

## Antiviral Strategies

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# Other Inhibitors of Viral Enzymes and Functions

H. Zimmermann, G. Hewlett, and H. Rübsamen-Waigmann

## Contents

|   |   |     |
|---|---|-----|
| 1 | Introduction .....  | 156 |
| 2 | Non-Nucleoside Inhibitors of HIV Reverse Transcriptase (NNRTIs) ..... | 157 |
| 3 | Integrase Inhibitors .....  | 159 |
| 4 | Helicase Inhibitors .....   | 162 |
| 5 | Terminase Inhibitors .....  | 164 |
| 6 | Maturation/Assembly Inhibitors .....                                  | 168 |
| 7 | Conclusions .....   | 170 |
|   | References .....  | 170 |

**Abstract** Until the end of the 1970s, the mainstays of antiviral chemotherapy were nucleoside analogues that targeted virus polymerase, in particular, the herpesvirus DNA polymerase. The scourge of HIV triggered an unprecedented commitment to identify novel antivirals, and these efforts transformed antiviral therapy into the modern, sophisticated treatment form described in this book, with targets such as the reverse transcriptase and the protease as well as the entry of the human immunodeficiency virus. As the regulation of human pathogenic virus growth cycles became more understandable, the realisation grew that these pathogens had more than one Achilles heel that might be suitable targets for small molecules with antiviral activity. This chapter addresses those “other” targets as well as other approaches to the tried and tested polymerase inhibitors, the so-called non-nucleoside inhibitors of reverse transcriptase.

## Abbreviations

|             |                                   |
|-------------|-----------------------------------|
| <i>DNA</i>  | Deoxyribonucleic acid             |
| <i>HIV</i>  | Human immunodeficiency virus      |
| <i>SARS</i> | Severe acute respiratory syndrome |

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|                 |  |
|-----------------|--|
| <i>NNRTI</i>    | Non-nucleoside reverse transcriptase inhibitors                            |
| <i>HAART</i>    | Highly active anti-retroviral therapy                                      |
| <i>NRTI</i>     | Nucleoside reverse transcriptase inhibitor                                 |
| <i>RT</i>       | Reverse transcriptase  |
| <i>LTR</i>      | Long terminal repeat   |
| <i>DKA</i>      | Diketo acid; PI: protease inhibitor  |
| <i>INSTI</i>    | Integrase strand transfer inhibitor  |
| <i>OBT</i>      | Optimised background therapy   |
| <i>SF</i>       | Superfamily  |
| <i>NTP</i>      | Nucleoside triphosphate  |
| <i>HCV</i>      | Hepatitis C virus  |
| <i>NTPase</i>   | Nucleoside triphosphatase  |
| <i>RNA</i>      | Ribonucleic acid   |
| <i>SARS-CoV</i> | Severe acute respiratory syndrome coronavirus                              |
| <i>HCMV</i>     | Human cytomegalovirus  |
| <i>ARV</i>      | Anti-retroviral  |
| <i>AIDS</i>     | Acquired immune deficiency syndrome  |
| <i>GCV</i>      | Ganciclovir  |
| <i>PFA</i>      | Foscarnet  |
| <i>CDV</i>      | Cidofovir  |
| <i>ORF</i>      | Open reading frame   |
| <i>ATP</i>      | Adenosine triphosphate   |
| <i>BDCRB</i>    | 2-Bromo-5,6-dichloro-1- $\beta$ -d-ribofuranosyl-1 <i>H</i> -benzimidazole |
| <i>TCRB</i>     | 2,5,6-trichloro-1- $\beta$ -d-ribofuranosyl-1 <i>H</i> -benzimidazole      |
| <i>HBV</i>      | Hepatitis B virus  |
| <i>HAP</i>      | Heteroaryl dihydropyrimidine   |
| <i>HSV</i>      | Herpes simplex virus   |

## 1 Introduction

In recent years, the spectre of a viral epidemic or even a pandemic has become more of a reality than ever before. The sudden appearance and the spread of HIV alerted the general public to the evils of pathogenic viruses and caught the attention of the media throughout the world. Since then, we have monitored the HIV outbreak and seen it growing and have been confronted with the threat of a SARS epidemic and, more recently, tried to prepare for a potential pandemic of H5N1 avian influenza variants. It is clear that there is an urgent need for more effective antiviral drugs, directed not only against what now can be termed the “classical” targets like polymerase and protease, but also against novel targets and with novel mechanisms of action. Such new approaches to antiviral therapy mainly targeting viral enzymes will be discussed in the following paragraphs.

## 2 Non-Nucleoside Inhibitors of HIV Reverse Transcriptase (NNRTIs)

Combination anti-HIV chemotherapy, commonly referred to as highly active anti-retroviral therapy (HAART), has led to a dramatic reduction in mortality and morbidity in HIV-infected patients (Lee et al. 2001). Thus far, over 20 anti-HIV drugs have been approved for the treatment of HIV infection. Despite the availability of these approved anti-retroviral drugs, there is still a need for new anti-retrovirals to improve convenience, reduce toxicity and, of particular and growing importance, to provide activity against drug-resistant HIV strains (Pauwels 2004), which not only emerge in infected individuals but are also being transmitted at increasing incidence.

