PCV retained good activity whereas ACV had much reduced activity.

In cell culture using hollow fiber cartridge, it is possible to simulate human PK (Hamzeh, Schaad, & Lietman, 1995). With twice-daily dosing with ACV, not only the levels of ACV in the circulating medium fall to a low level between doses but the intracellular levels of ACV-TP fall to <2 pmol/10^6 cells having risen to about 20 pmol/10^6 cells soon after dosing. In this hollow fiber cartridge system, the trough levels of PCV-TP increased over the four doses from about 100 pmol/10^6 cells at 12 h to about 350 pmol/10^6 cells at 48 h. Because the trough levels of PCV-TP increased over four doses, this model suggested that a loading dose would improve clinical efficacy. Over a decade later (since 2006), for recurrent HSV-1 and HSV-2 infections, loading dose regimens have been incorporated into clinical trial regimens. However, for primary HSV infections (and VZV), this has not yet been adopted into clinical practice.

The high stability of PCV-TP has been shown to give PCV an advantage in both cell culture assays and in animal models (Bacon & Schinazi, 1993; Field, Tewari, Sutton, & Thackray, 1995; Vere Hodge & Perkins, 1989). When looking for duration of antiviral protection after treatment for 18 h, virus was measured at days 2, 4, and 6. In ACV-treated cultures, there were high levels of virus at all the time points. It seems reasonable to suppose that the ACV-TP levels would have declined soon after the removal of ACV, so allowing virus replication to recommence. In contrast, no virus was detected in PCV-treated cultures. This experiment suggests that, once high levels of PCV-TP are formed within an infected cell, that cell dies naturally from the HSV infection before PCV-TP levels fall below inhibitory concentrations. In an HSV-1 murine immunosuppression model, following treatment for 5 or 10 days with VACV, there were resurgences of virus replication in both the ear and brain stem (Fig. 1.2). There were no such resurgences following treatment with FCV (Field et al., 1995; Thackray & Field, 1996). A similar explanation may account for this difference—once there is a high level of PCV-TP in an HSV-infected cell, that cell dies before viral DNA synthesis can restart.

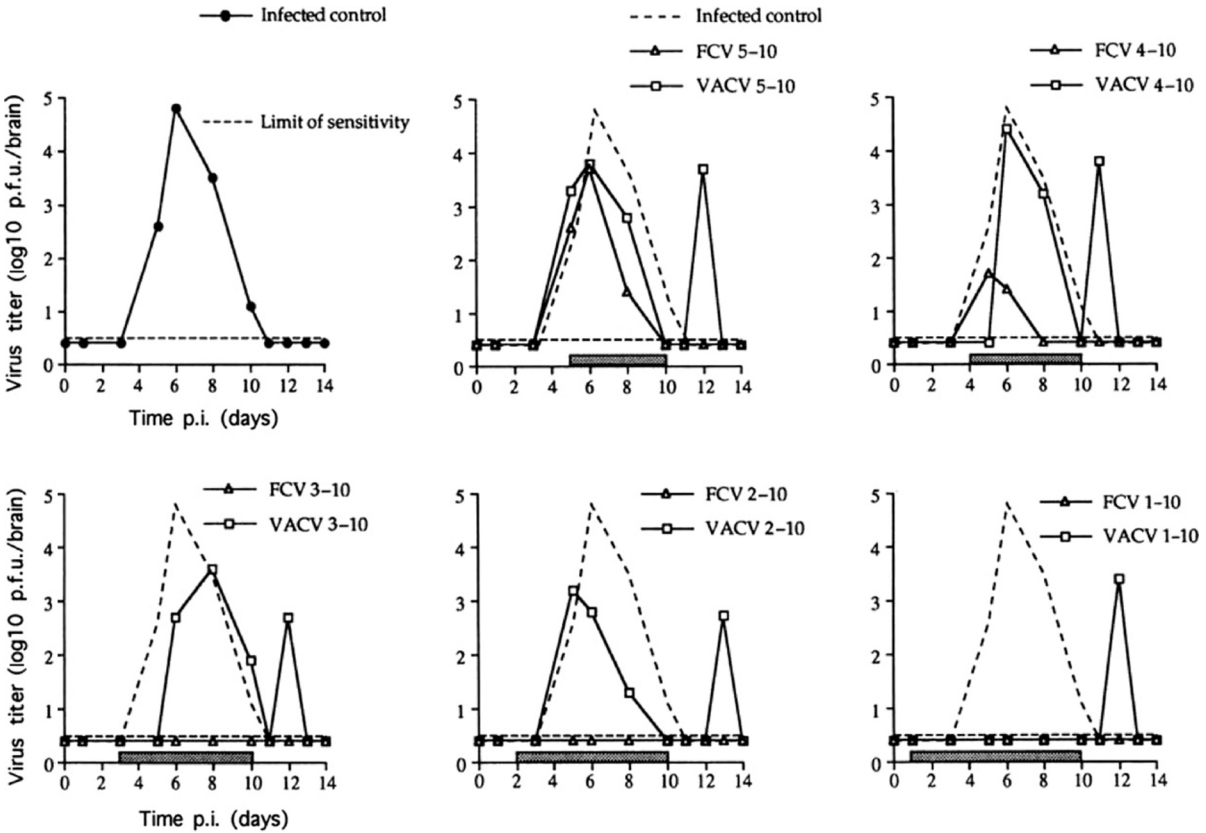


Figure 1.2 Mice were experimentally infected with HSV-1 and therapy commenced at various times after inoculation. These data show that, when therapy with VACV (squares) is terminated, there is a resurgence of infectious virus in the tissues. Resurgence was not observed on termination of FCV (triangles) therapy. The control, untreated, is shown in the first panel (solid circles) and the control curve is shown by a dotted line in the subsequent panels. *Adapted from Thackray and Field (1996) with permission from Oxford University Press.*

In a series of experiments in the HSV-1 murine immunosuppression model (Field et al., 1995; Thackray & Field, 1997), in addition to the resurgence of virus replication noted earlier, other marked differences between the efficacies of FCV and VACV were discovered. When dosing was started at days 1, 2, 3, 4, or 5 days after infection, FCV-treated mice promptly restored their weights, becoming similar to those of the sham-infected mice. In contrast, VACV-treated mice only partially regained their normal weight which remained lower than those of sham-infected controls (Fig. 1.3).

When investigating the effect on latency, neither FCV nor VACV were able to prevent the establishment of latency (as evidenced by the detection of HSV DNA) even when treatment was started early (0–5 days) after primary infection following virus inoculation in the left ear (Field & Thackray, 2000). However, there was a marked difference between the compounds when looking at rates of experimental reactivation from explanted trigeminal ganglia. VACV, with treatment starting 1 or 2 days after infection, had little effect on the ipsilateral (left) ganglia compared to controls although it clearly had an effect on the contralateral (right) ganglia. The proportion (%, $n = 16$/group) of mice positive for virus from contralateral ganglia with treatment starting at 1 or 2 days were 0% or 0%, respectively, versus placebo (63%). In contrast, FCV commencing from 0 to 5 days after infection significantly reduced reactivations from both ipsilateral and contralateral ganglia (Thackray & Field, 1996). With treatment starting 1, 2, or 3 days after infection, the proportion (%, $n = 16$/group) of mice positive for virus from ganglia (ipsilateral and contralateral) were 0% for all groups. With FCV treatment starting 4 or 5 days after infection, the proportion of mice positive for virus were 13% or 38% (ipsilateral ganglia) and 0% or 0% (contralateral ganglia), respectively. Very similar results were obtained from dorsal root ganglia.

A paper by LeBlanc, Pesnicak, Godleski, and Straus (1999) was critical of the above series of papers, stating that their own conclusions differed and that they found that there were no marked differences between FCV and VACV. In their model, both compounds showed comparable activities in increasing the proportions of mice surviving the HSV-1 infection but neither compound had any effect on reducing latency. This was an influential paper as the last named author was the late Stephen Straus, a highly respected figure in antiviral research. However, there were important differences between the models used by Field and Thackray and LeBlanc et al. The latter used a large inoculum of a highly virulent strain of HSV-1 which killed all the untreated mice by day 5. Most critically, in their model, both eyes were inoculated so that latency was probably already established in the ganglia

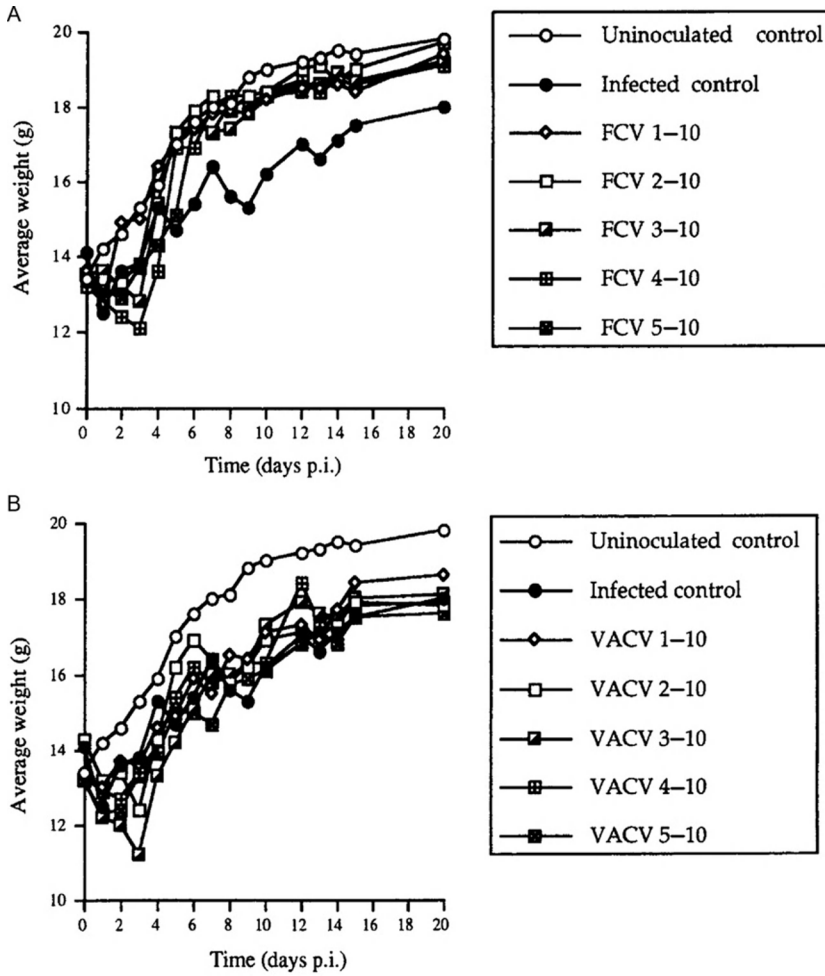


Figure 1.3 Mice lose weight following experimental infection with HSV-1 and weight is a sensitive measurement of disease severity. The graphs show how weight loss is affected by drug administration starting at various times after infection and continuing to day 10. (A) Famciclovir starting from day 1 to 5, weight of infected mice returns almost to weight of uninfected controls. (B) Valaciclovir, when treatment started early, gives some restoration of weight but, after the end of treatment (day 10), no group has weight restored to that of uninfected controls. *Adapted from Thackray and Field (1996) with permission from Oxford University Press.*

on both sides before the start of therapy on day 1 after infection. In their discussion, they say "The differences highlighted between these studies and the present one are few, but may underlie the distinct results. Thackray and Field infected mice with strain SC16 by the ear pinna route in which the virus ascends to the ganglia and then descends to induce an acute zosteriform

eruption. It is possible that the regional cutaneous spread of HSV in this model provides an extended period in which ganglia are seeded with virus, during which one antiviral might successfully intercede better than the other." We would wholly agree. They continue "We are confident, however, that our in vivo reactivation and PCR studies more accurately reflect levels of latent virus than the methods used in those earlier experiments" but they offer no explanation how their model reflects a clinical situation better than the Thackray and Field model.

2.2.1 Mechanism for latency efficacy: A hypothesis

The inability of FCV to prevent the establishment of latency but its activity in reducing the efficiency with which the virus could reactivate from the ganglia is similar to the results expected when using a TK-defective strain of HSV. When considering the difference in this latency mouse model between FCV and VACV, one of the marked differences in the mode of action of PCV and ACV may be important; PCV has a much higher (100-fold) affinity than ACV for HSV TK; inhibition constants, K_i 1.5 μM (Larsson et al., 1986) and 173 μM, respectively (Datema, Ericson, Field, Larsson, & Stenberg, 1987). Putting these factors together and building on the work by Sawtell (see below), a hypothesis was presented (Vere Hodge, 1999).

Sawtell (1997) was the first to show that individual neuronal cells may have between 1 and over 1000 HSV genomes. In a further study with TK-ve strain of HSV-1 (Thompson & Sawtell, 1999), it was shown that latently infected cells with high copy number could be established in the absence of significant virus genome replication within the neurons. This suggests that individual neurons can be infected by many HSV TK-defective virions. However, their results demonstrated that the functional TK is required for efficient replication within the ganglion for the effective establishment of latency.

In the hypothetical mechanism proposed by Vere Hodge (1999), the following terms were used. A latently infected cell has at least one viral genome (in a circular form); individual cells may have from 1 to >1000 viral genomes. An immature latently infected cell has one or only a few circular viral genomes of TK-positive HSV. A competent latently infected cell is formed from an immature latently infected cell via replication of the virus within that cell to give many viral genomes (e.g., >1000). Presumably, this process has to take place in the mouse early after infection, before the immune system controls further viral replication of the genome. Considering the FCV-dosing periods, this viral replication must be within about

10 days of infection. If this were not so, all latently infected cells would eventually have >1000 genomes. The proposed mechanism was that FCV, via inhibition of HSV TK by PCV, prevents the development of immature latently infected cells into competent latently infected cells. In summary, by inactivating the viral TK, FCV effectively converts a wild-type (wt) virus into a "TK-negative" virus. VACV does not have similar activity because the inhibition of viral TK by ACV is about 100-fold less than by PCV. This idea was given support by a review (Efstathiou et al., 1999) in which it was reported that compounds which inhibit viral TK, although they do not inactivate the virus, have been shown to decrease the rate of reactivation in cell cultures as well as in animal models (Watkins et al., 1998).

For FCV to have clinical activity, it is assumed that the inability of ganglia to reactivate in cell culture predicts a reduced potential to cause virus recurrences in patients. In mice, strong support for this link was reported by Sutton, Kingsley, Wells, Bartus, and Esser (1998). FCV or VACV treatment was started 2 days after infection. The proportions of ganglia able to reactivate were reduced from 100% in controls to 20% in FCV-treated mice. This was associated with a reduction of induced reactivations *in vivo* from 72% to 24%. In contrast, VACV had no effect.

For ACV and VACV, there is a very short window of opportunity for reducing latently infected cells, the treatment must be started at infection or very soon afterward (within hours). For FCV, the proposed mechanism suggests that there is an additional window of opportunity. The affinity of PCV for HSV TK inhibits the relatively slow process (within days) converting an immature latently infected cell into a competent latently infected cell.

Although it is difficult to extrapolate these latency results to man, especially the time scale, we give three clinical situations in which this unique activity of FCV could possibly lead to clinical benefit (see below). In addition, there is one published clinical observation that hints at a possible clinical effect on latency (see Section 3.2 below). One firm conclusion can be derived from all this preclinical work that there are sufficient differences between FCV and VACV that both compounds should be tested in each clinical situation. If a negative result is obtained with one antiviral, it should not be assumed that there is no need to test the other.

2.3. ACV, PCV, and their prodrugs: Clinical studies

2.3.1 Herpes labialis (cold sores) caused by HSV-1

Herpes labialis, when it occurs as a primary infection in children, is often subclinical or mild. However, in moderate or severe cases, therapy is

beneficial. Oral ACV suspension (15 mg/kg, five times daily for 7 days) was compared with placebo. ACV was shown to markedly reduce the duration of lesions from 9 days to 4 days and virus shedding from 5 days to 1 day, respectively. No trials have been done with FCV or VACV, but 500 or 1000 mg, respectively, twice daily for 7 days would be logical regimens (Cernik, Gallina, & Brodell, 2008).

Although ACV appeared from tissue culture and animal studies to lack toxicity, there was extreme caution at first regarding its use as an oral therapy for recurrent herpes labialis. Consequently, topical ACV was promoted for herpes labialis therapy even though its efficacy was limited. PCV cream, applied every 2 h while awake, did speed recovery in various lesion parameters; however, this was not a patient-friendly regimen. Oral ACV, VACV, and FCV are now regarded as extremely safe. Furthermore, there has been no evidence for increased incidence of antiviral resistance and therefore oral therapy is now considered appropriate for herpes labialis. From many trials, it eventually became clear just how important it was to start any therapy as soon as possible after the first symptoms were noticed by the patient. This meant that patients who suffer recurrent lesions must be given the medication with instructions keep the first dose at hand at all times and to initiate therapy as soon the first prodromal signs are noted (e.g., a tingling sensation of the lip).

A patient-initiated trial (ideally, within 1 h of the onset of symptoms) of FCV in comparison with placebo (1500 mg once or 750 mg twice for a 1 day only) reported that the single large dose was equally effective as two smaller doses (Spruance et al., 2006). Again, marked clinical benefits of therapy were observed with a significant reduction in the duration of pain and tenderness (each by 1 day or 41%). The times to healing of the primary lesions and of all lesions were accelerated (each by 2 days or about 30%) and the time to return of normal skin was reduced by 2.5 days or 36%.

Short patient-initiated treatment with VACV for herpes labialis has also been assessed. For example, VACV administered at 2000 mg twice for a single day or 2000 mg twice on day 1 followed by 1000 mg twice on day 2 days were compared with placebo (GlaxoSmithkline, 2011). Whereas the 2-day regimen did not provide additional benefit, the duration of the lesion was reduced by about 1 day.

In neither of these patient-initiated HSV-1 trials were there significant increases in the number of "aborted" lesions (prodromal signs or papule did not progress to lesions). Whereas only a small proportion of patients (<10%) experience frequent recurrences, for these cases suppressive therapy

may be appropriate. This is feasible given the perceived safety of the compounds and FCV (500 mg twice daily) or VACV (1000 mg once daily), having been shown to be effective in preventing recurrent lesions in such patients, would be reasonable regimens.

2.3.2 Genital herpes

2.3.2.1 Primary genital herpes (usually caused by HSV-2, sometimes caused by HSV-1)

Patients (who may or may not be already infected with labial herpes), when they encounter genital herpes for the first time, are described as having first-episode genital herpes. However, for patients already seropositive for HSV-1, patients are not protected from HSV-2 infection (Pebody et al., 2004) but their first-episode of HSV-2 is likely to be less severe than a true primary infection (defined as first infection with HSV-2 in an HSV-1-seronegative person). If the first-episode is caused by HSV-1, the disease can be severe but the likelihood of recurrences is less than with HSV-2. Therefore, it is helpful to test for the HSV type so that the patient can be advised about the future management of their infection. In the early days, oral ACV was the standard treatment although ACV has now been largely superseded by VACV. VACV (1000 mg twice daily) was compared with ACV (200 mg five times daily) for 10 days with physician-initiated treatment of first-episode genital herpes commencing within 72 h of onset of symptoms. In this trial, the times to cessation of virus shedding (median, 3 days) or cessation of pain (median 5 days) or time to healing (median 9 days) were similar for both treatment groups.

In a dose-ranging study, FCV (125-, 250-, and 500-mg doses, three times daily) was compared with ACV (200 mg five times daily) for 5 or 10 days of therapy. Both the 250- and 500-mg doses of FCV gave comparable benefit to ACV. Although not approved by the FDA, FCV (250 mg three times daily for 5–10 days) is recommended by the Centers for Disease Control and Prevention (CDC). VACV (1000 mg twice daily for 7–10 days) is both FDA approved and CDC recommended.

2.3.2.2 Therapy for recurrent genital herpes—Episodic therapy

As for herpes labialis discussed earlier, the key to successful therapy of genital herpes is to initiate therapy as early as possible after the onset of the first symptoms. Recently, there has been a focus on short treatment regimens (Aoki et al., 2006). In one placebo-controlled trial of FCV (1000 mg twice in 1 day only), patients were trained to take a pretreatment swab to enable

laboratory detection of virus shedding and were instructed to self-initiate therapy, preferably within 6 h of the first symptoms. Under the protocol for this trial, patients were required to attend the clinic within 24 h after starting their recurrence. In the event, the median time for starting therapy was just 2.5 h after the first prodromal sign. FCV therapy was shown to be beneficial with a reduction in the median time to healing by nearly 2 days (from 6.1 to 4.3 days for placebo and FCV, respectively), and there was a 30% reduction in each of the symptoms, burning and tingling and a 40% reduction in each of the symptoms pain, itching, and tenderness. In intention-to-treat analysis, an increase in the number of aborted lesions was reported (23% for FCV compared with 13% for placebo) and this was shown to be significant ($p = 0.003$). When just those patients, whose recurrence was confirmed by a PCR +ve swab for virus detection, the reduction was highly significant 21% compared with 5%, respectively ($p = 0.001$).

In another trial that was of similar design, single-day FCV treatment was compared with VACV treatment for 3 days (Abudalu, Tyring, Koltun, Bodsorth, & Hamed, 2008). For both groups, the times to resolution of symptoms and for healing were similar. However, there was a trend to more aborted lesions in those patients treated with FCV than VACV (virus culture positive, 22% vs. 13.5%, respectively, $p = 0.3$). From this study, it was concluded that both FCV (1000 mg twice for 1 day) and VACV (500 mg twice daily for 3 days) were equally effective in reducing the duration of the symptoms of genital herpes and both probably increased the likelihood that papules did not develop into lesions, with a trend in favor of FCV.

2.3.2.3 Therapy for recurrent genital herpes—Suppressive therapy

Mindel et al. (1988) tested ACV at different doses (200–800 mg) for time to first recurrence in a 12-week trial. The number of doses/day was more important than the dose/day. With 800 mg once daily, nearly 60% of the patients remained recurrence free, but this was much less than the 95% remaining recurrence free with 200 mg four times daily. Likewise, 400 mg twice daily was better (80% recurrence free) than 800 mg once daily but less good than 200 mg four times daily. However, the 400-mg dose twice daily became the accepted best compromise between efficacy and patient-friendly dosing regimen.

Using this optimal dose of ACV in a suppression trial which became extended to 6 years (Cernik et al., 2008), ACV therapy during the first year increased the chance of patients remaining recurrence free (44% vs. 2% with

placebo) and increased the median time to first outbreak (246 days vs. 18 days, respectively). There appeared to be a gradual improvement over the years so that, during the fifth year, 70% of the ACV-treated patients were recurrence free. This trial convincingly proved that ACV suppression therapy gave excellent clinical benefit to patients, greatly improving their lives; furthermore, this was achieved with remarkably few adverse events.

During the initial evaluation of FCV, various FCV regimens (125 mg once or twice daily, 250 mg once or twice daily, and 500 mg once daily) were compared. The most effective dose for prolonging the time to the first recurrence was 250 mg twice daily. In a larger, 1-year trial, this dose of FCV was compared with placebo. The proportions of patients remaining recurrence free during the year were 70% versus 20%, respectively (Tyring et al., 2003). In a further placebo-controlled study, in all FCV groups (125 mg three times daily, 250 mg three times daily, and 250 mg twice daily), the time to the first recurrence was increased (222–336 days vs. 47 days for placebo) (Diaz-Mitoma et al., 1998). However, the lower dose (125 mg) was less effective than that seen with either group of patients who received the 250-mg regimens.

VACV has also been shown to be highly effective in suppression of recurrent genital herpes. Initially, VACV (500 mg once daily) for 16 weeks was compared with placebo. The time to the first recurrence was >112 days compared to 20 days among those who received placebo. The proportions of patients who remained recurrence free at the end of the 16-week trial were 69% versus 9.5% (Cernik et al., 2008). A 12-month placebo-controlled trial compared various doses of VACV (250, 500, and 1000 mg once daily; 250 and 500 mg twice daily) with placebo. Of the patients receiving placebo, the proportion who remained free from recurrences was 5%. All patients taking VACV showed a benefit from therapy with the proportions remaining recurrence free being 22%, 40%, 48%, 50%, and 49%, respectively. When the data were analyzed in more detail, the interesting finding emerged that patients who suffered the most frequent rate of recurrences (10 or more recurrences in the previous year) required higher dose therapy in order to yield benefit compared to those patients who suffered fewer recurrences (Cernik et al., 2008). It was noted that patients with 10 or more recurrences per year required twice-daily dosing with 500 mg or 1 g once a day to be effective.

As well as causing suffering to the patient experiencing recurrent herpes lesions, shedding infectious virus clearly poses the risk of transmission. Importantly, transmission often happens during asymptomatic virus

shedding. There has been only one clinical trial to investigate the possible effects of suppressive therapy on transmission to a susceptible seronegative partner (referred to as discordant partners). In this placebo-controlled trial, VACV reduced transmission by about 50% (GlaxoSmithkline, 2011). However, it must be noted that, in several trials with either FCV or VACV, careful monitoring for asymptomatic virus shedding demonstrated that no therapy completely eliminates the shedding of virus. Even though some of that virus shedding may be due to dosing interruptions, it is important to provide adequate advice to patients concerning the risks of transmission during suppression therapy.

3. CLINICAL EXPERIENCE WITH ACV, VACV, AND FCV THROUGH TWO DECADES

3.1. Clinical benefit

Undoubtedly, the patients gaining the most benefit from antiviral therapy have been those who used the drugs to prevent the frequent genital herpes recurrences previously causing much distress. Some patients have been on antiviral therapy (ACV, VACV, or FCV) for years with remarkably few safety concerns. Severe primary herpes labialis is fortunately uncommon but primary genital herpes, usually HSV-2 but increasingly HSV-1, is often severe and antiviral therapy is useful in reducing symptoms quickly. The established multiday (usually 5 day) regimens for treating recurrent genital herpes gave rather modest reductions in symptoms, but the new high-dose single-day treatment regimens give useful reductions in many clinical symptoms. Herpes labialis used to be treated with topical therapies but these gave little benefit. Using the experience gained with genital herpes, high-dose single-day regimens have given patients a worthwhile option.

In this normally healthy patient population, studies (e.g., Boon et al., 2000; Bacon, Boon, Schultz, & Hodges-Savola, 2002; Bacon, Levin, Leary, Sarisky, & Sutton, 2003; Christophers et al., 1998; reviewed, Field, 2001) have looked for any increase in resistant virus but the levels have remained below 0.5% throughout this period. All three compounds have gained a reputation for a safety record remarkably free from adverse events.

In the rarer but more serious clinical situations, antiviral therapy has become mandatory. For example, in HK, although therapy has been sight-saving, viral resistance has emerged as a potential problem (see below).

Another rare but devastating, life-threatening disease is adult and neonatal HSV encephalitis. Routinely, ACV (i.v.) is used at its maximum tolerated dose, followed by VACV during the recovery phase. Although not FDA-approved (Richman, Whitley, & Hayden, 2010), the generally recommended regimen is ACV, 20 mg/kg i.v. every 8 h for 14–21 days in neonatal patients and similarly in adults but with 10–15 mg/kg. Therapy has been lifesaving but there is room for considerable improvement (Skelly, Burger, & Adekola, 2012). Although adult mortality has been reduced from about 50% to 10%, the majority of patients are left with neurological sequelae. There appears to be reluctance on the part of clinicians to try PCV/FCV, and in some places, including the United States, there is the difficulty that i.v. PCV is not licensed. However, there is good reason to think that a better outcome may be possible. First, PCV can be administered at a higher dose than ACV as PCV is less prone than ACV to crystallization in the kidneys. Second, in the preclinical data in mice described earlier, there were no resurgences of viral replication following FCV treatment whereas there were resurgences of viral replication in the brains stem of mice treated with VACV.

Author note: Current guide lines recommend continuing ACV or VACV therapy for six months.

3.2. New opportunities for FCV and VACV?

The preclinical work described earlier suggested that FCV may have activity in reducing the potential for latency if treatment is started soon after primary infection. As the research work suggested that VACV does not have the same activity, it is important that both drugs are tested and not allow a negative result with VACV to stop the evaluation of FCV.

With an incubation period for HSV-2 of 2–7 days, more commonly nearer 7 days (NIH Web site) together with a reluctance to see a physician quickly after feeling the first symptoms, it is difficult to set up a trial starting treatment within a few days of infection. Although one trial was attempted (patients could be enrolled up to 3 days of rash onset) to investigate an effect on latency, none was found. However, there was an observational study (Ahmed & Woolley, 1996) which gave a hint that FCV may be more effective than ACV. Whereas 12/63 (19%) of patients treated with ACV had a recurrence in the 6 months following the first episode, only 1/24 (4.2%) of FCV-treated patients had a recurrence. Although the authors looked for possible explanations based on differences between the two patient groups,

for example, symptom duration before start of therapy, length of time since presumed infection, and viral type, there was no easy explanation. So the authors concluded that their observation supported the animal data.

There is, perhaps, a greater chance of detecting an effect in those clinical situations for which daily dosing may be used. In the trial investigating HSV transmission between HSV-seropositive subjects to their seronegative partners (so-called discordant couples), VACV or placebo was taken daily by the seropositive partner (transmission was reduced by about 50%; Corey et al., 2004). One could use a similar trial design but ask the seronegative partner to take the drug daily. This would not be expected to prevent transmission of HSV nor of the virus becoming latent, but it might reduce the probability of recurrent lesions. This approach may provide couples, who do not want to use barrier contraceptive techniques, with an alternative option.

Another possibility, herpes gladiatorum plagues the sport of wrestling, especially in high school during summer camps. For example, Anderson (2006) reported on a camp in Minneapolis, Minnesota, USA, attended by about 300 students. In an attempt to prevent the spread of herpes gladiatorum, wrestlers known to have herpes were required to take suppressive antiviral therapy. In spite of this, there were about 15–20 new cases of infection each year. In 2001, there were 57 new cases. The camp director decided that ACV should be given prophylactically to all exposed students. This reduced the new cases to three and stimulated the start of a prospective study with VACV. It would be possible to compare FCV with VACV given prophylactically to all the exposed students and follow those new cases of infection for recurrences.

Recent research (Itzhaki, 2004) has provided evidence linking HSV-1 to Alzheimer's disease (AD). Clearly, AD is not caused by HSV replicating as in an acute disease because the progression of AD is so slow. Perhaps, the immune system is able to limit any acute infection of HSV-1 but some reactivating latently infected brain cells may, via cell to cell spread, lead to new latently infected cells? As both FCV and VACV have good safety records, clinical trials have little to lose and much to gain. To date, funding for clinical trials has been lacking and, to our knowledge, FCV and VACV have not been compared. Perhaps, the U.S. "Orphan drug" route should be explored for helping development.

CMX001 administered orally is readily taken up by cells and catabolized to cidofovir (CDV) diphosphate which is the active inhibitor of HCMV DNA pol. Persistence of antiviral activity *in vivo* is due to the stability (half-life of 8–10 days) of CDV diphosphate. Somewhat surprisingly,

the prodrug seems to have a much better safety profile than CDV. In a Phase II trial for CMV, about 230 patients were enrolled and CMX001 (100 and 200 mg) was administered either once or twice weekly versus placebo for about 10 weeks. Unlike CDV, no nephrotoxicity or renal complications have been reported for CMX001, the most common side effect being reversible diarrhea. Thus, the Phase II clinical trial of CMX001 was very encouraging. Chimerix Inc. could consider evaluating CMX001 for resistant HSV infections in immunocompromised patients.

Although antiviral resistance has not emerged as a problem in otherwise healthy herpes labialis and genital herpes patients, resistance has been a consistent concern in immunocompromised patients. HSV recurrences can be both frequent and prolonged. Antiviral therapy is required and often given as suppressive therapy for prolonged periods. For many years, virus resistance has been a significant clinical problem (see below).

Over a period of more than 20 years, there have been many reports of HSV drug resistance in immunocompromised patients. It appears that the frequency of resistance has been influenced by the degree of immunosuppression and there is no clear indication that resistance is increasing with time.

4. ANTIVIRAL DRUG RESISTANCE

4.1. Introduction

In the early days of HSV therapy for ophthalmic HSV infections, there was some confusion in the literature between "treatment resistance" and "biochemical resistance." Subsequently, it was shown that resistance mutations are readily selected in tissue culture in the presence of any of the nucleoside analogue inhibitors (Field, 2001). In the case of TFT, however, selection had to be carried out in TK-negative cells in order to detect resistance mutants. Resistant viruses occur at relatively high frequency as spontaneous mutations in the virus isolates and were selected by their ability to multiply in the presence of the inhibitor—there was no evidence that ACV or PCV is mutagenic. HSV TK is not essential for virus growth in tissue culture; therefore, the majority of mutations were associated with abnormal expression of this enzyme. Later, it was shown that resistance mutations occur as polymorphisms in the HSV *DNA pol* gene leading to single-amino acid residue substitutions in the enzyme which has reduced affinity for the nucleoside analogue triphosphates while retaining enzyme activity.

4.2. Drug-resistant strains tested in HSV laboratory animal infection models

Animal studies in the 1980s showed that the majority of TK-defective strains of HSV showed markedly reduced fitness in the infection models. In particular, TK-defective strains showed reduced neuropathogenicity and an inability to reactivate from latency. By contrast, mutants with TK having altered substrate specificity for nucleosides analogues or DNA pol mutants often showed normal or near-normal growth and production of clinical signs in the infection models. Moreover, occasional TK-defective mutants have been described that appear to retain pathogenicity (e.g., Chatis & Crumpacker, 1991; Sakuma, Yamamoto, Kumano, & Mori, 1988).

The pyrophosphate analogue, foscarnet (FOS) is a direct inhibitor of HSV DNA pol, and it selects for drug resistance mutations in this virus-induced enzyme. The compound is considered too toxic for administration to otherwise healthy HSV patients. However, the compound is used to combat isolates of HSV from immunocompromised patients that have acquired resistance to nucleoside analogues.

Following several very large surveys of clinical isolates taken from otherwise healthy patients with genital or labial herpes, there has been no trend to increasing isolation of drug-resistant strains or loss of sensitivity of clinical isolates over the past two decades. It is not clear why resistance *in vivo* is rare whereas resistant strains are so readily selected in tissue culture. Most likely, this relates to the loss of fitness demonstrated by the majority of resistant mutants together with the complex pathogenesis involving growth of virus in differentiated cells including reactivation in neurons and multiplication in epidermal cells.

4.3. HSV drug resistance in ophthalmic infections

There are at least two situations where HSV antiviral drug resistance is important. It appears that resistance is more common in eye disease following antiviral treatment (Duan, de Vries, Osterhaus, Remeijer, & Verjans, 2008). This suggests perhaps that the "treatment resistance" with first-generation nucleoside analogues reported many years ago was probably caused by the selection of resistant mutants. Use of alternative therapies, including potentially toxic TFT, may be employed but multiple resistant strains are described. In the future, the selection of resistance may be avoided by commencing therapy with combinations of three or more different compounds as is now the established strategy for HIV and hepatitis virus therapies.

4.4. Drug resistance in immunocompromised patients

The other situation, where HSV drug resistance is an even more serious problem, is infection in immunocompromised patients (Reyes et al., 2003). Generally, ACV-resistant strains have been successfully treated with FOS (Chatis, Miller, Schrager, & Crumpacker, 1989). Clinical isolates from treatment-experienced immunocompromised patients may comprise mixtures of DNA pol and TK-resistant mutants together with normal viruses (Christophers & Sutton, 1987; Christophers, Sutton, Noble, & Anderson, 1986). Furthermore, when different drugs are employed, isolates may become coresistant to a variety of compounds (e.g., ACV and PCV, FOS, CDV). Fortunately, after an episode caused by a drug-resistant strain in immunocompromised patients, generally, further episodes appear to be due to reactivations of the original sensitive virus (Piret & Boivin, 2011). This is an area where much improvement to current management is required. The development of compounds inhibiting new antiviral targets (e.g., helicase–primase inhibitors (HPI) see below) provides an opportunity to reconsider therapeutic strategies. Given the possibility of two nontoxic compounds with different target enzymes as sites of antiviral action suggests that combinations of two or more active compound may be introduced from the outset as opposed to sequential use of alternative compounds following treatment failure.

4.5. Concept of genetic barrier

To prevent viral resistance emerging, the concept of increasing the genetic barrier was developed largely in response to the huge challenge posed by HIV. The concept of a genetic barrier is simple although putting it into practice is hard. When there are many possible mutations which can give resistance to an antiviral compound, the genetic barrier is low—with HIV, resistance generally appears within days. With compounds targeting the catalytic site of an essential virus enzyme, there may be only a few, possibly only one, mutation(s) which gives drug resistance and retains good enzyme activity. In such cases, HIV may become resistant in months or years. To further increase the genetic barrier, it is essential to choose two or more compounds which inhibit the same viral target and which must have different resistance mutations. However, it is not sufficient to attempt to "overwhelm" a single viral target by combining two or more drugs. The difficult step is to find combinations so that there are no mutations, including multiple stepwise mutations, which can give resistance to the drugs while retaining enzyme

function. When that is achieved, the genetic barrier has been raised. What is remarkable is how close the best HIV therapies have come to this ideal.

This concept is still widely misunderstood—there are too many instances when researchers suggest that combining antiviral compounds with different viral targets will increase the genetic barrier but this is not so. The genetic barrier is no higher than the individual barriers associated with each compound. However, combining compounds with differing viral targets may be helpful, especially to treat naturally self-limiting infections. The combination may reduce the viral load more quickly than either agent alone and achieve a lower level of residual viral replication. Having less virus replicating will mean that there are fewer opportunities for the virus to spontaneously form a mutant that is drug resistant. Therefore, the emergence of resistance can be delayed until the immune system has gained control of the infection. Unfortunately, with a chronic infection such as HIV, the gain by this approach is modest; the emergence of resistance is delayed but certainly not prevented.

In considering how to raise the genetic barrier, it is necessary to consider the viral target—does it have a "back door escape route"? The HIV protease is a good example. By combining two or more protease inhibitors with differing associated resistant mutations, it may be that none of the spontaneously arising protease mutants can be both resistant to the drugs and be a competent enzyme. However, in this situation, both the protease and the viral RNA substrate comutate so that both the enzyme activity and the substrate change, thus enabling the virus to continue replication—the protease has "escaped through the back door."

With HIV, and it looks likely for HCV, the only successful way to usefully increase the genetic barrier is to combine two or more inhibitors of the viral polymerase. Although not increasing the genetic barrier, adding drugs with different targets may be useful by reducing viral replication more quickly. For HIV therapy, individual patients have been taking combination pills for years without the emergence of resistance. This represents a major scientific achievement. This concept of genetic barrier to resistance is discussed further by Vere Hodge and Field (2011). In otherwise healthy patients, HSV infections are self-limiting, and so it is probably unnecessary to consider combining compounds with the aim of raising the genetic barrier—it may be helpful to have any drug combination which reduces viral replication more quickly than the monotherapies. However, in severely immunocompromised patients, HSV infections may be so frequent and prolonged; the clinical situation becomes akin to a chronic infection.

5. NOVEL APPROACHES TO HSV CHEMOTHERAPY

5.1. Nucleoside analogues in combination with anti-inflammatory compounds

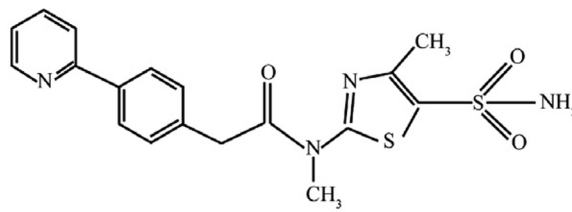
Following reactivation of latent HSV, the recurrence of herpes labialis is characterized by tingling, redness, and pain. It is generally believed that the majority of these and other clinical signs result from the response of the innate and acquired immune responses to virus antigen (Harmenberg, Oberg, & Spruance, 2010). Based on the premise that a highly effective inhibitor will not reverse the inflammatory responses which may have been triggered by minimal exposure to virus antigen, a series of topical formulations were prepared in which either ACV or FOS was combined with one of a variety of different anesthetic or anti-inflammatory compounds. These were then tested in a murine model for recurrent HSV disease. The optimal formulation in the model was 5% ACV plus 1% hydrocortisone. This formulation known as ME609 was first tested in a group of HSV patients in whom recurrent lesions were induced in response to UV light (Evans et al., 2002). The results were promising and a much larger trial was then conducted in 2437 herpes labialis patients (Sailer, Spruance, & Hull, 2011). The therapy (5% ACV–1% hydrocortisone cream applied five times daily) was self-initiated as soon as the patient recognized the prodromal signs of a recurrent lesion. During the trial, 1443 patients self-initiated the treatment and the number of "aborted" lesions was reported 42% versus 35% versus 26% for ACV-HC versus ACV versus placebo, respectively. The time to healing (the duration of ulcerative lesions up to the "loss of hard crust") was 5.7, 5.9, and 6.5 days, respectively, for the same treatment groups. The ACV/hydrocortisone combination is now licensed and available as a prescription medicine (called zovirax duo) in the United States for treatment of cold sores in people aged 12 years or older (Hull & Brunton, 2010). No problems with drug resistance have been reported so far; however, this formulation is not recommended for use in immunocompromised patients.

5.2. Helicase–primase: A new selective virus target for HSV

All the licensed HSV compounds ultimately target just one of the approximately 70 separate gene products encoded by HSV DNA, the HSV DNA pol which is essential for virus replication. When viral DNA synthesis is inhibited, virus replication ceases and no infectious progeny is produced. Compounds, which inhibit DNA pol, do so either directly or via their

respective nucleoside triphosphates. HSV DNA replication requires not only the two proteins that comprise the DNA pol but also, prior to the action of this enzyme, a heterotrimeric group of proteins comprising helicase, primase, and accessory proteins known as helicase–primase. These proteins act on HSV dsDNA to open the strands for synthesis of the RNA primers prior to the action of DNA pol. Several potent inhibitors of this essential virus function have been reported. ASP 2151 (amenamevir) was discovered in Japan by the Yamaguchi Corporation. Phase II clinical trials were conducted in man against genital herpes. However, these trials are currently on hold following undisclosed safety issues. A different compound was discovered by means of a screening process in the laboratories of Bayer Pharmaceutical Company. This compound is known as BAY 57-1293 or AIC316 recently named pritelivir (Fig. 1.4). In cell culture, the compound is about 10-fold more potent than ACV and antiviral concentrations are readily attained in serum following oral administration.

It was shown in a Phase-I dose-escalation study (Birkmann et al., 2010) that oral administration of pritelivir (5 mg up to 200 mg per day) was tested and the higher doses maintained serum concentration at a level which exceeded that required to inhibit HSV replication. The terminal half-life was calculated to be up to 80 h which is much greater than that for ACV or PCV. No deleterious side effects were reported. Patients with genital herpes were recruited for a Phase-II placebo-controlled trial in which once-daily oral doses of 5, 25, or 75 mg once per day or 400 mg once per week for a period of 28 days were compared with placebo (Vere Hodge, 2012; Wald et al., 2011). The rates of virus shedding were reported to reduce in a dose-dependent manner (21.2%, 9.2%, 2.0%, and 5.2%, respectively, vs. 16.5% for placebo) and the corresponding lesion rates were 13.3%, 4.6%, 1.1%, and 1.3%, respectively, versus 9.1%. Again no adverse side effects were reported and no resistant virus was detected among the



Pritelivir (BAY 57-1293, AIC316)

Figure 1.4 The structural formula of BAY 57-1293 (AIC316) or pritelivir, one of the first helicase–primase inhibitors to enter clinical trials.

patient samples analyzed. However, this was a relatively small trial (156 subjects, comprising105 females and 51 males). Albeit a small trial, significant benefit was claimed and further trials are planned.

All clinical isolates tested, to date, are equally sensitive to pritelivir. However, resistance mutations are readily selected in tissue culture. The majority of resistance mutations locate to a region in the gene just downstream from the IVth functional domain of the helicase gene (*UL5*) (Field & Biswas, 2011) as defined by Zhu and Weller (1992). As for nucleoside analogues, viruses bearing the resistance mutations exist in both laboratory and recent clinical isolates in some cases at relatively high frequency (e.g., 10^{-4}). One of the most common resistance mutations (K356N or K355N in HSV-2) conferred high resistance (>100-fold) and the mutant appeared to be fully fit when assessed in the laboratory murine infection model. In some cases, resistance mutations map to the primase protein; in this case, all the resistance mutants detected to date contain mutations at or near A899 or the corresponding A905 in HSV-2 (Field & Mickleburgh, 2013). We await the publication of further trial results and drug resistance data to confirm the utility of this new class of HSV antiviral.

5.3. TK bypass

To date, the effective inhibitors of HSV are nucleoside analogues and, once phosphorylated, inhibit DNA pol by direct interaction with active site. The initial phosphorylation step by HSV TK is the "safety gate" that is the key to the high selectivity of compounds such as ACV, BVDU, and PCV. This required activation step places a constraint on other potential inhibitors of HSV DNA pol, and to date, most compounds, which interact directly with HSV DNA pol, have been found to have unacceptable toxicity. An early compound of this type was the pyrophosphate analogue, phosphonoacetic acid, quickly superseded by phosphonoformic acid, FOS (Wagstaff & Bryson, 1994). This compound is often used in immunocompromised patients to combat HSV that has become resistant to nucleoside analogues.

A different approach to inhibitors that act independently of TK is the series of phosphonyl compounds synthesized by the late Anton Holy and colleagues in Prague (De Clercq et al., 1986). Although this approach has been most successful for inhibitors of reverse transcriptase, yielding drugs which have become the mainstay of HIV and HBV therapies, this approach has so far been only marginally successful in treating herpes viruses. Best known among these is the nucleotide analogue CDV (De Clercq, 2003)

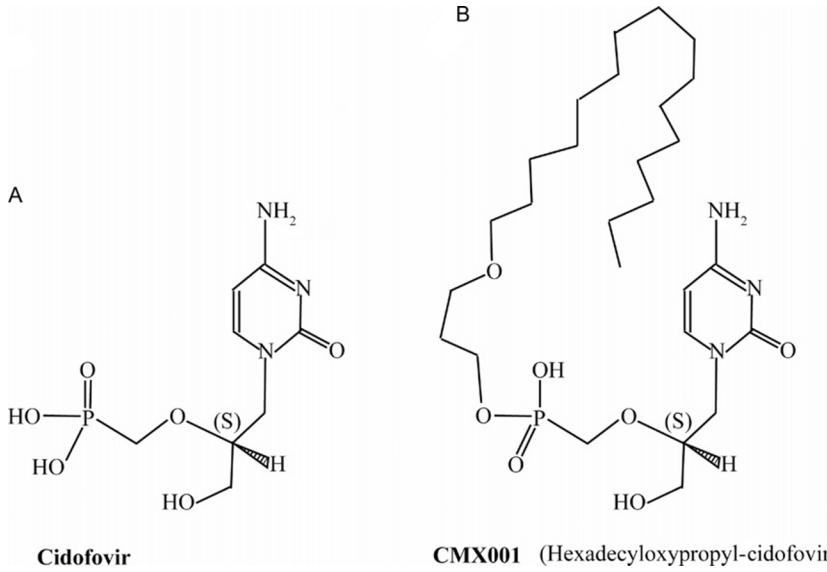


Figure 1.5 The structural formula of the phosphonyl nucleotide analogue, cidofovir and its orally bioavailable prodrug, CMX001 (hexadecyloxypropyl-cidofovir).

(Fig. 1.5A). The compound has broad-spectrum activity against DNA-containing viruses with an extremely long tissue half-life. CDV is used mostly in immunocompromised patients to combat HSV which has become resistant to VACV, FCV, and FOS. However, there are safety issues, in particular nephrotoxicity. The orally bioavailable prodrug of CDV CMX001 (Fig. 1.5B) appears to be safer and is being developed for treatment of HCMV (ClinicalTrials Web site). This may be a valuable addition to the armory of compounds for use against drug-resistant HSV.

6. PROSPECTS FOR NOVEL ANTIVIRAL COMPOUNDS AND VACCINES

6.1. Difficulties facing novel compounds

It is generally held that current therapy for HSV is far from ideal. The greatest obstacle being the fact that none of the antiviral compounds described so far are able to cure latency. As a consequence, when episodic therapy or a period of suppression therapy is completed, the disease is prone to further recurrence. Presently, we know of no effective strategies for eliminating latency—were such a strategy to emerge this would be a major

innovation. Furthermore, the short half-lives of the current standard compounds (ACV and PCV) notwithstanding the introduction of the orally bioavailable prodrugs, FCV and VACV, mean that at least once-daily dosing is required to maintain the antiviral effect. These are scientific constraints for the current compounds but provide potential opportunities for new compounds. However, a barrier to new introductions is a consequence of the extraordinary safety profile of these guanosine nucleoside analogues. Importantly, ACV, VACV, and FCV are now all available as relatively inexpensive generic drugs. The current oral formulations are convenient to administer giving good compliance and physicians are generally used to prescribing them. These factors and perceptions together add up to a considerable barrier to the introduction of novel compounds into the clinical arena. Furthermore, recurrent HSV is generally a self-limiting disease with considerable variation among episodes; therefore, trials to demonstrate superiority over existing therapies require large numbers of patients to be recruited at multiple centers, take many months or years to complete, and are consequently relatively expensive. For these reasons, new and potentially interesting compounds (e.g., HPIs) may remain as experimental drugs for many years before their clinical potential can be properly evaluated. Given the relatively short period during which the new compound is protected by patent, these facts make it understandable that commercial interest in the development of novel therapies for HSV has waned over the past two decades.

6.2. Lack of progress in the control of HSV by means of immunization

Notwithstanding the ease with which HSV can be cultured, the ready availability of animal infection models and the ability to engineer mutant viruses, no successful HSV vaccines have been developed, to date. The reasons underlying this failure are elegantly rehearsed in the recent review by Gershon (2013). One important difficulty in developing HSV vaccines may be the lack of a viremic phase in the pathogenesis of HSV infection that is a feature of varicella and several alphaherpes (varicellovirus) infections in domestic animals where there has been more success. Suffice to say that various candidate vaccines for HSV-1 and HSV-2 have been considered including subunit and DNA vaccines with adjuvant and gene-deleted live attenuated vaccines. None have been efficacious in clinical trials, and although the search for a successful HSV vaccine strategy continues, further discussion of HSV vaccines is beyond the scope of this review.

In contrast to HSV, the Oka vaccine for VZV is widely used and promising progress is being made with CMV vaccines (Field & Vere Hodge, 2013 in press).

7. CONCLUSION

It is timely to review herpes antiviral therapy, as research is emerging from a quiescent phase. Since VACV was introduced in the 1990s, no new anti-HSV drugs have become widely available. However, financial considerations have influenced prescribing choices. First, ACV became available generically which encouraged ACV use in preference to the prodrugs. Recently, generic FCV became available followed soon afterward by VACV. For oral therapy, there now seems little reason to use ACV when the prodrugs provide good efficacy with patient-friendly dosing protocols.

Experience over the past two decades with FCV and VACV has shown:

i. FCV and VACV are effective in reducing burden of HSV disease, both primary and recurrent.
ii. Although their parent compounds, PCV and ACV, have short (2–3 h) plasma half-lives, FCV and VACV are usually taken once or twice daily, leading to good compliance.
iii. For HSV-1 or HSV-2 recurrences, single-day treatments have been shown to be effective and should replace the current standard multiday therapies.
iv. During more than 20 years, there has been no increase in antiviral drug resistance among otherwise healthy patients (<0.5%), apart from those with ocular herpes.
v. Above all, they have established remarkably benign safety records. Even in individuals on long-term (several years) suppression therapy, no significant safety issues have emerged.

However, there remain further areas for exploration, even with FCV and VACV:

i. Preclinical studies revealed marked differences between VACV and FCV (e.g., affinity for HSV TK which may relate to an effect on latency, triphosphate stability which gives longer duration of activity and may relate to a lack of resurgences). We have suggested some areas for investigation.
ii. How can more financial support be enlisted to explore new avenues, for example, in possibly reducing herpes latency or AD?

iii. FCV was reclassified from prescription-only to pharmacist-controlled for herpes labialis in New Zealand in 2010 (see comment below) (Cunningham et al., 2012). Should this be repeated more widely?

The reclassification of FCV, from prescription-only to pharmacist-controlled for recurrent herpes labialis, raises several questions. What potential is there for harm? Is a patient subject to side effects by overdosing or likely to promote virus resistance by under dosing? All the experience with FCV suggests that neither of these risks is unacceptably large. For herpes labialis, the dosing regimens are simple (either one of two doses in a single day) which would help to avoid misuse. Although the initial diagnosis may not be by a physician, patient notes can give advice on how to use FCV to obtain maximum benefit, for example, to ensure the first dose is close at hand at all times so that the first pill can be taken as soon as the first prodromal sign is noticed. There is one major concern: the change of classification does not include genital herpes; it is only for herpes labialis. There is the distinct possibility that patients with genital herpes may feel that there is stigma associated with the disease, may choose to avoid consulting a physician, and opt to obtain FCV over-the-counter. This is especially likely for a patient with primary genital herpes when advice from a physician in most important. The dosing regimen for primary genital herpes is much longer than for recurrent herpes labialis. Also, the patient should have advice about the chances of having recurrences (typing the HSV infection will be needed to give appropriate advice) and informed about the risks of transmission especially during times of asymptomatic virus shedding. For patients with recurrent genital herpes, the problem is of much less concern because the dosing regimens for herpes labialis and genital herpes are essentially the same. As use for genital herpes is most likely to happen, this could be regarded as a reason for not allowing the reclassification. Alternatively, it may be better to allow the reclassification for both herpes labialis and genital herpes so that FCV would be issued with advice about both indications.

One area of recent research is to make better use of existing antivirals. ACV combined with hydrocortisone was shown to give faster resolution of herpes labialis symptoms, albeit rather marginally, but is now licensed in the United States. Except for patients who prefer topical to oral therapy, it seems likely that the new single-day oral treatments will become the therapies of choice. CDV use is limited by its toxicity but, remarkably, a prodrug, CMX001 seems to have both enhanced antiviral activity and reduced toxicity. Currently, it is being evaluated in Phase II trials for efficacy against HCMV, but it has activity against HSV. It would be a useful option to have available to treat ACV- and PCV-resistant virus in immunocompromised patients.

A new area of research showing promise is antiviral agents which target HSV helicase–primase complex. Pritelivir (AIC316) has given encouraging results in a Phase II HSV-2 trial. At the highest dose tested (75 mg once daily), the virus shedding rate was reduced to 2.0% from 16.5% with placebo and the corresponding lesion rates were 1.3% versus 9.1%. Certainly, this approach is breathing new life into the area of HSV research.

Against the background of two decades of successful anti-HSV therapy with ACV, VACV, and FCV, the experience with ACV as it came off patent indicates that it may not be sufficient to demonstrate noninferiority or even slight advantage with a new compound. In order to replace current therapies and become standard therapy, this will require the demonstration of significant superiority. This is a major hurdle. Large clinical trials are required to demonstrate antiviral superiority because natural HSV infections are so variable in both duration and severity. Thus, it will be very difficult to progress new classes of antiviral such as HPI for HSV. This raises the question how best to progress new compounds when the perceived market need is small but the medical need (to treat patients with resistant virus) is great.

A new compound, letermovir, has recently entered the HCMV clinical arena. It has been shown to inhibit a late stage of virus DNA processing: the terminase enzyme that is required to cleave newly replicated virus DNA into genome lengths for packaging into virions. This activity is performed by an enzyme complex comprising three HCMV proteins: the products of HCMV genes *UL56*, *UL89*, and *UL104* (Goldner et al., 2011). Letermovir has demonstrated that the terminase enzyme is a viable target and that similar compounds for HSV could be sought.

Antiviral chemotherapy is entering a new era. All three well-established compounds, ACV, VACV, and FCV, are now off patent and available generically. So efficacy, rather than price, should determine their relative use. Pritelivir (AIC316) targeting HSV helicase–primase is on the horizon having shown promise in a Phase II trial. CMX001 has demonstrated that prodrug approach can not only aid oral absorption but also reduce the toxicity of the parent compound. Letermovir has confirmed that herpes virus terminase enzyme is a viable target which could possibly be used to seek new HSV therapies. Research for better HSV therapies is returning after a quiet phase.

CONFLICT OF INTEREST

H. J. F. has a research collaboration with AiCuris GmbH & Co. for the development of HPIs. H. J. F. received research funding from both the former Glaxo Wellcome and SmithKline Beecham companies during the development of VACV and FCV, respectively. R. A. V. H. was an

employee of SmithKline Beecham (now GlaxoSmithKline) working within the antiviral field during the discovery of PCV, the selection of its prodrug, FCV, and their development.

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CHAPTER TWO

Nucleotide Prodrugs for the Treatment of HCV Infection

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Contents

Abstract

The HCV RNA-dependent RNA polymerase is an essential enzyme in HCV viral replication and has been a prominent target in the search for therapies to treat individuals infected with HCV. The development of both nucleoside and nucleotide HCV inhibitors has been pursued because of their potential for showing pangenotypic activity and because of their high barrier to resistance. Even though nucleoside inhibitors were shown to be effective in a clinical setting, their potency limited their effectiveness. The exploitation of prodrug strategies to deliver nucleoside 5′-monophosphates has resulted in the development of a number of very potent inhibitors of HCV replication. In addition, several of these nucleotide prodrugs have demonstrated liver-targeting characteristics when administered orally. Human clinical studies have shown that a number of nucleotide prodrugs are potent inhibitors of viral replication leading to significant reductions in viral load when given orally. Combinations of these nucleotide prodrugs with either pegylated interferon-α and ribavirin or another direct acting antiviral alone has lead to cure rates as high as 100% after only 12 weeks of therapy. The combination of a nucleotide prodrug and another direct-acting antiviral agent holds the promise of delivering an interferon-free therapy for HCV patients thus eliminating the undesirable side effects associated with taking interferon.

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ABBREVIATIONS

(*S*)-HPMPA (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine
CMV cytomegalovirus
DAA direct-acting antiviral agents
HBV hepatitis B virus
HCC hepatocellular carcinoma
HCV hepatitis C virus
HDP hexadecyloxypropyl
HIV human immunodeficiency virus
HSV-1 or HSV-2 herpes virus
NTP nucleoside triphosphate
ODE octadecyloxyethyl
PEG-IFN pegylated interferon-α
POLRMT human mitochondrial RNA polymerase
RBV ribavirin
RdRp RNA-dependent RNA polymerase
SATE *S*-acylthioethyl ester
SOC standard of care
SVR sustained virological response
VZV varicella-zoster virus

1. INTRODUCTION

The hepatitis C virus (HCV) is a single-stranded positive-sense RNA virus of the *Flaviviridae* family of viruses in the genus *Hepacivirus*. Other flaviviruses include bovine viral diarrhea virus, yellow fever virus, dengue virus, and West Nile virus. There are six major HCV genotypes (genotype 1–6) that are distinguished based on the genomic variability in a region of the gene that encodes the viral NS5B RNA-dependent RNA polymerase (RdRp). The distribution of these genotypes varies globally. In North America and Europe, genotypes 1, 2, and 3 are found with genotype 1 predominating. Africa shows almost exclusively genotypes 4- and 5-infected patients, and genotype 6 virus is the most prevalent in Asia and other parts of the world (Simmonds, 2004; Simmonds et al., 2005). In addition, HCV exhibits a high rate of viral replication that when coupled with the error-prone nature of the viral polymerase and the lack of a proof-reading function leads to a large population of preexisting mutations (Cuevas, Gonzalez-Candelas, Moya, & Sanjuan, 2009; Ogata, Alter, Miller, & Purcell, 1991).

HCV infection has been shown to impact approximately 180 million individuals worldwide and along with hepatitis B virus (HBV) infection is known to be a major cause of liver cirrhosis and hepatocellular carcinoma (HCC) (Lavanchy, 2009). In addition, HCV infection that leads to decompensated liver disease is a leading cause of liver transplants worldwide and thus a significant health economic burden. Until 2011, the standard of care (SOC) for treating patients with HCV infection was 48 weeks of pegylated interferon-α (PEG-IFN) injections and oral ribavirin (RBV) treatments. However, PEG-IFN/RBV treatments have proven to be effective at curing (providing a sustained virological response—SVR) only 40–50% of genotype 1 patients and 75% of genotype 2 and 3 patients (Fried et al., 2002; Zeuzem et al., 2009). It has also been shown that subpopulations of HCV patients respond poorly to PEG-IFN/RBV. African-American patients and patients that harbor a TT or TC allele 3-kb upstream of the *IL28B* gene in a region that encodes the type III interferon, IFN-λ3, demonstrate a diminished response to PEG-IFN/RBV therapy (Ge et al., 2009; Manns et al., 2007). These modest cure rates coupled with the prospect of facing significant adverse events such as fatigue, hemolytic anemia, depression, and flu-like symptoms that ultimately lead to high rates of discontinuations prompted the search for direct-acting antiviral agents (DAA) that are safe and effective. To a limited extent, this search was culminated with the introduction of the first-generation HCV protease inhibitors, telaprevir and boceprevir. Both of these agents when given in combination with PEG-IFN/RBV to HCV genotype 1-infected patients, improved SVR rates and shortened duration of therapy (Kwong, McNair, Jacobson, & George, 2008; Sulkowski, 2007). However, these new triple combination regimens came with significant added side effects, complicated dosing regimens, a low barrier to resistance, and only addressed genotype 1 patient populations. Consequently, the need for oral, pangenotypic DAAs that eliminate the use of PEG-IFN/RBV remained an objective for drug discovery efforts.

The HCV NS5B RdRp is one of several viral targets under investigation for the development of novel DAAs to treat patients infected with HCV. This viral-specific enzyme is essential for HCV replication. The NS5B RdRp is a 66 kDa protein of ~590 amino acids located at the C-terminus of the virally encoded HCV polyprotein of ~3000 amino acids (Penin et al., 2004). The HCV RdRp exhibits the typical palm–finger–thumb structural motif found in many viral polymerases (Miller &

Purcell, 1990). The Gly317-Asp318-Asp319 (GDD) catalytic motif found in the palm domain of the HCV NS5B RdRp is an invariant element in RNA viral polymerases and is highly conserved across all HCV genotypes (Poch, Sauvaget, Delarue, & Tordo, 1989). It is at this site that a ribonucleoside 5′-triphosphate (NTP) binds through the coordination of a divalent metal (Mg^{2+} or Mn^{2+}). Subsequently, this NTP is added to the 3′-end of the growing RNA chain through the formation of a 3′,5′-phosphodiester linkage. Consequently, the proposition that nucleos(t)ide inhibitors of the NS5B RdRp could address the requirement of a pangenotypic DAA with a high barrier to resistance appeared viable.

For a nucleoside to be effective as an inhibitor of a viral polymerase, it must first be converted to its corresponding nucleoside 5′-triphosphate. This process takes place by the action of three separate kinases. The activity of the nucleoside as a viral polymerase inhibitor can be dramatically affected by the efficiency at which phosphorylation occurs. In addition, the inhibitory potency of the nucleoside is determined by the half-life of the resulting NTP. The longer the intracellular half-life of the NTP, the more efficacious the nucleoside will appear to be. It is not uncommon that a nucleoside will demonstrate poor activity as an inhibitor of a viral polymerase simply because either the nucleoside or its mono- or diphosphate anabolite is a poor substrate for the requisite kinase in the phosphorylation cascade. It has been observed that the first kinase in the phosphorylation cascade is the most substrate selective, and therefore, the step where phosphorylation is frequently blocked. In order to overcome this nonproductive first phosphorylation step, delivery of a nucleoside 5′-monophosphate is required. However, nucleoside 5′-monophosphates are negatively charged, which severely limits their ability to cross biological membranes, and they are susceptible to degrading phosphatases. Consequently, they are typically poor drug candidates and usually require implementation of a prodrug approach if they are going to succeed as therapeutics (Hecker & Erion, 2008).

2. NUCLEOSIDE INHIBITORS

The development of inhibitors of the HCV RdRp has included the investigation of both nucleosides and nucleotides (Sofia, 2011; Sofia, Chang, Furman, Mosley, & Ross, 2012). A number of nucleosides have been prepared in the hope of identifying potent inhibitors of the HCV viral replication. These nucleosides include molecules with novel modifications

to the ribose sugar as well as modifications to the appended nucleobase moiety (Fig. 2.1; Sofia et al., 2012). Ribose sugar modifications have focused largely on substitutions at the C2′- and C4′ positions of the ribonucleoside sugar (Fig. 2.1). It was demonstrated that C2′ and C4′ modifications lead to active HCV inhibitors. To date, modifications at the C3′ or C1′ positions have not produced analogs that demonstrate activity at inhibiting HCV viral

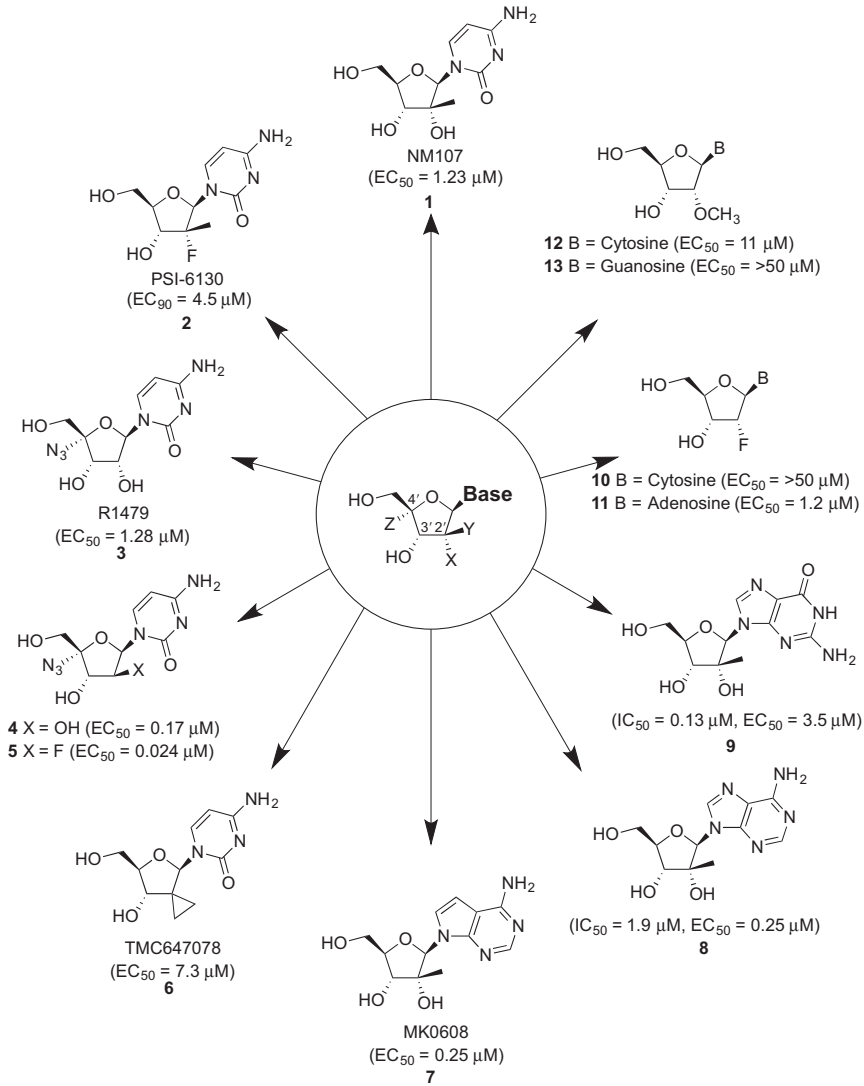


Figure 2.1 Nucleoside HCV inhibitors.

replication in the standard whole-cell replicon assay (Sofia et al., 2012). Also, a number of carbocyclic ribosides have been prepared, but none have garnered significant interest to develop beyond early *in vitro* studies (Liu et al., 2012; Sofia et al., 2012). In regard with the nucleobase, both pyrimidine and purine bases and their corresponding structural analogs have been explored in combination with either the natural ribose sugar or one of the modified sugars (Sofia et al., 2012).

Of the ribose sugar modifications, both the 2′-β-*C*-methyl substitution and the 4′-azido substitution have proven effective at producing active inhibitors of HCV replication. It is these two classes of nucleosides that have demonstrated the most promise and have progressed the furthest in the development of nucleoside drugs to treat HCV infection. The 2′-β-*C*-methyl substitution was shown to be compatible with both a 2′-α-OH (Fig. 2.1, **1**, **7**–**9**) and 2′-α-F (Fig. 2.1, **2**) substituent (Clark et al., 2005; Eldrup, Allerson, et al., 2004; Pierra et al., 2006; Prakash et al., 2005; Tomassini et al., 2005). The 4′-azido nucleosides can tolerate either a 2′-α- or 2′-β-OH group (Fig. 2.1, **3** and **4**) or a 2′-β-F substituent (Fig. 2.1, **5**) (Griffon, Dumas, Soter, Sommadossi, & Gosselin, 2009; Klumpp et al., 2008; Smith et al., 2007). In the case of 2′-β-*C*-methyl-2′-α-OH-substituted ribose sugars, compatible base substitutions include cytosine (Fig. 2.1, **1**), guanine (Fig. 2.1, **9**), and adenine (Fig. 2.1, **8**), including modified adenine bases such as 7-deaza (Fig. 2.1, **7**) and 7-deaza-7-F analogs (Eldrup, Prhavc, et al., 2004; Prhavc et al., 2008). For 2′-β-*C*-methyl-2′-α-F-substituted ribose sugars, cytosine (Fig. 2.1, **2**) was the preferred base and demonstrated the greatest activity, where guanine and adenine bases proved to be more weakly active in the whole-cell replicon assay (Clark et al., 2005, 2006). It should be noted that in combination with either the 2′-β-*C*-methyl-2′-α-OH or 2′-β-*C*-methyl-2′-α-F ribose modifications, substitution of a uracil base resulted in complete loss in whole-cell activity (Ding, Girardet, Hong, Shaw, & Yao, 2006; Shi et al., 2005). A similar structure–activity relationship regarding the base substitution was observed for the 4′-azido nucleosides.

Development of the 2′-β-*C*-methyl and 4′-azido classes of nucleosides led to the clinical investigation of three agents (Fig. 2.2): valopicitabine (NM283, Fig. 2.2, **14**), balapiravir (R1626, Fig. 2.2, **15**), and mericitabine (RG7128, Fig. 2.2, **16**). Each of these is an ester prodrug of the parent nucleosides NM107, R1479, and PSI-6130, respectively. Each of the parent nucleosides was shown to be a nonobligate chain terminator of HCV

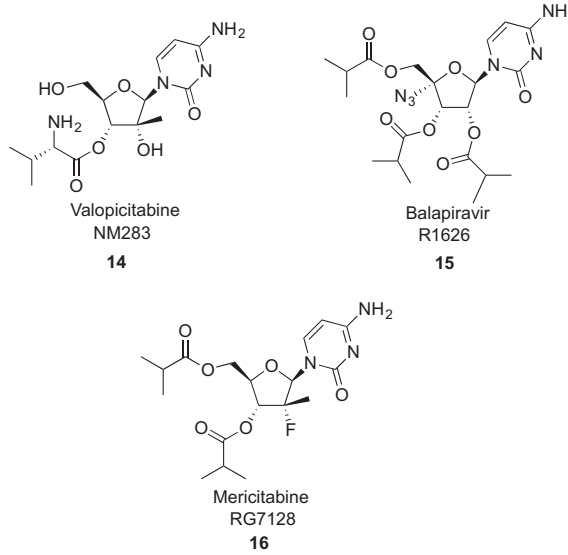


Figure 2.2 Nucleoside ester prodrug HCV inhibitors.

replication. In each case, the use of ester prodrugs was necessary to overcome poor oral bioavailability of the parent nucleoside (Brandl et al., 2008; Pierra et al., 2005, 2006; Sofia, Furman, & Symonds, 2010; Toniutto et al., 2008). *In vitro*, the S282T amino acid replacement in the HCV RdRp afforded resistance to the 2′-*C*-methyl nucleosides NM107 and PSI-6130, where the S96T amino acid replacement provided resistance to R1479. The resistant mutations leading to the S282T- and S96T-resistant phenotypes had not been observed as preexisting mutations in clinical isolates (Le Pogam et al., 2006; McCown et al., 2008).

In human clinical studies, valopicitabine demonstrated proof-of-concept for the reduction in HCV viral load. Valopicitabine administered twice daily at 800 mg over 14 days to genotype 1-infected patients led to a -1.2 $\log_{10}$ IU/mL reduction in viral load and when administered with SOC for 28 days delivered a >4 $\log_{10}$ IU/mL reduction in viral load (Brown, 2009; Pierra et al., 2005). Unfortunately, clinical studies were discontinued because of significant gastrointestinal toxicity. Similarly, the 4′-azido nucleoside prodrug, balapiravir, when administered twice daily to genotype 1-infected patients at doses of 500–4500 mg twice daily over 14 days produced mean viral load reductions of -0.3 to -3.7 $\log_{10}$ IU/mL (Brown, 2009; Zeuzem et al., 2010). When balapiravir was subsequently dosed at

1500 mg twice daily in combination with PEG-IFN/RBV over 28 days, a -5.2 $\log_{10}$ IU/mL reduction in viral load and an 81% rapid virological response (RVR) was observed. However, development of balapiravir was discontinued due to significant hematological adverse events observed in subsequent studies (Zeuzem et al., 2010).

Mericitabine was able to differentiate itself from the other nucleoside HCV inhibitors with a somewhat improved clinical profile and a decidedly improved safety profile. Clinical studies showed that mericitabine at a twice daily dose of 1500 mg produced a -2.7 $\log_{10}$ IU/mL reduction in viral load after 14 days of monotherapy in genotype 1 naïve patients (Brown, 2009; Burton, 2009; Sofia, Furman, et al., 2010). When combined with PEG-IFN/RBV for 28 days in genotype 1, 2, and 3 patients, RVR rates of 85–90% were observed with no reported significant adverse events and no viral breakthroughs. These results demonstrated for the first time that a DAA could be effective in patients infected with not only genotype 1 but also genotype 2 and 3 virus and that a nucleoside was capable of demonstrating a high barrier to resistance in multiple genotypes. Further studies investigating the concept of a PEG-IFN-sparing regimen showed that mericitabine combined with a HCV protease inhibitor, danoprevir, could produce viral load declines (-4.9 to -5.1 $\log_{10}$ IU/mL) comparable to regimens that include a DAA in combination with PEG-IFN/RBV, thus laying the groundwork for PEG-IFN-free regimens (Gane et al., 2010). In a study looking at whether cirrhotic patients respond differently to mericitabine + PEG-INF/RBV therapy, it was shown that neither drug pharmacokinetics nor a patient's early-rapid virological response was affected by their cirrhotic status (Moreira et al., 2012). These results added increased support that a nucleoside-based regimen could be useful in a wide and diverse patient population. In a treatment-naïve patient population, the interferon-free regimen of mericitabine + danoprevir + RBV given for 24 weeks produced a 71% SVR 12 weeks (SVR12) after cessation of therapy (Feld et al., 2012). In addition, this IFN-free regimen resulted in 39% and 55% SVR12 rates in genotype 1b prior partial responders and prior null responders (Feld et al., 2012). To date, no adverse events have been associated with mericitabine treatment. However, what emerged from these clinical studies was the observation that nucleoside NS5B inhibitors were not as effective as other inhibitors of HCV replication, such as HCV protease inhibitors, at rapidly reducing viral load during the early phase. The advantages that nucleoside-based therapy did have

over other classes of HCV inhibitors were pangenotypic coverage and a high barrier to resistance.

