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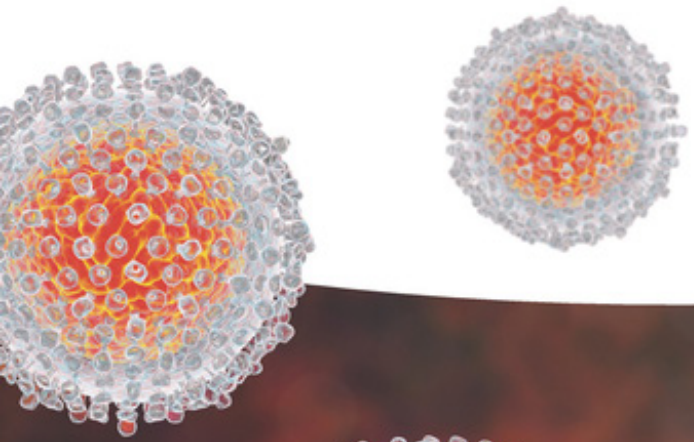
New Drug Development for Known and Emerging Viruses



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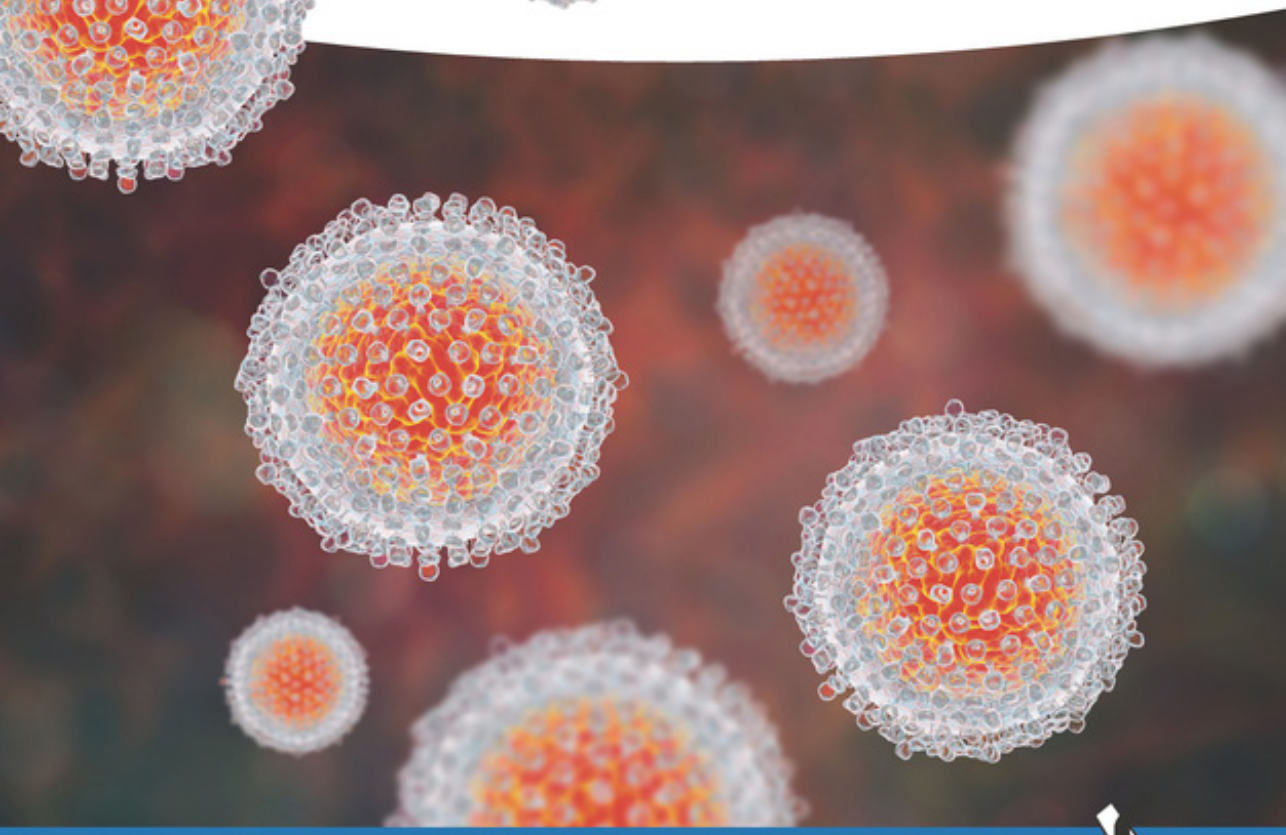

Methods and Principles in Medicinal Chemistry

New Drug Development for Known and Emerging Viruses

Methods and Principles in Medicinal Chemistry

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Preface

The area of Medicinal Chemistry aimed at fighting viral diseases has seen enormous progress in recent decades, making this updated reference from Helga Rübsamen-Schaeff and her team of expert authors more than overdue, after Eric De Clercq published last in this series back in 2010.

Many important, novel antiviral drugs have reached patients in recent years, making diseases such as AIDS or Hepatitis C manageable today, while only some years back, their diagnosis had been comparable to a death sentence. While we, the series editors, write this preface, our world is in the midst of a global pandemic caused by SARS CoV-2 (COVID-19), and promising, novel small molecule-based antiviral medicines have reached the clinical stage in record time, complementing vaccine and drug repurposing efforts to manage a global health crisis.

This impressive evolution is the result of the fascinating and relentless efforts of creative expert drug hunters, not accepting the status quo and pushing the boundaries of Medicinal Chemistry to combat the fascinating, yet often lethal efficiency of small fragments of viral RNA or DNA. Their success stories will be told in this fascinating book by Helga and her fellow authors, along with a thorough background review of all relevant viral biology and clinical pathology for the most important viral diseases. The reader will learn about cutting-edge drug discovery strategies to successfully develop antiviral agents, ranging from the use on non-classic elements, selective and kinetically controlled inhibition of viral enzymes, other viral proteins, and even of their RNA or capsids, often supported by the intelligent use of structure-enabled Medicinal Chemistry design.

The first two chapters focus on the science leading to the breakthrough in treating patients with HIV and Hepatitis C, followed by comprehensive overviews on Research and Development, and market entry for drugs against important viruses such as Influenza Virus, Respiratory Syncytial Virus, Herpes Simplex Virus, Human Cytomegalo Virus, Epstein Barr Virus, Kaposi's Sarcoma-associated Herpesvirus, Hepatitis B Virus, and Hepatitis E Virus (Chapters 3–10). Chapters 11–15 focus on Adeno-, Parvo-, Noro-, Picorna-, and Arboviruses RnD, respectively. Finally, a chapter on Biosafety Level 4 Pathogen Therapies and one on SARS CoV-2 virus conclude the reference book.

The combination of background, strategic insights, and applied case studies makes this volume a must read for every scientist involved in antiviral RnD, but also for the wider Medicinal Chemist and drug hunter community, interested in broadening expertise and skillsets.

Helga Rübsamen-Schaeff led Bayer's Virology Research functions from 1994 until 2001 and Bayer's whole Infectious Diseases Research from 2001 to 2006, when she became founder CEO of AiCuris, a German biotech company dedicated to delivering innovative antiviral and antibacterial drugs to patients. Helga has received numerous awards in her career, e.g. recently the Loeffler-Frosch-Medal of the German Society for Virology. Together with her research teams, she has delivered several important medicines and drug candidates, such as recently Letermovir against the cytomegalovirus. For the work on Letermovir, she received the Innovation Award of Northrhine-Westphalia and the Price for Technology and Innovation by the German President of State in 2018. She is member of the National Academy of Sciences, Leopoldina.

The editors would like to thank Helga and all contributing authors for what we believe to be the most complete and up-to-date reference book for antiviral research and development.

Boston, Aachen, and Düsseldorf
July 2021

Jörg Holenz
Helmut Buschmann
Raimund Mannhold

In Memoriam: Farah Elawar

On the day we received the proof pages for Chapter 4 of this book, we were shocked and deeply saddened to learn about the tragic passing of Farah Elawar. Farah is the lead author of our contribution: Respiratory Syncytial Virus Immunoreactivity, Vaccine Development, and Therapeutics. She was in the final stages of her Ph.D. studies on the diversity and drug resistance of Respiratory Syncytial Virus in the community, in the Department of Medical Microbiology and Immunology at the University of Alberta. From the beginning of her studies with the Marchant laboratory, she impressed all of us with her enthusiasm and everlasting energy. She was always three steps ahead of her supervisor with the results of her experiments. It was almost as though she could read minds. With this ability, she provided the leadership to conceptualize, write, and finalize this book chapter. With her warm personality, she has made so many friends and has given us so many joyous moments. Farah will be dearly missed and always remembered.

David Marchant and Matthias Gotte

Introduction

More than 60 years ago, in 1959, the first antiviral drug to be licensed was described by Prusoff [1]. It was marketed for the topical treatment of herpes simplex virus infections of the eye. In 1972, Ribavirin was described as a broad-spectrum antiviral agent [2]; however, it had significant side-effects. In contrast, Acyclovir, discovered in 1977, was very well tolerated [3, 4] and became the gold standard for treating infections caused by herpes viruses.

With the discovery of HIV in 1983 as the virus causing AIDS [5], a very active era of search for anti-HIV drugs began. In 1985, the antiviral activity of an existing drug, AZT (zidovudine, a polymerase inhibitor) was first described against HIV [6]. While up to that time, all drugs, with the exception of ribavirin, to which multiple modes of action are being ascribed, were inhibitors of viral polymerases (in case of AZT, the reverse transcriptase), the following years witnessed a very active search for drugs inhibiting other targets of HIV like the protease, fusion, or integrase or non-nucleosidic inhibitors of HIV reverse transcriptase as novel drug classes [7]. Finally, about 24 years after HIV had first been discovered, by combining drugs with different modes of action, the HIV-infection, which had been a death sentence, became a treatable, although chronic disease allowing patients to live a nearly normal lifespan. Likewise, for Hepatitis C (HCV), the discovery of the virus led to a worldwide search for specific antiviral drugs targeting its polymerase, protease, or NS5A. In this case, combining drugs with different modes of action even allowed to cure the chronic HCV infection in the vast majority of patients, 25 years after HCV had first been described. These unprecedented and major achievements against two of the most dangerous small RNA viruses demonstrate the enormous power of academic and industrial research, when combined and targeted toward a specific virus. They are described in the first two chapters of the book. Obviously, while HCV infections can now be cured this goal is still to be reached for HIV and many approaches are being pursued.

The next two chapters deal with two RNA viruses, which also pose significant health problems: Influenza and the Respiratory Syncytial Virus and describe the existing high medical need for therapies in these indications, as well as starting points for novel therapeutic options.