The first lead compounds for non-nucleoside reverse transcriptase (RT) inhibitors (NNRTI) were discovered about 15 years ago (Pauwels et al. 1990; Merluzzi et al. 1990; Goldman et al. 1991; De Clercq 1993; Rübsamen-Waigmann et al. 1997). Since then they have become an important ingredient of the drug combination schemes that are currently used in the treatment of human immunodeficiency virus type 1 (HIV-1) infections. Starting from the HEPT and TIBO derivatives, numerous classes of compounds have been described as NNRTIs. Four compounds (nevirapine, delavirdine, efavirenz and etravirine) have so far been approved for clinical use and several others are the subject of clinical trials (Balzarini 2004; Stellbrink 2007).

Of the NNRTIs that were first approved, nevirapine and, even more so, efavirenz became cornerstones of HIV therapy because of their potential as a component of HAART (Staszewski et al. 1999). The most commonly used NNRTI drug is efavirenz. In addition, nevirapine was shown to effectively prevent HIV transmission from mother to baby. NNRTIs have proven beneficial when included in drug combination (triple or quadruple) therapy, preferably in the presence of protease inhibitors and NRTIs.

Although the NNRTIs target HIV-1 RT, they are clearly different from the nucleoside RT inhibitors (NRTIs). They are highly selective for HIV-1 and do not inhibit HIV-2 or any other retrovirus. Moreover, the resistance spectrum of NNRTIs is different from that of NRTI, and, as a rule, NRTI-resistant mutant virus strains keep full sensitivity to the inhibitory effects of NNRTIs, and NNRTI-resistant mutant virus strains keep full sensitivity to the inhibitory effects of NRTIs. However, some influence of NRTI mutations on NNRTI susceptibility has been observed (Shulman et al. 2004).

The majority of NNRTIs share common conformational properties and structural features that allow them to fit into an asymmetric, hydrophobic pocket about 10 Å away from the catalytic site of the HIV-1 RT, where they act as non-competitive inhibitors (Kohlstaedt et al. 1992). However, the NNRTIs select for mutant virus strains with several degrees of drug resistance.

The first-generation NNRTIs, such as nevirapine, delavirdine and efavirenz, easily lose their inhibitory potential against mutant virus strains that contain single amino acid mutations in their RT. This resistance development is primarily based on the emergence of the K103N and Y181C mutations in the HIV-1 RT. So far, drug resistance has been seen for all anti-HIV drug classes and individual agents, including the NNRTIs (Deeks 2001; Wainberg 2003; Bachelier et al. 2001). This is important because antiretroviral drug resistance is the main cause and/or consequence of current therapy failure.

The second-generation NNRTIs usually require two or more mutations in the HIV-1 RT before a significant loss of antiviral potency occurs. Evidently, a markedly longer period of time is required before significant resistance against second-generation NNRTIs can arise, and therefore these compounds offer considerable promise as future anti-HIV-1 drugs.

Initial clinical trials with these new NNRTI drug candidates have provided the first in vivo evidence of their antiviral potency in both drug-naïve (Herandez et al. 2000; Gruzdev et al. 2003) and NNRTI-experienced patients (Hammond et al. 2003; Wolfe et al. 2001; Gazzard et al. 2003).

Examples of the new generation of NNRTIs are etravirine (TMC125) and rilpivirine (TMC278), with activity against both wild type and resistant viral isolates. Etravirine was approved by the US Food and Drug Administration in January 2008 and is indicated for the treatment of HIV-1 infection in antiretroviral treatment-experienced adult patients who have evidence of viral replication and HIV-1 strains resistant to an NNRTI and other ARV agents.

Rilpivirine shows a long half-life, excellent safety profile, and can be used once daily. Common NNRTI-resistance mutations, particularly K103N, do not appear to have substantial impact on the activity of etravirine and rilpivirine. Moreover, because of conformational changes, these compounds bind flexibly to the RT of HIV, presenting a higher genetic barrier for resistance. In a phase 2b dose-ranging study, rilpivirine was found to be generally safe and well-tolerated and showed a sustained 2.6 log decline in HIV RNA by week 12–16 that was maintained out to 48 weeks.

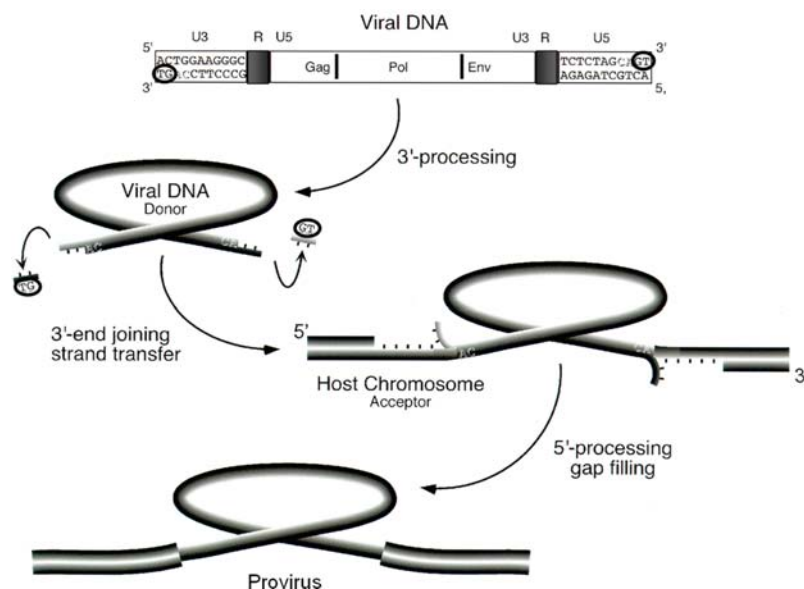
Given the increasing need for new NNRTIs in HAART regimes and the current interest in etravirine, the successor drug rilpivirine may become the next NNRTI for first-line therapy and may conceivably also have utility for people who harbour viruses resistant to nevirapine and efavirenz. Phase 3 clinical studies with rilpivirine are being initiated in 2008 and the results are eagerly awaited. The unmet medical need for NNRTIs with a higher barrier to resistance has led to increased interest in other next generation NNRTIs, which has in turn led to several more drugs currently in clinical development. Three drugs are currently in phase 2 (UK-453.061 from Pfizer, IDX 12899 from Idenix and RDEA-806 from Ardea Biosciences) and there are two more reported phase 1 activities (RDEA-427 from Ardea and MK-4965 from Merck). It will be interesting to see how these drugs will develop further and strengthen the importance of NNRTIs in HIV therapy.