3. NUCLEOTIDE PRODRUGS

The search for second-generation active-site inhibitors of the HCV polymerase focused on inhibitors that would improve on the profile exhibited by the first-generation nucleosides. The objective was to improve on potency and safety, provide once-daily dosing, maintain pangenotypic activity, show reduced treatment duration, and support combinations with other DAAs. The need to support DAA combinations stemmed from the hope that combining multiple potent DAAs would ultimately lead to eliminating the use of PEG-IFN. These objectives led to the investigation of nucleotide derivatives. Early studies showed that a number of nucleoside derivatives were either inactive or poorly active as inhibitors of HCV replication when studied in the whole-cell replicon system, but their corresponding 5′-triphosphates proved to be potent inhibitors of the HCV polymerase in biochemical assays (Sofia, 2011). In such cases, poor phosphorylation, particularly formation of the nucleoside 5′-monophosphate derivative, was determined to be the factor-limiting activity of the nucleoside. To circumvent this poor phosphorylation step and deliver the nucleoside 5′-monophosphate thus enabling efficient generation of the active NTP, phosphate prodrug approaches that masked the undesirable characteristics of the phosphate moiety were implemented. The application of prodrug strategies for delivery of nucleoside 5′-monophosphates in a liver-targeting fashion ultimately has had a major impact on the development of therapies to treat patients infected with HCV (Pockros, 2012; Sofia et al., 2012). No less than five nucleotide prodrugs have entered clinical development and several others are believed to be in early development. The impact of these agents has been profound in helping shape future HCV therapy and particularly in establishing the paradigm of IFN-free regimens.

A prodrug is a compound that undergoes biotransformation or chemical transformation *in vivo* resulting in the release of the biologically active agent that then elicits the desired pharmacological effect (Ettmayer, Amidon, Clement, & Testa, 2004; Stella, 2010). The prodrug is ideally pharmacologically inactive. This prodrug requirement can be achieved by masking a key pharmacophore of the active agent. This can be accomplished by either

attaching a removable promoiety to the active drug or by relying on a biochemical or chemical process *in vivo* that establishes a key pharmacophore on the molecule thus transforming the administered agent into the pharmacologically active drug. A promoiety is a temporary structural unit that is released after it has served the purpose of masking some undesired characteristic of the active drug. The promoiety generally functions by changing the properties of the molecule to help it function as an effective therapeutic.

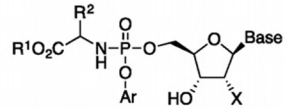
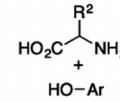
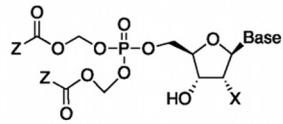
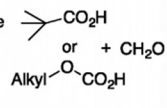
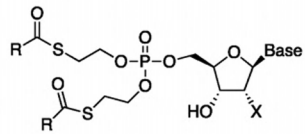
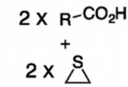
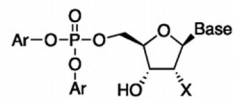
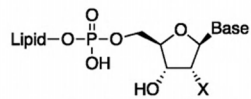
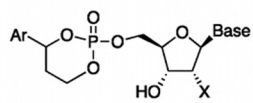
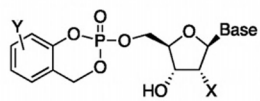
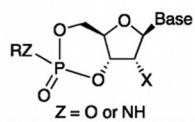
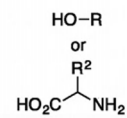
Many prodrug strategies have been developed for the delivery of nucleotide analogs (Hecker & Erion, 2008; Mackman & Cihlar, 2011; Sofia, 2011). This prodrug development effort stems from the fact that nucleotide analogs have shown significant promise for the development of antiviral therapies, yet their development was hampered because they lacked good drug characteristics. Nucleotide prodrugs have been approved for the treatment of many viral diseases such as human immunodeficiency virus (HIV), HBV, herpes virus (HSV-1, HSV-2), varicella-zoster virus (VZV), Epstein–Barr virus (EBV), and cytomegalovirus (CMV). Prodrug strategies that have been developed to deliver nucleotide analogs include the phosphoramidates, acyloxyalkyl esters, *S*-acylthioethyl esters (SATE), aryl- and lipid-phosphate esters, and cyclic esters comprising HepDirect, cycloSal, and 3′,5′-cyclic phosphates (Table 2.1) (Hecker & Erion, 2008; Hostetler, 2009; Mehellou, Balzarini, & McGuigan, 2009; Sofia, 2011).

In developing a nucleotide prodrug that would satisfy the requirements of an HCV therapeutic, several criteria need to be considered. The phosphate prodrug needs to demonstrate sufficient chemical stability to allow formulation into an oral dosage form. It must be stable to conditions of the gastrointestinal tract to reach the site of absorption intact. The prodrug requires good absorption properties without undergoing appreciable degradation during the absorption phase. Sufficient stability in the blood is necessary to allow the prodrug to reach the target organ. Transport of the prodrug into hepatocytes and release of the free nucleoside 5′-monophosphate should occur with minimal exposure to other organs, ideally providing live-targeting characteristics. The prodrug itself should not be pharmacologically active, and the metabolic byproducts resulting from promoiety release should have no associated toxicity and should be rapidly excreted from the body.

3.1. Nucleotide phosphoramidate prodrugs

The nucleotide phosphoramidate prodrug approach has been employed extensively in the attempt to deliver nucleoside 5′-monophosphates to

Table 2.1 Prodrug constructs

Prodrug name	Prodrug structure	Promoiety cleavage byproducts	Reference
Phosphoramidate			Mehellou, et al. (2009)
Z = Pivaloyl: Pivaloyloxymethyl (POM) Z = Alkoxy: Alkoxycarbonyloxymethyl (POC)			Hecker and Erion (2008) and Mackman and Cihlar (2011)
S-acylthioethyl ester (SATE)			Hecker and Erion (2008)
Aryl ester		2 X HO–Ar	Hecker and Erion (2008)
Lipid esters		Lipid–OH	Hostetler (2009)
HepDirect			Erion (2006)
cycloSal			Hecker and Erion (2008)
3′,5′-Cyclic phosphate or phosphoramidate			McGuigan et al. (2011) and Reddy et al. (2010)

the liver. There are many variants of the phosphoramidate construct. These include the most common construct where a 5′-phosphate group is derivatized with an amino acid and an aryloxy ester (Table 2.1) (Mehellou et al., 2009). A diamidate version incorporates two amide-linked amino acids and a less common version of the phosphoramidate construct

utilizes a simple amine formation of the amidate linkage (McGuigan et al., 2011). Nucleotide phosphoramidates were first disclosed by McGuigan as a strategy to deliver nucleotides for the treatment of HIV and cancer (Mehellou et al., 2009). More recently, this prodrug strategy was adopted extensively in the HCV field because it demonstrated the ability to enhance the potency of nucleosides in the whole-cell replicon assay and had the potential to enable liver targeting of nucleotides (Sofia, 2011; Sofia et al., 2012). The mechanism of nucleotide phosphoramidate cleavage to reveal the free nucleotide 5′-monophosphate has been extensively studied (Mehellou et al., 2009; Murakami et al., 2010). The first step involves hydrolysis of the terminal amino acid ester by either carboxylesterase or cathepsin A. Then a chemical transformation ensues that releases the aryloxy group to form a cyclic phosphoramidate intermediate. This cyclic intermediate is hydrolyzed under physiological conditions to give the monophosphate which following removal of the amino acid by the action of a phosphoramidase or histidine triad nucleotide-binding protein 1 reveals the nucleotide 5′-monophosphate.

The phosphoramidate prodrug approach was applied to 2′-*C*-methylcytidine 5′-monophosphate in an attempt to improve potency over the parent nucleoside and improve therapeutic index as a means to subvert adverse events seen with valopicitabine (Fig. 2.3; Donghi et al., 2009; Gardelli et al., 2009). Although improvement in intrinsic potency (Fig. 2.3, **17**; $EC_{50} \leq 0.5$ μM) and human hepatocyte triphosphate levels were observed, this did not translate *in vivo* where only a twofold improvement in hamster live triphosphate levels were detected (Gardelli et al., 2009). Application of the acyloxyethylamino phosphoramidate promoiety (Fig. 2.3, **18**) to delivery of 2′-*C*-methylcytidine 5′-monophosphate again resulted in improved intrinsic potency and levels of triphosphate in human hepatocytes but no increased levels of triphosphate *in vivo* (Donghi et al., 2009). An attempt at investigating phosphoramidate monoesters (Fig. 2.3, **19** and **20**) resulted in loss of replicon potency as expected but surprisingly showed high levels of NTP in human hepatocytes. Unfortunately, oral administration did not result in appreciable levels of NTP in liver.

Extending the phosphoramidate prodrug strategy to 2′-*C*-methylpurine nucleotides investigated both adenosine and guanine derivatives (Fig. 2.4). Development of 2′-*C*-methyladenosine analogs as HCV inhibitors appeared to be plagued by weak potency and adenosine deamination. It was hoped that enabling kinase bypass by use of a 5′-monophosphate phosphoramidate

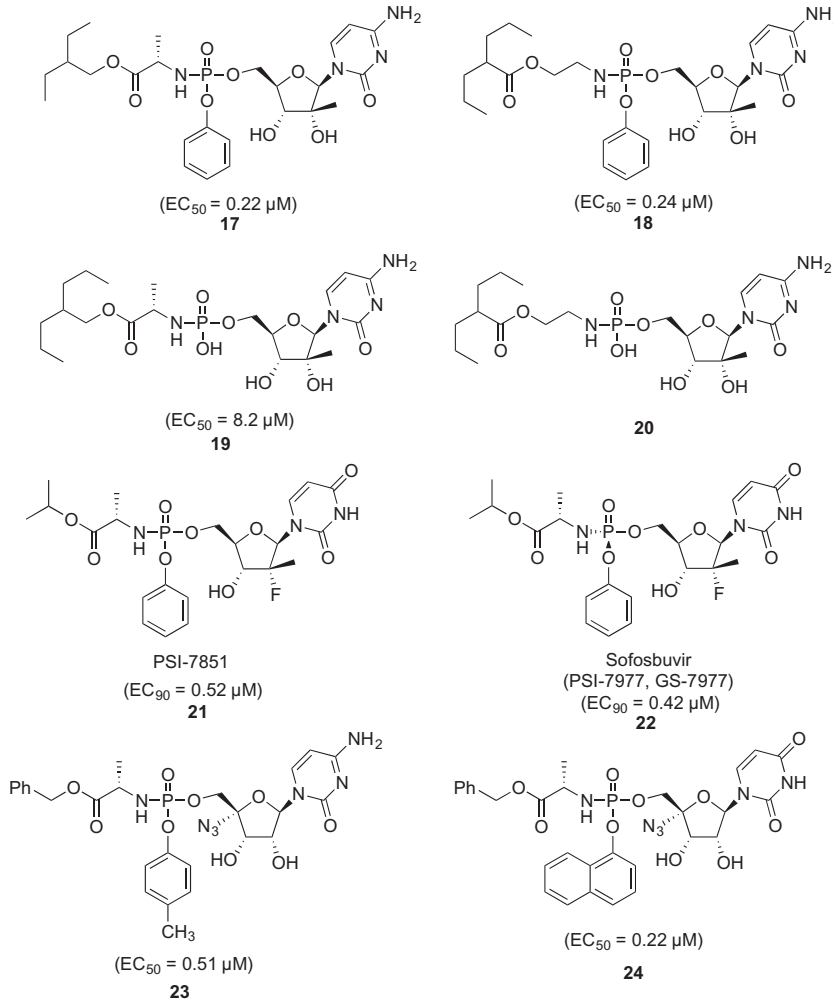


Figure 2.3 Phosphoramidate prodrug HCV inhibitors of pyrimidine nucleoside 5′-monophosphates.

prodrug could improve cell penetration and block deamination. However, improved activity over the parent nucleosides was not forthcoming (Fig. 2.4, **25**) (McGuigan, Perrone, Madela, & Neyts, 2009).

To overcome low whole-cell replicon potency resulting from inefficient nucleoside phosphorylation of 2′-*C*-methylguanosine, various phosphoramidate prodrugs were also examined (Fig. 2.4). Initially a 10- to 30-fold improvement in HCV replicon potency and acceptable *in vitro*

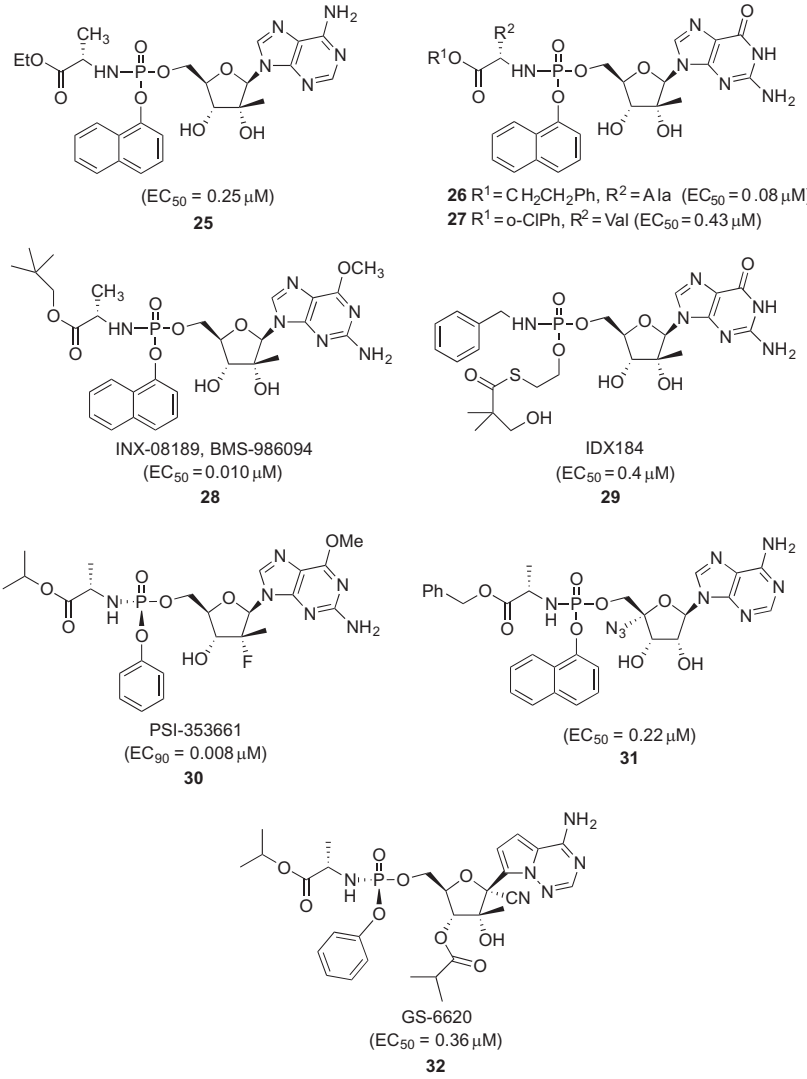


Figure 2.4 Phosphoramidate prodrug HCV inhibitors of purine nucleoside 5′-monophosphates.

stability characteristics were noted, but the liver levels of NTP observed in mice were not substantially different for that observed for the parent nucleoside (Fig. 2.4, **26** and **27**) (McGuigan, Gilles, et al., 2010). Ultimately, further modification at the C6 position of the guanosine base with a metabolically labeled methoxy group led to the double prodrug 28 (Fig. 2.4,

INX-08189, BMS-986094) (McGuigan, Madela, et al., 2010; Vernachio et al., 2011). This double prodrug was shown to be highly potent in the HCV replicon assay ($EC_{50} = 0.01$ μM) and showed activity against both genotype 1a and 2 replicons. Substantial levels of the corresponding NTP were observed in primary human hepatocytes, and like the general class of 2′-*C*-methyl nucleosides, INX-08189 showed moderate resistance in the replicon having the S282T NS5B mutation. High-dose proportional liver levels of 2′-*C*-methylguanosine triphosphate were observed in rats on oral administration (Vernachio et al., 2011). When dosed to portal vein-cannulated cynomolgus monkeys, the prodrug was observed in the portal vein but not in the systemic circulation where only 2′-*C*-methylguanosine was detected giving support to efficient liver extraction of the prodrug (McGuigan, Gilles, et al., 2010). INX-08189 was progressed into clinical development and early phase 1 studies showed that at doses of 25 mg once daily, a -1.3 $\log_{10}$ IU/mL reduction in viral load was observed in naïve genotype 1 patients with no adverse events reported (Patti et al., 2011). Unfortunately, in subsequent phase 2 studies where doses were increased to 200 mg once daily, severe cardiac adverse events that included a patient's death and a number of cases of congestive heart failure resulted in discontinuation of clinical trials. To date, the underlying cause of the cardiac adverse events is unknown. However, data from a recent study of anti-HCV ribonucleosides suggest that attrition during clinical studies may be a result of inhibited mitochondrial gene expression (Arnold et al., 2012). The study investigating whether HCV nucleos(t)ide inhibitors are substrates for human mitochondrial RNA polymerase (POLRMT) showed that 2′-*C*-methylguanosine triphosphate was a nonobligate chain terminator of POLRMT and thus inhibited elongation. This study also showed that other 2′-*C*-methyl nucleotides such as 2′-*C*-methyladenosine and the 4′-azidocytidine were nonobligate chain terminators of POLRMT. Such off-target activity can possibly lend some explanation to toxicities associated with these molecules.

Another variant of a 2′-*C*-methylguanosine 5′-monophosphate phosphoramidate prodrug was explored leading to the discovery of the compound IDX-184 (Fig. 2.4, **29**) (Zhou et al., 2011). The phosphoramidate prodrug construct employed in IDX-184 varied from that seen in INX-08189 by use of a SATE moiety and a benzylamine as the prodrug substituents. Although details of the mechanism of prodrug release have not been disclosed, it is believed to involve both a CYP450-dependent and -independent mechanism. It is reasonable to speculate that IDX-184

promoiety decomposition releases benzylamine, the breakdown products of the SATE group, episulfide, and 2,2-dimethyl-3-hydroxy-propanoic acid. IDX-184 was shown to have an $EC_{50} = 0.4$ μM in the HCV genotype 1b replicon assay and was active in the JFH1 genotype 2a replicon ($EC_{50} = 0.6$–11 μM) (Standring et al., 2008). When combined with a protease inhibitor, PEG-IFN or RBV, additive or synergistic effects were observed (Lallos et al., 2009). High triphosphate levels were detected *in vivo* in cynomolgus monkeys, and in HCV-1-infected chimpanzees a 10 mg/kg oral dose produced a -2.3 $\log_{10}$ IU/mL decline in mean viral load over 3 days. Following a phase 1 human clinical study where doses of 25–100 mg once daily over 3 days resulted in a -0.74 $\log_{10}$ IU/mL decline in viral load and no adverse events, a 14-day study combining IDX-184 (200 mg) with PEG-IFN/RBV showed a maximal viral load decline of -4.1 $\log_{10}$ IU/mL (Lalezari et al., 2009, 2010). IDX-184 is currently under clinical hold stemming from the adverse cardiac events associated with INX-08189 and the fact that INX-08189 and IDX-184 share the same end product 2′-*C*-methylguanosine triphosphate.

Investigation of the metabolism of 2′-α-F-2′-β-*C*-methylcytidine (PSI-6130, Fig. 2.1, **2**), the parent nucleoside of mericitabine, revealed that this cytidine nucleoside was extensively metabolized to the uridine derivative (PSI-6206) (Ma et al., 2007; Murakami et al., 2007). It was also determined that the uridine nucleoside was inactive as an inhibitor in the whole-cell replicon assay, but in a biochemical assay the uridine triphosphate ($IC_{50} = 1.19$ μM) was demonstrated to be an effective inhibitor of the NS5B RdRp (Murakami et al., 2008). In hepatocytes, this triphosphate exhibited a long intracellular half-life of 36 h making it an attractive inhibitor of the HCV NS5B RdRp. Since it was observed that the monophosphate of this uridine nucleoside was readily metabolized to its corresponding triphosphate, a phosphoramidate prodrug strategy was pursued to determine if successful delivery of the uridine monophosphate would result in an effective inhibitor of HCV replication. This was confirmed and subsequent development of the 2′-α-F-2′-β-*C*-methyluridine 5′-phosphoramidate led to the identification of the clinical candidate PSI-7851 (Fig. 2.3, **21**), a mixture of diastereoisomers (Sofia, Bao, et al., 2010). PSI-7851 ($EC_{90} = 0.52$ μM) demonstrated potent inhibition of HCV replication in the whole-cell replicon assay and produced high levels of the corresponding NTP in both isolated primary human hepatocytes and in a rat *in vivo* study. *In vitro* studies showed that PSI-7851 provided either additive or synergistic effects when combined with PEG-IFN and/or RBV

and other DAAs such as protease inhibitors, NS5A inhibitors, and non-nucleoside NS5B inhibitors (Lam et al., 2010). A phase 1 clinical study showed that PSI-7851 was safe and well tolerated up to a dose of 800 mg once daily and that PK results supported a profile that indicated rapid uptake by the liver. A 3-day monotherapy study in genotype 1 HCV patients given 400 mg once daily of PSI-7851 led to a -1.95 $\log_{10}$ IU/mL reduction in viral load with no treatment emergent or posttreatment viral resistance (Sofia, Furman, et al., 2010). With these positive clinical results supporting further development, the more potent *Sp* diastereoisomer of PSI-7851, PSI-7977 (Fig. 2.3, **22** (sofosbuvir, GS-7977)) was taken forward. A 14-day monotherapy study revealed that sofosbuvir (400 mg once daily) was dramatically more potent than the diastereomeric mixture PSI-7851 producing a -5.0 $\log_{10}$ IU/mL reduction in viral load with 85% of patients showing undetectable virus levels (Lawitz et al., 2011). Following demonstration of a 93% RVR rate when genotype 1 patients were administered sofosbuvir combined with PEG-IFN/RBV for 28 days, a protocol having 12- and 24-week treatment arms produced SVR rates at 12 weeks posttreatment (SVR12) of 90–92% in genotype 1, 82% in genotype 4, and 100% in genotype 6 patient populations with no difference in the 12- and 24-week treatment regimens (Hassanein et al., 2012; Lawitz et al., 2010; Nelson et al., 2011).

To investigate the potential of sofosbuvir, an ambitious clinical study investigating IFN-free regimens in genotype 1, 2, and 3 patients was undertaken. In a small population of treatment-naive genotype 2 and 3 patients treated for 12 weeks, a SVR12 of 60% was achieved with sofosbuvir alone and a dramatic 100% SVR12 was achieved with a simple sofosbuvir + RBV combination (Gane et al., 2012, 2013). An SVR24 of 68% was observed in a genotype 2/3 treatment experienced population on the sofosbuvir + RBV standard regimen for 12 weeks. Neither a shorter treatment duration of 8 weeks nor a reduced RBV dose performed as well as the standard regimen. In addition, a significant lessening of the affect on hemoglobin and absolute neutrophil count, as compared to PEG-IFN-containing regimens, was reported supporting the advantages of an IFN-free regimen. However, in a population of genotype 1 patients who were either naïve to therapy or prior null responders to PEG-IFN therapy and which had unfavorable IL28B allele types, a significant difference in response rates was observed relative to that seen in genotype 2/3 patients (Gane et al., 2012). In the genotype 1 naïve population, a SVR12 of 84% was achieved, but in the null responder group only a 10% SVR24 was achieved. These results seemed

to indicate a difference in response to sofosbuvir in genotype 1 and genotype 2 and 3 patient populations and thus implied that a more aggressive regimen employing more than one potent DAA may be required in genotype 1 difficult to treat populations. To investigate this need for combining multiple potent DAAs, several studies that include sofosbuvir with an NS5A inhibitor, with or without RBV, were initiated (Sulkowski et al., 2012). A study combining sofosbuvir with the NS5A inhibitor daclatasvir achieved a 100% SVR12 irrespective of a 12- or 24-week treatment duration (Sulkowski et al., 2012). Similarly, the combination of sofosbuvir plus RBV and the NS5A inhibitor GS-5885 resulted in a SVR4 rate of 100% (Gane et al., 2012). In both sofosbuvir–NS5A combinations the treatment regimens were well tolerated with no drug-related adverse events. Even in a population of difficult to treat genotype 1 mostly African-American patients with various stages of liver fibrosis and a high CT/TT IL28B allele frequency, the sofosbuvir + RBV combination administered for 24 weeks achieved a SVR4 of 75% for the cohort having a large percentage of advanced fibrotics and a SVR12 of 90% for the cohort with no advanced fibrotic patients (Osinusi et al., 2012). In addition, preliminary studies show promise for the use of sofosbuvir in HIV/HCV coinfected patients (Rodriguez-Torres et al., 2012). Sofosbuvir is currently in late stage phase 3 clinical trials for the treatment of HCV-infected patients of all genotypes, and a US NDA filing is expected in mid-2013.

In the 2′-α-F-2′-β-*C*-methyl nucleoside series of inhibitors, the phosphoramidate prodrug approach was extended to include purines. A study of 2′-α-F-2′-β-*C*-methylguanosine ($EC_{90} = 69.2\ \mu M$) showed it to be weakly active as an inhibitor of HCV replication due to poor first-step phosphorylation yet its corresponding triphosphate was an effective inhibitor of the HCV polymerase (Chang et al., 2011; Clark et al., 2006). It was also revealed that the triphosphate was equipotent at inhibiting both the wild-type polymerase and the S282T-mutant polymerase known to be resistant to the 2′-methyl class of nucleosides. This observation was the first example where a 2′-methyl nucleos(t)ide retained activity against the S282T-mutant polymerase. In order to take advantage of this finding, a 2′-α-F-2′-β-*C*-methylguanosine 5′-phosphoramidate prodrug was developed resulting in a dramatic improvement in replicon potency. Further optimization to increase lipophilicity and improve cell penetration led to the C-6 methoxypurine double prodrug derivative (Fig. 2.4, **30** (PSI-353661) $EC_{90} = 0.008\ \mu M$) (Chang et al., 2011). Combination studies with PSI-353661 and either PEG-IFN, RBV, or other DAAs resulted in additive

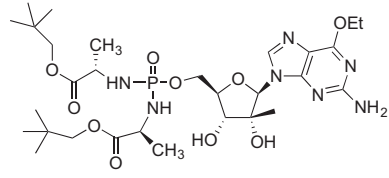
or synergistic effects (Furman et al., 2011). Studies investigating the unique resistance profile of this 2′-α-F-2′-β-*C*-methylguanosine class of nucleotides showed that these derivatives selected for a number of amino acid changes in a genotype 2 replicon that included S15G, R222Q, C223Y, C223H, L320I, and V321I and that mutations encoding for the C223Y and C223H amino acid substitutions were lethal to genotype 1 replicons (Lam et al., 2011a, 2011b). Phenotypic analysis showed that any single amino acid change was insufficient to significantly reduce the activity of PSI-353661 and that a combination of three amino acid changes, S15G/C223H/V321I was needed to confer a high level of resistance. Further analysis revealed that this triple mutation remained fully susceptible to 2′-*C*-methylguanosine derivatives INX-08189 and IDX-184 (Lam et al., 2011a, 2011b). Unfortunately, for unknown reasons, PSI-353661 was never progressed into clinical development.

The application of phosphoramidate prodrug technology was also shown to be effective in the development of novel C-linked nucleosides. The phosphoramidate prodrug GS-6620 (Fig. 2.4, **32**) contains a 2′-*C*-methyl ribose core to which is linked, via a carbon–carbon bond at the 1′-position, an adenosine base mimic (Cho et al., 2012). GS-6620 showed pangenotypic activity using subgenomic replicons from genotype 1a, 1b, and 2a and in chimeric replicons encoding NS5B from GT2b, 3a, 4a, 5a, and 6a. It also showed high intestinal absorption and hepatic extraction in dogs and high levels of the active triphosphate when administered to dogs and hamsters (Ray et al., 2011). GS-6620 entered human clinical studies and was determined to be safe and well tolerated; however, it demonstrated a variable intra- and interpatient pharmacokinetic and pharmacodynamic profile across an HCV genotype 1 patient population (Lawitz et al., 2012). Consequently, the development of GS-6620 was terminated because of its poor overall efficacy profile.

When the phosphoramidate prodrug strategy was applied to the 4′-azido class of HCV nucleoside inhibitors, variable results were achieved (Figs. 2.3 and 2.4). In the case of 4′-azidocytidine, phosphoramidate prodrug technology was utilized in an attempt to find a way to circumvent some of the hematological toxicity issues observed with R1626 (McGuigan, Kelleher, et al., 2009). It was hoped that a phosphoramidate prodrug of the 5′-monophosphate of R1626 would enable liver targeting and thereby reduce systemic exposure and dosage. However, exploration of R1626 phosphoramidates did not identify any inhibitors of HCV replication with improved potency over the parent nucleoside, thus implying that

phosphorylation was not an issue in the nucleoside's potency (Fig. 2.3, **23**) (McGuigan, Kelleher, et al., 2009). Further investigation of 4′-azido nucleosides focused on uridine and adenosine derivatives, which were inactive as inhibitors of HCV replication. When their phosphoramidate prodrugs were examined, active inhibitors such as **24** (Fig. 2.3) and **31** (Fig. 2.4) ($EC_{50} = 0.22$ μM and $EC_{50} = 0.22$ μM, respectively) were identified, but no further development of these series has been reported (Perrone et al., 2007).

With nucleotide phosphoramidate prodrugs such as PSI-7851, IDX-184, and INX-08189, the phosphate prodrug motif possessed a chiral phosphorus center. In the case of PSI-7851, the more potent diastereoisomer PSI-7977 (GS-7977, sofosbuvir) was progressed into late-stage clinical development and a stereoselective synthesis was ultimately achieved for this molecule (Ross, Reddy, Zhang, Rachakonda, & Sofia, 2011). For IDX-184 and INX-08189, the diastereomeric mixtures were progressed into development because there was no observed activity difference between the isomers. However, the development of an isomeric mixture can be problematic because of the need to carefully control the isomeric ratio and the potential difficulty in developing an amorphous solid versus a single isomer crystalline material. To develop a prodrug having an achiral phosphoramidate core, the phosphorodiamidates were explored. It was also suggested that the phosphorodiamidates would eliminate any potential toxicity concerns associated with the release of an aryloxy substituent found in the most common version of phosphoramidate prodrug constructs. Several reports that investigated the use of phosphorodiamidates studied their application in the case of 2′-*C*-methyl-6-alkoxyguanosine derivatives (Fig. 2.5, **35**) (Dousson et al., 2012; McGuigan et al., 2011). In these cases, the achiral diamidate prodrugs were shown to be as potent in the replicon assay and delivered equally



(EC_{50} = 0.04 μM)
33

Figure 2.5 Phosphonodiamidate prodrug HCV inhibitor of a 2′-*C*-methyl-*C*-6-ethoxy-2-aminopurine-5′-monophosphate.

significant levels of the active NTP to the liver *in vivo* as was seen for their corresponding chiral phosphoramidate cousins.

3.2. HepDirect liver-targeting prodrugs

Another prodrug construct that offers the potential for nucleotide liver targeting is the HepDirect technology (Erion, Bullough, Lin, & Hong, 2006). HepDirect technology relies on the use of a 1-aryl-1,3-propanyl moiety to effect liver targeting by relying on liver-specific enzymes that cleave the promoiety from a phosphate group. Removal of the HepDirect promoiety is accomplished via a cytochrome P450-mediated oxidation which results in hydroxylation of the C-4 tertiary carbon of the promoiety by CYP3A4 followed by ring opening and β-elimination to expose the nucleotide monophosphate. As a result of the prodrug cleavage process, an aryl vinyl ketone is released. It has been suggested that this potential cytotoxic byproduct can be rapidly scavenged by high levels of glutathione in the liver, thus minimizing the associated risk. The first clinical demonstration of the HepDirect prodrug technology was achieved with pradefovir, a prodrug of adefovir, as a treatment for HBV infection (Tillmann, 2007). However, reports of increased cancer risk when rats and mice were given high doses of pradefovir during long-term toxicology studies raised concerns about this prodrug approach.

HepDirect prodrugs of the 5′-monophosphates of both 2′-*C*-methylcytidine and 2′-*C*-methyladenosine were studied as inhibitors of HCV replication. Optimization of the prodrug moiety for each nucleotide led to the identification of prodrugs **34** and **35** (Fig. 2.6) (Carroll et al., 2011; Hecker et al., 2007). Studies in two HCV-infected chimpanzees showed that the cytidine derivative **34** when administered at a 10 mg/kg once-daily dose led to a −1.3 and −1.5 $\log_{10}$ IU/mL reduction in viral load (Carroll et al., 2011). A follow-up study that evaluated a 4 mg/kg intravenous dose over 6 days resulted in a −4.8 and −3.6 $\log_{10}$ IU/mL decline in viral load

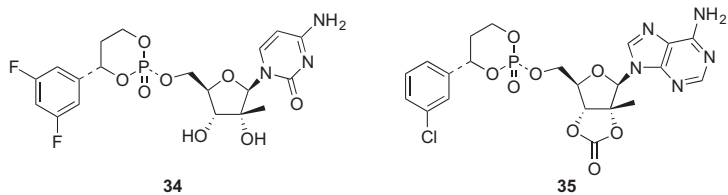


Figure 2.6 HepDirect prodrugs of 2′-*C*-methylcytidine-5′-monophosphate (**34**) and 2′-*C*-methyadenosine-5′-monophosphate (**35**).

with one animal reaching the detectability limit on day 2. These results showed an improvement over the chimpanzee study results seen for the nucleoside NM283 (Fig. 2.2, **14**).

Application of the HepDirect prodrug approach to 2′-*C*-methyladenosine 5′-monophosphate was intended to help address the poor phosphorylation rate and the problem of adenosine deamination. However, even after applying the HepDirect promoiety, oral bioavailability in the rat was observed to be low (5%). To help address this poor PK result, the 2′-and 3′-hydroxyl groups of the ribose sugar were eventually masked as a 2′,3′-cyclic carbonate providing compound **35** (Fig. 2.6) (Hecker et al., 2007). This double prodrug displayed a 2- to 10-fold improvement in rat liver triphosphate levels relative to the use of HepDirect alone. No subsequent reports addressing the development of HepDirect prodrugs for HCV have appeared.

3.3. 3′,5′-Cyclic phosphate nucleotide prodrugs

Several early reports have documented the use of 3′,5′-cyclic phosphates as an approach to deliver nucleoside 5′-monophosphates; however, none have disclosed the use of this prodrug strategy in a clinical setting (Beres, Bentrude, Kruppa, McKernan, & Robins, 1985; Beres et al., 1986; Girardet et al., 1995). The 3′,5′-cyclic phosphate approach was developed with the expectation that by both reducing the rotational degrees of freedom with a conformationally constrained structure and blocking the C3′ hydroxyl group to reduce polarity, improved cellular uptake would result. In the area of HCV nucleotide drug development, the 3′,5′-cyclic phosphate prodrug construct has seen significant application by marrying it with a variety of prodrug activation mechanisms. These activation mechanisms have included the phosphoramidate, the SATE, the pivaloyloxymethyl (POM), carbonate, and the simple alkyl ester group as promoieties.

Use of the 3′,5′-cyclic phosphoramidate construct was applied to 2′-*C*-methylcytidine (Fig. 2.7, **36**) (Meppen et al., 2009). Although several 2′-*C*-methylcytidine 3′,5′-phosphoramidates produced improved levels of NTP (two to sevenfold) when incubated with human primary hepatocytes, none showed improved activity in the replicon assay relative to the parent nucleoside. In addition, these cytidine cyclic phosphoramidates lacked sufficient oral bioavailability and produced low liver levels of triphosphate.

Application of the cyclic phosphate construct to purine nucleotides showed mixed results. 2′-*C*-methyladenosine derivatives that incorporated

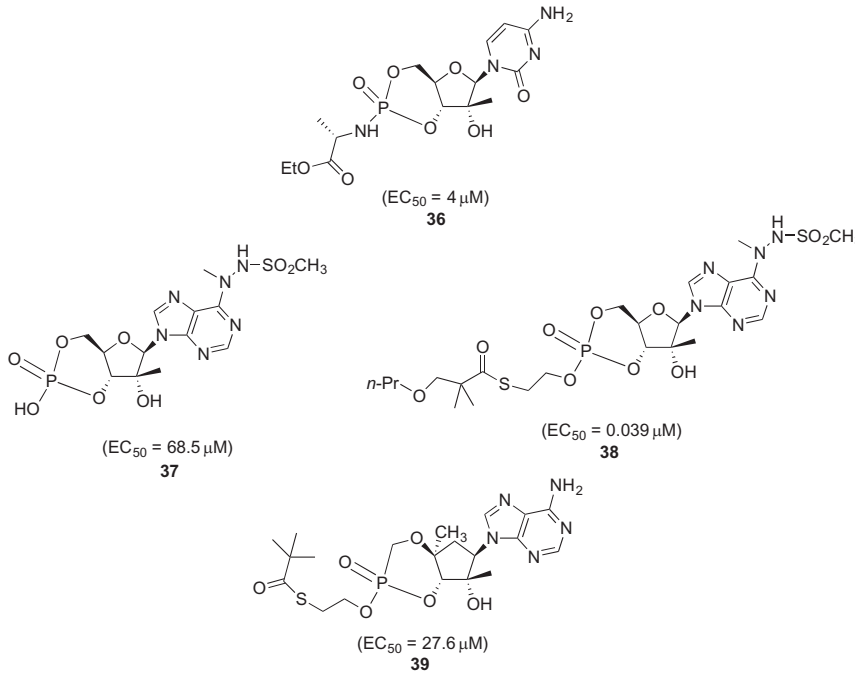


Figure 2.7 Nucleotide derivatives having the 3′,5′-cyclic phosphate prodrug construct containing either a free phosphate monoacid (**37**), a phosphoramidate (**36**), or SATE (**38** and **39**) promoiety.

both a C-6 hydrazidosulfonamide group as an amine promoiety and the 3′,5′-cyclic phosphate construct showed only weak activity in the case of the simple cyclic phosphate derivative **37** (Fig. 2.7) but potent replicon activity when using the SATE ester **38** (Fig. 2.7, $EC_{50} = 0.039$ μM) (Gunic et al., 2007). Attempts to further explore these adenosine cyclic phosphates by applying POM or carbonate ester promoieties were decidedly unsuccessful. Similarly, a 3′,5′-cyclic phosphonate with at SATE ester promoiety **39** (Fig. 2.7) was shown to be only modestly active in the HCV replicon assay and >14-fold less active than 2′-*C*-methyladenosine (Fig. 2.1, **8**) (Gunic et al., 2007).

When the 3′,5′-cyclic phosphate prodrug strategy was applied to guanosine nucleoside analogs, more promising results were forthcoming than that observed for either cytidine or adenosine analogs. Like in the case of the 2′-*C*-methyl-6-hydrazidosulfonamidopurine derivatives **38** (Fig. 2.7), the cyclic phosphate of the 2′-*C*-methyl-2-amino-6-hydrazidosulfonamidopurine analog **41** (Fig. 2.8) which contained the SATE ester promoiety was shown

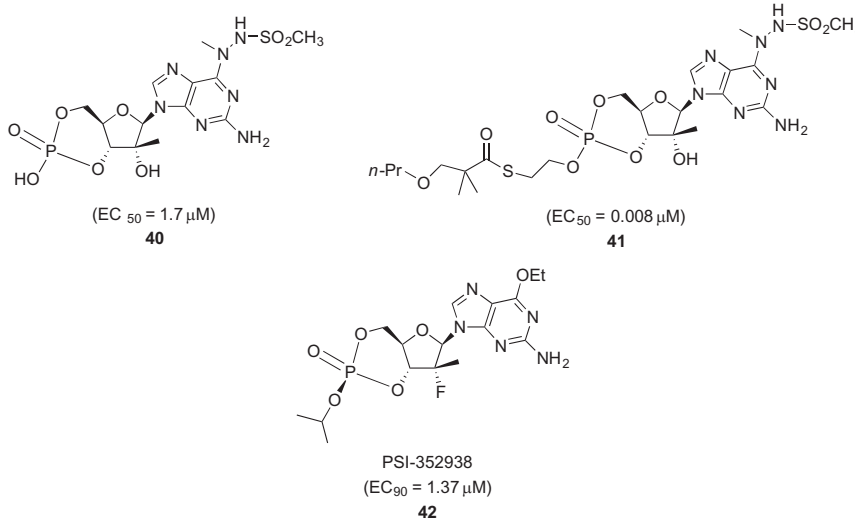


Figure 2.8 Purine nucleotide derivatives having the 3′,5′-cyclic phosphate prodrug construct containing either a free phosphate monoacid (**40**), a SATE (**41**) or an alkyl ester (**42**) promoiety.

to be quite potent ($EC_{50} = 0.008$ μM) (Gunic et al., 2007). However, the most exciting results were obtained for the 2′-α-F-2′-β-C-methyl class of nucleosides. In this case, double prodrugs that combined an alkyl 3′,5′-cyclic phosphate construct with a C-6 alkoxy substituent on the 2-aminopurine base demonstrated good *in vitro* replicon potency and generally good overall safety and stability characteristics (Du et al., 2012; Reddy et al., 2010). As in the case of their phosphoramidate counterparts (Fig. 2.4, **30**), this series of cyclic phosphates were shown to be equipotent in both the wild-type HCV replicon and the replicon containing the S282T amino acid change in the NS5B polymerase known to be resistant to 2′-*C*-methyl nucleosides. Resistance was observed in the case of the replicon containing the combination of three amino acid changes S15G/C223H/V321I in the NS5B polymerase (Lam et al., 2011a, 2011b). Broad genotype coverage and additive or synergistic activity was also observed when these cyclic phosphates were combined with other PEG-IFN, RBV, or other DAAs. In both rat and dog, high liver levels of the active triphosphate were detected after oral dosing, but evidence of rapid first pass metabolism was not detected since high-circulating levels of intact prodrug was observed. Ultimately, the cyclic phosphate PSI-352938 (Fig. 2.8, **42**) was selected for clinical development, and in a phase 1 study, it was shown to be safe and well

tolerated at doses of up to 1600 mg once daily (Rodriguez-Torres et al., 2011). A 7-day multiascending dose monotherapy study in genotype 1 HCV patients administered 100, 200, and 300 mg once daily and 100 mg twice daily led to viral load declines of −4.31, −4.65, −3.94, and −4.59 $\log_{10}$ IU/mL, respectively (Rodriguez-Torres et al., 2011). These results represented the first proof-of-concept for the use of a 3′,5′-cyclic phosphate prodrug for the delivery of a nucleotide in a human clinical setting. Subsequently, a 14-day study that examined an IFN-free regimen combining two nucleotides (sofosbuvir and PSI-352938) showed that there was no PK interactions between the two drugs and that a −4.6 to −5.5 $\log_{10}$ IU/mL reduction in viral load was achieved with 94% of patients having viral load levels below the limit of detection with rapid first-phase viral kinetics (Lawitz et al., 2011). This study provided proof-of-concept for a dual nucleotide combination regimen that is similar to that seen in HIV highly active antiretroviral therapy drug regimens. Detailed mechanistic studies eventually delineated the metabolic pathway by which these cyclic phosphates are converted to their guanosine nucleoside 5′-monophosphates. It was determined that in the case of PSI-352938, a CYP3A4-mediated oxidation first removes the phosphate isopropyl ester followed by phosphodiester cleavage of the 3′ phosphate–oxygen bond. Once the 5′-monophosphate was revealed, hydrolytic cleavage of the C-6 ethoxyl group of the purine base proceeded via adenosine deaminase like protein-1-mediated hydrolysis producing the 2′-α-F-2′-β-*C*-methylguanosine-5′-monophosphate. These metabolism results and the abundance of CYP3A4 enzymes in the liver were able to explain the liver-targeting profile of this class of cyclic phosphate prodrugs. Unfortunately, in extended human clinical study elevated liver enzymes were detected in patients after 28 days on PSI-352938. These adverse events resulted in suspension of clinical trials.

3.4. Phosphate and phosphonate ester prodrugs

Several phosphate ester prodrug strategies have been applied to nucleotides in an attempt to mask a phosphate group and enhance its ability to penetrate biological membranes (Hecker & Erion, 2008). These strategies include the well-known SATE promoiety and lipid-phosphate esters. As observed with the phosphoramidate promoieties of INX-184 and the 3′,5′-cyclic phosphates **38** and **41**, the SATE promoiety has been incorporated into other prodrug constructs. However, its use as a stand-alone promoiety has also

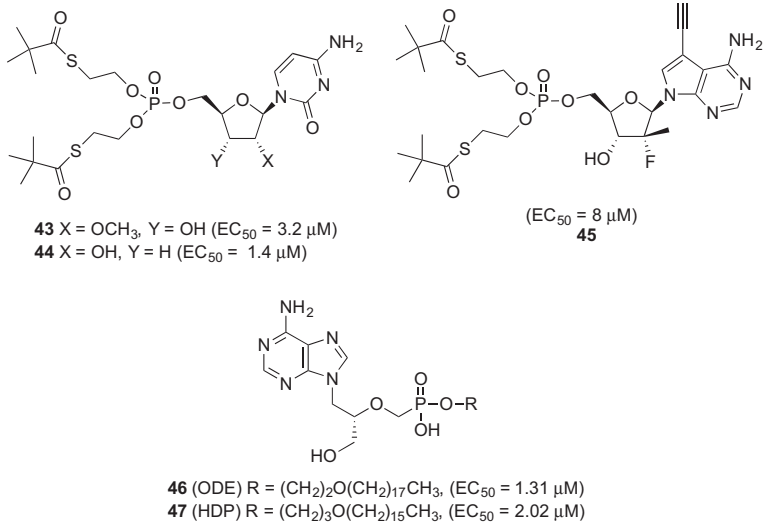


Figure 2.9 Phosphate ester prodrug HCV inhibitors containing the SATE promoiety (**43**, **44**, and **45**) or a lipid monoester promoiety (**46** and **47**).

been reported. In an attempt to improve on the potency and cellular stability of 2′-*O*-methylcytidine and 3′-deoxycytidine, the SATE prodrugs of their corresponding 5′-monophosphate derivatives were investigated (Prakash et al., 2005; Tomassini et al., 2005). Relative to the parent nucleosides, the SATE prodrugs of 2′-*O*-methylcytidine and 3′-deoxycytidine (Fig. 2.9, **43** and **44**) demonstrated a 7- and 25-fold improvement in replicon potency, respectively. This was consistent with the observation of a 3- and 10-fold increase in intracellular triphosphate formation. The SATE prodrug approach was also applied in the case of 2′-α-F-2′-β-*C*-methyl 7-ethynyl-7-deaza adenosine 5′-monophosphate (Prhavc et al., 2008). The activity of the SATE prodrug **45** (Fig. 2.9; $EC_{50} = 8$ μM) was shown to be threefold better in the whole-cell replicon assay than the parent nucleoside. Since no additional information has been reported regarding the development of these SATE prodrugs, it is assumed that these compounds were not progressed further into development. Much of the concern regarding the SATE prodrug approach stems from the prodrug cleavage products (Table 2.1). The SATE cleavage products include the release of an equivalent of episulfide. Episulfide is known to react rapidly with nucleophiles and therefore can alkylate proteins and nucleic acids and has been identified as a mutagen (Jones & Bischofberger, 1995).

Acyclic nucleotides have been extensively studied as inhibitors of viral replication (Mackman & Cihlar, 2011). These acyclic nucleotides include the drugs tenofovir and adefovir for the treatment of HIV and HBV infection. Each of these drugs is an acyclic phosphonate that requires a prodrug strategy to make it optimally effective. The acyclic nucleotide antiviral (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine ((*S*)-HPMPA) which was shown to be active against herpes and orthopoxvirus was evaluated as an inhibitor of HCV replication by first preparing the octadecyloxyethyl (ODE) and hexadecyloxypropyl (HDP) phosphate-lipid prodrugs (Wyles et al., 2009). These phosphate-lipid prodrugs were demonstrated to be effective at improving cellular uptake of acyclic nucleotides in the case of acyclic inhibitors of HIV and CMV. They also appeared to have utility in delivering acyclic nucleotides for treating HCV. ODE (Fig. 2.9, **46**) (genotype 1b, $EC_{50} = 1.31$ μM, genotype 2a, $EC_{50} = 0.69$ μM) and HDP (Fig. 2.9, **47**) ($EC_{50} = 2.02$ μM) esters of (*S*)-HPMPA proved to be modest inhibitors of HCV replication.

4. CONCLUSION

The HCV NS5B RdRp has proven to be a rich and productive target for the development of drugs to treat patients infected with HCV. In particular, the development of nucleos(t)ide inhibitors to treat HCV infection has seen an explosion of activity over the past 10 years. This activity has resulted in the discovery of a number of novel nucleoside and nucleotide inhibitors that have proven to be effective at inhibiting HCV. These discoveries have been successfully translated into the human clinical setting. Nucleos(t)ides have differentiated themselves from other direct-acting antiviral therapies to treat HCV by being pangenotypic, by having a high barrier to development of resistant virus, and by their successful use in DAA combination regimens. The implementation of prodrug strategies to enable the intracellular and *in vivo* liver-targeting oral delivery of nucleoside 5′-monophosphate HCV inhibitors led to the identification of potent agents that have revolutionized the way clinicians are thinking about the future of HCV treatment. It is now possible to contemplate that HCV patients of all genotypes can be treated with therapies that no longer include IFN. Several studies have shown that combining a nucleotide prodrug HCV inhibitor with only one other DAA can lead to cure rates of as high as 100% after only 12 weeks of therapy and without the undesirable side effects associated with IFN-containing treatments. Even previously difficult to treat

patients who did not respond to historic SOC treatment regimens or who demonstrated significant fibrotic liver disease responded to IFN-free nucleotide prodrug-containing regimens. Therefore, it is possible to imagine that in the near future an HCV-infected individual may be cured by a simple 12-week therapeutic regime consisting of a single pill comprising a coformulation of a nucleotide prodrug and one other DAA. Future work will determine which nucleotide prodrug/DAA combinations will be both effective and safe and what will be the optimal and shortest treatment duration to guarantee a cure.

CONFLICT OF INTEREST

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CHAPTER THREE

HIV Integrase Inhibitors: 20-Year Landmark and Challenges

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Contents

Abstract

Since the discovery of HIV as the cause for AIDS 30 years ago, major progress has been made, including the discovery of drugs that now control the disease. Here, we review the integrase (IN) inhibitors from the discovery of the first compounds 20 years ago to the approval of two highly effective IN strand transfer inhibitors (INSTIs), raltegravir (Isentress) and elvitegravir (Stribild), and the promising clinical activity of dolutegravir. After summarizing the molecular mechanism of action of the INSTIs as interfacial inhibitors, we discuss the remaining challenges. Those include: overcoming resistance to clinical INSTIs, long-term safety of INSTIs, cost of therapy, place of the INSTIs in prophylactic treatments, and the development of new classes of inhibitors (the LEDGINs) targeting IN outside its catalytic site. We also discuss the role of chromatin and host DNA repair factor for the completion of integration.

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ABBREVIATIONS

3′-P 3′-processing
CCD catalytic core domain
CTD C-terminal domain
DTG dolutegravir
EVG elvitegravir
HAART highly active antiretroviral therapy
IN integrase
INSTI integrase stand transfer inhibitor
LEDGIN LEDGF/p75-integrase inhibitor
NNRTI nonnucleoside reverse transcriptase inhibitor
NRTI nucleoside reverse transcriptase inhibitor
NTD N-terminal domain
PIC preintegration complex
RAL raltegravir
RT reverse transcriptase
ST strand transfer

1. INTRODUCTION

Human history has been marked by pandemics resulting from parasitic (malaria), bacterial (plague), and viral (flu) infections. The Center for Disease Control and prevention (CDC) alerted about acquired immunodeficiency syndrome (AIDS) in 1981, and the etiological agent, human immunodeficiency virus (HIV), was isolated in 1983 (Barre-Sinoussi et al., 1983). Azidothymidine (AZT) was approved shortly after on March 1987 as the first nucleoside reverse transcriptase (RT) inhibitor (NRTI) for the treatment of AIDS (Yarchoan & Broder, 1987). As resistance to AZT quickly emerged by mutations of its target RT (Larder & Kemp, 1989), it became clear that resistance could be minimized by combining drugs together in a highly active antiretroviral therapy (HAART) to raise the genetic barrier (Larder, Darby, & Richman, 1989). The first bitherapy was made publicly available in 1997 [AZT in combination with lamivudine (3TC)], 10 years after AZT approval. The number of drugs approved increased at an unprecedented speed (Fig. 3.1), making available new classes of molecules (nonnucleoside RT inhibitors, NNRTI) as well as drugs acting on new viral targets [protease (PR) inhibitors, PI; entry/fusion inhibitors; integrase (IN) strand transfer (ST) inhibitors, INSTIs]. Today, with 27 drugs available (Table 3.1), the therapeutic choices are multiple and have changed the prognosis of HIV

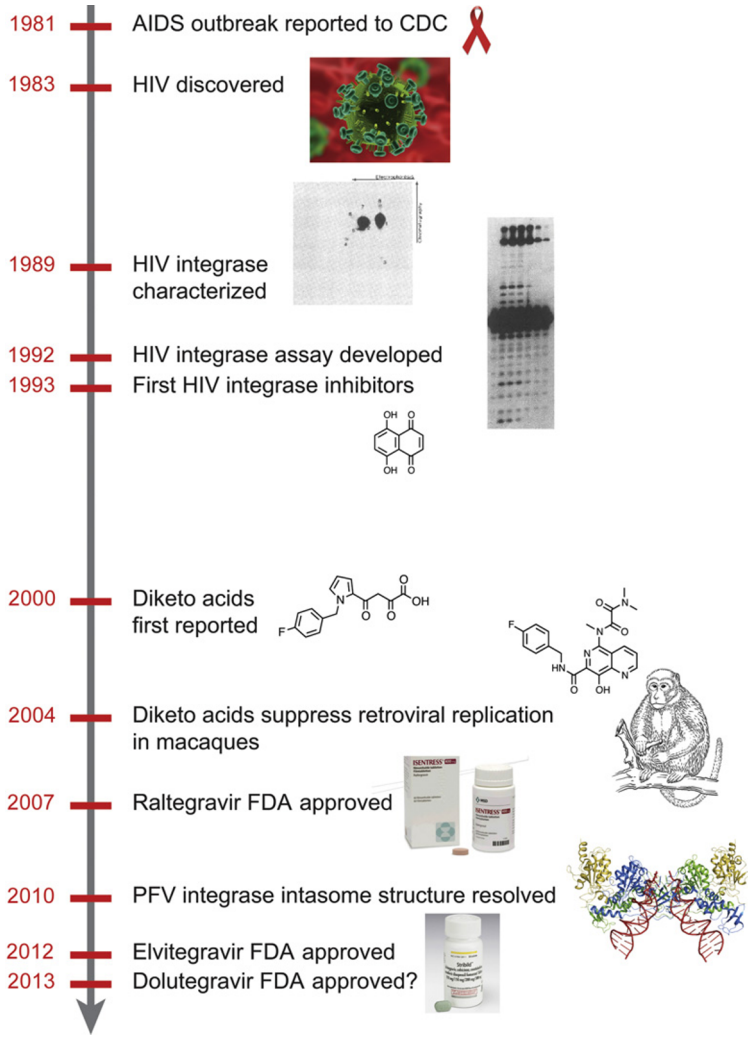

Figure 3.1 Timeline focusing on HIV-1 integrase inhibitor discovery.

infections in the developed world. Recently, single daily pills with high efficacy and practical convenience are becoming the norm (Marchand, 2012). Providing access to the drugs in low- and middle-income countries remains a major challenge (globally, only 54% of people eligible to therapy actually are under treatment; Vazquez, 1996).

Table 3.1 List of the FDA-approved anti-HIV drugs and formulations (www.fda.org)

Approval date	Brand name	Class	Category	In combination with		Manufacturer
2012	Stribild	Multitarget combination	INI + NRTI	Elvitegravir Cobicistat Emtricitabine Tenofovir disoproxil fumarate	QD	Gilead Sciences
2011	Edurant		NNRTI	Rilpivirine	QD	Tibotec Therapeutics
2011	Complera	Multiclass combination	NRTI + NNRTI	Rilpivirine Emtricitabine Tenofovir disoproxil fumarate	QD	Gilead Sciences
2008	Intelence		NNRTI	Etravirine	BID	Tibotec Therapeutics
2007	Selzentry		CCR5 antagonist	Maraviroc	BID[a]	Pfizer
2007	Isentress		INI	Raltegravir	BID	Merck & Co.
2006	Atripla	Multiclass combination	NRTI + NNRTI	Efavirenz Emtricitabine Tenofovir disoproxil fumarate	QD	Bristol-Myers Squibb Gilead Sciences
2006	Prezista		PI	Darunavir	QD[b]	Tibotec Therapeutics

2005	Aptivus		PI	Tripranavir (TPV)	BID[b]	Boehringer Ingelheim
2004	Epzicom	Combination	NRTI	Abacavir Lamivudine	QD[a]	GlaxoSmithKline
2004	Truvada	Combination	NRTI	Emtricitabine Tenofovir disoproxil fumarate	QD	Gilead Sciences
2003	Emtriva		NRTI	Emtricitabine (FTC)	QD	Gilead Sciences
2003	Lexiva		PI	Fosamprenavir calcium (FOS-APV)	BID[b]	GlaxoSmithKline
2003	Fuzeon		Fusion inhibitor	Enfuvirtide (T-20)	BID[c]	Hoffmann-La Roche Trimeris
2003	Reyataz		PI	Atazanavir sulfate (ATV)	QD[b]	Bristol–Myers Squibb
2001	Viread		NRTI	Tenofovir disoproxil fumarate	QD	Gilead Sciences
2000	Trizivir	Combination	NRTI	Abacavir Lamivudine Zidovudine	BID	GlaxoSmithKline
2000	Kaletra	combination	PI	Lopinavir Ritonavir	BID	Abbott Laboratories

Continued

Table 3.1 List of the FDA-approved anti-HIV drugs and formulations (www.fda.org)—cont'd

Approval date	Brand name	Class	Category	In combination with		Manufacturer
1999	Agenerase		PI	Amprenavir (APV)	BID[a]	GlaxoSmithKline
1998	Ziagen		NRTI	Abacavir sulfate (ABC)	BID[a]	GlaxoSmithKline
1998	Sustiva		NNRTI	Efavirenz (EFV)	QD[a]	Bristol–Myers Squibb
1997	Combivir		NRTI	Lamivudine Zidovudine	BID	GlaxoSmithKline
1997	Rescriptor		NNRTI	Delavirdine (DLV)	TID[a]	Pfizer
1997	Fortovase[d]		PI	Saquinavir	TID or BID[b]	Hoffmann-La Roche
1997	Viracept		PI	Nelfinavir mesylate (NFV)	TID or BID	Agouron Pharmaceuticals
1996	Viramune[e]		NNRTI	Nevirapine (NVP)	QD[a]	Boehringer Ingelheim
1996	Crixivan		PI	Indinavir (IDV)	QID	Merck & Co.
1996	Norvir		PI	Ritonavir (RTV)	BID[a]	Abbott Laboratories
1995	Epivir		NRTI	Lamivudine (3TC)	QD[a] or BID[a]	GlaxoSmithKline

1995	Invirase	PI	Saquinavir mesylate (SQV)	BID[b]	Hoffmann-La Roche
1994	Zerit	NRTI	Stavudine (d4T)	BID	Bristol–Myers Squibb
1992	Hivid[d]	NRTI	Zalcitabine (ddC)	QID[a]	Hoffmann-La Roche
1991	Videx[e]	NRTI	Didanosine (ddI)	QD or BID	Bristol–Myers Squibb
1987	Retrovir	NRTI	Zidovudine (ZDV, AZT)	BID[a] or TID[a]	GlaxoSmithKline

QD, *quaque die* (once a day); BID, *bis in die* (twice daily); TID, *ter in die* (three times a day); QID, *quater in die* (four times a day); INI, integrase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor.
[a]Must be given in combination with other antiretroviral medications.
[b]Must be administered with ritonavir.
[c]Injection.
[d]No longer marketed.
[e]Newer formulation available (i.e., extended release or enteric coated).

2. REQUIREMENT OF IN FOR HIV REPLICATION

HIV is a retrovirus coding for structural (*env*), nonstructural (*gag*-pol), and accessory proteins (Nef, Rev, Tat, Vif, Vpr, and Vpu; Cullen, 1991). Its replication requires both viral and cellular enzymes. IN is one of the three viral enzymes encoded by the *POL* gene, together with RT and PR. To replicate, the virus first attaches to cells harboring the membrane receptor CD4 (Fig. 3.2), explaining its selectivity for dendritic cells, macrophages, and T-lymphocytes. Interactions with a coreceptor (either CCR5 or CXCR4) initiate conformational changes of the viral gp120–gp41 complex, enabling membrane fusion and release of the viral core into the cytoplasm. Viral RNA is reverse-transcribed into a double-stranded viral DNA (vDNA) copy (Fig. 3.2), which is assembled into a large nucleoprotein complex with IN bound to its ends (preintegration complex, PIC; Bukrinsky et al., 1993).

IN carries out vDNA integration following two consecutive steps: 3′-processing (3′-P) in the cytoplasm and ST in the nucleus. For 3′-P, IN processes vDNA by cleaving its 3′-end immediately after a conserved CA dinucleotide, thereby releasing a GT dinucleotide from each long terminal repeats (LTRs) 3′-ends (Figs. 3.2 and 3.3). The PIC then translocates to the nucleus, where IN binds to the cellular target DNA. ST is carried out by IN tetramers (see Section 3), allowing the concerted integration of both extremities of the linear vDNA, five bases from each other on opposite strands, producing a 5-nucleotide sequence that is repeated at each side of the fully integrated proviral DNA (Fig. 3.3). Thus, to complete the integration process, ST products need to be processed and fully sealed with the host genome. This "repair" step requires removal of the two mispaired nucleotides at the 5′-ends of the vDNA and gap filling (Fig. 3.3 and Section 5.5). Once repaired, transcription, translation, and maturation of the different viral components lead to the assembly of new particles budding out of the cell. Of note, a small but consistent fraction of PIC (around 1%) can undergo different processes after nuclear import (Jurriaans, de Ronde, Dekker, Goudsmit, & Cornelissen, 1992). Those include end-joining, homologous recombination, or autointegration (IN dependent) and produce circular forms of vDNA (1-LTR and 2-LTR circles, Fig. 3.3; Craigie & Bushman, 2012). Inhibition of IN markedly increases the proportion of those forms, raising the question of potential episomal expression or DNA reservoirs for later integration (Cara & Klotman, 2006).

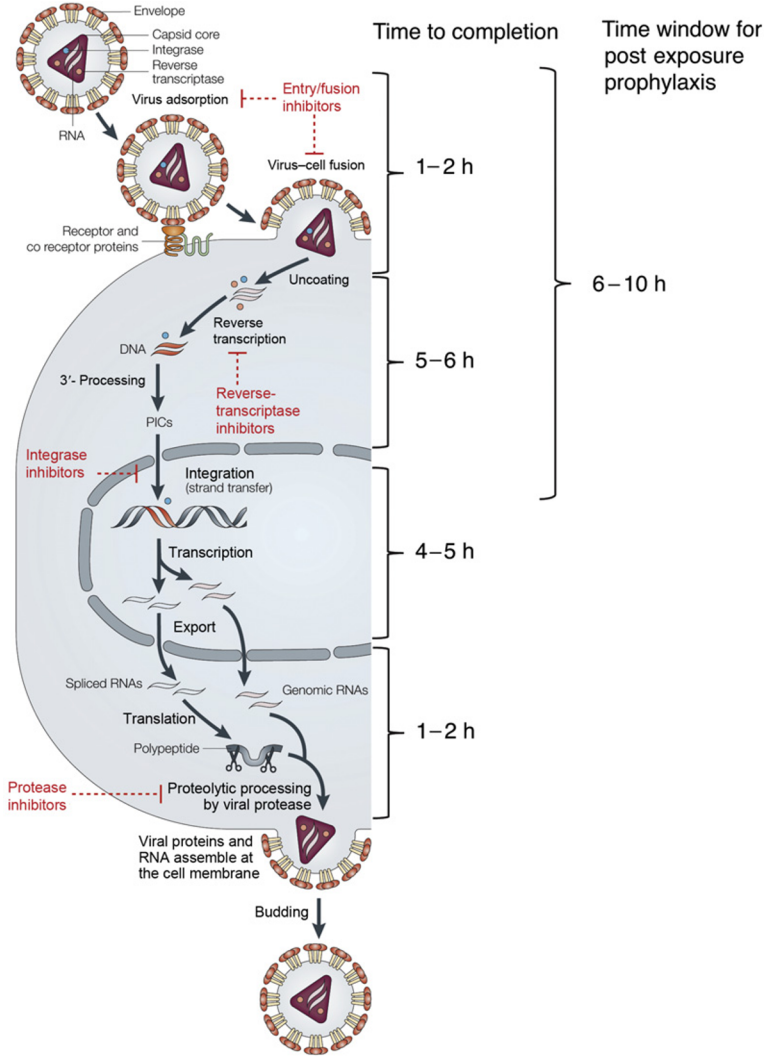

Figure 3.2 HIV-1 replication cycle. Clinically targeted steps are highlighted in red/dark gray. Time frame for postinfection prophylaxis with INSTIs is indicated on the right. *Adapted from Metifiot, Marchand, Maddali, and Pommier (2010) and Pommier et al. (2005).*

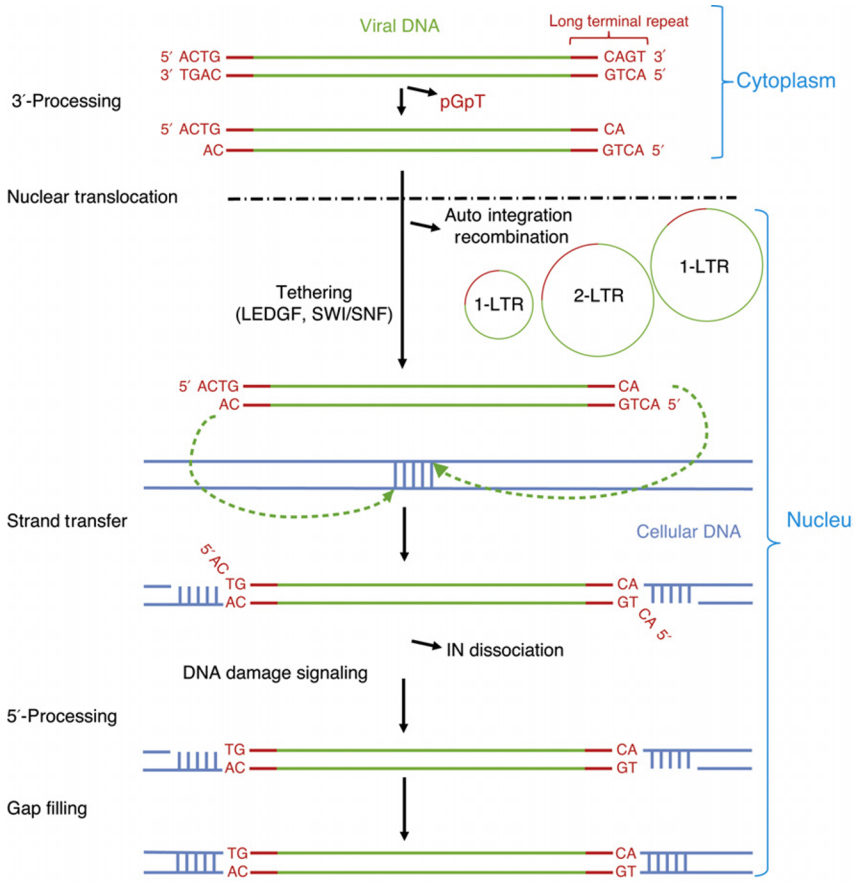

Figure 3.3 Schematic steps for integration.

3. IN STRUCTURE

HIV-1 IN is a 288 amino acid long (32 kDa) polypeptide belonging to a family of proteins including RNase H, Ruv C, transposases, and other retroviral INs (Engelman & Cherepanov, 2012). IN contains three domains. The N-terminal domain (NTD, amino acids 1–49) comprises a zinc-binding motif, HHCC, important for oligomerization. IN residues 50–212 correspond to the catalytic core domain (CCD), including a D-D$_{35}$-X motif ($D_{64}D_{116}E_{152}$) conserved among the IN superfamily. The C-terminal domain (CTD, amino acids 213–288) contains an SH3-like

domain involved in nonsequence-specific DNA binding. Overall, the three domains of IN bind DNA as an oligomer.

IN, in its active form, the intasome, is a tetramer with two active sites (Fig. 3.4), each using two magnesium ions coordinated by a triad of conserved acidic residues (the DDE motif). To date, no crystal structure of the full-length HIV-1 IN has been resolved. However, studies involving the three isolated domains demonstrated that all three form homodimers in solution. Resolution of the combinations NTD–CCD and CCD–CTD structures in addition to electron microscopy experiments have permitted the modeling of a full-length structure(Chen et al., 2000; Ren, Gao, Bushman, & Yeager, 2007; Wang, Ling, Yang, & Craigie, 2001). However, in those structures lacking the vDNA, a flexible loop surrounding the active site (amino acids 140–149) is poorly resolved, voiding the possibility of rational drug design.

After 20 years, the first crystal structure of a full-length IN in complex with its DNA substrate (intasome) has been solved (Fig. 3.4; Hare, Gupta, Valkov, Engelman, & Cherepanov, 2010). This intasome is from the prototype foamy virus (PFV), a Spumavirus quite divergent from its HIV-1 counterpart outside of the active site (Yin & Craigie, 2010). Nonetheless, this first insight in the complete architecture of the intasome enabled the generation of more accurate homology models for HIV-1 IN (Johnson, Metifiot, Pommier, & Hughes, 2012; Krishnan et al., 2010). These models include the vDNA and allow docking and rational design of inhibitors in the active site of HIV-1 IN.

4. THE INSTIs

The first IN inhibitors (INI) were reported 20 years ago (Fesen, Kohn, Leteurtre, & Pommier, 1993), only 10 years after the discovery of HIV as the agent responsible for AIDS, and approximately 15 years before the Food and Drug Administration (FDA)'s approval of raltegravir. These inhibitors belonged to various pharmacological categories including DNA binding drugs, antimalarial agents, naphthoquinones, and flavones (Fig. 3.1). Of note, a common feature of these compounds was a metal chelating catechol group (Fesen et al., 1993). The next milestone was reached 7 years later with the report by Hazuda and coworkers from the Merck research laboratories of the diketo acids as the first INSTIs to inhibit viral replication at nanomolar concentrations in infected cells (Hazuda, Iwamoto, & Wenning, 2009; Hazuda et al., 2000). Four years later, the same group provided the proof

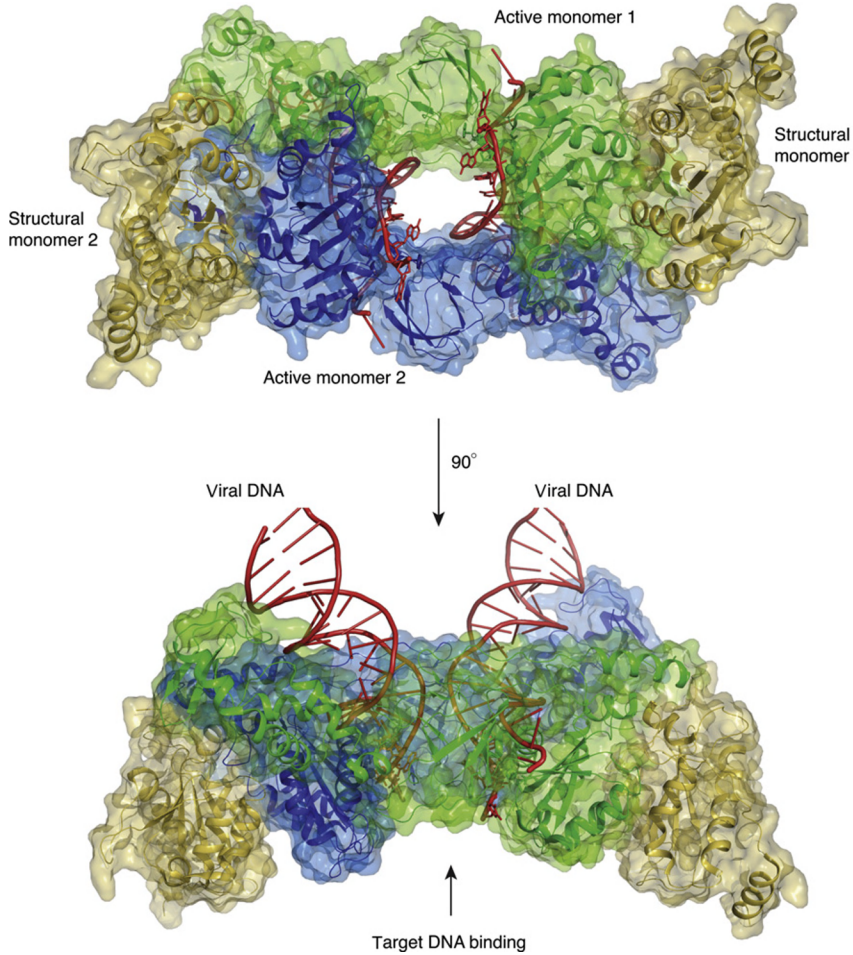

Figure 3.4 Structure of the intasome. The tetrameric PFV IN structures containing the viral DNA are derived from the PDB ID 4E71 (Hare et al., EMBO 2012). Proteins are shown in surface and cartoon, and the magnesium cofactors as spheres. Structural outer subunits (noncatalytically active) are represented in yellow/light gray and inner active subunits are represented in blue/dark gray and green/medium gray, respectively. The viral DNA is shown in red cartoon (dark gray) and the last four bases on the cleaved strand are presented as sticks. The bottom representation corresponds to a 90° rotation of the top view.

of principle that INSTIs can efficiently suppress viral replication in rhesus macaques (Hazuda et al., 2004). Raltegravir (RAL, Isentress®, Merck & Co.) was approved in 2007 by the FDA as the first INSTI for heavily treated AIDS patients with multidrug resistance. Two years later, RAL was approved for naïve patients in first-line therapy, and since, the use of RAL has been extended to pediatric patients and patients with co-hepatitis B or C infections. RAL is well tolerated but in a small fraction of patients selects for resistance mutations in the IN gene. Resistance to RAL arises from three major mutation sites at positions 155 (N155H), 143 (Y143R), and 148 (G140S/Q148H double mutant). RAL is used in a twice-daily administration, which does not favor treatment adherence. Therefore, new INSTIs have been developed to overcome RAL resistance with a once-daily administration.