Chapters 5–8 deal with large DNA viruses like Herpes Simplex, Cytomegalovirus, Epstein–Barr Virus, and the Human Herpes Virus 8, all widespread and implicated in a number of severe or fatal conditions. The successful development of novel generations of drugs against Herpes Simplex and Cytomegalovirus using novel targets for attacking these viruses will be described (Chapters 5 and 6). The next chapters highlight potential targets

and strategies to be addressed in the search of therapeutics against the Epstein–Barr Virus or the Human Herpes Virus 8 (Chapters 7 and 8).

Chapter 9 describes antiviral efforts against a small DNA virus, Hepatitis B, which causes significant morbidity and mortality worldwide, especially in Asia, and against which strategies for a cure are being sought as well. Here, inhibitors against the viral capsid could be one potential avenue.

The following chapters address small DNA viruses (Hepatitis E, Adeno, and Parvo) and RNA viruses (Noro, Picorna), followed by chapters on emerging viruses like arbovirus infections and biosafety 4 level viruses like EBOLA.

Outbreaks of HIV, SARS-1 Coronavirus, MERS Coronavirus, EBOLA, and Zika clearly have indicated that the globalized world has become very vulnerable to epidemics of often zoonotic viruses infecting humans and spreading quickly due to the strong and multiple connections in to-day's world. While containment of several outbreaks or eventually treatment (HIV) and even cure (HCV) has been possible in the past, we are now facing an unprecedented outbreak of SARS CoV-2 causing the disease COVID-19. Infections with the virus were first documented in 2019, and the next two years saw a rapid spread leading to a pandemic with millions of cases worldwide and 5 million deaths by the fall of 2021. Efficient transmission by air makes containment of SARS CoV-2 particularly difficult and resulted in very significant economical downturns worldwide. SARS-CoV-2 causes severe respiratory symptoms, but also pathological inflammation and multi-organ-dysfunction, including the acute respiratory distress symptom, cardiovascular events, coagulopathies, nephropathy, and neurological symptoms [8–11]. While several highly active vaccines have meanwhile been discovered and vaccination campaigns are pushed worldwide, there is still a great need for highly potent and well-tolerated direct acting antiviral agents. We will give medicinal chemists insights into targets and strategies for the discovery of these urgently needed therapies against SARS-Cov-2 in the final chapter of this book.

Most of this book will deal with small molecular weight drugs and their targets, but where appropriate and potentially also the better strategy, immune modulators or immune therapies will be discussed as well.

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1

HIV—Disease Overview, Targets for Therapy and Open Issues

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Nearly 40 years have passed since the first patients were discovered to have the previously unknown "acquired immunodeficiency syndrome" (AIDS) [1] and 38 years, since the causative virus was discovered [2]. The time since, has been characterized by a dynamically evolving body of epidemiological and basic science that is unique in medical history. Preliminary result is the development of antiretroviral treatments and social medicine progress, accompanied and catalyzed by the predominantly affected risk groups' emancipation, with reintegration into the society.

1.1 HIV—Disease Overview

There has not been a significant change in the **natural history of HIV-infection**; however, today in industrialized countries, disease manifestation in the form of AIDS can only be observed in undiagnosed, late presenting patients, who already show manifestations of immunological deterioration. Figure 1.1 demonstrates the course of the disease, according to the two most important measurable surrogate markers—CD4-cell count and HIV-RNA (viral load).

After initial HIV transmission, the retrovirus spreads throughout the human body and infects potentially all CD4-receptor positive cells. Consequently, during the first weeks of infection, there is a substantial fall in CD4-positive T-lymphocyte count and a rise in viral load—up to a turning point. Thereafter, CD4-cells rise again and viral load decreases, due to regain of a partial immunological control. At this time, anti-HIV antibodies can be found in plasma, and the patient will now respond positively to serological HIV-tests. Elimination of HIV, however, will not occur due to the rapid variation of viral surface receptors which may hide infected cells from the immune system and lead to divergent virus populations, including in a single patient [3, 4, 5, 6]. This continuous change of HIV stems from proofreading failures which lead to the evolution of many

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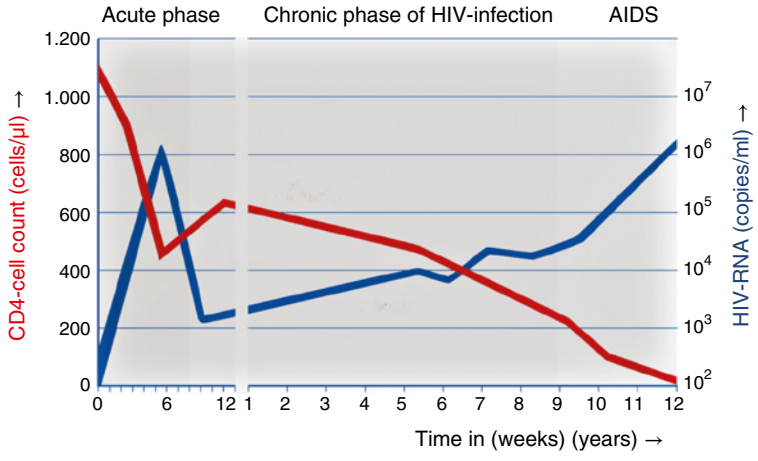


Figure 1.1 Shows the course of both most important measurable surrogate markers from blood count, i.e. CD4-cell count and HIV-RNA (viral load), during untreated HIV infection.

HIV quasispecies. Another reason for the inability to eliminate HIV is the infection of durable reservoir and "archive" cells, e.g. edaphic CD4-receptor-positive macrophage and monocyte cells, leading to the chronic phase of the infection. For approximately 3–10 years, the patient will experience a relatively stable period, marked by individually solid CD4-cell count- and HIV-RNA viral load- "setpoints" [7]. However, after months or years, an immunological exhaustion will occur. Then, the CD4-cell count is substantially decreasing and viral load is rising again. The result may be AIDS, as defined by the emergence of at least one of 26 opportunistic infections and/or tumors, including pneumocystis pneumonia, cerebral toxoplasmosis, tuberculosis, cytomegalovirus retinitis, Kaposi's sarcoma, or B-cell non-Hodgkin lymphoma.

1.2 Targets for Antiretroviral Therapy

The replication of the retrovirus in the human host cell is well described and offers targeted treatment options, in order to prevent viral replication. Figure 1.2 shows the passage of HIV through the human host CD4-receptor positive T-cell. Antiretroviral drugs are able to address specific points in the HIV replication cycle and used in combination antiretroviral therapy (cART) aims to completely suppress HIV-1 replication long term. This will give the immune system a chance to recover and overcome opportunistic infections and tumors and/or to avoid significant deterioration from the beginning, when applied early after infection.

During the retrovirus replication in the human cell, specific cART-drug classes offer to interfere with different therapeutic intervention targets (see Table 1.1, presently favored drugs are printed in bold and Figure 1.2). Such interventions comprise: inhibition of first contact of HIV with the CD4-positive cell (attachment), the cell entry, intracellular reverse

transcriptase-enzyme and -activity, DNA-integration, virus assembling by proteases, and virus maturation; the latter leaves immature, noninfectious virus particles. The chemical structures of the different HIV drugs can be found in Table 1.2.

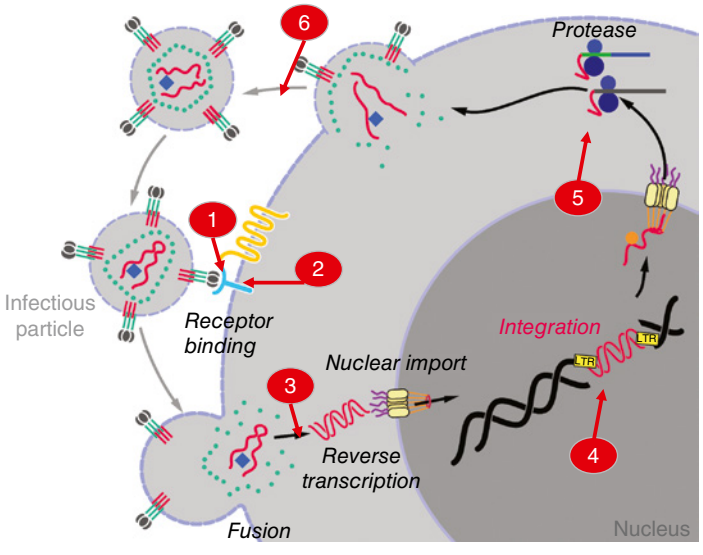


Figure 1.2 Shows the viral replication cycle for HIV in the human target cell, i.e. the CD4-receptor positive cell, and six treatment targets for antiretroviral therapy classes (for numbers in red, see Table 1.1/row 1).

Table 1.1 Explains the mode of action for available cART options, according to the target area in HIV cell passage (for numbers: see Figure 1.2).

No. (Figure 1.2)	Drug class: inhibitor of…	Generic names of available cART-drugs	Mode of action: inhibition of the…	Formula	CAS registry number
1	Attachment	Fostemsavir[a]	HIV-binding site gp120, used by HIV for first contact with CD4-receptor	$C_{25}H_{26}N_7O_8P$	864953-29-7
		Ibalizumab (TNX-355)	Human CD4-receptor binding site for HIV (whole antibody)	n/a	680188-33-4
2	Entry-/fusion	Enfuvirtid (T20)	HIV-gp41-fusion protein (36 amino acids-containing polypeptide)	$C_{204}H_{301}N_{51}O_{64}$	159519-65-0
		Maraviroc (MVC)	Human CCR5-coreceptor	$C_{29}H_{41}F_2N_5O$	376348-65-1

(Continued)

Table 1.1 (Continued)

No. (Figure 1.2)	Drug class: inhibitor of…	Generic names of available cART-drugs	Mode of action: inhibition of the…		Formula	CAS registry number
3	Nucleosidal reverse transcriptase (NRTI)	Zidovudin (AZT)	Thymidin-analogue	Nucleobase replacement during RNA–DNA-transcription as faulty chip, leading to early chain termination	$C_{10}H_{13}N_5O_4$	30516-87-1
		Lamivudin (3TC)[b]	Cytidin-analogue		$C_8H_{11}N_3O_3S$	134678-17-4
		Emtricitabine (FTC)[b]			$C_8H_{10}FN_3O_3S$	143491-57-0
		Tenofovir (TAF/TDF)[b]	Adenosine-analogue		$C_9H_{14}N_5O_4P$	147127-20-6
		Abacavir (ABC)[b]	Gunaosine-analogue		$C_{14}H_{18}N_6O$	136470-78-5
4	Non-nucleosidal reverse transcriptase (NNRTI)	Efavirenz (EFV)	Reverse transcriptase-enzyme binding site		$C_{14}H_9ClF_3NO_2$	154598-52-4
		Nevirapine (NVP)			$C_{15}H_{14}N_4O$	129618-40-2
		Rilpivirine (RPV)[b]			$C_{22}H_{18}N_6$	500287-72-9
		Doravirin (DOR)[b?>]			$C_{17}H_{11}ClF_3N_5O_3$	1338225-97-0
5	Integrase strand transfer (INSTI)	Raltegravir (RGV)	Viral DNA integration in human host DNA, in cell nucleus		$C_{20}H_{21}FN_6O_5$	518048-05-0
		Elvitegravir (EVG)			$C_{23}H_{23}ClFNO_5$	697761-98-1
		Dolutegravir (DGT)[b]			$C_{20}H_{19}F_2N_3O_5$	1051375-16-6
		Bictegravir (BTG)[b]			$C_{21}H_{18}F_3N_3O_5$	1611493-60-7
		Cabotegravir[a]			$C_{19}H_{17}F_2N_3O_5$	1051375-10-0
6	Protease (PI)	**Darunavir (DRV)**[b]	gag-pol-polyprotein cleavage		$C_{27}H_{37}N_3O_7S$	206361-99-1
		Atazanavir (ATV)			$C_{38}H_{52}N_6O_7$	198904-31-3
		Lopinavir (LPV)			$C_{37}H_{48}N_4O_5$	192725-17-0
7	Maturation/capsid	Lenacapavir[a]	Extracellular capsid arrangement[a]		$C_{39}H_{32}ClF_{10}N_7O_5S_2$	2189684-44-2
		GSK3640254[a]	Last protease cleavage event: CA-p24/SP1[a]		n/a	n/a

a) In clinical study development—also refer to public study registry online-resource, available at: https://www.clinicaltrials.gov.