### 3 Integrase Inhibitors

The HIV integrase is one of the three virally encoded enzymes required for HIV-1 replication and catalyses the integration of viral DNA into a host chromosome (Esposito and Craigie 1999; Asante-Appiah and Skalka 1999). The integrase of HIV-1 is a 32-kDa enzyme that is encoded together with the reverse transcriptase and the protease by the *pol* gene of HIV. It is generated during virion maturation by proteolytic processing of the Gag–Pol precursor, and approximately 40–100 integrase molecules are packaged into each HIV particle.

HIV integrase consists of three distinct domains. The N-terminal domain contains a HHCC motif that coordinates a zinc atom that is required for viral cDNA integration. Three highly conserved amino acids (D,D-35-E) are embedded in the core domain, which form the acidic catalytic triad coordinating one or possibly two divalent metals ( $Mn^{2+}$  or  $Mg^{2+}$ ). The C-terminal domain (residues 213–288) is responsible for unspecific DNA binding and adopts an overall SH3 fold (Chiu and Davies 2004). The enzyme functions as a multimer and to this end all three domains can form homodimers.

The integration of newly synthesized viral DNA into the host chromosome is a multi-step process (Anthony 2004; Van Maele and Debyser 2005) that relies on the integrity of the last 10–20 base pairs at both ends of the viral cDNA besides a fully functional integrase (see Fig. 1). Initially, integrase recognizes the long-terminal repeat (LTR) of the retro-transcribed viral DNA and performs endonucleolytic



**Fig. 1** Integration of HIV DNA into the host genome

processing (3'-processing) of the 3' ends of both strands via recognition of an absolutely conserved CA dinucleotide and specific cleavage of the terminal GT dinucleotide downstream, thereby generating two recessed CA-3'-hydroxyl DNA ends that serve as nucleophiles in the following strand transfer step. During and after 3'-processing, a multimeric pre-integration complex (PIC) is formed, which comprises the integrase still bound to the viral cDNA as well as viral (reverse transcriptase, matrix, nucleocapsid and Vpr) and cellular factors (e.g. lens epithelium-derived growth factor (LEDGF/p75), barrier-to-autointegration factor (BAF) and HMGA1) (for review see Turlure et al. 2004). Subsequently, the HIV PIC is translocated to the nucleus via the intact nuclear envelope. The karyophilic property enables HIV to replicate in non-proliferating cells, such as terminally differentiated macrophages.

After cleavage of the host DNA, both viral 3'-hydroxyl DNA ends are ligated to opposite strands of the acceptor DNA in a trans-esterification reaction. Finally, for ligation to the acceptor DNA, the last two nucleotides at the 5'-end of the viral cDNA are trimmed and gap filling is performed, probably carried out by host cell repair (Pommier et al. 2005).

Since integration of viral DNA into the cellular chromosome is an essential step in the viral replication cycle, ensuring the stable maintenance of the viral genome in the host organism (Chiu and Davies 2004; Wiskerchen and Muesing 1995), it represents an attractive target for therapeutic intervention (Anthony 2004; Debyser et al. 2002; Witvrouw et al. 2005; Kehlenbeck et al. 2006). Accordingly, the search for integrase inhibitors has been ongoing for a long time, but only recently met with success. Early drug development mainly focussed on in vitro screening for inhibitors of 3' processing which, however, showed only low potency against viral replication.

The discovery of a series of diketo acid (DKA) containing HIV-1 integrase inhibitors provided the first proof of concept for HIV-1 integrase inhibitors as antiviral agents (Hazuda et al. 2000; Wai et al. 2000). DKA derivatives act as specific strand-transfer inhibitors and trap selectively a catalytic transition state (the 3'-processing intermediate) of the PIC. They target the catalytic motif D,D,-35-E of the core domain and compete in binding with the acceptor DNA by chelating the divalent metal ions ( $Mg^{2+}$  and  $Mn^{2+}$ , respectively) at the interface of the integrase – viral cDNA complex (Espeseth et al. 2000; Grobler et al. 2002; Hazuda et al. 2004). Because of their mode of inhibition, DKAs have been classified as *interfacial inhibitors* of macromolecular complexes (Pommier et al. 2005). As expected from their novel mode of action, DKA-like inhibitors were also shown to be effective against clinical isolates that were resistant to reverse transcriptase and protease inhibitors (PIs) (Hazuda et al. 2001).