Elvitegravir (EVG) was approved as the second INSTI for the treatment of HIV/AIDS in August 2012. It is formulated as the "quad pill" (Stribild®, Gilead Sciences) together with the pharmaco-enhancer cobicistat, and the two NRTIs emtricitabine and tenofovir disoproxil fumarate (Marchand, 2012). Cobicistat, by boosting the blood concentration of EVG, allows EVG to be administered at 150 mg once daily with a safety and efficacy comparable to raltegravir 400 mg twice daily. Because EVG does not structurally interact with the HIV-1 IN residue Y143, it alleviates RAL resistance resulting from mutation at this position (Table 3.2; Metifiot, Vandegraaff, et al., 2011). Unfortunately, RAL resistance mutation at position 148 (double mutant G140S/Q148H) remains highly cross-resistant to EVG (Table 3.2; Metifiot, Vandegraaff, et al., 2011).

Dolutegravir (DTG) is in advanced stage clinical development as a once-daily single formulation in the 572-Trii pill (Shionogi-ViiV Healthcare, LLC). Like the "quad pill," 572-Trii combines both IN and RT inhibitors. DTG (50 mg, unboosted) is packaged with two NRTIs, lamivudine and abacavir (van Lunzen et al., 2012). DTG (formerly S/GSK1349572) has a higher genetic barrier than RAL and EVG with respect to selecting for drug-resistance mutations in IN (Hare et al., 2011; van Lunzen et al., 2012). The double mutant G140S/Q148H is less than 4-fold resistant to DTG, whereas it is resistant to RAL by several 100-fold (Table 3.2). Hightower et al. recently used RAL, EVG, and DTG to measure each drug's residence time within the IN active site. Surprisingly, EVG seems to be the weakest binder in the context of the WT enzyme, and the reported residence time of the drugs are about 3, 9, and 71 h for EVG, RAL, and DTG, respectively (Hightower et al., 2011). More importantly, mutations involved in INSTIs resistance increase the dissociation rate of the clinical drugs, and only DTG retains a long

Table 3.2 Clinically relevant integrase mutations conferring resistance to INSTIs used in the treatment of HIV/AIDS

Resistance mutations	Raltegravir	Elvitegravir	Dolutegravir	References
N155H	30–50	50–60	<4	Hare et al. (2011), Metifiot, Vandegraaff, et al. (2011), and Quashie et al. (2012)
Y143R	42–50	2	<4	Hare et al. (2011), Metifiot, Johnson, et al. (2011), Metifiot, Vandegraaff, et al. (2011), Quashie et al. (2012), and Wainberg et al. (2012)
G140S/ Q148H	400–770	2200	<4	Hare et al. (2011), Metifiot, Johnson, et al. (2011), Metifiot, Vandegraaff, et al. (2011), Quashie et al. (2012), and Wainberg et al. (2012)
E138K/ Q148K	>150	>150	≈20	Fransen et al. (2009), Johnson et al. (2006), Kobayashi et al. (2011), and Quashie et al. (2012)
Q148R/ N155H	>140	>300	≈10	Kobayashi et al. (2011)
G118R	2–10	<2	2–10	Hare et al. (2011), Quashie et al. (2012), and Wainberg et al. (2012)
R263K	1–2	6	11	Margot et al. (2012) and Quashie et al. (2012)
R263K/ H51Y	NR	NR	15	Mesplede et al. (2012)

Antiviral resistance is expressed as fold-change compared to wild type. NR, not reported. Note the importance of Q148 mutations for INSTI resistance.

retention time (>5 h), which correlates with its better activity against those mutants. However, the improved resistance profile of DTG is not yet fully understood (Johnson et al., 2012). Clinical resistance to DTG has not yet been reported, but IN mutations G118R, E138K, Q148K, R263K, and H51Y have been selected in infected cells cultured in the presence of DTG (Table 3.2; Quashie et al., 2012; Wainberg, Mesplede, & Quashie, 2012).

RAL, EVG, and DTG have all been cocrystallized in the PFV IN active site (Hare et al., 2010, 2011). They all share the same interfacial inhibition mechanism. Interfacial inhibitors are drugs that target macromolecular complexes by binding at the interface of at least two macromolecular components, thereby stabilizing a conformation intermediate that interferes with the dynamic activity of the macromolecular complex (Fig. 3.5; Pommier, Johnson, & Marchand, 2005; Pommier & Marchand, 2012). In the case of INSTIs, the macromolecular complex is the intasome consisting of the catalytic site of one IN polypeptide, the vDNA, and the two catalytic magnesium cations (Fig. 3.5D). All the INSTIs establish molecular contacts with each single component of the intasome complex (Metifiot, Marchand, Maddali, & Pommier, 2010). For all INSTIs, three oxygen or nitrogen atoms coordinate the two magnesium ions in the IN catalytic triad residues (DDE motif; Fig. 3.5). At the same time, the INSTIs also bind by stacking their halobenzyl group with the 3′-penultimate cytosine of the vDNA, while the terminal adenosine of the vDNA is displaced from the active site (Fig. 3.5).

5. CHALLENGES

This section is a selected list of questions, challenges, and possible answers regarding IN biology and drug targeting (see Table 3.3).

5.1. Resistance to INSTIs

The HIV genome is highly dynamic due to low fidelity of RT, and RAL resistance can evolve readily in the clinic (Malet et al., 2008; Wainberg et al., 2012). Drug-resistance mutations are determined both by drug selection and viral fitness (Fransen et al., 2009; Hazuda, 2010; Metifiot, Marchand, et al., 2010; Quercia, Dam, Perez-Bercoff, & Clavel, 2009; Wainberg et al., 2012). However, viral fitness is impacted by deleterious mutations in key catalytic residues and in residues involved in IN structural stability. Thus, emergence of resistant viruses depends on the selective advantage of such mutants, defined by the resistance level and viral fitness. The probability of such deleterious mutations increases with the number of mutations, which explains why combination of INSTIs with RT inhibitors is likely the most effective way to impede the occurrence of drug-resistance mutations. Indeed, to overcome three different classes of drugs, HIV must undergo at least three independent mutations (at least one for each drug), not counting additional secondary mutations to compensate for lack of fitness

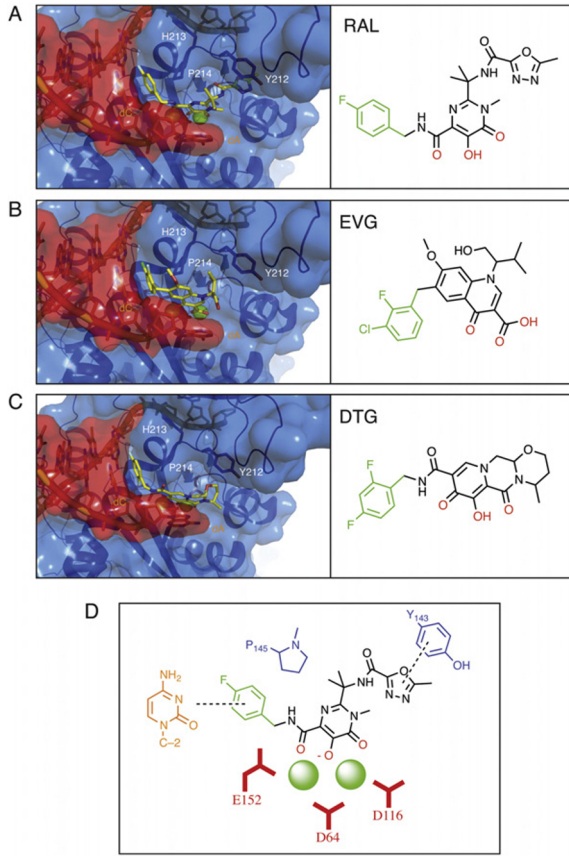

Figure 3.5 INSTIs interfacial binding to the intasome. (A–C, left panels) zoom-in view of the PFV IN active site containing metal, DNA, and drugs [pdb ID 3OYA (RAL, panel A), 3L2U (EVG, panel B), and 3S3M (DTG, panel C)]. (A–C, right panels) chemical structures of RAL, EVG, and DTG. INSTIs' main features are colored in red/dark gray and green/medium gray to highlight the chelating triad and the halobenzyl ring, respectively. (D) Scheme showing the interfacial inhibition mechanism of RAL. Π-stacking of the halobenzyl ring with the penultimate cytosine of the viral DNA and of the oxadiazole ring with the tyrosine residue of IN are indicated by dashed lines.

due to the primary drug-resistance mutations (for instance, the associated 140 mutations that rescue the deleterious effects of 148 mutations and confer full drug resistance; Fransen et al., 2009; Metifiot, Maddali, et al., 2010; Quercia et al., 2009). Treatment adherence is another key factor since the probability of mutations is directly linked to viral replication, which can resume if drug levels fall below therapeutic range. Compared to

Table 3.3 Challenges for the discovery and use of IN inhibitors

Challenges	Possible answers (new approaches)
Resistance to INSTIs	• Combination treatments • Treatment adherence: multidrug once-daily formulations • Novel INSTIs with different genetic barriers • Drugs targeting different regions of IN • Dual IN and RT inhibitors?
Long-term safety of INSTIs	• Follow cancer incidence and long-term side effects
Prophylaxis and cost	• INSTIs for pre- and postinfection prophylaxis • Microbicides • Regulatory agreements for price determination • Novel inhibitors (competition, nonprofit drug discovery such as FightAIDS@Home)
Novel IN inhibitors targeting different IN sites	• LEDGINs • Target IN dimerization • Discovery of IN cofactors and targeting their IN interface • Allosteric inhibitors of IN (Flexible loop site) • 3′-processing inhibitors with IN selectivity (RNase H)
Role of DDR and repair pathways for complete integration	• Model systems and techniques to elucidate the molecular DNA repair pathways and cellular responses following ST (5′-processing, gap filling, and provirus chromatinization) • New therapeutic opportunities?

raltegravir, both the recently approved EVG quad pill (Stribild®) and the DTG 572-Trii pill in late clinical trials are multidrug (IN + RT) once-daily pills, addressing the combination and treatment adherence issues, and therefore should reduce the risk of drug resistance.

Yet, when drug resistance due to IN mutations occurs, new drugs are necessary (Hazuda, 2010). EVG can overcome one of the RAL mutations (Y143R) but neither the G140S/Q148H nor the N155H mutations

(Metifiot, Vandegraaff, et al., 2011). Fortunately, DTG partially overcomes those mutations (Hare et al., 2011) and could therefore be used as salvage therapy in RAL- or EVG-resistant viruses. It is still unknown which resistance mutations will be seen in DTG-treated patients. None have been reported so far, and the genetic barrier for DTG appears higher than for RAL. The known DTG-resistance mutations have been generated in cell lines exposed for months to suboptimal concentrations of the drug (Table 3.2; Quashie et al., 2012). Clinical use of DTG will reveal whether and which IN mutations are clinically relevant. The next question will then be whether those mutations confer cross-resistance to RAL and ELG. In any case, it is clear additional INSTIs will be useful to overcome DTG-resistance (Agrawal et al., 2012; Metifiot et al., 2013; Taoda et al., 2012). It also appears that mutations at IN residue Q148 are a common determination of high levels of drug resistance (Table 3.2), suggesting that Q148 is a critical determinant of the IN catalytic site where the INSTIs bind (Johnson et al., 2007, 2006).

Another approach to overcome INSTI resistance is to develop drugs that target IN out of its active site (Table 3.3). LEDGINs, discussed in detail below are not cross-resistant with INSTIs and combination experiments demonstrate that LEDGINs and INSTIs act additively, suggesting a rationale for combining both INIs together (Christ & Debyser, 2013).

5.2. Long-term safety and tolerance for INSTIs

After 5 years of clinical use, it is clear that RAL (Isentress®) remains remarkably well tolerated. Rare side effects (less than 5% of patients) include insomnia, headache, nausea, and fatigue. One case of severe cerebral ataxia under RAL therapy has been reported (Reiss, Bailey, Pham, & Gallant, 2010). Because EVG is administered in the Quad pill (together with cobicistat, emtricitabine, tenofovir disoproxil fumarate) (Stribild®), it will be more difficult to determine EVG-specific long-term side effects. Yet, Stribild® is remarkably well tolerated. Diarrhea and nausea occur in less than 15% of patients and headaches and insomnia in less than 10% of patients.

In spite of the absence of a cellular equivalent of IN, human proteins structurally related to IN can be inhibited by some INSTIs at relatively high concentrations. This justifies testing new INSTIs against RAG1/2 recombinase, RNase H, or Metnase (Marchand et al., 2008; Melek et al., 2002; Williamson et al., 2012). Interfering with V(D)J recombination, which depends on RAG1/2 (Melek et al., 2002) could lead to immune disorders and opportunistic infections. Inhibition of Metnase

(Williamson et al., 2012), which is involved in DNA repair, topology, and stability (De Haro et al., 2010; Ponder et al., 2011; Wray et al., 2010), might produce neurotoxicity, immune disorders, and increase cancer risk. Another consideration is the accumulation of abortive unintegrated viral copies (2-LTR circles; see above) in infected cells treated with INSTIs. Such circular vDNA copies might insert into functionally important genomic sites, potentially giving rise to secondary malignancies or immune defects. Long-term follow-up studies will reveal the incidence of cancers and immune or neurological disorders in INSTIs-treated patients.

5.3. Economical considerations and prophylactic treatments

Cost is a major limitation for HIV therapies, especially in low-income countries. While the price of a single antiretroviral varies from a couple of hundred to tens of thousands of dollars per year (Hill, Cho, & Mrus, 2011), combination therapies are expensive ($30,000–40,000 per year). For RAL alone, the cost exceeds $10,000 per year. For Stribild®, the price tag is $28,500 per year. Low-cost generics have reduced the price of anti-HIV therapies by threefold (Venkatesh, Mayer, & Carpenter, 2012). Yet, generics remain a complex legal issue. It is questionable whether new INSTIs or other classes of INIs can be developed at low cost.

Preventing HIV infections with drugs is economically sound as the cost of treatments is offset by their short duration. RT inhibitors effectively prevent mother to child transmission. However, RT inhibitors are not optimum for postexposure prophylaxis unless they can be given immediately after exposure. Indeed, RT is completed within 2–4 h following viral entry. On the other hand, integration takes between 5 and 10 h (Hazuda et al., 2009; Fig. 3.2). Therefore, INSTIs should be advantageous compared to RT inhibitors in postexposure prophylaxis. INIs in combination with RT and PR inhibitors could also be used for local prophylaxis. In which case, cheaper drugs that do not fulfill oral bioavailability might be useful.

The use of antiretroviral agents as microbicides has been considered recently for INIs (Crucitti, Botta, & Di Santo, 2012; Terrazas-Aranda et al., 2008). However, formulation will be a key determinant for ease of use and efficacy over time. A main constraint in preexposure prophylaxis is the safety of and resistance to available drugs (Abraham & Gulick, 2012). Similar to HAART, it seems that microbicides should combine multiple drugs to prevent transmission of resistant viruses. Of note, S/GSK1265744 (ViiV Healthcare), which is now in clinical phase 2b as

an oral pill, has an apparent residence time of 30 h (Taoda et al., 2012). Recent conferences contained presentations that showed an impressive time of residence of 21–50 days by injection, which would be compatible with a one every 1–3 months administration. This approach could be advantageous in developing countries, where access to people at risk is limited. One could imagine a prophylactic dose for every person coming to be tested, protecting that individual for months to come.

5.4. Novel INIs targeting IN outside its catalytic site

5.4.1 The LEDGINs

Even in the most biochemical reductionist view, IN functions as a multimer (Fig. 3.4) with several protein–protein interfaces moving around each other to confer IN oligomeric flexibility and productive formation of the intasome. In a broader physiological view, IN functions with cofactors that regulate its activity. LEDGF/p75 was identified as an IN cofactor by coimmunoprecipitating overexpressed IN from human cells (Cherepanov et al., 2003). LEDGF/p75 is critical for HIV replication because it tethers IN to a host chromosome for integration, protects IN from proteasomal degradation, and stimulates its catalytic activity (reviewed in Christ & Debyser, 2013). A crystal structure of the LEDGF/p75 IN binding domain with a dimer of the IN catalytic core identified two regions of the IN dimer interface mediating the interaction: A128, W131, W132 and G168, E170, T174, M178 (Cherepanov, Ambrosio, Rahman, Ellenberger, & Engelman, 2005). This dimer interface region provides a defined binding pocket for LEDGF/p75 with multiple hydrophobic and hydrogen bond interactions favorable for drug binding.

The LEDGINs are small molecules rationally designed to bind to the LEDGF/p75 IN dimer interface binding pocket (reviewed in Christ & Debyser, 2013). Cocrystal structures confirmed that LEDGINs bind at the IN dimer interface in the LEDGF/p75 pocket (Christ et al., 2010). By stabilizing IN multimerization nonproductively and restricting IN oligomeric flexibility, LEDGINs inactivate allosterically (at a distance) the IN catalytic site (Christ & Debyser, 2013; Christ et al., 2012; Kessl et al., 2012; Tsiang et al., 2012). Thus, LEDGINs can be viewed as interfacial inhibitors (Pommier & Marchand, 2012; as they bind at the interface of 2 IN monomers) that act allosterically on the IN active site. They can also be viewed as competitive inhibitors with respect to LEDGF/p75. In contrast to INSTIs, LEDGINs are not selective for ST over 3′-P. They also must be

added prior to the DNA substrate to observe biochemical IN inhibition (Christ et al., 2012; Kessl et al., 2012; Tsiang et al., 2012). Several LEDGINs, derived from CX014442, have nanomolar antiviral potencies and are in advanced preclinical development (Christ & Debyser, 2013). Yet, the successful clinical development of LEDGINs will require optimization for bioavailability, pharmacokinetics, and toxicological studies. Moreover, LEDGINs will remain susceptible to drug resistance. *In vitro* selection of resistant viruses showed that single mutations in the IN LEDGF binding site, such as H99Y and A128T are sufficient to confer drug resistance (Christ et al., 2012, 2010). To limit the occurrence of IN resistance mutations, targeting LEDGF/p75 directly has recently been reported. Phage display screening led to the discovery of a cyclic peptide inhibitor of HIV replication (Desimmie et al., 2012).

5.4.2 Peptides targeting the LEDGF/p75 binding site

Short peptides targeting the LEDGF/p75 IN binding site have also been studied (Al-Mawsawi, Christ, Dayam, Debyser, & Neamati, 2008; Hayouka et al., 2012, 2007; Rhodes, Peat, Vandegraaff, Jeevarajah, Newman, et al., 2011). However, their potency remains inferior to LEDGINs and cellular delivery is even more challenging than for the LEDGINs. Peptides corresponding to IN dimerization interfaces (α5) or derived from interacting proteins (LEDGF, SNF5) were identified as potent inhibitors (Cherepanov, Devroe, Silver, & Engelman, 2004; Maroun et al., 2001; Yung et al., 2001).

5.4.3 Inhibitors targeting other cellular cofactors

IN binds other protein cofactors beside LEDGF/p75 (Cherepanov et al., 2003), including RT (Wu et al., 1999), INI1/hSNF5 (Cano & Kalpana, 2011; Kalpana, Marmon, Wang, Crabtree, & Goff, 1994), Rev (Rosenbluh et al., 2007), and Vpr (Bischerour et al., 2003; Fletcher et al., 1997). Based on the LEDGF/p75 example, defining such interactions might offer opportunities to discover inhibitors targeting these additional IN–protein interfaces by designing inhibitors mimicking the structural segments of the cofactor binding to IN. This approach recently led to the discovery of IN inhibitory peptides derived from RT (Armon-Omer et al., 2008) and Vpr (Gleenberg, Herschhorn, & Hizi, 2007; Suzuki, Maddali, et al., 2010; Suzuki, Urano, et al., 2010). In addition, inhibiting IN–cofactor interactions could influence other stages of viral replication, as evidenced by class II IN mutants, which are defective in viral assembly, particle

production, reverse transcription, or nuclear import, while retaining catalytic integration activity (Engelman, Englund, Orenstein, Martin, & Craigie, 1995; Leavitt, Robles, Alesandro, & Varmus, 1996; Li, Koh, & Engelman, 2012; Lu et al., 2004; Wiskerchen & Muesing, 1995). A recent study indicates that an acetylated form of IN could interact with transportin 3 and Nup358 (Allouch & Cereseto, 2011). Targeting IN at these cofactor binding sites will potentially lead to novel classes of integration inhibitors.

5.4.4 Allosteric inhibitors of IN

A novel inhibitor binding site has recently been reported outside the IN catalytic site. This new binding site accommodates a new series of INIs discovered using catalytic assays, surface plasmon resonance, and saturation transfer difference NMR to screen a fragment-based library (Rhodes, Peat, Vandegraaff, Jeevarajah, Le, et al., 2011). Cocrystallographic experiments have confirmed that these inhibitors bind in proximity to the IN flexible loop (residues 140–149) and contact the three IN residues: Q62, S147, and H183 (Rhodes, Peat, Vandegraaff, Jeevarajah, Le, et al., 2011). Further structural, binding, and mechanistic studies should provide more insight on this new binding pocket, which may represent an alternative to alleviate clinical resistance to INSTIs.

5.5. Chromatin and DNA repair cofactors completing integration

IN-mediated ST leaves two nicked DNA segments in a host chromosome, each with a 2-base 5′-vDNA mismatch and a 5-base gap in the host sequence at the junction of the viral and host DNAs (Fig. 3.3). Processing of these intermediates relies on cellular repair (Yoder & Bushman, 2000) and chromatin factors following disassembly of the intasome.

A parallel can be drawn between retroviral integration and Mu transposition (Mizuuchi & Craigie, 1986). Following integration into the bacterial chromosome, the transposome formed by MuA, which carries out a reaction similar to IN, needs to be disassembled by action of the host factor ClpX, which mediates MuA unfolding and destabilization to permit completion of Mu transposition (Burton & Baker, 2003). IN posttranslational modifications include phosphorylation and acetylation (Cereseto et al., 2005; Francis, Di Primio, Allouch, & Cereseto, 2011; Manganaro et al., 2010; Terreni et al., 2010). However, their relationship with IN dissociation is unknown. PARP-1 (poly-ADP ribose polymerase) has also been implicated in HIV

replication (Ariumi, Turelli, Masutani, & Trono, 2005; Gaken et al., 1996; Ha et al., 2001). PARP-1 is involved in the recognition and repair of single-stranded DNA regions by two mechanisms. PARP catalytic activation forms large poly (ADP ribose) polymer networks attached to chromatin (histones, topoisomerase I, and itself), which tends to dissociate PARylated proteins from DNA, regulate protein–protein interactions and repair enzyme activities. In addition, PARP binds directly to DNA repair complexes and controls base excision repair (BER; including XRCC1 and ligase III). PARP-1 has also been implicated in the nuclear proteasome activation responsible for histone degradation (Ullrich et al., 1999). Thus, a potential role of PARP-1 in HIV replication could be related to the dissociation process involving either PARylation or degradation of IN or key components of the integration complex. Because PARP inhibitors have recently emerged in the anticancer armamentarium (Rouleau, Patel, Hendzel, Kaufmann, & Poirier, 2010) and role of PARP in HIV replication remains controversial (Ariumi et al., 2005; Gaken et al., 1996; Ha et al., 2001; Kameoka et al., 2004; Kameoka et al., 2005; Siva & Bushman, 2002), further studies are warranted to determine the potential usefulness of PARP inhibitors as anti-HIV drugs.

Recent studies using siRNA screens have identified key DNA repair factors for integration (Brass et al., 2008; Espeseth et al., 2011; Konig et al., 2008; Zhou et al., 2008). The two most critical pathways for integration are BER and HR (Espeseth et al., 2011). The 14 BER genes identified (and detailed below) encode a coherent group of factors that can repair the proviral DNA gapped ends: (1) PARP1 might recognize the single-stranded gaps and recruit the BER complex; (2) the five glycosylases (MUTYH, NEIL2, NEIL3, NTHL1, and OGG1) can remove the two mismatched bases and generate abasic sites (see Fig. 3.3); (3) the major abasic site endonuclease (APEX1) can convert the abasic sites into breaks that would trim the 5′-ends of the vDNA; (4) any of the four identified repair polymerases (POLB, POLL, POLE, and POLI) can fill the gaps and remove the 5′-dRP residues resulting from the action of glycosylases with AP lyase activity; and (5) XRCC1 together with ligase III (LIG3) can finish the gap filling process by relegating the new DNA patch to the end of the vDNA. The importance of BER for proviral gap joining is consistent with earlier biochemical studies (Yoder & Bushman, 2000). It is notable that the glycosylases identified by genetic screening (Espeseth et al., 2011) are oxidative damage glycosylases, suggesting that oxidation of the bases at the ends of the vDNA might be important to initiate gap repair and allow full integration.

The implication of genes in the HR pathway (RAD51C, RAD51L3, RAD52, RAD50, MUS81, DMC1, RECQL4, and SMC6L1; Espeseth et al., 2011) suggests alternative pathways for the proviral DNA end processing. In which case, it remains to be shown whether the single-stranded gaps might be converted to double-strand breaks after breakage of the intact strand opposite to the gap. This possibility might explain the phosphorylation of histone gamma-H2AX in response to retroviral integration (Daniel et al., 2004) and the activation of DNA-dependent protein kinase during retroviral integration (Daniel, Katz, & Skalka, 1999). Insertion of the viral ends without the canonical 5-base-pair repeats might also reflect integration following double-strand breakage of the viral–host DNA junctions.

Activation of cellular processes involved in DNA damage response may also interfere with the normal vDNA fate. Components of NHEJ (non-homologous end-joining) including Ku70/Ku80, Ligase IV, and XRCC4 promote the production of 2-LTR circles (Jeanson et al., 2002; Li et al., 2001). Components of the MRN complex, including Mre11, NBS1, and Rad50 can induce the formation of 1-LTR circles through recombination (Kilzer et al., 2003).

6. CONCLUSION

During the past 30 years, AIDS evolved from a death sentence after only few months' survival to an asymptomatic disease with long-term remissions controlled by continuous drug treatments. The development of HAART played a crucial role in this transition and the approval of two INSTIs is a major step forward. The development of new, more efficient INIs that overcome drug resistance by viral mutations is warranted not only to cure and control the disease but also to prevent infections.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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CHAPTER FOUR

Advances in the Treatment of Varicella-Zoster Virus Infections

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Contents

Abstract

Varicella-zoster virus (VZV) causes two distinct diseases, varicella (chickenpox) and shingles (herpes zoster). Chickenpox occurs subsequent to primary infection, while herpes zoster (usually associated with aging and immunosuppression) appears as a consequence of reactivation of latent virus. The major complication of shingles is

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postherpetic neuralgia. Vaccination strategies to prevent varicella or shingles and the current status of antivirals against VZV will be discussed in this chapter. Varivax®, a live-attenuated vaccine, is available for pediatric varicella. Zostavax® is used to boost VZV-specific cell-mediated immunity in adults older than 50 years, which results in a decrease in the burden of herpes zoster and pain related to postherpetic neuralgia. Regardless of the availability of a vaccine, new antiviral agents are necessary for treatment of VZV infections. Current drugs approved for therapy of VZV infections include nucleoside analogues that target the viral DNA polymerase and depend on the viral thymidine kinase for their activation. Novel anti-VZV drugs have recently been evaluated in clinical trials, including the bicyclic nucleoside analogue FV-100, the helicase–primase inhibitor ASP2151, and valomaciclovir (prodrug of the acyclic guanosine derivative H2G). Different candidate VZV drugs have been described in recent years. New anti-VZV drugs should be as safe as and more effective than current gold standards for the treatment of VZV, that is, acyclovir and its prodrug valacyclovir.

ABBREVIATIONS

5-FU 5-fluorouracyl
ACV acyclovir
ACV^r acyclovir resistant
AIDS acquired immune deficiency syndrome
ANP acyclic nucleoside phosphonate
APs amphipathic polymers
ARN acute retinal necrosis
AUC area under the curve
BCNAs bicyclic nucleoside analogues
bid twice daily
BOI burden of illness
BVaraU sorivudine
BVDU brivudin
BVU (E)-5-(2-bromovinyl)uracil
CC_{50} 50% cytostatic concentration
cCDV cyclic cidofovir
CDKs cyclin-dependent kinases
CDV cidofovir
CDVp cidofovir monophosphate
CDVpp cidofovir diphosphate
$C_{log}P$ calculated log *P* values
CL_t clearance
C_{max} peak plasma concentration
CMI cell-mediated immunity
dGTP deoxyguanosine triphosphate
DP diphosphate

DPD dihydropyrimidine dehydrogenases
DPPIV/CD26 dipeptidyl peptidase IV
dTMP deoxythymidine monophosphate
dTTP deoxythymidine triphosphate
EBV Epstein–Barr virus
EC_{50} 50% effective concentration
ED_{50} 50% effective dose
FAM famciclovir
***F*DA** Food and Drug Administration
Fpo oral bioavailability
GMP guanosine monophosphate
HCV hepatitis C virus
HCMV human cytomegalovirus
HDP-CDV hexadecyloxypropyl-CDV
HHV-6 human herpesvirus 6
HHV-8 human herpesvirus 8
HIV human immunodeficiency virus
HSV herpes simplex virus
HSV-1 herpes simplex virus type 1
HSV-2 herpes simplex virus type 2
IC_{50} 50% inhibitory concentration
IDU 5-iodo-2′-deoxyuridine
MCP major capsid protein
MP monophosphate
NDP nucleoside diphosphate
NDP-K NDP kinase
PCV penciclovir
PK pharmacokinetics
PFA foscarnet, Foscavir
PHN postherpetic neuralgia
PMEA adefovir
PMPA tenofovir
PORN progressive outer retinal necrosis
QD once daily
SI selectivity index
ssDNA single-stranded DNA
SVV simian varicella virus
$t_{1/2}$ biological half-life
TID three times a day
TK thymidine kinase
TP triphosphate
TPase thymidine phosphorylase
VACV valacyclovir
V_D volume of distribution
VZV varicella-zoster virus
ZAP zoster-associated pain

1. INTRODUCTION

Primary infection with varicella-zoster virus (VZV) is associated with chickenpox (varicella), a common childhood illness. Similar to other members of the Herpesviridae family, following primary infection VZV undergoes a lifelong latent state. In VZV, latency is associated with persistence of the viral DNA in the dorsal root ganglia and cranial root ganglia. Reactivation of the virus produces skin lesions characteristic of herpes zoster (shingles) (Breuer & Whitley, 2007). Herpes zoster presents as a localized rash in a unilateral, dermatomal distribution that is often associated with severe neuropathic pain.

VZV is highly infectious and is transmitted via the respiratory tract. In the prevaccine era, primary VZV infection was associated with a typical winter–spring seasonality pattern of infection in countries with a temperate climate (Gershon et al., 2010). VZV can also be transmitted by fomites from skin lesions present in varicella and shingles. In contrast to varicella, herpes zoster does not display a seasonal pattern because it is the consequence of virus reactivation from latency that occurs when there is a decline in cell-mediated immunity (CMI). VZV enters the body via the airborne route and spreads rapidly from the pharyngeal lymphoid tissue to circulating T lymphocytes (Ku et al., 2004). Following an incubation period of 10–21 days, the virus reaches the skin, causing the typical vesicular rash of varicella. Lifelong immunity against clinically apparent second episodes of varicella is observed in most individuals.

Primary VZV infection typically occurs in childhood and is normally mild. However, complications can occur in adults and among immunocompromised patients. Disseminated varicella infections with multiorgan failure in immunocompetent adults (Anderson et al., 1994; Beby-Defaux et al., 2009) and in immunocompromised patients (Chilek, Routhouska, & Tamburro, 2010; Lu, Fan, Wang, & Cheng, 2010; Plisek et al., 2011; Zampogna & Flowers, 2001) have been described. VZV reactivation leading to herpes zoster may occur at any age but herpes zoster incidence is highest in older people and among immunocompromised individuals who suffer from human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) or malignancies, received organ or hematopoietic stem cell transplantation, or high-dose corticosteroid therapy. Age-related impaired CMI facilitates reactivation of VZV from latency. Herpes zoster affects up to 25% of individuals during their lifetime, but about 50% of persons aged

80 years or more (Johnson, 2010; Johnson & McElhaney, 2009). The disease is rarely life threatening and it is associated with a number of acute syndromes such as a vesicular rash and pain. Herpes zoster can result in prolonged pain, known as postherpetic neuralgia (PHN), a debilitating condition that can be very difficult to manage, especially in the elderly where herpes zoster tends to be more severe. PHN can have a considerable impact on the patient's quality of life and functional ability (Johnson et al., 2010; Opstelten, McElhaney, Weinberger, Oaklander, & Johnson, 2010; Pickering & Leplege, 2010). PHN causes a loss of physical function and patients complain of fatigue, anorexia, weight loss, reduced mobility, physical inactivity, sleep disturbance (especially insomnia), and loss of social contact as a result of reduced independence. A clear correlation between increasing severity of pain and greater interference with daily activities has been found (Johnson, 2010). Other neurological complications of herpes zoster include motor neuropathy, particularly in patients with zoster ophthalmicus (Sanjay, Huang, & Lavanya, 2011).

VZV infections among immunocompromised patients may be more prolonged and severe with unusual clinical presentation. Multidermal involvement and hyperkeratotic skin lesions seem to occur specifically in immunocompromised patients (Grossman & Grossman, 1993; Vafai & Berger, 2001; Wauters, Lebas, & Nikkels, 2010). Central nervous system (CNS) diseases attributed to VZV in this patient population can appear without skin rash. In patients with AIDS and other immunocompromised individuals, VZV can be associated with severe acute retinal necrosis (ARN), a disease with poor prognosis (Mueller, Gilden, Cohrs, Mahalingam, & Nagel, 2008). ARN is uncommon but can be devastating and blinding syndrome may occur not only in immunocompromised patients but also in immunocompetent individuals (Hillenkamp et al., 2009). In fact, ARN is mostly seen in immune competent individuals of either gender at any age. VZV is recognized as the leading cause of ARN although other herpesviruses [herpes simplex virus (HSV) and Epstein–Barr virus (EBV)] have been implicated in the pathogenesis of ARN. VZV necrotizing retinitis needs to be differentiated from progressive outer retinal necrosis (PORN), a form of VZV chorioretinitis found almost exclusively in severely compromised people, such as those at end-stage HIV infection (Austin, 2000; Pavesio et al., 1995; Yin et al., 2007). Exceptionally, some cases of PORN have been described in immunocompetent persons (Benz, Glaser, & Davis, 2003). PORN has a very fast evolution that may lead to blindness within a few days or weeks and treatment strategies have had limited success.

2. VACCINATION STRATEGIES AND POSTEXPOSURE PROPHYLAXIS

The development of a live-attenuated Oka vaccine (V-Oka) for varicella (Varivax®) was first reported in 1974 (Schmid & Jumaan, 2010). The introduction of a single-dose varicella vaccination program was first initiated in Japan (in 1986) and in Korea (in 1988). Later on, it was started in the United States (in 1995) for children aged 12–18 months, resulting in a significant decrease in the incidence of varicella disease and in the morbidity and mortality associated with the disease (Vazquez, 2004; Vazquez & Shapiro, 2005). Prelicensure and postlicensure studies demonstrated an excellent safety profile of the varicella vaccine. Only a low percentage of vaccine recipients (<5%) can develop a papular or vesicular rash, usually at the site of infection within 6 weeks following vaccination. Very rarely, generalized varicella-like rashes can develop within 14 days after vaccine administration and they were shown to be due to wild-type VZV incidentally acquired soon after vaccination. V-Oka also establishes latent infection in vaccine recipients and the virus may very rarely reactivate to cause herpes zoster. Vaccine-related herpes zoster occurs less frequently and is also less severe than that caused by wild-type VZV reactivation and it always manifests as herpes zoster of one dermatome.

Several issues have emerged that could prevent or delay the introduction of broad varicella vaccination programs in other countries (Schmid & Jumaan, 2010). Indeed, mild breakthrough infections were commonly observed among vaccinated children in day care and primary schools following a single dose of V-Oka. To prevent mild breakthrough infections, a recommendation for a 2-dose schedule was given in 2006. This results in improved immunity to VZV in vaccinated individuals but it is also associated with increased costs that can make the application of vaccination programs cost-prohibitive in some countries. Also, the emergence of vaccine-wild-type recombinant strains in some cases of herpes zoster in vaccinated children was shown in a few reports. Likewise most live-attenuated viral vaccines, varicella vaccine includes a mixture of variant strains. The analysis of the genomic variation among specimens from cases of postvaccination rash and herpes zoster in vaccine recipients was performed to determine the polymorphisms between vaccine Oka strains and the parental Oka strain, allowing the identification of a set of wild-type markers among vaccine adverse event isolates (Breuer & Schmid, 2008; Breuer et al., 2007;

Loparev, Rubtcova, Seward, Levin, & Schmid, 2007). In case of recombination between vaccine-wild-type viruses, the surveillance for adverse events associated with the varicella vaccine should be complicated. Additionally, it has been speculated that a reduced rate of VZV reexposure among adults with latent VZV infections could result in an earlier waning of VZV immunity levels in the elderly, leading to a younger average onset of herpes zoster.

A zoster vaccine (Zostavax®) that boosts the CMI to VZV is now available. The zoster vaccine has proved safe and partially effective in preventing both herpes zoster and PHN (Gnann, 2008; Levin, Gershon, Dworkin, Brisson, & Stanberry, 2010; Sanford & Keating, 2010). A randomized double-blind placebo-controlled trial enrolling 38,546 women and men aged 60 and older, performed by the Shingles Prevention Study, showed that the vaccine significantly diminished the herpes zoster burden of illness (BOI) by 61.1%, lowered the incidence of PHN by 66.5%, and reduced the incidence of herpes zoster by 51.3%. Although some of the vaccinated individuals developed herpes zoster, the mean duration of their pain and the mean severity of illness score were lower compared to nonvaccinated individuals (Oxman et al., 2005). The zoster vaccine prevented not only herpes zoster but also reduced the burden of herpes zoster-related interference with daily activities and health-related quality of life (Schmader et al., 2010). The zoster vaccine was licensed in the United States for use in all adults $\geq$50 years and is expected for licensure in other countries. Issues about the zoster vaccine include the costs of administration, the overall health care costs to society, and the acceptance and implementation of the vaccine in the elderly. Another concern for a herpes zoster vaccine is that the vaccine efficacy and the level of VZV-CMI also decline with increasing age. An increase in the dose of the vaccine that results in an increase in the immune response could be envisaged to overcome this problem. However, this is not feasible with VZV due to the limitations of VZV growth in cell culture. On the other hand, additional doses of the herpes zoster vaccine did not give improved immune responses in elderly vaccines. A new adjuvant-containing VZV subunit vaccine is currently being developed to enhance vaccine-induced immune responses (Levin et al., 2010).

It needs to be mentioned that a live vaccine cannot be given to immunosuppressed patients and this patient population is at high risk for developing herpes zoster. VariZIG is a VZV immune globulin preparation available for postexposure prophylaxis of varicella in persons at high risk for severe disease, who lack immunity to VZV and are ineligible for varicella vaccine

(Centers for Disease Control, Prevention (CDC), 2012; Cohen, Moschopoulos, Stiehm, & Koren, 2011; Sauerbrei, 2011). The period after exposure to VZV during which a patient may receive VariZIG, which had been 96 h (4 days), has been extended to 10 days. VariZIG should be administered as soon as possible after exposure to the virus. Patient groups recommended to receive VariZIG include (a) immunocompromised patients; (b) neonates whose mothers have signs and symptoms of varicella around the time of delivery (i.e., 5 days before and 2 days after); (c) premature infants born at $\geq$28 weeks of gestation who are exposed during the neonatal period and whose mothers do not have evidence of immunity; (d) premature infants born at <28 weeks of gestation or who weigh $\leq$1 kg at birth and were exposed during the neonatal period, regardless of their mothers' immunity status; and (e) pregnant women.

3. EXISTING ANTIVIRAL TREATMENTS

The implementation of safe and effective antiviral therapy dramatically improved the morbidity and mortality associated with varicella and herpes zoster, especially in the immunocompromised host. In the United States, three drugs are approved for the treatment of VZV infections, that is, acyclovir (ACV), valacyclovir (VACV), and famciclovir (FAM) (Fig. 4.1). Also, brivudin (BVDU) has been licensed for the therapy of herpes zoster in several countries in Europe.

ACV [9-(2-hydroxyethoxymethyl)guanine, Zovirax®] (Fig. 4.1), a structural analogue of the natural 2′-deoxyguanosine, was the first described really specific antiviral agent with potent activity against VZV and herpes simplex type 1 (HSV-1) and type 2 (HSV-2) and EBV and modest activity against human cytomegalovirus (HCMV) (Elion et al., 1977; Schaeffer et al., 1978). Soon after its discovery, ACV became the drug of choice for the treatment of HSV and VZV infections in immunosuppressed patients. Nowadays, ACV and its prodrug VACV (the L-valyl ester of ACV) have become the gold standard for prophylaxis and treatment of diseases caused by HSV and VZV.

The selective activity of ACV against HSV and VZV is based on its preferential phosphorylation by the virus-encoded thymidine kinase (TK) (Fyfe, Keller, Furman, Miller, & Elion, 1978; Keller et al., 1981). This enzyme converts ACV to ACV monophosphate (ACV-MP) which is then phosphorylated by the cellular GMP (guanosine monophosphate) kinase to ACV diphosphate (ACV-DP) and further to ACV triphosphate

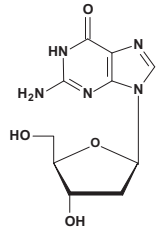
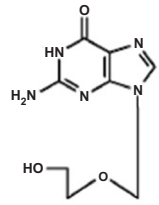
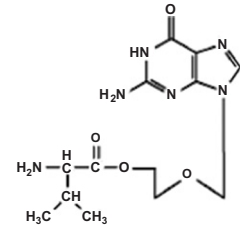
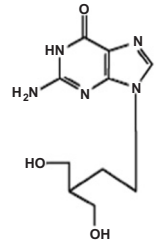
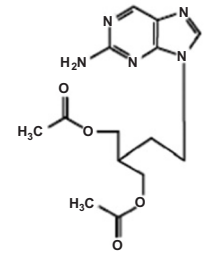
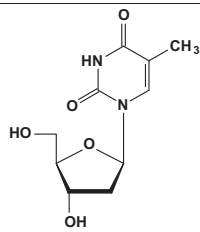
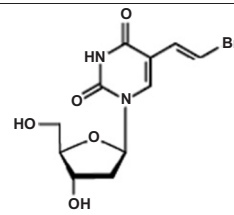
Natural nucleoside	Nucleoside analogue	Prodrug
 **2′-Deoxyguanosine**	 **Acyclovir (ACV)** 9-(2-hydroxyethoxymethyl)guanine **Zovirax®**	 **Valacyclovir (VACV)** L-valine ester of acyclovir **Valtrex®, Zelitrex®**
	 **Penciclovir (PCV)** 9-(4-hydroxy-3-hydroxymethyl-but-1-yl)guanine **Denavir®, Vectavir®**	 **Famciclovir (FAM)** Diacetyl ester of 9-(4-hydroxy-3-hydroxymethyl-but-1-yl)-6-deoxyguanine **Famvir®**
 **2′Deoxythymidine**	 **Brivudin** (E)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU), bromovinyldeoxyuridine **Zostex®, Zonavir®, Zerpex®**	

Figure 4.1 Compounds approved for the management of VZV infections.

(ACV-TP) by nucleoside diphosphate (NDP) kinase (NDP kinase) (Miller & Miller, 1980, 1982). ACV-TP, the active form of ACV, is a competitive inhibitor with respect to the natural substrate dGTP (deoxyguanosine triphosphate). High concentrations of dGTP can reverse the antiviral activity of ACV (Fig. 4.2). In addition, ACV-TP can also serve as a substrate for the DNA polymerase and then be incorporated into DNA at its 3′-terminal. As the 3′-terminal ACV-MP residues cannot be excised by

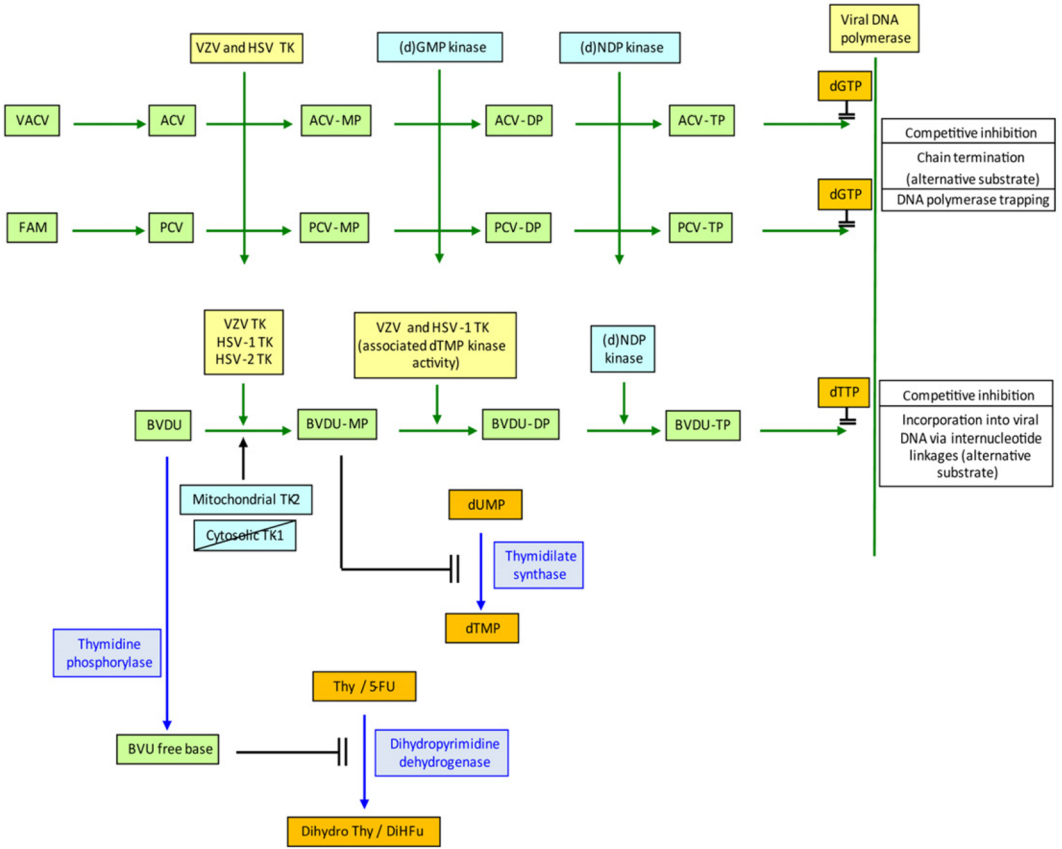


Figure 4.2 Metabolism and mode of action of approved anti-VZV compounds.

the DNA polymerase-associated 3′–5′exonuclease (Derse, Cheng, Furman, St Clair, & Elion, 1981), they prevent further chain elongation and thus act as DNA chain terminators because ACV does not contain the 3′-hydroxyl group required for DNA elongation.

Because ACV has limited oral bioavailability (15–30%) and limited solubility in water (~0.2%, 25 °C), relatively large doses and frequent administration are necessary to maintain plasma levels of ACV high enough to achieve viral inhibition. To improve the solubility and oral bioavailability (F_{po}) of ACV, several water-soluble esters of ACV were investigated. The valine ester of ACV, VACV (Valtrex®, Zelitrex®) (Fig. 4.1), proved to be a safe and efficacious drug (Darby, 1994; Perry & Faulds, 1996). The absolute oral bioavailability of ACV following oral administration of VACV is 54% (Weller et al., 1993). The increased oral bioavailability of VACV is due to a carrier-mediated intestinal absorption, via the human intestinal peptide transporter hPEPT1 (Guo, Hu, Balimane, Leibach, & Sinko, 1999), followed by the rapid conversion to ACV by ester hydrolysis in the small intestine (De Clercq & Field, 2006; Perry & Faulds, 1996). Following oral administration, VACV is rapidly metabolized to yield ACV and the essential amino acid L-valine (Perry & Faulds, 1996).

Several clinical studies have demonstrated that VACV has a safety profile comparable to that of ACV in patients with herpes zoster (Beutner, 1995; Beutner, Friedman, Forszpaniak, Andersen, & Wood, 1995; Corey et al., 2004; DeJesus et al., 2003; Gupta et al., 2004; Warren, Harris, & Brennan, 2004). VACV appears a more attractive option in the treatment of VZV infections due to a less frequent dosing regimen, which may contribute to increased patient adherence to therapy.

Penciclovir [PCV, 9-(4-hydroxy-3-hydroxymethyl-but-1-yl)guanine Denavir®, Vectavir®] (Fig. 4.1) is also a 2′-deoxyguanosine analogue resembling ACV in chemical structure, mechanism of action, and spectrum of antiviral activity (Perry & Wagstaff, 1995). Like ACV, PCV depends on the VZV-encoded TK for activation to the monophosphate form (Fig. 4.2). Cellular enzymes are responsible for further phosphorylation to the triphosphate form. PCV-TP inhibits then the viral DNA polymerase through competition with 2′-dGTP and is incorporated in the viral DNA. Unlike ACV-TP, PCV-TP is not an obligate chain terminator because PCV has two hydroxyl groups on the acyclic chain and can thus be incorporated into the extended DNA chain. Intracellular concentrations of PCV-TP are at least 30-fold higher than those of ACV-TP (Vere Hodge, Sutton, Boyd, Harnden, & Jarvest, 1989). However, the VZV polymerase has a higher

affinity for ACV-TP than for PCV-TP. As a result, the relative activities of ACV and PCV against HSV and VZV in cell culture are quite similar. PCV-TP is more stable within infected cells than ACV-TP and therefore it has a prolonged antiviral activity (Boyd, Bacon, Sutton, & Cole, 1987).

Similar to ACV, PCV is very poorly absorbed when given orally. Famciclovir (FAM) (Fig. 4.1), the diacetylester of 6-deoxypenciclovir, was developed as the oral prodrug. Following oral administration, FAM is rapidly and extensively absorbed and efficiently converted to PCV in two steps: (i) removal of the two acetyl groups (the first acetyl side chain of FAM is cleaved by esterases found in the intestinal wall and the second acetyl group is removed on first pass through the liver) and (ii) oxidation at the six position catalyzed by aldehyde oxidase that account for the conversion of 6-deoxypenciclovir to PCV (Perry & Wagstaff, 1995; Rashidi, Smith, Clarke, & Beedham, 1997). The total oral bioavailability of PCV from FAM is 77%. Data collected from several clinical studies demonstrated that FAM is well tolerated in patients and is effective against HSV-1 and HSV-2 for both therapy and long-term suppression of recurrences and is also efficacious in the treatment of herpes zoster (Mertz et al., 1997; Sacks, Aoki, Martel, Shafran, & Lassonde, 2005; Simpson & Lyseng-Williamson, 2006; Wald et al., 2006).

In the late 1970s, BVDU [(*E*)-5-(2-bromovinyl)-2′-deoxyuridine, bromovinyldeoxyuridine Zostex®, Zonavir®, Zerpex®] (Fig. 4.1) was described as a highly selective antiviral agent against HSV-1 and VZV (De Clercq, 2004; De Clercq et al., 1979). Several congeners of BVDU were synthesized, including BVaraU (sorivudine), the arabinofuranosyl counterpart of BVDU. The selective activity of BVDU against HSV-1 and VZV is dependent on a specific phosphorylation of the compound by the HSV-1 and VZV TK to its monophosphate (BVDU-MP) and diphosphate (BVDU-DP) (Fig. 4.2). The latter is then converted to the triphosphate BVDU-TP by a NDP or other cellular kinase, whereupon BVDU-TP enters in competition with the natural substrate deoxythymidine triphosphate (dTTP) for the viral DNA polymerase. It can inhibit the incorporation of dTTP into the viral DNA or as an alternate substrate it can be itself incorporated, thus leading to the formation of a structurally and functionally disabled viral DNA (De Clercq, 2004).

Following oral administration of BVDU, approximately 90% is absorbed and about 70% of the oral dose is rapidly transformed to bromovinyluracil (BVU) during the first passage through the liver (Wutzler, 1997). Clinical studies have confirmed that BVDU is effective in the treatment of herpes zoster, both in short-term (formation of new lesions) and long-term effects

(prevention of PHN), being as efficient and/or convenient as the other anti-VZV drugs ACV, VACV, and FAM (De Clercq, 2004). There is one limitation for the use of BVDU: it should not be given to patients under 5-fluorouracil therapy since BVU, the degradation product of BVDU, is a potent inhibitor of dihydropyrimidine dehydrogenase (DPD), the enzyme responsible for the first step in the catabolic pathway of pyrimidines. Because DPD is also needed for the degradation of 5-fluorouracil, concomitant administration of this drug together with BVDU results in increased exposure to 5-fluorouracil (since BVU protects 5-fluorouracil against breakdown and significantly increases its half-life) (Desgranges et al., 1986). Like BVDU, BVaraU is metabolized to BVU and therefore, its administration with 5-fluorouracil is contraindicated. BVaraU was licensed in Japan in 1993 for the treatment of herpes zoster, but the product was withdrawn following several deaths related to coadministration with 5-fluorouracil (Okuda et al., 1997; Okuda, Ogura, Kato, Takubo, & Watabe, 1998).

4. MEDICAL NEED FOR NEW ANTI-VZV AGENTS

4.1. Management of PHN and other complications

Current antiviral drugs approved for the treatment of herpes zoster significantly decrease the incidence of new lesion formation, accelerate healing, and shorten the duration of viral shedding, thereby reducing the incidence, severity, and duration of pain (Whitley, Volpi, McKendrick, Wijck, & Oaklander, 2010). The effect on the resolution of pain is a fundamental component of antiviral therapy. There are three different ways of measuring pain associated with herpes zoster: (i) pain at presentation (acute pain) that can be quantified over the first 30 days; (ii) PHN defined by the U.S. Food and Drug administration (FDA) as "pain that has not resolved 30 days after disease onset," although an alternative definition is pain that persists after healing or pain 90 days after rash onset; and (iii) zoster-associated pain (ZAP) whereby pain is viewed from the time of acute zoster until its complete resolution, if it occurs (Whitley et al., 2010).

Three oral antivirals (ACV, VACV, and FAM) are approved worldwide for the treatment of herpes zoster in immunocompetent and in immunocompromised patients, while BVDU is available in some countries. All drugs are able to decrease the incidence of new lesion formation and accelerate healing and the resolution of acute pain. Also, antiviral therapy reduced the duration of viral shedding, limiting neuron damage and thereby decreasing the incidence, severity, and duration of pain. VACV was shown to be

superior to ACV according to ZAP analysis from different clinical studies. Thus, in one of these studies, it was demonstrated that the time to ZAP resolution was significantly longer with ACV (800 mg 5× daily for 5 days) compared to VACV (1000 mg 3× daily for 7 days or 1000 mg 3× daily for 14 days) (Whitley et al., 2010). A direct head-to-head comparison of famciclovir and VACV in immunocompetent patients aged >50 years showed that the two drugs were therapeutically equivalent in terms of healing rate and pain resolution. In a large multicenter study that included patients with acute herpes zoster aged ≥50 years, BVDU proved similar efficacy on pain and rash as well as a similar tolerability compared to FAM (Whitley et al., 2010). Existing therapies are not completely effective in preventing PHN, probably because therapy with antivirals should be started within 72 h of rash appearance. One of the presumed major causes of reduced efficacy of antiviral therapy is due to the delay between onset of symptoms and start of treatment. Clinical trials of antiviral drugs for herpes zoster have enrolled patients within 72 h from rash onset; however, there are no well-controlled clinical trials that have compared early-onset therapy with later therapy (>72 h) (Whitley et al., 2010). Therefore, therapy with more active antiviral agents may be beneficial in achieving a rapid decrease in viral replication and subsequent neural damage, and consequently may reduce both acute and chronic symptoms of herpes zoster.

4.2. Treatment of rare but important side effects caused by the VZV vaccine

Varicella vaccine in children has indeed diminished the consequences of chickenpox in terms of both health care and economical burden, while zoster vaccine was shown to protect immunocompetent adults from herpes zoster and also reduces the severity of herpes zoster in those who develop the disease. Although adverse events were not seen in clinical trials performed with the vaccine, rare but important side effects are being seen due to the increasing number of vaccine doses administered worldwide. A few cases of disseminated varicella infections due to the vaccine strain of VZV have been described in immunocompromised patients requiring treatment with antiviral agents (Galea et al., 2008; Levy et al., 2003). Also, VZV vaccine can occasionally reactivate in healthy children and cause herpes zoster (Ota et al., 2008; Uebe, Sauerbrei, Burdach, & Horneff, 2002). Recently, a disseminated varicella-zoster infection with CNS involvement directly following vaccine administration has been reported in a previously healthy elderly woman (Fusco et al., 2010). Also, a case of vaccine-associated herpes

zoster ophthalmicus and encephalitis in an immunocompetent child that required ACV therapy has been described (Chouliaras, Spoulou, Quinlivan, Breuer, & Theodoridou, 2010). Thus, antiviral therapy will still have a major role in the treatment of diseases caused by VZV, even following widespread implementation of vaccination programs for both varicella and herpes zoster.

4.3. Emergence of drug-resistant viruses

Similar to HSV, ACV treatment for VZV infections does not generate acyclovir-resistant (ACV^r) viruses in immunocompetent individuals. However, in the immunocompromised hosts, VZV infection tends to be severe and prolonged and ACV^r mutants have been isolated after long-term treatment with ACV (Boivin et al., 1994; Pahwa et al., 1988; Snoeck et al., 1994; Talarico, Phelps, & Biron, 1993). Among hematological patients, VZV-related complications occur frequently and antiviral resistance is a relevant factor in persistent infections and needs to be investigated in various affected body sites (van der Beek et al., 2012).

Resistance to ACV in VZV appears as a consequence of mutations either in the TK or the DNA polymerase genes, being the most frequent mutants isolated both in cell culture and in the clinic TK mutants (Gilbert, Bestman-Smith, & Boivin, 2002). Mutations conferring resistance to nucleoside analogues have been found all along the VZV TK gene, although specific regions including ATP- and nucleoside-binding sites are recognized as mutagenic hot spots as well as amino acid 231 (Boivin et al., 1994; Morfin et al., 1999; Talarico et al., 1993). The pyrophosphate analogue foscarnet (FOS, Foscavir®) is a direct inhibitor of the viral DNA polymerase and does not require an activation step by the viral TK. Therefore, FOS is the treatment of choice for ACV^r VZV infections. Indeed, several reports have documented the use of FOS as a salvage therapy for ACV^r VZV infections in immunocompromised patients (Bryan et al., 2008; Hatchette et al., 2008; Safrin et al., 1991). However, resistance to FOS associated with mutations in the VZV DNA polymerase gene has also been described in immunocompromised patients (Fillet et al., 1995; Visse, Dumont, Huraux, & Fillet, 1998; Visse, Huraux, & Fillet, 1999). The amino acid substitutions in the VZV DNA polymerase that confer resistance to FOS^r were cross-resistant to ACV. The mutations in the VZV DNA polymerase were found to correspond with changes described in the HSV DNA polymerase, although some of the mutants exhibited a discrepancy in their sensitivity to

ACV or aphidicolin in comparison with the corresponding HSV-1 mutants (Kamiyama, Kurokawa, & Shiraki, 2001; Visse et al., 1998, 1999). Interestingly, it appeared that ACV and PCV select *in vitro* for different drug-resistant VZV genotypes: ACV selected for TK mutants, while PCV selected for DNA polymerase mutants (Andrei, De Clercq, & Snoeck, 2004). Several reports have indicated that PCV remains active against some HSV-1, VZV TK, and DNA polymerase mutants that are resistant to ACV (Andrei et al., 2004; Boyd et al., 1987; Hasegawa, Kurokawa, Yukawa, Horii, & Shiraki, 1995; Pelosi, Mulamba, & Coen, 1998). These findings indicate that the interactions between HSV or VZV TK and PCV or ACV, and likewise between the viral polymerases and the triphosphates of PCV or ACV, are distinct and may account for the differences observed between ACV^r and PCV^r VZV strains. Furthermore, the emergence frequency of resistant VZV mutants proved to be significantly higher following ACV exposure than following PCV exposure (Ida et al., 1999). The nucleotide analogue cidofovir (CDV) (which is activated to the active form by cellular kinases) could also be used for the treatment of VZV infections resistant to ACV, PCV, and/or foscarnet. This compound has proven to be effective in the treatment of progressive mucocutaneous infections due to ACV- and/or FOS-resistant HSV in immunocompromised patients (Piret & Boivin, 2011; Snoeck & De Clercq, 2002).

Development of resistance to ACV during chronic infection with the Oka vaccine strain of VZV has also been reported in an immunosuppressed child who was vaccinated just prior to the discovery of a tumor that required intensive antitumor therapy (Levin et al., 2003). Genotyping of the viral DNA recovered from this patient demonstrated a mutation in the viral TK that was responsible for the clinical resistance to ACV. Successful resolution of both local and systemic VZV infection was observed following PFA therapy. A second report on ACV-resistant VZV caused by Oka strain virus was described in a child with neuroblastoma (Bryan et al., 2008). Thus, these studies showed that the Oka vaccine strain of VZV is capable of causing severe herpes zoster and systemic disease in immunocompromised patients after reactivation from latency, requiring chronic therapy with ACV with the consequent risk of selection of drug-resistant viruses.

5. DEVELOPMENT OF NOVEL ANTI-VZV AGENTS

Current approved anti-VZV drugs are nucleoside analogues (i.e., ACV, PCV, and BVDU) that depend on the viral TK for their activation

and all of them share the same viral target, that is, the viral DNA polymerase. The alternative drug of choice for treatment of VZV infections resistant to nucleoside analogues is FOS, which also targets the viral DNA polymerase and may be associated with significant renal toxicity. Eventually the nucleotide analogue CDV may be used for the treatment of VZV strains resistant to ACV, PCV, and/or FOS, but this drug is also associated with renal toxicity. These limitations of the standard treatment for VZV diseases highlight the need for development of novel anti-VZV agents with potent antiviral activity based on different mechanisms of action. Inhibition of virus replication using drugs with different mechanisms of action can help not only in controlling drug-resistance infections but also in reducing the probability of antiviral resistance.

Significant research efforts have been and are currently being made to discover and develop anti-VZV agents with a superior activity compared to the standard of treatment (i.e., VACV) for herpes zoster. Additionally, work toward new classes of inhibitors targeting other viral enzymes than the DNA polymerase has been favored. These new agents are expected to be active against the currently available anti-VZV agents. Recently, three different anti-VZV agents have entered clinical trials to evaluate their efficacy, compared to VACV, in reducing herpes ZAP and severity. A summary of drugs approved for the treatment of VZV infections as well as those under development or considered as candidate anti-VZV drugs in presented in Table 4.1.

5.1. Bicyclic nucleoside analogues

5.1.1 Structure–activity relationship of BCNAs

In 1999, McGuigan and collaborators reported on the potent and selective anti-VZV activity of some unusual bicyclic nucleoside analogues (BCNAs) (McGuigan et al., 1999). Among BCNAs containing a simple alkyl side chain on the bicyclic base ring, the optimal carbon chain length to display the most potent anti-VZV activity ranked between 8 and 10. Thus, among this first series of 6-substituted furopyrimidine derivatives, the 6-octyl-substituted derivative **Cf1368** (Fig. 4.3) was able to inhibit the replication of wild-type VZV reference strains with 50% effective concentration (EC_{50}) value of 0.008 μM, while not being toxic up to a 50% cytostatic concentration (CC_{50}) of >50 μM (McGuigan et al., 1999). This compound showed a promising selectivity index (SI, ratio CC_{50} to EC_{50}) of >5000. Like ACV and BVDU, the BCNAs were inactive against TK-deficient strains, pointing to a crucial role of the VZV-encoded TK in the activation (phosphorylation)

Table 4.1 Status of varicella-zoster virus inhibitors

Type of molecule	Drug and oral prodrug	Comment
FDA (Food and Drug administration) or EMA (European Medicines Agency) approved anti-VZV drugs		
Nucleoside analogues	Acyclovir (oral prodrug valaciclovir)	
	Brivudin	
	Penciclovir (oral prodrug Famciclovir)	
Anti-VZV drugs used off-label		
Pyrophosphate analogue	Foscavir	Drug of choice for the treatment of ACV^r infections
Nucleotide analogue	Cidofovir	Drug of choice for the treatment of ACV^r/PFA^r infections
Drugs for which Phase II/III studies have been performed		
Bicyclic nucleoside analogue (BCNA)	Cf1743 (prodrug FV-100)	Under development
Helicase–primase inhibitor	ASP2151	Development halted because of toxicity in Phase I
Carbocyclic nucleoside analogue	H2G (omaciclovir) (oral prodrug valomaciclovir)	Under development
Candidate anti-VZV drugs		
N-alpha-methylbenzyl-*N*′-arylthiourea analogues	Comp I, Comp II, and Comp III	Target the ORF54 protein, most likely essential for cleavage and packaging of viral DNA into capsids
Amphipathic polymers (APs)	REP9	No studies on mechanism of action against VZV described; it can be assumed that similar to other viruses, virus entry should be inhibited

Table 4.1 Status of varicella-zoster virus inhibitors—cont'd

Type of molecule	Drug and oral prodrug		Comment
5-Substituted 4′-thiopyrimidine nucleosides	5-Iodo-4′-thio-2′-deoxyuridine (4′-thioIDU)		Mechanism of action appeared to be similar as IDU
Pyrazolo[1,5-*c*]1,3,5-triazin-4-one derivative	Compound 35B2		Targets the herpesvirus major capsid proteins resulting in inhibition of normal capsid formation
Nonnucleoside DNA polymerase inhibitors	4-Oxo-dihydroquinolines (4-oxo-DHQs)		Competitive inhibitors of dTTP by viral DNA polymerases. Drug resistance associated with mutations in the viral DNA polymerase
Nucleotide analogues	Esters of acyclic nucleoside phosphonates	CMX001 (HDP-CDV)	Reduced renal toxicity and increased oral bioavailability compared to cidofovir
	O-linked acyclic nucleoside phosphonates and triazine analogues of cidofovir	HPMPO-DAPy, PMEO-DAPy, HPMP-5-azaC	Further studies are needed to determine the clinical potential of these compounds to become new drug candidates to treat VZV infections
Cellular targets	CDK inhibitors: roscovitine, olomoucine, and flavopiridol		Clinical studies are needed to determine the potential of CDK inhibitors in the treatment of viral diseases since concerns exist on the potential toxicity of CDK inhibitors
Immunomodulators	Antimicrobial peptides: human β-defensin-2 and human cathelicidin LL-37		Mechanism of action based on the disruption of the viral envelope or on the interaction with viral glycoproteins

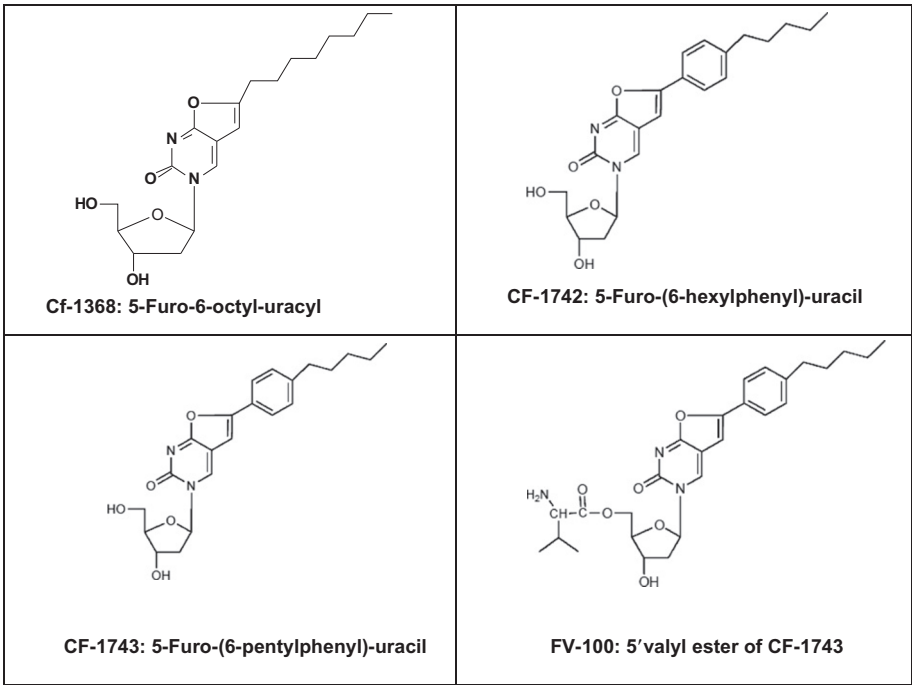


Figure 4.3 Bicyclic pyrimidine nucleoside analogs (BCNAs).

of the BCNAs. Although BCNAs are structurally related to BVDU, they differ significantly in their spectrum of antiviral activity. In contrast to BVDU that possesses potent anti-HSV-1 and anti-VZV activity, the BCNAs solely inhibit VZV.

In the next step, a variety of BCNAs were synthesized containing a terminal alkyl unsaturation in the side chain on the furanyl ring of the bicyclic base (Srinivasan et al., 2001). Compared to the *n*-octyl-BCNA derivative (Cf1368), a clear decrease in the antiviral efficiency was observed for the *n*-alkenyl- and *n*-alkynyl-BCNAs (e.g., ∼10- and ∼300-fold decreased activity for the *n*-octenyl- and *n*-octynyl-BCNAs relative to the corresponding parental *n*-octyl-BCNA).

BCNAs with a terminal halogen substitution (i.e., replacement of the terminal methyl group of the alkyl side chain of the BCNAs by a halogen such as fluoro, chloro, bromo, or iodo) resulted in activity and selectivity comparable to that of the parent compound Cf1368 (Brancale, Mcguigan, et al., 2000).

When the heteroatom in the bicyclic pyrimidine ring of the 6-alkyl-substituted derivatives was changed from oxygen to sulfur (thus converting the *furo* into a *thieno* moiety), anti-VZV activity was conserved (Brancale et al., 2001). In contrast, replacement of *furo* by *pyrrolo* resulted in a significant loss in antiviral activity (McGuigan, Pathirana, et al., 2000).

In order to restrict the conformational freedom of the alkyl side chain of the prototype BCNAs, an aromatic ring system (phenyl) was introduced in the side chain. Several alkylaryl-BCNAs proved exquisitely inhibitory against VZV replication. Among these derivatives, the *n*-pentylphenyl- and *n*-hexylphenyl-BCNAs (**Cf1742** and **Cf1743**, respectively) (Fig. 4.3) emerged as the most potent compounds with EC_{50} values as low as 0.0001–0.0005 μM against reference VZV strains as well as clinical isolates (McGuigan, Barucki, et al., 2000). Shortening or lengthening the optimal pentylphenyl group of the BCNAs led to decreased anti-VZV activity. Indeed, a strong correlation between the size (length) of the *n*-alkyl and *n*-alkylaryl moiety of the BCNAs and their optimal antiviral activity was observed. Thus, for the alkyl derivatives, this appeared to be 8–10 carbons, and for the alkylaryl derivatives 5 or 6 carbons, the phenyl group being equivalent to 3–4 carbons (Balzarini & McGuigan, 2002). The fact that the *n*-alkylaryl-BCNAs are invariably more potent than the *n*-alkyl-BCNAs can be explained by the reduced conformational freedom of the alkylaryl side chain relative to the long alkyl side chain, fitting the *n*-alkylaryl-BCNAs more optimally to their target enzyme than the more flexible *n*-alkyl-BCNAs.

Cf1742 and Cf1743 were markedly more potent in inhibiting VZV replication *in vitro* than the currently used anti-VZV drugs. The compounds were 10–25-fold more active against wild-type VZV clinical isolates than BVDU and showed up to 4000–7800-fold higher efficacy than ACV and PCV (Andrei et al., 2005). These results were comparable to those obtained for Oka and YS laboratory strains. Due to their lack of toxicity *in vitro*, Cf1742 and Cf1743 presented extremely high-selectivity indices, which were calculated to be >100,000. Akin to BVDU, ACV, and PCV, the BCNAs were not able to inhibit TK-deficient VZV strains. Cf1743 was also able to reduce the replication and spread of VZV in organotypic epithelial raft cultures infected with VZV after 4–5 days of differentiation as measured by morphological changes induced by the virus and quantification of the viral DNA load (McGuigan et al., 2007). In this three-dimensional culture system, Cf1743 gave the most pronounced drop of viral DNA within a broad concentration range (0.00004–4 μg/mL). At the highest Cf1743 concentration tested, the drop of viral DNA load was up to 3 orders of magnitude compared to 1–1.5 orders of magnitude decreased viral DNA levels at the highest concentrations tested of ACV, BVDU, and CDV.

In contrast to the arabinosyl derivative of BVDU (designated BVaraU or sorivudine) that is ∼10-fold more potent than BVDU against VZV, the sugar-modified arabinosyl and ribofuranosyl derivatives of the prototype *n*-octyl-BCNA (Cf1368) were markedly less potent than its 2′-deoxy derivative (Balzarini & McGuigan, 2002). As also seen for BVU, the free base of BVDU, the free *n*-octyl-bicyclic base did not present any measurable anti-VZV activity.

5.1.2 Physical and pharmacokinetic (PK) properties of BCNAs

A relatively long alkyl or alkylaryl side chain at C-6 of the bicyclic furano or thieno pyrimidine ring of the BCNAs is required for optimal anti-VZV activity. This property results in a relatively high lipophilicity of the compounds, represented by their calculated log *P* values ($C_{\log}P$) that ranged between 2.5 and 3.5 (Balzarini & McGuigan, 2002). Considering that a higher lipophilicity of BCNAs more or less corresponds with higher bulkiness of the side chain, it can be assumed that an optimal $C_{\log}P$ value required for best anti-VZV activity reflects an optimal lipophilic interaction in the active site of the target enzymes or an optimal size of the side chain that must fit in a lipophilic pocket of the particular enzymes.

The high $C_{\log}P$ values correlate with a low water solubility (<1 mg/L) of the BCNAs, representing a challenge for an appropriate oral formulation

(Balzarini & McGuigan, 2002). Attempts to enhance water solubility of the lead BCNAs, such as replacement of one of the methylene units in the *n*-decyl-BCNA chain by an oxygen (resulting in glycol/ether derivatives of the *n*-decyl-BCNA) or unsaturation at the terminus of the alkyl side chain of lead bicyclic *furo* compounds (alkene and alkyne derivatives), resulted not only in decreased $C_{\log}P$ values but also in a lower anti-VZV activity (Brancale, Srinivasan, et al., 2000; Srinivasan et al., 2001).