b) Modern, recommended first-line combination antiretroviral therapy components: printed in **bold**. For treatment guidelines from the European AIDS Clinical Society (EACS), version 10, from November 2019, please refer to online-resource, available at: https://www.eacsociety.org/files/2019_guidelines-10.0_final.pdf.

Table 1.2 Illustrates chemical formula details and CAS registry numbers for available cART-drugs.

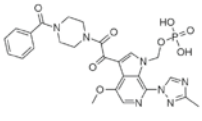
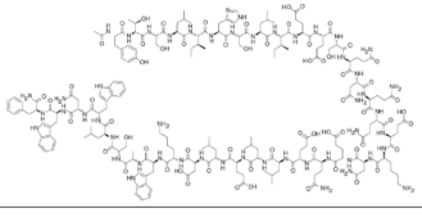
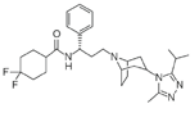
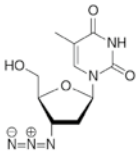
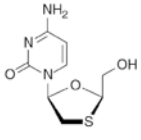
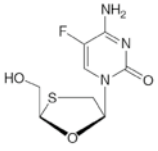
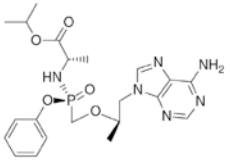
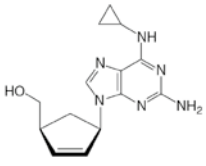
Drug class: inhibitor of...	Generic drug name/chemical formula/CAS registry no./graphic structure formula	Generic drug name/chemical formula/CAS registry no./graphic structure formula
Attachment	Fostemsavir[a] $C_{25}H_{26}N_7O_8P$ 864953-29-7 	Ibalizumab (TNX-355) n/a 680188-33-4 n/a—chemical formula not yet published, the drug is a humanized mouse whole antibody
Entry-/fusion	Enfuvirtid (T20) $C_{204}H_{301}N_{51}O_{64}$ 159519-65-0 	Maraviroc (MVC) $C_{29}H_{41}F_2N_5O$ 376348-65-1 

Table 1.2 (Continued)

Drug class: inhibitor of...	Generic drug name/chemical formula/CAS registry no./graphic structure formula	Generic drug name/chemical formula/CAS registry no./graphic structure formula	
Nucleosidal reverse transcriptase (NRTI)	Zidovudin (AZT) Thymidin-analogue $C_{10}H_{13}N_5O_4$ 30516-87-1 	Lamivudin (3TC) Cytidine-analogue $C_8H_{11}N_3O_3S$ 134678-17-4 	Emtricitabine (FTC) Cytidine-analogue $C_8H_{10}FN_3O_3S$ 143491-57-0 
	Tenofovir (TAF/TDF) Adenosine-analogue $C_9H_{14}N_5O_4P$ 147127-20-6 As tenofovir alafenamid (TAF) 	Abacavir (ABC) Guanosine-analogue $C_{14}H_{18}N_6O$ 136470-78-5 	

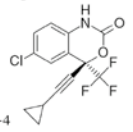
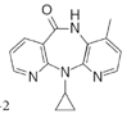
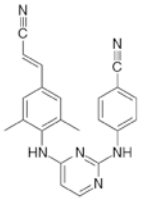
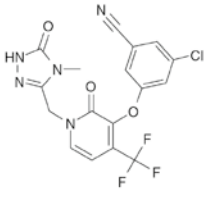
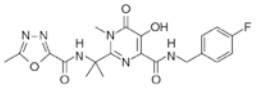
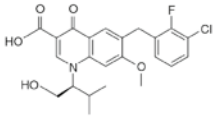
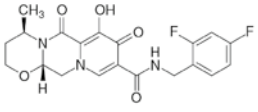
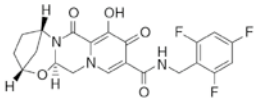
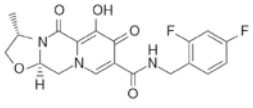
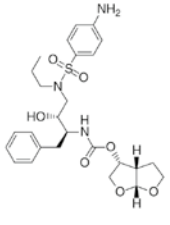
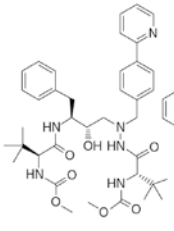
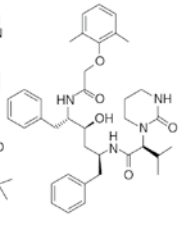
Drug class: inhibitor of...	Generic drug name/chemical formula/CAS registry no./graphic structure formula	Generic drug name/chemical formula/CAS registry no./graphic structure formula
Non-nucleosidal reverse transcriptase (NNRTI)	Efavirenz (EFV) $C_{14}H_9ClF_3NO_2$  154598-52-4	Nevirapine (NVP) $C_{15}H_{14}N_4O$  129618-40-2
	Rilpivirine (RPV) $C_{22}H_{18}N_6$ 500287-72-9 	Doravirin (DOR) $C_{17}H_{11}ClF_3N_5O_3$ 1338225-97-0 

Table 1.2 (Continued)

Drug class: inhibitor of...	Generic drug name/chemical formula/CAS registry no./graphic structure formula	Generic drug name/chemical formula/CAS registry no./graphic structure formula
Integrase strand transfer (INSTI)	Raltegravir (RGV) $C_{20}H_{21}FN_6O_5$ 518048-05-0 	Elvitegravir (EVG) $C_{23}H_{23}ClFNO_5$ 697761-98-1 
	Dolutegravir (DGT) $C_{20}H_{19}F_2N_3O_5$ 1051375-16-6 	Bictegravir (BTG) $C_{21}H_{18}F_3N_3O_5$ 1611493-60-7 
	Cabotegravir[a] $C_{19}H_{17}F_2N_3O_5$ 1051375-10-0	

Protease (PI)	Darunavir (DRV) $C_{27}H_{37}N_3O_7S$ 206361-99-1 	Atazanavir (ATV) $C_{38}H_{52}N_6O_7$ 198904-31-3 	Lopinavir (LPV) $C_{37}H_{48}N_4O_5$ 192725-17-0 
Maturation/capsid	GS-6207[a] GSK3640254[a]	Chemical formula not yet published, CAS registry-no. not yet allocated (as of search on 30 December 2019)	

Source: WIKIPEDIA, the free encyclopedia, as accessed online on 30 December 2019, please also see https://en.wikipedia.org.

a) In clinical study development—refer to public study registry online-resource, available at: https://www.clinicaltrials.gov.

1.3 Currently Open Issues in HIV/AIDS Research

Important milestones in cART development have been achieved in the recent decades as standard of care: complete virus suppression, side-effect- and drug-interaction control, and convenience in taking antiretroviral regimens. Recently observed trends in HIV-treatment include the development of long-acting cART drugs, which are administered alternatively, e.g. injected every eight weeks, or once even less frequently implanted periodically.

When in July 2015 the first results from the START-study (Strategic Timing of Antiretroviral Treatment) were published, the benefit from modern cART for patients with early HIV-infection was evident for the first time [8]. Subsequently, antiretroviral therapy guidelines have changed worldwide and recommend cART for everybody with an HIV-infection, independent from the individual clinical category and CD4-cell count. Thereafter, the global focus of interest was to establish programs that could allow every infected person access to cART. Therefore, the United Nations Program on HIV/AIDS (UNAIDS) have established the 90–90–90-targets, in order to end AIDS as a disease and to control HIV transmissions on a society level [9]. Data on beneficial effects of programs, which lowered the barriers to cART, i.e. linked to HIV transmission control, were published before [10]. Moreover, the exciting confirmation of the SWISS STATEMENT hypothesis (Undetectable HIV leads to zero transmissions) [11] was a major step forward to realize antiretroviral treatment as most effective prevention [12] in the absence of a protective vaccine.

Another major open issue is cure from HIV/AIDS. Albeit individual cases of cure from HIV have been reported [13, 14], e.g. by stem cell transplantation from donors with the rare, intrinsic HIV-resistance due to homozygous CCR5-Δ32/Δ32-gene mutations, stem cell transplantation will hardly be feasible for many patients, as this is associated with substantial risks. Alternative therapeutic procedures using the CRISPR-CASP-technique have been tried, but still require further developments [15]. Beyond stem cell manipulation and/or transplantation, the efforts to induce broadly neutralizing antibodies (BNAPs) remain a second scientific approach to achieve at least "functional cure" from HIV [16].

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2

Curing Hepatitis C with Direct-Acting Antiviral Therapy

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2.1 Introduction

Viral hepatitis is an inflammation of the liver that progressively leads to liver cirrhosis and eventually liver cancer. Every third person on the planet shows evidence of infection with viral hepatitis and some 500 million individuals are chronically infected. It is the leading cause of liver cancer worldwide and accounts for more than 80% of liver transplants globally. While mortality rates are dropping for many other infectious diseases, there has been an increase in mortality for those individuals diagnosed with chronic viral hepatitis [1].

It was not until the 1960s and 1970s that through a series of epidemiological studies and the work of several laboratories the hepatitis B virus (HBV) was discovered and linked to the occurrence of liver cancer. The development of a diagnostic test and ultimately of a vaccine dramatically reduced the incidence of HBV infection in the western world even though a cure remained far off on the horizon [2]. However, it turns out that HBV was not the only culprit that caused a chronic viral hepatitis that led to liver cancer. A mysterious non-A non-B hepatitis (NANBH) was identified by Harvey Alter and coworkers as a transfusion-associated hepatitis [2]. However, it was not until 1989 and the work of Michael Houghton and coworkers that the hepatitis C virus (HCV) was ultimately identified and its genome was mapped (Figure 2.1) [3, 4]. This work led to a diagnostic test that would allow for the screening of the donated blood supply and virtually eliminate acquiring HCV from blood transfusions [5]. Even with this breakthrough, acquisition of HCV infection via injection drug use and other means of contact with infected blood continued to propagate the disease such that the worldwide prevalence remained significant at approximately 75 million individuals. Because the development of a vaccine was and still is elusive and no cure was available, the spread of HCV continued and those who were infected had little hope that their future would be better.