Consequently, S-1360, a triazole analogue of DKA, was the first integrase strand transfer inhibitor (INSTI) to enter clinical trials, but the development was stopped during phase I/II (Billich 2003). Subsequently, a novel series of potent INSTIs, which replaced the 1,3-diketo acid moiety by an isosteric 8-hydroxy-1,6-naphthyridine core, showed improved metabolic stability (Zhuang et al. 2003). The compound L-870,810 moved into clinical trials, where it provided proof of concept in antiretroviral therapy-experienced and antiretroviral therapy-naïve

patients. However, recently the development of L-870,810 was discontinued in favour of MK-0518 (raltegravir), which represents another member of the naphthyridine carboxamide series characterized by an improved pharmacokinetic profile (Embrey et al. 2005).

In a phase II placebo-controlled study, this most advanced INSTI demonstrated an unexpectedly fast decay of HIV viral load in treatment-naïve patients: monotherapy with raltegravir over 10 days resulted in extensive monophasic decay for all dosage groups (i.e. 100–600 mg twice daily), with a median decrease of 2.2 log<sub>10</sub> HIV RNA copies/ml (Markowitz et al. 2007; Grinsztejn et al. 2007; Murray et al. 2007). Similarly, in a 48-weeks combination therapy study in patients receiving optimized background therapy, individuals taking raltegravir were significantly more likely to have HIV RNA < 50 copies/ml from day 15 to day 57 than those taking the NNRTI efavirenz. Plasma viral loads were 70% lower at initiation of second-phase decay of viremia for patients receiving the INSTI compared to the NNRTI. In addition, raltegravir has demonstrated a favourable side-effect profile in treatment-naïve and -experienced patients (for review see Evering and Markowitz 2007). In October 2007, raltegravir was approved by the U.S. Food and Drug Administration (FDA) for the treatment of HIV-1 as part of combination antiretroviral therapy in treatment-experienced patients.

The reason for the apparently superior antiretroviral activity of raltegravir compared with efavirenz is currently not understood. Several hypotheses have been advanced: first, it has been proposed that raltegravir may have superior pharmacokinetic properties that allow it to penetrate more efficiently into HIV sanctuaries such as the gut-associated lymphatic tissue and may thus be more potent at targeting major *in vivo*-reservoirs of HIV replication (Murray et al. 2007).

Second, the INSTI, but not an RTI, may conceivably inhibit the virus production from the pool of resting CD4 T cells that are in a state of pre-integration latency (Murray et al. 2007). Upon activation, the preformed pro-viral DNA that is already located in the nucleus integrates into the genome of these cells, allowing them to contribute to the viral load.

Third, an accumulation of unintegrated HIV-1 cDNA can promote apoptosis under certain experimental conditions *in vitro* (Temin 1980; Li et al. 2001). It has been hypothesized that an INSTI could induce the destruction of long-lived, productively infected cells such as macrophages *in vivo* by accumulation of episomal HIV-1 cDNAs following superinfection.

Fourth, Sedaghat et al. (2008) have used mathematical modelling to study the decay dynamics of HIV in relation to the stage of the replication cycle that is inhibited by a certain drug. These authors provide provocative evidence that the rapid HIV RNA decay in patients receiving an INSTI-containing regimen is not necessarily an indication of greater drug efficacy, but may rather be a consequence of the fact that this drug acts later in the replication cycle than an RTI. Ongoing clinical studies and experimental studies in animal models may shed more light on this question. The thus far unsurpassed potency of short-term viral load decay in raltegravir-containing regimens may also be of importance for the long-term performance of patients on



HAART, since the time to suppression of viremia has been identified as an important prognostic indicator (Louie et al. 2003; Polis et al. 2001).

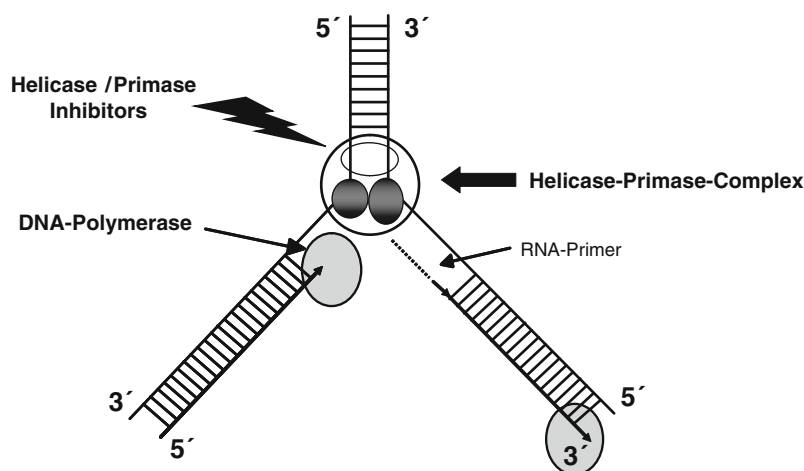
However, as with drugs against other targets of HIV, integrase inhibitors can also lead to resistance development. Resistance to raltegravir can develop along two different pathways, at positions 148 and 155 of the HIV integrase. They are associated with clusters of other mutations. As expected, so far, no cross-resistance was observed with any of the approved classes of HIV drugs.