Pharmacokinetic studies were performed in mice with Cf1743 formulated at 2 mg/mL in a mixture of 11% DMSO, 22% crematophore, and 67% PBS (McGuigan et al., 2007). At an intravenous bolus of 2.5 mg/kg, Cf1743 was cleared at a CL_t (clearance) of ~4 L/h/kg, the V_D (volume of distribution) was ~4 L/kg, and the $t_{1/2}$ (biological half-life) was 41 min. In contrast, at an oral dose of 20 mg/kg, CF1743 showed a remarkably long plasma $t_{1/2}$ of 281 min, a CL_t of 28 L/h/kg, and a V_D of 108 L/kg. The F_{po} was 14%. Interestingly, following a single oral dose of 20 mg/kg to adult mice, plasma drug levels of 0.02 μg/mL were observed, a concentration that still exceeded by 200-fold the EC_{50} value of the compound *in vitro*. The parent drug and its metabolites could be separated and quantified in plasma with a fluorescence detector-equipped HPLC because of the intrinsic fluorescence of the BCNAs. For CF1743, five different metabolites were detected following intravenous or oral administration to mice. Even if the drug was metabolized, the parental Cf1743 remained the predominant BCNA derivative detected in plasma.

In order to enhance the oral bioavailability of Cf1743, alternative formulations were evaluated. Although water solubility was increased when CF1743 was formulated in Captisol®, only limited (approximately twofold) enhancements in AUC (area under the curve) and C_{max} (peak plasma concentration) were observed when oral bioavailability was examined (McGuigan et al., 2007). In view of the need to enhance oral bioavailability, a prodrug approach was envisaged. In a first attempt, the 5′-monophosphate of Cf1743 was synthesized. As expected, the monophosphate derivative of Cf1743 was considerably more water soluble than the parent compound, but in PK assays in mice it showed only limited enhancements in AUC and C_{max}. Based on the efficacy of 5′-valyl prodrugs of ACV and ganciclovir, the synthesis of 5′-valyl prodrugs of Cf1743 was taken into account. Based on stability profile and pronounced water solubility, the HCl salt of the 5′-valine ester (designated FV-100) was chosen as a clinical candidate drug (McGuigan et al., 2007). Oral bioavailability and pharmacokinetics of FV-100 showed a significant, ~10-fold, boost in both AUC and C_{max}

compared to Cf1743. When evaluated against VZV reference strains and several clinical isolates, EC_{50} values for FV-100 were 2.5-fold to 4-fold higher than those for Cf1743.

The dipeptidyl peptidase IV (DPPIV/CD26)-based prodrug approach was applied to the BCNAs in order to improve their physicochemical and pharmacokinetic properties (Diez-Torrubia et al., 2011). Stability data demonstrated that the prodrugs efficiently release Cf1743 upon selective conversion by purified DPPIV/CD26 and by soluble DPPIV/CD26 present in bovine, murine, and human serum. This conversion was inhibited by vildagliptin, a specific inhibitor of DPPIV/CD26. Several novel prodrugs showed increased water solubility (up to more than 3 orders of magnitude) compared to the poorly soluble parent drug Cf1743 and also enhanced oral bioavailability in mice.

5.1.3 Mechanism of action of BCNAs

BCNAs need to be activated (phosphorylated) by the viral encoded TK before they can inhibit their antiviral target(s). Thus, ACV-, BVDU-, and BVaraU-resistant mutants bearing mutations in the VZV TK gene proved to be no longer sensitive to BCNAs (Andrei et al., 2005). Conversely, VZV-mutant strains resistant to BCNAs showed cross-resistance to BVDU, BVaraU, and ACV and they were also demonstrated to have mutations in the viral TK gene. Moreover, Cf1742 and Cf1743 were unable to inhibit the replication of two VZV clinical isolates recovered from an AIDS patient that bear mutations in the viral TK gene.

The crucial role of the VZV TK in the metabolic activation of BCNAs has been clearly demonstrated (Sienaert et al., 2002). A kinetic study with purified enzymes revealed that BCNAs were recognized by VZV TK as a substrate, but not by HSV-1 TK, nor by cytosolic TK-1 or mitochondrial TK-2. VZV TK was also shown to be able to phosphorylate the BCNAs not only to their corresponding 5′-mono- but also to their 5′-diphosphate derivatives due to the intrinsic deoxythymidine monophosphate (dTMP) kinase activity of the VZV TK. Thus, BCNAs are selectively phosphorylated to their 5′diphosphates by the two successive enzyme activities of VZV TK (TK and dTMP kinase (dTMP-K)). These data undoubtedly explained the anti-VZV selectivity of the BCNAs. No clear-cut correlation between the antiviral potency of the compounds and their affinity for VZV TK could be demonstrated, pointing to a different structure/activity relationship of the eventual antiviral target of these compounds.

BCNAs strikingly differ from BVDU in their mechanism of activation (Fig. 4.4) (Balzarini et al., 2002; Sienaert et al., 2003). Thus, BVDU is known to be converted to its 5′monophosphate derivative (BVDU-MP) by the TKs encoded by HSV-1, HSV-2, and VZV and by mitochondrial TK-2, but not by cytosolic TK-1. Also, BVDU-MP is known to inhibit thymidilate synthase. BVDU-MP is then converted to its 5′-diphosphate derivative (BVDU-DP) by the TKs encoded by VZV and HSV-1, but not by HSV-2 or cytosolic dTMP-K. The last step of activation of BVDU to its 5′-triphosphate derivative (BVDU-TP) is carried out by NDP-K and the latter metabolite is recognized by cellular and herpetic DNA polymerases, and incorporated into viral and cellular DNA. In contrast, human NDP-K was unable to convert BCNAs to their triphosphate derivatives (BCNA-TP) (Sienaert et al., 2002). This is in agreement with studies reporting no traces of BCNA-TP in VZV-infected cells. These data clearly indicated that the mechanism of action of BCNAs may be entirely different

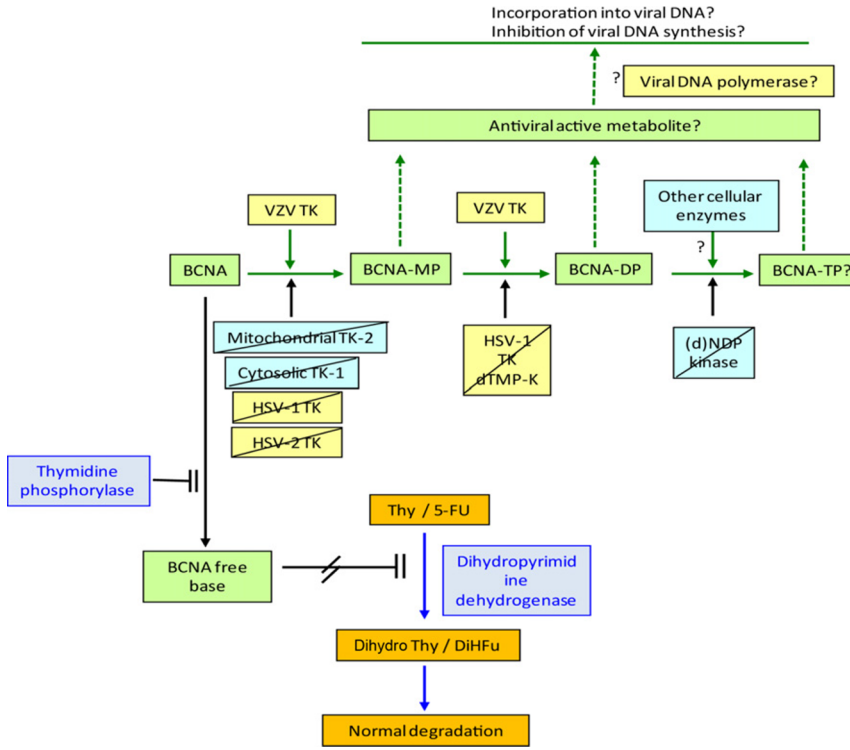


Figure 4.4 Anabolic and catabolic steps involved in the metabolism of BCNAs and possible mechanism of action.

from that of BVDU and suggested that BCNAs exert their inhibitory effect via their monophosphate or diphosphate derivative, being the DNA polymerase virtually excluded as the molecular target of the BCNAs. Although further studies are required to completely eliminate the viral DNA polymerase as target of the BCNAs.

The exact molecular target of the BCNAs has not been identified yet and is currently under investigation. Selection and characterization of mutants resistant to BCNAs were not able to provide insights into the molecular target of the BCNAs because the mutants presented alterations in the VZV TK, the enzyme responsible for activation of the compounds. Time-of-addition experiments showed that a delay of administration of ACV, BVDU, and Cf1743 for up to 49 h postinfection did not affect the antiviral efficacy of the drugs (McGuigan et al., 2007). At longer delayed time periods, all three drugs started to lose their activity. Studies aimed to investigate the effect of a limited exposure time of BCNAs to VZV-infected cell cultures showed that a limited exposure time to the BCNAs, but not to ACV or BVDU, is still highly effective.

BCNAs differ from BVDU and BVaraU also in their catabolic pathways (Fig. 4.4), providing BCNAs with significant advantages. While pyrimidine nucleoside analogues are susceptible to pyrimidine catabolic enzymes (such as uridine phosphorylase or thymidine phosphorylase (TPase)) and are hydrolyzed to their free base metabolites that lack antiviral activity, BCNAs were not recognized as substrates by human TPases and therefore not converted to their inactive free base derivatives (Balzarini et al., 2002). Furthermore, the free bases of BCNAs were not inhibitory to human DPD (dihydropyrimidine dehydrogenases), the catabolic enzyme involved in the degradation of pyrimidines and pyrimidines analogues. As mentioned above, the BVU free base is inhibitory to DPD, an enzyme necessary for the catabolic inactivation of the chemotherapeutic pyrimidine analogue 5-fluorouracyl (5-FU). Experiments performed in mice with Cf1368, administered either intravenously or orally, showed no indication of release of the free base, and a pronounced appearance of unaltered drug in plasma upon oral administration. In addition, when Cf1368 and Cf1743 were given to mice in combination with 5-FU, no increase in 5-FU plasma levels were seen, while both BVDU and BVU afforded a marked and extended rise of 5-FU plasma levels (Balzarini et al., 2002).

Given the natural and inherent UV fluorescence of the BCNAs, fluorescent microscopy was used to trace the entry of FV-100 in living cells. Cells treated with FV-100 for only 2 min showed very evident blue fluorescence, mostly in reticular-like structures (McGuigan et al., 2007). When the

incubation period was increased to 1 h, prominent sequestration of FV-100 in punctuate structures that resembled endocytic organelles such as endosomes and lysosomes was observed.

5.1.4 *Toxicology and* in vivo *efficacy*

Preliminary mouse toxicology studies were performed with Cf1743 (McGuigan et al., 2007). The compound was administered by the intraperitoneal route at 100 mg/kg/day for 10 consecutive days or by the peroral route at 50 mg/kg (twice a day) for 5 consecutive days. No signs of toxicity were observed upon visual examination of the drug-treated animals, nor indications of body weight loss, change of behavior of the animals nor microscopically visible histological abnormalities in the organs examined (i.e., heart, lung, liver, kidney, spleen, thymus, brain, testes, and ovaries).

Due to the lack of small animal models for VZV-induced disease, *in vivo* studies to evaluate the efficacy of BCNAs against VZV disease could not be performed in animals.

5.1.5 *Clinical studies*

FV-100 is currently being developed by Inhibitex for the treatment of herpes zoster. In order to characterize the pharmacokinetics and safety of oral FV-100, single and multiple doses were administered to healthy young adults and elderly adult volunteers. After a pilot study performed in human subjects receiving single oral doses (10, 20, and 40 mg) of FV-100 and in which FV-100 showed a favorable safety profile, three additional randomized, double-blind, placebo-controlled clinical trials were conducted: (i) a single ascending-dose study in 32 healthy subjects aged 18–55 years (100-, 200-, 400-, and 800 mg doses) with an evaluation of the food effect in the 400 mg group; (ii) a multiple ascending-dose study in 48 subjects aged 18–55 years (100 mg once daily (QD), 200 mg QD, 400 mg QD, 400 mg twice a day, and 800 mg QD for 7 days); and (iii) a two-part study in subjects aged 65 years and older with a single 400 mg dose in 15 subjects and a 400 mg QD dosing regimen for 7 days in 12 subjects (Pentikis et al., 2011). These studies showed that FV-100 was rapidly and extensively converted to CF-1743. Renal excretion of CF-1743 was very low. A high-fat meal reduced exposure to CF-1743, while a low-fat meal did not. The pharmacokinetic profile of FV-100 in elderly subjects was similar to that of younger subjects. Additionally, pharmacokinetic data demonstrated that all doses maintained mean drug plasma levels of the active form of FV-100 that exceeded the EC_{50} for approximately 24 h, supporting the potential for once-a-day

dosing in Phase II trials. There were no serious adverse events reported in the two trials and FV-100 appeared to be well tolerated at all dose levels.

Based on the good safety profile of the drug, Inhibitex started a **Phase II** clinical trial studying FV-100 as a treatment for herpes zoster. The purpose of this well-controlled, double-blind clinical trial was to compare the safety and efficacy of two doses of FV-100 to VACV (Valtrex®, GSK) in patients with herpes zoster. In December 2010, Inhibitex announced results from this study (http://www.inhibitex.com). Patients aged 50 years and older were randomized to one of the three treatment arms: 200 mg FV-100 administered QD; 400 mg FV-100 administered QD; and 1000 mg VACV administered three times per day (TID) (the standard of care). The primary endpoint of the study was a reduction in herpes ZAP and severity as measured by the Zoster BOI scale after 30 days of treatment. The trial was 80% powered to detect an approximate 20–25% difference between the FV-100 and Valtrex cohorts. Secondary endpoints included the BOI after 90 days, incidence of PHN, mean time to lesion crusting and healing, and use of concomitant pain medications.

As summarized in Table 4.2, improvements in the reduction in the severity and duration of shingles-associated pain at 30 days (Zoster BOI Day 30) of 3% and 7% for the 200 and 400 mg patient cohorts were seen, but this was far from the 20–25% reduction called as the primary endpoint, and not statistically significant. The data also showed a 4% and 14% reduction on the BOI-90 for FV-100 200 mg QD and FV-100 400 mg QD versus VACV 1000 mg TID. Interestingly, FV-100 appeared to be more effective in reducing PHN since patients on FV-100 200 mg also had a 12% reduced incidence of PHN (17.8% vs. 20.2%) versus VACV. Patients on FV-100 400 mg QD also had a 39% reduced incidence of PHN (12.4% vs. 20.2%) versus VACV.

Although this Phase II trial missed the primary endpoint, encouraging results were (1) continued separation from FV-100 cohorts and VACV between BOI-30 and BOI-90 [FV-100 went from 3% improvement at BOI-30 to 4% at BOI-90 (FV-100 200 mg QD) and from 7% improvement at BOI-30 to 14% improvement at BOI-90 (FV-100 400 mg QD)]; (2) a clear dose–response, the FV-100 400 mg arm showed reduced BOI-30 and BOI-90 scores versus the FV-100 200 mg arm; (3) reduced incidence of PHN, the most clinically meaningful endpoint; and (4) reduced use of additional pain medication (opioids) for the management of PHN in the FV-100 arm versus VACV arm. Additionally, FV-100 offers secondary benefits over VACV because FV-100 is QD dosing versus VACV TID dosing.

Table 4.2 Results of the Phase II clinical trial evaluating FV-100 for shingles treatment against valacyclovir

	FV-100 (200 mg QD, $n=107$)	FV-100 (400 mg QD, $n=113$)	Valacyclovir (1000 mg TID, $n=109$)
Efficacy			
Zoster BOI Day 30	114.49	110.31	117.96
Zoster BOI Day 90	221.53	196.94	229.59
Incidence of PHN (%)	17.8	12.4	20.2
Safety			
Excluded as zoster negative	$N=10$	$N=4$	$N=7$
Any adverse events (%)	46	55	42
Treatment-related adverse events (%)	21	26	20
Discontinuation of drug for adverse events (%)	1.7	1.7	1.7
Serious adverse events (%)	0	4	3
Treatment-related serious adverse events (%)	0	0	2

BOI, burden of illness.

Safety and tolerability of FV-100 looked generally similar to VACV in this Phase II clinical trial (Table 4.2). Adverse events in any cohort that exceeded 10% were headache and nausea. Serious adverse events were low and generally unrelated to drug treatment. Given the safety and tolerability profile of FV-100 and the clear dose–response seen in the Phase II data above, testing a higher dose for the Phase IIb or Phase III should be appropriate. Full data analysis of this Phase II clinical trial will be provided during next spring. Inhibitex, the company performing the clinical studies was acquired by Bristol-Myers Squibb, and Synergy Pharma has recently acquired all assets related to FV-100.

5.2. Helicase–primase inhibitors

Herpesviruses encode a helicase and a primase that assemble with a cofactor protein in a heterotrimeric complex with 1:1:1 stoichiometry. The herpesvirus helicase–primase possesses multiple enzymatic activities including DNA helicase, single-stranded DNA (ssDNA)-dependent ATPase and

primase, all of which are essential for viral DNA replication and viral growth. Therefore, agents that target the helicase–primase complex have the potential to represent novel antiherpes agents. The helicase–primase complex is well conserved among members of the herpesvirus family. Thus, the genes encoding the HSV helicase subunit (*UL5*), primase subunit (*UL52*), and cofactor subunit (*UL8*) share homology with the HCMV genes *UL105*, *UL70*, and *UL102* and the VZV *ORF55*, *ORF6*, and *ORF52* genes of VZV.

Three related classes of compounds, that is, 2-amino-thiazole, thiazolylphenyl (e.g., BILS 179 BS), and thiazole urea (e.g., BAY 57-1293) derivatives have been shown to target the HSV helicase–primase complex (Kleymann et al., 2002; Spector, Liang, Giordano, Sivaraja, & Peterson, 1998). Both the thiazolylphenyl and thiazole urea derivatives exhibited low toxicity and were generally superior to currently approved drugs to treat HSV infections in different animal models (Baumeister et al., 2007; Crute et al., 2002). Because these helicase/primase inhibitors (HPI) have a completely different mechanism of action and do not require activation by viral TK, they retain activity against HSV mutants that are resistant to nucleoside analogues. Furthermore, combination of HPI with nucleosidic drugs showed a synergistic effect *in vitro* (Kleymann, 2003). BILS 179 BS and BAY 57-1293 had a limited antiviral activity spectrum, as both compounds inhibited only HSV-1 and HSV-2 but not other herpesviruses. Also, specific inhibitors of HCMV helicase–primase complex (i.e., the nitropyrimidine T-0902611 and imidazolyl-pyrimidines) were shown to hamper HCMV replication and infection (Chen et al., 2007; Cushing et al., 2006).

Recently, a novel oxadiazolephenyl derivative, that is, **ASP2151** (amenamevir), has been described as a novel class of HPI that possesses potent antiviral activity against not only HSV-1 and HSV-2 but also VZV (Chono et al., 2010).

5.2.1 Antiviral activity and selectivity of ASP2151 in cell culture

The oxadiazolephenyl derivative ASP2151 (Fig. 4.5) was able to inhibit the replication of HSV-1 and HSV-2 with EC_{50} values of, respectively, 0.047 and 0.028 μM. The EC_{50} values of ASP2151 against HSV-1 and HSV-2, as determined by plaque reduction assay in human embryonic lung cells, were comparable to those calculated for BILS 179 BS and BAY 57-1293. In contrast, the EC_{50} values of BILS 179 BS and BAY 57-1293 for VZV were 4.1 and 11 μM, respectively, compared to 0.047 μM for ASP2151. In addition, EC_{90} values of ASP2151 for VZV, HSV-1, and HSV-2 confirmed the antiviral potency against these viruses. Importantly, ASP2151 showed no

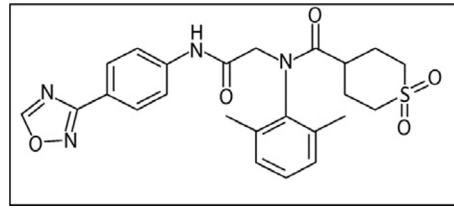


Figure 4.5 Helicase-primase inhibitor ASP2151.

toxicity up to a concentration of 30 μM, resulting in an SI of at least 638. No antiviral activity was observed against HCMV.

The anti-VZV activity of ASP2151 compared to ACV was further evaluated using several strains of VZV, including clinical isolates and an ACV-resistant mutant. ASP2151 inhibited the replication of all the wild-type VZV strains tested with EC_{50} values in the range of 0.038–0.10 μM compared to 1.3–5.9 μM for ACV. ASP2151 was also active against an ACV-resistant VZV strain (EC_{50} values of 27 μM for ACV compared to 0.082 μM for ASP2151). The CC_{50} of ASP2151 was determined to be 200 μM in these experiments, which provided a SI higher than 2000. ASP2151 can be considered as the first HPI exerting more potent anti-VZV activity than ACV.

In order to study the effect of ASP2151 on viral DNA replication, the quantity of viral DNA in virus-infected cells exposed to ASP2151 was measured. At concentrations of $\geq$0.03 mM, ASP2151 inhibited the DNA synthesis of VZV, HSV-1, and HSV-2. In agreement with the plaque reduction assays, no effect on viral DNA replication was observed in HCMV-infected cells at ASP2151 concentrations of up to 1 mM. The EC_{50} value for VZV DNA replication was determined using real-time PCR. Both ASP2151 and ACV reduced the quantity of VZV DNA in a concentration-dependent manner, with EC_{50} values of 0.057 and 0.44 μM, respectively, which is consistent with the plaque reduction assays (Chono et al., 2010).

Recently, combined therapy of ASP2151 with ACV and other nucleoside analogue antiherpes drugs demonstrated a synergistic/additive effect against HSV and VZV infections, suggesting that combination therapy may be a useful approach for treating severe disease conditions such as encephalitis or infections in patients with immunosuppression (Chono, Katsumata, Suzuki, & Shiraki, 2012).

5.2.2 Activity of ASP2151 against helicase–primase complex

The inhibitory activity of ASP2151 against helicase–primase complex was assayed using the recombinant UL5–UL52–UL8 complex of HSV-1. The

helicase–primase complex of HSV has multiple enzymatic activities, being DNA helicase and ssDNA-dependent ATPase activity catalyzed by the UL5 helicase subunit and primase activity catalyzed by the UL52 primase subunit. ASP2151 inhibited the DNA helicase activity of the complex at concentrations of $\geq$0.1 μM. Similarly, the ssDNA-dependent ATPase activity was inhibited in a concentration-dependent manner with a mean EC_{50} value of 0.078 μM. In addition, ASP2151 inhibited the primase activity at concentrations of $\geq$0.03 μM.

5.2.3 *Characterization of ASP2151-resistant VZV mutants*

To confirm that the anti-VZV activity of ASP2151 was due to inhibition of the VZV helicase–primase complex, a VZV mutant able to replicate in the presence of ASP2151 at concentrations up to 60 μM was isolated. Sequencing analysis of *ORF55* (helicase gene) and *ORF6* (primase gene) of this mutant indicated three amino acid changes from the parent strain. Substitution of Asn-336 to Lys (N336K) was found in helicase motif IV, one of the six well-conserved sequence motifs in *ORF55*. The other substitutions were Arg-446 to His (R446H) in *ORF55* and Asn-939 to Asp (N939D) in *ORF6*. Notably, this mutant showed a marked defect in viral replication.

5.2.4 In vivo *antiviral activity*

The *in vivo* efficacy of ASP2151 was evaluated in hairless mice cutaneously infected with HSV-1, which leads to a progressive disease course due to virus spread (Katsumata et al., 2012). ASP2151 at doses of 0.3, 1, 3, 10, and 30 mg/kg or VACV at doses of 3, 10, 30, and 100 mg/kg (suspension in 0.5% methylcellulose solution) were orally administered twice daily (bid) for 5 days starting 3 h after viral inoculation. Ten mice per test group were used. Disease course was monitored daily for 17 days and scored based on the severity of lesions and general symptoms. Oral administration of ASP2151 and VACV significantly reduced mortality on day 17, the cumulative disease score and area under the disease score–time curve for the period days 0–17 postinfection ($AUCday_{0–17}$) at doses of $\geq$1 mg/kg bid and $\geq$10 mg/kg bid, respectively ($P < 0.05$). Based on the $AUCday_{0–17}$, ED_{50} (50% effective dose) values (95% confidence interval) of ASP2151 and VACV were calculated as 1.9 (0.9–3.4) mg/kg bid and 27 (14–74) mg/kg bid, respectively. ASP2151 was 14-fold more potent than VACV in the model, being the difference statistically significant.

Pharmacokinetic and pharmacodynamic parameters of ASP2151 in a murine model of HSV-1 infection indicated that a plasma ASP2151

concentration exceeding 100 ng/mL for 21–24 h per day provides the maximum efficacy in this animal model (Katsumata et al., 2012).

5.2.5 Toxicology in mice

ASP2151 was well tolerated and did not reveal obvious safety concerns when administered for 5 days to mice at dosing of up to 30 mg/kg bid. Furthermore, no safety issues were evidenced in toxicology tests performed in mice that received ASP2151 for 4 weeks up to 500 mg/kg.

5.2.6 Clinical studies

Astellas Pharma planned two randomized, double-blind, multiple dose, multicenter studies to investigate the efficacy and safety of three different doses of ASP2151, as compared to VACV and placebo. One of the studies included subjects with recurrent episodes of genital herpes and the primary endpoint was comparison of the efficacy and safety of ASP2151 with VACV and placebo in the acute treatment of recurrent genital HSV. The second study was supposed to recruit persons with herpes zoster and the primary endpoint was comparison of the efficacy and safety of ASP2151 with VACV and the secondary endpoint was comparison of improvements in cutaneous symptoms and pain between the two drugs. However, due to treatment-emergent serious adverse events, a Phase I study comparing the safety of ASP2151 to VACV in healthy volunteers had to be terminated and development of the drug was halted (ClinicalTrials.gov). Astellas Pharma did not provide information about the nature of the adverse events that led to cessation of trials with ASP2151 but the development of this compound has been completely abandoned.

5.3. Carbocyclic nucleoside analogues: H2G (omaciclovir) and its prodrug (valomaciclovir)

H2G, (*R*)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine, omaciclovir (Fig. 4.6), is a carbocyclic nucleoside analogue that has shown potent activity against different herpesviruses, being especially active against VZV (Abele, Eriksson, Harmenberg, & Wahren, 1988). The EC_{50} for omaciclovir against HSV-1 (0.62 ± 0.3 μM) was similar to that seen for ACV, whereas the EC_{50} against HSV-2 (5.8 ± 1.2 μM) was significantly higher than that seen for ACV. Against 20 clinical isolates of VZV, the average EC_{50} for H2G was 2.3 ± 0.8 μM, compared to 46.8 μM for ACV and 78.7 μM for PCV, pointing to a superior activity of omaciclovir against this virus (Lowe et al., 1995). H2G had no activity against HCMV up to 200 μM, but showed

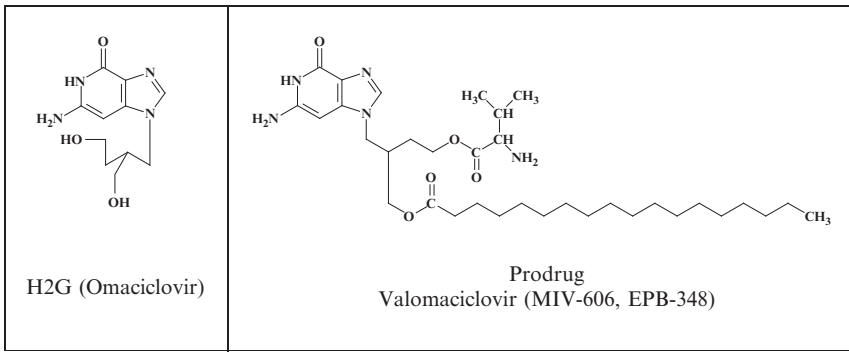


Figure 4.6 H2G and its prodrug valomaciclovir stearate.

a good activity against EBV, with EC_{50} of 0.9 μM. In cellular cytotoxicity studies, the compound gave a CC_{50} of >500 μM in MRC-5 cells (human embryonic lung fibroblasts), >2000 μM in Vero cells, and >50 μM in human hematopoietic stem cells, the results being comparable to those obtained with ACV (Lowe et al., 1995). Omaciclovir was reported to inhibit human herpesvirus 6 (HHV-6) with variable EC_{50}'s ranging from less than 5 μM to more than 197.4 μM and selectivity indices from 1 to 47. This variability would be explained by the diversity of cells, viral strains, and methods used (Bonnafous & Agut, 2010; De Clercq et al., 2001).

This compound has a mode of action similar to that of ACV, but with less selectivity as a substrate for TK. Also, resistance to H2G has been mapped in the TK (Ng et al., 2001). In contrast to ACV, H2G is not an obligate chain terminator, although incorporation of the triphosphate form (H2G-TP) results in limited chain elongation. Another difference with ACV-TP is the longer intracellular half-life of H2G-TP. This feature could provide dosing advantages over ACV. The TP form of H2G is a potent inhibitor of VZV DNA polymerase, but less active than ACV-TP. The antiherpesvirus activity of H2G was demonstrated to be markedly enhanced by the immunosuppressive agent mycophenolate mofetil (Neyts, Andrei, & De Clercq, 1998).

H2G was reported to have an oral bioavailability of 17% in cynomolgus monkeys, which is similar to that of ACV (Soike, Bohm, Huang, & Oberg, 1993). Valomaciclovir, MIV-606 [L-valine, (3*R*)-3-[(2-amino-1,6-dihydro-6-oxo-purin-9-yl)methyl]-4[(1-oxooctadecyl)oxo]butylester] (Fig. 4.6) is an example of a double prodrug of H2G. The combination of the valine and stearyl esters lead to a bioavailability improvement in rats compared to H2G. MIV-606 has an oral bioavailability of >70% in rats and monkeys, and >60% in humans (De Clercq & Field, 2006). MIV-

606 was licensed from the Swedish biotech company Medivir AB to Epiphany Biosciences for further development under the name EPB-348. Phase I/II clinical trials have been performed with valomaciclovir (http://www.epiphanybio.com). The Phase IIb trial enrolled 373 immunocompetent patients suffering from acute herpes zoster, randomized into three arms: 1 g once-a-day valomaciclovir, 2 g once-a-day valomaciclovir, and 1 g three times per day VACV (Tyring et al., 2012). Eighteen patients also received 3 g of QD valomaciclovir. The primary endpoint was noninferiority of QD valomaciclovir compared to three times VACV in terms of time to complete crusting of the shingles rash. QD valomaciclovir at 2 g met the primary endpoint; it was not only more convenient than three times daily VACV for the treatment of herpes zoster but also equally safe. Valomaciclovir was also noninferior to VACV in the secondary endpoints (time to complete pain resolution, time to rash resolution, and time to cessation of new lesion formation). Furthermore, the highest dose of valomaciclovir (3 g QD) demonstrated superiority to VACV with regard to the primary endpoint (*P* value <0.007). There were no significant adverse event differences between valomaciclovir and VACV groups. The most common adverse event was nausea in all patient groups. No patient was reported to have to discontinue treatment due to adverse events related to valomaciclovir.

Valomaciclovir has also been shown to be effective against acute infectious mononucleosis, for which there is no FDA-approved treatment. The results of a randomized, placebo-controlled, double-blind trial of valomaciclovir for infectious mononucleosis were reported in 2009 (Balfour et al., 2009). Subjects over the age of 15 with acute mononucleosis were randomized to 21 days of valomaciclovir 4 g/day or placebo and followed by 6 months. The 11 individuals who received valomaciclovir had significantly faster clinical improvement than placebo recipients as documented by comparing the slopes of the plots of their physical exam/symptoms score for each visit during the treatment period. The drug produced a significant decrease in median EBV load in the oral compartment versus placebo. No differences were found in CL_t of EBV DNAemia, CD8:CD4 ratios, CD8 lymphocytosis, or CD8 responses to lytic and latent EBV tetramers in the valomaciclovir versus placebo subjects.

6. CANDIDATE ANTI-VZV DRUGS

Novel classes of antiviral compounds able to target different steps of the viral replicative cycle have been identified. Moreover, a new strategy

based on the discovery of specific cellular targets required for viral replication has been developed. We focus on novel anti-VZV compounds described in the past 5 years that can be considered as candidate anti-VZV drugs.

6.1. *N*-alpha-methylbenzyl-*N*′-arylthiourea analogues

A series of nonnucleoside, *N*-alpha-methylbenzyl-*N*′-arylthiourea analogues were described with selective activity against VZV but were inactive against other human herpesviruses (Visalli et al., 2003). Three *N*-methylbenzyl-*N*′-arylthiourea analogues (Comp I, Comp II, and Comp III) (Sergerie, Rivest, & Boivin, 2007) were selected for further study (Fig. 4.7). These compounds had potent activity against VZV early-passage clinical isolates and an ACV-resistant isolate (EC_{50} values of all three compounds consistently ranged from 0.1 to 1.5 μM, while no apparent cytotoxic effects at compound concentrations of >20 μM were observed). The compounds were not able to inhibit VZV immediate-early or late antigen expression. In an effort to identify the target of these compounds, resistant viruses were generated against compound I, which were also resistant to other compounds in the series, suggesting that this group of related small molecules was acting on the same virus-specific target. Resistance to the *N*-alpha-methylbenzyl-*N*′-arylthiourea analogues was mapped to the VZV ORF54 protein. Sequencing of the VZV *ORF54* gene from two

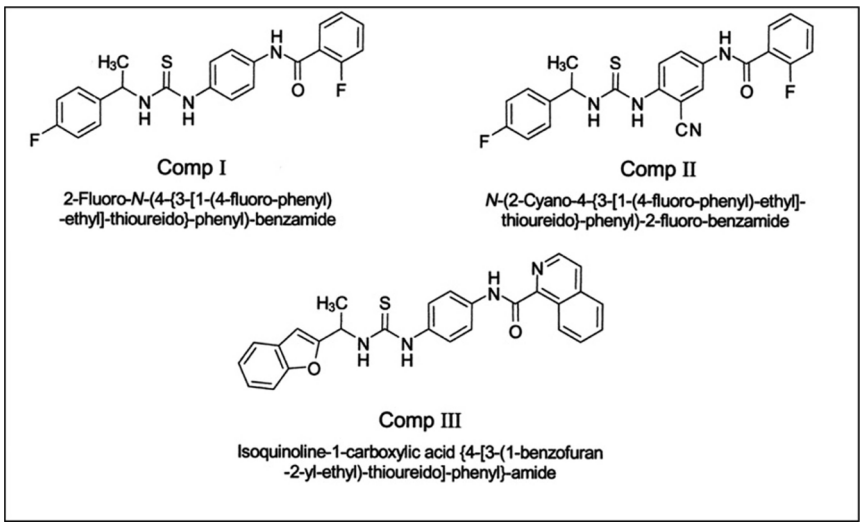


Figure 4.7 N-alpha-methylbenzyl-N′-arylthiourea analogs.

independently derived resistant viruses revealed mutations in *ORF54* compared to the parental VZV strain sequence. Recombinant VZV in which the wild-type *ORF54* sequence was replaced with the *ORF54* gene from either of the resistant viruses became resistant to the series of inhibitor compounds. The VZV ORF54 protein shows 44% amino acid identity to its HSV-1 UL6 homolog, and it was assumed that the ORF54 protein might perform a functional role similar to that of UL6 during VZV replication. HSV-1 UL6 is a capsid-associated protein that is essential for cleavage and packaging of viral DNA into capsids. Indeed, treatment of VZV-infected cells with the inhibitor impaired morphogenesis of capsids. Furthermore, DNA-containing dense-core capsids in the nucleus were not observed in inhibitor-treated cells and only incomplete virions were present on the cell surface. These data indicated that the VZV-specific thiourea inhibitor series block virus replication by interfering with the function of the ORF54 protein and/or other proteins that interact with the ORF54 protein.

6.2. Amphipathic polymers

Phosphorothioated oligonucleotides (PS-ONs) have a sequence-independent, broad-spectrum antiviral activity as amphipathic polymers (APs). They exhibit potent *in vitro* antiviral activity against a broad spectrum of herpesviruses, including HSV-1, HSV-2, HCMV, VZV, EBV, and HHV-6 (Bernstein et al., 2008; Cardin et al., 2009; Guzman et al., 2007). The activity of APs is dependent on phosphorothioation, and this is not simply due to the stabilization of oligonucleotides by this modification, as evidenced by the lack of activity of randomer 3 ONs, which were not phosphorothioated but fully stable. Thus, the antiviral activity is dependent on the chemical nature of PS-ONs APs, independent of the stability that phosphorothioation provides to oligonucleotides. The 40-mer degenerate parent compound (REP 9) (Fig. 4.8), demonstrated *in vitro* activity against a broad spectrum of herpesviruses and inhibited VZV replication with EC_{50} values <0.02 μM while its CC_{50} (concentration that results in 50% toxicity in uninfected cells compared to untreated, uninfected controls) was >100 μM (Bernstein et al., 2008). A murine microbiocide model of genital HSV-2 infection was used to demonstrate the *in vivo* activity of REP 9 and REP 9C (an acid stable analogue) (Fig. 4.8) (Bernstein et al., 2008). Thus, APs are considered as candidate vaginal microbicides. Recently, the activity of these agents against murine and guinea pig cytomegalovirus *in vivo* was also shown (Cardin et al., 2009).

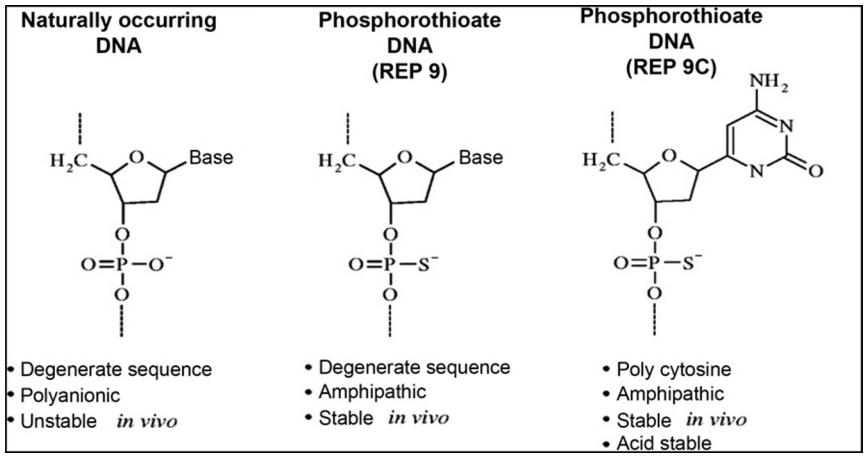


Figure 4.8 Amphipathic DNA polymers.

PS-ONs are also polyanionic, and the antiviral activities of other classes of polyanions against enveloped viruses are well documented. The mechanism of action of these APs suggests that their antiviral activity may be derived from their hydrophobic interactions with multiple target sites along proteins involved in viral entry. The antiviral activity was independent of the sequence but was dependent on size: the most potent activity against HSV was observed for analogues of 40 nucleotides in length. It was shown that they were able to block HSV-2 binding, entry and gene expression, and HSV-induced apoptosis when added postentry (Guzman et al., 2007). PS-ONs were also demonstrated to interact with a peptide derived from the N-terminal heptad repeat region of HIV gp41. The HIV-1 fusion-inhibitory activity of PS-ONs closely correlated with their ability to block gp41 six-helix bundle formation, a critical step during the process of HIV-1 fusion with the target cell (Vaillant et al., 2006). The increased hydrophobicity of PS-ONs appeared to contribute to their inhibitory activity against HIV-1 fusion and entry, because longer PS-ONs ($\geq$30 bases), which have a greater hydrophobicity, were more potent in blocking the hydrophobic interactions involved in the gp41 six-helix bundle formation and inhibiting HIV-1 mediated cell–cell fusion than shorter PS-ONs ($<$30 bases). Amphipathic DNA polymers were also demonstrated to inhibit hepatitis C virus (HCV) infection by blocking viral internalization; they had no effect on viral replication in the HCV replicon system or binding to the cells but inhibited internalization (Matsumura et al., 2009). The antiviral activity of PS-ONs *in vivo* (in the presence or absence of CpG motifs) appeared not to be derived

from stimulation of the immune system but more likely due to direct hydrophobic interactions preventing the attachment and fusion of virions to the host cell (Bernstein et al., 2008). No studies on the mechanism of action of the APs against VZV have been described, but it can be assumed that, as in other viruses, virus entry should be inhibited.

6.3. 5-Substituted 4′-thiopyrimidine nucleosides

The 5-iodo-4′-thio-2′-deoxyuridine (4′-thioIDU) (Fig. 4.9) was selected from a series of 5-substituted 4′-thiopyrimidine nucleosides as the most potent and selective inhibitor, not only of herpesviruses but also of orthopoxviruses (Kern et al., 2009; Prichard et al., 2009). This compound selectively inhibited HSV-1, HSV-2, VZV, and HCMV with EC_{50} values of, respectively, 0.1, 0.5, 2, and 5.9 μM but not EBV, HHV-6, or human herpesvirus 8 (HHV-8). The reported CC_{50} (concentration that reduced the proliferation of human foreskin fibroblasts by 50%) values varied between 70 and >100 μM, resulting in selectivity indices of >1, 250 (HSV-1), >220 (HSV-2), >17 (HCMV), 35 (VZV). The mechanism of action of 4′-thioIDU appeared to be similar to that of 5-iodo-2′-deoxyuridine (IDU) because the compound is phosphorylated preferentially by the viral TK and the triphosphate metabolite is a substrate of the viral DNA polymerase. This suggests that mutations conferring resistance to 4′-thioIDU may occur either in the TK or DNA polymerases. In agreement with this, some ganciclovir-resistant strains of HCMV were found to exhibit reduced susceptibility to the compound, which appeared to be related to specific mutations in the viral DNA polymerase. 4′-ThioIDU could be also incorporated into DNA in uninfected cells, indicating that a cellular kinase, such as TK-1, can also phosphorylate the compound and that the triphosphate metabolite is also a substrate for a cellular polymerase. This would also be consistent with the inhibition of cell proliferation seen with 4′-thioIDU (50% inhibitory concentration (IC_{50}) values of 3.0 μM in cell proliferation assays vs. >100 μM

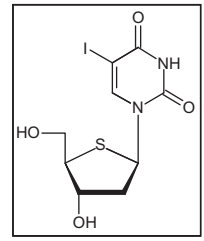


Figure 4.9 5-iodo-4′-thio-2′-deoxyuridine (4'-thioIDU).

in stationary cells). This compound proved to be a much more potent inhibitor of orthopoxvirus replication both *in vitro* and *in vivo* than of herpesvirus replication. Therefore, 4′-thioIDU may have its greater potential for the treatment of orthopoxvirus infections. There is less enthusiasm for further development of 4′-thioIDU as a therapeutic for herpesvirus infections, but other analogues may have improved antiherpesvirus activity.

6.4. Pyrazolo[1,5-*c*]1,3,5-triazin-4-one derivative: Compound 35B2

A derivative of pyrazolo[1,5-*c*]1,3,5-triazin-4-one (coded as 35B2) (Fig. 4.10) was identified from a library of 9600 random compounds as an inhibitor of VZV (Inoue et al., 2012). This compound was also active against ACV^r. In a plaque reduction assay performed in human fibroblasts with the Oka strain, the EC_{50} value was 0.75 μM for 35B2 compared to 4.25 μM for ACV and the selective index of the compound was >200. The compound was only weakly active against HSV-1 and had no effect on HHV-6 or HCMV. Treatment with 35B2 inhibited neither immediate-early gene expression nor viral DNA synthesis. Different drug-resistant virus clones resistant to 35B2 were isolated, all of which had a mutation(s) in the amino acid sequence of open reading frame 40, which encodes the major capsid protein (MCP). Infected cells treated with 35B2 showed the lack of capsid formation as determined by electron microscopic studies. These data indicate that targeting the herpesvirus MCPs results in inhibition of normal capsid formation and this can be achieved with a small molecule by a mechanism that differs from those of the known protease and encapsidation inhibitors.

6.5. Nonnucleoside DNA polymerase inhibitors

Brideau and colleagues (Brideau et al., 2002) identified 4-oxo-dihydroquinolines (4-oxo-DHQs), represented by PNU-183792 (Fig. 4.11), as specific inhibitors of herpesvirus polymerases. PNU-183792

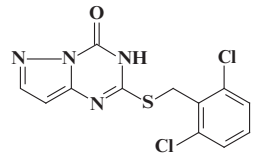


Figure 4.10 Pyrazolo[1,5-*c*]1,3,5-triazin-4-one derivative: compound 35B2.

was highly active against HCMV ($IC_{50} = 0.69$ μM), VZV ($IC_{50} = 0.37$ μM), and HSV ($IC_{50} = 0.58$ μM) polymerases but was inactive ($IC_{50} > 40$ μM) against human α, γ, or δ polymerases. *In vitro* antiviral activity of PNU-183792 (determined as EC_{50} values using cytopathic effect, plaque reduction, and virus-yield reduction assays) was in the range of 0.3–2.4 μM (HCMV), 0.1 μM (VZV), and of 3–5 μM (HSV). PNU-183792 was also active (EC_{50} ranging 0.1–0.7 μM) in cell culture assays against simian varicella virus (SVV), MCMV, and rat cytomegalovirus. PNU-183792 was also active against drug-resistant herpesvirus strains. Toxicity assays performed with four different species of proliferating mammalian cells showed that PNU-183792 was not cytotoxic up to >100 μM. PNU-183792 was inactive against unrelated DNA and RNA viruses, indicating specificity for herpesviruses. In animals,

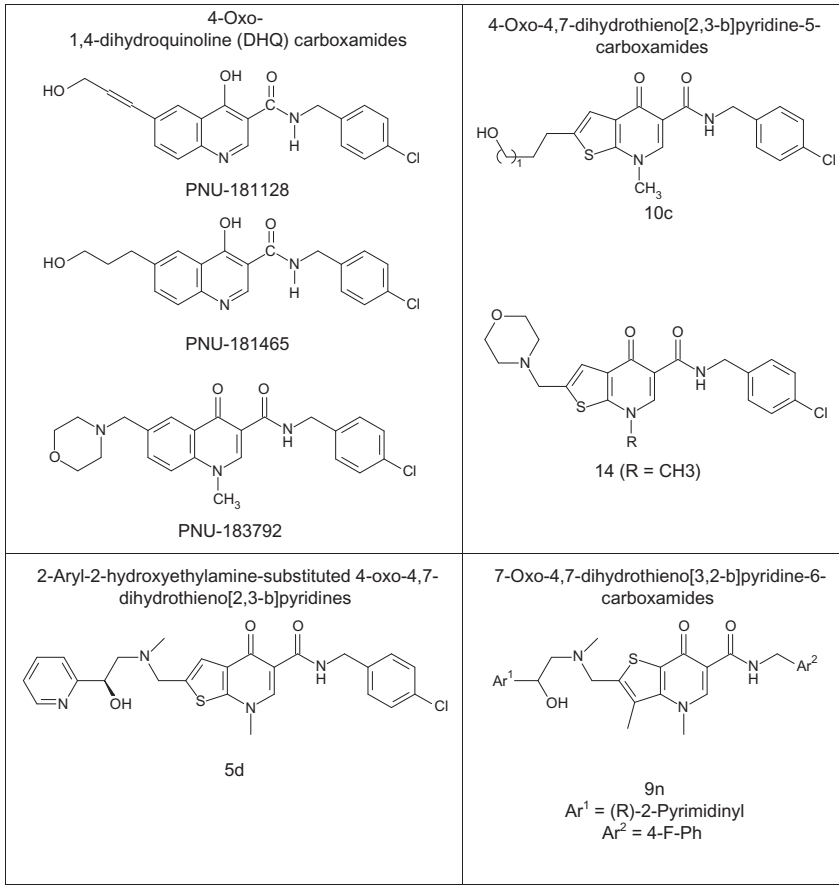


Figure 4.11 Non-nucleoside DNA polymerase inhibitors.

PNU-183792 was orally bioavailable and was efficacious in a model of lethal MCMV infection.

Schnute and colleagues described a series of 4-oxo-4,7-dihydrothieno [2,3-b]pyridine-5-carboxamides as potential antivirals against human herpesvirus infections, including HCMV, VZV, and HSV. In the 4-oxo-4,7-dihydrothienopyridines (DHTPs), the quinolone ring of the 4-oxo-DHQ was replaced by the thieno[2,3-b]pyridine ring (Schnute et al., 2005). Among these nonnucleoside analogues, compounds 10c and 14 (Fig. 4.11) demonstrated broad-spectrum inhibition of herpesvirus polymerases HCMV, VZV, and HSV-1 (Schnute et al., 2005) with high specificity for the viral polymerases compared to human α polymerase. The antiviral activity of compounds **10c** and **14** was comparable or superior to that of the existing antiherpesvirus drugs. Drug resistance to compound **14** was associated with point mutations in the conserved region III of the herpesvirus DNA polymerase, but they did not confer resistance to reference nucleoside analogues. In contrast to the kinetics determined for the 4-oxo-DHQs, DHTPs proved to be competitive inhibitors of dTTP incorporation into the primer template by HCMV DNA polymerase (Schnute et al., 2005).

Further SAR studies led to the discovery of compound **5d** (Fig. 4.11) which demonstrated broad-spectrum inhibition of the herpesvirus polymerases HCMV, HSV-1, EBV, and VZV. This compound was selected for advanced clinical development (Schnute et al., 2007). Later on, the 7-oxo-4,7-dihydrothieno[3,2-b]pyridine-6-carboxamides were synthesized (Larsen et al., 2007), leading to the identification of compound **9n** (Fig. 4.11) which exhibited an antiviral activity equivalent to compound **5d** but had better solubility and markedly improved hERG (human ether-á-go-go-related gene) profile (Larsen et al., 2007). hERG potassium channel blockage is considered to be a significant cardiovascular risk factor when selecting compounds for advanced development.

Despite promising results *in vitro*, these nonnucleoside DNA polymerase inhibitors have not been evaluated for any herpesvirus indication in clinical trials.

6.6. Nucleotide analogues

6.6.1 Esters of acyclic nucleoside phosphonates

Cidofovir (CDV, HPMPC) (Fig. 4.12), an acyclic nucleoside phosphonate (ANP), is approved for systemic treatment of HCMV infections. In contrast to nucleoside analogues, it does not require activation by the viral enzymes.

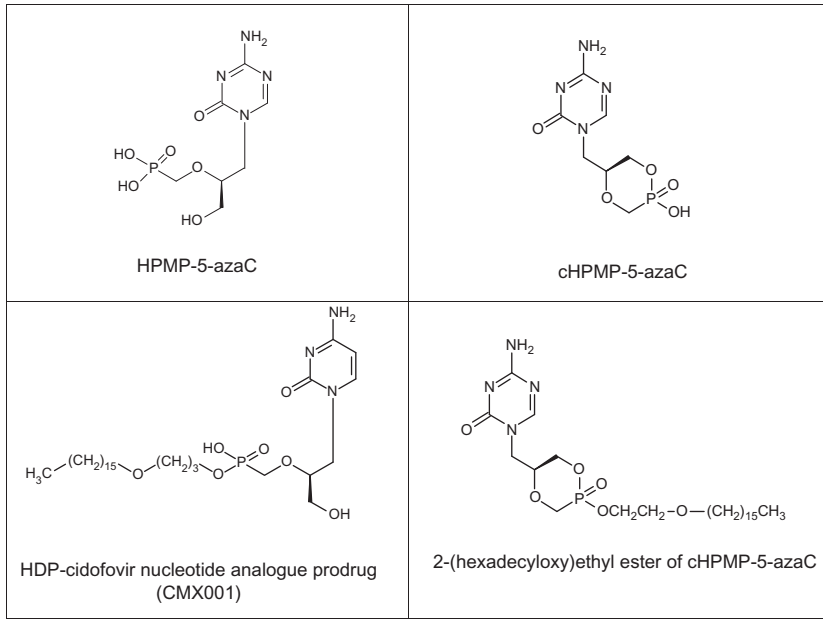


Figure 4.12 Nucleotide analogues.

CDV (Vistide®, Gilead) possesses broad-spectrum activity against DNA viruses and has been licensed for the intravenous treatment of HCMV retinitis in AIDS patients. Due to the broad activity of CDV against a variety of human DNA viruses, this compound is also used "off-label" for the intravenous or topical treatment of various herpes-, polyoma-, and adenovirus infections, poxvirus infections (i.e., molluscum contagiosum and orf virus), and papillomavirus-associated diseases such as genital warts and laryngeal papillomas (De Clercq & Holy, 2005; Snoeck & De Clercq, 2002). Because CDV has a phosphonate group, it bypasses the first phosphorylation step and therefore is independent of activation by the viral HSV or VZV TK or the HCMV pUL97 (protein kinase responsible for activation of ganciclovir). Cellular kinases convert the drug to the active diphosphate form, which acts as a competitive inhibitor of the viral DNA polymerase, causing premature chain termination during viral DNA synthesis (Fig. 4.13). Incorporation of cidofovir diphosphate (CDVpp) into HCMV DNA slows down elongation and results in chain termination when two consecutive CDVpp residues are incorporated in a row (Xiong, Smith, & Chen, 1997; Xiong, Smith, Kim, Huang, & Chen, 1996). The diphosphate forms of ANPs inhibit viral polymerases more potently than cellular DNA polymerases. The metabolites of

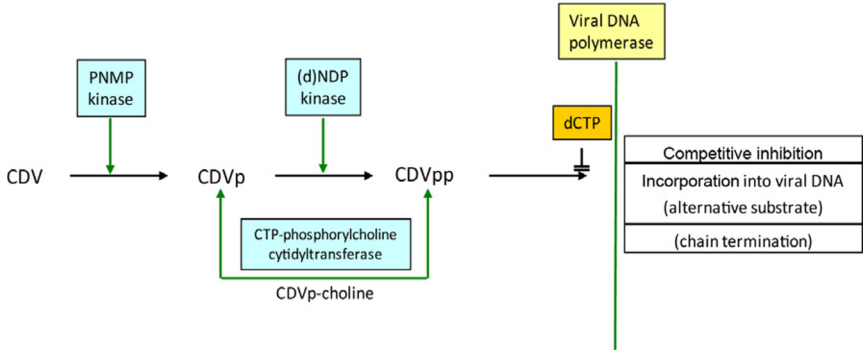


Figure 4.13 Metabolism and mode of action of cidofovir (CDV).

the ANPs show an unusually long intracellular half-life; this may account for the long-lasting antiviral activity of the compounds. This prolonged antiviral action may be attributed to the formation of the cidofovir monophosphate (CDVp)-choline adduct, which could serve as an intracellular reservoir for the mono- and diphosphate derivatives of CDV (i.e., CDVp and CDVpp). CDV presents two major disadvantages that have restrained its use: (i) low oral bioavailability (<5%) requiring IV administration, usually once a week, and (ii) dose-dependent nephrotoxicity that can be limited by prehydration and coadministration of probenecid.

Because of the limitations associated with the use of CDV, that is, poor oral bioavailability and renal toxicity, alkoxyalkyl esters of CDV and of its cyclic form (cCDV) have been synthesized (Beadle et al., 2002; Hostetler, 2009). In these prodrugs, a natural fatty acid (lysophosphatidylcholine) molecule is used as carrier to facilitate drug absorption in the gastrointestinal tract. Different alkoxyalkyl esters (i.e., hexadecyloxypropyl (HDP) and octadecyloxyethyl) of CDV and cCDV proved much more active *in vitro* than the parent compounds against several herpesviruses, including HSV, VZV, CMV, EBV, HHV-6, and HHV-8, and poxviruses. In addition to herpes- and poxviruses, the increased activity of alkoxyalkyl esters of CDV compared to the parent compound CDV was also shown against adenovirus, polyomavirus, and papillomavirus (Andrei, Snoeck, Schols, & De Clercq, 2000; Andrei, Snoeck, Vandeputte, & De Clercq, 1997; Hartline et al., 2005; Hostetler et al., 2006; Lebeau et al., 2007; Snoeck, 2006).

The alkoxyalkyl ester derivatives of CDV showed improved uptake and absorption, and had oral bioavailabilities in mice of 88–97%, compared to less than 5% for CDV. Increased cell penetration (10–20-fold) and higher intracellular levels (100-fold) of CDV-DP (the active form of the

compound) than those measured following treatment of the cells with CDV were confirmed in studies using radiolabeled compound (Aldern, Ciesla, Winegarden, & Hostetler, 2003). *In vivo*, oral administration of the HDP-CDV (Fig. 4.12) proved as effective as the parental compound CDV in the treatment of herpes- and poxvirus infection in several mouse models (Bidanset, Beadle, Wan, Hostetler, & Kern, 2004; Keith et al., 2004; Kern et al., 2004; Quenelle et al., 2004; Wan et al., 2005). Importantly, diminished accumulation of the drug in the kidney was reported according to studies evaluating tissue distribution of radiolabeled HDP-CDV and other alkoxyalkyl esters of CDV in mice (Ciesla et al., 2003; Kern et al., 2004). Thus, these produgs are supposed to avoid the dose-limiting toxicity of CDV.

HDP-CDV (CMX001) (Fig. 4.12), an oral formulation of CDV, is being developed for prevention and treatment of smallpox and for various double-stranded DNA virus infections by Chimerix. A Phase I clinical study to evaluate the safety and pharmacokinetics of orally administered CMX001 in healthy volunteers showed dose-proportional C_{max} and AUC with single doses ranging (Hostetler, 2009; Painter & Hostetler, 2004). Plasma concentrations of CMX001 in normal volunteers were as much as 20-fold greater than those observed in animals at comparable doses per kg of body weight. Currently, Chimerix is conducting a Phase I clinical trial of multidose CMX001 in transplant recipients with polyoma BK viruria and a Phase II clinical trial of CMX001 in immunocompromised patients with HCMV. CDV and CMX001 could be valuable drugs for the treatment of ACV-resistant and/or foscarnet VZV strains in immunosuppressed patients.

A similar prodrug strategy was applied to other ANPs, such as (*S*)-HPMPA, the enhancement of antiviral potency being similar to that of CDV (Beadle et al., 2006; Dal Pozzo et al., 2007; Lebeau et al., 2006; Ruiz et al., 2006, 2007). Thus, the HDP ester of (*S*)-HPMPA showed 40–270-fold increase in anti-CMV activity *in vitro* compared to the parent compound. The HDP derivatives of PME analogues of diaminopurine (PMEDAP) and of *N*6-cyclopropyldiaminopurine (PME-*N*6-cPr-PMEDAP) also showed increased anti-HCMV activity *in vitro* (Hostetler, 2009).

6.6.2 New generations of acyclic nucleoside phosphonates

The discovery of ANPs represented a breakthrough in the treatment of DNA viruses and retroviruses (De Clercq, 2007; De Clercq & Holy, 2005). According to their activity spectrum, the first generation of ANPs can be classified in three categories: (i) the "HPMP" derivatives represented

by HPMPC (CDV), which displays activity against a broad variety of DNA viruses; (ii) the "PME" derivatives represented by PMEA (adefovir) with activity against retro-, hepadna-, and herpesviruses; and (iii) the "PMP" derivatives represented by PMPA (tenofovir) active against retro- and hepadnaviruses. These three compounds have been licensed for the treatment of HCMV retinitis in AIDS patients (CDV, Vistide®), chronic hepatitis B virus infections (adefovir dipivoxil, Hepsera®, the oral prodrug form of PMEA), and HIV infections (tenofovir disoproxil fumarate, Viread®, the oral prodrug form of PMPA). Following the success of this first generation of ANPs, two new generations have been described. The "second generation" ANPs includes the "open ring" or "*O*-linked" ANP analogues or 6-[2-phosphonomethoxyalkoxy]-2,4-diaminopyrimidines (DAPys), which showed substantial potential for the treatment of a broad range of DNA virus and retrovirus infections (Balzarini et al., 2004; De Clercq et al., 2005; Hockova et al., 2003, 2004). HPMPO-DAPy presented a spectrum of activity similar to that of HPMPC except for HCMV that was poorly inhibited. The "third generation" of ANPs encompasses HPMP derivatives with a 5-azacytosine moiety such as HPMP-5-azaC (Fig. 4.12) and its cyclic form (i.e., cHPMP-5-azaC) (Krecmerova, Holy, Piskala, et al., 2007; Krecmerova, Holy, Pohl, et al., 2007). These compounds were at least as potent as the parent compounds against several DNA viruses, including HCMV. The antiviral activity of HPMP-5-azaC was comparable to that of the reference drug (*S*)-HPMPC against HSV-1, HSV-2, and vaccinia virus, or two- to sevenfold more active against VZV, HCMV, HHV-6, and adenovirus 2. HPMP-5-azaC proved to be two-fold less cytotoxic for human embryonic lung fibroblasts than (*S*)-HPMPC [CC_{50} 140 μg/mL for HPMP-5-azaC compared to 61 μg/mL for (*S*)-HPMPC], but two-fold more toxic for human T-lymphoblast HSB-2 cells [CC_{50} 16 μg/mL for HPMP-5-azaC compared to 37 μg/mL for (*S*)-HPMPC]. For all these DNA viruses, HPMP-5-azaC showed a 2–16-fold higher antiviral SI than (*S*)-HPMPC. The spectrum of activity of cHPMP-5-azaC was comparable to that of HPMP-5-azaC and the reference compounds HPMPC and cHPMPC. The cyclic form of HPMP-5-azaC did not affect cell morphology or cell growth of HEL cells up to a concentration of 100 μg/mL. This resulted in selectivity indices varying from >47 (vaccinia virus) to >1500 (HCMV). Furthermore, HPMPC and HPMP-5-azaC proved equally potent in pathogenic models of HSV and poxvirus infections in mice. Among several prodrugs of cHPMP-5-azaC synthesized, the hexadecyloxyethyl ester (Fig. 4.12) proved to be about 250-fold more active than the parent compound (Krecmerova, Holy, Pohl, et al., 2007). Not only was

an improvement in the antiviral activity observed for the hexadecyloxyethyl ester of cHPMP-5-azaC but also an increase in selectivity. Thus, selectivity indices for this ester were 43 (HHV-6A), 70 (HHV-6B), 173 (vaccinia virus), 1160 (HSV), ≥5800 (VZV), and ≥24,600 (HCMV). Recently, the potent and selective activity of HPMP-5-azaC, its cyclic form, and the hexadecyloxyethyl ester derivative of cHPMP-5-azaC was shown against polyomavirus BK in human renal cells (Topalis, Lebeau, Krecmerova, Andrei, & Snoeck, 2011). Further studies are needed to determine the clinical potential of these compounds to become new drug candidates to treat VZV infections.

6.7. Drugs that target cellular proteins

As an alternative approach to traditional antiviral agents that are designed to target specific viral proteins, cellular proteins that are essential for viral replication may serve as novel targets to specifically inhibit virus replication (Schang, 2002). Classical antiviral agents are active against a narrow spectrum of viruses and development of resistance has been considered a hallmark of the specificity of these antiviral drugs. On the contrary, antivirals that target cellular proteins indispensable for viral replication are expected to be active against a broader range of viruses because several unrelated viruses may require the same cellular proteins. Also, antivirals that target cellular events might select for drug-resistant virus mutants less rapidly than antivirals that target viral proteins. Furthermore, they should retain activity against viral mutants resistant to conventional antiviral agents. However, one of the limitations of targeting cellular proteins might be the risks for increased cytotoxicity and undesirable side effects.

Many viruses such as papillomaviruses, polyomaviruses, adenoviruses, poxviruses, herpesviruses, and HIV require cyclin-dependent kinases (CDKs) for their replication. HCMV has a dramatic stimulatory impact on permissive cells and is also associated with dysregulation of host cell cyclin levels and disruption of cell cycle progression. The upregulation of CDK activity during the infection implies that the virus is dependent on these enzymes for viral transcription, replication, and/or assembly of virus particles. Roscovitine (a reversible inhibitor of CDK-1, -2, -5, -7, and -9) and other CDK inhibitors such as olomoucine and flavopiridol (Fig. 4.14) are able to inhibit herpesviruses, as well as other DNA viruses and retroviruses, underlying the importance of host cell protein kinases in viral replication (Guendel, Agbottah, Kehn-Hall, & Kashanchi, 2010; Orba et al., 2008; Schang, St Vincent, & Lacasse, 2006). In the past years, the activity of

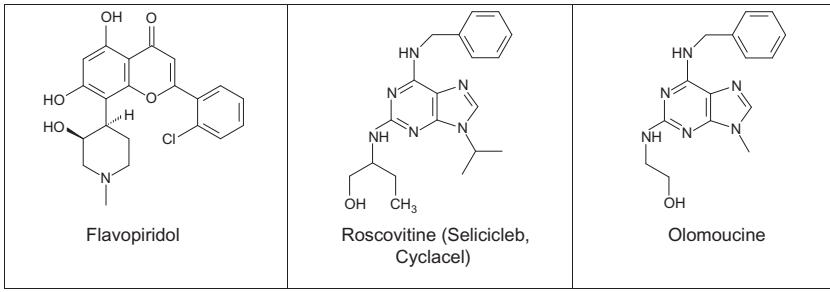


Figure 4.14 Drugs that target cellular proteins.

CDK inhibitors has been assessed *in vivo* (Schang, 2006). Using mouse models, Yang and colleagues (Yang et al., 2005) and Reeves and colleagues (Reeves et al., 2005) demonstrated that imatinib (inhibitor of c-abl tyrosine kinase) and CI-1033 (inhibitor of EGFR tyrosine kinase) were able to hamper poxvirus pathogenesis. CDK inhibitors were also able to prevent VZV replication in culture, *ex vivo*, and in SCID-Hu mice (Rowe, Greenblatt, Liu, & Moffat, 2010). Larger clinical studies are needed to determine the potential of CDK inhibitors in the treatment of viral diseases since concerns exist on the potential toxicity of CDK inhibitors.

6.8. Immunomodulators

Antimicrobial peptides are known to have an important role in cutaneous defense. Crack and colleagues demonstrated that human β-defensin-2 and human cathelicidin LL-37 had an antiviral effect on VZV replication in keratinocytes (and B cells) as evidenced by reduction in viral load (Crack, Jones, Malavige, Patel, & Ogg, 2012). Interestingly, significant inhibition of viral replication was not seen until day 10 postinfection. A crucial difference between these two antimicrobial peptides was that preincubation of VZV with LL-37 but not with β-defensin-2 further reduced viral load. The mechanism of action of antimicrobial peptides is based either on the disruption of the viral envelope or on the interaction with viral glycoproteins. Elucidation of the morphological changes following exposure of VZV to antimicrobial peptides is necessary to determine whether these peptides directly disrupt the viral envelope.

7. PERSPECTIVES

The major development issue for a new VZV inhibitor is the lack of a good animal model to study virus pathogenesis and efficacy of antiviral

agents because of the host specificity of VZV. Although experimental animal models of latency and pathogenesis exist for closely related viruses, such as HSV-1 and HSV-2, VZV causes disease exclusively in humans. Several attempts to produce disease by experimental inoculation of animals have led to seroconversion without clinical symptoms. Therefore, preclinical development of potential drug candidates is very limited. Novel inhibitors can be only tested in *ex vivo* models such as organotypic epithelial raft cultures or organ cultures and in SCID-Hu mice. The use of this model is also hindered by the fact that these are immunocompromised animals and the model does not reproduce the type of VZV diseases observed in humans. The lack of an *in vivo* model for VZV appears to be particularly difficult in cases of compounds that specifically inhibit VZV and not other herpesviruses (such as HSV or SVV) for which animal models exist. SVV is a simian counterpart of VZV that infects nonhuman primates. A study has shown for the first time that infection of rhesus macaques with SVV recapitulates key aspects of VZV infection from chickenpox and the resolution of acute viremia to the establishment of latency in ganglia, and the development of B and T cell responses to virus (Messaoudi et al., 2009). This model may be of importance to improve our understanding of the immune response to VZV, and to develop and test vaccines and antiviral drugs.

When considering the development of new anti-VZV compounds, it is important to keep in mind that the potential drug candidate will need to be at least as effective as the standard of treatment VACV for herpes zoster, present an acceptable safe profile since ACV has been used for more than 25 years and has a very safe profile. The new drug candidate will also have to possess some advantages over ACV and VACV, such as longer intracellular half-life allowing once-a-day dosing, superior efficacy, independence of TK for activation or target another enzyme than the DNA polymerase. Host targets may also be attractive but the major obstacle for their development will be the selectivity for virally infected cells.

Antiviral agents will still have a role in the treatment of VZV-associated diseases even after the widespread implementation of vaccination programs for both chickenpox and herpes zoster. Treatment with currently approved antiviral agents (i.e., ACV, now replaced by VACV, and FAM) is recommended for VZV-associated infections in immunocompromised individuals, where VZV infections can be very severe. Among immunocompetent patients, antiviral therapy is recommended for adolescents and adults suffering from varicella and for patients (especially those older than 50 years) with herpes zoster. It should be noted that antiviral therapy will also be necessary to

treat rare but important side effects caused by VZV vaccine. Novel antiviral chemotherapeutics with different mode of action than the current available anti-VZV drugs are required for the treatment of ACV-resistant strains in the immunocompromised host. Furthermore, compounds with a better activity than the currently approved drugs will be extremely useful if they are able to shorten the time to complete crusting of the shingles rash and the time to complete pain resolution and prevention of PHN. It should be kept in mind that new anti-VZV drugs should be as safe as and more effective than ACV to be able to replace ACV (and its prodrug VACV) as the gold standard for the treatment of VZV infections.

8. CONCLUSION

Despite the availability of a live-attenuated vaccine for the prevention of pediatric varicella in children (Varivax) and for the prevention of herpes zoster in adults (Zostavax), there will continue to be a need for antiviral drugs. Some elderly people are not able to mount a strong response to the vaccine. In immunocompromised persons, including patients with advanced HIV infection, varicella vaccination is currently contraindicated for fear of disseminated vaccine-induced disease. On the other hand, the vaccine coverage is still quite limited and childhood immunization with lower coverage could theoretically shift the epidemiology of the disease and increase the number of severe cases in older children and adults.

Indeed, infections with VZV are a serious cause of morbidity and mortality among immunosuppressed patients and in the elderly, PHN (the major complication of herpes zoster) can be very difficult to manage. Existing antiviral agents shorten the duration of herpes zoster and promote rash healing, but they are not totally effective in preventing PHN, probably because of the delay between diagnosis and the start of antiviral treatment. New antiviral chemotherapeutics with a different mechanism of action are under development for the management of herpes zoster, including the bicyclic nucleoside analogue FV-100, the HPI ASP2151, and the carbocyclic nucleoside analogue valomaciclovir. Although the development of ASP2151 has been halted due to problems of toxicity, the results of Phase II trials with FV-100 and valomaciclovir demonstrated the potential of these drugs as effective, well-tolerated, once-daily therapy for the treatment of herpes zoster. Further studies are needed to prove an advantage of FV-100 and/or valomaciclovir over the standard of care VACV. The development of other HPI against VZV would be of value due to their capacity to inhibit ACV-resistant strains.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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CHAPTER FIVE

Hepatitis C Virus: Standard-of-Care Treatment

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Contents

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Abstract

Hepatitis C virus (HCV) infection is curable by therapy. The antiviral treatment of chronic hepatitis C has been based for decades on the use of interferon (IFN)-α, combined with ribavirin. More recently, new therapeutic approaches that target essential components of the HCV life cycle have been developed, including direct-acting antiviral (DAA) and host-targeted agents (HTA). A new standard-of-care treatment has been approved in 2011 for patients infected with HCV genotype 1, based on a triple combination of pegylated IFN-α, ribavirin, and either telaprevir or boceprevir, two inhibitors of the HCV protease. New triple and quadruple combination therapies including pegylated IFN-α, ribavirin, and one or two DAAs/HTAs, respectively, are currently being evaluated in Phase II and III clinical trials. In addition, various options for all-oral, IFN-free regimens are currently being evaluated. This chapter describes the characteristics of the different drugs used in the treatment of chronic hepatitis C and those currently in development and provides an overview of the current and future standard-of-care treatments of chronic hepatitis C.

ABBREVIATIONS

2′,5′OAS 2′,5′-oligoadenylate synthetase
2-5A 2′,5′-oligoadenylate
5′UTR 5′-untranslated region
ADAR1 RNA-specific adenosine deaminase 1
AUC area under the time–concentration curve
DAA direct-acting antiviral
DRESS drug reaction with eosinophilia with systemic symptoms
DVR delayed virological response
eIF2α eukaryotic translation initiation factor 2α
EVR early virological response
HCV hepatitis C virus
HTA host-targeted agent
IFN interferon
IFNAR IFN-α receptor
IFNGR IFN-γ receptor
IFNLR IFN-λ receptor
IL interleukin
IL-10R2 IL-10 receptor-2
IMPDH inosine monophosphate dehydrogenase
IRES internal ribosome entry site
ISG interferon-stimulated gene
ISG20 20-kDa ISG product
ISGF3 IFN-stimulated gene factor 3
ISRE IFN-stimulated response element
IU international unit
Mx myxovirus
NS nonstructural

PEG polyethylene glycol
PKR double-stranded RNA-activated protein kinase
RdRp RNA-dependent RNA polymerase
RVR rapid virological response
SCID severe combined immunodeficiency
Stat signal transducer and activator of transcription
SVR sustained virological response
uPA urokinase-type plasminogen activator

1. INTRODUCTION

Approximately 120–130 million individuals are chronically infected by hepatitis C virus (HCV) worldwide. Acute infection becomes chronic in approximately 80% of cases, and patients with chronic hepatitis C are at high risk of life-threatening complications, including cirrhosis in 20% of cases and hepatocellular carcinoma (primary liver cancer) at an incidence of 4–5% per year in cirrhotic patients. In the Western world, HCV infection is the first cause of chronic liver disease, the first indication of liver transplantation, and it is becoming the first etiology of hepatocellular carcinoma. Thus, HCV infection represents a major public health burden.

In contrast to other chronic viral infections in man, HCV infection is curable by therapy. After antiviral treatment, the sustained virological response (SVR), defined as an undetectable HCV RNA 12 (SVR12) or 24 weeks (SVR24) after the end of therapy, has been shown to correspond to a definitive clearance of infectious HCV from the patient's body, with no risk of early or late recurrence, even in case of immune suppression (Swain et al., 2010). The antiviral treatment of chronic hepatitis C has been based for decades on the use of interferon (IFN)-α. IFN-α was initially used alone, three times per week subcutaneously. Tremendous progress has been made with the introduction of pegylated forms of IFN-α that can be administered once a week and with the coadministration of ribavirin, the precise mechanism of action of which in combination with IFN-α remains elusive. More recently, better understanding of the viral life cycle and development of study models have made it possible to develop new therapeutic approaches that target essential components of the HCV life cycle. These new drugs include direct-acting antiviral (DAA) agents that specifically block a viral enzyme or functional protein, and host-targeted agents (HTA) that block interactions between host proteins and viral components that are essential to the viral life cycle.