In 1991, the first approved therapy for treating HCV was the cytokine interferon-α (IFN). First administered three times weekly, this therapy produced very low cure rates that came along with significant side effects such as anemia, neurological complications, and flu-like

New Drug Development for Known and Emerging Viruses, First Edition.
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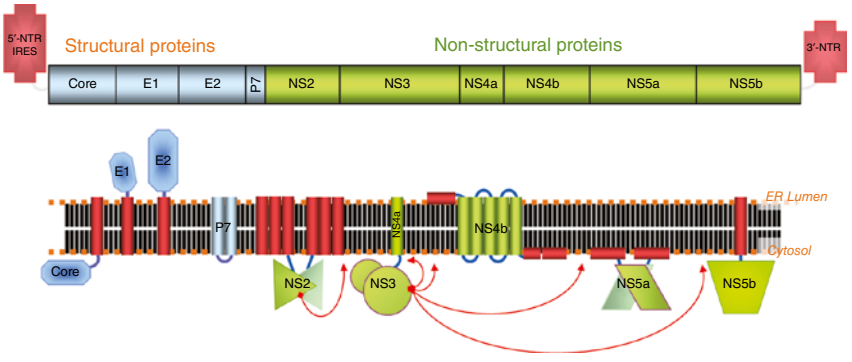


Figure 2.1 HCV Genome organization. The open reading frame (ORF) codes for a ~3000 amino acid precursor polyprotein that lies between the 3′ and 5′ nontranslated region (NTR). The NS2/3 protease and NS3/4A protease are involved in the polyprotein processing.

symptoms. The introduction of the nucleoside ribavirin (RBV), a nonspecific antiviral agent, led to improved cure rates but also added to the side-effect profile of this combination. The advent of pegylated-IFN (PEG-IFN) in combination with RBV, further improved cure rates and reduced the frequency of administration. However, cure rates remained modest and the serious side effects persisted [6].

Although IFN-containing regimens did provide a modest cure rate, the side effects were sufficiently severe that many patients could not complete the 48-week regimen. It became an aspirational objective to eliminate IFN from drug regimens that cured HCV, but in the early 2000s, it was not obvious how to accomplish this objective. In addition, there were six different viral genotypes (GT) known for HCV and the virus was shown to have a very high mutation rate because of the poor proof-reading function of the viral polymerase. These other factors complicated the outlook and increased the complexity of the task because the most desirable cure therapy would have the characteristics of being pangenotypic, with a high barrier to the formation of resistant virus and IFN-free.

2.2 Tools to Enable Drug Discovery

With the genome of the HCV virus fully delineated, the field was now ready to attempt to identify agents that could impact the virus directly with direct-acting antivirals (DAAs) and see if DAAs could deliver on some of the objectives. Early progress was slow because drug hunters were using surrogate systems such as bovine virus diarrhea virus (BVDV) as a way to evaluate potential drug candidates. Therefore, a new tool needed to be developed, a cell-based assay that drug discovery scientist could use to screen for inhibitors targeting the nonstructural proteins essential for the HCV virus to replicate itself. This was eventually achieved with the development of the HCV replicon cell system pioneered by Bartenschlager and coworkers [7]. By eliminating the gene sequence for the structural proteins, this noninfectious cell system allowed for screening of inhibitors of viral replication against the druggable nonstructural proteins. It also provided a convenient way to

assess drug resistance. Eventually, the JFH-1 cell line was developed and provided a way to assess drug candidates in a real infectious system [7].

Another challenge that hampered the discovery of curative therapies was the availability of an accessible and cost-effective animal model. Because of the narrow host tropism, the chimpanzee was the only animal model available for testing new therapeutic agents, but the cost, availability, and ethical concerns limited its use and therefore limited the impact on the discovery and development of new drugs. Although other animal models were eventually developed, their impact was not significant in the scheme of HCV drug discovery.

2.3 Drug Discovery Targets

HCV is a positive-sense single-stranded RNA virus whose genome is approximately 9.6 kb in length. The genome encodes over 3000 amino acids which after polyprotein processing produces three structural proteins and seven nonstructural proteins. The mapping of the HCV virus genome by Houghton and coworkers provided an initial list of viral targets against which drug discovery efforts could be launched (Figure 2.1) [8]. Several of the seven viral nonstructural proteins were considered druggable targets. They included the NS3/4A protease and NS5B viral polymerase around which most of the early drug discovery work focused. Subsequently, efforts targeting the NS4B and NS5A proteins began to emerge with significant focus on agents that bind to NS5A.

In addition to directly targeting the HCV nonstructural proteins, a number of efforts were initiated to target host-related targets that had effects on viral replication or on host immune responses. These targets included cyclophilins, MiR122, and interferon-γ [9–11]. Although targeting these nonviral targets did produce clinical agents that led to reductions in HCV viral load in early clinical trials, it was the work on the DAAs targeting NS3/4A protease, NS5A and NS5B polymerase that ultimately, in various combinations, produced the highly efficacious, safe, and short duration curative interferon-free therapies. The very rapid and successful development of DAA combination therapies made it difficult for host targeting agents to be competitive, especially since they would also have had to be combined with a DAA and could not stand alone.

2.4 NS5B Polymerase Inhibitors

2.4.1 Nucleoside and Nucleotide Inhibitors of NS5B Polymerase

The HCV NS5B RNA-dependent RNA polymerase (RdRp) is responsible for two RNA polymerization steps that are necessary for replicating the viral genome. The RdRp uses the HCV genomic RNA as a template from which the complementary negative RNA strand intermediate is generated and then this negative RNA strand becomes a template for the synthesis of a positive RNA strand [8]. The HCV NS5B polymerase is well conserved across all GTs and contains characteristics common among known viral RdRps [12]. It maintains the characteristic finger, palm, and thumb domains and contains the Asp, Gly, and Asp catalytic triad at the active site with the requirement of needing two divalent metal ions to initiate polymerization (Figure 2.2) [15].

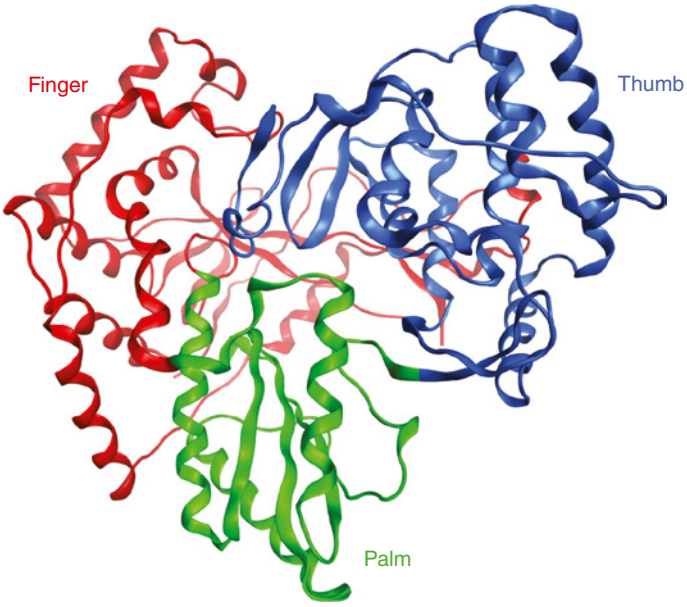


Figure 2.2 HCV NS5B RdRp crystal structure with palm, finger, and thumb domains designated. PDB code 1C2P (1.9Å) [13, 14].

To target the HCV NS5B polymerase, two distinct approaches were pursued. These included the investigation of nucleosides and nucleotides as alternate substrates and the pursuit of non-nucleoside small-molecule allosteric modulators that bind to either the palm or thumb domains of NS5B [16].

The development of nucleos(t)ide inhibitors largely revolved around 2′-α-fluoro-2′-*C*-methyl and 2′-α-hydroxy-2′-*C*-methyl substitution on the furanose ring system of the nucleos(t)ide (Figures 2.3 and 2.4, Tables 2.1 and 2.2) [16]. The 2′-methyl substitution was shown to be important for anti-HCV activity. Particularly in combination with the 2′-α-F substitution, 2′-*C*-methyl substitution induced a level of selectivity for HCV versus other viruses and human polymerases. Also, it was demonstrated that the 2′-α-F substitution provided a profound benefit as it related to specificity for HCV and safety profile. Some of the early 2′-hydroxyl nucleosides having an adenosine or cytosine base, MK-0608 and NM-283 (valopicitabine), suffered from adverse safety observations either in preclinical testing or in human clinical studies resulting in termination of their further development (Figure 2.3 and Table 2.1) [17–19, 48–50]. This is in contrast to the early fluorinated cytosine nucleosides PSI-6130 and its prodrug RG7128 which were not hampered by safety concerns but were ultimately not taken forward in development because of efficacy and other development challenges (Figure 2.3 and Table 2.1). However, it was the study with RG7128 that first demonstrated clinical efficacy in GT 1, 2 and 3 HCV patients, thus establishing the possibility for development of a pangenotypic DAA HCV cure strategy [51].

Resistance was not a significant issue with the 2′-methyl-2′-F or 2′-methyl-2′-OH nucleosides. The S282T amino acid substitution that conferred resistance to these

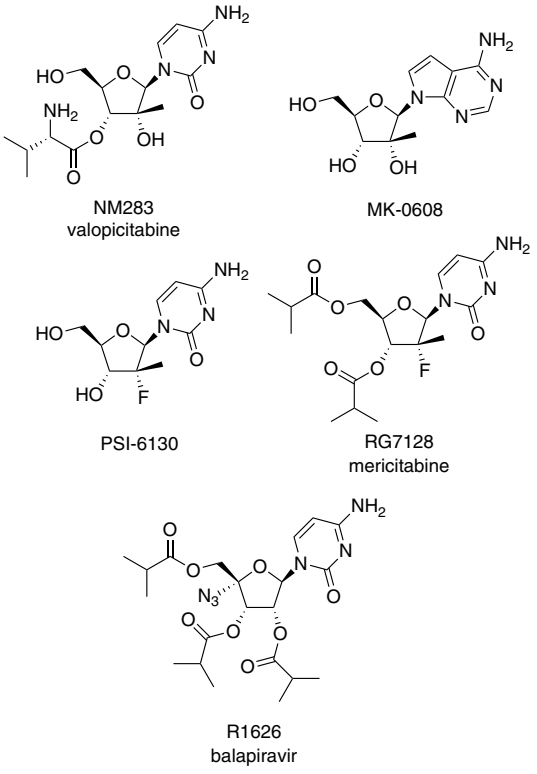


Figure 2.3 Nucleoside HCV NS5B inhibitors.

nucleosides was shown not to be a pre-existing variant and was also shown to be quite unfit [20, 52–54].