A second clinical development compound named GS-9137/JTK-313 (elvitegravir) belongs to the structurally related class of 4-oxoquinoline integrase inhibitors (Sato et al. 2005; Shimura et al. 2008). Elvitegravir, which needs to be boosted with ritonavir, was recently shown to be as effective as a boosted protease inhibitor regimen at cutting viral load in heavily pre-treated HIV-positive patients, according to phase II results. The drug–drug interaction studies are already completed and showed no interactions. Among the observed IN mutations, T66I and E92Q substitutions mainly contributed to elvitegravir resistance. Some resistance mutations conferred reduced in vitro-susceptibility to other IN inhibitors, including raltegravir, suggesting that a common mechanism is involved in resistance and potential cross-resistance. Based on the currently still limited data set, resistance to INSTI appears to develop faster than with protease inhibitors, but not quite as fast as with some of the NNRTIs. Furthermore, elvitegravir has to be boosted with ritonavir in contrast to raltegravir, and raltegravir has to be given twice daily. Without doubt, integrase inhibitors will add an important new weapon to the anti-HIV armamentarium. Currently, they are primarily used in salvage therapy regimens in multi-drug resistant patients, but may soon replace other drug classes in first-line HAART.

## 4 Helicase Inhibitors

The helicases are enzymes central to life itself. The nature of double-stranded DNA means that before a polymerase can begin to copy the appropriate region of the nucleic acid, the two strands have to be unwound; the separation of the two strands is the function of the helicase (Fig. 2). An indication of the significance of this family of enzymes is seen in the so-called Werner syndrome, where the helicase function required in the suppression of inappropriate recombination events is defective and causes genomic instability and cancer (for a review see Cobb and Bjergbaek 2006).

Helicases can be divided into two structural groups: those that form rings to surround the nucleic acid strand and those that do not. From an evolutionary point of view, helicases can also be grouped into three superfamilies (Gorbalenya and Koonin 1993): non-ring helicases are usually in SF1 or SF2 and ring helicases are in SF3. Helicases exhibit domains that are similar to a domain first identified in the RecA protein of *E. coli*. This domain has been identified as the motor of the helicase that is powered by the hydrolysis of NTP and drives the protein along the nucleic acid molecule. An NTP-binding site is usually found in the vicinity of the RecA-like domain.



**Fig. 2** Herpesvirus DNA-replication fork showing the site of action of helicase/primase inhibitors

The herpes simplex virus genome expresses two helicases during its replication cycle, encoded by the viral genes UL5 and UL9. The former is found in a protein complex that also contains the primase protein (coded by UL52). As reported in 2002 (Crute et al. 2002; Kleymann et al. 2002), both Boehringer/Biomega and Bayer developed highly specific inhibitors of the helicase–primase complex, and it is hoped that clinical trials will reflect the excellent *in vitro* activity and the *in vivo* efficacy already observed in several animal models of herpesvirus disease. More recently, Phase 2 studies with ASP2151, an inhibitor of the Herpes Virus helicase–primase that is under development by Astellas Pharma, have been initiated in patients with herpes zoster and genital herpes, in Japan and the USA (see [info@astellas.com](mailto:info@astellas.com)).

Since the pioneering work of Kleymann et al. (2002), Betz et al. (2002), Baumeister et al. (2007), and Crute et al. (2002), who showed that compounds identified as inhibitors of the helicase–primase enzyme complex could alleviate herpesvirus-induced disease in animal models, the attention of researchers developing antiviral compounds has been drawn more and more towards the virus-encoded helicases, particularly those of Herpes viruses and of RNA viruses such as Hepatitis C Virus (HCV) and SARS coronavirus (SARS-CoV). Enzyme activity is usually assayed by measuring NTPase activity in the presence of an appropriate nucleic acid co-substrate although, more recently, novel fluorimetric and luminescence principles have been applied to the measurement of strand unwinding and/or translocation of the protein along the nucleic acid (Frick 2003, 2006).

Much of the literature pertaining to putative inhibitors of HCV helicase has recently been discussed in the excellent review published by Frick (2007). As he points out, one of the main problems with a helicase as target for antiviral drugs is the potential for general toxicity related to the highly conserved nature of the

helicase motor domains. However, if the potential inhibitor is directed towards an allosteric regulatory site, this problem might be overcome.

In general, one may expect that nucleoside analogues that compete for the NTP binding site will provide useful information about the role of the HCV helicase in viral replication, but it is doubtful whether any of these compounds will achieve success as an inhibitor of the disease process. Another mechanism of action that has been explored is competition for the nucleic acid substrate (Maga et al. 2005). Borowski et al. (2003) described a tetrabromobenzotriazole that inhibited the unwinding activity of HCV helicase, albeit at quite high  $\mu\text{M}$  concentrations, but did not inhibit its NTPase activity. Whether this compound also competes for the nucleic acid substrate is not clear, but this molecule and compound QU663 reported by Maga et al. (2005) both represent interesting leads for more specific inhibitors of the HCV enzyme. The patent literature lists many more small molecules that appear to be inhibitors of the helicase (see Frick 2007), but there have been no reports on their further development.

One exciting approach is the development of short sequences of RNA that bind specifically to HCV helicase and/or the protease activity found in the same hepatitis C virus-encoded non-structural protein, NS3, and inhibit helicase at sub-micromolar concentrations (Umehara et al. 2005). These molecules could provide the basis for developing potent helicase inhibitors with improved pharmacotherapeutic properties.

Helicase has also been a focal point for the development of antiviral chemotherapy of the coronavirus associated with severe acute respiratory syndrome (SARS) in humans. Although several experimental compounds with nucleic acid binding activity showing effective inhibition of SARS-CoV helicase were reported in 2005, there have been no reports of any further development since that time (Kesel 2005). It remains to be seen whether the SARS-CoV compounds will be developed further, especially since no new infections have been observed in recent years.