A new standard-of-care treatment was approved in 2011 for patients infected with HCV genotype 1, the most prevalent HCV genotype worldwide. It is based on a triple combination of pegylated IFN-α, ribavirin, and either telaprevir or boceprevir, two DAA inhibitors of the HCV nonstructural 3-4A (NS3-4A) protease. This new treatment yields improved HCV infection cure rates, but at the cost of frequent side effects and drug–drug interactions. New triple and quadruple combination therapies including pegylated IFN-α, ribavirin, and one or two DAAs/HTAs, respectively, are currently being evaluated in Phase II and III clinical trials. They could reach the market soon. However, the proof-of-concept that IFN is not absolutely required to achieve high cure rates has been recently brought. Various options for all-oral, IFN-free regimens are currently being evaluated in a number of Phase II and III clinical trials and will likely become the basis of HCV therapy within the next 2–5 years.

This chapter describes the characteristics of the different drugs used in the treatment of chronic hepatitis C and those currently in development and provides an overview of the current and future standard-of-care treatments of chronic hepatitis C.

2. CURING HCV INFECTION

In order for antiviral therapy to definitively cure HCV infection, three steps are required (Neumann et al., 1998). Indeed, it is necessary (i) to shut down virus production to achieve a rapid initial reduction of circulating HCV RNA levels (first-phase decline); (ii) to maintain viral inhibition throughout treatment, that is, to prevent viral breakthrough due to the selection of resistant viral variants or to poor adherence to therapy; (iii) to induce a significant, slower second-phase decline in HCV RNA level, resulting in gradual clearance of HCV-infected liver cells through cell death or, more often, HCV removal from infected cells (Dahari, Guedj, Perelson, & Layden, 2011). The latter is due to restoration of the cellular innate immune response as a result of the inhibition of viral protein production. An SVR can be achieved only if the second-phase decline is gradual and treatment is administered for a sufficient duration, ensuring that every infected cell has been cleared or cured when it is stopped.

3. INTERFERONS

IFNs were discovered in 1957 by Isaacs and Lindenmann (Isaacs & Lindenmann, 1957). They are natural glycoproteins produced by the cells of most vertebrates in response to challenge by foreign agents, such as infectious organisms, and by tumor cells. IFNs can be produced by cells of the innate and adaptive immune systems and by nonimmune cells such as fibroblasts and epithelial cells (Chevaliez & Pawlotsky, 2009).

3.1. The different types of IFNs

The three major classes of human IFNs are type I IFNs that bind to a receptor complex that consists of two subunits, IFN-α receptor-1 (IFNAR-1) and IFNAR-2 (Mogensen, Lewerenz, Reboul, Lutfalla, & Uze, 1999); type II IFN that binds to a receptor complex composed of two chains, IFN-γ receptor-1 (IFNGR-1) and IFNGR-2 (Bach, Aguet, & Schreiber, 1997); and type III IFNs that bind to a specific receptor complex composed of IFN-λ receptor-1 (IFNLR-1) and the interleukin-10 (IL-10) receptor-2 (IL-10R2) chain (Dumoutier, Lejeune, Hor, Fickenscher, & Renauld, 2003; Kotenko et al., 2003; Sheppard et al., 2003).

3.1.1 Type I IFNs

Type I IFNs form a superfamily of innate cytokines that comprise IFN-α (13 subtypes), IFN-β, IFN-ω, IFN-κ, and IFN-ε (Pestka, Krause, & Walter, 2004). All IFN-α subtypes are secreted by leukocytes, whereas IFN-β is also produced by fibroblasts. Type I IFNs bind to their specific receptor complex composed of IFNAR-1 and IFNAR-2, which are intimately associated with two Janus family tyrosine kinases, Tyk2 and Jak1, both located on the cytoplasmic side of the cell membrane (Domanski & Colamonici, 1996). Type I IFN interaction with the specific receptor complex activates a transcription factor, IFN-stimulated gene factor 3 (ISGF3), which induces the expression of IFN-stimulated genes (ISGs) (van Boxel-Dezaire, Rani, & Stark, 2006). Different receptor subunit affinities and interaction kinetics lead to differential signal activation, thereby explaining the different biological activities of type I IFNs (Jaks, Gavutis, Uze, Martal, & Piehler, 2007).

3.1.2 Type II IFNs

There is only one known type II IFN, IFN-γ, discovered in 1965 (Wheelock & Sibley, 1965). IFN-γ is exclusively produced by immune cells,

such as activated thymus-derived T cells and natural killer cells, after stimulation by foreign antigens or mitogens in the early stages of the innate immune response (Boehm, Klamp, Groot, & Howard, 1997). The type II IFN receptor that consists of two subunits, IFNGR1 and IFNGR2, is primarily required for signaling. Each α-chain is constitutively associated with Janus kinases Jak1 and Jak2, and IFN-γ binding to the two-α-chain receptor complex leads to transphosphorylation and reciprocal activation of the Jak proteins (Bach et al., 1997).

3.1.3 Type III IFNs

Recently, a novel class of type I-like human IFN, named IFN-λ1 or IL-29, IFN-λ2 (IL-28A), and IFN-λ3 (IL-28B), was identified (Dumoutier et al., 2003; Sheppard et al., 2003). The type III IFN receptor is composed of two chains, IFNLR-1 (specific for IFN-λ) and IL10R-2 (shared with the IL-10, IL-22, and IL-26 receptors). The formation of a ternary complex including IFN-λ, IFNLR-1, and IL10R-2 is necessary to trigger the transduction signal. As with the type I IFN receptor, Tyk2 and Jak1 are the two receptor-associated tyrosine kinases that mediate activation of the signal transducer and activator of transcription (Stat) proteins (Ank, West, & Paludan, 2006; Uze & Monneron, 2007). A novel protein, IFN-λ4, was recently identified, the expression of which seems to be related to the ability of patients with HCV infection to cure acute infection or eliminate chronic infection on antiviral therapy. IFN-λ4 is related to, but distinct from, IFN-λ3 and is able to induce ISGs (Prokunina-Olsson et al., 2013).

3.2. Mechanisms of antiviral action of IFNs in HCV infection

So far, only type I and III IFNs have been used for the treatment of chronic hepatitis C. The vast majority of patients have been treated with IFN-α, with two molecules approved for HCV treatment worldwide. A few clinical studies assessed the antiviral efficacy of IFN-β and, more recently, IFN-λ.

3.2.1 Responses to type I and III IFNs

The transcriptional responses induced by type I and type III IFNs are similar (Doyle et al., 2006; Dumoutier et al., 2004). On binding to its receptor, the IFN molecule is sandwiched between the two chains of the receptor, thus forming a ternary complex that activates the canonical Jak/Stat pathway through activation of the transduction elements located in the intracytoplasmic tail of each receptor subunit. Jak1 and Tyk2 are activated through tyrosine transphosphorylation. The activated kinases induce the

formation of the ISGF3, itself composed of two elements, ISGF3α and ISGF3γ. ISGF3α is formed of two cytoplasmic peptides, Stat1 and Stat2. ISFG3γ is a member of the IRF family that has been renamed IRF-9. It comprises two domains: a conserved amino-terminal DNA-binding domain and a carboxy-terminal Stat-binding domain. IRF-9 is mostly cytoplasmic and inactive in untreated cells. It migrates to the nucleus after IFN treatment.

The phosphotyrosyl residues of activated Jak proteins serve as docking sites for Stat1 and Stat2, both of which are phosphorylated by activated Jak proteins. Stat1 and Stat2 phosphorylation induces their release from the IFNARs and their dimerization. Stat1–Stat2 heterodimers then move to the nucleus to form the ISGF3, together with IRF-9. ISGF3 then interacts directly with the IFN-stimulated response element (ISRE), a DNA sequence that characterizes ISGs. ISGs may contain one or several ISRE sequences in their promoter sequence.

3.2.2 IFN antiviral end-effectors

The cellular actions of the different types of IFNs are mediated by various IFN-induced proteins. The best-known antiviral effectors produced as a result of IFN cascade induction include 2′,5′-oligoadenylate synthetase (2′,5′OAS), double-stranded RNA-activated protein kinase (PKR) and myxovirus (Mx) proteins. Other, minor effectors include RNA-specific adenosine deaminase 1 (ADAR1), 20-kDa ISG product (ISG20), ISG54, and ISG56.

3.2.2.1 2′,5′-Oligoadenylate synthetase

Three forms of 2′,5′OAS exist in humans, namely, OAS1, OAS2, and OAS3. They comprise one, two, or three OAS units, respectively, that share substantial sequence homology. Each form can be induced by INF-α, IFN-β, and IFN-γ. However, the three OAS forms are associated with different subcellular fractions and their expression is regulated by cell-specific factors. When activated, OAS1 and OAS2 mediate cell resistance to viral infections via the production of 2′,5′-oligoadenylate (2-5A) that consists of a mixture of 5′-triphosphorylated oligoadenylates with 2′,5′-phosphodiester bonds. The only known function of 2-5A is to activate a latent endoribonuclease, RNAse L, which degrades viral and cellular mRNAs and rRNAs (Hovanessian & Justesen, 2007; Hovanessian & Wood, 1980).

RNAse L is composed of three domains: an N-terminal ankyrin repeat domain with nine complete or partial ankyrin motifs, some of which are essential for 2-5A binding; a protein kinase homology domain; and a

C-terminal ribonuclease domain (Tanaka et al., 2004; Zhou, Hassel, & Silverman, 1993). 2-5A binding induces a conformational change in the ankyrin domain that unmasks the C-terminal ribonuclease domain and leads to homodimerization of RNAse L and activation of its nuclease activity. RNAse L plays an important role in the antiviral and antiproliferative activities of IFNs by regulating viral and cellular RNA expression.

3.2.2.2 Double-stranded RNA-activated protein kinase

PKR is a serine–threonine kinase composed of three domains: a kinase domain common to the other members of the family and two dsRNA-binding domains connected by a linker that regulate the enzyme's activity. PKR is activated by intracellular dsRNAs more than 30 nucleotides long. dsRNA binding to PKR induces its activation through homodimerization and its autophosphorylation; this further stabilizes PKR dimerization and in turn increases the catalytic activity of the kinase. Activated PKR phosphorylates eukaryotic translation initiation factor 2α (eIF2α). This prevents the eIF2α recycling required for ongoing translation, thus leading to a general inhibition of translation, the basic mechanism by which PKR exerts its antiviral activity.

3.2.2.3 Myxovirus proteins

Mx proteins are IFN-induced GTPases (Haller & Kochs, 2002). In humans, two distinct Mx GTPases have been identified, MxA and MxB, both of which are localized in the cytoplasm. Mx proteins comprise both a highly conserved amino terminus that constitutes the GTPase domain and is a dynamin family signature, and a less conserved carboxy terminus that contains a leucine zipper motif and serves as the effector domain. Important functional domains, including a GTP-binding sequence, a central interactive domain, and the leucine zipper motif, form a "self-assembling sequence" essential for GTPase activity and antiviral specificity. Only MxA has antiviral activity against a broad spectrum of viruses, irrespective of their intracellular replication site. Its antiviral properties appear to be due to blockade of intracellular transport of viral components that have been trapped by MxA in infected cells (Haller & Kochs, 2002).

3.2.2.4 Adenosine deaminase acting on RNA 1

The ADAR family comprises three members, named ADAR1, ADAR2, and ADAR3. ADAR proteins are involved in RNA editing, substituting adenosines by inosines in cellular mRNA and viral dsRNA targets. The

inosine residues are transcribed and translated into guanosine residues (Bass, 1997; Wang, Khillan, Gadue, & Nishikura, 2000). ADAR1 appears to exert potent antiviral properties through adenosine to inosine editing (Taylor, Puig, Darnell, Mihalik, & Feinstone, 2005).

3.2.2.5 IFN-stimulated gene product of 20 kDa (ISG20)

ISG20 is a member of the DEDD exonuclease superfamily, which has both RNAse and DNAse activities (Horio et al., 2004). ISG20 expression is strongly induced by the activation of TLR receptors recognizing CpG motifs or dsRNAs, independently of IFN secretion, but also by IFNs, thereby exerting potent antiviral activities, with a preference for RNA viruses (Degols, Eldin, & Mechti, 2007; Espert et al., 2003). Recent reports suggest that ISG20 may play a role in RNA processing because of its presence at the major site of early rRNA processing in the nucleus (Espert et al., 2006; van Hoof, Lennertz, & Parker, 2000).

3.2.2.6 IFN-stimulated gene products of 54 (ISG54) and 56 kDa (ISG56)

ISG54 and ISG56 are tetratricopeptide proteins encoded by two closely related IFN-induced genes. Both proteins bind to the translation initiation factor eIF3 and inhibit translation by inhibiting eIF3-mediated stabilization of the eIF2–GTP–Met-tRNA ternary complex. In addition, ISG54 is able to inhibit the formation of the 48S preinitiation complex between the 40S ribosomal subunit and the 20S complex composed of eIF3, the ternary complex eIF4F, and mRNA (Terenzi, Hui, Merrick, & Sen, 2006).

3.2.3 Immunomodulatory properties of IFNs

In addition to their antiviral properties described above, IFNs exhibit potent immunomodulatory properties. They can stimulate the effector function of natural killer cells, cytotoxic T lymphocytes, and macrophages, upregulate the expression of major histocompatibility complex class I and class II molecules, induce immunoglobulin synthesis by B cells, and stimulate the proliferation of memory T cells. However, these properties do not appear to play any role in HCV clearance on IFN-based therapy, which is primarily due to the antiviral properties of the drug.

3.3. Effects of IFNs on HCV models

Type I IFNs, especially IFN-α, have been shown to inhibit HCV replication in various productive and nonproductive cellular and animal models.

3.3.1 Cellular models

IFN-α significantly inhibits HCV replication in subgenomic and genomic replicons in the Huh7 hepatoma cell line (Frese, Pietschmann, Moradpour, Haller, & Bartenschlager, 2001; Guo, Bichko, & Seeger, 2001; Guo, Zhu, & Seeger, 2003; Lanford et al., 2003). This inhibition is related to the induction of multiple IFN-dependent pathways. It is concentration dependent, unrelated to IFN toxicity, and has been observed with several HCV genotypes (Miyamoto, Kato, Date, Mizokami, & Wakita, 2006). IFN-α resistance has been observed and is attributed to cellular factors (intracellular signaling) rather than to intrinsic properties of the viral replicons (Naka et al., 2005; Zhu, Nelson, Crawford, & Liu, 2005). HCV subgenomic replicon replication can also be inhibited by IFN-λ, although through signaling pathways different from those induced by IFN-α (Marcello et al., 2006; Robek, Boyd, & Chisari, 2005).

IFN-α was also reported to inhibit the replication of various chimeric forms of the Japanese HCV strain JFH1 that can generate infectious viral particles after transfection of Huh7 cells (Kim, Jung, Wakita, Yoon, & Jang, 2007; Lindenbach et al., 2005; Sakamoto et al., 2007; Targett-Adams & McLauchlan, 2005; Wakita et al., 2005; Zhong et al., 2005). This inhibition is concentration dependent (Targett-Adams & McLauchlan, 2005) and different infected cells may respond differently to IFN-α (Kim et al., 2007). Finally, we have shown that IFN-α potently inhibits HCV replication in primary human hepatocytes infected by incubation with infectious human plasma, the model closest to natural HCV infection (Castet et al., 2002).

3.3.2 Animal models

Although human IFN-α is able to induce IFN responses in the liver of HCV-infected chimpanzees, neither IFN-α administration (with or without ribavirin) nor adenovirus-based gene therapy inducing high-level hepatic IFN-α expression has been shown to reduce HCV RNA levels in experimentally infected chimpanzees (Demers et al., 2002; Lanford et al., 2006).

Mouse models have been developed to assess drug efficacy on HCV infection. They include a humanized mouse line generated by engrafting human hepatocytes into chimeric urokinase-type plasminogen activator (uPA)–severe combined immunodeficiency (SCID) mice. This model can be infected with HCV-containing human plasma and serum, with cell culture-produced virus, and also by intrahepatic injection of transcribed RNA (Hiraga et al., 2007; Mercer et al., 2001). HCV replication levels are similar to or slightly lower than those observed during human infection.

Another xenograft model has been developed from gamma-irradiated SCID mice implanted with a mouse-adapted luciferase replicon-containing Huh7 cell line. In this model, HCV replication is monitored *in vivo* by real-time, noninvasive whole-body imaging (Zhu et al., 2006). IFN-α significantly inhibits HCV replication in both these *in vivo* models (Kneteman et al., 2006; Zhu et al., 2006).

3.4. Therapeutic forms of IFN

3.4.1 Standard IFN-α

Different forms of IFN-α have been available for the treatment of chronic hepatitis B and C, including IFN-α2a and IFN-α2b. The administered dose was of 3 to 5 million units three times a week subcutaneously. Standard IFN-α is no longer used for the treatment of chronic hepatitis C because of its inadequate pharmacokinetic profile.

3.4.2 Pegylated IFN-α

Polyethylene glycol (PEG) is formed by linking repeating units of ethylene glycol to form polymers that are linear or branched molecules with different masses. Pegylation is the process by which PEG chains are covalently attached to IFN molecules. Pegylation confers a number of properties on IFN molecules, such as sustained blood levels that enhance antiviral effectiveness and reduce adverse reactions, as well as a longer half-life and improved patient convenience (Kozlowski, Charles, & Harris, 2001).

Two forms of pegylated IFN-α have been approved by the Food and Drug Administration and by the European Medicines Agency for the treatment of chronic hepatitis C in adults, namely, pegylated IFN-α2a and pegylated IFN-α2b, in which the IFN-α molecules are linked to PEG molecules of different sizes. More recently, a pegylated formulation of IFN-λ has been developed, which is under clinical evaluation for the treatment of HCV infection.

3.4.2.1 Pegylated IFN-α2a

Pegylated IFN-α2a (Pegasys®, Roche) is a 40-kDa branched monomethoxy PEG conjugate of IFN-α2a, consisting of four major positional isomers (Bailon et al., 2001). The pegylated IFN-α2a molecule contains a large, branched PEG moiety attached at a single point to the native protein by means of a hydrolytically stable amide bridge. There are four main potential IFN-α2a pegylation sites, at lysine positions 31, 121, 131, and 134. The molecule is very stable. However, at least *in vitro*, the specific antiviral activity of

Table 5.1 Pharmacological parameters of pegylated IFN-α molecules used in the treatment of chronic hepatitis C

	Pegylated IFN-α2a	**Pegylated IFN-α2b**
Chemical parameters		
Molecular weight (kDa)	60	31
PEG structure	Branched	Linear
Production	Recombinant DNA technology in *E. coli*	
Pharmacokinetic parameters		
C_{max}	Dose related	Dose related
T_{max} (h)	72–96	15–44
Half-life (h)	80[a]	40[a]
Apparent volume of distribution (L)	6–14	69
Clearance	Principally renal	Not fully elucidated
Posology and administration		
Approved dose	180 μg qw	1.5 μg/kg qw
Route of administration	Subcutaneous	Subcutaneous

[a]After subcutaneous injection.

the 40-kDa branched pegylated IFN-α2a molecule is only 7% that of the nonpegylated molecule (Bailon et al., 2001).

The pharmacokinetics of pegylated IFN-α2a have been studied in healthy volunteers after a single subcutaneous injection. In comparison with standard IFN-α2a, the pegylated form shows sustained absorption, reduced systemic clearance, and a longer half-life (Table 5.1). High serum levels of pegylated IFN-α2a are reached 3–8 h after subcutaneous administration and the maximum serum concentration is reached approximately 80 h post-injection (Reddy, Modi, & Pedder, 2002). The steady-state serum concentration is reached within 5–8 h after weekly subcutaneous administrations and a uniform drug concentration is present throughout the dosing interval. The pharmacokinetic properties of pegylated IFN-α2a appear to be similar in patients with chronic hepatitis C (with and without cirrhosis) and in healthy volunteers (Reddy et al., 2002).

In rats, pegylated IFN-α2a is distributed to both the liver and kidney, whereas standard IFN-α localizes principally in the kidney. Pegylated IFN-α2a requires hepatic metabolism by nonspecific proteases. Active renal

excretion is limited (<5%). Thus, pegylated IFN-α2a has significantly lower renal clearance than standard IFN-α and this prolongs antiviral activity (Reddy et al., 2002). OAS activity is induced within 48 h after pegylated IFN-α2a administration, and values are higher than with standard IFN-α 2a and are maintained for about a week.

3.4.2.2 Pegylated IFN-α2b

Pegylated IFN-α2b (PegIntron®, Merck) is a linear, 12-kDa monomethoxy PEG conjugate of IFN-α2b (Grace et al., 2001; Wang, Youngster, et al., 2000). The pegylated IFN-α2b molecule is essentially monopegylated (95%) and 50% of the monopegylated isomers are linked through the histidine residue at position 34 (Grace et al., 2001). The *in vitro* antiviral activity of the 12-kDa linear pegylated IFN-α2b molecule is 28% that of the non-pegylated molecule (Foser et al., 2003).

When compared with standard IFN-α2b, pegylated IFN-α2b also has sustained absorption, reduced systemic clearance, and an increased half-life (Table 5.1). After subcutaneous injection, the maximal serum concentration of pegylated IFN-α2b occurs at 15–44 h and lasts for 48–72 h. It is followed by a slow elimination phase. The maximum concentration (C_{max}) of pegylated IFN-α2b and the area under the time–concentration curve (AUC) increase in a dose-related manner (Glue et al., 2000). Pegylated IFN-α2b is excreted in urine. After initial dosing, pegylated IFN-α2b produces a dose-unrelated increase in the serum 2′,5′OAS concentration (Glue et al., 2000).

3.4.2.3 Pegylated IFN-λ

Pegylated IFN-λ (Bristol–Myers Squibb) is a conjugate of a recombinant form of human IFN-λ1 and a 20-kDa linear PEG chain, currently under clinical development for the treatment of chronic HCV infection. As a member of the type III IFN family, IFN-λ binds to a receptor complex with more restricted distribution than the receptor for type I IFN, thus having the potential for similar efficacy to other IFNs with a more favorable tolerability profile.

The estimated half-life of pegylated IFN-λ is 50–80 h and the time to the maximum concentration is 8–24 h. Pegylated IFN-λ demonstrated dose-dependent biological activity with the induction of increases in serum β2-microglobulin that correlated with antiviral efficacy (Muir et al., 2010).

4. RIBAVIRIN

4.1. General properties

Ribavirin is a guanosine analogue with a broad spectrum of activity against DNA and RNA viruses (Sidwell et al., 1972). Ribavirin modestly and transiently inhibits HCV replication *in vivo* (Pawlotsky et al., 2004). Its mode of action in chronic hepatitis C is to efficiently prevent relapses during IFN–ribavirin combination therapy (Bronowicki et al., 2006). This effect is the result of ribavirin-induced acceleration of the second-phase decline of viral replication, through molecular mechanisms that remain unknown. As a result, the addition of ribavirin increased the SVR rate to 41% and 43%, respectively, compared to 16% and 19% with standard IFN-α2a and IFN-α2b monotherapy (McHutchison et al., 1998; Poynard et al., 1998). Similarly, in patients without cirrhosis, pegylated IFN-α plus ribavirin gave global SVR rates of 54–56%, compared to 18–39% with pegylated IFN-α monotherapy (Fried et al., 2002; Hadziyannis et al., 2004; Manns et al., 2001). More recent studies indicated that ribavirin exerts the same effect on the second-phase decline of HCV RNA levels and prevention of relapses in patients receiving DAAs without IFN (Zeuzem, Buggisch, et al., 2012).

4.2. Pharmacological properties

Ribavirin is administered orally at a dose of 0.8–1.4 g/day, depending on body weight and the HCV genotype (AASLD, 2002). Higher doses may be necessary for heavy patients. Interestingly, in patients infected with HCV genotype 1, the probability of an SVR increases with the area under the curve of ribavirin concentrations (Snoeck, Wade, Duff, Lamb, & Jorga, 2006). In addition, the proportion of patients achieving an SVR is higher with a standard weight-based dose of ribavirin than with a low dose (Jensen et al., 2006).

Ribavirin is absorbed intestinally and then circulates with a bioavailability of approximately 50%. The steady-state plasma concentration is achieved in approximately 4 weeks (Glue, 1999; Preston et al., 1999). Nucleotide transporters are involved in ribavirin penetration into cells (Kong, Engel, & Wang, 2004). Ribavirin is activated by intracellular phosphorylation into its triphosphate form, which appears to be the active form of the molecule. It is finally excreted in urine.

Ribavirin accumulates in erythrocytes. Thus, hemolytic anemia is the most frequent adverse effect of ribavirin administration, and this side effect is dose dependent and amplified in patients who also receive IFN-α (Fried et al., 2002; Hadziyannis et al., 2004; Manns et al., 2001).

4.3. Mechanisms of ribavirin action in HCV therapy

Ribavirin primarily acts by accelerating the second-phase viral level decline in the context of efficient inhibition of virus production. This suggests that ribavirin enhances the effects of the restored innate immune response in infected hepatocytes that do not express viral proteins any more. The underlying mechanisms have been debated for years and remain elusive (Thomas, Ghany, & Liang, 2012). Several hypotheses have been raised. The direct inhibitory role of ribavirin on HCV RNA polymerase activity is unlikely to play a major role. This was suggested by our observation that ribavirin only modestly and transiently inhibits viral replication when used as a monotherapy (Pawlotsky et al., 2004). It was confirmed by the demonstration that replacing ribavirin by a potent DAA in combination with IFN-α results in lower SVR rates than with the combination of pegylated IFN-α and ribavirin (Hezode et al., 2009). Ribavirin inhibits the enzyme inosine monophosphate dehydrogenase (IMPDH), resulting in depletion of GTP pools. However, this mechanism is unlikely to be involved in the therapeutic HCV clearance because potent, specific IMPDH inhibitors have not been able to reproduce the effect of ribavirin *in vivo*, alone, or in combination with pegylated IFN-α (Rustgi et al., 2009). It has also been suggested that ribavirin can induce T-helper 1 responses and thereby increase the immune response against HCV (Hultgren, Milich, Weiland, & Sallberg, 1998). However, the adaptive immune response does not appear to play a major role (if any) in HCV clearance on antiviral therapy, which appears to be mainly due to the innate intracellular response.

Currently, three nonmutually exclusive hypotheses remain credible to explain the effect of ribavirin in accelerating the cure of infected cells in the context of efficient inhibition of HCV replication. (i) Ribavirin could act as a mutagen agent, inducing "error catastrophe," that is, the generation of nonviable HCV quasispecies. Although conflicting results have been reported in the context of HCV infection, recent *in vitro* data with another viral model showing that ribavirin can induce error catastrophe in the context of efficient inhibition of virus production by another agent (Perales, Iranzo, Manrubia, & Domingo, 2012) support a role for this

mechanism in the treatment of chronic hepatitis C. (ii) Ribavirin could inhibit the production of ISGs known to be preactivated in hepatocytes from patients who do not respond to IFN-based therapy, thereby switching these cells to IFN sensitivity (Testoni, Durantel, Levrero, & Zoulim, 2012). (iii) Ribavirin could induce the expression of a number of ISGs involved in viral elimination from infected cells in the context of efficient suppression of viral production (Feld et al., 2010, 2007; Thomas et al., 2012). Further studies in various models will be needed to definitively identify the mechanism(s) involved in the antiviral effect of ribavirin in the treatment of chronic hepatitis C with IFN-based or IFN-free regimens.

5. DIRECT HCV INHIBITORS (DAAs AND HTAs)

Virtually every step of the HCV life cycle can be the target of one or several families of drugs that block virus production. So far, only molecules that target polyprotein processing (i.e., NS3-4A protease inhibitors) and inhibitors of HCV replication through various targets and mechanisms have reached clinical development. The latter include nucleoside/nucleotide analogue inhibitors of HCV RNA-dependent RNA polymerase (RdRp), nonnucleoside inhibitors of RdRp, NS5A inhibitors, cyclophilin inhibitors, and a microRNA-122 antagonist. The DAAs and HTAs currently in clinical development are presented in Table 5.2.

5.1. The HCV cell cycle, a target for direct inhibition

5.1.1 Viral attachment, entry, and fusion

HCV displays two glycoproteins, E1 and E2, at its surface. The latter is involved in receptor binding at the surface of target cells. Several cell-surface molecules mediate HCV binding and internalization, including glycosaminoglycans and the low-density lipoprotein receptor, which could serve as the initial docking site for HCV attachment (Agnello, Abel, Elfahal, Knight, & Zhang, 1999; Albecka et al., 2012; Barth et al., 2005, 2003); the tetraspanin CD81, which could act as a postattachment entry coreceptor (Cormier et al., 2004; Pileri et al., 1998); the scavenger receptor B1, an essential component of the cellular HCV receptor complex (Bartosch et al., 2003; Scarselli et al., 2002; Voisset et al., 2005); claudin-1 and occludin, which appear to act late in the entry process, after the interaction with CD81 (Evans et al., 2007; Ploss et al., 2009). After attachment, HCV's

Table 5.2 DAAs and HTAs in clinical development

Drug	Manufacturer	Current phase	Dose	Duration	Mean/median log HCV RNA reduction	Genotypic coverage	Barrier to resistance
NS3-4A protease inhibitors							
Telaprevir	Vertex & Janssen	Approved	750 mg q8h	14 days	−4.4	Genotype restricted	Low barrier
Boceprevir	Merck	Approved	400 mg tid	7 days	−1.6		
Simeprevir	Janssen	III	200 mg qd	7 days	−4.1		
Faldaprevir	Boehringer–Ingelheim	III	240 mg qd	14 days	−4.0		
Danoprevir/r	Roche/ Genentech	II	200 mg q8h	14 days	−3.8		
Vaniprevir	Merck	II	700 mg bid	8 days	−4.7		
Narlaprevir/r	Merck	II	400 mg bid	7 days	−4.2		
Asunaprevir	Bristol–Myers Squibb	III	300 mg bid	3 days	−3.3		
Sovaprevir	Achillion	II	600 mg qd	5 days	−4.2		
GS-9451	Gilead	II	400 mg qd	3 days	−3.5		
ABT-450/r	Abbott	III	200 mg qd	3 days	−4.1		
MK-5172	Merck	II	400 mg qd	7 days	−5.4	Pangenotypic	Higher barrier

Continued

Table 5.2 DAAs and HTAs in clinical development—cont'd

Drug	Manufacturer	Current phase	Dose	Duration	Mean/median log HCV RNA reduction	Genotypic coverage	Barrier to resistance
Nucleoside/nucleotide analogue inhibitors of HCV RNA-dependent RNA polymerase							
Sofosbuvir	Gilead	III	400 mg qd	3 days	−3.7	Pangenotypic	High barrier
Mericitabine	Roche/ Genentech	II	1500 mg bid	14 days	−2.7		
VX-135	Vertex	II	200 mg qd	7 days	−4.5		
Nonnucleoside inhibitors of HCV RNA-dependent RNA polymerase							
Tegobuvir	Gilead	II	20 mg bid	8 days	−1.4	Genotype restricted	Low barrier
Setrobuvir	Roche/ Genentech	II	800 mg bid	3 days	−2.9		
BI207127	Boehringer–Ingelheim	II	800 mg q8h	3 days	−3.1		
ABT-333	Abbott	III	600 mg bid	2 days	−1.5		
ABT-072	Abbott	III	600 mg qd	3 days	−1.6		
VX-222	Vertex	II	750 mg bid	3 days	−3.7		
BMS791325	Bristol–Myers Squibb	?	?	?	?		

NS5A inhibitors							
Daclatasvir	Bristol–Myers Squibb	III	10 mg qd	1 day	−3.2	Pangenotypic	High barrier (except subtype 1a)
PPI-461	Presidio	II	100 mg qd	3 days	−3.7		
PPI-668	Presidio	Ib	240 mg qd	3 days	−3.7		
Ledipasvir	Gilead	III	30 mg qd	3 days	−3.3		
BMS-824393	Bristol–Myers Squibb	II	50 mg qd	3 days	−3.9		
ACH-2928	Achillion	II	60 mg qd	3 days	−3.7		
ABT-267	Abbott	III	200 mg qd	3 days	−3.1		
GSK2336805	Glaxo SmithKline	II	60 mg qd	3 days	−3.0		
IDX719	Idenix	II	50 mg qd	3 days	−3.7		
ACH-3102	Achillion	Ib	50 mg qd	1 day	−3.8		High barrier
Cyclophilin inhibitors							
Alisporivir	Novartis	III	1200 mg bid	14 days	−3.6	Pangenotypic	High barrier
SCY-465	Scynexis	II	900 mg qd	15 days	−2.2		
microRNA-122 antagonist							
Miravirsen	Santaris	Ib	7 mg/kg/wk SC	4 weeks	−3.0	Pangenotypic	High barrier

The table shows the mean or median HCV RNA level reduction in Phase Ib trials. The dosages in these trials could be different from those in subsequent Phase II or III clinical trials. Durations of administration differed between the different drugs. ? means that the data has not been reported.

entry into cells has been shown to be pH dependent and related to clathrin-mediated endocytosis. Entry is followed by a fusion step within an acidic endosomal compartment.

5.1.2 RNA translation

Decapsidation of viral nucleocapsids liberates free positive-strand genomic RNAs in the cell cytoplasm, where they serve, together with newly synthesized RNAs, as messenger RNAs for synthesis of the HCV polyprotein. The HCV 5′-untranslated region (5′UTR), the most conserved region of the HCV genome, contains several domains that are highly structured in numerous stem-loops and a pseudoknot and constitute, together with the first 12–30 nucleotides of the core-coding region, the internal ribosome entry site (IRES), which controls HCV genome translation (Honda, Beard, Ping, & Lemon, 1999). The IRES mediates cap-independent internal initiation of HCV polyprotein translation by recruiting both cellular proteins, including eIF2 and eIF3, and viral proteins.

5.1.3 Polyprotein processing

The large precursor polyprotein generated by HCV genome translation is targeted to the endoplasmic reticulum membrane where the processing events take place. The co- and posttranslational processing of the HCV polyprotein results in the generation of at least 11 proteins, including 3 structural proteins (C or core, E1, and E2); a viroporin, p7; 6 nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B); and the so-called "F" protein that results from a frameshift in the core-coding region (Moradpour, Penin, & Rice, 2007). At least two host cellular peptidases are required for processing of the HCV structural proteins, including host signal peptidase and signal peptide peptidase. Two viral peptidases are involved in the processing of HCV nonstructural proteins: NS2, a zinc-dependent metalloprotease that cleaves the site between NS2 and NS3; and NS3-4A, a serine protease that catalyzes HCV polyprotein cleavage at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B junctions. The viral proteins remain associated with intracellular membranes after processing (Moradpour et al., 2007).

5.1.4 Replication

Replication is catalyzed by the HCV RdRp or NS5B protein. The NS5A protein plays an important regulatory role in virus replication, but the mechanisms are still unclear. The NS3 helicase–NTPase domain of the NS3

protein has several functions important in replication, including RNA-stimulated NTPase activity, RNA binding, and unwinding of RNA regions of extensive secondary structure (Gwack, Kim, Han, & Choe, 1997; Tai, Chi, Chen, & Hwang, 1996). Finally, NS4B is an integral membrane protein which serves as a membrane anchor for the replication complex and plays an important role in membrane rearrangements leading to the formation of the "membranous web" or replication complex that supports and compartmentalizes HCV replication (Egger et al., 2002; Elazar, Liu, Rice, & Glenn, 2004; Gretton, Taylor, & McLauchlan, 2005; Moradpour et al., 2007).

The replication complex associates viral proteins, cellular components, and nascent RNA strands. It is believed that the positive-strand genome RNA serves as a template for the synthesis of a negative-strand intermediate of replication. Then, negative-strand RNA serves as a template to produce numerous strands of positive polarity that will subsequently be used for polyprotein translation, synthesis of new intermediates of replication, or packaging into new virus particles (Bartenschlager, Frese, & Pietschmann, 2004; Moradpour et al., 2007). A number of host cellular factors are involved in HCV replication, such as, for instance, cyclophylin A, a peptidyl-prolyl *cis/trans* isomerase required for HCV replication through its interaction with NS5A and the RdRp at the replication complex level, microRNA-122, which augments HCV replication through its binding at two specific sites within the 5′UTR of the HCV genome, or phosphatidylinositol-4 kinase III-α, which plays an important role in replication complex formation. Like viral functions, host-cell factors represent potential targets for anti-HCV therapies (Nagy & Pogany, 2012).

5.1.5 Assembly and release

Viral particle formation is probably initiated by the interaction of the core protein with genomic RNA (Shimoike, Mimori, Tani, Matsuura, & Miyamura, 1999; Tanaka et al., 2000). HCV uses the lipoprotein production pathway to generate mature viral particles and exports them. Cytoplasmic lipid droplets serve as virus assembly platforms and several nonstructural proteins appear to play a role in the late steps of the HCV life cycle, including p7, NS2, NS3, and NS5A. The very low-density lipoprotein synthesis/secretion machinery appears to be involved in infectious HCV production (Bartenschlager, Penin, Lohmann, & Andre, 2011). The mechanisms underlying exportation of mature virions in the pericellular space or their transfer to neighboring cells have yet to be understood.

5.2. NS3-4A protease inhibitors

A large number of NS3-4A protease inhibitors have reached clinical development, including two drugs, telaprevir and boceprevir, that have been approved for use in combination with pegylated IFN-α and ribavirin in patients infected with HCV genotype 1 (Zeuzem et al., 2011). NS3-4A protease inhibitors have closely related chemical structures. They are peptidomimetic compounds that target the catalytic site of the enzyme and block posttranslational processing of the viral protein that gives birth to nonstructural proteins NS4A, NS4B, NS5A, and NS5B. NS3-4A protease inhibitors inhibit viral replication by 3.5–4.5 log international units (IU)/mL when administered alone for a few days (Table 5.2) (Detishin, Haazen, Robison, Robarge, & Olek, 2010; Forestier et al., 2011; Goldwater et al., 2010; Hotho et al., 2009; Lawitz, Gaultier, et al., 2010; Lawitz, Hill, et al., 2010; Lawitz et al., 2008; Manns et al., 2011, 2008; Reesink et al., 2009). Telaprevir (Janssen and Vertex) and boceprevir (Merck) are active against genotypes 1 and 2 only, whereas other protease inhibitors have broader genotype coverage; however, none of the first-generation NS3-4A protease inhibitors is fully active against genotype 3. With some protease inhibitors, dosing intervals have been extended while enhancing patient exposure and reducing side effects by means of ritonavir boosting (100 mg/day) (Lawitz, Gaultier, et al., 2010; Reesink et al., 2009; Rouzier et al., 2011).

First-generation protease inhibitors have a low barrier to resistance. Amino acid substitutions conferring resistance to these compounds preexist at generally low levels in infected patients and are selected within a few days to weeks of drug monotherapy (Chevaliez et al., 2011; Kieffer et al., 2007; Sarrazin et al., 2007; Suzuki, Sezaki, et al., 2012). Major differences have been reported between HCV genotypes in their susceptibility and resistance profiles to protease inhibitors (Imhof & Simmonds, 2011). Different resistance profiles have also been reported for subtypes 1a and 1b with both telaprevir and boceprevir. Indeed, NS3-4A protease variants V36M, R155K, and V36M+R155K emerge frequently in patients with HCV genotype 1a infection versus V36A, T54A, and A156S/T in patients with genotype 1b infection (Kieffer et al., 2012).

Cross-resistance among first-generation protease inhibitors is conferred by most of these amino acid substitutions. Indeed, R155K and D168V were the most frequently observed resistant variants with faldaprevir (Boehringer–Ingelheim) *in vitro* (Manns et al., 2012). The resistance profiles may,

however, differ between different members of the protease inhibitor family (Welsch, Shimakami, et al., 2012). For instance, amino acid substitutions selected by boceprevir also include Q41R, F43C, V55A, and V170A (Vermehren et al., 2012; Welsch, Schweizer. et al., 2012). In contrast, changes at V36, T54, F43, and Q80 do not confer resistance to faldaprevir (Lagace et al., 2012).

Second-generation NS3-4A protease inhibitors have pangenotypic coverage (including genotype 3) and a higher barrier to resistance than first-generation drugs (Brainard et al., 2010). The most advanced second-generation protease inhibitor is MK-5172 (Merck), a P2-P4 quinoxaline macrocyclic compound. MK-5172 inhibits HCV replication of genotypes 1a, 1b, 2a, 2b, and 3a at subnanomolar concentrations *in vitro*. MK-5172 is active against viral variants bearing the R155K and D168Y substitutions, known to confer resistance to first-generation protease inhibitors. In contrast, the A156T substitution confers reduced susceptibility to MK-5172 *in vitro* (Summa et al., 2012). This variant is known to have reduced replication capacity; it is still unclear whether its selection *in vivo* will lead to clinically meaningful virological breakthroughs.

5.3. Nucleoside/nucleotide analogues

Nucleoside/nucleotide analogues target HCV RNA formation within the catalytic site of the HCV RdRp. They act as false substrates for the RdRp, leading to chain termination after incorporation into the newly synthesized RNA chain. Nucleoside analogues need three phosphorylation steps to be activated. In contrast, nucleotide analogues are already phosphorylated and need only two additional phosphorylations, making them more rapidly active at the target site. Several drugs have reached clinical development, including purine and pyrimidine analogues (Table 5.2) (Lawitz, Rodriguez-Torres, et al., 2011; Reddy et al., 2007). They are active on all known genotypes and subtypes.

2′-Substituted nucleoside/nucleotides have a high barrier to resistance, as a result of the mutational bias of HCV RdRp in favor of transitions over transversions (Powdrill et al., 2011), that makes the resistance mutation unlikely to occur spontaneously, and of the extremely low fitness of the selected substitution, S282T, making it unlikely to grow and become clinically meaningful. This was initially shown in studies with mericitabine (Roche), a nucleoside analogue (Pawlotsky, Najera, & Jacobson, 2012).

Sofosbuvir (GS-7977, Gilead) is the most advanced nucleotide analogue inhibitor of HCV RdRp in clinical development. S282T was selected by sofosbuvir in genotype 1a, 1b, and 2a models. However, it conferred a substantial level of viral resistance only for genotypes 1a and 1b. Additional amino acid changes were selected, which appeared to enhance resistance to sofosbuvir and/or the replication capacity of resistant variants. Whether variants with multiple substitutions can be selected *in vivo* upon sofosbuvir administration and be fit enough to lead to virological breakthroughs remains to be established (Lam et al., 2012).

5.4. Nonnucleoside inhibitors of HCV RdRp

Nonnucleoside inhibitors of HCV RdRp are a heterogeneous group of drug families targeting one of four allosteric sites at the surface of the viral enzyme, including "thumb" domains I and II and "palm" domains I and II (Table 5.2) (de Bruijne et al., 2010; Erhardt et al., 2009; Larrey et al., 2009; Lawitz et al., 2009; Rodriguez-Torres et al., 2010; Wagner et al., 2011). Their binding alters the three-dimensional structure of the RdRp, thereby altering its catalytic function and blocking RNA replication. Their antiviral action is thus far restricted to HCV genotype 1 in most instances. Different nonnucleoside inhibitors have different antiviral potencies (Table 5.2) (de Bruijne et al., 2010; Erhardt et al., 2009; Larrey et al., 2009; Lawitz et al., 2009; Rodriguez-Torres et al., 2010; Wagner et al., 2011).

Nonnucleoside inhibitors of RdRp have a low genetic barrier to resistance and select amino acid substitutions that are generally located in close vicinity to their target site. The corresponding variants are generally fit. Cross-resistance exists between drugs targeting the same site and, sometimes, between drugs targeting different sites. For instance, resistance to filibuvir (Pfizer, halted) has been characterized in treated patients. The most frequently observed substitutions were M423I/T/V. These substitutions were associated with reduced replication capacity *in vitro* (Troke et al., 2012). Other substitutions at positions R422 and M426 were observed in a small number of patients (Troke et al., 2012). Experiments in the subgenomic genotype 1b replicon identified substitutions C316Y, Y448H, Y452H, and C445F as associated with *in vitro* resistance to tegobuvir (Gilead); this pattern was different from other nonnucleoside inhibitors (Shih et al., 2011). Double and triple mutant viruses that are resistant to two or three nonnucleoside inhibitors of HCV RdRp can be selected *in vitro* when a stepwise resistance selection protocol is used (Delang, Vliegen, Leyssen, & Neyts, 2012).

New nonnucleoside inhibitors of HCV RdRp are at the preclinical developmental stage. For example, thumb pocket site I inhibitor TMC647055 (Janssen) was recently shown to efficiently inhibit HCV genotypes 1a, 1b, 3a, 4a, and 6a (but not genotypes 2a and 2b) in a replicon assay (Devogelaere et al., 2012). *In vitro* selection experiments were performed, which identified substitutions L392I and P495S/T/L as frequent with HCV subtype 1b, while P495S/L, Q309R/K, F574V/S/A, and C575F/S were most frequently selected with subtype 1a, with changes in 50% effective concentrations of the drug of the order of 10- to 400-fold (Devogelaere et al., 2012). Another nonnucleoside inhibitor of HCV RdRp, JTK-853 (Japan Tobacco), associates with the palm site and β-hairpin region of the enzyme. This compound exhibited decreased antiviral activity in the presence of C316Y, M414T, Y452H, and L466V substitutions in the palm site region of HCV RdRp (Ando et al., 2012).

5.5. NS5A inhibitors

NS5A inhibitors bind to domain I of the NS5A protein and block its ability to regulate HCV replication within the replication complex, through an as-yet-unclear mechanism. They could inhibit both the *cis* and *trans* functions of NS5A and perturb the function of newly formed replication complexes through redistribution of this viral protein from the endoplasmic reticulum to lipid droplets (Fridell, Qiu, et al., 2011; Targett-Adams et al., 2011). NS5A inhibitors are potent and have pangenotypic coverage (Table 5.2).

The barrier to resistance of first-generation molecules is low for HCV subtype 1a, high for other genotypes/subtypes (Brown et al., 2010; Gao et al., 2010; Lawitz, Gruener, et al., 2011; Nettles et al., 2008, 2010; Wang et al., 2012). There is good correspondence between amino acid substitutions selected in the replicon system *in vitro* and those reported in patients receiving daclatasvir (Bristol–Myers Squibb), one of the most advanced NS5A inhibitors, currently in Phase III clinical development, which are essentially at positions 28, 30, 31, and 93 for subtype 1a and at positions 31 and 93 for subtype 1b (Fridell, Wang, et al., 2011). Amino acid substitutions not responsible for resistance by themselves can substantially improve the fitness of resistant variants, as recently shown (Sun et al., 2012). Second-generation NS5A inhibitors with improved barrier to resistance will soon reach early clinical development.

5.6. Cyclophilin inhibitors

Cyclophilin A plays an important role in the HCV replication cycle by binding to both NS5A and the RdRp within the viral replication complex. Blocking its peptidyl-prolyl *cis–trans* isomerase enzyme activity is associated with a significant inhibition of HCV replication, through mechanisms that remain unclear (Coelmont et al., 2008). Because their target is a host protein, cyclophilin inhibitors have pangenotypic coverage and a high barrier to resistance (Coelmont et al., 2009). Variants bearing amino acid substitutions in the NS5A protein have been selected after numerous passages in cell culture. However, these substitutions confer low-level resistance and the corresponding variants exhibit low fitness both *in vitro* and *in vivo* (Coelmont et al., 2008). Their contribution to treatment failure in patients receiving cyclophylin inhibitors is unlikely.

5.7. miRNA-122 inhibitors

miR-122 binds at two sites in the 5′UTR of HCV genome and this binding is required for efficient replication. miR-122 antagonists inhibit HCV replication (Lanford et al., 2010; Reesink et al., 2012). No resistance to these compounds has been described thus far (Patick et al., 2012).

6. INTERFERON-BASED STRATEGIES IN 2013

6.1. Dual IFN-based therapy (HCV genotype non-1)

In patients infected with HCV genotypes 2, 3, 4, 5, and 6, the standard treatment of chronic hepatitis C is still the combination of pegylated IFN-α and ribavirin (EASL, 2011). Pegylated IFN-α2a and pegylated IFN-α2b must be administered at the doses of 180 μg/week or 1.5 μg/kg/week, respectively. For patients infected with genotype 2 or 3, the dose of ribavirin is 0.8 g/day and treatment duration 24 weeks; however, patients with genotypes 2 and 3 with baseline factors suggesting low responsiveness should receive weight-based ribavirin. In patients infected with genotypes 4, 5, and 6, the dose of ribavirin is based on body weight and treatment should last up to 72 weeks, depending on the on-treatment virological response (EASL, 2011).

HCV RNA levels should be measured at baseline, weeks 4, 12, and 24, at the end of treatment, and 24 weeks after treatment withdrawal (Chevaliez, Rodriguez, & Pawlotsky, 2012; EASL, 2011). A rapid virological response (RVR) is defined as undetectable HCV RNA at week 4; an early virological response (EVR) as HCV RNA detectable at week 4 but undetectable at

week 12; and a slow or delayed virological response (DVR) as HCV RNA detectable at week 12 but undetectable at week 24. Patients who have an RVR probably need no more than 24 weeks of therapy, except for those infected by an HCV genotype other than 2 or 3 and who have a high baseline HCV RNA level (>400,000–800,000 IU/mL). In patients infected with genotype 2 or 3 who have a low baseline HCV RNA level and who experience an RVR, 16 weeks of therapy could be sufficient. Patients who achieve an EVR require 48 weeks of therapy, whereas patients with a DVR appear to benefit from 72 weeks of treatment. Patients with less than a 2-$\log_{10}$ decline in HCV RNA at week 12 are unlikely to experience sustained viral eradication and should be taken off therapy (Chevaliez et al., 2012; EASL, 2011).

6.2. Triple IFN-based therapy

The current standard-of-care for patients infected with HCV genotype 1 is the combination of pegylated IFN-α, ribavirin, and an NS3-4A protease inhibitor, either telaprevir or boceprevir (Ghany, Nelson, Strader, Thomas, & Seeff, 2011).

6.2.1 Telaprevir-based therapy (HCV genotype 1)

Telaprevir is administered for 12 weeks in combination with pegylated IFN-α and ribavirin, followed by pegylated IFN-α and ribavirin alone for a duration that depends on the virological response (Ghany et al., 2011). HCV RNA levels should be determined at baseline, week 4, 12, and 24, at the end of treatment, and 24 weeks after the end of treatment (Chevaliez et al., 2012; Ghany et al., 2011). According to the European and American labels, treatment-naïve patients and responder–relapsers must stop therapy at week 24 if they achieve an eRVR; if not, they should continue on pegylated IFN-α and ribavirin until week 48. Treatment-naïve patients with cirrhosis who have undetectable HCV RNA at weeks 4 and 12 may benefit from an additional 36 weeks of pegylated IFN-α and ribavirin (48 weeks total). Partial responders and null-responders to a first course of pegylated IFN-α and ribavirin should receive 12 months of the triple combination followed by 36 weeks of pegylated IFN-α and ribavirin alone, regardless of their virological response. In all instances, it is recommended to halt treatment if the HCV RNA level is >1000 IU/mL at week 4 or 12 or if HCV RNA remains detectable at week 24 (Chevaliez et al., 2012). HCV-resistance testing based on sequence analysis of the protease region has no utility in clinical practice,

because the results will have no impact on subsequent treatment decisions (Pawlotsky, 2011).

Telaprevir use was associated with two key adverse reactions: skin disorders, including rash and pruritus, and anemia. These adverse events were frequent (more than 50% of cases), sometimes severe, and in some cases treatment limiting. Indeed, approximately 90% of all rashes were mild or moderate (grades 1 and 2), whereas 6% of patients experienced severe (grade 3) rash, leading to telaprevir discontinuation. Among the more than 3000 patients treated with telaprevir worldwide, three cases suggestive of Stevens–Johnson syndrome and 11 cases suggestive of drug reaction with eosinophilia with systemic symptoms (DRESS syndrome) have been reported, none of which were lethal (Picard & Cacoub, 2012). The majority of rashes occurred during the first 4 weeks, with a median time of onset at 22 days. After approval of the drug, two lethal cases have been reported, one due to toxic epidermal necrolysis and one due to DRESS syndrome, in patients who did not stop therapy after the onset of the symptoms.

On the other hand, hemolytic anemia induced by ribavirin is aggravated by the addition of telaprevir, as a result of bone-marrow suppression. Patients treated with the triple combination of pegylated IFN-α, ribavirin, and telaprevir more often had severe anemia, defined by hemoglobin $\leq$10.0 g/dL than those receiving pegylated IFN-α and ribavirin alone (36% vs. 14%, respectively). In Phase III trials, nausea and diarrhea were also more frequent in the telaprevir arms than in the control arm (difference $\geq$10%) (Jacobson et al., 2011; Zeuzem et al., 2011).

Telaprevir is a substrate of cytochrome P450 3A (CYP3A) and of the drug transporter P-glycoprotein. As a result, numerous drug–drug interactions have been reported with this drug. In patients infected with HCV, special care should be taken with immunosuppressive drugs, in particular, cyclosporine and tacrolimus, some antiretroviral drugs, inhibitors of HMG-CoA reductase, oral contraceptives, antidepressants, antipsychotics, anxiolytics and sleep aids, opioid replacement, and antihypertensive agents. The main telaprevir drug–drug interactions are presented on the Website of the University of Liverpool (www.hep-druginteractions.org).

Failure to eradicate HCV results primarily from an inadequate response to pegylated IFN-α and ribavirin, which leads to uncontrolled outgrowth of resistant variants selected by the protease inhibitor (Bacon et al., 2011; Chevaliez et al., 2011; Poordad et al., 2011; Zeuzem et al., 2011). Preexisting resistant variants do not appear to influence the results of triple combination therapy (Chevaliez et al., 2011; Suzuki, Sezaki, et al., 2012).

A recent analysis of treatment failures in treatment-naïve and treatment-experienced patients has shown a consistent, subtype-dependent resistance profile in patients failing to eradicate HCV (Kieffer et al., 2012). The levels of resistant variants declined over time after treatment cessation, as recently suggested (Sullivan et al., 2011).

6.2.2 Boceprevir-based therapy (HCV genotype 1)

The triple combination of pegylated IFN-α, ribavirin, and boceprevir is indicated in patients infected with HCV genotype 1. Boceprevir must be administered after a 4-week "lead-in" period with pegylated IFN-α and ribavirin alone (Ghany et al., 2011). Like with telaprevir, the duration of treatment depends on the virological response. HCV RNA levels should be determined at baseline, weeks 8 (i.e., week 4 of boceprevir administration), 12 and 24, at the end of treatment, and 24 weeks after the end of treatment (Chevaliez et al., 2012). Noncirrhotic, treatment-naïve patients should receive the triple combination of pegylated IFN-α, ribavirin, and boceprevir until week 28 if they have undetectable HCV RNA at week 8 or otherwise until week 36 and continue on pegylated IFN-α and ribavirin until week 48 (Ghany et al., 2011). The European label recommends that noncirrhotic responder–relapsers and partial responders receive the triple combination of pegylated IFN-α, ribavirin, and boceprevir until week 36 and then continue on pegylated IFN-α and ribavirin until week 48, whereas the American label recommends that patients receive the triple combination until week 36 and continue on pegylated IFN-α and ribavirin only if HCV RNA is still detectable at week 8. The European and American labels agree that null-responders to a first course of pegylated IFN-α and ribavirin (whatever their fibrosis score) and cirrhotic patients should receive the triple combination until week 48. Treatment should be stopped if the HCV RNA level is >100 IU/mL at week 12 or if HCV RNA is detectable at week 24. HCV-resistance testing based on sequence analysis of the protease region has no utility in clinical practice, because the results will have no impact on subsequent treatment decisions (Pawlotsky, 2011).

Hemolytic anemia due to ribavirin is aggravated by the addition of boceprevir, as a result of bone-marrow suppression. Patients treated with the triple combination of pegylated IFN-α, ribavirin, and boceprevir more often had severe anemia, defined by hemoglobin ≤10.0 g/dL, than those receiving pegylated IFN-α and ribavirin alone (50% vs. 30%, respectively) (Bacon et al., 2011; Poordad et al., 2011). The utility of erythropoietin has been challenged in a recent Phase III trial showing no difference in

SVR in two groups of patients who experienced anemia under the triple combination of pegylated IFN-α, ribavirin, and boceprevir who were randomized to receive EPO or dose-reduced ribavirin (Poordad et al., 2012). In addition, mild or moderate dysgeusia was more frequently reported in patients receiving boceprevir (Bacon et al., 2011).

Like telaprevir, boceprevir is a substrate of cytochrome P450 3A (CYP3A) and of the drug transporter P-glycoprotein. Drug–drug interactions have been reported with immunosuppressive drugs, in particular, cyclosporine and tacrolimus, some antiretroviral drugs, inhibitors of HMG-CoA reductase, oral contraceptives, antidepressants, antipsychotics, anxiolytics and sleep aids, opioid replacement, and antihypertensive agents. Boceprevir drug–drug interactions are presented on the Website of the University of Liverpool (www.hep-druginteractions.org).

Failure to eradicate HCV on boceprevir-based therapy is governed by the same rules as treatment failure with telaprevir.

6.2.3 Other triple IFN-based therapies

A number of other triple therapies combining pegylated IFN-α, ribavirin, and one DAA or HTA currently are in Phase II or III evaluation. Several of these combinations should reach the market in 2014–2016. They include combinations with protease inhibitors, such as simeprevir (Janssen), faldaprevir, asunaprevir (Bristol–Myers Squibb), danoprevir (Roche); nucleotide analogue inhibitors of HCV RdRp, such as sofosbuvir and mericitabine; NS5A inhibitors, such as daclatasvir; or cyclophilin inhibitors, such as alisporivir (Novartis), currently on clinical hold. The results of a Phase III study with pegylated IFN-α, ribavirin, and sofosbuvir administered for 12 weeks in treatment-naïve patients infected with HCV genotypes 1, 4, 5, and 6 have been recently released. Overall, 90% of 327 patients achieved an SVR, including 89% of those infected by HCV genotype 1, 97% of those infected by HCV genotypes 4, 5, and 6, and 80% of the 56 patients with compensated cirrhosis (http://www.gilead.com/pr_1780873).

6.3. Quadruple IFN-based therapy

Interest was recently raised about quadruple combination treatment regimens that combine pegylated IFN-α, ribavirin, and two DAAs belonging to different drug classes without cross-resistance. In a study in patients who previously experienced a null response to pegylated IFN-α and ribavirin, 10 out of 10 patients receiving a quadruple combination including an NS5A inhibitor and an NS3-4A protease inhibitor achieved an SVR

(Lok et al., 2012). The number of patients in this study was too small to firmly conclude and it also remains to be established whether two drugs with a low barrier to resistance do better in combination with pegylated IFN-α and ribavirin than one drug with a high barrier to resistance, or whether quadruple combinations may be further improved by including at least one drug with a high barrier to resistance.

7. INTERFERON-FREE REGIMENS IN 2014 AND ONWARD

7.1. Achieving an SVR with IFN-free regimens

High SVR rates can be achieved with all-oral, IFN-free drug regimens if the drug or drug combination is potent enough to efficiently shut down virus production, has a high enough barrier to resistance to maintain viral inhibition throughout treatment, and is able to induce a steep and consistent second-phase decline that ultimately leads to the definitive clearance or cure of HCV-infected liver cells.

7.1.1 Antiviral effectiveness

The first-phase decline is rapid and profound with most available DAAs used as monotherapies. The antiviral effectiveness of the drugs presented in Table 5.2 is difficult to compare, because different dosages and durations have been applied. However, antiviral effectiveness of drugs from the four families of DAAs having reached clinical development can reach −3.0 to −4.0 $\log_{10}$ IU/mL at day 3 in many instances (Table 5.1). Similar antiviral effectiveness can be achieved after more days of administration with drugs that need to be activated, such as, for instance, mericitabine, a nucleoside analogue that requires to be phosphorylated three times, or with HTAs, such as cyclophylin inhibitors.

7.1.2 Barrier to resistance

DAAs and HTAs are characterized by their barrier to resistance, which is influenced by three major related factors *in vivo* (Pawlotsky, 2011). (i) The genetic barrier to resistance, which can be defined at several levels: the number of nucleotide substitutions needed to generate a resistant variant, which may vary according to the genotype or subtype for a given drug or class of drugs; the likelihood that a nucleotide mutation responsible for an amino acid change associated with resistance occurs, which is influenced by the mutational bias of HCV RdRp in favor of transitions over transversions (Powdrill et al., 2011); the number of amino acid substitutions needed for

a viral variant to acquire full resistance to the drug, which may also vary according to the genotype or subtype. (ii) The *in vivo* fitness of the resistant viral population, defined as the ability of the variant to survive and grow in the replication environment in the presence of drug. (iii) Drug exposure: resistant variants will be inhibited if the drug levels achieved *in vivo* are far above their 90% inhibitory concentration against these resistant variants.

HCV drugs in development can be split into two groups according to their barrier to resistance. HCV DAAS with a low barrier to resistance include first-generation NS3-4A protease inhibitors, nonnucleoside inhibitors of HCV RdRp and, for certain subtypes such as subtype 1a, first-generation NS5A inhibitors. HCV drugs with a high barrier to resistance include nucleoside/nucleotide analogues, possibly second- and third-generation protease and NS5A inhibitors, and HTAs, such as cyclophilin inhibitors. The combination of two oral drugs with a low barrier to resistance was recently shown to result in early virological breakthroughs due to the selection of viral populations that were resistant to both drugs (Lok et al., 2012; Zeuzem, Buggisch, et al., 2012). In contrast, the use of combinations including at least one drug with a high barrier to resistance, such as a nucleoside/nucleotide analogue regardless of the HCV genotype/subtype, or an NS5A inhibitor in patients infected with subtype 1b, was shown to reach a high barrier to resistance (Chayama et al., 2011; Gane et al., 2010, 2011, 2012b). The combination of three potent drugs which, individually, have a low barrier to resistance was also shown to achieve a sufficiently high barrier to resistance.

7.1.3 Infected cell clearance

The second-phase decline is the combined result of the natural death rate of infected cells and the rate of loss of the ability of the remaining infected cells to produce virus as their intracellular RNA degrades (cell cure) (Dahari et al., 2011). The second-phase decline is under the influence of several parameters, including antiviral treatment effectiveness, that is, the first-phase decline (Guedj & Perelson, 2011), the genetic background of the host (IL28B genotype) (Chu et al., 2012), and the severity of liver disease. Therefore, the duration of treatment required to eradicate infection varies from one patient to another, and a fixed duration of treatment may not fit all patients.

In combination with pegylated IFN-α, ribavirin has been shown to accelerate the second-phase decline and shorten the required treatment duration (Bronowicki et al., 2006; Herrmann, Lee, Marinos, Modi, & Zeuzem, 2003; Pawlotsky et al., 2004). Recent findings suggest that this

effect is not IFN dependent and can be obtained when ribavirin is combined with potent DAAs (Gane et al., 2012b; Zeuzem, Buggisch, et al., 2012). Therefore, ribavirin addition is likely to be useful to shorten treatment duration with future all-oral, IFN-free regimens.

7.2. Current approaches for IFN-free regimens

Given the available families of DAAs and their characteristics, distinct approaches have been developed by the different drug companies. These include the combination of a nucleotide analogue with ribavirin; the combination of a nucleotide analogue with a lower barrier to resistance drug, such as an NS5A inhibitor (low barrier for genotype 1a only) or a protease inhibitor, with or without ribavirin; the combination of a nucleoside analogue with two drugs with a low barrier to resistance, with or without ribavirin; the combination of three drugs with a low barrier to resistance, such as a protease inhibitor, an NS5A inhibitor, and a nonnucleoside inhibitor of HCV RdRp, with or without ribavirin. Other combinations including a drug with a high barrier to resistance, such as a cyclophilin inhibitor, or a second-generation protease or NS5A inhibitor are also in progress.

The results of several trials with IFN-free regimens have been reported recently. Twenty-one out of 21 null-responders to a prior course of pegylated IFN-α and ribavirin infected with HCV genotype 1b achieved an SVR with the combination of daclatasvir (NS5A inhibitor) and asunaprevir (NS3-4A protease inhibitor), whereas 7 out of 22 patients who were considered IFN–ribavirin ineligible or intolerant broke through or relapsed with the same drug regimen (Suzuki, Ikeda, et al., 2012).

Two Phase III trials with sofosbuvir (nucleotide analogue) and ribavirin have been conducted in patients infected with HCV genotypes 2 and 3. In the treatment-naïve patients from the FISSION trial, the SVR rate was 67% ($n = 257$), versus 67% in the 243 patients who received 24 weeks of pegylated IFN-α and ribavirin (noninferiority). The SVR rates with sofosbuvir and ribavirin were 97% versus 78% in genotype 2 patients, and 56% versus 63% in genotype 3 patients, respectively. They were 47% versus 38% in the patients with compensated cirrhosis, who represented 20% of the treated population (http://www.gilead.com/pr_1780873). In the treatment-experienced patients from the FUSION trial, the SVR rate was 50% ($n = 100$) versus 73% ($n = 95$) in the patients who received sofosbuvir and ribavirin 12 and 16 weeks, respectively. The SVR rates were 86%

versus 94% in genotype 2 patients, and 30% versus 62% in genotype 3 patients, respectively. They were 31% versus 66%, respectively, in the patients with compensated cirrhosis, who represented 34% of the treated population (http://www.gilead.com/pr_1786260).

In patients infected with HCV genotype 1 included in the ELECTRON trial, the SVR rates with sofosbuvir and ribavirin were 84% in 25 treatment-naïve patients, and 10% in 10 null-responders to a prior course of pegylated IFN-α and ribavirin. In contrast, 100% of 25 treatment-naïve and 10 null-responders achieved an SVR when ledipasvir, an NS5A inhibitor, was added to the sofosbuvir–ribavirin combination (Gane et al., 2012a). Phase III trials with this combination are underway. Similarly, in 44 treatment-naïve patients infected with genotypes 2 and 3, 100% of patients achieved an SVR with the combination of sofosbuvir and daclatasvir (NS5A inhibitor), and 93% with the same combination plus ribavirin (the patient who did not achieve an SVR was lost for follow-up) (Sulkowski et al., 2012).

The SOUND-C2 trial was conducted in patients infected with HCV genotype 1 with a combination of faldaprevir (protease inhibitor) and BI207127 (nonnucleoside inhibitor of HCV RdRp) in combination with ribavirin. With 28 weeks of this combination, the SVR rates were 43% in patients infected with HCV genotype 1a, and 85% in those infected with genotype 1b, suggesting that this therapy should be restricted to genotype 1b (Zeuzem, Soriano, et al., 2012).

The AVIATOR study was performed on patients infected with HCV genotype 1, with various combinations of ABT-450, a ritonavir-boosted protease inhibitor, ABT-267, an NS5A inhibitor, ABT-333, a nonnucleoside inhibitor of HCV RdRp, and ribavirin. In treatment-naïve patients, 8 weeks of the quadruple combination induced an 89% SVR rate ($n=80$); with 12 weeks of therapy, the SVR rates were 85% for ABT-450 plus ABT-333 plus ribavirin ($n=41$), 90% for ABT-450 plus ABT-267 plus ribavirin ($n=79$), 88% for ABT-450 plus ABT-267 plus ABT-333 ($n=79$), and 97% for the quadruple combination ($n=79$). In null-responders to a prior course of pegylated IFN-α and ribavirin-treated 12 weeks, the SVR rates were 89% for ABT-450 plus ABT-267 plus ribavirin ($n=45$), and 93% for the quadruple therapy ($n=45$) (http://www.abbott.com/news-media/press-releases/abbott-presents-promising-phase-2b-interferonfree-hepatitis-c-results-at-2012-liver-meeting.htm).

A large number of trials with IFN-free combinations of oral DAAs are ongoing and other results will be presented soon.

8. CONCLUSION

Recent data have shown that it is relatively easy to cure HCV infection without IFN, provided that a potent combination of DAAs and/or HTAs with a high barrier to resistance is used for a sufficient duration, with or without ribavirin that may reduce the required treatment duration. Very high cure rates have been achieved in Phase II and III clinical trials in relatively easy-to-treat populations. Twelve weeks of therapy with such potent IFN-free regimens appear well suited as universal first-line therapy of chronic hepatitis C in the future. The ideal combination(s) of first-line drugs, however, remain(s) to be defined, based on cost, side effects, and potential interactions with concomitantly administered drugs. This approach will considerably simplify HCV therapy that will essentially require an HCV RNA assessment prior to therapy and after its end while yielding high infection cure rates. Nevertheless, access to care will need to be substantially improved, notably through large-scale screening and funding for therapy in experienced settings. The minority of patients who will not respond to this first-line therapy will subsequently need to be taken care of in highly specialized centers that will use other, more potent/longer therapeutic approaches. This may be particularly important for cirrhotic patients, who need long-term follow-up to detect the appearance of liver complications even after clearing HCV infection and for patients with complex comorbidities. The current path of HCV drug development suggests that, for all patients infected with HCV, the IFN era has almost come to an end.

CONFLICT OF INTEREST

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CHAPTER SIX

Influenza Virus Resistance to Antiviral Therapy

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Contents

Abstract

Antiviral drugs for influenza therapy and prophylaxis are either of the adamantane or neuraminidase inhibitor (NAI) class. However, the NAIs are mainly prescribed nowadays, because of widespread adamantane resistance among influenza A viruses and ineffectiveness of adamantanes against influenza B. Emergence and spread of NAI resistance would further limit our therapeutic options. Taking into account the previous spread of oseltamivir-resistant viruses during the 2007/2008 season preceding the last pandemic, emergence of yet another naturally NAI-resistant influenza virus may not be an unlikely

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event. This previous incident also underlines the importance of resistance surveillance and asks for a better understanding of the mechanisms underlying primary resistance development. We provide an overview of the major influenza antiviral resistance mechanisms and future therapies for influenza. Here, we call for a better understanding of the effect of virus mutations upon antiviral treatment and for a tailored antiviral approach to severe influenza virus infections.

ABBREVIATIONS

DANA 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid
HA (H1–H17) hemagglutinin types 1–17
IC_{50} 50% inhibitory constant
NA (N1–N10) neuraminidase types 1–10
RBS receptor-binding site
US United States
vRNA viral RNA
WHO World Health Organization

1. INTRODUCTION

1.1. Influenza virus

Influenza is an infection of the human respiratory tract caused by an influenza virus (Smith, Andrewes, & Laidlaw, 1933). Each year, approximately 5–10% of the world population is infected with this virus. In nonrisk individuals, influenza usually causes a self-limiting upper respiratory tract infection that is controlled by innate and adaptive immune responses. Virus replication starts rapidly after infection with titers peaking within the first 3 days. Influenza-like symptoms typically include headache, sore throat, muscle ache, fatigue, and fever. Usually, the total course of illness does not take more than a week, although complete recovery and normal daily activities may take much longer.

Influenza virus infections are well known for causing complications, ranging from bronchitis and otitis media to death (Fiore et al., 2010; Short et al., 2013). Perhaps most feared is when replication of the virus expands into the lower respiratory tract and (severe) pneumonia develops (Kaiser et al., 2003). This may then lead to severe damage of the lungs, hypoxemia, shock, renal failure, and acute respiratory disease syndrome (ARDS) (Bautista et al., 2010; Fraaij & Heikkinen, 2011). Individuals at increased risk

of severe complications are children <2 years of age, pregnant women, the elderly, and patients of any age with a concomitant morbidity (Fiore et al., 2010).

In the United States (US) alone, influenza virus infections result in an estimated 200,000 hospitalizations each year and 3000–49,000 influenza related deaths (Thompson et al., 2010).

The highest fatality rates (88%) are found among people over 65 years. This percentage has slowly increased over the past decade, probably as a consequence of an aging population (Thompson et al., 2010). Therefore, the World Health Organization (WHO) recommends vaccination of people over 65 as well as those at risk (Klimov et al., 2012; WHO, 2013). In addition to vaccination, antiviral drugs are available for influenza antiviral therapy and prophylaxis.

Influenza virus types A, B, and C are enveloped viruses with a segmented negative-sense RNA genome (Palese & Shaw, 2007). Only influenza virus A and B are considered to be of major clinical importance. Nevertheless, type C virus infections have been associated with severe infections in children (Moriuchi, Katsushima, Nishimura, Nakamura, & Numazaki, 1991; Wright & Neumann, 2007). Influenza viruses of type A are further subtyped by their hemagglutinin (HA) and neuraminidase (NA) (Palese & Shaw, 2007). These are two integral membrane glycoproteins and seen by electron microscopy as the spike-like structures on the virus (Gamblin & Skehel, 2010). So far, 16 HAs (H1–H16) and 9 NAs (N1–N9) have been identified on viruses isolated from a wide variety of animals, including horses, seals, pigs, and birds (Fouchier et al., 2005). Aquatic birds are considered the natural host of these viruses (Webster, Bean, Gorman, Chambers, & Kawaoka, 1992). Recently, a new HA (H17) was identified on a virus identified in a bat (Tong et al., 2012). It also contained a novel NA-like protein "N10" (Li et al., 2012; Zhu et al., 2012). However, it does not appear to have sialidase activity (Garcia-Sastre, 2012).

Two antigenically distinct influenza B virus lineages are currently circulating in humans. These are represented by the virus strains B/Victoria/2/87 and B/Yamagata/16/88 (Rota et al., 1990). Influenza B virus infections are, in contrast to influenza A, more restricted to humans (Osterhaus, Rimmelzwaan, Martina, Bestebroer, & Fouchier, 2000).

Two other influenza viruses currently circulating in humans are influenza A viruses of subtypes H3N2 and H1N1. Influenza virus A/H3N2 has circulated since the 1968 pandemic. The A/H1N1 virus caused the

2009 pandemic and has replaced the previous A/H1N1 subtype, which had circulated in man since the 1977 pandemic (CDC, 2013). Other pandemics of the past century were in 1918 (H1N1), 1957 (H2N2), and 1968 (H3N2) (Neumann & Kawaoka, 2012). These outbreaks have been associated with a higher case fatality rate compared to the seasonal influenza virus epidemics (Dawood et al., 2012; Wright & Neumann, 2007).

Of pandemic potential are the occasionally reported sporadic cases of highly pathogenic avian influenza A/H5N1 virus infections which were first identified in 1997 (Claas et al., 1998; WHO, 2012). The threat posed by this virus, which might become a pandemic virus, has prompted WHO's advice toward countries to stockpile antiviral drugs, as part of their pandemic preparedness programs. Although these avian influenza viruses do not spread efficiently among humans yet, only few mutations were shown to be sufficient to tune up A/H5N1 virus transmissibility in ferrets (Herfst et al., 2012; Imai et al., 2012; Russell et al., 2012). Most recently, more than 20 fatal human cases have been reported in China due to infection with an avian influenza A/H7N9 virus that appears to be low pathogenic in poultry (Gao et al., 2013; Uyeki & Cox, 2013).