It was the discovery of the liver-targeted nucleotide prodrug PSI-7851 and its single isomer PSI-7977 (sofosbuvir, SOF) that had a profound impact on the development of nucleos(t)ide drugs for HCV and ultimately on the development of IFN-free HCV cure regimens (Figure 2.4 and Table 2.2) [25, 55, 56]. Sofosbuvir was a 2′-α-F-2′-*C*-methyluridine 5′-phosphoramidate prodrug that leveraged liver first-pass metabolism to remove the promoiety and deliver the 2′-α-F-2′-*C*-methyluridine monophosphate to the liver. This prodrug strategy produced high concentrations of the active drug in the liver with virtually no systemic exposure. It demonstrated a high barrier to resistance and was shown to be active against all viral GTs.

Fast follow-on approaches based on sofosbuvir produced several uridine nucleotide prodrug clinical agents. These included IDX21427 (uprifosbuvir) and AL-335. Each of these agents demonstrated proof-of-concept in the clinic but were terminated because of a lack of competitive clinical efficacy (Figure 2.4 and Table 2.2).

Attempts to leverage the liver-targeted phosphoramidate prodrug strategy employing purine bases in place of the uridine base of sofosbuvir either with the 2′-α-fluoro-2′-*C*-methyl or 2′-α-hydroxy-2′-*C*-methyl furanose substitution proved unsuccessful. The drug INX-08189 was ultimately terminated because of fatal cardiovascular toxicity in the clinic

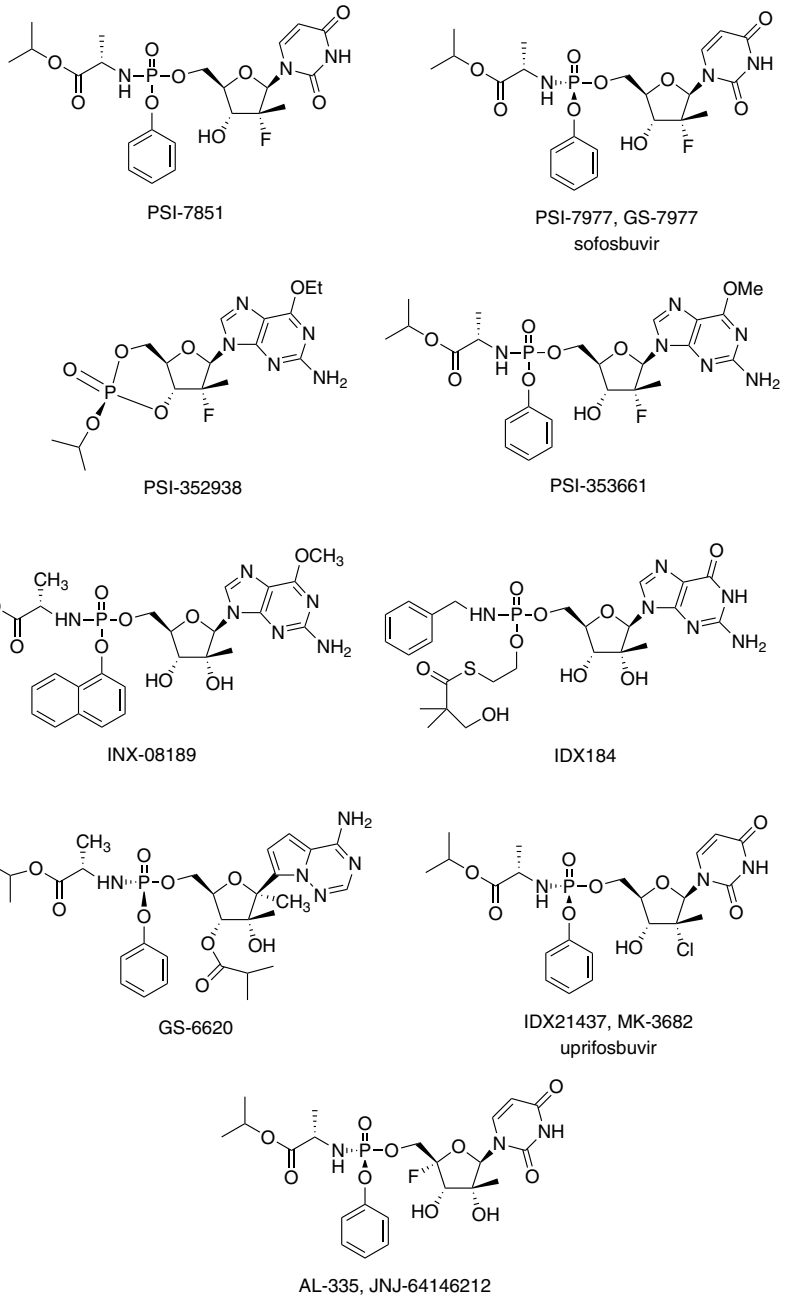


Figure 2.4 Nucleotide HCV NS5B polymerase inhibitors.

and PSI-352938 was terminated because of liver toxicity (Figure 2.4 and Table 2.2) [28, 33, 34, 57, 58]. Development of IDX-184 was ultimately discontinued because of lack of competitive efficacy [36–38, 59].

Table 2.1 Nucleoside HCV NS5B polymerase inhibitors.

		Phase I monotherapy				
Drug	**Replicon potency GT1**	**HCV RNA reduction Log_{10} IU/mL**	**Dosage**	**Dosing period**	**GT**	**Status**
NM283 [17–19] Valopicitabine	EC_{50} = 1.23 μM	1.2	800 mg b.i.d	14 days	1	Discont'd PhII GI tox
PSI-6130 [20–22]	EC_{90} = 4.5 μM	ND	ND	ND	ND	Discont'd PhI Poor PK
RG7128 [18, 19, 22] Mericitabine	EC_{90} = ~4.5 μM	2.7	1500 mg b.i.d.	14 days	1	Discont'd PhIII, Efficacy
R1626 [19, 23, 24] Balapiravir	EC_{50} = 1.28 μM	3.7	4500 mg b.i.d.	14 days	1	Discont'd PhII Hematological tox

ND, not determined.
Replicon potency, phase I monotherapy data with development status.

Over a 12-year period, a number of other nucleos(t)ides (see Tables 2.1 and 2.2) were taken into development without success. These failures were attributed to either safety, efficacy, or other development issues such as lack of a competitive market profile.

In a 2011 phase II clinical study, sofosbuvir in combination with RBV was shown to deliver a 100% cure rate, sustained virological response (SVR), after only 12 weeks of once-a-day oral therapy [60]. This groundbreaking result led to the further development of SOF. In a phase III clinical study, SOF plus RBV delivered greater than a 93% cure rate in GT2 and 3. For GT1, 4, 5, or 6 patients, SOF plus RBV was not sufficient to deliver high cure rates; therefore, the triple combination of SOF plus PEG-INF/RBV for 12 weeks gave 89% SVR in GT1 patients and 97% SVR in GT4, 5, and 6 patients [61, 62]. Sofosbuvir was approved by the US FDA on 6 December 2013 as the first IFN-free HCV curative regimen for GT2 and 3 patients and to this day stands as the only nucleos(t)ide HCV cure agent approved for clinical use.

2.4.2 Non-nucleoside NS5B Inhibitors

The identification of non-nucleoside NS5B inhibitors was fueled by high-throughput screening and the use of structure-based drug design tools. The availability of HCV NS5B polymerase protein co-crystal structures with small molecule inhibitors allowed for identification of the allosteric binding sites and subsequent optimization of inhibitor binding. Ultimately, four allosteric binding sites were identified for non-nucleoside inhibitors.

Table 2.2 Nucleotide inhibitors of HCV NS5B polymerase.

		Phase I monotherapy				
Drug	**Replicon potency GT1**	**HCV RNA reduction Log_{10} IU/mL**	**Dosage**	**Dosing period**	**GT**	**Status**
PSI-7851 [25, 26]	$EC_{90} = 0.42$ μM	1.95	400 mg q.d.	3 days	1	Discont'd Moved to single isomer PSI-7977
PSI-7977 [25, 27] GS-7977 Sofosbuvir	$EC_{90} = 0.42$ μM	4.7	400 mg q.d.	14 days	1	Approved Sovaldi® Combo: Harvoni®, Epclusa®, Vosevi®
PSI-352938 [28–32]	$EC_{90} = 1.37$ μM	4.65	200 mg q.d.	7 days	1	Discont'd PhI Liver tox
INX-08189 [33–35]	$EC_{50} = 0.01$ μM	1.31	25 mg q.d.	3 days	1	Discont'd PhII Cardiac tox
IDX184 [36–39]	$EC_{50} = 0.4$ μM	0.74	100 mg q.d.	3 days	1	Discont'd PhII Efficacy
GS-6620 [40–42]	$EC_{50} = 0.36$ μM	1.73	900 mg b.i.d	5 days	1	Discont'd PhI PK/PD Variability
IDX21437 [43–45] MK-3682 Uprifosbuvir	$EC_{50} = 0.32$ μM	4.2	300 mg q.d.	7 days	1	Discont'd PhIII Efficacy
AL-335 [46, 47] JNJ-64146212	$EC_{50} = 0.075$ μM	2.76	400 mg q.d.	7 days	1	Discont'd PhII Efficacy

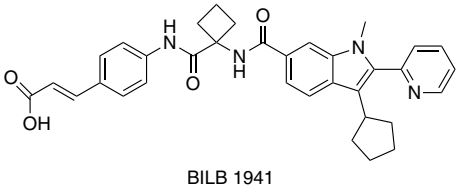
Replicon potency, phase I data, and development status.

These sites are located in the palm or thumb domains of the NS5B polymerase [16]. No allosteric inhibitors have been identified that bind to the finger domain; however, several inhibitors with undisclosed binding sites have been reported [16].

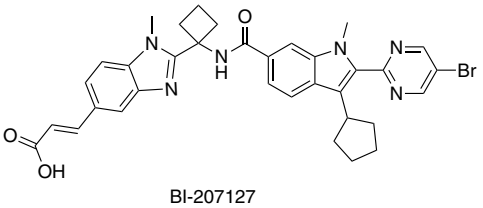
The development of non-nucleoside HCV NS5B inhibitors faced a number of challenges, most notably narrow genotype coverage and rapid development of drug-resistant variants. Yet, there was significant activity in the exploration of non-nucleoside inhibitors because of the ease with which they could be identified by biochemical or phenotypic screening approaches (Figures 2.5–2.8 and Tables 2.3–2.6). The first non-nucleoside to enter the clinic and demonstrate clinical proof-of-concept was HCV-796 (nesbuvir) (Figure 2.8 and

Table 2.6) [106–108]. It demonstrated a 1.2–1.5 $\log_{10}$ IU/ml drop in HCV RNA levels; however, development was halted because of liver toxicity.