A recent review stated "There are no HCV helicase inhibitors currently in development. Most experts believe that it will be difficult, if not impossible, to develop helicase inhibitors" (Hepatitis C Support project 2006). Whether or not this is a valid statement remains to be seen, but the potential success of compounds with a similar target in the herpesviruses suggests that the possibility of developing inhibitors of HCV helicase should not be dismissed quite so lightly.

## 5 Terminase Inhibitors

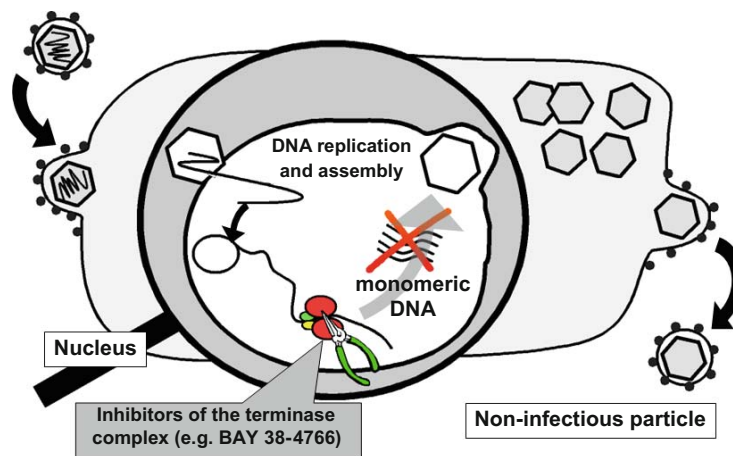
HCMV is widespread in the human population. In immunocompetent individuals, the infection is inapparent or associated with mild symptoms. However, HCMV is frequently transmitted perinatally and is the leading cause of neurological disease and hearing loss in congenitally infected newborns, affecting some 8,000 newborns per year in USA alone (Arvin and Alford 1990). Furthermore, following the first 100 days after transplantation, HCMV-induced pneumonia develops in about 50%

of heterologous bone marrow transplants, with an 80% mortality rate if left untreated (de Jong et al. 1998). Approximately 15–70% of kidney, liver, bone marrow and heart/lung transplant recipients are affected by HCMV hepatitis and pneumonia, resulting in decreased graft and patient survival (Falagas et al. 1998). Before the advent of highly active antiretroviral therapy (HAART), HCMV retinitis occurred in about 10–45% of patients with late-stage AIDS (Jabs 1995). While the use of HAART has diminished the impact of HCMV disease significantly, cessation of treatment in patients with virological and immunological failure under potent antiretroviral therapy led to the recurrence of HCMV retinitis (Casado et al. 1998; Torriani et al. 2000). In addition, antiviral resistance emerges in 14–37% of AIDS patients with HCMV retinitis treated for 9 months with ganciclovir, cidofovir or foscarnet (Jabs et al. 1998a, b).

Currently, only inhibitors of herpesviral DNA polymerases are licensed for the prophylaxis and treatment of HCMV infections (Drew et al. 2001), but these anti-HCMV therapies do not eliminate virus or eradicate infection (Field 1999). Current HCMV therapies, including ganciclovir (GCV) and its orally bio-available prodrug valganciclovir, foscarnet (PFA) and cidofovir (CDV), are associated with multiple side effects such as dose-limiting bone marrow and kidney toxicity, as well as the emergence of single and double drug resistance (Sarasini et al. 1995; Harada et al. 1997). The antisense oligonucleotide fomivirsen (ISIS 2922) for the treatment of HCMV retinitis in AIDS patients has been a very innovative approach, but could only be applied intravitreally and is associated with increased intraocular pressure and ocular inflammation in 25% of treated patients (Azad et al. 1993). Today its use is limited since it is no longer marketed in several countries due to commercial reasons. Clearly, better tolerated human cytomegalovirus (HCMV) therapies with novel mechanisms of action are needed to allow broader and longer application and to treat drug-resistant HCMV that arises during therapy with currently approved agents.

The process of viral DNA packaging is multifunctional and determined by specific interactions of protein–DNA and protein–protein. Portal proteins play an important role during this process. Portals are large macromolecular complexes and are found throughout herpesviruses as well as in those double-stranded DNA bacteriophages examined to date (Black 1988). Portal proteins provide, on the one hand, the channel for entry of the DNA during packaging and, on the other hand, the exit for releasing DNA during infection.

The enzymes involved in the packaging process and responsible for site-specific duplex nicking and insertion of the DNA into the procapsids are called terminases (Fig. 3). The human cytomegalovirus (HCMV) terminase is composed of two subunits, the large pUL56 and the small pUL89, each with a different function (Bogner et al. 1993, 1998; Bogner 2002). While the large subunit mediates sequence-specific DNA binding and ATP hydrolysis, pUL89 is required only for duplex nicking (Hwang and Bogner 2002; Scheffczik et al. 2002; Scholz et al. 2003). The hydrolysis of ATP has multiple functions during the packaging process. It is also involved in the formation of the packaging complex.



**Fig. 3** Representation of the replication cycle of cytomegalovirus showing the site of action of terminase inhibitors

The large subunit pUL56 is stably associated with the capsid, represents a structural component and forms a dimer with C-2 symmetry (Beard et al. 2004; Catalano 2000; Sheaffer et al. 2001; Yu and Weller 1998; Savva et al. 2004). This structure is the prerequisite for the formation of a protein–DNA complex required for packaging into the procapsid.