1.2. Influenza virus replication cycle

The influenza virus replication cycle starts when an influenza virus particle binds to sialic acid receptors, which are found on glycosylated cellular membrane proteins at the surface of epithelial cells (van Riel et al., 2010). Binding to sialic acids is established at the receptor-binding site (RBS) of HA (Wilson, Skehel, & Wiley, 1981). After binding, the virus becomes internalized by endocytosis. A critical step during virus internalization takes place at the stage when the virion-trapped endosome acidifies (Palese & Shaw, 2007). Acidification induces an influx of protons into the virion across the viral M_2-channel and this triggers uncoating of the viral RNA (vRNA) (Pinto, Holsinger, & Lamb, 1992; Wharton, Belshe, Skehel, & Hay, 1994). In addition, the decreased pH in the endosome induces HA to undergo a conformational change, which forces fusion of the virus and endosome membranes. The uncoated vRNA is then released into the cytoplasm and transported into the nucleus (Wharton et al., 1994). In the nucleus, vRNA reverse transcribed into complementary RNA (cRNA), from which the new viral proteins are translated. Since both cellular and virus proteins at the cell surface are glycosylated, sialic acid removal is essential for efficient

release of newly formed virus particles from the host cell (Colman, Varghese, & Laver, 1983). For this, NA sialidase activity is required to prevent premature HA binding and aggregation of progeny viruses.

1.3. Current antiviral drugs

1.3.1 Adamantanes

The adamantanes, amantadine and rimantadine, act by binding to the M_2 proton channel and blocking proton transport across the membrane (Cady et al., 2010; Pielak & Chou, 2010). Although amantadine and rimantadine have been around for many years (Davies et al., 1964), these drugs are hardly prescribed nowadays, because of their ineffectiveness against influenza B virus, their considerable side effects, and the naturally occurring adamantane resistance of virtually all circulating influenza A viruses (Deyde et al., 2007; Jefferson, Demicheli, Di Pietrantonj, & Rivetti, 2006; WHO, 2013). Adamantane resistance mutations either destabilize channel assembly and lower adamantane binding affinity or allow an influx of protons despite the presence of these drugs (Pielak & Chou, 2010). The major amino acid changes causing adamantane cross-resistance are at positions 26, 27, 30, 31, and 34 of the M_2-proton channel and do not affect viral fitness (Nelson, Simonsen, Viboud, Miller, & Holmes, 2009). Several high-resolution structures of M_2 protein channels have been published recently (Cady et al., 2010; Du, Huang, Wang, & Chou, 2010; Pielak & Chou, 2010; Rosenberg & Casarotto, 2010). These structures may drive the development of new effective M2-proton channel inhibitors.

1.3.2 Neuraminidase inhibitors

Removal of sialic acids from the infected cell surface by NA is an essential and final step in the influenza virus replication cycle. When this process is inhibited, viruses tend to aggregate and remain attached to the cell surfaces (Colman, 1994). The neuraminidase inhibitors (NAIs) were designed on the basis of an early transition state analogue of sialic acid, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (DANA) (Fig. 6.1). This compound was recognized as a weak NAI in the 1970s (Meindl, Bodo, Palese, Schulman, & Tuppy, 1974; Palese & Compans, 1976; Palese, Schulman, Bodo, & Meindl, 1974). The development of the NAIs was accelerated when the first NA crystal structures were solved in 1983 (Colman et al., 1983). Subsequently, structure-based design of these initial weak-binding compounds led to the synthesis of zanamivir and oseltamivir (Kim et al., 1997; von Itzstein et al., 1993) (Fig. 6.1). Since 1999, these two

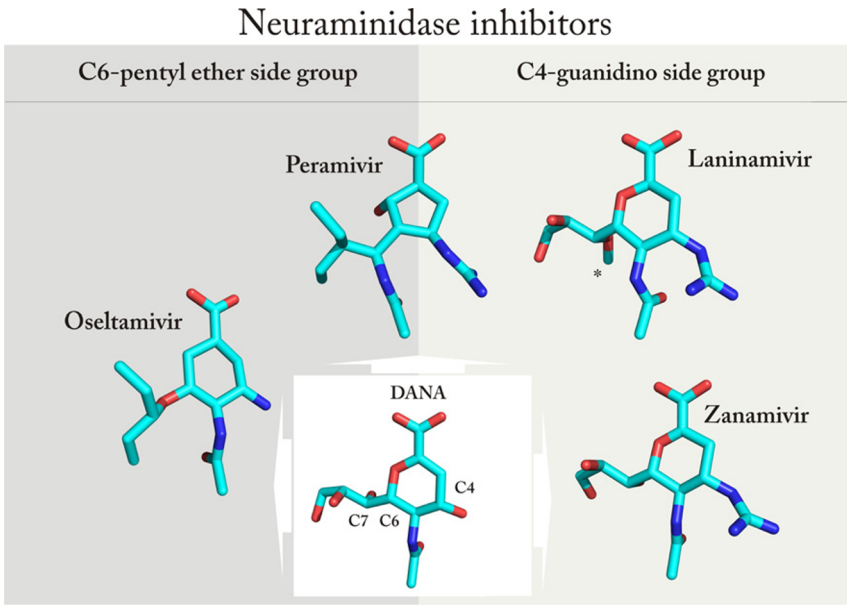

Figure 6.1 Chemical structure of DANA with neuraminidase inhibitors. As compared to a transition state analogue of sialic acid, DANA (2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid), oseltamivir has a hydrophobic pentoxil ether side group, the C6-carbon of the cyclohexane ring, where DANA has a polar glycerol. At the C4-carbon, the hydroxyl of DANA is substituted for a guanidino group in both zanamivir and laninamivir. Peramivir has a cyclopentane ring with both a hydrophobic as well as a guanidino side group. As compared to zanamivir, laninamivir has an additional methoxyl group at the C7-carbon (marked with an asterisk). *Adapted from Collins et al. (2008) and McKimm-Breschkin (2013). Image was generated using PYMOL.*

compounds have been approved by the US Food and Drug Administration and later by other regulatory bodies for the treatment and prophylaxis of influenza A and B virus infections. Guidelines for antiviral drug prescriptions are found elsewhere (WHO, 2013).

As compared to the initial sialic acid transition state analogue DANA, zanamivir has a 4-guanidino group (Varghese, McKimm-breschkin, Caldwell, Kortt, & Colman, 1992) (Fig. 6.1). In the structure of oseltamivir, this 4-guanidino group is substituted by a 4-amino group and the 6-glycerol by a hydrophobic pentyl ether side chain. These adjustments resulted in enhanced binding to the NA active site approximately 10,000-fold (McKimm-Breschkin, 2013). Oseltamivir phosphate shows better bioavailability compared to zanamivir and is administered orally. This prodrug is converted in the liver to the active form oseltamivir carboxylate (Moscona, 2005).

Bioavailability of zanamivir is poor and therefore administered by inhalation directly into the respiratory tract (Hayden et al., 2000). However, for those patients who are critically ill, or on mechanical ventilation, this administration route may be challenging (Fraaij et al., 2011; Kiatboonsri, Kiatboonsri, & Theerawit, 2010). Therefore, alternative intravenous zanamivir and oseltamivir formulations are in phase III clinical trials (Brennan et al., 2012; Gaur et al., 2010; Kohno et al., 2011; Wathen, Barro, & Bright, 2013). More recently, peramivir and laninamivir were added to the NAI class of drugs. So far, peramivir has been approved only in Japan and South Korea (Kohno et al., 2011; Yamashita, 2010). Peramivir has the 4-guanidino group of zanamivir and the hydrophobic pentoxil group of oseltamivir and is administered intravenously (Fig. 6.1) (Kohno et al., 2011). Laninamivir octanoate has been developed as a long-lasting prodrug with the 4-guanidino and polar glycerol groups of zanamivir. This prodrug is administered by a single inhalation and is converted directly to the active form laninamivir in the respiratory tract (Watanabe et al., 2010). Laninamivir octanoate has been approved only in Japan, but is entering phase II studies in the United States (Ishizuka, Toyama, Yoshiba, Okabe, & Furuie, 2012; Ishizuka, Yoshiba, Okabe, & Yoshihara, 2010).

2. ANTIVIRAL DRUGS UNDER DEVELOPMENT

Currently, several new strategies, inhibiting various stages of the influenza virus life cycle, are under development. These include virus inhibition at the stage of attachment, membrane fusion, and gene transcription (Hayden, 2013; Wathen et al., 2013). Three examples are highlighted here.

2.1. DAS181

DAS181 is a fusion protein of a bacterial sialidase with a human epithelium anchor domain (DAS181) (Belser et al., 2007; Malakhov et al., 2006; Nicholls et al., 2008). The sialidase removes sialic acids from cellular receptors, thus preventing attachment of influenza viruses to uninfected cells (Triana-Baltzer et al., 2009). DAS181 48 h postexposure therapy reduced mortality and virus titers in mice (Hedlund, Aschenbrenner, Jensen, Larson, & Fang, 2010). Unfortunately, no significant clinical effect could be obtained in a first phase II clinical trial (Moss et al., 2012), although antiviral treatment significantly reduced viral loads in pharyngeal and nose swabs in treated patients compared to placebo. Interestingly, DAS181 has

been used on a compassionate, emergency basis in three immunocompromised patients severely infected with parainfluenza type 3 (PIV-3) (Chen et al., 2011; Drozd et al., 2013; Guzman-Suarez et al., 2012). All three patients showed clinical improvement after start of DAS181 therapy. DAS181 antiviral resistance mutations have been selected by *in vitro* passaging of the virus under increasing concentrations of the drug (Triana-Baltzer et al., 2011). Mutations were found in the HA and NA genes. However, these were found to compromise *in vitro* viral fitness (Triana-Baltzer et al., 2011).

2.2. Monoclonal antibodies

Recently, exciting new monoclonal antibodies have been identified which were shown to neutralize a wide range of influenza viruses (Laursen & Wilson, 2013; Wilson & Andrews, 2012). These either target the HA head or stem domain. The HA head domain may be less attractive as a target, because it is highly immunogenic and subject to continuous antigenic drift. Variability at this region leads to the annual influenza epidemics (Laursen & Wilson, 2013). Nevertheless, several monoclonal antibodies have been identified, all binding to the relatively conserved RBS (Ekiert et al., 2012; Laursen & Wilson, 2013; Lee et al., 2012; Tsibane et al., 2012; Whittle et al., 2011). The HA stalk region is far more conserved even between different HA subtypes. Several monoclonals directed against this region have now been identified. An advantage of these antibodies is the cross-reactive multiple HA subtypes (Corti et al., 2011; Dreyfus et al., 2012; Ekiert et al., 2009, 2011). These antibodies may be an attractive therapeutic option for immunocompromised patients, although cost aspects may be limiting.

2.3. Favipiravir (T-705)

The RNA polymerase inhibitor favipiravir (T-705) is a broad-spectrum antiviral candidate drug with activity against a number of RNA viruses, including arenavirus (Mendenhall et al., 2011), bunyaviruses (Gowen et al., 2007), flaviviruses like West Nile virus (Morrey et al., 2008), and yellow fever virus (Julander, Shafer, Smee, Morrey, & Furuta, 2009), as well as influenza A and B viruses (Furuta et al., 2009). Favipiravir induces a rapid mutation rate of the virus RNA polymerase complex, which results in a large proportion of

nonviable viruses within the total virus population (Baranovich et al., 2013; Lauring & Andino, 2010). Such "error catastrophe" was previously also observed for the broad-spectrum antiviral ribavirin (Crotty et al., 2000). In poliovirus, ribavirin-induced enrichment of the virus quasispecies was found to affect the pathogenicity of the virus in mice (Vignuzzi, Stone, Arnold, Cameron, & Andino, 2006). However, the use of ribavirin to treat influenza is debatable when considering clinical benefit and the substantial side effects (Chan-Tack, Murray, & Birnkrant, 2009; Riner, Chan-Tack, & Murray, 2009). Reports on ribavirin antiviral resistance are rare, but resistance has been reported, for instance, for hepatitis C- and enteroviruses (Pfeiffer & Kirkegaard, 2003; Young et al., 2003). No influenza antiviral resistance to favipiravir has been reported so far (Baranovich et al., 2013).

2.4. Combination therapy

The use of a combination of drugs, preferably with a nonoverlapping resistance profile, may decrease the chance of antiviral resistance emergence and may act synergistically (Hoopes et al., 2011). Combination therapies have been implemented successfully for treatment of patients with chronic virus infections such as those living with HIV. For influenza, *in vitro* data show synergistic antiviral efficacy when different combinations of antiviral drugs are used (Bantia, Kellogg, Parker, & Babu, 2010; Ilyushina et al., 2008; Ilyushina, Hoffmann, Salomon, Webster, & Govorkova, 2007; Nguyen, Hoopes, et al., 2010; Smee, Hurst, Wong, Bailey, & Morrey, 2009; Smee et al., 2010; Smee, Wong, Bailey, & Sidwell, 2006). In mice, combination therapy studies showed significant reduction in mortality compared to monotherapy when treated with ribavirin and peramivir or oseltamivir (Ilyushina et al., 2008; Smee, Bailey, Morrison, & Sidwell, 2002) and oseltamivir with rimantidine or amantadine (Galabov, Simeonova, & Gegova, 2006; Ilyushina et al., 2007). A triple therapy consisting of amantadine, oseltamivir, and ribavirin was found superior to dual or monotherapy (Nguyen et al., 2012). In patients, therapy with this combination of drugs was well tolerated in healthy volunteers and in six immunocompromised patients (Seo et al., 2012). However, clinical studies addressing effectiveness of combination therapy are still very limited. A clinical study by Duval et al. (2010) reported an adverse effect of oseltamivir–zanamivir combination therapy compared to oseltamivir and zanamivir monotherapy. In contrast,

triple (amantadine, ribavirin, oseltamivir) therapy was suggested to be more effective than oseltamivir monotherapy in critically ill 2009 influenza A/H1N1 virus-infected patients (Kim et al., 2011). More preclinical (e.g., in immunocompromised ferrets; Van der Vries et al., 2013) and clinical efficacy studies analyzing different combinations of antiviral compounds are clearly needed, the latter especially for immunocompromised patients (Duval et al., 2010; Hayden, 2009; Pukrittayakamee et al., 2011).

3. RESISTANCE TO ANTIVIRAL THERAPY

To date, most circulating influenza viruses are either inherently adamantane resistant (influenza A) or not sensitive to this drug class (influenza B) (Nelson et al., 2009; WHO, 2013). In a recent paper by Whitley et al. (2013), we have reported on the incidence of current NAI resistance in a global multicentre study carried out between 2008 and 2012 (WHO, 2013). For influenza A, oseltamivir resistance was found to be rare (2.9%) and detected only in relation with therapy (16/656 patients). Emergence of oseltamivir resistance was highest in children between 1 and 5 years of age (14/138 (10.1%)). The study did not detect NAI-resistant influenza B viruses. No zanamivir resistance was detected either in this study. However, others have reported reduced zanamivir susceptibility for influenza A viruses (Hurt, Lee, et al., 2011; LeGoff et al., 2012; Nguyen, Fry, Loveless, Klimov, & Gubareva, 2010; van der Vries, Stelma, & Boucher, 2010) and B virus (Wang et al., 2013).

In addition to the high incidence of oseltamivir resistance in children (Kiso et al., 2004; Whitley et al., 2013), antiviral resistance in immunocompromised patients is not uncommon (Alonso et al., 2011; Carr et al., 2011; Khanna et al., 2009; Renaud et al., 2011; Van der Vries et al., 2013). In both patient groups, virus shedding is not controlled by adequate immune responses. This may lead to prolonged virus persistence in these patients (Kimberlin et al., 2013; Van der Vries et al., 2013). This increases the chance of developing a severe infection and emergence of an antiviral resistant virus.

Oseltamivir-resistant variants of the 2009 influenza A/H1N1 subtype have been isolated primarily from patients on therapy (Alonso et al., 2011; Carr et al., 2011; Hurt, Deng, et al., 2011; Memoli, Hrabal, Hassantoufighi, Eichelberger, & Taubenberger, 2010; Nguyen, Fry, et al.,

2010; Petersen et al., 2011; Speers et al., 2010; Tamura et al., 2011; van der Vries, Stelma, & Boucher, 2010). However, three studies reported the detection of oseltamivir-resistant variants in untreated patients (Hurt et al., 2012; Meijer et al., 2012; Storms et al., 2012).

A dramatic increase in oseltamivir resistance was observed during the influenza season of 2007–2008 (Hauge, Dudman, Borgen, Lackenby, & Hungnes, 2009), which became the dominant virus by the end of that same year (Besselaar et al., 2008; Meijer et al., 2009). Fortunately, the oseltamivir-resistant A/H1N1 virus was replaced by the oseltamivir sensitive pandemic H1N1, but the oseltamivir-resistant pandemic variants detected in untreated patients are alarming (Ghedin et al., 2012; Hurt et al., 2012; Meijer et al., 2012; Storms et al., 2012).

4. MECHANISMS OF RESISTANCE TO NAIs

The active site of the NA is highly conserved among different influenza viruses (Russell et al., 2006). However, the combination of small differences in the active sites of N1, N2, and B neuraminidases, and those between the NAIs (Fig. 6.1), lead to different options for development of resistance. Most amino acid changes causing NAI resistance are located in or are in close proximity to the active site (van der Vries, Schutten, et al., 2011). A detailed overview of resistance patterns found *in vitro* in the clinic and in surveillance programs is reviewed elsewhere (Samson, Pizzorno, Abed, & Boivin, 2013). The amino acids described below are numbered according to those in N2 neuraminidase.

4.1. N1 neuraminidase

In N1 neuraminidases, the major amino acid change causing antiviral resistance is a histidine to tyrosine change at position 274 (H274Y) and causes an increase of the 50% inhibitor constant (IC_{50}) of about 150-fold oseltamivir and 80-fold for peramivir (Samson et al., 2013), but no increase in IC_{50} for zanamivir. The H274Y change is the major oseltamivir resistance change for A/H1N1 and highly pathogenic avian influenza A/H5N1 viruses (Beigel et al., 2005; de Jong, Tran, et al., 2005; de Jong, Van Cam, et al., 2005; Le et al., 2005). Another A/H5N1 NA mutation (Earhart et al., 2009; Ilyushina, Seiler, Rehg, Webster, & Govorkova, 2010; Kiso et al., 2011; Le et al., 2005), an asparagine to serine change at position

294 (N294S), was found to cause an 80-fold increase in IC_{50} for oseltamivir and sevenfold for and zanamivir (Collins et al., 2008). Shortly after the outbreak of the 2009 pandemic A/H1N1 virus, several reports described the emergence of alternative resistance changes isoleucine to arginine at position 222 (I222R) and serine to asparagine at position 246 (S246N) (Hurt, Lee, et al., 2011; van der Vries et al., 2010). The I222R causes a 50-fold increase in oseltamivir, sevenfold in peramivir, and 10-fold in zanamivir resistance. The S246N change increases the IC_{50} for oseltamivir by sevenfold and zanamivir by threefold with no increase in IC_{50} for peramivir (Hurt, Lee, et al., 2011). Interestingly, in combination with H274Y, both I222R and S246N changes elevate the levels of resistance to the NAIs dramatically (Hurt, Lee, et al., 2011; Nguyen, Fry, et al., 2010).

In detail, all amino acids at positions 222, 246, 274, and 294 interact with the hydrophobic pentoxil group at the carbon ring position C6 of oseltamivir (Fig. 6.2). This group interacts with a hydrophobic pocket in the active site of NA (van der Vries, Schutten, et al., 2011) and is different from the polar glycerol group of zanamivir and sialic acid. For binding of the pentoxil group of oseltamivir into the hydrophobic pocket, reorientation is required of a glutamic acid residue at position 276 (E276) toward H274. In case of the H274Y change, this reorientation is blocked by the larger tyrosine residue (Collins et al., 2008; van der Vries et al., 2012). Since the C6-group of zanamivir is a polar glycerol binding to the active site does not involve E276 reorientation (Collins et al., 2008; Russell et al., 2006; van der Vries et al., 2012). The I222R, S246N changes alter the hydrophobic pocket and make binding of oseltamivir to a H274Y mutant even more difficult.

A glutamine to lysine change at position 136 (Q136K) was previously shown to cause zanamivir (300-fold) and peramivir (70-fold) resistance in H1N1 viruses (Hurt, Holien, Parker, Kelso, & Barr, 2009). Fortunately, more recently it has been shown that the presence of the Q136K mutation seemed to be an artifact of virus propagation in Madin Darby Canine Kidney cell cultures (Kaminski, Ohnemus, Staeheli, & Rubbenstroth, 2012).

4.2. N2 neuraminidase

The major NAI resistance changes are the glutamic acid to valine change at position 119 (E119V) and the arginine to lysine change at position 292

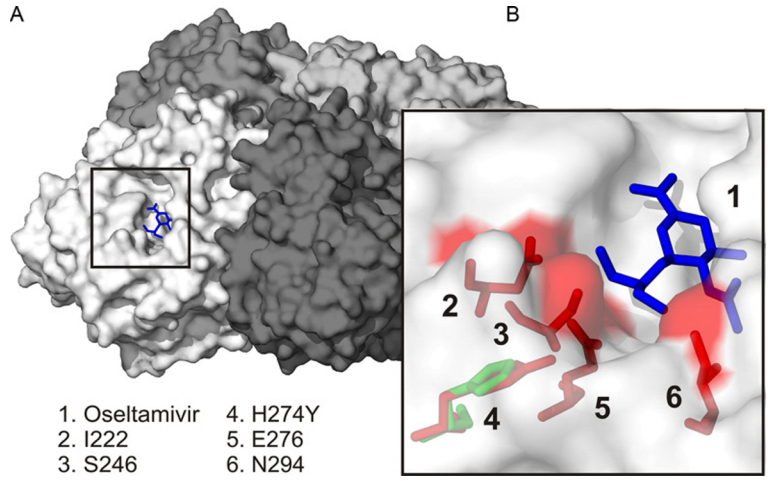

Figure 6.2 Amino acid residues in N1 neuraminidase associated with oseltamivir resistance. Surface presentation of (homo) tetrameric neuraminidase (gray) in complex with oseltamivir carboxylate (blue) (A). In an enlarged image of the active site (B, inset), oseltamivir and amino acids associated with NAI resistance are presented as red sticks (1–6). These are located close to the active site of NA to which oseltamivir carboxylate is bound. At position 274 a histidine (H, green) to tyrosine (Y, red) change prevents reorientation of glutamic acid (E) side chain at position 276 when oseltamivir carboxylate binds. At position 294 (6), an asparagine (N, green) to serine (S, red) substitution weakens oseltamivir binding affinity. Surface was made transparent to visualize otherwise hidden residues and colored red if a residue is a surface occupant. *Figure adapted from van der Vries, Schutten, et al. (2011). Image was generated using PYMOL.*

(R292K). These mutations cause resistance to oseltamivir only and not to zanamivir. Compared to the H274Y change in N1 neuraminidase these mutations are less frequently detected in virus isolates (Whitley et al., 2013). This may indicate that the E119V and R292K mutations are more deleterious than the H274Y in the currently circulating influenza viruses. Like the resistance mechanism of H274Y, the R292K change also prevents rotation of glutamic acid at position 276 (Smith et al., 2002). For the E119V oseltamivir-resistant mutant, no X-ray crystal structure is available to determine its exact resistance mechanism. However, another NA structure of a glutamic acid to glycine mutant (E119G) reveals that this change involves interaction with the C4 groups of zanamivir (C4-guanidino) and oseltamivir (C4-amino) (Smith et al., 2002) (Fig. 6.1).

4.3. B neuraminidase

Several oseltamivir-resistant B viruses have been isolated from patients (Hurt et al., 2006; Monto et al., 2006; Sleeman et al., 2012). Oseltamivir resistance may be caused by amino acid changes, D198N, R371K (Moscona & McKimm-Breschkin, 2007; Sheu et al., 2008) and by an isoleucine to valine or threonine change at position 222 (I222V/T) (Garg et al., 2012; Sleeman et al., 2012). These mutations cause only a two- to threefold increase in IC_{50} to the NAIs. Recently, an influenza B H274Y mutant virus was isolated from an untreated patient causing oseltamivir and peramivir resistance (Higgins et al., 2012). An arginine to lysine change at position 152 (R152K) was identified in an influenza B virus infected patient on zanamivir (Gubareva, Matrosovich, Brenner, Bethell, & Webster, 1998).

5. PERMISSIVE AND COMPENSATORY MECHANISMS GOVERNING PRIMARY RESISTANCE

It remains highly speculative why naturally antiviral resistance influenza viruses have emerged. Some suggest that the emergence of adamantane resistance might have resulted from large scale use of these drugs in the Chinese poultry industry (Cyranoski, 2005). However, other data do not support this claim (Nelson et al., 2009). Adamantane resistance of the 2009 A/H1N1 virus was inherited from a swine-lineage virus that had become adamantane resistant in the 1980s (Garten et al., 2009).

If massive drug use is the cause of antiviral resistant virus emergence, one would not expect that in 2007 the H275Y H1N1 viruses would emerge in Norway first. Oseltamivir prescription in Norway is one of the lowest in the world, in contrast to Japan where NAI prescription is highest (Hauge et al., 2009; Soderstrom et al., 2009). In Japan, the oseltamivir levels detected in sewage waters were found high enough to induce oseltamivir resistance in birds when experimentally infected with influenza virus (Jarhult, 2012; Jarhult et al., 2011).

5.1. Loss of viral fitness

In the first few years after the approval of the NAIs, it was generally believed that selection of antiviral resistance would go hand in hand with a reduction of NA activity and viral fitness (Carr et al., 2002; Ives et al., 2002). Therefore, antiviral resistance was thought not to be of major clinical significance. Indeed, the H274Y oseltamivir-resistant H1N1 viruses that were isolated early after the introduction of zanamivir and oseltamivir were compromised in their fitness

(Carr et al., 2002; Ives et al., 2002). Because nonfitness compromised H274Y mutant H1N1 viruses emerged during the 2007–2008 season, several research groups studied the impact of this change on the fitness of the 2009 pandemic virus (Duan et al., 2010; Hamelin et al., 2010; Memoli et al., 2011; Pinilla, Holder, Abed, Boivin, & Beauchemin, 2012). In ferrets, the H274Y mutant viruses were found to be only slightly compromised compared to their wild-type counterparts. Because virus fitness reduction by the H274Y change was found to be limited, only little additional changes seem to be necessary to facilitate the H274Y change without loss of virus fitness.

5.2. Gain of fitness

A gain of fitness may either be a result of permissive mutations in the influenza genome, including HA, which facilitate emergence of antiviral resistance or a secondary mutation and compensate for the initial loss of virus fitness due to a primary resistance mutation (Chao, Bloom, Kochin, Antia, & Longini, 2012). In case of the emergence of the H274Y mutation in the seasonal A/H1N1 viruses that circulated in 2007–2008, several permissive amino acid changes have been suggested to have facilitated the emergence of the H274Y change. An aspartic acid to asparagine change at position 343 (D343N) increased the enzymatic properties of the NA (Collins et al., 2009; Rameix-Welti, Enouf, Cuvelier, Jeannin, & van der Werf, 2008). In addition, in an *in vitro* cell system, it was shown that changes V233M and R221Q maintained expression of NA on the cell surface, which was reduced when NA was expressed only with the H274Y change (Abed, Pizzorno, Bouhy, & Boivin, 2011; Bloom, Gong, & Baltimore, 2010). For H3N2 viruses, important roles for amino acid changes at position 222 were assigned, compensating for the fitness costs of the E119V oseltamivir resistance mutation (Richard et al., 2011; Simon et al., 2011). Recently, several clusters of 2009 A/H1N1 viruses have been detected with a H274Y change, which do not seem to be affected by this change (Hurt et al., 2012; Meijer et al., 2012). These viruses contain, in addition to the H274Y change, mutations at amino acid residues 240, 368, and 385. Whether these mutations also have an impact on viral permissiveness remains to be determined (Hurt et al., 2012).

6. EFFECTIVENESS OF NAIs

There has been an intense discussion in the scientific community regarding the effectiveness of NAIs and the evidence available (Godlee, 2012; Jefferson, Jones, Doshi, & Del Mar, 2009). Several systematic reviews

have recently been published that have addressed these issues (Burch et al., 2009; Jefferson, Demicheli, Rivetti, et al., 2006; Michiels, Van Puyenbroeck, Verhoeven, Vermeire, & Coenen, 2013; Muthuri, Myles, Venkatesan, Leonardi-Bee, & Nguyen-Van-Tam, 2013; Tappenden et al., 2009).

In nonrisk patients, oseltamivir and zanamivir therapies were shown to reduce the duration of illness by approximately 0.5–1 day when started within 48 h. Both drugs were found to be effective as a postexposure prophylaxis. The impact of therapy increases when initiated soon after onset of symptoms (Muthuri et al., 2013). Clinical studies with peramivir and laninamivir octanoate showed data comparable to those found for oseltamivir and zanamivir (Kohno et al., 2011; Shobugawa et al., 2012; Sugaya & Ohashi, 2010; Watanabe et al., 2010).

Although there is lack of randomized clinical trials including patients at risk, numerous observational studies have shown improved outcome, including reduced mortality with the use of NAIs (Hernan & Lipsitch, 2011; Hiba et al., 2011; Kumar et al., 2010; Louie et al., 2012; Rodriguez et al., 2011). For those patients who eventually require intensive medical care, antiviral treatment remains a challenge (Chan-Tack et al., 2012; Fraaij et al., 2011; Fraaij, van der Vries, & Osterhaus, 2013), especially when extracorporeal membrane oxygenation is ultimately required to compensate for influenza-associated lung failure. Since mortality and emergence of antiviral resistance in these patients are high, current antiviral therapies leave room for further improvement (Carr et al., 2011; Khanna et al., 2009; Van der Vries et al., 2013).

In addition, little is known about the impact of antiviral resistance mutations on the effectiveness of antiviral therapies. Antiviral therapy was shown to be less effective for treatment of H274Y mutant H1N1 viruses (Kawai, Ikematsu, Hirotsu, et al., 2009; Saito et al., 2010). A similar impact on the effectiveness was seen for the adamantanes (Kawai et al., 2007). For many other mutations, especially those that do not cause a dramatic shift in IC_{50}, the impact of these mutations is unclear.

7. CONCLUSION

Antiviral therapy against influenza virus infections is currently approved for mono-therapeutic treatment of uncomplicated influenza virus infections (WHO, 2010). Although some clinical studies have been performed in hospitalized patients, the available data is limited and most of it

comes from observational, retrospective studies (Lee & Ison, 2012). In addition, similar to antiviral therapy in other RNA virus infections such as those with HIV and hepatitis C virus, treatment with a low genetic barrier mono- or combination therapy may lead to rapid selection of primary antiviral resistance and subsequent secondary compensatory mutations (van der Vries, Schutten, et al., 2011). Especially in very young children and immunocompromised patients, this appears to be the case: resistant viruses are frequently detected in these patients and virus replication is only partially inhibited (Carr et al., 2011; Kiso et al., 2004; Van der Vries et al., 2013; Whitley et al., 2013). Therefore, there is a clear need for improved antiviral therapies with a high genetic resistance barrier. New therapeutic options may include higher dosing of the existing drugs (Dutkowski, Smith, & Davies, 2010) and/or combination therapies that include existing and/or novel antiviral therapies, preferably with distinct modes of action. Although several combination studies have been performed to address pharmacokinetic interactions and tolerance in humans (Atiee et al., 2011; Morrison et al., 2007; Pukrittayakamee et al., 2011), studies on their efficacy are still to be performed (Hayden, 2013).

Finally, influenza antiviral surveillance networks, such as the Neuraminidase Inhibitor Susceptibility Network and Influenza Resistance Information Study (IRIS), are important for monitoring the incidence of primary resistance in circulating influenza viruses. A better understanding of the impact of antiviral resistance mutations on the therapeutic effectiveness will be useful for treatment of critically ill patients. A better understanding of the potential permissive amino acid changes in the circulating viruses may aid in a better understanding of the emergence of noncompromising antiviral resistance and may even be of predictive value.

CONFLICT OF INTEREST

E. V. D. V., M. S., C. B., and A. O. are investigators in the IRIS study. The Erasmus Medical Center receives funding from Hoffmann-La Roche for the IRIS study. A. O. is CSO of Viroclinics Biosciences BV, an Erasmus MC spin out, that collaborates with pharmaceutical companies.

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CHAPTER SEVEN

The Natural Course of Chronic Hepatitis B Virus Infection and Its Management

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Contents

Abstract

Chronic infection with the hepatitis B virus (HBV) runs a long natural course during which underlying changes in liver histology can progress to cirrhosis and hepatic

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decompensation, as well as to hepatocellular carcinoma. Therapeutic intervention is currently aiming at suppression of HBV replication by applying a number of pharmacological agents. For an optimum use of available therapies, good knowledge of the natural course of chronic infection, as well as of the role played by several viral, host, and environmental factors, is mandatory. The larger part of this chapter deals with how to treat the various subsets of patients with chronic hepatitis B (CHB), using mainly three first-line drugs: pegylated interferon-α2a, entecavir, and tenofovir, administered either in finite courses or indefinitely. The frequency of virological, serological, biochemical, and histological responses in the various subsets of patients, both during and after stopping treatment, is reviewed. It is stressed that the application of the highly potent antivirals entecavir and tenofovir, with acceptable safety records and with a high barrier to HBV resistance, represents major progress in the treatment of CHB. Despite the hitherto important developments in the treatment of viral hepatitis B, clinical cure of chronic HBV infection with HBsAg loss is achievable only in a few treated patients while eradication of HBV infection appears unrealistic. Development of new pharmacological agents acting at multiple targets of the replicative cycle of HBV may achieve higher efficacy and even cure of CHB.

ABBREVIATIONS

ADV adefovir dipivoxil
CHB chronic hepatitis B
ETV entecavir
LAM lamivudine
LdT telbivudine
NA nucleos(t)ide analogue
TDF tenofovir

1. INTRODUCTION

Hepatitis B is a common viral disease with higher global prevalence than that of the human immunodeficiency virus (HIV) and of hepatitis C virus (HCV) infection (Lavanchy, 2004; Mulkay, 2012; WHO, 2012).

Infection with the hepatitis B virus (HBV) can cause acute and chronic necroinflammation and fibrosis of the liver known as acute and chronic hepatitis B (CHB), respectively. In both conditions, hepatitis B surface antigen (HBsAg), a glycoprotein of the envelope of HBV itself, initially referred to as Australia Antigen (Au Ag) (Blumberg, Alter, & Visnich, 1965), is positive in serum (Blumberg, Sutnick, & London 1969; Hadziyannis, 2011a). It has also been realized that in chronic HBV infection, the liver usually exhibits

necroinflammatory changes and fibrosis that may deteriorate and progress to the development of cirrhosis and even of hepatocellular carcinoma (HCC) (Bianchi & Gudat, 1979; El-Serag, 2012; Hadziyannis, 2007b; Hadziyannis, Merikas, & Afroudakis, 1970; Sherlock, Fox, Niazi, & Scheuer, 1970).

Despite the fact that these important observations on liver pathology in chronic HBV infection were already reported from the 1970s, no treatment whatsoever for CHB could be envisioned for several years. On the other hand, a safe and effective HBV vaccine aiming at primary prevention of HBV infection was developed in the 1970s and started to be applied first in high risk groups and subsequently in the general population of several countries, particularly those of high HBV endemicity (Krugman, McAuliffe, & Purcell, 1981; Maupas et al., 1981; Szmuness et al., 1980). Of course, vaccination and other measures aiming at prevention of HBV infection were not expected to protect from progressive liver damage in persons already harboring chronic HBV infection. In such individuals with CHB, liver-related morbidity and mortality were found to be high with a risk for early death either from end-stage liver disease (cirrhosis) or from HCC, ranging between 15% and 25% (Colvin et al., 2010). The current WHO and other estimates continue to be alarming, indicating that globally there continues to exist a very large pool of chronic HBV infection with 350–400 million people affected and with an annual number of HBV-related deaths exceeding 600,000 (Lavanchy, 2004; WHO, 2012). In a recent report, it was estimated that worldwide, HBsAg seroprevalence has increased from 223 million in 1990 to 240 million in 2005 (Ott, Stevens, Groeger, Wiersma, 2012).

Certainly, the improvement in the socioeconomic and hygiene level globally combined with vaccination programs against HBV, and other measures of prevention of HBV transmission have already been shown to be associated with a decrease in the incidence of new HBV infections and of HCC, at least in young age groups (Chang et al., 1997; McMahon et al., 2011; Zanetti, Van Damme, & Shouval, 2008). However, though morbidity and mortality from chronic viral B liver disease and HCC emerge from the early years of the infection, their magnitude increases several decades later (Lok, 2011; Szpakowski & Tucker, 2012). Thus, looking at the future, even in an optimistic way, a significant reduction in the overall HBV morbidity and mortality derived from effective worldwide HBV vaccination programs can be expected to be achieved several years or even decades from now (Hadziyannis, 2011a; Zanetti et al., 2008). Therefore, and for the time being, expectations for further reduction in HBV-related morbidity and

mortality from advanced stages and complications of CHB, including HCC, are linked with the efficacy and availability of existing treatment options (Liaw, 2011) and their application to large numbers of chronically HBV-infected people. In consequence, the question of "whom, when, and how to treat" has become the most clinically relevant one, and all treatment guidelines are renewed and revised at frequent intervals (EASL, 2009, 2012; Liaw et al., 2012; Lok & McMahon, 2007, 2009a, 2009b). Of course, if treatment of CHB can contribute to significant reduction in morbidity and mortally, it has to be accompanied by appropriate screening programs for identifying HBV-infected persons in the community and with adequate monitoring of the infected individuals in the various geographical areas of the world. In connection with such treatment strategies, it should be taken into account that even in countries of originally low HBV endemicity such as the United States, France, and Germany, and despite a further significant decrease in the incidence of new HBV infections, the overall HBV prevalence has not decreased, this being an effect of a lot of immigration from areas of high HBV endemicity, such as Asia, Africa, and Eastern Europe (Cohen et al., 2008; Wasley et al., 2010).

In view of the above remarks, it is understandable why the present clinical chapter had to deal mainly with current treatment efforts in CHB with hitherto approved drugs focusing on their short-, medium-, and even long-term efficacy, their tolerance, side effects, resistance profiles; and other properties permitting their current classification and ranking in clinical practice as first- or second-line and first or second choice therapies for patients with CHB. However, depending on the natural course of CHB infection and on several viral (Lin & Kao, 2011; Sonneveld et al., 2012), host (Sonneveld et al., 2012), and other factors, patients may present with greatly differing clinical, serological, biochemical, and liver histological profiles that determine the decision for treatment or no treatment (Bonino, Piratvisuth, Brunetto, & Liaw, 2010; Perrillo, Hou, Papatheodoridis, & Manns, 2010), which may remain stable or change significantly over time (Hadziyannis, 2011b). It was, therefore, thought necessary first to review when and how HBV infection becomes chronic, then summarize the various phases in its natural course and their characteristics, including associated and superimposed conditions, comorbidities and complications, and then proceed to a critical review of the various facets of current anti-HBV treatment. However, prior to the description of the natural course of chronic HBV infection, a flashback subchapter from the discovery of HBV to the recognition of the wide spectrum of chronic HBV infection was thought to be of order.

2. A FLASHBACK FROM THE DISCOVERY OF HBV TO THE RECOGNITION OF THE WIDE SPECTRUM OF CHRONIC HBV INFECTION

Up to the mid-1960s, nothing was really known about viral hepatitis B and its natural history. Then in 1967, the scenery of hepatitis B was opened up by the demonstration that Australia antigen, discovered in the early 1960s by Blumberg et al. (1965), is specifically associated with viral hepatitis type B and that it actually represented the coat protein of HBV itself (Bayer, Blumberg, & Werner, 1968; Blumberg, Gerstley, Hungerford, London, & Sutnick, 1967; Giles, McCollum, Berndtson, & Krugman, 1969). Worldwide seroepidemiological studies of Au Ag, subsequently renamed HBsAg, disclosed in the 1970s that HBV infection is a major global public health problem, that individuals persistently positive for HBsAg harbor chronic HBV infection, and that on the basis of their characteristics they can be divided in two categories: (a) those with chronic liver disease referred as CHB patients and (b) those without any clinical, biochemical, or other evidence of underlying liver damage, referred as "healthy" HBsAg carriers (Bianchi & Gudat, 1979; Hadziyannis, Gerber, Vissoulis, & Popper, 1973; Hadziyannis, Merikas, Panetsos, & Kourepi, 1972; Hoofnagle & Alter, 1984).

Then, another HBV protein, the hepatitis B e antigen (HBeAg) discovered in 1972 (Magnius & Espmark, 1972), was added as a serological marker of HBV replication, and the concept that patients with CHB were always HBeAg-positive while the so-called healthy HBsAg carriers were invariably HBeAg-negative/anti-HBe-positive was advanced (Hoofnagle et al., 1981). Chronic HBV infection was thus divided in two groups one labeled as "replicative" and the other as "nonreplicative," respectively, and a proposal was made and became widely accepted that they represented two sequential phases in the natural history of chronic HBV infection rather than independent types of CHB (Hoofnagle & Alter, 1984; Yim & Lok, 2006). It was also proposed and largely accepted that after HBeAg loss and development of seroconversion to anti-HBe, chronic HBV infection becomes inactive and that ongoing HBsAg seropositivity was the result of secretion of surface proteins of the virus expressed in hepatocytes harboring HBV DNA sequences integrated in their genome (Hadziyannis et al., 1987; Shafritz, Shouval, Sherman, Hadziyannis, & Kew, 1981; Yim & Lok, 2006). During the same period of the 1980s, studies in Asian patients also revealed that a

number of patients with chronic HBV infection, mostly children and young adults, were HBeAg-positive and had very high HBV DNA levels, but serum aminotransferases and liver histology were nearly normal (Chu et al., 1985; Yuen & Lai, 2011). This was considered to be an immune tolerant state, and then the natural history of chronic HBV infection consisting of three phases was proposed (Chu et al., 1985). At the same time, the existence of CHB with negative HBeAg and positive anti-HBe was recognized in the Mediterranean area, first on the basis of immunofluorescence studies and then by assays of serum HBV DNA, and this second type of CHB was added to the natural history of chronic HBV infection as a late phase usually resulting from reactivation of HBV replication (Fattovich, Bortolotti, & Donato, 2008; Hadziyannis & Papatheodoridis, 2006; Hadziyannis & Vassilopoulos, 2001a; Fig. 7.2). The application of more sensitive assays for the measurement of serum HBV DNA by polymerase chain reaction (PCR) methods confirmed the earlier Mediterranean findings on anti-HBe-positive CHB and the earlier terms "replicative" and "nonreplicative", phases, proposed in 1981, were modified to "high" and "low" replicative, respectively (Yim & Lok, 2006). Then, to a scheme of the natural history of chronic HBV infection proposed in 1995 by Hadziyannis that consisted of four phases (Hadziyannis, 1995), a fifth phase of HBsAg clearance was added (EASL, 2012; McMahon, 2009), and a nomenclature of the various phases, based both on the HBeAg/anti-HBe status and on host immune activity against the replicating virus, was adopted (Hoofnagle, Doo, Liang, Fleischer, & Lok, 2007; Liaw & Chu, 2009; Lok, Heathcote, & Hoofnagle, 2001; McMahon, 2009).

3. DEVELOPMENT OF CHRONIC HBV INFECTION AND THE PHASES IN ITS NATURAL COURSE

Depending on the age and modes of HBV transmission, acute HBV infection may become chronic at highly variable rates. In the case of materno-fetal and vertical neonatal transmission of HBV, the rates of transition of the acute infection to chronic have been reported to be extremely high exceeding 80%. Acute HBV infection in infancy and childhood may become chronic at much lower rates ranging between 20% and 30%, while in healthy nonimmunocompromised adults, the rates of transition to chronicity have been well documented to be extremely low (Hadziyannis, 2011b). Moreover, data from sequencing of the infecting HBV strains have revealed that in order of an acute HBV infection to become chronic, the sine

qua non prerequisite is that the infecting HBV strain is of wild type (wt) at least in its precore and/or basic core promoter region and, therefore, producing HBeAg. Otherwise, acute infection will run a self-limited course regardless the age at and the mode of its acquisition (Cote et al., 2000; Hadziyannis & Vassilopoulos, 2001b).

Once HBV infection becomes chronic, its subsequent course can be divided into four or even five phases of variable severity, duration and outcome of the underlying liver disease (Figs. 7.1 and 7.2). These phases are usually, but not necessarily, sequential and evolution from one to the next and backward to the previous can occur. All phases have been linked pathogenetically to the level of HBV replication, the strength of the host immune reactivity against the replicating HBV, and the interplay between the host and the virus (Bertoletti et al., 2010; Hadziyannis & Vassilopoulos, 2001b).

The first two phases are associated with HBeAg seropositivity (Feld & Heathcote, 2006), while the others develop after clearance of HBeAg and development of anti-HBe immunity (Chen & Yang, 2011; Milich, 1989,

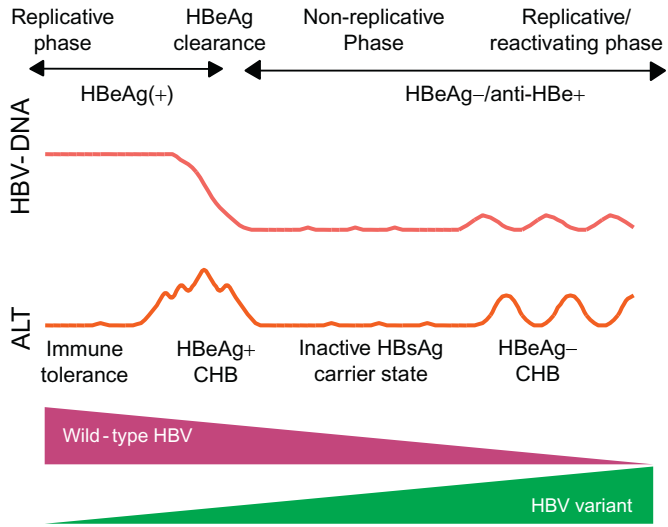

Figure 7.1 The four phases of the natural course of chronic HBV infection are depicted in this figure in a sequential way together with a schematic configuration of the molecular changes of the virus that result in prevention of HBeAg formation without affecting its replicative efficacy. *With permission from Hadziyannis, 2011b.*

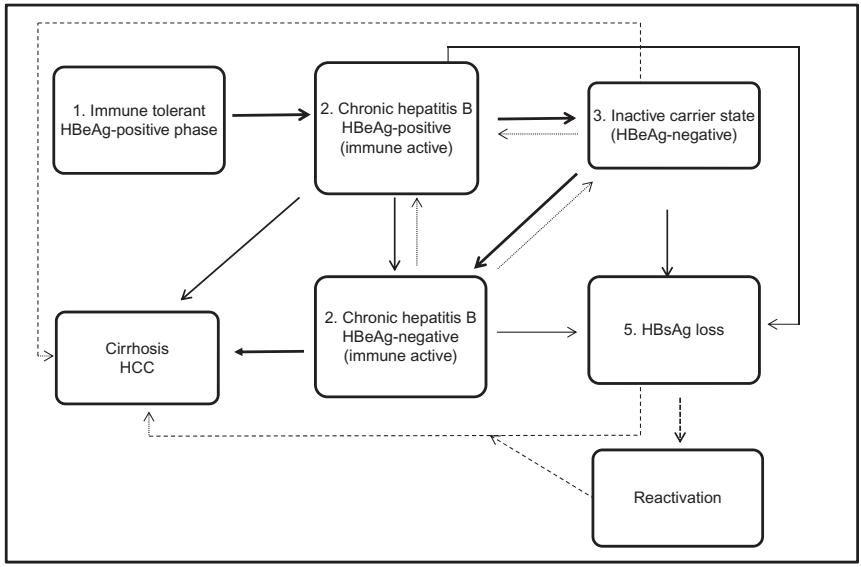

Figure 7.2 Algorithmic configuration of the natural history of chronic HBV infection. The four phases in the natural history of chronic HBV infection as well as the fifth one of HBsAg clearance are not necessarily sequential ones and jumping from one to another and backward may occur as depicted in the algorithm of this figure. In HBeAg seronegative patients remaining in a persistently inactive state, HBsAg clearance may occur at a rate ranging from 0.5% to 1% per year (Chu & Liaw, 2007). As shown in this figure, the underlying liver disease may progress to cirrhosis and HCC. Such an unfavorable outcome cannot be ruled out even in persons who have cleared HBsAg.

1991). Loss of HBsAg signals a most favorable outcome of chronic HBV infection and is generally regarded as the closest one to cure of the infection. This may occur spontaneously or can be induced by treatment in any phase of the natural history of chronic infection at different, however, rates. Moreover, transition from one to the next phase of chronicity is not recognizable in all patients either because it may not be an obligatory step in the overall natural course of the infection or because it is of very short duration.

3.1. The first phase

This phase is considered to represent a state of host immune tolerance against the virus, permitting its replication usually at very high levels. During this phase, HBeAg is positive, serum HBV DNA levels are very high, HBV infectivity is high, but serum alanine aminotransferase (ALT) activity is normal, and hepatic histology reveals little if any liver damage. Despite the close

link of the immune tolerant phase to vertical HBV transmission in neonates, such a phase can also occur in horizontal HBV transmission from HBeAg-positive individuals to toddlers but at significant lower rates ranging from 20% to 30% (Liaw, Brunetto, & Hadziyannis, 2010).

The duration of the immune tolerant phase is variable. In vertical HBV transmission from HBeAg-positive mothers, it is usually long and may last for more than three decades, while under other conditions, as in horizontal HBV spread among children, it appears to be very short and hardly recognizable (Yim & Lok, 2006). The infecting HBV genotypes and racial, nutritional, or environmental factors also appear to play an important role in the development and duration of the first phase (Hadziyannis, 2011b).

3.2. The second phase

The initial immune tolerance phase of HBeAg-positive chronic HBV infection leads to a second one of immune reactivity against HBV that is implicated pathogenetically in the development of liver necroinflammation and fibrosis. In clinical practice, this phase is referred as HBeAg-positive CHB but the terms "immune reactive," "immune active," or "HBeAg clearance phase" are also used (Hadziyannis, 2011b; Liaw et al., 2010; McMahon, 2009). Serum continues to be HBeAg-positive, HBV DNA levels are usually high, ALT levels are increased and liver histology reveals necrosis, inflammation, and variable stages of fibrosis. The severity, duration, and outcome of this phase and of the underlying liver damage are also variable. The annual rate of spontaneous HBeAg clearance in this phase of CHB is ranging from 3% to 12% and increases by two to three times with interferon alpha (IFN-α) treatment. The phase of HBeAg-positive CHB may end not only in HBeAg seroconversion but also in HBsAg clearance and seroconversion to anti-HBs (Buster et al., 2008; Hansen, Buster, Steyerberg, Lesaffre, & Janssen, 2010). However, in a number of patients, HBV replication may continue despite HBeAg loss and development of anti-HBe antibodies (see Fig. 7.2).

3.3. The third phase

Although the previous phase of immune reactivity against HBV may have unfavorable outcomes with progression of the underlying liver necroinflammation and fibrosis to cirrhosis and even to development of HCC and death (Fig. 7.2), it terminates, sooner or later in HBeAg clearance and transition to a third phase. During this third phase, anti-HBe is positive,

there is little residual viral replication (Manesis et al., 2003), and liver histology is essentially normal, except for the presence of "ground-glass" hepatocytes (Hadziyannis et al., 1973) harboring cytoplasmic HBsAg, mainly attributed to its translation from HBV DNA sequences integrated into the genome of hepatocytes. This third phase actually corresponds to the so-called inactive HBsAg carrier state (Kumar et al., 2008).

3.4. The fourth or reactivation phase

The previous phase of HBeAg-negative/anti-HBe-positive inactive HBsAg carrier state is not synonymous with permanent termination of HBV replication and of HBV-induced chronic liver damage. Although the majority of patients may remain for life in an inactive HBsAg carrier state, and a number of them (around 2% per year) may also lose HBsAg and enjoy a complete recovery, others retain or redevelop over time significant HBV replication and progressive liver damage (Chu, Hung, Lin, Tai, & Liaw, 2004; Fattovich et al., 2008; Hadziyannis, 1995; Hadziyannis et al., 1983; Hadziyannis & Papatheodoridis, 2006; Liaw et al., 2010; Liaw & Chu, 2009; Lok et al., 1984).

This state of HBV-induced liver damage has been first referred to as "HBeAg-negative/anti-HBe-positive CHB," and similarly to HBeAg-positive CHB, it also represents an immune active phase in the natural course of chronic HBV infection. It is generally viewed as a fourth phase in the natural history of chronic HBV infection usually developing because of reactivation of HBV replication, though in some patients, it may immediately follow the second phase of HBeAg-positive CHB despite clearance and even seroconversion of HBeAg (Liaw et al., 2010; McMahon, 2009; Fig. 7.2).

It is noteworthy that up to the early 1980s, all HBsAg+/anti-HBe+ patients were considered to harbor a completely inactive HBV infection without liver necroinflammation, with integrated HBV sequences coding for HBsAg production. In such individuals, episomal HBV replication was thought as completely and permanently eliminated (Hadziyannis, 2011a; Lai & Liaw, 2010). It took almost 20 years to realize that this concept was an oversimplification of the natural course of chronic HBV infection and that development of CHB can also occur after HBeAg loss and seroconversion to anti-HBe. Thus, for historical reasons, it may be of interest to note that while HBeAg-negative CHB was first recognized in the Mediterranean area in 1981 (Hadziyannis, 1981; Hadziyannis et al., 1983), its existence was

largely disputed at least up to the discovery in 1989 of precore HBV mutants with a G1896A mutation resulting in a novel precore translational stop codon at position 28 that prevents HBeAg formation without affecting HBV replication (Brunetto et al., 1993; Carman et al., 1989). These molecular findings gave a convincing answer to those hepatitis B experts who for almost 3 years were rejecting from publication the above mentioned early observations of Hadziyannis et al. because they believed that " . . . after loss of HBeAg and development of anti-HBe immunity the HBV can neither continue to replicate or to reactivate and therefore that after HBeAg clearance development of CHB is not possible." Of course, these old beliefs eventually proved completely wrong, and in recent reviews (Fattovich et al., 2008; Yim & Lok, 2006), it is clearly stated that understanding the molecular basis of HBV replication in HBeAg-negative patients represents a major milestone in hepatitis B research and in revealing its natural history. Nevertheless, it was only in the 2000s with a delay of two decades that HBeAg-negative CHB was formally accepted as a phase in the natural history of chronic infection with the wt HBV (Lok et al., 2001; McMahon, 2009).

HBeAg-negative/anti-HBe-positive CHB being usually a late phase in the course of chronic HBV infection is manifested among relatively older age groups of patients with a mean age difference from HBeAg-positive CHB of 10 or more years (Hadziyannis & Vassilopoulos, 2001a). Its prevalence, initially thought to be low and geographically restricted, has subsequently been found to increase and to become predominant worldwide. Currently, HBeAg-negative CHB represents the far commonest type of CHB particularly in European, African, and Middle East countries of the Mediterranean Basin (EASL, 2009; Fattovich et al., 2008; Funk, Rosenberg, & Lok, 2002).

3.5. The fifth or HBsAg-negative phase

In the natural history of CHB, a number of patients lose HBsAg and may also develop anti-HBs either spontaneously or as a favorable outcome of treatment (Hadziyannis, Sevastianos, Rapti, Vassilopoulos, & Hadziyannis, 2012). This outcome that is currently considered as the closest to cure is not actually equivalent with complete elimination of the risk for development of the life-threatening complication of CHB. Low-level HBV replication may persist with detectable HBV DNA in liver tissue and with either completely negative or very low levels in serum. Such patients are

considered to harbor occult HBV infection (EASL, 2012), and under immunosuppression, particularly under rituximab-containing chemotherapeutic regimens, they may experience dangerous HBV reactivation (Ferri, Govoni, & Calabrese, 2010). Moreover, chronic liver disease may have already progressed to cirrhosis prior to the loss of HBsAg, and thus the risk for development of HCC though significantly lower compared to that in patients without HBsAg loss does not become eliminated (Chu & Liaw, 2007). In fact, even in patients with HBsAg loss and only mild or no changes in liver histology, there is always a risk for hepatocarcinogenesis attributed to the integration of HBV sequences into the genome of hepatocytes (Shafritz et al., 1981).

4. COMPLICATING AND ASSOCIATED CONDITIONS AND COMORBIDITIES

Chronic HBV infection, either *ab initio* or in the long years of its natural history, may be associated with or complicated by several other conditions and comorbidities that have to be taken into account in the diagnostic and therapeutic approach. CHB patients with concomitant, superimposed, and complicating conditions have been excluded from the large registration trials of developing therapies but have been included in subsequent randomized controlled trials (RCTs), observational and other studies and are usually referred to as "special populations." Actually, several of the special populations may be in greater need for treatment compared to those fulfilling the strict inclusion and exclusion criteria adopted in phase III RCTs of anti-HBV agents under development (Rapti & Hadziyannis, 2011). The rational for exclusion of such patients from the large registration trials of drugs under development has been that either CHB was already far advanced to a non-reversible stage (decompensated cirrhosis) or that existing comorbidities could seriously affect the underlying liver disease or that in the presence of certain comorbidities the treatment of CHB might be contraindicated. In the group of special populations of patients belong those with hepatitis D virus, HCV, and HIV coinfections, patients who have undergone transplantation or are immunosuppressed due to chemotherapy or to other treatments, patients with end-stage renal disease under hemodialysis, and also, children and pregnant women (Peters, 2009; Pollicino et al., 2011). In numerous original and review articles, all aspects of treatment of these special categories are discussed, since, as stated, for many of these patients treatment is of a greater importance

compared with the standard patient with CHB. Moreover, in real life, they constitute a significant percentage of the total number of patients with CHB (Rapti & Hadziyannis, 2011).

5. TREATMENT OF CHB

This section dealing with the treatment of patients with chronic HBV infection has been divided into six subsections:

5.1. The rationale for treatment, its aims and ultimate goals.
5.2. Development of the current anti-HBV armamentarium.
5.3. Properties of the hitherto approved drugs.
5.4. When to treat and when to wait under appropriate monitoring.
5.5. How to treat CHB and how to monitor anti-HBV treatment applying appropriate endpoints.
5.6. Limitations and uncertainties of current therapies.

5.1. The rationale for treatment, its aims and ultimate goals

In view of the above described natural history of chronic HBV infection with frequent progression of the underlying changes in hepatic histology to advanced stages of fibrosis, to full blown cirrhosis and liver failure, as well as to development of HCC and death, the administration of drugs with a potential for suppression and, hopefully, elimination of underlying HBV replication has been viewed as a fundamental and sine non qua prerequisite for efficacious treatment (Liaw, 2011, 2013). The initial aim of such a treatment would be to achieve certain changes in biochemical, serological and virological markers of HBV replication and of liver disease activity and in the long run to reach the ultimate goal to prevent or delay the development of advanced fibrosis/cirrhosis and of its life-threatening complications including HCC (Hadziyannis, 2006, 2007a; Lee & Keeffe, 2011; Liaw et al., 2011, 2004; Perrillo et al., 2010). Efforts toward these goals have lead to the development of the current anti-HBV armamentarium consisting of seven approved drugs.

5.2. The development of the current Anti-HBV armamentarium

Although the need for therapy of CHB has been recognized already from the 1970s, it was only in 1992 that a therapeutic armamentarium against hepatitis B started to be constructed. Actually up to the late 1980s, all treatment efforts in chronic HBV infection had been failing one after the other. Prednisone

administration proved to be of no benefit and even harmful to patients, though after its discontinuation some beneficial effects were reported (Perrillo, 2009). Immunotherapeutic efforts with BCG and levamisole were also of no benefit, and attempts for antiviral therapy with adenine arabinoside (ARA-A), ARA-AMP, and with other agents proved noneffective and even toxic.

The first fruitful clinical efforts in the treatment of CHB are linked with the availability in sufficient amounts of IFN-α, a substance with antiviral and immunomodulatory properties, produced by the recombinant DNA technology as well as by lymphoblastoid cell cultures (Dusheiko et al., 1986; Hoofnagle et al., 1988; Thomas, Scully, & McDonald, 1986). Several RCTs of IFN-α were conducted in those days, and eventually in 1992, conventional IFN-α was approved for the treatment of CHB (Perrillo, 2009). For many years, the indication for IFN-α therapy in CHB was restricted only to HBeAg-positive patients with increased ALT levels aiming at HBeAg loss and its seroconversion and at return of ALT to normal (Dusheiko et al., 1986; Hoofnagle et al., 1981; Thomas et al., 1986; Weller et al., 1982). Promising results on the efficacy of conventional IFN-α in HBeAg-negative CHB were also reported in relatively small trials and observational studies mostly from Europe (Brunetto et al., 2003; Hadziyannis, 1995; Lampertico et al., 1997; Lok et al., 2001; Manesis & Hadziyannis, 2001), but no large multicenter RCT was carried out in the HBeAg-negative subset of CHB patients. Then in 2001, a large multicenter RCT was conducted on the efficacy of Peg-IFN-α-2a in HBeAg-negative CHB (Marcellin et al., 2004), and the formal indications for treatment of CHB with conventional IFN-α (Lok et al., 2001) were expanded with Peg-IFN-α-2a to include both HBeAg-positive and HBeAg-negative CHB (Lau et al., 2005; Marcellin et al., 2004).

In the meantime, in 1998, 6 years after the approval of conventional IFN-α, the nucleoside analogue (NA) lamivudine (LAM), a reverse transcriptase inhibitor, originally designed for HIV infection, was approved for CHB and opened a new treatment scenery. It is important to stress, at least from an historical point of view, that during the period of the late 1990s, when LAM entered the field of therapy of CHB and of prophylaxis of graft infection in the setting of liver transplantation, the pharmacological intervention in hepatitis B was actually revolutionized (Lok et al., 2001). That period represents the most remarkable time in the history of anti-HBV treatment in clinical practice. The drug worked and lives were saved. However, soon thereafter, it became obvious that development of resistance to LAM, due to selection of HBV mutants resistant to the antiviral activity of

this drug, was a major problem culminating in failure of treatment (Lok et al., 2007; Thai et al., 2012). Fortunately, things improved significantly with the introduction of newer NA, including initially adefovir dipivoxil (ADV) (Hadziyannis et al., 2003; Mailliard & Gollan, 2003), and subsequently entecavir (ETV) (Hirsch, 2007) and tenofovir (TDF) (Heathcote et al., 2011; Marcellin et al., 2008), both possessing strong anti-HBV activity (Fig. 7.3), very high barriers to HBV resistance, and no cross resistance (Table 7.1; Yuen & Lai, 2011). The latter two oral antivirals and peg-IFN-α currently represent the first-line therapies for CHB making things relatively easy for practicing clinicians (Perrillo et al., 2010). The information that has accumulated over the years on their indications, efficacy, and side effects is quite ample, and most treatment guidelines for hepatitis B, formulated in most geographical areas and countries, are regularly revised and adapted on the basis of strength and quality of new evidence for efficacy of the approved medications (EASL, 2009, 2012; Liaw et al., 2012; Lok & McMahon, 2009a, 2009b).

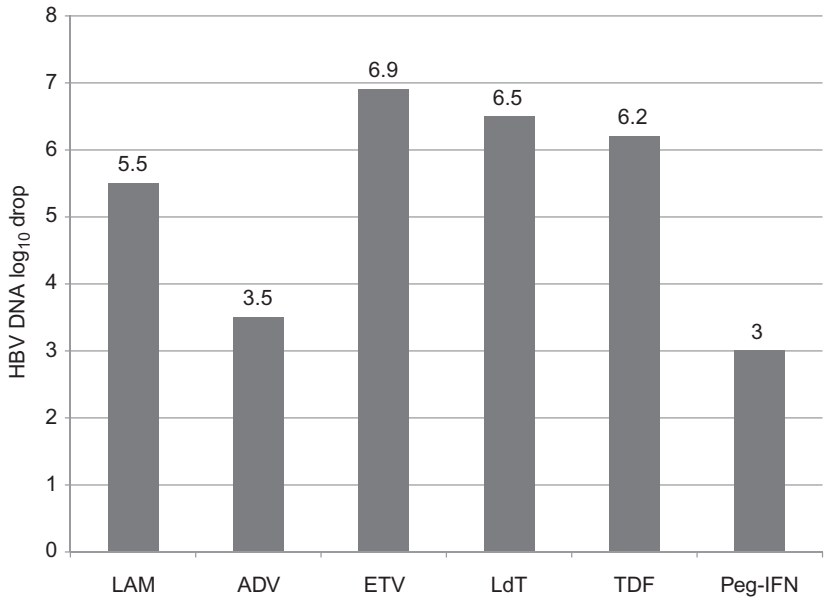

Figure 7.3 The potency of the seven approved drugs for the treatment of chronic hepatitis B as measured on the basis of decline of serum HBV DNA levels in log10 achieved at 48 weeks of therapy is shown in this histogram. The data are derived from different studies and not from head to head comparisons (Lok, 2012). LAM, lamivudine; ADV, adefovir; ETV, entecavir; TDF, tenofovir; LdT, telbivudin; Peg-IFN, pegylated interferon-α-2α.

Table 7.1 The most frequent HBV mutations that confer resistance and cross resistance to nucleoside analogues are shown in the first column in terms of amino acid changes

	Drug susceptability				
HBV aa substitutions	**LAM**	**LdT**	**ETV**	**ADV**	**TDF**
Wild type	S	S	S	S	S
M2014V	R	S	I	I	S
M204I	R	R	I	I	S
L180M + M204V	R	R	I	I	S
A181T/V	I	S	S	R	S
N236T	S	S	S	R	I
L180M + M204V/I ± I169T ± V173L ± M250V	R	R	R	S	S
L180M + M204V/I ± T184G ± S202I/G	R	R	R	S	S

The level of susceptibility to the five approved nucleos(t)ide analogues is indicated as S = sensitive, I = intermediate susceptibility, and R = resistant.
Adapted from EASL (2012).

During the same period, significant progress has also been made in the understanding of the pathogenetic mechanisms involved in acute and chronic HBV infection and in chronic liver damage induced by the HBV. Moreover, meaningful primary and secondary endpoints of treatment have been introduced in the evaluation of the efficacy of new and old anti-HBV drugs (Hadziyannis & Vassilopoulos, 2012). Several endpoints such as sustained suppression in HBV replication, reduction in histological activity, HBeAg seroconversion, and loss of HBsAg, have been introduced and are currently applied in clinical practice as surrogate markers toward the achievement of the mentioned ultimate goals of treatment: to prevent/delay the development of advanced fibrosis/cirrhosis and of its life-threatening complications as well as the development of HCC and death (Hadziyannis, 2006, 2007a; Lee & Keeffe, 2011; Perrillo et al., 2010).

Yet, despite the hitherto important developments and progress in the treatment of CHB, clinical cure of chronic HBV infection with HBsAg loss is achievable only in few treated patients while eradication of HBV infection appears as a completely unrealistic goal.

5.3. Properties of the hitherto approved drugs

In this part of the chapter, the overall properties of the seven currently approved anti-HBV drugs, including their mechanisms of action, safety, dosage and dose adjustments, modes of action, tolerance, side effects, and

potency, are reviewed while strategies and options for their application in the treatment of the various subsets of patients with CHB are dealt with in Section 5.5 on how to treat.

5.3.1 Interferons

The two IFNs approved for the treatment of CHB are conventional IFN-α-2a and -2b and Peg-IFN-α-2a. They possess a number of antiviral, antiproliferative, and immunomodulatory properties (Thimme & Dandri, 2012). Conventional IFN-α-2b has been used for almost 30 years in the treatment of CHB, while over the past decade, it has been practically replaced by Peg-IFN-α-2a.

IFN-α exerts its multiple actions, after binding to its specific receptor (IFN-α/β receptor complex) on target cells, through the transcription of more than 100 genes (IFN-stimulated genes) (Garcia-Sastre & Biron, 2006). These genes code for different proteins involved in inflammation, antiviral activities, signal transduction, mediation of apoptosis, and inflammation. In general, IFN-α induces an antiviral state in target cells, rendering them resistant to new viral infection. In hepatitis B, studies in cell cultures, transgenic, and humanized mice have shown that IFNs lead to decrease of core particle formation, degradation of pregenomic RNA and core particles, as well as to decreased cccDNA transcription of genomic and subgenomic HBV RNAs (Belloni et al., 2012; Thimme & Dandri, 2012). At the same time, the therapeutic effect of IFNs in HBV infection appears to be mainly due to their immunomodulatory action. IFNs make infected cells more susceptible to apoptosis and lysis from cytotoxic T and/or natural killer (NK) cells, while at the same time, they lead to activation and proliferation of NK cells (Thimme & Dandri, 2012). The effect of IFNα on CD8+ T cells appears to be more complicated, with some recent studies showing reduction of CD8+ T cells (end-stage effector) without restoration of their effector function in CHB (Thimme & Dandri, 2012).

The main side effects of IFNs include cytopenias (anemia, leucopenia, thrombocytopenia), flu-like syndrome after injections, mood changes, induction of autoantibodies, and induction or exacerbation of autoimmune diseases such as Hashimoto's thyroiditis and systemic lupus erythematosus (Perrillo, 2009). During or after completion of therapy, 25–40% of patients may develop flares of hepatitis which may lead to HBeAg seroconversion or even to HBsAg clearance, but in certain cases, they are associated with liver decompensation (especially in patients with advanced cirrhosis). Moreover, IFNs should be used with caution in patients with renal dysfunction and are contraindicated in clinically overt cirrhosis (Lok & McMahon, 2009a, 2009b).

The need for daily subcutaneous injections is another disadvantage of the use of conventional IFNs.

Overall, Peg-IFN-α-2a is better tolerated than conventional IFN and is applied only once weekly (Perrillo, 2009). In the registration trials, drug discontinuation has been reported in 6–9% of patients, while dose modifications were necessary in 31–47%. Serious adverse events have been recorded in 4–5% of patients.

The approved dose of Peg-IFN-α-2a for HBeAg CHB (positive or negative) is 180 mcg given subcutaneously weekly for 1 year. Although there have been specific recommendations for the use of Peg-IFNs in patients with renal impairment and in patients with chronic hepatitis C, there are no such recommendations available for CHB (Ghany, Strader, Thomas, Seeff, 2009). Peg-IFN is contraindicated during pregnancy.

5.3.2 Nucleoside and nucleotide analogues

5.3.2.1 Lamivudine (LAM or 3TC)

LAM or 3TC is the first L-NA that has been approved for the treatment of CHB in 1998. It is the negative enantiomer of 2′,3′-dideoxy-3′-thiacytidine that is being phosphorylated by host kinases in resting peripheral blood mononuclear cells to form its active compound, the active LAM triphosphate form (Jarvis & Faulds, 1999). LAM triphosphate causes the termination of chain elongation (negative and positive DNA strand) when incorporated in DNA transcripts during reverse transcription. The target of LAM is the reverse transcriptase domain of the HBV polymerase. The drug does not interfere with HBV DNA integrated in host DNA (Jarvis & Faulds, 1999). There is no evidence also of interference with human DNA synthesis, and thus, no cytotoxicity has been observed in human cell lines or mitochondria (Jarvis & Faulds, 1999). The medication is well absorbed after oral intake with a terminal half-life of 6 h and is then mainly excreted by the kidneys.

A number of *in vitro* and *in vivo* studies in animals have demonstrated the antiviral activity of LAM (Jarvis & Faulds, 1999). In humans, treatment with LAM for 1 year results in suppression of HBV DNA by 5.5 $\log_{10}$ copies/ml in HBeAg-positive and by 4.7 $\log_{10}$ copies/ml in HBeAg-negative CHB, whereas undetectable HBV DNA levels (<300–400 copies/ml) were seen in 44% and 73% of patients, respectively (Dienstag, 2009). Short- or long-term HBV viral suppression by LAM has been also associated with partial restoration of HBV-specific CD8+ T cell antiviral action (Boni et al., 2012, 2003; Thimme & Dandri, 2012).

LAM has an excellent safety profile with very few side effects reported. During treatment, mild elevations in ALT have been noted with similar frequency as in the control population.

The medication is given per os at a dose of 100 mg/day. The dose should be adjusted in patients with renal impairment. More specifically, for creatinine clearance (CrCl) between 30 and 49 ml/min, the recommended dose is 50 mg/day, for 15–29 ml/min is 25 mg/day, for 5–14 ml/min is 15 mg/day, while for <5 ml/min is 10 mg/day (Lok & McMahon, 2009a, 2009b). During pregnancy, LAM is categorized a category C drug (risk cannot be ruled out in humans).

5.3.2.2 Telbivudine (LdT or TBV)

Similar to LAM, LdT is an L-nucleoside analog (β-L-2′-deoxythymidine). The drug is rapidly absorbed after oral intake and is converted in liver cells by cellular enzymes to its phosphate form. This active form acts as a chain terminator leading to the inhibition of synthesis of DNA strands (negative and positive). No effect in human DNA polymerases has been observed.

LdT given as monotherapy in CHB has an excellent short- and long-term safety profile, with very few side effects reported (Hadziyannis & Vassilopoulos, 2008). Rare instances of LdT-induced myopathy manifested mainly by asymptomatic elevations in creatine phosphokinase have been reported, although in certain cases, symptomatic myopathy has developed (Fontana, 2009).

The medication is given at a dose of 600 mg/day pos. The dose should be adjusted in patients with renal insufficiency. More specifically, for CrCl between 30 and 49 ml/min, the recommended dose is 600 mg every 48 h, for <30 ml/min is 600 mg every 72 h, and for patients requiring hemodialysis 600 mg every 96 h (administered after hemodialysis) (Lok & McMahon, 2009a, 2009b). During pregnancy, LdT is categorized as category B drug (no evidence of risk in humans).

5.3.2.3 Adefovir dipivoxil (ADV)

It is an oral nucleotide analogue of adenosine monophosphate. The drug is administered orally in its prodrug form, ADV, which is rapidly converted to adefovir and then after phosphorylation to its diphosphate form (dATP analogue). This medication acts also as a chain terminator of HBV DNA strands. Its plasma half-life is approximately 5–7 h, and the majority of the drug is excreted in the urine by 24 h (90%).

Prolonged administration of ADV in CHB (HBeAg-positive or -negative) is well tolerated. Three to eight percent of patients treated for 5 years developed mild elevations in creatinine levels, which in certain cases was accompanied by hypophosphatemia (Hadziyannis et al., 2006; Marcellin et al., 2008). These side effects were more common in patients undergoing liver transplantation. For patients receiving ADV for more than 1 year, serum creatinine measurement every 3 months is recommended (especially, if renal impairment is present) (Gara et al., 2012).