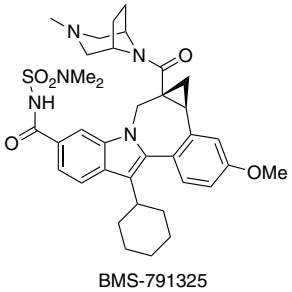
Ultimately, two approved drugs emerged from the non-nucleoside class of inhibitors. The first was beclabuvir (Figure 2.5 and Table 2.3), which was approved only in Japan as part of the fixed-dose combination (Ximency®) that included the NS3 protease inhibitor asunaprevir and the NS5A inhibitor daclatasvir [113, 114]. The other was dasabuvir (Exviera®) (Figure 2.7 and Table 2.5), which was part of a complex multi-tablet combination therapy (Viekira Pak®) that included the NS5A inhibitor ombitasivir, the NS3/4 protease inhibitor paritaprevir, and ritonavir [115]. These agents were only approved for the treatment of GT1 patient populations, and ultimately, neither played a significant role in



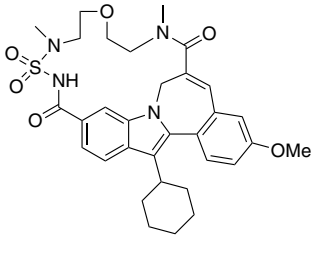
BILB 1941



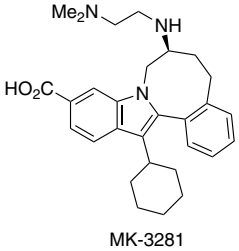
BI-207127
deleobuvir



BMS-791325
beclabuvir



TMC647055



MK-3281

Figure 2.5 Non-nucleoside HCV NS5B polymerase thumb domain inhibitors.

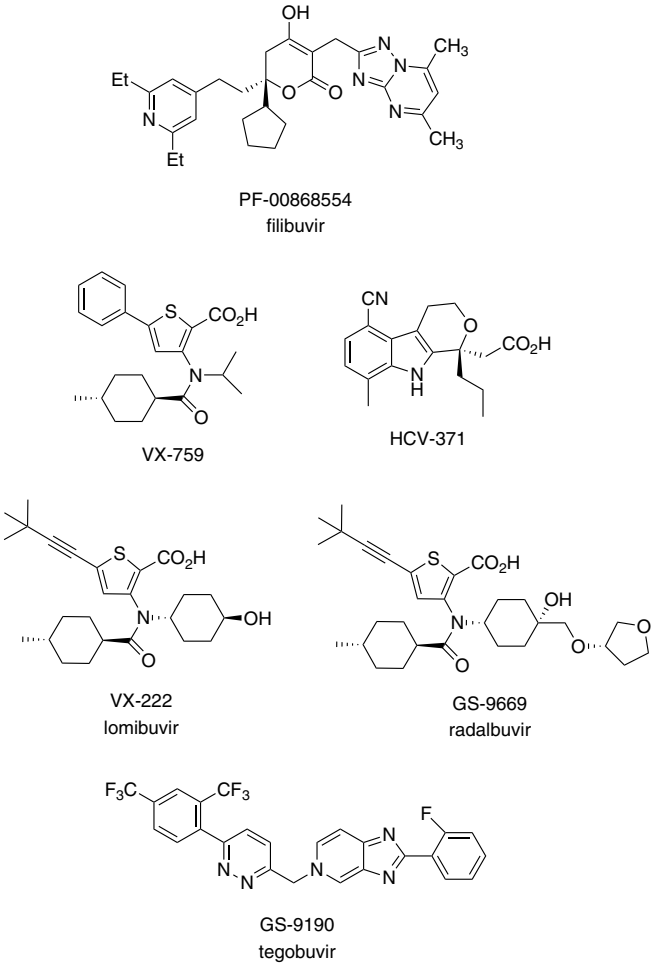


Figure 2.6 Non-nucleoside HCV NS5B polymerase thumb domain inhibitors.

widely adopted curative combination therapies nor were their market penetration significant. In general, because of their limited genotype coverage and high occurrence of resistance, the non-nucleoside class of inhibitors could not compete with the nucleotide sofosbuvir as the NS5B polymerase component of curative combination regimens and ultimately faded from use.

2.5 HCV NS3/4A Protease Inhibitors

The discovery and development of small-molecule HCV NS3/4A protease inhibitors was aggressively pursued with 10 drugs receiving regulatory approval either as single agents to be used in combination with IFN or as part of DAA combination regimens. The NS3/4A protease is a member of the chymotrypsin family of serine proteases. It is formed by

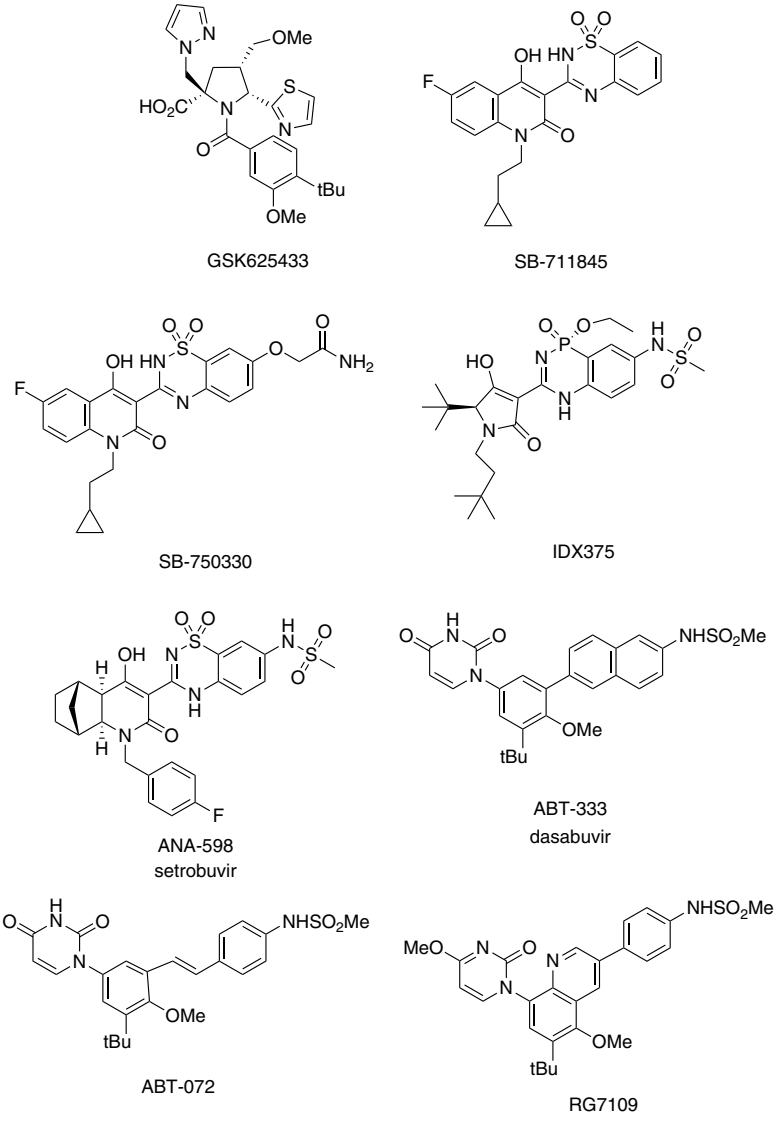
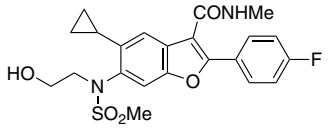
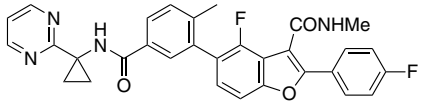


Figure 2.7 Non-nucleoside HCV NS5B polymerase palm domain inhibitors.

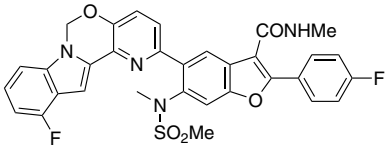
proteolytic cleavage of the HCV genome-derived ~3000 amino acid viral polypeptide by the HCV NS2/3 cysteine protease. It plays a critical role in viral polyprotein processing to generate the nonstructural proteins NS3, NS4A, NS4B, NS5A, and NS5B. The NS3 protein is bifunctional having a serine protease domain and an NTPase/helicase domain. It associates noncovalently with the NS4A cofactor forming the NS3/4A protease [8]. The crystal structure of the NS3/4A protease has been solved including structures having truncated NS3 domains (Figure 2.9). The substrate binding groove is extremely shallow, elongated, and solvent exposed with significant hydrophobic character making inhibitor design and optimization challenging [116].



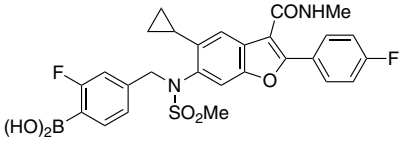
HCV-796
nesbuvir



BMS-929075



MK-8876



GSK2485852

Figure 2.8 Non-nucleoside HCV NS5B polymerase palm domain inhibitors.

Table 2.3 Non-nucleoside HCV NS5B polymerase thumb domain inhibitors.

		Phase I monotherapy				
Drug	**Replicon potency GT1**	**HCV RNA reduction Log_{10} IU/mL**	**Dosage**	**Dosing period**	**GT**	**Status**
BILB 1941 [63, 64]	EC_{50} = 84 nM	>1	450 mg t.i.d.	5 days	1	Discont'd PhII GI & Liver Tox
BI-207127 [65, 66] Deleobuvir	EC_{50} = 11–23 nM	3.8	600 mg t.i.d.	5 days	1	Discont'd PhIII Efficacy
BMS-791325 [67–69] Beclabuvir	EC_{50} = 3–6 nM	2.5	300 mg q.d.	24 h	1	Approved Combo Regimen Japan Ximency®
TMC647055 [70, 71]	EC_{50} = 166 nM	2.4–3.3	1000 mg b.i.d.	6 days	1	Discont'd PhI
MK-3281 [72, 73]	EC_{50} = 38 nM	1.95	800 mg b.i.d.	7 days	1	Discont'd PhI

Replicon potency, phase I data, and development status.

Development of early generation HCV NS3/4A protease inhibitors was limited by several factors. Their genotype coverage was restricted to GT1, and they suffered from the rapid emergence of clinical resistance. Through tenacious medicinal chemistry optimization, subsequent generation protease inhibitors were able to achieve broader genotype coverage and an increased barrier to resistance.

Table 2.4 Non-nucleoside NS5B thumb domain inhibitors.