Current evidence suggests that viral DNA is packaged into a procapsid consisting of major capsid protein (UL86), minor capsid protein (UL85), minor capsid protein-binding protein (UL46), smallest capsid protein (UL47/48), assembly protein (UL80.5) and proteinase precursor protein (UL80a) (Gibson 1996). The translocation of concatenated viral DNA into procapsids and its cleavage at packaging sites is not understood. Recent studies with herpes simplex virus type 1 (HSV-1) mutants defective in UL6, UL15, UL25, UL28, UL32 or UL33 suggest that these genes are essentially involved in viral DNA cleavage and packaging, since cells infected with these mutants produce only B capsids lacking DNA (Al-Kobaisi et al. 1991; Baines et al. 1997; Lamberti and Weller 1996, 1998; McNab et al. 1998; Patel et al. 1996; Tengelsen et al. 1993; Yu et al. 1997). The respective homologues of these genes in HCMV are UL104, UL89, UL77, UL56, UL52 and UL51 (Chee et al. 1990). By analogy to gp17, a known ATP-dependent endonuclease from bacteriophage T4, the HCMV UL89 gene may encode an endonucleolytic subunit of a putative HCMV terminase (Bhattacharyya and Rao 1993, 1994). Studies by Bogner et al. (1998) suggest that the gene product of HCMV open reading frame (ORF) UL56 has specific nuclease activity, as well as specific binding affinity to packaging elements.

Inhibitors targeting the viral terminase complex may offer an attractive alternative to present drugs, since mammalian cell DNA replication does not appear to involve such processing mechanisms. Drugs targeted to terminase-like proteins should therefore be safe and highly selective. The status of their development is reviewed in the following paragraphs.

2-Bromo-5,6-dichloro-1- $\beta$ -D-ribofuranosyl-1*H*-benzimidazole (BDCRB) and its 2-chloro homologue, 2,5,6-trichloro-1- $\beta$ -D-ribofuranosyl-1*H*-benzimidazole (TCRB), are nucleoside analogues active against HCMV, which were originally synthesized by Townsend et al. (1995). Unlike most currently marketed anti-HCMV agents, BDCRB and TCRB do not inhibit viral DNA synthesis, even at concentrations that completely prevent generation of infectious virus, but instead exert antiviral activity by inhibition of HCMV DNA maturation (Townsend et al. 1995). Genetic mapping experiments showed that inhibition of viral DNA maturation is mediated by interactions involving the products of the HCMV ORFs UL89 and UL56 (Underwood et al. 1998; Krosky et al. 1998). However, clinical development was not pursued after preclinical pharmacokinetic studies demonstrated that both BDCRB and TCRB are cleaved *in vivo* to produce the less active but more cytotoxic aglycones (Chulay et al. 1999).

The sulphonamide BAY 38–4766 is another representative of a non-nucleosidic class of inhibitors of HCMV that targets virus-specific proteins known to be required for the cleavage and packaging of viral DNA by processing high-molecular-weight viral DNA to monomeric genome length (Reefschlaeger et al. 1999). A large panel of laboratory HCMV strains and clinical isolates was shown to be several times more sensitive to BAY 38–4766 than to ganciclovir. Ganciclovir-resistant as well as ganciclovir/foscarnet and ganciclovir/cidofovir double-resistant clinical isolates were as susceptible to BAY 38–4766 as wild-type strains. These latter results suggested that BAY 38–4766 acts by a mode of action distinct from all DNA polymerase inhibitors.

Sequence analyses of the genomes of two BAY 38–4766-resistant HCMVs generated by selection *in vitro* revealed several amino acid exchanges in UL89, encoding part of the putative viral terminase and UL104, a minor structural component of virions and capsids (Underwood et al. 1998; Krosky et al. 1998). These data together with DNA cleavage analysis indicate that both UL89 and UL104, alone or by interaction, represent the molecular antiviral drug target (Bürger et al. 2001).

Although it was proposed that inhibition of HCMV DNA maturation by the benzimidazole ribonucleoside BDCRB is mediated through the *UL89* gene product, and resistance to TCRB maps to the two ORFs UL89 and UL56, there was no cross-resistance of an HCMV AD169 sulphonamide-resistant strain to BDCRB (Reefschlaeger et al. 1999).

It can be expected that the requirement to accumulate multiple mutations to generate a resistant phenotype may translate into a relatively slow development of clinical HCMV resistance. In addition, a mechanism that is distinct from those of the marketed drugs will offer the possibility of treating patients who have acquired resistance to these agents.

Apart from offering a new and highly specific approach to the inhibition of herpesviruses, this new mechanism of action could potentially also have beneficial immunological consequences. During treatment with BAY 38–4766, viral protein synthesis continues, but due to the lack of monomeric genomic length DNA, only empty particles (dense bodies) can be formed. It is conceivable that these non-infectious viral particles could aid the establishment of an antiviral immune response, leading to better control of the virus by the host. This mechanism appears

possible in all cases where an immuno-incompetent host (re)gains immune competence (newborns, transplant recipients). However, proof of this theoretical benefit will have to await clinical studies.

To summarize, terminase inhibitors point the way toward a switch in strategy for developing HCMV inhibitors, with the aim of achieving a quality different from that of established DNA polymerase inhibitors. Intervention with viral DNA maturation arrests the replicative cycle at the DNA cleavage and packaging step, leading to an accumulation of empty procapsids and unprocessed concatemeric DNA.

Terminase inhibition is an antiviral approach that may also be of consequence for other members of the herpesvirus group. In addition, since a similar DNA maturation process does not occur in higher cells, this principle offers the potential for high selectivity, in contrast to many of the viral DNA polymerase inhibitors, which also interact with cellular enzymes and hence can have severe side effects.