ADV is given at a dose of 10 mg/day pos. In patients with renal dysfunction, dose adjustment is required; thus, for CrCl of 20–49 ml/min, the dose should be 10 mg every other day, for 10–19 ml/min 10 mg every third day, and for hemodialysis patients 10 mg every week following dialysis (Lok & McMahon, 2009a, 2009b).

5.3.2.4 Entecavir (ENT)

ETV is a carbocyclic analogue of 2′-deoxyguanosine which after phosphorylation to its active triphosphate form inhibits HBV DNA polymerase and the synthesis of the negative and positive strand of HBV DNA (Lok & McMahon, 2009a, 2009b). Following oral administration, the drug reaches peak concentrations in 1 h with an accumulation half-life of approximately 24 h (Lok & McMahon, 2009a, 2009b). The drug is excreted mainly by the kidneys by glomerular filtration and tubular secretion.

Its safety profile is excellent, similar to what has been reported in patients receiving LAM.

For nucleoside-naïve patients, the recommended dose is 0.5 mg/day pos which should be adjusted for the CrCl. Thus, for CrCl of 30–49 ml/min, the recommended dose is 0.25 mg/day or 0.5 mg every 48 h, for 10–29 ml/min is 0.15 mg/day or 0.5 mg every 72 h, and for <10 ml/min or hemodialysis patients is 0.05 mg/day or 0.5 mg every 7 days (Lok & McMahon, 2009a, 2009b).

For LAM-refractory or resistant patients, the approved dose is 1.0 mg/day which is adjusted for CrCl <50 ml/min; for CrCl 30–49 ml/min, the dose is 0.5 mg/day or 15 mg every 48 h, for 10–29 ml/min is 0.3 mg/day or 1 mg every 72 h, and for < 10 ml/min or hemodialysis patients is 0.1 mg/day or 1 mg every 7 days (Lok & McMahon, 2009a, 2009b).

ENT has been classified as a drug of pregnancy risk category C, meaning that a risk cannot be ruled out. (EASL, 2012).

5.3.2.5 Tenofovir (TDF)

TDF is an oral NA of adenosine monophosphate. The drug is administered orally in its prodrug form, TDF disoproxil fumarate, which is rapidly converted to TDF. Its mode of action and pharmacokinetics are similar to ADV (see above).

Most data regarding the safety of TDF were available until recently in HIV-treated patients. In these patients, mild nephrotoxicity and hypophosphatemia developed in 4% of the cases, while rarely, cases of low bone mineral density or osteomalacia, Fanconi syndrome, nephrogenic diabetes insipidus, and acute renal failure have been reported (Fontana, 2009; Gupta, 2008). In recent long-term trials of 3 or 5 years duration in patients with CHB, the prevalence of elevated creatinine or hypophosphatemia in patients treated with TDF was much lower (~1%) (Heathcote et al., 2011; Marcellin et al., 2012). In HIV patients, regular monitoring of serum creatinine, phosphate, and urinalysis is recommended (Fontana, 2009).

The approved dose of TDF is 300 mg/day pos. The dose should be adjusted for renal insufficiency as follows: CrCl: 30–49 ml/min = 300 mg every 48 h, 10–29 ml/min = 300 mg every 72–96 h, <10 ml/min in hemodialysis = 300 mg once a week or after 12 h of dialysis. Similarly to LdT the risk of ENT in pregnancy, has been classified in category B with both drugs representing the preferred NAs if treatment is considered necessary (EASL, 2012).

The potency of the approved drugs in terms of $\log_{10}$ reduction in serum HBV DNA levels as reported in various studies is depicted in Fig. 7.3, while the resistance and cross resistance profile of the five NAs is included in Table 7.1.

5.4. When to treat and when to wait under appropriate monitoring

In view of the natural history of chronic HBV infection, it is conceivable that ideally all HBsAg-positive patients should be viewed as potential candidates for curative treatment. However, considering the properties of currently available anti-HBV drugs and the fact that their application in CHB is not curative of the infection, initiation of therapy is justifiable and is generally recommended only for patients in the immune active phases of its natural course who fulfill certain inclusion criteria and for all those with clinically overt cirrhosis, either compensated or decompensated. Thus, for patients with chronic HBV infection, the question is not on whether to treat or not to treat but on when and how to treat and how to monitor (EASL, 2012; Lok & McMahon, 2009a, 2009b).

The decision to treat a patient with chronic HBV infection is based on several factors, including the patient's age, ALT values, liver histology, presence of cirrhosis (compensated or decompensated), HBV DNA levels, presence of certain contraindications to antiviral treatment, and the patient's preferences (EASL, 2012; Keeffe, Dieterich, Pawlotsky, & Benhamou, 2008; Lok & McMahon, 2007, 2009a, 2009b; Vassilopoulos & Hadziyannis, 2008). Among these factors, ALT and HBV DNA levels, as well as the grade/stage of the underlying liver disease as assessed by liver biopsy, are the most critical for therapeutic decision making.

HBeAg-positive patients with normal or nearly normal ALT levels and high serum HBV DNA levels usually belong to young age groups and are in an early immune tolerant phase of chronic HBV infection. Treatment is not effective and is not indicated in this phase of chronic HBV infection. If applied, it is rarely, if ever, followed by HBeAg loss and seroconversion. Thus, they must be monitored biochemically, virologically, and serologically and treatment initiated when the immune active phase of HBeAg-positive CHB develops.

For HBeAg-positive patients with elevated ALT and HBV DNA values, an initial observation period of 3 to 6 months is recommended before initiation of treatment, because spontaneous HBeAg seroconversion may occur (EASL, 2012). Following such an observation period, patients with elevated ALT ($>2\times$ the upper limit of normal ULN), moderate/severe necroinflammation and/or fibrosis on liver biopsy, and with high HBV DNA levels ($>20{,}000$ IU/ml or $>10^5$ copies/ml) should be treated (EASL, 2012; Liaw et al., 2012; Lok & McMahon, 2007, 2009a, 2009b). In these patients, the endpoint of treatment is loss of HBeAg and development of anti-HBe (HBeAg seroconversion) followed by a consolidation period of 6 and, possibly more months (Tong, Hsien, Hsu, Sun, & Blatt, 2008; Vassilopoulos & Hadziyannis, 2009).

HBeAg-negative patients with persistently normal or nearly normal ALT levels, undetectable or low HBV DNA levels ($<2{,}000$ IU/ml), and absence of moderate/severe necroinflammation and/or fibrosis on liver biopsy (inactive HBsAg carrier state) do not benefit from current antiviral therapies so treatment is not recommended for them (Chen, Huang, Chu, & Liaw, 2012; EASL, 2012; Lok & McMahon, 2009a, 2009b; Papatheodoridis, Manolakopoulos, Liaw, & Lok, 2012). However, close monitoring of ALT and HBV DNA levels every 3–6 months is essential for these patients.

For HBeAg-negative patients, initiation of therapy is indicated if ALT values are $>2\times$ ULN, serum HBV DNA levels are $>2{,}000$ IU/ml or

10,000 copies/ml, and on liver biopsy, there is moderate/severe necro-inflammation and/or fibrosis (Papatheodoridis et al., 2012). Suppression of HBV replication sustained after stopping IFN-based regimens given for 1 or 2 years or maintained during or after stopping prolonged therapy with oral NAs represents the virological endpoints of therapy (EASL, 2012; Hadziyannis et al., 2012).

Both for treated and untreated patients appropriate and long-term monitoring is most important and relevant for clinical decision making regarding further management of the disease.

5.5. How to treat and monitor CHB

Treatment strategies and choice of drugs may differ according to the HBeAg/anti-HBe status, the levels and patterns of serum HBV DNA and of ALT, and the stage of underlying liver disease, as well as by the presence of complications, superimposed conditions, comorbidities (coinfection with HIV, HCV, hepatitis D virus), and other factors (Peters, 2009; Rapti & Hadziyannis, 2011; Thomas & Zoulim, 2012; Zhang , Liu, & Jia, 2010).

This part of this chapter deals with how to treat the various subsets of patients with CHB, except special populations, using the approved anti-HBV drugs either in finite courses of therapy or indefinitely. Finite courses of therapy usually last for 1 and up to 2 years, and all approved drugs can be used. They are aiming at virological suppression and biochemical remission that can be sustained after stopping treatment (SVR, sustained virological response).Long-term/indefinite drug administration (even for lifetime) applies only to NAs and is aiming at suppression of HBV replication maintained under ongoing treatment referred as MVR (maintained virological response).

Both HBeAg-positive and HBeAg-negative CHB patients have been included in the pivotal phase III registration trials of hitherto approved anti-HBV agents and comprise individuals with histologically mild, moderate, and even advanced fibrosis (cirrhosis). Treatment strategies and options in patients with decompensated cirrhosis and in other so-called special populations have been evaluated in subsequent trials, and observational studies have been reviewed critically in several articles (Rapti & Hadziyannis, 2011), while the quality and strength of the accumulated evidence is evaluated carefully in most recent treatment guidelines (EASL, 2012; Liaw et al., 2012; Lok & McMahon, 2009a, 2009b).

5.5.1 How to treat HBeAg-positive CHB

5.5.1.1 Finite courses

Finite courses of therapy of 1-year duration with any of the approved drugs represents a reasonable choice aiming at HBeAg seroconversion, which is usually associated with virologic and biochemical responses that can be sustained after stopping therapy. Currently, 48 weeks of treatment with Peg-IFN-α has the best chance for achieving an SVR with an estimated HBeAg seroconversion rate of 32% (Janssen et al., 2005; Lau et al., 2005), compared to 12–18% with LAM, 12% with ADV, 21% with ETV, 22% with TBV, and 21% with TDF (EASL, 2009, 2012; Lok & McMahon, 2007). Also, compared with oral NAs, IFN-α is superior in terms of HBsAg clearance, which occurs in 3–4% of patients treated with Peg-IFN-α and 0–3% of those receiving NAs (Lok & McMahon, 2007). In 40–50% of those HBeAg-positive patients who achieve, mostly by Peg-IFN-α therapy, sustained HBeAg seroconversion and remain in virological remission, HBsAg loss and development of anti-HBs occurs in 5- to 10-year time (Buster et al., 2008; EASL, 2012).

Since the chances for HBeAg seroconversion in HBeAg-positive patients treated for 1 year or so with ADV is only 12% and in view of the fact that the risk for development of HBV resistance is high among highly viremic patients treated with LAM or LdT, particularly if HBeAg seroconversion is not achieved during the first year of therapy, it appears reasonable that NA monotherapy with any of these three compounds cannot be a first choice treatment option. However, if other compounds are not available or affordable, then these three NAs can be used as short-term treatment options aiming at HBeAg seroconversion. This is particularly true for patients who harbor baseline viramia levels lower than 10^7 copies/ml provided that they achieve HBV DNA nondetectability at week 24 of treatment (Lai et al., 2006; Lok et al., 2012). In clinical practice, a final decision on how to treat cannot be taken unless the patient is properly informed about treatment options and on contraindications, side effects, inconveniences, and cost of IFN versus oral NAs.

5.5.1.2 Longer treatment options

Longer treatment options for HBeAg-positive CHB patients usually represent an extension of short-term courses under which HBeAg seroconversion could not be achieved. Prolongation of NA treatment has been associated with gradually increasing HBeAg seroconversion rates, provided that resistance to the applied NAs does not develop. With LAM and ADV treatment

of 5-year duration, HBeAg seroconversion reached levels of 50% and 48%, respectively (Lok & McMahon, 2007; Marcellin et al., 2008). In the case of prolongation of treatment with the potent first-line compounds ENT or TDF of high barrier to HBV resistance, >90% of patients under ongoing therapy remain with practically nondetectable serum HBV DNA, and approximately, half of them may also lose HBeAg (Gish et al., 2007; Heathcote et al., 2011; Pan et al., 2012).

5.5.2 How to treat HBeAg-negative CHB

5.5.2.1 Short-term treatment options

In the vast majority of patients with HBeAg-negative CHB, none of the currently approved NAs administered for 1 year can achieve the goal of sustained virologic and biochemical response after stopping therapy. However, 1- to 2-year courses of conventional IFN-α and 48 weeks of Peg-IFN-α-2a represent a reasonable first-line therapeutic option because they induce sustained biochemical and virological responses in approximately 20% of patients. Moreover, within 3–4 years after stopping treatment, HBsAg loss progressively increases to levels above 10% reaching 32% at post treatment year 7 among sustained biochemical responders (Lampertico et al., 2003; Manesis & Hadziyannis, 2001). It is noteworthy that even patients with histologically developed but clinically compensated cirrhosis may achieve an SVR, and many have been reported to achieve improvement in fibrosis of more than two histological stages (Schiff et al., 2008).

5.5.2.2 Long-term treatment options

As in HBeAg-positive CHB, long-term treatment options for patients with HBeAg-negative disease are restricted to oral NAs. Currently, these agents are applied in the treatment of HBeAg-negative patients who do not respond or do not consent to finite courses of IFN-α therapy. As stated, oral NAs also represent the only treatment option for patients with decompensated cirrhosis.

The best results of long-term NA therapy in HBeAg-negative CHB have been reported with a 4–5 years administration of ADV during which approximately 70% of patients achieved normal ALT and nondetectable serum HBV DNA levels without development of HBV resistance (Hadziyannis et al., 2006). The highly potent antiviral compounds, ENT and TDF with a high barrier to HBV resistance, have induced even higher response rates maintained under treatment exceeding 90% (Lai et al., 2006; Marcellin et al., 2012). These data taken together with the backbone safety and efficacy information derived from the long-term trials of ADV

(Hadziyannis et al., 2006), ENT, and TDF (Chang, Lai, et al., 2010; Chang, Liaw, et al., 2010; Heathcote et al., 2011) clearly support the concept that in the absence of curative therapies, long-term administration of first-line NAs in treatment-naïve HBeAg-negative patients is the most suitable way to treat effectively without development of HBV resistance.

5.5.3 How to treat patients with cirrhosis regardless of their HBe status

Viremic patients (HBV DNA >2,000 IU/ml) with compensated cirrhosis, regardless of their HBeAg/anti-HBe status, can be treated safely with IFNs or oral NAs without or with negligible resistance rates. Most studies so far have not demonstrated a significant difference in response rates between patients with and those without cirrhosis (Liaw et al., 2004). However, when oral NAs with a high resistance rate (e.g., LAM) are administered, their benefit (i.e., decrease in the rate of hepatic decompensation and HCC) is frequently offset by the appearance of virologic and biochemical breakthroughs terminating to life-threatening complications. Therefore, the potent first-line NAs ENT and TDF with very high barriers to HBV resistance clearly represent the first-line and first choice therapies for treatment-naïve cirrhotics (EASL, 2012; Lok & McMahon, 2009a, 2009b). Moreover, in patients already under NAs of low barrier to development of HBV resistance, treatment should be changed to a first-line NA lacking cross-resistance with the previously already administered one. Practically, this means switching to TDF in patients previously receiving LAM or LdT and switching to ENT in those already receiving ADV (Perrillo et al., 2010).

In NA-treated cirrhotic patients, either with compensated or with decompensated disease, monitoring of kidney function is mandatory.

5.5.4 How to treat patients with HBV mutants resistant to NAs

The development of drug resistance after long-term monotherapy with oral NAs has been a major problem with significant clinical sequelae (Keeffe et al., 2008). This issue has been illustrated best by long-term studies of LAM and ADV showing that 65–70% and 20–29%, respectively (Hadziyannis et al., 2006; Keeffe et al., 2008), of treated patients develop virologic resistance after 5 years of therapy. LdT treatment has been associated with a resistance rate of approximately 9–22% after 2 years, whereas resistance to ENT and TDF has been reported so far to be negligible or nonexistent (Chang, Lai, et al., 2010; Keeffe et al., 2008). The best treatment strategy for patients who develop drug-resistant mutations has been debated for several years. However, the lack of cross resistance: (a) between ENT and

Table 7.2 Treatment options in cases of drug resistance

Drug resistance	Treatment
LAM	Switch to TDF (add on ADV if TDF not available)
ADV	*NA naïve prior to ADV* Switch to ETV (preferred in high viremia) or TDF *Prior LAM resistance* Switch to ETV + TDF
LdT	Switch to or add on TDF (add on ADV if TDF not available)
ETV	Switch to or add on TDF (add on ADV if TDF not available)
TDF	Not detected to date—genotypic and phenotypic analysis required for confirmation Switch to ETV or add on ETV, LdT, or LAM is suggested

Recommendations on how to manage patients with HBV mutants resistant to nucleos(t)ide analogue therapy.

ADV or TDF and (b) between TDF and LAM or LdT has been well documented (Tables 7.1 and 7.2). On the other hand, it should be emphasized that the detection of viral resistance requires frequent (every 3–6 months) monitoring of HBV DNA levels with sensitive assays. When increases, >1 $\log_{10}$ from the lowest level achieved during therapy are observed, then a more detailed laboratory analysis is recommended to identify the type of mutation and the *in vitro* susceptibility of the mutant viral isolates to the different NAs (see Table 7.1).

In clinical practice, LAM resistance is the most common and best studied type of HBV drug resistance. With the approval of ADV in 2002, treatment with this compound was documented to be quite effective particularly in HBeAg-negative patients, with adding ADV on LAM being superior than switching to it (Rapti, Dimou, Mitsoula, & Hadziyannis, 2007; Sheng et al., 2011; Vassiliadis et al., 2010). But newer evidence in such patients indicates that the most effective and safe option is to switch to TDF (EASL, 2012; Liaw et al., 2012).

The management of CHB in patients with HBV resistance to the various NAs is dealt with in several clinical practice guidelines (EASL, 2012; Lok & McMahon, 2009a, 2009b). In general, the latest data are favoring the switch rather than the addition of the first-line nucleotide analogue TDF in cases of development of resistance to any of the nucleoside analogues and also of the switch to the first-line nucleoside analogue ENT in cases of development of resistance to the nucleotide analogues ADV or TDF.

5.5.5 Monitoring and endpoints of treatment

Since the ultimate goal of treatment in CHB is to prevent the progression of underlying liver necroinflammation and fibrosis to cirrhosis, to end-stage liver disease, and to development of HCC, and since these clinical outcomes usually occur after a long course of infection lasting for several decades, it becomes practically unfeasible to evaluate prospectively the efficacy of various therapies toward preventing such unfavorable long-term outcomes. Therefore, the short- and intermediate-term efficacy of treatments, as evaluated on the basis of virological, biochemical, serological, and histological responses achieved under treatment or after its discontinuation, has been extrapolated to the future of treated patients, and these "endpoints" of therapy are applied as surrogate markers of a final clinical outcome of CHB (Chotiyaputta & Lok, 2010; Hadziyannis & Vassilopoulos, 2012).

Endpoints of therapy in CHB have first been used in the treatment of HBeAg-positive CHB with conventional IFN-α and have been extended thereafter to the evaluation of the efficacy of NAs both in HBeAg-positive and HBeAg-negative CHB (Hadziyannis & Vassilopoulos, 2012). Because of the relatively late description and recognition of HBeAg-negative CHB (Hadziyannis & Papatheodoridis, 2006), all major multicenter trials in this type of CHB have been conducted with delay, and the relevant reports have appeared in the world literature mostly during the past 5 years or so (Chotiyaputta & Lok, 2010; Hadziyannis & Vassilopoulos, 2012). In general, endpoints applied to the treatment of HBeAg-negative CHB patients have been similar to those in HBeAg-positive ones except, of course, for the nonapplicable endpoint of HBeAg seroconversion. However, their predictive value regarding medium- and long-term outcomes of CHB has been found to exhibit several differences. A typical example is that in NA therapy of HBeAg-negative but not of HBeAg-positive CHB, on-treatment endpoints are lacking of any predictive value at least regarding virological and biochemical responses sustained after stopping therapy (de Franchis et al., 2003; Lok & McMahon, 2007). Moreover, HBeAg-negative CHB differs from the HBeAg-positive one not only in the HBeAg/anti-HBe status but also in other aspects and particularly in its natural course (Hadziyannis & Papatheodoridis, 2006). Therefore, endpoints of NA therapy and their application during and after treatment differ between HBeAg-positive versus HBeAg-negative CHB patients as well as in their predictive values as surrogate biomarkers of short-, medium-, and long-term outcomes of the disease.

On-treatment endpoints may or may not predict what the intermediate posttreatment (medium-term) outcome is going to be. Therefore, endpoints of therapy should not be viewed as indicators for termination of treatment unless they possess a high positive predictive value for sustained response.

Endpoints actually correspond to the goals of therapy to suppress HBV replication, to normalize serum ALT levels, to decrease liver necroinflammation and reverse fibrosis; and in the long run, to prevent cirrhosis, liver failure, and HCC.

First, in order comes the achievement of an optimum *virological endpoint* which is a profound suppression of HBV replication, without development of HBV resistance and with serum HBV DNA becoming and remaining undetectable by sensitive PCR assays (EASL, 2009, 2012; Lok & McMahon, 2007). Then come the *serological endpoints*, also referred by some authors as virological endpoints, being HBeAg loss and seroconversion to anti-HBe (in HBeAg-positive CHB) and HBsAg loss with or without seroconversion to anti-HBs (both in HBeAg-positive and HBeAg-negative CHB). Loss of HBsAg is the only endpoint that can predict reliably a favorable long-term outcome of CHB without cirrhosis and hepatic decompensation and with very rare development of HCC (see Fig. 7.2). However, HBsAg loss rarely occurs in NA treatment both of HBeAg-positive and HBeAg-negative CHB (Chotiyaputta & Lok, 2010; Tana & Hoofnagle, 2012). The achievement of profound suppression of HBV replication is associated with the *biochemical endpoint* of return of ALT levels to normal, and in clinical practice, this is referred as biochemical remission. Finally, *histological endpoints* consist of improvement in the hepatic necroinflammatory score by 2 or more points without deterioration of fibrosis (Chotiyaputta & Lok, 2010; Feld, Wong, & Heathcote, 2009). It is noteworthy that in a number of patients treated with NAs, significant regression of fibrosis and even reversion of cirrhosis can be achieved (Chang, Liaw, et al., 2010; Gish et al., 2007; Hadziyannis et al., 2006; Marcellin et al., 2012; Ono et al., 2012; Tana & Hoofnagle, 2012), and this actually represents a strong and most desirable endpoint. In an ongoing very long-term trial of TDF, such favorable outcome has been reported at year 5 of treatment in 87% of patients, with an apparent regression of cirrhosis in 71% of those with an Ishak score of 5 or 6 (cirrhosis) at baseline (Marcellin et al., 2013; Tana & Hoofnagle, 2013).

5.6. Limitations and uncertainties of current therapies

Regardless of the favorable overall results and of the general progress achieved in the treatment of CHB, it is important that clinicians treating and monitoring patients with CHB are aware of uncertainties, limitations, and drawbacks of current therapies and consider certain problems that may be encountered and result in reduced efficacy and even in treatment failure.

5.6.1 HBeAg-positive patients

a. The goal of HBeAg loss can be achieved by finite courses of NA treatment, of 1- or 2-year duration, only in approximately 20% of patients. It increases to 25–30% and to almost 50% after 2 and 5 years of therapy, respectively (Chotiyaputta & Lok, 2010; Lau, 2010), but treatment can be stopped only if anti-HBe develops and after a consolidation period of 6–12 months has been completed.

b. A stable HBeAg seroconversion is usually followed by sustained virological and biochemical remission but monitoring of patients should be continued since HBV reactivation and development of HBeAg-negative CHB is also possible. It should also be noted that the results are better in younger age groups and in genotype B and D infections compared to genotype C (Chotiyaputta & Lok, 2010; Lau, 2010).

c. In patients not achieving HBeAg seroconversion under the second line NAs as LAM and ADV, treatment should be extended in time using compounds with a high genetic barrier to HBV resistance, while adherence to therapy should be carefully evaluated. Good news is that in a clinical trial of long-term NA therapy with TDF reaching year 7, no instances of HBV resistance were encountered and that up to year 5, in addition to HBeAg seroconversion, 10% of patients had lost HBsAg (Heathcote et al., 2011).

d. HBeAg-positive patients who do not achieve seroconversion to anti-HBe under NA therapy with TDF or ENT may remain in virological (undetectable HBV DNA by sensitive PCR assays) as well as biochemical remission during treatment (Chang, Lai, et al., 2010; Heathcote et al., 2011), and a significant proportion of them can also achieve histological endpoints with improvement of liver necroinflammation as well as with reversion of fibrosis (Chang, Lai, et al., 2010; Chang, Liaw, et al., 2010). Even so, these promising endpoints cannot predict that such responses under long-term therapy will be sustained after treatment is discontinued.

e. On-treatment HBeAg seroconversion may be a good surrogate marker of *intermediate* post-treatment outcomes, but it remains questionable whether the unfavorable long-term ones of development of cirrhosis and of HCC are actually prevented and to what extent.

5.6.2 HBeAg-negative CHB patient

a. Finite courses of antiviral treatment with NAs, even with the newer ones that combine high antiviral potency and high genetic barrier to HBV resistance, fail to achieve virological and biochemical responses that are sustained after stopping treatment. This is true although all on-treatment endpoints are easily reached and maintained in the vast majority of patients (Ayoub & Keeffe, 2011; Fung, Lai, & Yuen, 2011).

b. Finite course of treatment with IFN-α may achieve a good number of sustained responses, including HBsAg loss in small percentages, but they have various side effects and are contraindicated in a number of patients.

c. Virological and biochemical remission maintained under long-term antiviral therapy in HBeAg-negative patients is associated with impressive rates of histological response in terms of liver necroinflammation and reversal of fibrosis and cirrhosis (Hadziyannis et al., 2006; Marcellin et al., 2012). Achievement of these histological endpoints under NA therapy actually represents the best hitherto treatment paradigm in HBeAg-negative CHB. Even so, the risk for the development of HCC or HBV reactivation may not be eliminated, and therefore, discontinuation of treatment cannot be advocated regardless of costs and of possible long-term side effects (Tana & Hoofnagle, 2012).

d. Discontinuation of long-term (for 4 and 5 years) NA treatment in HBeAg-negative patients with CHB genotype D has been attempted in a recent study in which the included patients had compensated liver disease without histological or other evidence of cirrhosis. Approximately, 30% of the prospectively followed-up patients have experienced HBsAg loss within 5 years after discontinuation of treatment (Hadziyannis et al., 2012). However, such a promising new treatment paradigm has to be further evaluated in larger number of patients infected with various HBV genotypes, and if validated, it may become clinically applicable at least for patients who have not developed cirrhosis.

6. FUTURE TREATMENTS FOR CHB

From the above remarks on current therapies in CHB, it becomes obvious that the current state-of-the-art of antiviral treatment in this disease has clearly progressed to a significant extent but it still remains far from being satisfactory. Thus, in HBeAg-positive patients, the rate of HBeAg seroconversion is relatively low, HBV may reactivate after stopping therapy both in HBeAg-positive and HBeAg-negative patients, and long-term administration of antiviral agents increases the medical costs and the adverse effects of the applied drugs (Thomas & Zoulim, 2012). Possibly long-term HBV suppression by treatment with NAs could be increased if followed by or combined with IFN-α or with efficient immunotherapies (Ayoub & Keeffe, 2011; Liaw, 2013; Perrillo, 2009; Wang et al., 2013; Zhang et al., 2012). However, in any case, eradication of HBV infection is not foreseeable by current pharmacological or other forms of treatment (Thomas & Zoulim, 2012). Of course, the goal of a long-term outcome with serum HBV clearance, HBsAg loss and seroconversion is generally viewed as almost equivalent to cure (EASL, 2012; Hadziyannis et al., 2012; McMahon, 2009). However, this goal is achievable with currently available drugs only in a limited percent of treated patients. It is, therefore, imperative that, similar to other chronic viral diseases such as HCV and HIV infection, new safe drugs acting at other targets of the life and replication cycle of HBV (and not only at its reverse transcription) become available (Abdul et al., 2012; Beck & Nassal, 2007; De Clercq, 2012; Kim, Kim, & Seong, 2010; Wen, Lin, & Ma, 2003; Zheng et al., 2012).

New treatment options may include novel pharmacological agents in the pipeline acting at several targets inhibiting the replication cycle of the HBV from its entry to hepatocytes to the secretion of new hepatitis B virions from the cytoplasm of infected hepatocytes (Kock et al., 2010; Nassal, 2009; Fig. 7.4). Examples of efforts for development of such antiviral agents are: (a) Myrcludex, CPP, and other HBV entry inhibitors (Abdul et al., 2012; Krepstakies et al., 2012; Lucifora, Esser, & Protzer, 2012; Schieck et al., 2013; Volz et al., 2013); (b) epigenetic factors acting at cccDNA (Guo et al., 2007; Guo, Li, Mu, Zhang, & Yan, 2009); (c) SiRNA (in the generation of the minimichrosome) (Zhang, Cheng, et al., 2010); (d) IFN-λ in the formation of pregenomic HBV RNA and its encapsidation (Hong et al., 2007; Pagliaccetti, Chu, Bolen, Kleinstein, & Robek, 2010); (e) capsid

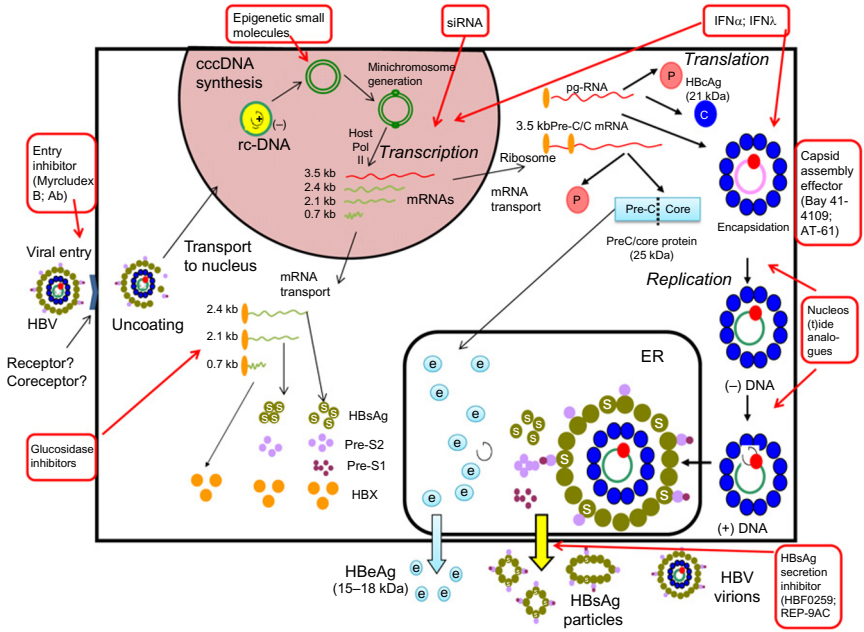

Figure 7.4 Replicative cycle of the HBV and targets of current and future treatments. This figure has been designed to show the following steps of HBV replication: attachment of HBV on hepatocytes, viral entry and uncoating, nuclear entry, formation of cccDNA from relaxed circular HBV DNA, transcription, translation, capsid formation, reverse transcription, viral maturation, and coating with surface protein and release to the circulation. Current targets of treatment and potential future ones are also depicted.

assembly effectors (Bay 41-4109, At-61, HAP12, HAP13, AT-130) (Billioud, Pichoud, Puerstinger, Neyts, & Zoulim, 2011; Brezillon et al., 2011; Li, Chirapu, Finn, & Zlotnick, 2013); (f) glycosidase inhibitors acting at the formation of small-, medium-, and large surface proteins (Kaur, Gupta, & Puri, 2011); (g) inhibitors of secretion of HBsAg spheres and tubules and of HBV particles (HB FO 359, REP-9AC, and CPP) (Abdul et al., 2012; see Fig. 7.4).

In the above context, advances in our knowledge of the molecular mechanisms regulating cccDNA stability and its transcriptional activity at the RNA, DNA, and epigenetic levels in the course of chronic HBV infection may also reveal new potential therapeutic targets for anti-HBV drugs aiming at silencing and eventually depleting the cccDNA reservoir, thus succeeding not only in long-term suppression but also in eradication of

HBV from the liver of chronically infected patients (Levrero et al., 2009; Locarnini & Zoulim, 2010; Thomas & Zoulim, 2012).

7. CONCLUSION

The treatment of CHB has been enriched over the past decade with highly potent oral NAs with high barriers to HBV resistance and the pegylated form of IFN-α-2a that provide the clinicians who take care of CHB patients with an expanding array of safe and efficacious therapeutic options. Current oral treatments of CHB can suppress, in the vast majority of patients, HBV replication to serum HBV DNA levels close to or under the level of its detectability by real-time PCR assays. Under their long-term administration, virological, biochemical, and histological improvement is maintained in the vast majority of treated patients and with extension of treatment to 5 and more years, even developed cirrhosis may regress in two-thirds of patients.

The acquisition of new knowledge on the natural course and pathogenesis of chronic HBV infection and the results of short- and long-term trials have contributed significantly in the formulation of specific guidelines for best treating the different subsets of chronically infected patients. Furthermore, the experience gained from treating patients with HIV infection has underscored the importance of monitoring HBV DNA levels and looking for drug resistance before and during treatment with oral NAs. However, despite these important steps toward more efficacious therapies of chronic HBV infection, outcomes of treatment are still far from satisfactory, and the answers to several questions on the optimal duration of therapy with oral NAs, the role of combination therapy, and the long-term effects of current treatment options on the hard endpoints of therapy, for example, mortality, rate of HCC, transplantation still remain uncertain. Clearance of HBsAg, the closest to cure outcome of chronic HBV infection can be achieved only in a small proportion of treated patients and treatment-induced eradication of HBV infection still remains at the level of wishful thinking. Safe, potent and affordable new anti-HBV agents and strategies are needed to further improve the outcomes of treatment, while clinicians must be able to select the most appropriate agents and schemes of treatment in the various groups and subsets of patients with CHB as well as to evaluate the efficacy of new and old drugs on the basis of realistic and meaningful primary and secondary endpoints of treatment applied in the frame of therapeutic algorithms.

The following recent review articles are proposed to clinicians for further reading: Antonelli and Turriziani (2012), Bonino et al. (2010), Chan et al. (2011), De Clercq (2013, 2012), Doo and Ghany (2010), Frenette and Gish (2009), Hadziyannis (2011b), McMahon et al. (2012), Seto, Fung, Yuen, and Lai (2012), Wong and Sung (2012), and Zoulim and Locarnini (2012).

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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CHAPTER EIGHT

The Holý Trinity: The Acyclic Nucleoside Phosphonates

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Contents

Abstract

The Holý Trinity was named after Dr Antonín Holý (the Holý Trinity being an Unesco recognized monument in Olomouc, Czech Republic), who together with Dr John C. Martin (Gilead Sciences) and myself pioneered a new class of antiviral agents, the acyclic nucleoside phosphonates. These compounds have revolutionized the antiviral drug field with several drugs that have been approved for the treatment of various DNA virus infections (cidofovir), hepatitis B (adefovir), and AIDS (HIV infection; tenofovir). The latter is also available as its oral prodrug, tenofovir disoproxil fumarate, for the treatment of hepatitis B and in combination with emtricitabine ((-)FTC) for the treatment and prophylaxis of HIV infections and in combination with (-)FTC and other HIV inhibitors, that is, efavirenz, rilpivirine, or elvitegravir (and a pharmacoenhancer thereof, cobicistat), for the treatment of AIDS.

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ABBREVIATIONS

CMV cytomegalovirus
DHPA (*S*)-9-(2,3-dihydroxypropyl)adenine
EBV Epstein–Barr virus
(-)FTC emtricitabine
HDP hexadecyloxypropyl
HPMPA (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine
HPMPC (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine
HSV herpes simplex virus
NHL non-Hodgkin's lymphoma
ODE octadecyloxyethyl
PFA foscarnet
PMEA adefovir [9-(2-phosphonylmethoxyethyl)adenine]
PMEG 9-(2-phosphonomethyloxy)guanine
PMPA tenofovir [(*R*)-9-(2-phosphonylmethoxypropyl)adenine]
SAH *S*-adenosylhomocysteine
TDF tenofovir disoproxil fumarate
TFV-DP tenofovir diphosphate
TK$^-$ thymidine kinase deficient
VZV varicella-zoster virus

1. INTRODUCTION

From May 3 to 5, 1976, I participated in a *Symposium on Synthetic Nucleosides, Nucleotides, and Polynucleotides*, organized by the late Karl-Heinz Scheit at the Max-Planck Institut für Biophysikalische Chemie, Göttingen, Germany, where I made the biggest discovery of my life, that is, I met there a small but selected group of nucleoside chemists who, for the rest of my life, would become close collaborators and even close friends. It was in Göttingen, when I met for the first time and started the collaboration with Dr Antonín Holý from the Czechoslovak Academy of Sciences in Prague. In 1982, I met Dr Holý again at the *International Symposium on Nucleic Acid Chemistry in Kyoto*, Japan from November 24 to 26, 1982, and on numerous later occasions, either in Prague or Leuven or elsewhere.

The collaboration I had started with Antonín Holý resulted within 2 years in the identification of an acyclic nucleoside analogue, (*S*)-9-(2,3-dihydroxypropyl)adenine (DHPA) with broad-spectrum antiviral activity (De Clercq, Descamps, De Somer, & Holý, 1978). This was just a few months after acyclovir [9-(2-hydroxyethoxymethyl)guanine] had been described as a selective anti-herpes simplex virus (HSV) agent (Schaeffer

et al., 1978), depending for its antiviral activity on a specific phosphorylation by the virus-encoded thymidine kinase (Elion et al., 1977). From the beginning, it was clear that DHPA, unlike acyclovir, did not depend for its antiviral activity on a virus-induced thymidine kinase. Its activity spectrum, encompassing, among other viruses, specifically vaccinia virus and vesicular stomatitis virus, was clearly different from that of acyclovir and was later found to correlate with an inhibitory effect on *S*-adenosylhomocysteine hydrolase (Cools & De Clercq, 1989; De Clercq & Cools, 1985). Albeit without clinical data, DHPA as Duvira gel was marketed in the Czechoslovak Republic for the topical treatment of herpes labialis.

2. HPMPA

The broad-spectrum antiviral activity of (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) was first reported in 1986 (De Clercq et al., 1986). It was found active against virtually all DNA viruses against which it was tested. Together with its nonenantiomeric sister compound, adefovir 9-(2-phosphonylmethoxyethyl)adenine (PMEA), it was the first isosteric and isoelectronic nucleotide analogue reported to be antivirally active (Fig. 8.1). Although synthesized as an acyclic isosteric nucleotide analogue of dAMP (Fig. 8.1), HPMPA could be structurally conceived as a hybrid molecule between two antiviral compounds [DHPA (see *supra*) and foscarnet (PFA, phosphonoformic acid, foscavir®)]. Although HPMPA was not marketed for clinical use, it has served as the prototype of the acyclic nucleoside phosphonates.

3. CIDOFOVIR

HPMPA paved the way for (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC, cidofovir) which was first described in 1987 as a broad-spectrum anti-DNA virus agent (De Clercq et al., 1987) and approved in 1996 for the treatment of cytomegalovirus (CMV) retinitis in AIDS patients (registered name Vistide®; Fig. 8.2). Cidofovir is active against a broad spectrum of viruses (polyoma-, papilloma-, adeno-, herpes- [herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella-zoster virus (VZV), Epstein–Barr virus (EBV), CMV, human herpesvirus type 6 (HHV-6), type 7 (HHV-7), type 8 (HHV-8), and thymidine kinase-deficient strains of HSV-1, HSV-2, and VZV)] and poxviruses (vaccinia, variola, cowpox, monkeypox,

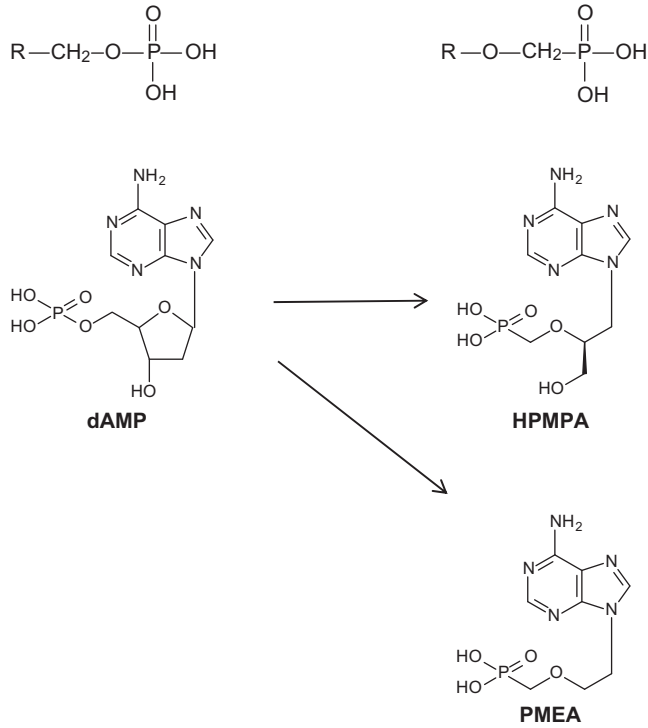


Figure 8.1 First antiviral isosteric and isoelectronic nucleotide analogues.

Figure 8.2 (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC, cidofovir, Vistide®).

camelpox, molluscum contagiosum, orf) (De Clercq, 2004). The target of action is the viral DNA polymerase, which can allow two consecutive incorporations into the viral DNA after the compound has been metabolized intracellularly to its diphosphate (which then acts as an alternative substrate of dCTP; Fig. 8.3). Neither HPMPC nor HPMPA thus acts as obligate chain terminator in the DNA polymerase reaction (Magee et al., 2011), which can obviously be expected from the presence of the 3-hydroxyl group in their acyclic side chain.

From a clinical viewpoint, numerous anecdotal case reports have pointed to the efficacy of cidofovir by local injection in the treatment of hypopharyngeal papilloma (Fig. 8.4; Van Cutsem et al., 1995), laryngeal papilloma (Snoeck et al., 1998), recurrent respiratory papillomatosis in children (Pransky, Magit, Kearns, Kang, & Duncan, 1999), topical treatment of plantar warts (Davis et al., 2000), intravenous injection in the treatment of molluscum contagiosum in AIDS patients (Erickson et al., 2011; Meadows, Tyring, Pavia, & Rallis, 1997; Fig. 8.5), and topical treatment of orf (ecthyma contagiosum) (Fig. 8.6; Geerinck et al., 2001).

Two problems associated with the systemic use of cidofovir are: (i) its nephrotoxicity, that is, when it is administered intravenously at a dosage of ≥ 5 mg/kg and (ii) its lack of oral bioavailability, necessitating topical application. To circumvent these problems, the hexadecyloxypropyl (HDP) and octadecyloxyethyl (ODE) derivatives have been synthesized

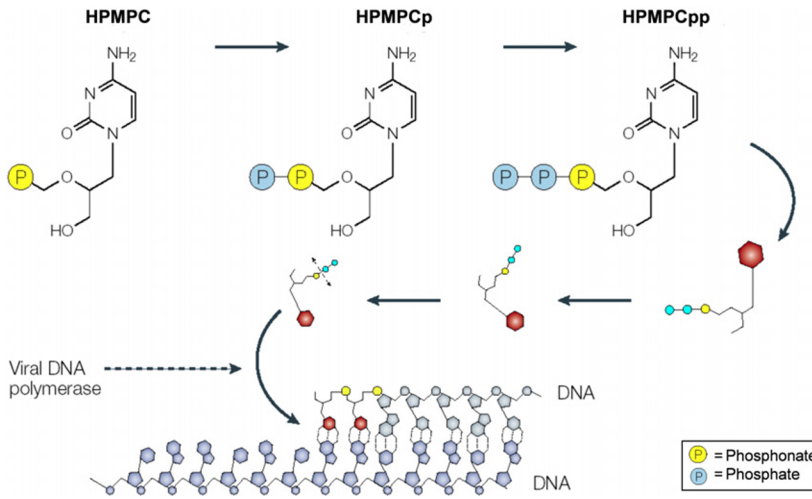


Figure 8.3 Mechanism of action of cidofovir (De Clercq, 2004).

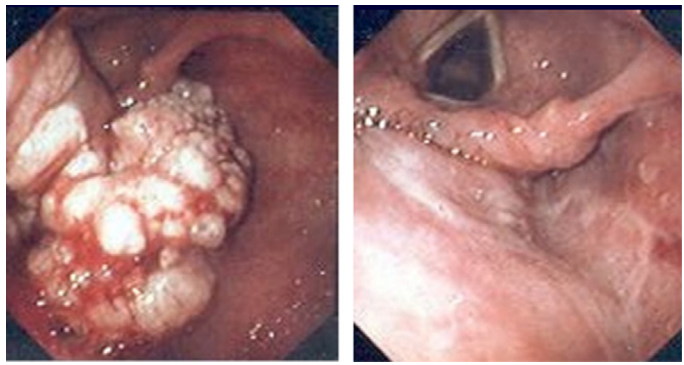


Figure 8.4 Hypopharyngeal papilloma before and after treatment with cidofovir (local injection). Cidofovir was administered by local injection (directly into the tumor) at 1.25 mg/kg at weekly intervals. Complete regression of the tumor van achieved after seven injections (Van Cutsem et al., 1995).

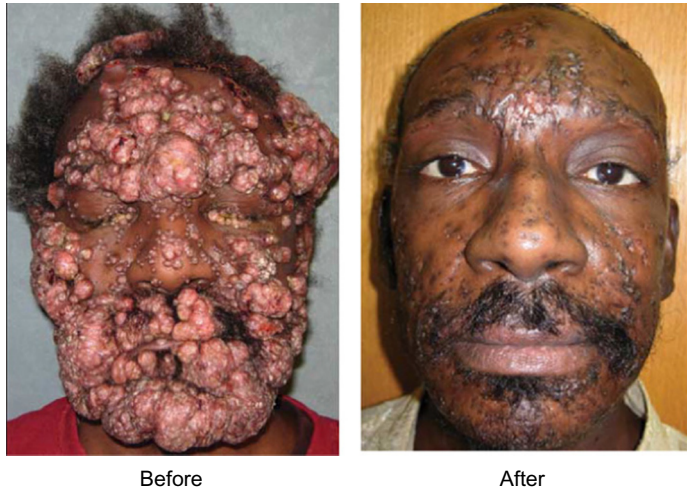


Figure 8.5 Efficacy of intravenous cidofovir (3–5 mg/kg) in the treatment of giant molluscum contagiosum in a patient with human immunodeficiency virus (Erickson, Driscoll, & Gaspari, 2011).

(Hostetler, 2010; Lanier et al., 2010). HDP-cidofovir and ODE-cidofovir (Fig. 8.7) may be useful in the oral treatment of poxvirus infections and other virus infections that are normally amenable to cidofovir therapy and so could be the HDP and ODE derivatives of HPMPA in the treatment of those virus infections falling within the activity spectrum of HPMPA.

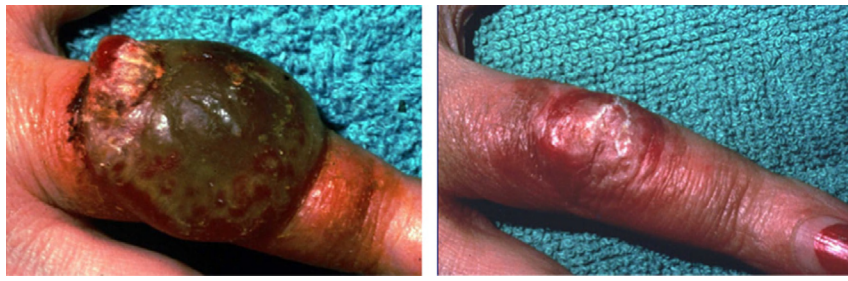


Figure 8.6 Orf (ecthyma contagiosum) before and after treatment with cidofovir (topical 1% cream). Cidofovir was administered topically as a 1% cream in Beeler basis once daily for repeated courses (5 days on/5 days off therapy) with complete resolution of the lesions after seven courses (2–3 months) (Geerinck et al., 2001).

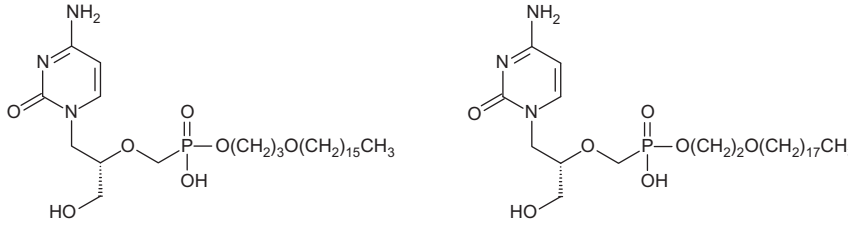


Figure 8.7 Hexadecyloxypropyl (HDP) and octadecyloxyethyl (ODE) derivatives of cidofovir.

4. ADEFOVIR

PMEA was originally described in 1986, concomitantly with HPMPA (De Clercq et al., 1986). In 2002, it was licensed for clinical use for the treatment of hepatitis B in its oral prodrug form [adefovir dipivoxil, the bis (pivaloyloxymethyl)ester of PMEA or bis(POM)PMEA; Hepsera®; Fig. 8.8]. Like HPMPC (cidofovir), adefovir is also active against herpesviruses but much less than cidofovir; however, it is more active against hepadnaviruses, including hepatitis B virus (HBV) as well as retroviruses [human immunodeficiency virus type 1 (HIV-1), human immunodeficiency virus type 2 (HIV-2), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), visna/Maedi virus, feline leukemia virus, murine AIDS (LP-BM5) virus, and murine (Moloney) sarcoma virus; De Clercq, 2004]. After intracellular phosphorylation to its diphosphate, it acts, in competition with dATP, as a straight chain terminator of the DNA synthesis in the reverse transcriptase reaction (Fig. 8.9).

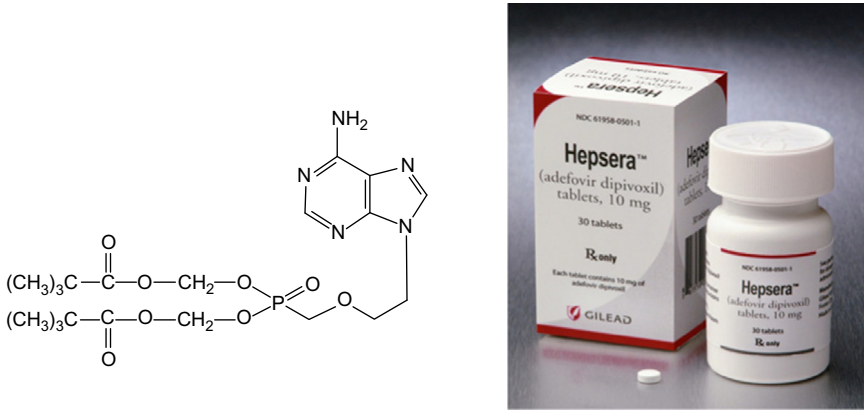


Figure 8.8 Adefovir dipivoxil [Bis(pivaloyloxymethyl)ester of 9-(2-phosphonylmethoxyethyl) adenine, bis(POM)PMEA, Hepsera®].

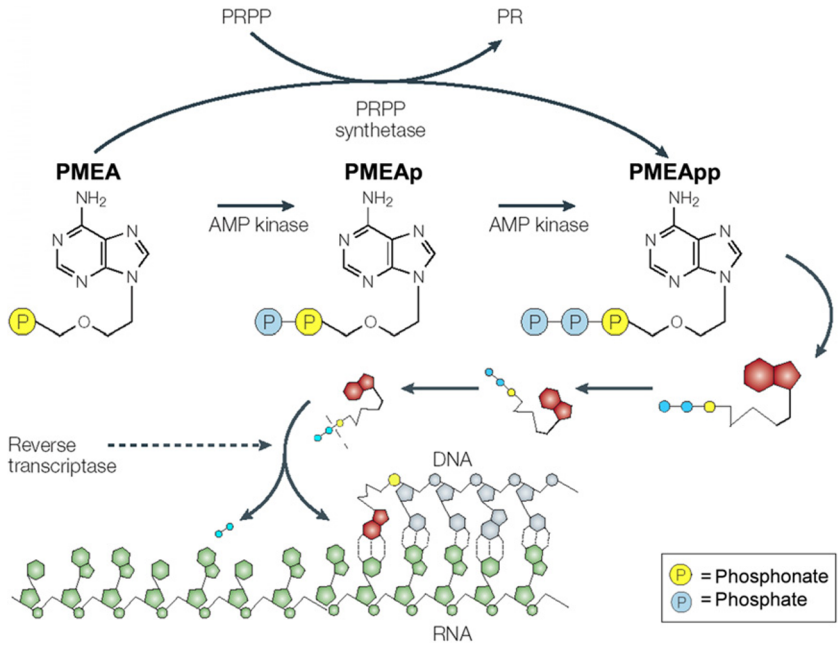


Figure 8.9 Mechanism of action of adefovir (De Clercq, 2004).

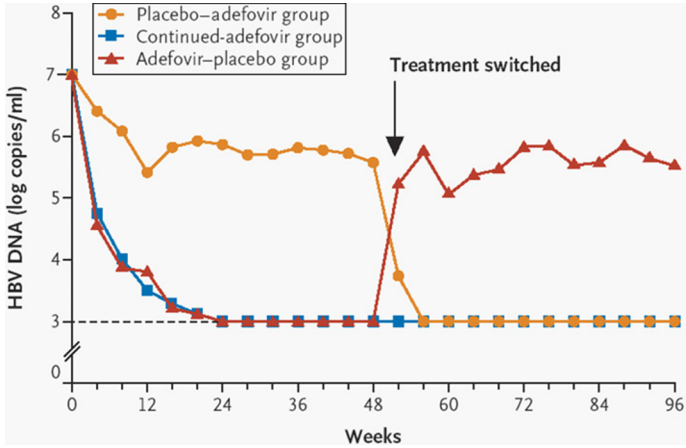


Figure 8.10 Median serum HBV DNA levels through weeks 96 (HBe Ag-negative chronic hepatitis B). The dashed line indicates 3 log copies/ml; which was the lower limit of detection for the assay (Hadziyannis et al., 2005).

From a clinical viewpoint, Hadziyannis et al. (2003) and Marcellin et al. (2003) described the potent antiviral activity demonstrated by adefovir dipivoxil at a daily dose as low as 10 mg in the hepatitis B e antigen-negative chronic HBV patients and antigen-positive chronic HBV patients, respectively. Most convincing were Hadziyannis' results of a crossover study (in HBe antigen-negative chronic HBV patients; Fig. 8.10), which clearly confirmed the efficacy of adefovir dipivoxil in reducing the serum HBV DNA levels over a period of 0–48, 48–96, or 0–96 weeks (Hadziyannis et al., 2005).

5. TENOFOVIR

Tenofovir [(*R*)-9-(2-phosphonylmethoxypropyl)adenine, PMPA] was originally described in 1993 (Balzarini et al., 1993) concomitantly with (*R*)-9-(2-phosphonylmethoxypropyl)-2,6-diaminopurine, which while being more potent and more selective in its anti-HIV activity than PMPA was not further developed. PMPA, on the other hand, was approved for clinical use in its oral prodrug form, tenofovir disoproxil fumarate (TDF) (Viread®) (Fig. 8.11), in 2001 for the treatment of AIDS (HIV infections) and in 2008 for the treatment of chronic hepatitis B (HBV infections). The antiviral activity spectrum of tenofovir is similar to that of adefovir (De Clercq, 2004). Although not considered active against herpesviruses (Balzarini et al., 1993), tenofovir would retain some activity against HSV-1, albeit in the order of (decreasing)

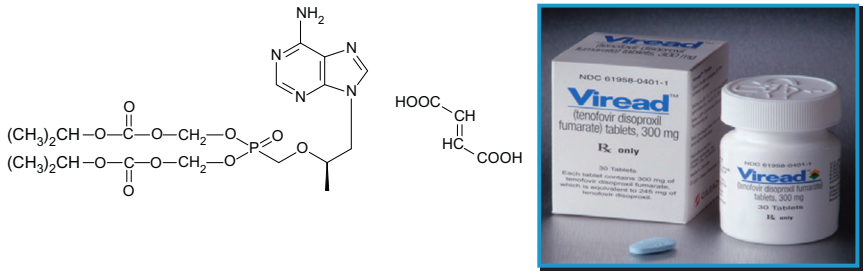


Figure 8.11 Tenofovir disoproxil fumarate (TDF) [Fumarate salt of bis (isopropoxycarbonyloxymethyl) ester of (*R*)-9-(2-phosphonylmethoxypropyl)adenine, bis(POC)-PMPA, Viread®].

potency: cidofovir >adefovir > tenofovir (Andrei et al., 2011). Its mechanism of action is similar to that of adefovir; in its diphosphate form, tenofovir thus acts as an obligate chain terminator of the reverse transcriptase reaction (Fig. 8.12; De Clercq, 2003).

From a clinical viewpoint, TDF was subsequently approved in several single once-daily pill formulations, in 2004, in combination with emtricitabine, Truvada®; in 2006, in combination with emtricitabine and efavirenz, Atripla®; and in 2011, in combination with emtricitabine and rilpivirine (which in the meantime has been registered under the trade name of Edurant®), Complera® (US), or Eviplera® (EU) (De Clercq, 2012). Complera® has been found to maintain HIV suppression in patients switching to Complera from a protease inhibitor-based regimen (Palella et al., 2012).

6. TENOFOVIR COMBINATIONS

For more than a decade, antiretroviral drugs have been recommended for postexposure prophylaxis to prevent sexual transmission of HIV; TDF in combination with emtricitabine ((-)FTC) and raltegravir was recently advocated for this purpose (Mayer, Mimiaga, Gelman, & Grasso, 2012). Similarly, the combination of TDF/(-)FTC with either efavirenz or atazanavir (boosted with ritonavir) has been recommended for initial treatment of HIV, particularly in those patients with a higher initial viral load (Hull & Montaner, 2011; Sax et al., 2011).

Of the four tenofovir-containing regimens recommended by the World Health Organization (WHO) for initial HIV therapy (TDF/lamivudine (3TC)/nevirapine (NVP); TDF/emtricitabine ((-)FTC)/NVP; TDF/3TC/

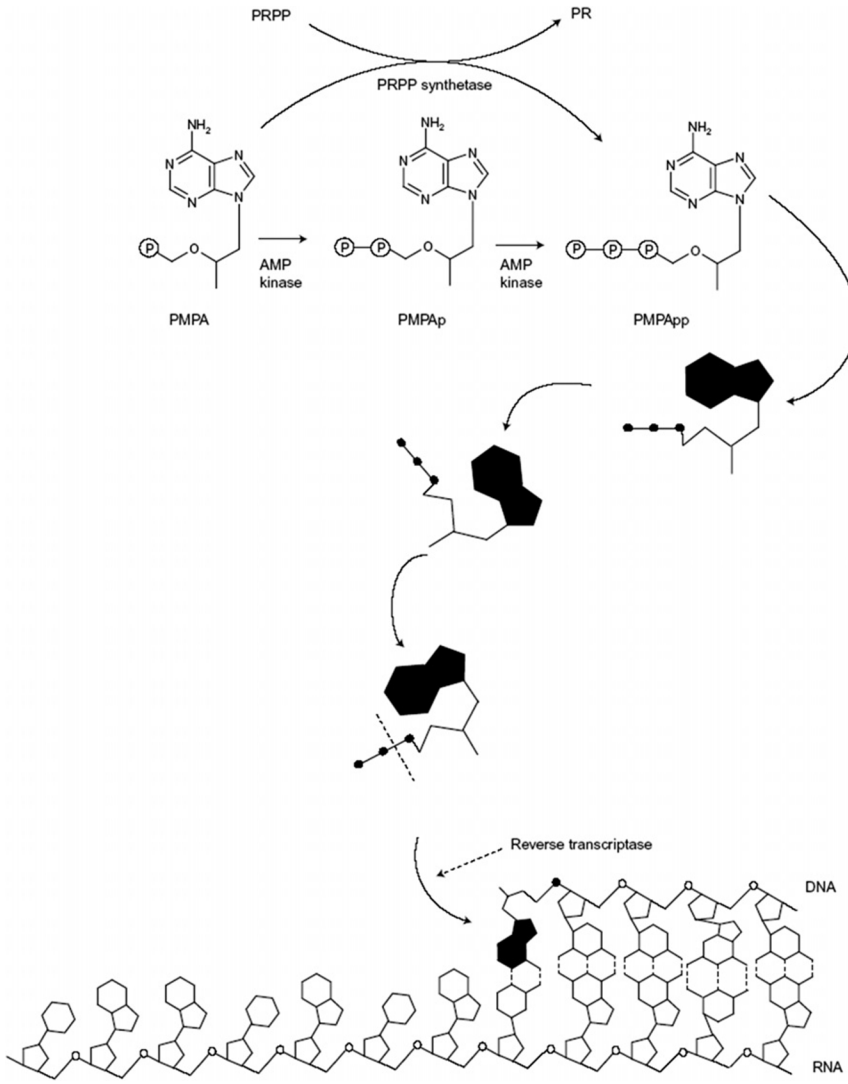


Figure 8.12 Mechanism of action of tenofovir (De Clercq, 2003).

efavirenz (EFV); and TDF/(-)FTC/EFV), the latter proved equivalent or superior to the other combinations (Tang, Kanki, & Shafer, 2012).

Following the approval of Complera® in 2011, the Quad pill Stribild®, containing TDF, emtricitabine, elvitegravir (an HIV integrase inhibitor), and cobicistat (a pharmacoenhancer or "booster" of elvitegravir), was approved by the U.S. FDA on August 27, 2012. The Quad pill dosed

once-daily (QD) proved as effective as twice-daily raltegravir over 2 years of therapy in a pivotal phase 3 trial (Study 145) (Elion et al., 2012). Once daily dosing offers an advantage over the twice-daily dosing of raltegravir (Wills & Vega, 2012). In addition to this Quad, which could be termed Quad no. 1, several other Quads are forthcoming: Quad no. 2, containing Truvada®, atazanavir, and cobicistat; Quad no. 3, containing GS-7340, emtricitabine, darunavir, and cobicistat; and Quad no. 4, containing GS-7340, emtricitabine, elvitegravir, and cobicistat (De Clercq, 2012). GS-7340 is an oral phosphonoamidate prodrug of tenofovir (Lee et al., 2005) that will be further discussed (see Section 11).

The Quad pill Stribild® would seem the preferable regimen for the initial treatment of HIV infection: 90% and 83% of HIV-1 patients showed suppression of HIV-1 RNA to less than 50 copies/ml both at 24 and 48 weeks with the Quad regimen and Atripla regimen, respectively; and in addition, there were fewer central nervous system and psychiatric adverse events with the Quad than with the Atripla regimen (Cohen, Elion, et al., 2011).

7. TENOFOVIR FOR THE PREVENTION OF HIV INFECTIONS

That tenofovir (PMPA) might be effective in the prevention of HIV infections was heralded by the findings of Tsai et al. (1995), who demonstrated that tenofovir could completely prevent SIV in rhesus macaques if the compound was administered within 24 h after viral infection (Fig. 8.13; Tsai et al., 1995). We had to wait another 15 years before it was demonstrated that a 1% vaginal gel formulation of tenofovir could reduce HIV acquisition by up to 54% in women with high gel adherence (Abdool Karim et al., 2010). A 44% reduction in the incidence of HIV was noted with the combination of emtricitabine with TDF (Truvada®) administered orally once daily in the preexposure chemoprophylaxis for HIV prevention in men having sex with men (Grant et al., 2010). In a substudy of the iPrEx trial (Grant et al., 2010), it was found that an intracellular concentration of tenofovir diphosphate of 16 fmol/million PBMCs was associated with a 90% reduction in HIV acquisition relative to the placebo arm (Anderson et al., 2012). The observations of Grant et al. (2010) played a crucial role in the final approval of Truvada® by the US Food and Drug Administration on July 16, 2012 for the prevention of HIV infection, the first compound ever to be approved in this capacity.

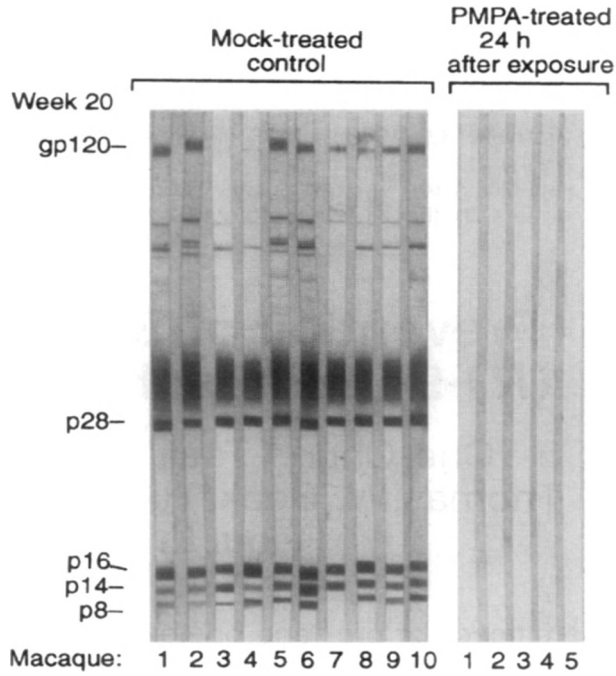


Figure 8.13 Protein immunoblot analysis of SIV-specific antibody response in macaques 20 weeks postinfection with SIV. Mock-treated control macaques ($n = 10$) and macaques treated with PMPA (tenofovir) starting at 24 h postinfection ($n = 5$) are presented. Antibodies to *env* glycoprotein gp120, gag proteins p28, p16 and p8, and *vpx* protein p14 were detected in all of the control macaques. None of the macaques treated with PMPA starting 24 h postinfection showed SIV-specific antibodies (Tsai et al., 1995).

To be effective in the prevention of sexual HIV transmission, antiretroviral drugs should readily penetrate into the (male and female) genital tract, semen, and cervicovaginal secretions (Else, Taylor, Back, & Khoo, 2011). With tenofovir and TDF, diphosphate concentrations have been detected in rectal tissue for 14 days, which were 100-fold higher than those achieved in vaginal and cervical tissues (Patterson et al., 2011). Tenofovir, whether alone or combined with other compounds such as emtricitabine ((-)FTC), should yield great potential as colorectal microbicides (Herrera, Cranage, McGowan, Anton, & Shattock, 2011).

When antiretroviral drugs (including Truvada) were given therapeutically, they could prevent HIV transmission in 96% of the cases (Cohen, Chen, et al., 2011), a procedure which has now been called HIV "treatment as prevention," chosen by the journal *Science* as its "Breakthrough of the Year 2011."

The use of antiretrovirals (ARVs) as a potential HIV prevention strategy, as reported by Granich, Gilks, Dye, De Cock, and Williams (2009), was originally met with a mix of excitement and disbelief (El-Sadr, Affrunti, Gamble, & Zerbe, 2010; Garnett & Baggaley, 2009). Because of the many *caveats*, that is, identifying infected and uninfected people at high risk, missing very early infections, acceptance and long-term adherence, drug resistance, more risky behavior, drug toxicity, costs, and human resources, ARVs as HIV prevention has been considered a tough road to wide impact (Shelton, 2011).

8. TENOFOVIR FOR THE TREATMENT OF HBV INFECTIONS

TDF (Viread®) has virtually replaced adefovir dipivoxil (Hepsera®) in the treatment of HBV infections after TDF at a daily dose of 300 mg had proven superior to adefovir dipivoxil at a daily dose of 10 mg in suppressing HBV DNA levels in both HBe antigen-negative patients and HBe antigen-positive patients (Lee et al., 2010; Marcellin et al., 2008; Snow-Lampart et al., 2010). These studies (study 102, study 103) when originally published were carried out for a period of 48 weeks but have in the meantime been extended to a period of 4 years (Fig. 8.14). No development of resistance to tenofovir was noted, even after 5 years. In patients with chronic HBV infection, 5 years of treatment with TDF was safe and effective, leading to a complete suppression of HBV replication, accompanied by the regression of fibrosis and cirrhosis (Marcellin et al., 2013).

Besides pegylated interferon, five antivirals, that is, lamivudine, adefovir dipivoxil, entecavir, telbivudine, and TDF have been approved for the therapy of chronic hepatitis B (Scaglione & Lok, 2012): in terms of response rates at weeks 48–52, and undetectable HBV DNA (percent), HBsAg loss (percent), and genotypic resistance upon extended treatment, in both HBeAg-positive and -negative patients, TDF proved as effective, if not more so, than the other licensed compounds.

9. TENOFOVIR FOR PMTCT

The majority of perinatal HIV transmissions occur during labor and delivery. Zidovudine has been shown previously to reduce the risk of transmission of HIV from mother to child (Connor et al., 1994; Sperling et al., 1996) and so does nevirapine (Guay et al., 1999). However, for toxicity

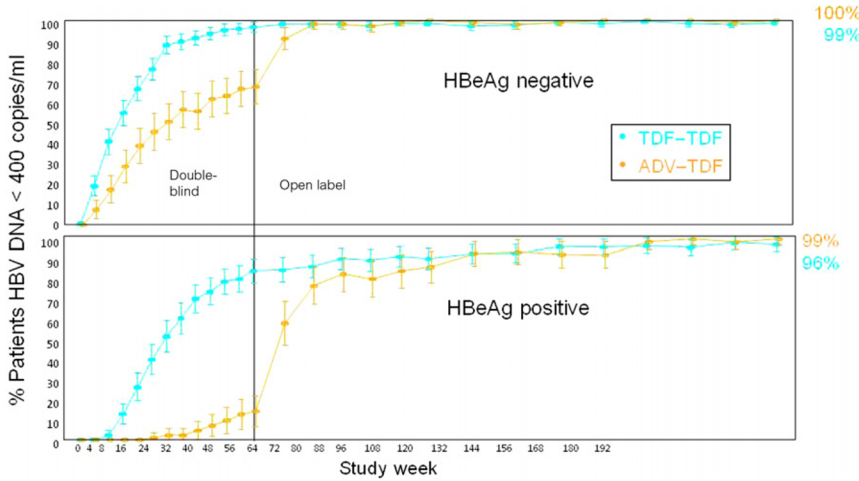


Figure 8.14 Suppression of HBV DNA levels by tenofovir disoproxil fumarate (daily dose: 300 mg through week 48) and adefovir dipivoxil (daily dose: 10 mg through week 48) in HBe Ag-positive and -negative patients with chronic hepatitis B. Viral suppression was defined as an HBV DNA level of less than 400 copies/ml (Lee et al., 2010; Marcellin et al., 2008; Snow-Lampart et al., 2010). The virologic response is mentioned for a period of 4 years, but is similar out to year 6 and the study is being extended to 10 years.

(zidovudine) reasons and resistance development (nevirapine), neither compound seems ideal for prevention of mother-to-child transmission (PMTCT).

TDF, with or without (-)FTC, would seem of potential use in PMTCT (Flynn et al., 2011). *In vitro* exposure to TDF does not impair growth patterns, bone health, and markers of bone metabolism in HIV-uninfected children born to HIV-infected mothers (Viganò et al., 2011), and, similarly, no increase in congenital, renal, or growth abnormalities has been observed following *in utero* tenofovir exposure (Gibb et al., 2012). TDF should therefore be further pursued for PMTCT.

10. TENOFOVIR TOXICITY

All antiretroviral agents are linked with toxic side effects, particularly after long-term (many years of) use. For tenofovir (TDF), a persistent decline in estimated but not measured glomerular filtration rate may reflect tubular rather than glomerular toxicity (Vrouenraets et al., 2011). Tenofovir

(TDF) with ritonavir-boosted lopinavir (LPV/r) or boosted atazanavir leads to a greater decline in estimated glomerular filtration rate than tenofovir with efavirenz (Young et al., 2012). Yet, over a 4- to 5-year period in Africa, differences in eGRF changes were small, suggesting that first-line antiretroviral therapy, including tenofovir (TDF), can be given safely without routine renal function monitoring (Stöhr et al., 2011).

Cumulative tenofovir (TDF) exposure has been associated with decreased bone density and an increased risk of osteoporotic fracture risk, but so has been LPV/r (Bedimo, Maalouf, Zhang, Drechsler, & Tebas, 2012).

On the other hand, chronic HIV infection impairs endothelial function, and tenofovir (TDF) protects against endothelial dysfunction (Francisci et al., 2011), apparently because tenofovir selectively decreases the production of cardiovascular disease-related inflammatory cytokines (Melchjorsen et al., 2011).

11. GS-7340, GS-9131, GS-9191, GS-9219

Prominent among the new oral prodrugs of tenofovir is GS-7340 (Fig. 8.15; Lee et al., 2005). GS-7340 is 400-fold more potent than tenofovir in PBMCs and GS-7340 is 200-fold more stable in plasma than TDF (Lee et al., 2005). GS-7340 is rapidly metabolized inside the lysosomes of lymphoid cells by the enzyme cathepsin A (Birkus et al., 2007). Compared to Viread (at a dose of 300 mg), GS-7340 at a dose of 50 or 150 mg causes a much greater decrease in viral load after 14 day once-daily (QD) dosing (Fig. 8.16; Markowitz et al., 2011).

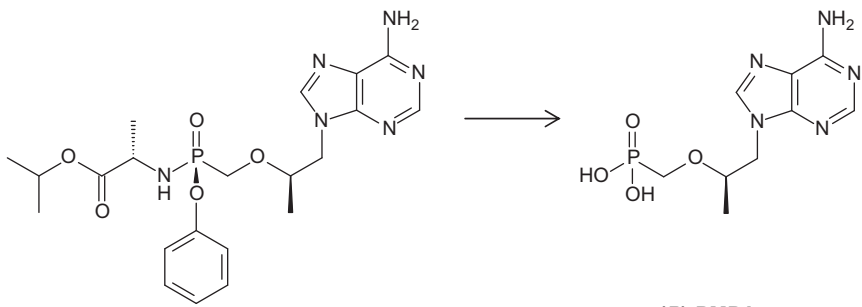


Figure 8.15 GS-7340 and (*R*)-PMPA (tenofovir).