		Phase I monotherapy				
Drug	**Replicon potency GT1**	**HCV RNA reduction Log_{10} IU/mL**	**Dosage**	**Dosing period**	**GT**	**Status**
PF-00868554 [74–77] Filibuvir	EC_{50} = 75 nM	2.13	300 mg t.i.d.	8 days	1	Discont'd PhII
HCV-371 [78]	EC_{50} = 4.8 μM	Not significant	—	—	1	Discont'd PhI Efficacy
VX-759 [79–81]	EC_{50} = 300 nM	2.5	800 mg t.i.d.	10 days	1	Discont'd
VX-222 [82, 83] Lomibuvir	EC_{50} = 5.9 nM	3.5	750 mg b.i.d.	3 days	1	Discont'd PhII
GS-9669 [84–86] Radalbuvir	EC_{50} = 11 nM	>3	500 mg q.d.	3 days	1	Discont'd PhII
GS-9190 [87–91] Tegobuvir	EC_{50} = 0.7 nM	1.61–1.95	40–120 mg b.i.d.	8 days	1	Discont'd PhII

Replicon potency, phase I data, and development status.

2.5.1 HCV NS3/4A Protease Acyclic Reversible Inhibitors

The discovery of HCV NS3/4A protease inhibitors employed both peptidomimetic approaches and co-crystal structure-guided approaches. Early work in substrate-based peptide optimization led to the identification of several key compounds (Figure 2.10) that spawned an entire class of acyclic reversible inhibitors shown in Figure 2.11 and Table 2.7. A number of these agents entered clinical development demonstrating proof-of-concept antiviral activity in HCV patients. The only approved drug that emerged from this acyclic peptidomimetic class was asunaprevir (Figure 2.11 and Table 2.7). Asunaprevir was eventually launched in Japan as part of a combination regimen for a narrow GT1b patient population [114]. However, this class paved the way for next-generation inhibitors.

2.5.2 HCV NS3/4A Protease Acyclic Covalent Binding Inhibitors

Simultaneously, with the development of reversible acyclic peptidomimetic inhibitors, a class of acyclic peptidic inhibitors that bound covalently to the active-site serine residue of HCV protease were being developed. This class is represented primarily by the approved agents telaprevir (VX-950) and boceprevir (SCH503034) (Figure 2.12 and Table 2.8). As part of their design, each employed a reactive ketoamide warhead that reacted with the active-site serine. These two agents were developed as combination regimens with PEG-IFN/RBV, the standard of care at the time and were the first DAAs approved for therapy. They

Table 2.5 Non-nucleoside HCV NS5B polymerase palm domain inhibitors.

		Phase I monotherapy				
Drug	**Replicon potency GT1**	**HCV RNA reduction Log_{10} IU/mL**	**Dosage**	**Dosing period**	**GT**	**Status**
GSK625433 [92–94]	EC_{50} = 3 nM	NA	NA	NA	1	Discont'd PhI
SB-750330 [95]	EC_{50} = 2 nM	NA	NA	NA	1	Discont'd PhI
IDX375 [96]	EC_{50} = 18 nM	0.5 – 1.1	200 mg q.d.	2 days	1	Discont'd
ANA-598 [97, 98] Setrobuvir	EC_{50} = 3–18 nM	2.9	800 mg b.i.d.	3 days	1	Discont'd PhII
ABT-333 [99–102] Dasabuvir	EC_{50} = 1.8–7.7 nM	NA	300 – 1200 mg b.i.d.	2 days	1	Approved as combo Viekira Pak®
ABT-072 [103, 104]	EC_{50} = 0.3–1.1 nM	NA	400 mg q.d.	NA	1	Discont'd
RG7109 [105]	EC_{50} = 1.1 nM	NA	NA	NA	NA	Discont'd PhI

NA, data not available.
Replicon potency, phase I data, and development status.

Table 2.6 Non-nucleoside NS5B polymerase palm domain inhibitors.

		Phase I monotherapy				
Drug	**Replicon potency GT1**	**HCV RNA reduction Log_{10} IU/mL**	**Dosage**	**Dosing period**	**GT**	**Status**
HCV-796 [106–108] Nesbuvir	EC_{50} = 13 nM	1.2–1.5	50–1500 mg b.i.d.	14 days	1	Discont'd PhII Safety
BMS-929075 [94, 109]	EC_{50} = 3–20 nM	NA	NA	NA	NA	Discont'd PhIa
MK-8876 [94, 110]	EC_{50} = 1–7 nM	3.4	800 mg q.d.	7 days	1	Discont'd PhII
GSK2485852 [111, 112]	EC_{50} = 1–8 nM	NA	NA	NA	NA	Discont'd PhIa

NA, data not available.
Replicon potency, phase I data, and development status.

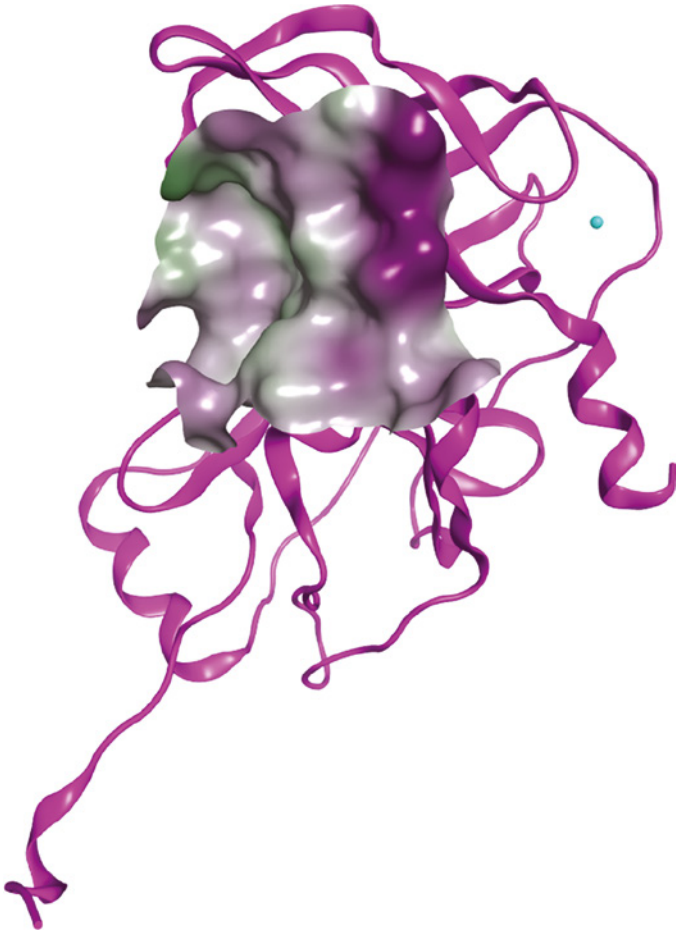


Figure 2.9 Crystal structure of the protease domain of the HCV NS3/4A protease showing the shallow substrate binding groove in gray (PDB Code: 3RC6).

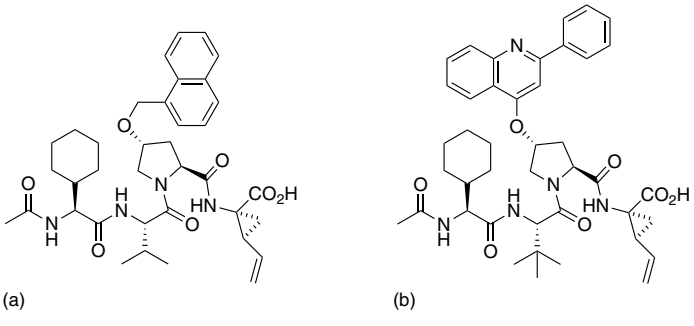
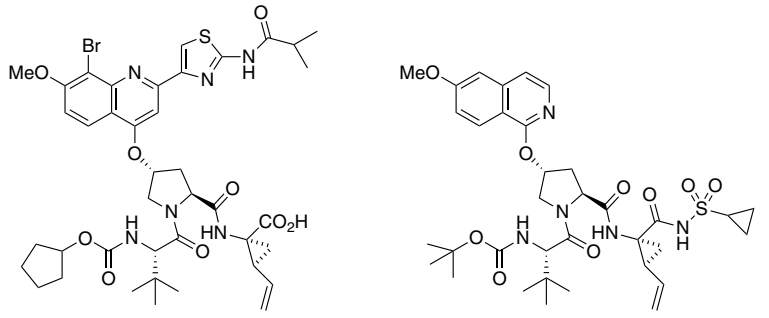
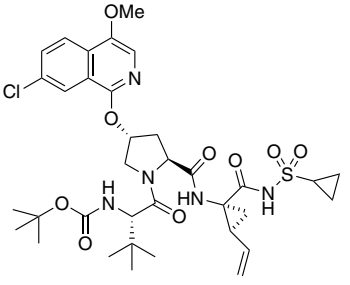


Figure 2.10 HCV NS3/4A protease inhibitor early leads that derived from substrate-based peptide optimization.

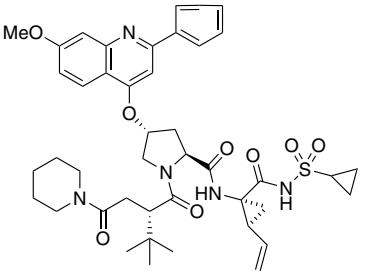


BI 201335
faldaprevir

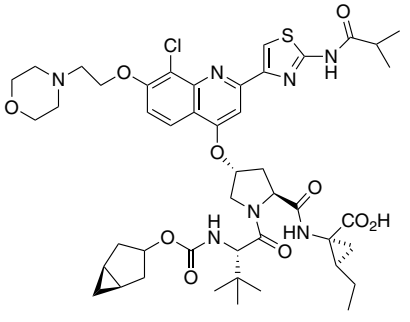
BMS-605339



BMS-650032
asunaprevir



ACH-1625
sovaprevir



GS-9451
vedroprevir

Figure 2.11 HCV NS3/4A protease acyclic reversible binding inhibitors.

displayed limited genotype coverage and thus were approved to treat only GT1 patients but were able to achieve a ~70–80% SVR (sustained virological response off therapy) cure in this patient population [134, 139]. However, because they required twice-daily or three times-a-day administration, exhibited significant side effects in addition to those presented with PEG-IFN/RBV co-therapy and demonstrated a low barrier to resistance with V36, T54, R155, and A156 clinically observed mutations, they presented significant limitations in the clinic [140].

Table 2.7 HCV NS3/4 protease acyclic reversible inhibitors.