The terminase inhibitors so far tested in the clinic have shown excellent safety, tolerability and pharmacokinetic data after single oral doses in healthy male subjects (Nagelschmitz et al. 1999; Reefschlaeger et al. 1999).

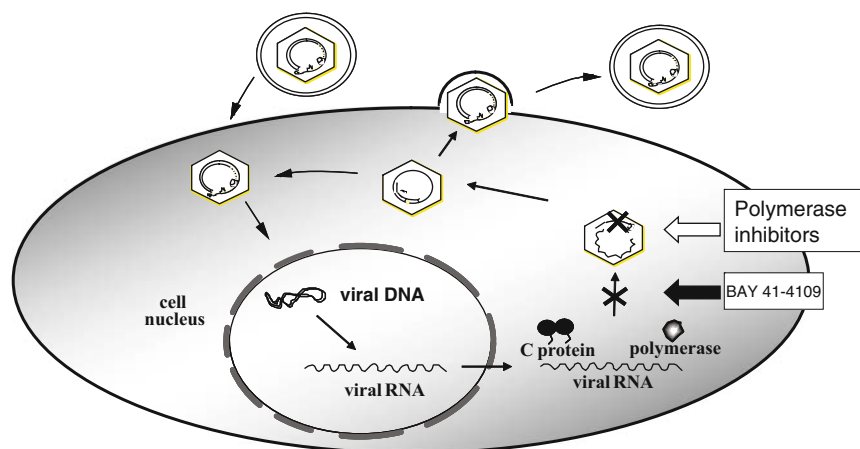
## 6 Maturation/Assembly Inhibitors

Virus maturation and assembly at the cell membrane or the nuclear membrane has long been seen as a potential target for antiviral compounds. For the virus to mature and be released in a conformation that will insure stability and survival of the viral genome in the extracellular environment, the protein subunits of the capsid or nucleocapsids have to be transported to the assembly point where they will form the final particles around the viral nucleic acid. If this process does not occur in an orderly and programmed manner, the capsid subunits will not form the required multimers and the viral components will become targets for the cellular disposal mechanisms.

In 2003, Deres and colleagues published an intriguing paper describing the inhibition of Hepatitis B Virus (HBV) by drug-induced depletion of nucleocapsids (Deres et al. 2003). The principal compound described in that paper, BAY 41–4109, is a non-nucleosidic, heteroaryl dihydropyrimidine (or HAP) inhibitor that appeared to block the replication of HBV by preventing the formation of high molecular weight viral core particles that are the site of DNA replication and are aggregates assembled from HBV core protein subunits (Fig. 4). The authors concluded that the compound inhibited particle assembly and that there was an increased degradation of core protein that involved proteasome-related mechanisms. In HBV transgenic mice, this class of compound caused a dose-dependent reduction in viral DNA in the liver and blood plasma after oral application. Furthermore, it reduced the amount of core protein in the liver in contrast to the anti-HBV compound lamivudine (Weber et al. 2002).

The HIV capsid is made up of auto-assembled protease-cleaved Gag polyprotein. This self-assembly cannot take place when appropriately positioned mutations are present, resulting in a drastically reduced infectivity of the progeny virus. Recently,





**Fig. 4** Replication cycle of HBV illustrating the site of action of polymerase inhibitors and heteroaryl dihydropyrimidine (HAP) assembly inhibitors such as BAY 41-4109

three groups have published details of inhibitors of capsid formation: Tang and colleagues described a chlorinated urea compound (Tang et al. 2003) that was well-tolerated in cell culture and inhibited assembly of the capsid subunits. Sticht and colleagues reported a 12-mer peptide that binds to the capsid protein altering the dimer interface and prevents self-assembly in this way (Sticht et al. 2005). However, there have not been any reports of further development of these compounds and they should perhaps be regarded primarily as indicators of the feasibility of this approach to the chemotherapy of HIV infections.

Another group described a betulinic acid derivative (bevirimat; PA-457) that blocks the cleavage of the Gag polyprotein by the viral protease at the CA-Sp1 site (Zhou et al. 2004). This compound thus acts as a specific inhibitor of a single cleavage site and does not affect protease cleavage at other sites. By blocking this cleavage site, bevirimat blocks viral maturation and infectivity in tissue culture. It was granted fast-track development status by the US FDA in 2005 and has since completed a Phase 2b clinical study of five treatment-experienced patient cohorts. Unfortunately, Gag polymorphisms and pharmacokinetic factors appear to affect the response to bevirimat. However, when effective blood levels were achieved and the target virus lacked Gag polymorphisms, more than 90% of the patients responded to bevirimat, with a mean reduction in viral load of 1.26 log units (Panacos Pharmaceutical Inc., press release).

The identification of inhibitors of virus subunit assembly has been an objective of virologists for several years but it is only recently that papers have been published that demonstrate the validity of this approach to antiviral chemotherapy. It is hoped that the information provided by the compounds described above will provide the foundation for the generation of potent antiviral drugs to combat diseases caused by HIV, HBV and other viruses.



## 7 Conclusions

In this chapter, we have described the spectrum of antiviral activities that have been discovered beyond the world of nucleoside analogues, protease and fusion inhibitors. The compounds and mechanisms described here may one day add significantly to the armamentarium of antiviral agents, not only against Herpes Simplex, Hepatitis B and Human Immunodeficiency Virus, but also against Hepatitis C and Human Cytomegalovirus.

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