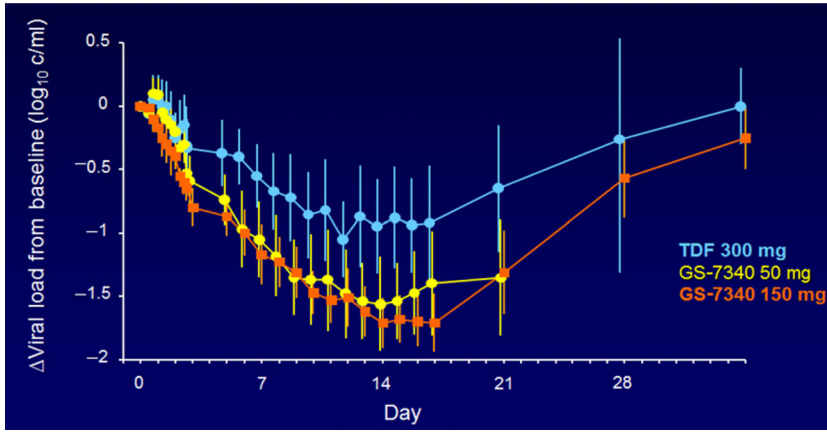
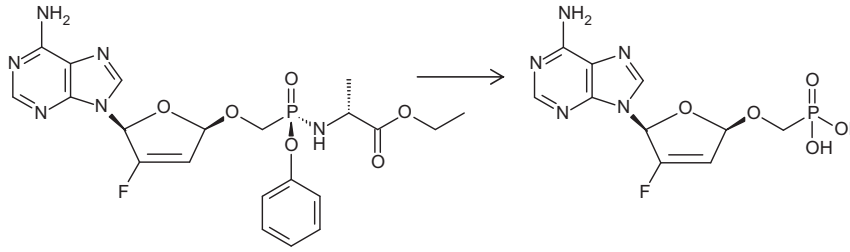


Figure 8.16 GS 7340. Dose response versus Viread. Phase 2a—14 day QD dosing (Markowitz et al., 2011).



GS-9131 **GS-9148**

Figure 8.17 GS-9131 and GS-9148.

Starting from a cyclic nucleoside phosphonate, GS-9148, a phosphonoamidate prodrug (GS-9131) was conceived, which serves as the oral prodrug of GS-9148 (Cihlar et al., 2008; Ray et al., 2008; Fig. 8.17). Unlike tenofovir, GS-9148 would be active against nucleoside-resistant HIV-1 variants (Cihlar et al., 2008). GS-9148 and its prodrug, GS-9131, should be considered alternative drug candidates for the treatment of HIV infections.

GS-9191 can be considered as a pro-prodrug of 9-(2-phosphonylmethoxyethyl)guanine (PMEG). It is first converted to cPrPMEDAP (the *N*-cyclopropyl-2,6-diaminopurine counterpart of PMEA) before it is further converted to PMEG (Fig. 8.18). GS-9191 is further pursued by Graceway Pharmaceuticals for the topical treatment of papillomatous (i.e., condylomatous) lesions in humans (Wolfgang et al., 2009).

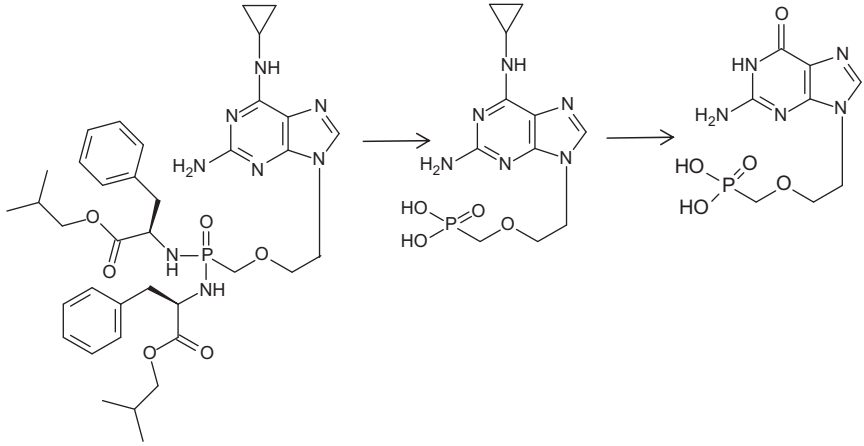


Figure 8.18 GS-9191, cPrPMEDAP, and PMEG.

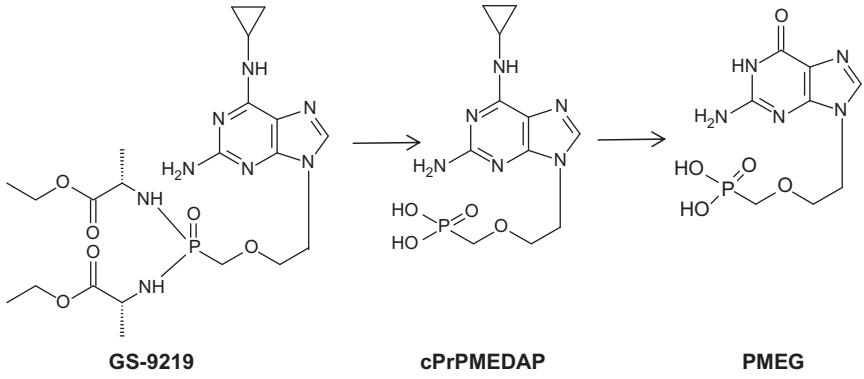


Figure 8.19 GS-9219, cPrPMEDAP, and PMEG.

Concomitantly with GS-9191, GS-9219 has been developed for the treatment of non-Hodgkin's lymphoma (NHL) in dogs (Reiser et al., 2008; Vail et al., 2009; Fig. 8.19). This potential application is being pursued by Vet DC in dogs.

In both cases (GS-9191 and GS-9219), the active metabolite is PMEG (De Clercq et al., 1987), which, upon its phosphorylation to its diphosphate (PMEGpp), competes with the natural substrate, dGTP, for the inhibition of cellular DNA synthesis (Fig. 8.20).

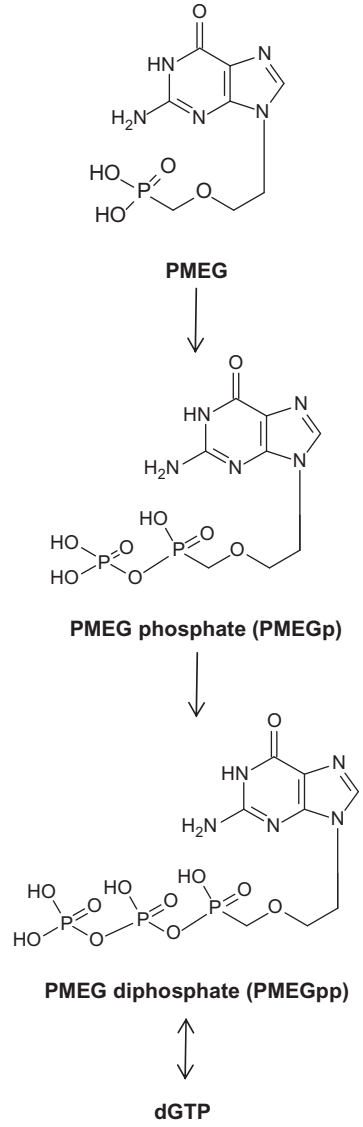


Figure 8.20 PMEG, PMEG phosphate, and PMEG diphosphate.

12. CONCLUSION

Tenofovir has become the cornerstone in the treatment and prevention of HIV infections. In combination with emtricitabine [(-)FTC], TDF has been approved for both the therapy and prophylaxis of HIV infections (Truvada®). For the treatment of HIV infections, Truvada has become

available, in combination with efavirenz (Sustiva®), as Atripla®; in combination with rilpivirine (Edurant®), as Complera®/Eviplera®; and in combination with elvitegravir and cobicistat, as Stribild®. A forthcoming "quad" combination is based on the use of Truvada with atazanavir and cobicistat. In the future, TDF will be replaced by GS-7340, a phosphonoamidate prodrug of tenofovir, and new "quad" combinations based on darunavir plus cobicistat plus (-)FTC plus GS-7340 and elvitegravir plus cobicistat plus (-)FTC plus GS-7340 have been planned.

CONFLICT OF INTEREST

The author is coinventor of cidofovir, adefovir, and tenofovir.

ACKNOWLEDGMENTS

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CHAPTER NINE

The Nucleoside Reverse Transcriptase Inhibitors, Nonnucleoside Reverse Transcriptase Inhibitors, and Protease Inhibitors in the Treatment of HIV Infections (AIDS)

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Contents

Abstract

The majority of the drugs currently used for the treatment of HIV infections (AIDS) belong to either of the following three classes: nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs). At present, there are 7 NRTIs, 5 NNRTIs, and 10 PIs approved for clinical

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use. They are discussed from the following viewpoints: (i) chemical formulae; (ii) mechanism of action; (iii) drug combinations; (iv) clinical aspects; (v) preexposure prophylaxis; (vi) prevention of mother-to-child transmission; (vii) their use in children; (viii) toxicity; (ix) adherence (compliance); (x) resistance; (xi) new NRTIs, NNRTIs, or PIs in (pre)clinical development; and (xii) the prospects for a "cure" of the disease.

ABBREVIATIONS

(−)FTC emtricitabine
3TC 2′,3′-dideoxy-3′-thiacytidine
ART antiretroviral therapy
AZT 3′-azido-3′-deoxythymidine
CRIs coreceptor inhibitors
d4T 2′,3′-didehydro-2′,3′-dideoxythymidine
FIs fusion inhibitors
HEPT 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine
INIs integrase inhibitors
NNRTIs nonnucleoside reverse transcriptase inhibitors
NRTIs nucleoside reverse transcriptase inhibitors
NtRTIs nucleotide reverse transcriptase inhibitors
PIs protease inhibitors
PMTCT prevention of mother-to-child transmission
RT reverse transcriptase
TDF tenofovir disoproxil fumarate
TIBO tetrahydro-imidazo[4,5,1-jk][1,4]-benzodiazepine-2(1*H*)-one and -thione

1. INTRODUCTION

The replicative cycle of HIV (Fig. 9.1) (De Clercq, 2002) can be schematically divided into 10 steps: (i) virus adsorption, mediated by the interaction of the viral envelope gp120 glycoprotein with the cellular CD4 receptor, followed by the interaction of gp120 with the cellular coreceptor (CXCR4 for T-lymphotropic virus strains; CCR5 for macrophage (M)-tropic virus strains); (ii) thereupon, the viral envelope gp41 glycoprotein anchors into the cell membrane, resulting in virus–cell fusion, upon which (iii) the viral nucleocapsid, stripped from the viral glycoprotein, enters the cell (uncoating, penetration); (iv) the viral RNA genome (2 copies per virus particle) is transcribed through the virus-associated reverse transcriptase (RT) (RNA-dependent DNA polymerase) to an RNA/DNA hybrid intermediate that upon degradation of the RNA component by ribonuclease H, which is physically part of the RT, is duplicated, again by the RT to

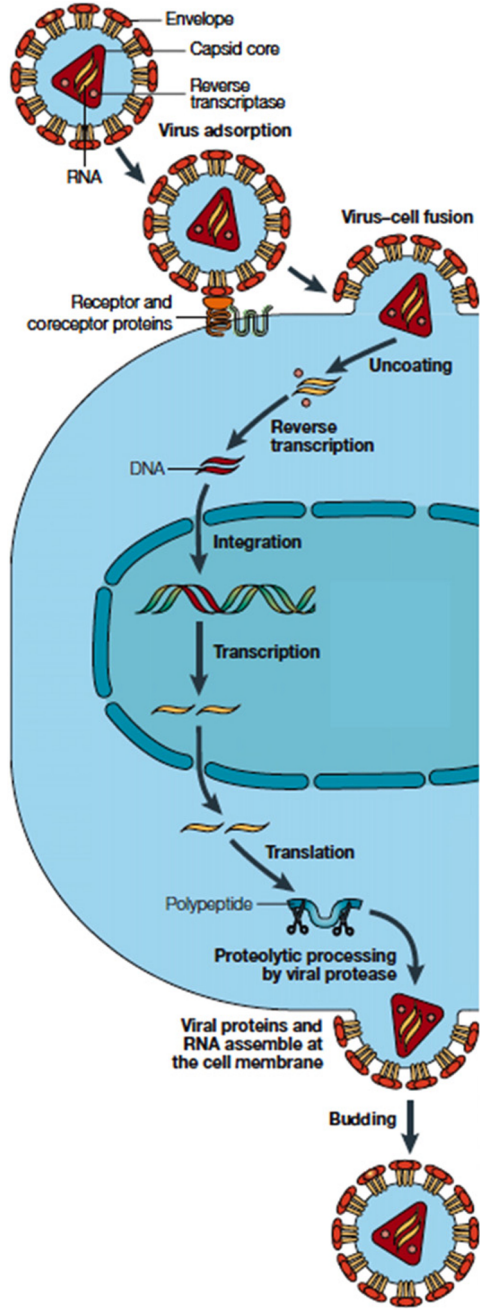


Figure 9.1 Replicative cycle of HIV, highlighting the principal targets for therapeutic intervention: (co)receptor interaction, virus–cell fusion, reverse transcription (by the reverse transcriptase), integration, and proteolytic processing (by the viral protease). *According to De Clercq (2002).*

double-stranded DNA; (v) the latter is then incorporated (integration) by another virus-associated enzyme, the integrase, into the cellular genome, from where the viral genome, now called proviral DNA, can be transmitted together with the cellular genome to all daughter cells; (vi) the proviral DNA can be transcribed to viral mRNA (transcription), and (vii) subsequently, translated to viral precursor polypeptide (translation) which (viii) is then cleaved by a viral-encoded protease (proteolytic processing) to mature viral proteins (i.e., protease, RT, integrase), which are then assembled together with the viral RNA genome into progeny virus particles (ix) that are released from the infected cell through a process called budding (x).

Several of these processes have proven to be adequate targets for anti-HIV drugs: that is, virus adsorption, virus–cell fusion, uncoating (these three processes are collectively referred to as viral entry), reverse transcription, integration, and proteolytic processing (by the viral protease). In fact, the RT offers several sites for chemotherapeutic intervention: the catalytic (substrate binding) site for the nucleoside RT inhibitors (NRTIs) and nucleotide RT inhibitors (NtRTIs), and an allosteric site for the nonnucleoside RT inhibitors (NNRTIs). The NtRTIs will be the subject of a separate chapter dealing with the acyclic nucleoside phosphonates. The NRTIs and NNRTIs will be the subject of this chapter. So will also be the protease inhibitors (PIs), targeted at the proteolytic processing. Inhibitors of viral entry (i.e., coreceptor inhibitors (CRIs) and fusion inhibitors (FIs)) and integration (i.e., integrase inhibitors (INIs)) will be dealt with in separate chapters. The antiretroviral drug classes reviewed here (NRTIs, NNRTIs, PIs) represent the three major classes of drugs used for the treatment of HIV infections in the past decade (2000–2009) (Pursuing Later Treatment Option II (PLATO II) Project Team et al., 2012).

2. REVERSE TRANSCRIPTASE

The RT is a heterodimer, consisting of two subunits, p66 and p51 (Fig. 9.2) (Tantillo et al., 1994). The p66 subunit contains several domains: the fingers, palm, thumb, connection, and ribonuclease H domain. The active sites of the RT are located in the palm domain, with the binding site for both the NRTIs (following their phosphorylation to the triphosphate) and NtRTIs (following their phosphorylation to the diphosphate, as they already contain a phosphonate entity) and the NNRTIs.

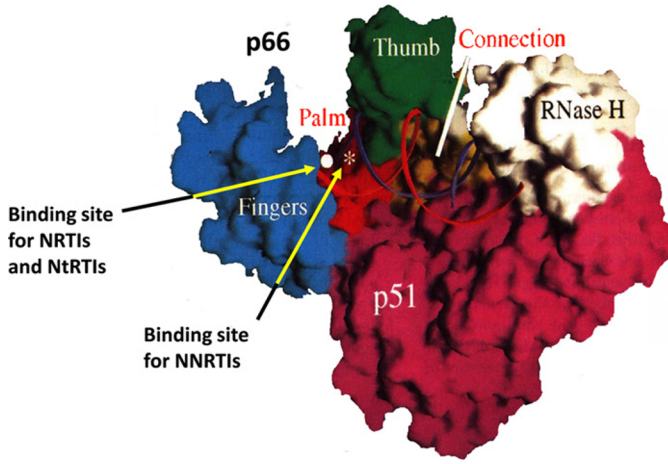
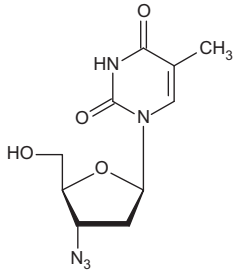


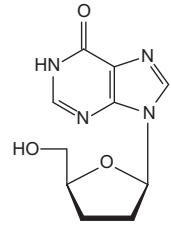
Figure 9.2 HIV reverse transcriptase with the binding site for the NRTIs and NtRTIs and the binding site for the NNRTIs. *According to De Clercq (2009b); structure of the enzyme according to Tantillo et al. (1994).*

3. NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

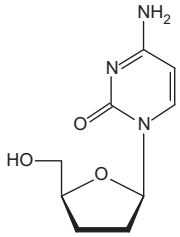
There are, at present, seven NRTIs licensed for clinical use in the treatment of HIV infections: zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir, and emtricitabine ((−)FTC) (Fig. 9.3). The first of the series, originally made public under a code number (BW A509U), was 3′-azido-3′-deoxythymidine (AZT) (Mitsuya et al., 1985). When it was announced as an anti-HIV agent, it was second to suramin, an hexasulfonate, which had been reported just 1 year before AZT (Mitsuya et al., 1984) to inhibit the infectivity and cytopathic effect of HIV (then called T-lymphotropic virus type III/lymphadenopathy-associated virus). Following AZT, several other 2′,3′-dideoxynucleosides, including 2′,3′-dideoxyadenosine (ddA), 2′,3′-dideoxyguanosine (ddG), 2′,3′-dideoxycytidine (ddC), 2′,3′-dideoxythymidine (ddT), and 2′,3′-dideoxyinosine (ddI), were reported to inhibit the infectivity of HIV (Mitsuya & Broder, 1986). Two of these 2′,3′-dideoxynucleosides, that is, ddI (didanosine) and ddC (zalcitabine), would eventually be developed as anti-HIV drugs. The discovery of AZT as an inhibitor of HIV replication, how serendipitous it may seem, unleashed a worldwide search for new



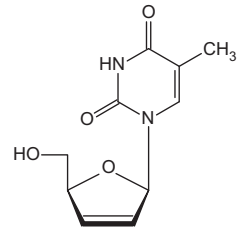
Zidovudine
3′-Azido-2′,3′-dideoxythymidine,
azidothymidine (AZT)
Retrovir®



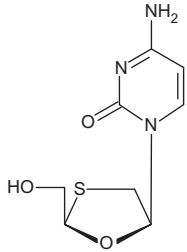
Didanosine
2′,3′-Dideoxyinosine (ddI)
Videx®, Videx® EC



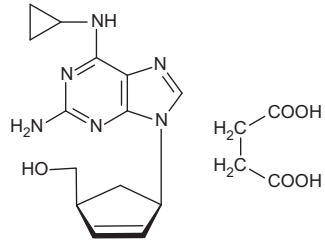
Zalcitabine
2′,3′-Dideoxycytidine (ddC)
Hivid®



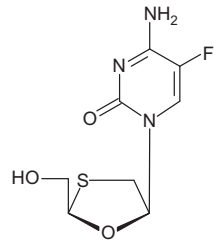
Stavudine
2′,3′-Didehydro-2′,3′-dideoxythymidine
(d4T)
Zerit®



Lamivudine
2′,3′-Dideoxy-3′-thiacytidine
3TC
Epivir®



Abacavir
(1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-
9*H*-purin-9-yl]-2-cyclopentene-1-methanol
succinate (ABC)
Ziagen®



(-)FTC
Emtricitabine
(-)-β-L-3′-thia-2′,3′-dideoxy-5-fluorocytidine
Emtriva®

Figure 9.3 Structural formulae of zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir, and emtricitabine (NRTIs).

nucleoside analogues as potential anti-HIV agents. This led to the identification of 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T) as a potent anti-HIV agent in a number of laboratories all over the world (Baba et al., 1987; Hamamoto et al., 1987; Lin, Schinazi, & Prusoff, 1987). Then followed 3TC (2′,3′-dideoxy-3′-thiacytidine), as the result of the pioneering work of the late Bernard Belleau (Soudeyns et al., 1991), and (−)FTC, originating from the efforts of D. Liotta and R.F. Schinazi from Emory University (Schinazi et al., 1992). Meanwhile, abacavir (ABC) had been described by the late Susan Daluge and her colleagues (Daluge et al., 1997).

All NRTIs act in a similar fashion as AZT (Furman et al., 1986): following intracellular phosphorylation to their 5′-triphosphate, they serve as alternate substrates/competitive inhibitors with respect to the natural substrates (dTTP, dCTP, dATP, or dGTP) in the RT reaction. This requires for AZT, ddI, ddC, d4T, 3TC, ABC, and (−)FTC, the following intracellular conversions, respectively: AZT → AZT-MP → AZT-DP → AZT-TP; ddI → ddI-MP → ddA-MP → ddA-DP → ddA-TP; ddC → ddC-MP → ddC-DP → ddC-TP; d4T → d4T-MP → d4T-DP → d4T-TP; 3TC → 3TC-MP-→ 3TC-DP → 3TC-TP; ABC → ABC-MP → carbovir (CBV)-MP → CBV-DP → CBV-TP; (−)FTC → (−)FTC-MP → (−)FTC-DP → (−)FTC-TP. For AZT, the prototype of the NRTIs, the mechanism of action is depicted in Fig. 9.4 (De Clercq, 2002).

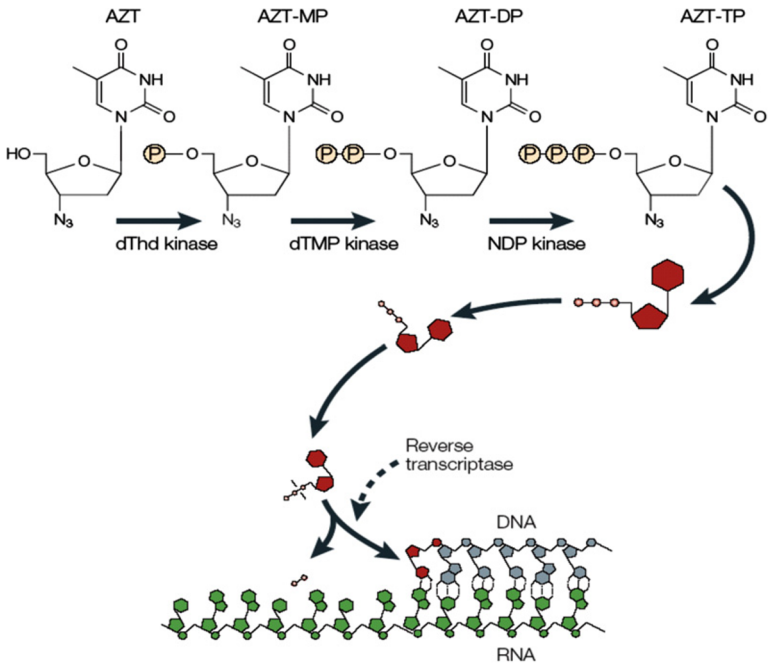


Figure 9.4 Mechanism of action of AZT. Following phosphorylation to its triphosphate form, AZT acts as a competitive inhibitor/alternative substrate with respect to dTTP in the reverse transcriptase reaction. *According to De Clercq (2002).*

4. NONNUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

The era of the NNRTIs started with the discovery of the anti-HIV-1 activity of the 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) derivatives (Baba et al., 1989; Miyasaka et al., 1989) and virtually simultaneous discovery of the anti-HIV-1 activity of the tetrahydro-imidazo[4,5,1-jk][1,4]-benzodiazepine-2(1*H*)-one and -thione (TIBO) derivatives (Pauwels et al., 1990). Subsequent papers ascertained that both the HEPT and TIBO derivatives inhibited the HIV-1 RT by specifically interacting with an allosteric site located at a close distance (about 15 Å) from the catalytic (dNTP-binding) site (Baba et al., 1991a, 1991b; Debyser et al., 1991; Debyser, Pauwels, Baba, Desmyter, & De Clercq, 1992; Pauwels et al., 1994). Following HEPT and TIBO, nevirapine (Koup et al., 1991; Merluzzi et al., 1990), delavirdine (Romero et al., 1991, 1993), and efavirenz

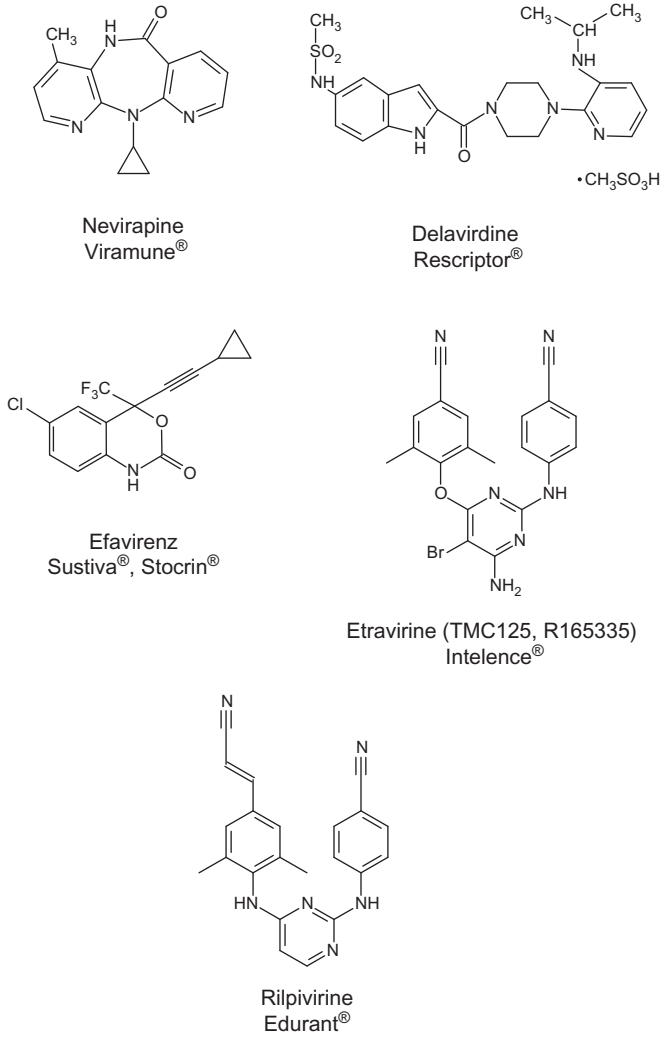


Figure 9.5 Structural formulae of nevirapine, delavirdine, efavirenz, etravirine, and rilpivirine.

(Corbett et al., 1999) were reported (De Clercq, 2004) as specific HIV-1 inhibitors targeted at the HIV-1 RT. All three compounds (i.e., nevirapine, delavirdine, and efavirenz) (Fig. 9.5) were approved and commercialized for clinical use (delavirdine is no longer commercially available). From HEPT originated emivirine (Coactinon®) (MKC-442) (Baba et al., 1994), which was eventually abandoned after it had proceeded to phase III clinical trials.

The TIBO path led successively to TIBO R82150, TIBO R82913, TIBO R86183 (tivirapine), α-APA (R89439, loviride), ITU R100943, DATA R106168, DAPY R147681 (TMC 120, dapivirine), DAPY R165335 (TMC 125, etravirine), and DAPY R278474 (TMC 278, rilpivirine) (De Clercq, 2012a). Etravirine (Intelence®) and rilpivirine (Edurant®) complete the panel of the five NNRTIs which have so far been approved for clinical use (Fig. 9.5). The route from TIBO (tivirapine) to rilpivirine can be considered as a prime example of the sustained efforts of medicinal chemistry over a time period of 20 years (Janssen et al., 2005). It resulted in a durable anti-HIV drug.

Both etravirine and rilpivirine can be positioned in the NNRTI-binding site of the HIV-1 RT (Fig. 9.6) (Janssen et al., 2005; Pauwels, 2004). Within the NNRTI-binding site, etravirine and rilpivirine assume a "butterfly" or "horseshoe"-type of conformation. They interact with crucial amino acids of the NNRTI-binding site: L100, K101, K103, V106, V108, Q138, Y181, Y188, F227, W229, L234, H235, and Y318 (Janssen et al., 2005; Pauwels, 2004).

Several events occur in the binding of NNRTIs to their "pocket" in the HIV-1 RT: (i) restriction of thumb mobility (Kohlstaedt, Wang, Friedman, Rice, & Steitz, 1992); (ii) distortion of the catalytic triad (Ren et al., 1995); (iii) repositioning of the primer grip (Das et al., 1996); and (iv) loosening of

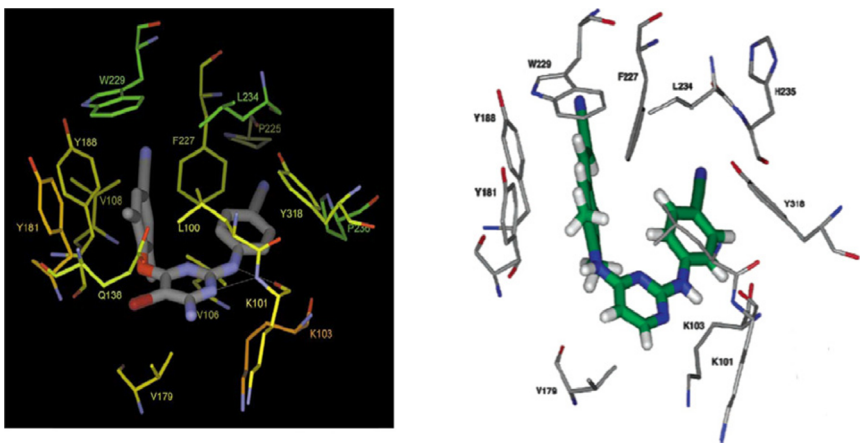


Figure 9.6 Left panel. Etravirine (TMC125) positioned in NNRTI-binding site of HIV-1 RT. Right panel. Molecular modeling of rilpivirine (TMC 278) docked into the NNRTI-binding site of HIV-1 reverse transcriptase. *Panel (A): According to Pauwels (2004). Panel (B): According to Janssen et al. (2005).*

the thumb and fingers clamp (Liu, Abbondanzieri, Rausch, Le Grice, & Zhuang, 2008). The polymerase domain of the HIV-1 RT could undergo conformational changes so as to accommodate either AZT triphosphate (AZT-TP) at the dNTP-binding site, or nevirapine at the NNRTI-binding pocket, but not both simultaneously (Fig. 9.7) (Das et al., 2012).

Additional complexities might occur during the reverse transcription process such as enzyme-templates dissociation and association and reversal of orientation to perform distinct tasks, such as RNase H cleavage of the viral RNA (Liu et al., 2008, Liu, Harada, Miller, Le Grice, & Zhuang, 2010). *In vivo*, an excess of RT (50–200 enzymes per virion) in comparison to the RNA template may be present (Thomas et al., 2007), such that different enzymes could perform different tasks (polymerization/RNase H) at the same time *in vivo* (von Kleist, Metzner, Marquet, & Schütte, 2012).

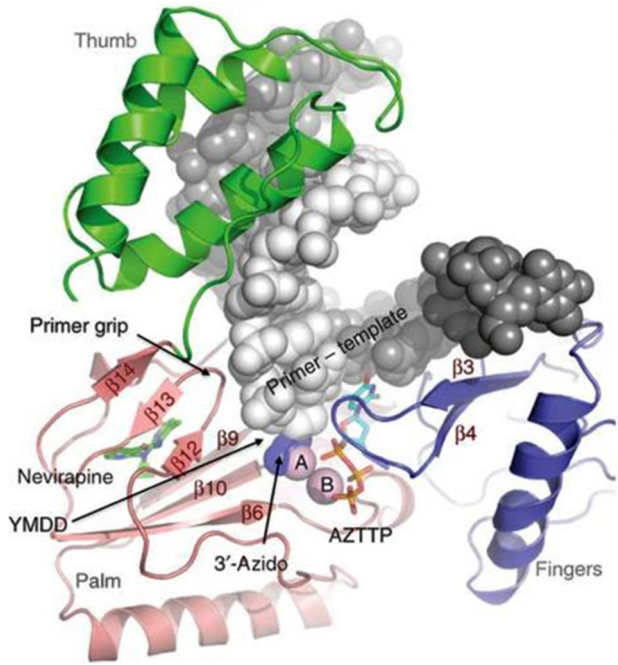


Figure 9.7 Polymerase domain of HIV-1 RT in complex with DNA. Nevirapine and AZT-TP are placed based on the superposition of the palm subdomain of nevirapine- and AZTTTP-ternary structures, respectively, in the RT-DNA structure. *According to Das, Martinez, Bauman, and Arnold (2012).*

5. PROTEASE INHIBITORS

There are currently 10 PIs that have been approved by the U.S. FDA (Food and Drug Administration) for the treatment of HIV infections: saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, atazanavir, fosamprenavir, tipranavir, and darunavir (Fig. 9.8). Except for tipranavir, which is built on a coumarin scaffold, all other PIs are built on the same peptidomimetic principle, that is, they contain a nonhydrolyzable hydroxyethylene core $\left[R_1{-}\underset{\displaystyle OH}{\underset{|}{CH}}{-}CH_2{-}R_2\right]$ instead of a peptide linkage $\left[R_1{-}CO{-}NH{-}R_2\right]$ which would otherwise be cleaved by hydrolysis (via the intermediary $\left[R_1{-}\underset{\displaystyle HO\quad OH}{C}{-}NH{-}R_2\right]$ to $R_1{-}COOH$ and $H_2N{-}R_2$).

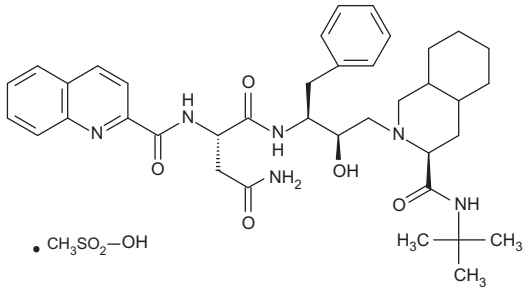
Through their inhibitory effect on the HIV protease, PIs prevent the cleavage of the precursor polyprotein which is required for the formation of the mature viral proteins needed for the infectivity of the progeny virions. The HIV protease is a homodimeric aspartyl protease, and the PIs fit snugly within the cleft left between the active sites of the two subunits (Fig. 9.9) (Pauwels, 2006).

A clinically derived multidrug-resistant protease (PR20) (Louis, Aniana, Weber, & Sayer, 2011), bearing 20 mutations (Dierynck et al., 2007) (Fig. 9.10), would exhibit extreme resistance to clinical inhibitors through coordinated structural rearrangements (Agniswamy et al., 2012).

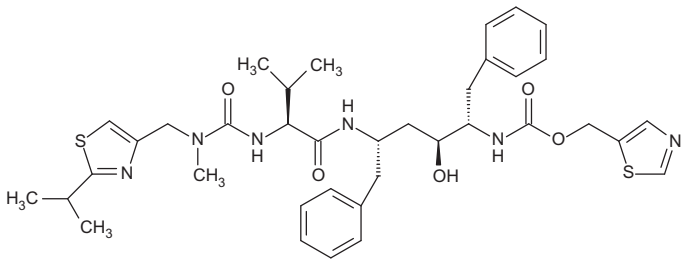
6. DRUG COMBINATIONS

Given the wealth of anti-HIV drugs now available for the treatment of HIV infections (i.e., 7 NRTIs, 1 NtRTI, 5 NNRTIs, 10 PIs, 1 CRI, 1 FI, and 1 INI; De Clercq, 2009a, 2009b), numerous drug combinations are theoretically possible to treat AIDS (Fig. 9.11). As in the treatment of *Mycobacterium tuberculosis*, such drug combinations can now be considered as standard of care for the treatment of AIDS, principally for three reasons: (a) to obtain a synergistic action between different drugs interacting with different molecular targets; (b) to reduce the risk of emergence of HIV drug resistance; and (c) to diminish the toxic side effects by lowering the doses of the individual drugs.

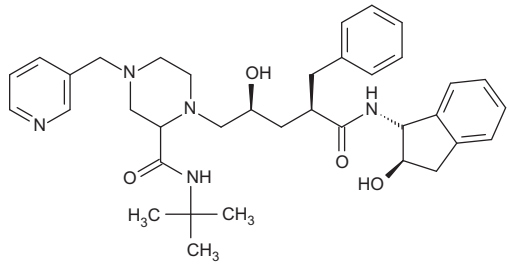
The first fixed-dose drug combinations used for the treatment of HIV infections were Combivir® (AZT + 3TC) launched in 1997 and Trizivir®



Saquinavir
hard gel capsules, Invirase®
soft gelatin capsules, Fortovase®



Ritonavir
Norvir®



Indinavir
Crixivan®

Figure 9.8 Structural formulae of the protease inhibitors (PIs): saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, atazanavir, fosamprenavir, tipranavir, and darunavir.

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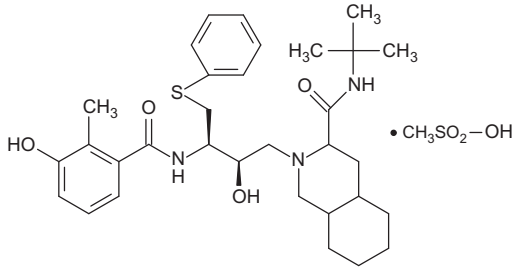
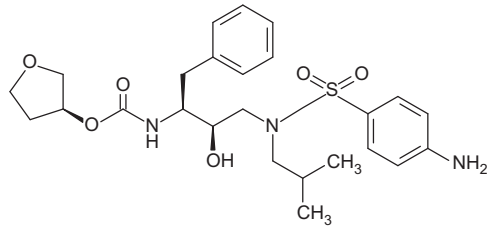
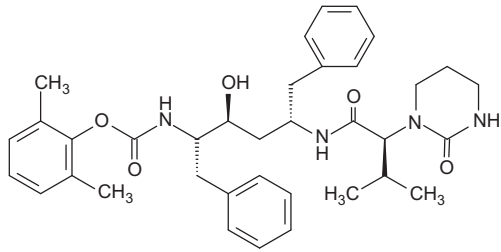


Nelfinavir
Viracept®

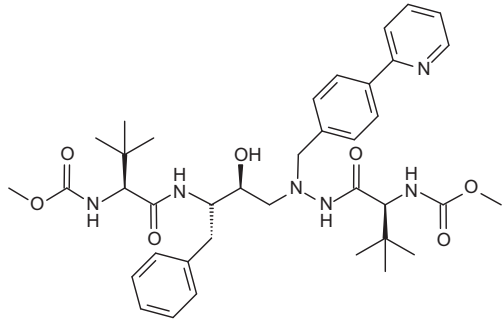


Amprenavir
Agenerase®, Prozei®

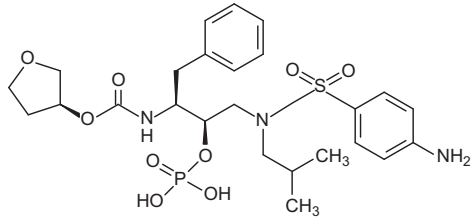


Lopinavir
combined with ritonavir at 4/1 ratio
Kaletra®

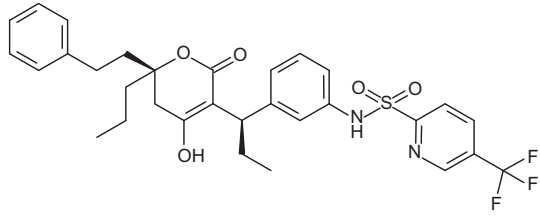
Figure 9.8—cont'd



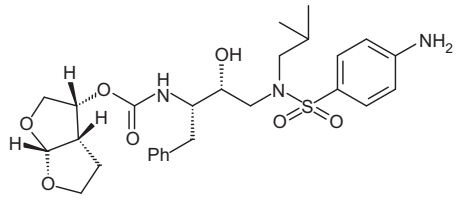
Atazanavir
Reyataz®



Fosamprenavir
Lexiva®, Telzir®



Tipranavir (U-140690)
Aptivus®



Darunavir (TMC-114)
Prezista®

Figure 9.8—cont'd

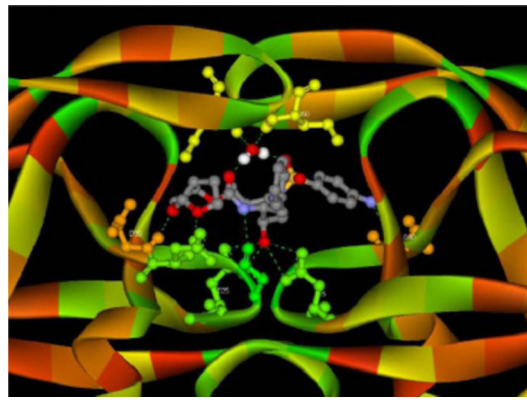


Figure 9.9 HIV protease structure with darunavir (TMC114) in the active site. *According to Pauwels (2006).*

(AZT + 3TC + ABC) launched in 2000. Then followed in 2004, Epzicom® (3TC + ABC) and, also in 2004 Truvada® [(−)FTC and TDF (tenofovir disoproxil fumarate)]; in 2006, Atripla® [(−)FTC, TDF, and efavirenz]; and in 2011, Complera®/Eviplera® [(−)FTC, TDF, and rilpivirine]. As single anti-HIV drugs, zidovudine was launched in 1987, lamivudine (3TC) in 1995, abacavir (ABC) in 1998, efavirenz (Sustiva®) in 1998, tenofovir (TDF, Viread®) in 2001, emtricitabine [(−)FTC, Emtriva®] in 2003, and rilpivirine (Edurant®) in 2011.

The latest fixed-dose drug combination formally approved (on August 27, 2012) for the treatment of HIV infections was Stribild®. It contains a fixed-dose drug combination of TDF, (−)FTC, the INI elvitegravir, and the "booster" cobicistat. This is the first quadruple drug combination in one single once-daily pill (De Clercq, 2012b). Other forthcoming combinations involve atazanavir + cobicistat + (−)FTC and TDF, darunavir + cobicistat + (−)FTC + GS7340 (prodrug of tenofovir), and elvitegravir + cobicistat + (−)FTC + GS7340 (De Clercq, 2012b).

Given the number of licensed anti-HIV drugs currently available, numerous triple-drug combinations could theoretically be conceived. Yet, the combination of TDF and (−)FTC has become the cornerstone in most of these drug combinations. Virologic failure was less likely to occur with efavirenz (an NNRTI) than with lopinavir/ritonavir (a PI) (Riddler et al., 2008). In combination with TDF/(−)FTC, extended-release nevirapine (NVP XR) was shown to be noninferior in efficacy to immediate-release nevirapine

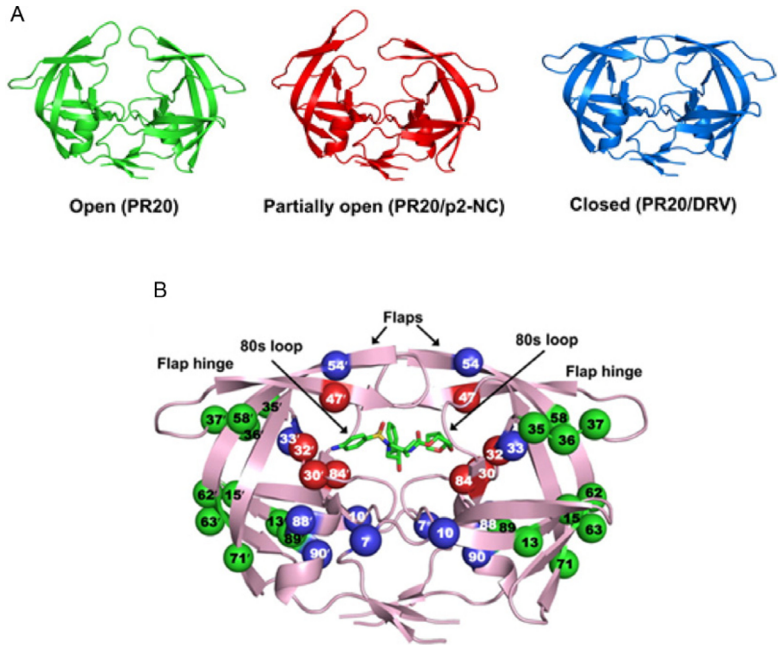


Figure 9.10 Crystal structure of PR20 exhibits three different conformations: open, partially open, and closed (A). (B) Sites of the 20 multidrug-resistant mutations are mapped on HIV-1 PR (pink cartoon presentation) with darunavir (DRV) colored with green sticks for carbon atoms. The mutations with direct interaction to inhibitors are colored as red systems, while second shell mutations are shown as blue spheres, and more distal mutations are shown as green spheres. *According to Agniswamy et al. (2012).*

(NVP IR) (Gathe et al., 2011). Nevirapine demonstrated at week 48 noninferior antiviral efficacy compared with atazanavir/ritonavir, when given along with TDF/(−)FTC (Soriano et al., 2011). When combined with TDF/(−)FTC, etravirine or efavirenz would lead to a similar rate of HIV RNA suppression (Gazzard et al., 2011).

An N(t)RTI-sparing regimen existing of efavirenz combined with lopinavir/ritonavir gave a similar efficacy as the efavirenz/TDF/(−)FTC regimen, but a higher frequency of drug-resistant mutants (Riddler et al., 2008). A similar N(t)RTI-sparing regimen existing of darunavir/ritonavir and etravirine could be particularly cost-effective in some conditions (Mauskopf, Brogan, Talbird, & Martin, 2012). The combination of

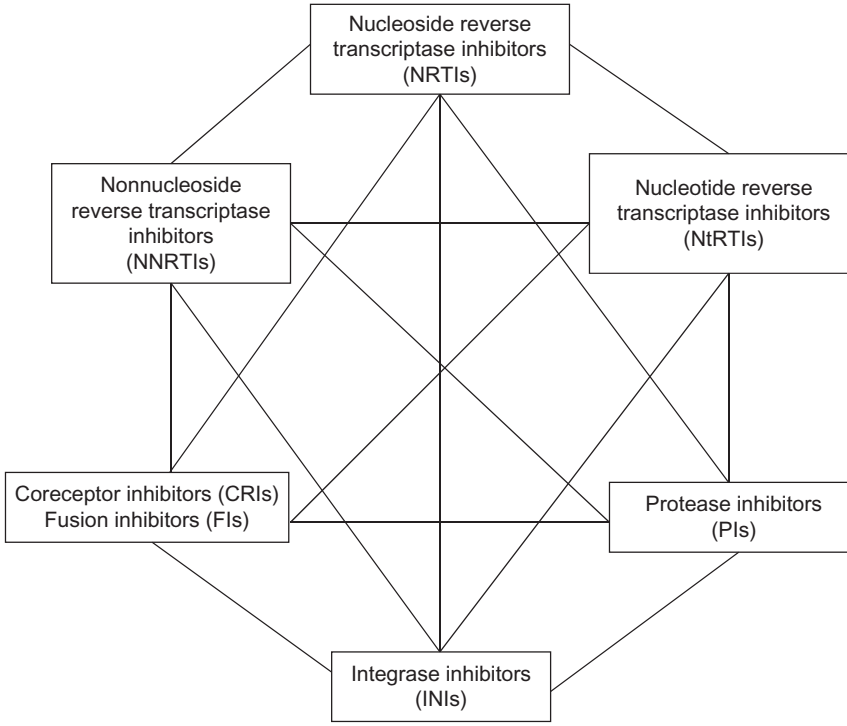


Figure 9.11 Anti-HIV drug combinations.

darunavir/ritonavir plus raltegravir may lead to virologic failure particularly in patients with baseline viral load higher than 100,000 copies/ml (Taiwo et al., 2011), but greater efficacy could be expected if etravirine would be added to this drug regimen (Fagard et al., 2012).

Atazanavir plus ritonavir and efavirenz have similar antiviral activity when used with abacavir/lamivudine or TDF/(−)FTC (Daar et al., 2011). Boosting with ritonavir produces higher atazanavir plasma levels which are beneficial in terms of efficacy, especially in patients with high plasma HIV RNA levels (Focà, Ripamonti, Motta, & Torti, 2012).

In the future, ritonavir may be replaced as a boosting agent by cobicistat, which has been proved noninferior to ritonavir in a pivotal phase III study (Study 114) (Gallant et al., 2012) and which has been scheduled for use in combination with elvitegravir and Truvada® (TDF/(−)FTC) as part of the quad pill Stribild®, and in combination with atazanavir and Truvada® as part of another quad pill.

7. CLINICAL ASPECTS

Efavirenz, in comparison with nevirapine, was estimated to lower mortality, lower incidence of AIDS-defining illness, and cause a larger 12-month increase in CD4 cell count and a smaller risk of virologic failure at 12 months (The HIV-CAUSAL Collaboration, 2012). For first-line therapy of HIV infection, efavirenz gave a faster initial viral decay than lopinavir (Haubrich et al., 2011). As compared to efavirenz (at a dose of 600 mg), however, rilpivirine (at a dose of 25 mg) proved noninferior (James, Preininger, & Sweet, 2012), and in addition, it was also better tolerated than efavirenz, especially with regard to the psychiatric–neurologic adverse events and blood lipid abnormalities (Sanford, 2012).

Of the PI regimens, an atazanavir/ritonavir-based regimen is not inferior in antiviral efficacy to a lopinavir/ritonavir-based regimen (Zhu et al., 2012); fosamprenavir may be useful to control HIV replication in the central nervous system (CNS) (Croteau et al., 2012); and tipranavir (boosted with ritonavir) may be a valuable option in highly antiretroviral treatment-experienced patients (Allavena et al., 2012). Ritonavir-boosted tipranavir and darunavir showed similar short-term safety and efficacy (Elgadi & Piliero, 2011).

A darunavir-based regimen appears to be a sequential option in the case of lopinavir/ritonavir failure (Lambert-Niclot, Masquelier, et al., 2012). Since darunavir exhibits efficacy against viruses with significant PI resistance mutations, darunavir may be critically important in the treatment of experienced patients (Robertson & Feinberg, 2012). As the majority of patients receiving second-line antiretroviral therapy started to accumulate protease resistance mutations, darunavir might be advocated as the drug of choice for third-line regimens, at least in India (Saravanan et al., 2012).

8. PREEXPOSURE PROPHYLAXIS

The CAPRISA 004 and iPrEx studies have indicated the efficacy of anti-HIV drugs, in particular, tenofovir gel and TDF/(−)FTC, in, respectively, the topical application (as a microbicide) and oral administration for the prevention of HIV infections (Abdool Karim et al., 2010; Grant et al., 2010), but the early termination of other studies, that is, FEM-preexposure prophylaxis (PrEP) and one arm of VOICE, has tempered the enthusiasm

(Person & Hicks, 2012). Many questions have remained, among which the best route for administering the PrEP products.

Microbicides are topical PrEP products, such as gels, capsules, tablets, films, and intravaginal rings, and the positive data from the CAPRISA 004 trial of tenofovir gel represented a turning point in the field (Shattock & Rosenberg, 2012). Intravaginal rings would allow simultaneous delivery of several drugs, that is, tenofovir and acyclovir (Moss et al., 2012). Various combinations of antiretroviral drugs could be concocted as microbicides (Balzarini & Schols, 2012), and PIs could be included in such combination microbicides (Selhorst et al., 2012). Saquinavir could be considered for this purpose as it was found capable of blocking viral maturation and transmission of HIV-1 at mucosal surfaces (Stefanidou, Herrera, Armanasco, & Shattock, 2012).

One of the major questions is who should get PrEP (Person & Hicks, 2012). Stable heterosexual HIV-1 discordant couples in Africa have high HIV-1 transmission rates and seem a critical population for evaluation of PrEP strategies (Mujugira et al., 2011). PrEP use in stable couples may be associated with improved adherence and thus increased effectiveness (Ware et al., 2012). Policymakers, healthcare workers, and NGOs, particularly from Sub-Saharan Africa, would be willing to support PrEP if proven cost-effective (Wheelock et al., 2012). Cost-effectiveness has remained a critical issue for the use of TDF/(−)FTC for PrEP (Keller & Smith, 2011).

9. PREVENTION OF MOTHER-TO-CHILD TRANSMISSION

For the prevention of mother-to-child transmission (PMTCT) of HIV-1, administration of a single dose of nevirapine has been shown to reduce the transmission risk by 40% or more (Guay et al., 1999; Stringer et al., 2003). Transmission of HIV is more effectively reduced if nevirapine (NVP) is combined with zidovudine (AZT) and lamivudine (3TC) (Dabis et al., 2005; Lallemant et al., 2002). Given the role of nevirapine resistance, even after a single dose of the compounds in mothers or children (Arrivé et al., 2007), it has been recommended by the World Health Organization (WHO) to use AZT + 3TC + NVP as the standard PMTCT regimen wherever feasible (McIntyre et al., 2009).

In neonates whose mothers did not receive antiretroviral therapy (ART) during pregnancy, prophylaxis with a two-drug ART regimen (AZT/NVP) or three-drug ART regimen (AZT/3TC/nelfinavir) is superior to zidovudine alone for the prevention of intrapartum HIV transmission; rate of

in utero transmission of HIV-1 were 2.2% and 2.4% with the two-drug group and three-drug group, respectively, as compared to 4.8% for zidovudine alone (Nielsen-Saines et al., 2012). A maternal triple-ART regimen of AZT, 3TC, and NVP or nelfinavir from 34 to 36 weeks gestation to 6 months postpartum during breast feeding would seem safe and feasible in a resource-limited setting (Thomas et al., 2011). A 1-week "tail" of lamivudine and zidovudine following the single intrapartum dose of nevirapine would prevent most NVP resistance (Van Dyke et al., 2012).

Besides antiretroviral therapy (ART), caesarian delivery is another protective factor for mother-to-child transmission of HIV (Delicio et al., 2011), and so is valaciclovir, apparently because of its inhibitory activity against a concurrent herpes simplex virus type 2 infection, which would otherwise stimulate HIV infection (Drake et al., 2012).

10. CHILDREN

In resource-limited settings, nevirapine-based ART is often the only therapeutic regimen available for children. Nevirapine resistance after exposure to the drug for PMTCT of HIV transmission is a common problem that has led to the recommendation of using ritonavir-boosted lopinavir in such settings (Violari et al., 2012). In HIV-infected children of 2–36 months of age who had no prior exposure to nevirapine, ritonavir-boosted lopinavir provided a better outcome than nevirapine, when combined with zidovudine and lamivudine (Violari et al., 2012). In the meantime, the safety of a new pediatric fixed-dose combination (FDC) of zidovudine/lamivudine/nevirapine in HIV-infected children has been established (Chokephaibulkit et al., 2011).

11. TOXICITY

The successful use of anti-HIV agents, particularly HIV PIs, has greatly contributed to the transformation of HIV infection from a once fatal disease to a chronic illness (Palella et al., 1998). This has been tempered by an increased risk for the development of proatherogenic metabolic side effects, including dyslipidemia and insulin resistance (Grinspoon, 2005; Mulligan et al., 2000). In fact, the "older" HIV PIs directly alter glucose homeostasis (Brown et al., 2005), as they act as competitive inhibitors of the insulin-responsive glucose transporters (Hresko & Hruz, 2011).

The main risks associated with the use of the NNRTIs are rash for nevirapine (Dong et al., 2012) and neuropsychiatric adverse events for efavirenz (Muñoz-Moreno et al., 2009). CNS adverse events may be an acceptable reason for switching from an efavirenz-containing regimen (such as Atripla(R); Scourfield et al., 2012) to a rilpivirine-containing regimen (such as Complera®). Also, lipid elevations were higher in efavirenz-containing regimens than in those containing etravirine (Fätkenheuer et al., 2012).

Stavudine has been associated with significant (long-term) morbidity, that is, lipoatrophy, lactic acidosis, hyperlactatemia, and peripheral neuropathy (Pujades-Rodríguez et al., 2011), which could be partially remedied by a dosage reduction (from 40 to 30 mg), but should eventually lead to a phasing-out of stavudine in rollout programs in resource-limited countries (Menezes, Maskew, Sanne, Crowther, & Raal, 2011; Phan, Thai, Choun, Lynen, & van Griensven, 2012).

For abacavir (ABC), hypersensitivity reactions have been noted, which can be screened for by HLA-B5701 testing (Mallal et al., 2008). Of greater concern is the still debated connection between ABC and the increased risk for myocardial infarction (Behrens, 2011; Sabin et al., 2008). While most patients may be expected to safely continue their often long-lasting ABC therapy, it would be wise not to start ABC as part of a new regimen in patients with high risk for coronary heart disease (Behrens, 2011). Abacavir leads to the formation of some reactive aldehyde metabolites (Charneira et al., 2011), and, through inhibition of guanylyl cyclase, increases platelet reactivity (Baum, Sullam, Stoddart, & McCune, 2011), which might explain the risk of myocardial infarction. *In vitro*, neither abacavir nor didanosine or tenofovir had any direct effect on coronary endothelial cells; if abacavir increases the cardiovascular risk, it must be through a mechanism other than the coronary endothelial activation (Kim et al., 2011).

For PIs, concerns have arisen about their potential adverse effects on cardiac conductivity, as manifested by QT and PR interval durations on the standard electrocardiogram (Anson et al., 2005; Ly & Ruiz, 2007; Singh, Arora, & Jawad, 2010). However, Worm et al. (2012) found no significant association between recent PI exposure and sudden death or nonhemorrhagic stroke, although the authors stated further that "cumulative exposure to PIs was associated with these outcomes" (Worm et al., 2012).

There was no increased risk of liver toxicity in patients who had been treated with NNRTIs (nevirapine or efavirenz) for at least 3 years (Van Welzen, Mudrikova, Arends, & Hoepelman, 2012). The frequency of grade 3 or 4 transaminase elevations in HIV/HCV-coinfected patients treated

with an efavirenz-based combination was low and similar to that found in patients receiving ritonavir-boosted PIs (Neukam et al., 2011). Similarly, efavirenz appeared to be safe in HIV/HCV-coinfected patients with advanced liver fibrosis (Pineda et al., 2012). However, in another study, the hepatic adverse events in HIV patients treated with efavirenz or rilpivirine were higher in HBV/HCV-coinfected patients than in those that were not coinfected (26.7% vs. 4.1%) (Nelson et al., 2012).

12. ADHERENCE (COMPLIANCE)

The success of antiretroviral treatment (ART) for HIV infection is primarily determined by adherence (Bärnighausen et al., 2011). The latter depends on a number of factors which have been categorized as either behavioral, cognitive, affective, biologic, structural (or combination, thereof). Use of an FDC such as Epzicom® (abacavir + lamivudine) appears to substantially improve adherence to a third regimen component, which has been labeled as a spillover adherence effect of the FDC combination HIV therapy (Kauf, Davis, Earnshaw, & Davis, 2012).

Both the presence of minority NNRTI resistance mutations and NNRTI adherence could influence virologic failure (Li, Paredes, et al., 2012). Adherence may be facilitated by reducing perceptual and practical barriers to ART, favoring the use of Truvada once daily over Combivir (zidovudine plus lamivudine) twice daily (Cooper et al., 2011). Adherence is further increased by the use of Atripla (efavirenz/(−)FTC/TDF fixed-dose regimen), thereby showing the positive impact of single-daily oral medication on pill adherence (Juday, Gupta, Grimm, Wagner, & Kim, 2011).

The importance of counseling (Chung et al., 2011), HIV testing and treatment (Rosen & Fox, 2011), and loss of follow-up (LTFU) in ART programs (Wandeler et al., 2012) has proved to be of mandatory adherence, especially in Sub-Saharan Africa, and so is adherence to ART treatment in both HIV-infected women (Musiime et al., 2011) and HIV-infected children (Haberer et al., 2011; van Dijk et al., 2011).

13. RESISTANCE

Mutations engendering resistance toward NRTIs and NNRTIs in the HIV-1 RT and toward PIs in the protease are schematically presented in Fig. 9.12 (Cortez & Maldarelli, 2011). NRTI, NNRTI, and PI resistance mutations have also been reviewed by Arts and Hazuda (2012).

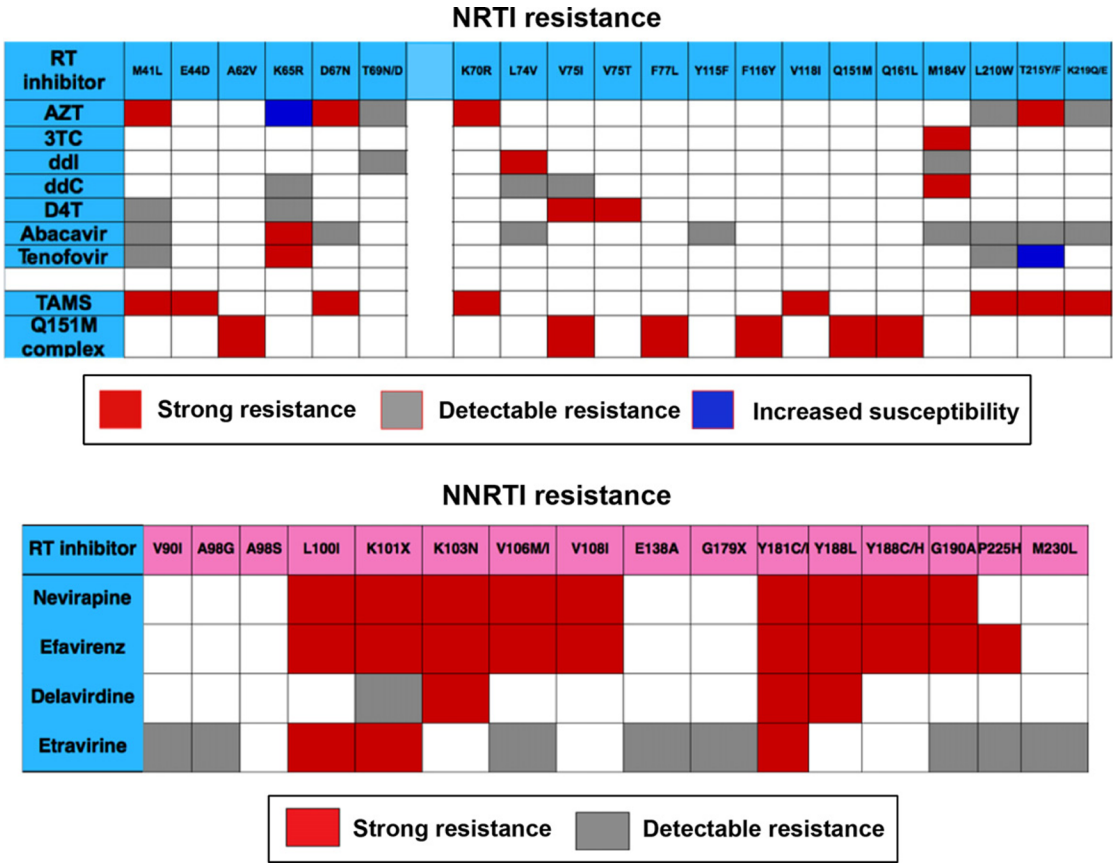


Figure 9.12—cont'd

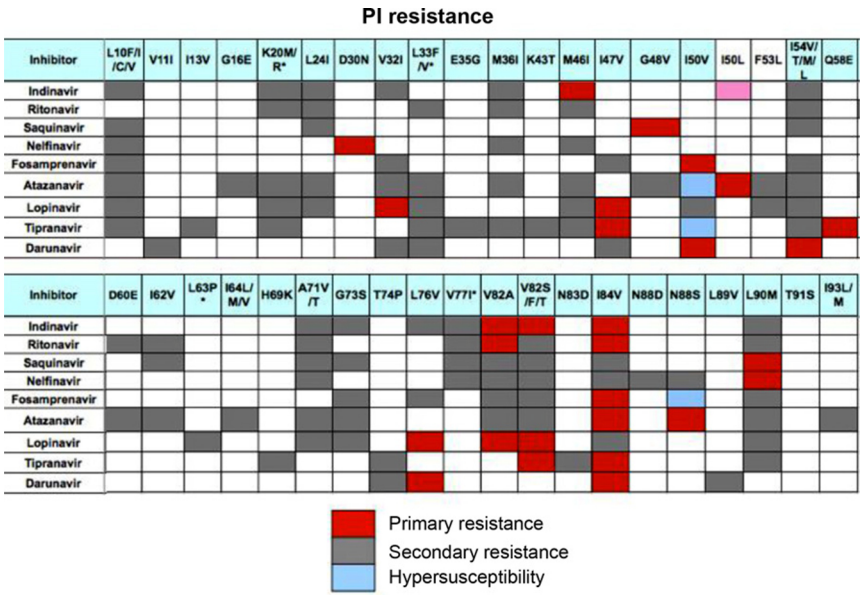


Figure 9.12 NRTI resistance, NNRTI resistance, and PI resistance mutations in HIV-1 RT (NRTIs, NNRTIs) and protease (PIs), respectively. *According to Cortez and Maldarelli (2011).*

Resistance to NRTIs is mediated by two mechanisms: ATP-dependent pyrophosphorolysis, that is, removal of NRTIs from the 3′end of the nascent chain (Arion, Kaushik, McCormick, Borkow, & Parniak, 1998; Boyer, Sarafianos, Arnold, & Hughes, 2001; Meyer, Matsuura, Mian, So, & Scott, 1999), and prevention of the incorporation of the NRTI into the nascent chain. Mutations associated with the second mechanism include M184V/I and K65R. The M184V mutation emerges with 3TC or (−) FTC therapy (Schinazi et al., 1993), whereas treatment with tenofovir, ddC, ddI, d4T, and ABC can select K65R (Wainberg et al., 1999). M184V counteracts K65R so that K65R is rarely seen in patients on both (−)FTC and TDF. In fact, the M184V/I mutation was significantly lower in patients receiving (−)FTC and TDF than in those receiving 3TC and TDF (Marcelin, Charpentier, et al., 2012).

Resistance to AZT and other thymidine analogues is generally imparted by the first mechanism (pyrophosphorolysis), which leads to excision of the chain-terminating residue, that is, AZT-MP, after it has been incorporated (Scott, 2011). The mutations (thymidine-associated mutations) leading to excision include M41L, D67N, K70R, L210W, T215Y/F, and K219Q. An excision-based resistance mechanism has been noted only with HIV-1 RT and not with HIV-2 RT, which would act only by the exclusion pathway (Boyer, Clark, & Hughes, 2012).

NNRTI resistance generally results from amino acid substitutions such as L100, K101, K103, E138, V179, Y181, and Y188 in the NNRTI-binding pocket of RT (Tantillo et al., 1994). The most common NNRTI mutations are K103N and Y181C (Bacheler et al., 2000, 2001). The majority of NNRTI resistance mutations selected under NNRTI treatment are commonly found as wild type in HIV-1 group O and HIV-2 and are linked to a Cys, Ile, or Tyr at position 181 (Tebit et al., 2010).

Although etravirine retains significant activity against RT mutants containing K103N or Y188C, emerging mutations are associated with etravirine failure, that is, at position V179 (Marcelin, Descamps, et al., 2012). In patients with HIV-1 subtype CRF01_AE infection, etravirine resistance may be seen in up to 60% of patients (Bunupuradah et al., 2011). In these cases, there was also high cross-resistance to rilpivirine.

In fact, etravirine and rilpivirine are likely to select for E138K as the major resistance mutation (Asahchop et al., 2013), but this resistance may be counteracted by the Y181C mutation (Xu, Oliveira, Asahchop, et al., 2012). On the other hand, the resistance engendered by the E138K mutation could be enhanced by the M184I mutation (in response to (−)FTC

present in the rilpivirine/(−)FTC/TDF combination) (Kulkarni et al., 2012). While E138K abrogates the polymerase defect of M184I, it reduces rilpivirine-binding affinity mainly by increasing its dissociation rate (Singh et al., 2012). These mutational effects occurred via both subunits (Xu, Oliveira, Quashie, et al., 2012).

Mutations in the connection domain of the HIV-1 RT have also been linked to resistance to NRTIs and NNRTIs, that is, the A360V mutation which was selected by AZT monotherapy (Brehm et al., 2012). Other mutations in the connection domain, that is, Y318F/W, N348I, A376S, and F369I/V, may be involved in resistance to NNRTIs (Menéndez-Arias, Betancor, & Matamoros, 2011).

As it has a vital role in the life cycle of HIV-1, the HIV protease was initially not expected to be a cradle for resistance development. However, the protease has great plasticity with polymorphisms observed in 48 of the 99 codons, and more than 20 substitutions known to be associated with resistance (Fig. 9.12). For PI resistance, HIV appears to follow a "stepwise" pathway to overcome drug sensitivity: (i) acquisition of primary resistance mutations in the protease gene, (ii) selection of secondary/compensatory protease mutations to repair the enzymatic function and rescue viral fitness, and (iii) selection of mutations in the major cleavage sites of the gag and gag-pol polyprotein precursors that restore protein processing (Arts & Hazuda, 2012).

For the mutations conferring resistance to PIs (Fig. 9.12), distinction is made between primary mutations, generally drug specific, and secondary mutations, which by themselves confer little resistance but in the presence of primary mutations lead to cross-resistance to various PIs (Shafer & Schapiro, 2008; Turner, Schapiro, Brenner, & Wainberg, 2004).

Minor PI mutations at baseline do not increase the risk for virologic failure in HIV-1 subtype B-infected patients (Scherrer et al., 2012). In patients with poor or intermittent adherence, there may be a low prevalence of PI resistance (Fisher et al., 2012). Especially in patients with failure on darunavir/ritonavir monotherapy, darunavir-resistant minority species are rarely observed (Lambert-Niclot, Flandre, et al., 2012). Yet, preexisting HIV-1 drug resistance mutations in drug-naïve patients should be carefully monitored (Aghokeng et al., 2011).

14. NEW NRTIs, NNRTIs, AND PIs

Among the new NRTIs worth exploring for their potential as anti-HIV drugs is (-)-β-D-(2*R*,4*R*)-dioxolane thymine (DOT) (Chu, Yadav,

Chong, & Schinazi, 2005; Lennerstrand, Chu, & Schinazi, 2007). However, it is poorly phosphorylated to the 5′-monophosphate, a problem that can be circumvented by phosphoramidate prodrugs of DOT (Wang, Rachakonda, et al., 2012). Another promising NRTI is 4′-ethynyl-2-fluoro-2′-deoxyadenosine (EFdA) (Hattori et al., 2009; Kawamoto et al., 2008; Kohgo et al., 2003; Michailidis et al., 2009; Murphey-Corb et al., 2012; Nakata et al., 2007; Ohrui et al., 2007). EFdA (Sohl, Singh, et al., 2012) and its thymine counterpart, Ed4T, have a low potential for mitochondrial toxicity (Sohl, Kasiviswanathan, et al., 2012).

Various strategies for the design of new NNRTIs have been described (Li, Zhan, De Clercq, & Liu, 2012). Representative examples of such new NNRTIs are the dihydro-alkoxyl-benzyl-oxopyrimidine derivatives (Yang, Chen, & De Clercq, 2012), 1-benzyl-3-(3,5-dimethylbenzyl)uracil derivatives (Isono et al., 2011), truncated reverse isoxazolidinyl nucleosides (Romeo et al., 2012), thiazolidin-4-ones (Murugesan et al., 2011), imidazole-5-one derivatives (Mokale, Lokwani, & Shinde, 2012), and catechol diethers (Bollini et al., 2011). Among the latter, one compound showed an EC_{50} as low as 0.055 nM (Bollini et al., 2011).

For the next generation of NNRTIs, compounds are sought that possess both broad spectrum antiviral activity against key mutant strains and a high genetic barrier to the selection of new mutant viral strains (Gomez, Jolly, Williams, Tucker, et al., 2011). In this perspective, conformationally constrained NNRTIs were synthesized (Gomez, Jolly, Williams, Vacca, et al., 2011). Attempts have been mainly focused to improve activity, in particular, against the RT single or double mutants (K103N and Y181C), that is, with aryl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazones (Ma et al., 2011), difluoromethylbenzoxazole pyrimidine thioether derivatives (Boyer et al., 2011), 1-[(2-benzyloxy/alkoxyl)methyl]-5-halo-6-aryluracils (Wang, Zhang, et al., 2012), and new indolylaryl sulfone derivatives-bearing nitrogen-containing substituents at the indole-2-carboxamide (La Regina et al., 2012). Although these attempts have been encouraging, activity against the K103N and Y181I (single and double mutants) could still be much improved upon (Samuele et al., 2011).

There are, at present, no new HIV-1 PIs forthcoming for clinical use, given the multitude (10 compounds!) that have already been licensed. Yet, the same principle, the 2-hydroxyethyl scaffold replacing the peptidomimetic core, has remained the basis for the design of new HIV-1 PIs (Parai et al., 2012). This principle also remains valid for novel amprenavir-based P1-substituted bi-aryl derivatives accredited as ultrapotent HIV PIs (Yan et al., 2012).

Ritonavir, being approved as an HIV PI (600 mg BID (=twice a day)), is rarely used as such but more frequently as a pharmacokinetic enhancer, as inhibitor of the cytochrome P450 enzyme CYP3A4, to enhance ("boost") the efficacy of other PIs (such as lopinavir or atazanavir). As ritonavir as such can lead to gastrointestinal side effects and changes in serum lipids, insulin resistance, lipoatrophy, and PI resistance mutations, it should in the future be replaced by cobicistat (GS-9350), a compound structurally related to ritonavir, with a CYP3A4 inhibition comparable to that of ritonavir, but no anti-HIV activity. This prevents the formation of PI resistance. Cobicistat should enhance ("boost") the anti-HIV activity of other compounds, depending on CYP3A4 for their liver metabolism, including elvitegravir, and, hence, cobicistat has stimulated the synthesis of other, novel pharmacokinetic enhancers of HIV PIs (Jonckers et al., 2012).

15. CONCLUSION

As early in 1995, David Ho reasoned that to hit HIV, treatment should be early and hard (Ho, 1995). The benefit achieved would be highest when viral turnover would be at its peak. With more effective treatment modalities becoming available, immediate treatment (IT) should be preferred over deferred treatment (Hogan et al., 2012; Tossonian & Conway, 2012).

Through IT with the currently available antiretrovirals, a "functional" cure would be achievable; to this end, three conditions should be fulfilled: (i) no disease progression, (ii) no virus transmission, and (iii) a life expectancy close to that of uninfected individuals (not treated with antiretrovirals) (Lafeuillade, 2011). Is there a chance of eliminating the HIV reservoir, thus achieving a "sterilizing" HIV cure? A case in point is the so-called Berlin case, that is, the long-term control of an HIV-infected patient following two allogeneic bone marrow transplants for leukemia from a donor with a homozygous CCR5 Δ32 deletion (Hütter et al., 2009); the patient became seronegative after more than 4 years of follow-up and was declared "cured" (Allers et al., 2011).

Lafeuillade (2012) has discussed several strategies for HIV eradication by purging its reservoirs, using, for example, histone deacetylase inhibitors (Choi et al., 2010; Matalon, Rasmussen, & Dinarello, 2011), vorinostat (Cohen, 2011), bryostatin-1, a protein kinase C activator (Pérez et al., 2010), or quinolinol (Gallastegui et al., 2012; Xing et al., 2011, 2012). All these compounds are capable of reactivating latent HIV-1 from its reservoirs, but this is only part of the equation, as concomitantly with the reactivation, the virus should also be eliminated, and this remains to be ascertained.

CONFLICT OF INTEREST

The author has no conflicts of interest to declare.

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