		Phase I monotherapy				
Drug	**Replicon potency GT1**	**HCV RNA reduction Log_{10} IU/mL**	**Dosage**	**Dosing period**	**GT**	**Status**
BI 201335 [117–120] Faldaprevir	EC_{50} = 3 nM	4.2	240 mg q.d.	14 days	1	Discont'd PhIII Efficacy
BMS-605339 [121]	EC_{50} = 12 nM	1.8	120 mg q.d.	12 h	1	Discont'd Ph1 Cardio tox
BMS-650032 [122–124] Asunaprevir	EC_{50} = 6 nM	2.7 – 3.5	200–600 mg b.i.d	3 days	1	Approved Combo Ximency®
ACH-1625 [125, 126] Sovaprevir	EC_{50} = 11 nM	3.81	600 mg q.d.	5 days	1	Discont'd PhI Liver tox
GS-9451 [127–130] Vedroprevir	EC_{50} = 2 nM	3.6	400 mg q.d.	3 days	1	Discont'd PhI

Replicon potency, phase I data, and development status.

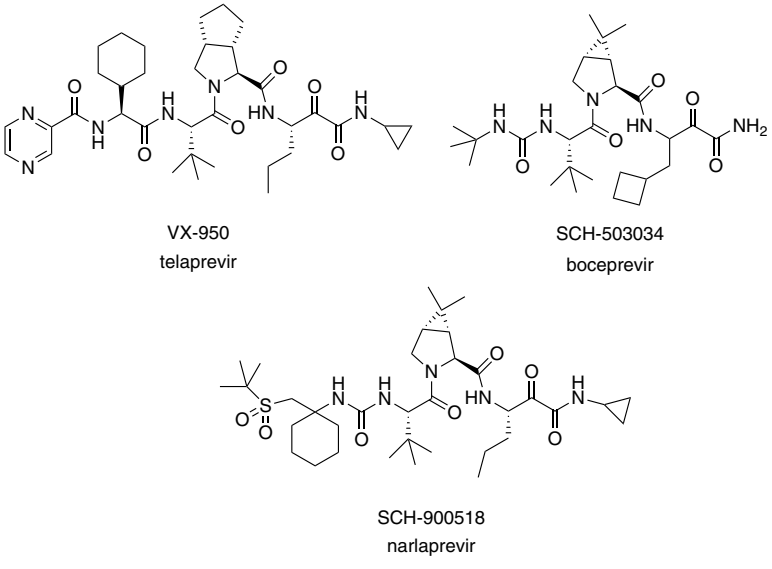


Figure 2.12 HCV NS3/4A protease covalent binding inhibitors.

Table 2.8 HCV NS3/4 protease covalent binding inhibitors.

		Phase I monotherapy				
Drug	**Replicon potency GT1**	**HCV RNA reduction Log_{10} IU/mL**	**Dosage**	**Dosing period**	**GT**	**Status**
VX-950 [131–133] Telaprevir	EC_{50} = 83–280 nM	4.4	750 mg t.i.d.	14 days	1	Approved Incivek®
SCH-503034 [134, 135] Boceprevir	EC_{50} = 220–233 nM	2.06	400 mg t.i.d.	7 days	1	Approved Victrelis®
SCH-900518 [136–138] Narlaprevir	$EC9_0$ = 40 nM	4.5	800 mg t.i.d.	7 days	1	Discont'd PhII

Replicon potency, phase I data, and development status.

2.5.3 HCV NS3/4A Protease Macrocyclic Reversible Inhibitors

Further work on the acyclic peptidic inhibitors and particularly on inhibitor B (Figure 2.10) led to ciluprevir (BILN-2061, Figure 2.13 and Table 2.9), a cyclic inhibitor. Ciluprevir became the foundation for all subsequent work on cyclic reversible inhibitors of HCV NS3/4A protease (Figure 2.13 and Table 2.9). A number of these agents entered clinical development but only two, simeprevir (TMC435) and parateprevir (ABT-450) (Figure 2.13 and Table 2.9), were ultimately approved for clinical use either in combination with PEG-IFN/RBV or as part of a DAA combination regimen.

2.5.4 HCV NS3/4 Protease P2–P4 Macrocyclic Inhibitors

The other major class of macrocyclic HCV NS3/4A protease inhibitors were the P2–P4 macrocycles (Figure 2.14 and Table 2.10). This class was identified by structure-based approaches and the realization that there was potential to access additional interactions with the helicase domain of NS3/4A. Developments in this class of inhibitors led to the identification of molecules that expanded the genotype coverage beyond GT1 and increased the barrier to resistance. However, resistance concerns would still remain and as with other protease inhibitors, this class required combination with either PEG-IFN/RBV or other potent DAAs such as the nucleotide sofosbuvir or a potent NS5A inhibitor which could protect against resistance and enhance genotype coverage. The approved commercial combinations became vaniprevir + PEG-IFN/RBV (Vanihep®, Japan only, GT1), grazoprevir + elbasvir (NS5A) (Zepatier®, GT1 and 4), voxilaprevir + sofosbuvir (NS5B) + velpatasvir(NS5A) (Vosevi®,pangenotypic), and glecaprevir + pibrentasvir (NS5A) (Mavyret®, pangenotypic).

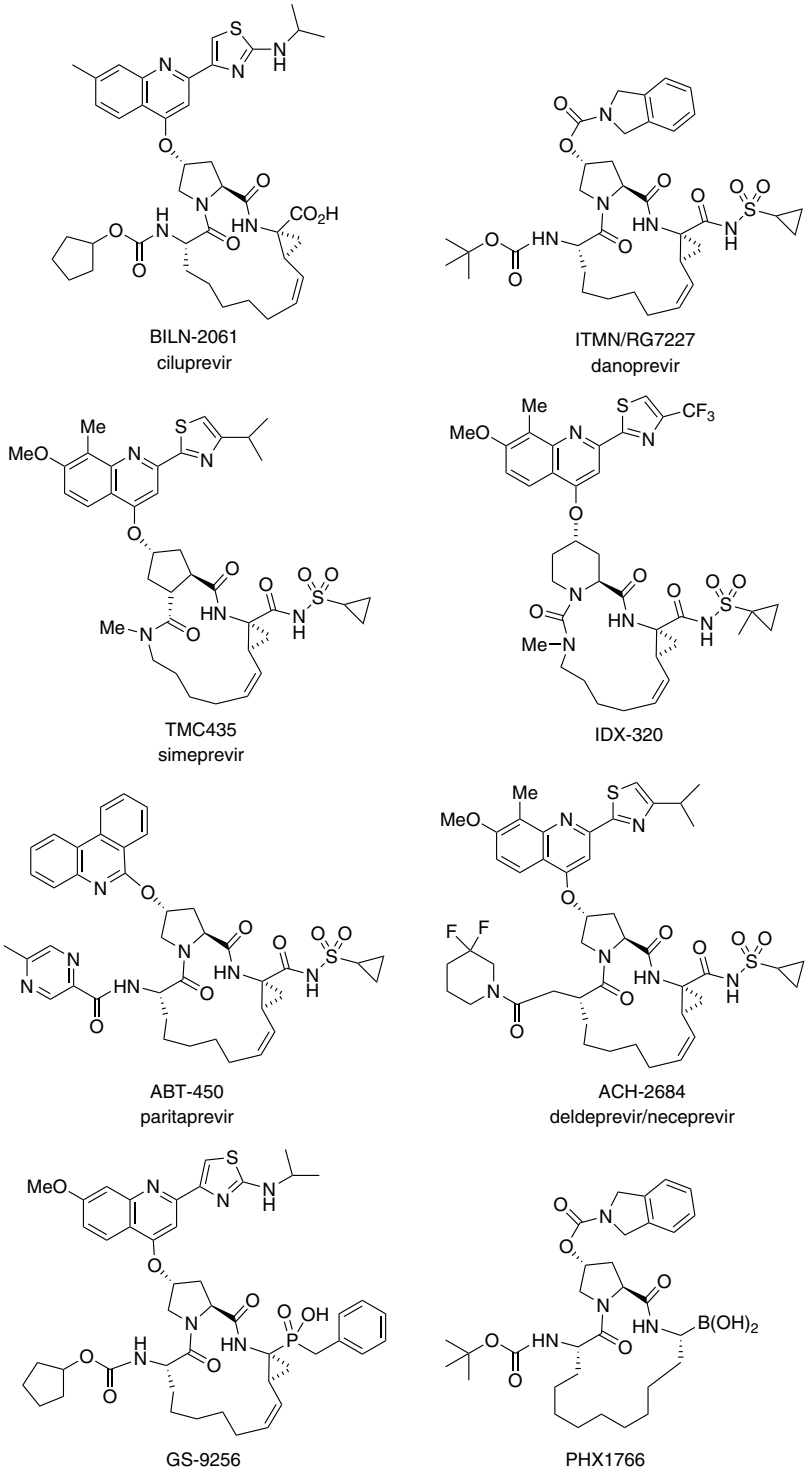


Figure 2.13 HCV NS3/4A protease macrocyclic reversible binding inhibitors.

Table 2.9 HCV NS3/4 protease macrocyclic reversible binding inhibitors.

		Phase I monotherapy				
Drug	**Replicon potency GT1**	**HCV RNA reduction Log_{10} IU/mL**	**Dosage**	**Dosing period**	**GT**	**Status**
BILN-2061 [141] Ciluprevir	EC_{50} = 1.2 nM	>2	200 mg b.i.d.	2 days	1	Discont'd PhI, Cardio tox
ITMN-191 [51, 142, 143] RG7227 Danoprevir	EC_{50} = 1.8 nM	3.8	200 mg t.i.d.	14 days	1	Approved Ganovo® (China)
TMC435 [144, 145] Simperevir	EC_{50} = 9.4 nM	3.91	150 mg q.d.	6 days	1	Approved Olysio®
IDX-320 [146, 147]	EC_{50} = 0.5–3.4 nM	3.3	400 mg q.d.	3 days	1	Discont'd PhII Liver tox
ABT-450 [148–150] Paritaprevir	EC_{50} = 0.21–1.0 nM	4.0	200 mg q.d.	3 days	1	Approved Viekira Pak®, Technivie®
ACH-2684 [151–153] Deldeprevir, Neceprevir	EC_{50} = 0.04–0.7 nM	3.73	400 mg q.d.	3 days	1	Discont'd PhII
GS-9256 [154–156]	EC_{50} = 20 nM	2.8	450 mg b.i.d.	1 day	1	Discont'd PhII
PHX1766 [157]	EC_{50} = 8 nM	1.5	800 mg b.i.d.	6 days	1	Discont'd PhI Efficacy

Replicon potency, phase I data, and development status.

2.6 HCV NS5A Inhibitors

The HCV NS5A nonstructural protein has been shown to play several roles in the virus life cycle, yet it has no known enzymatic activity (Figure 2.15). It was shown to be important in the process of viral genome replication and modulation of host cell factors that support viral persistence [8]. Small molecule agents that inhibit the function of NS5A were identified by phenotypic screening of large compound libraries and resistance mapping of early leads (Figure 2.16) [172]. Additional studies that included pull-down experiments, X-ray crystallography, and affinity labeling studies helped solidify the nature of the inhibitor mode of binding and complex stoichiometry of the inhibition [171, 173–175].

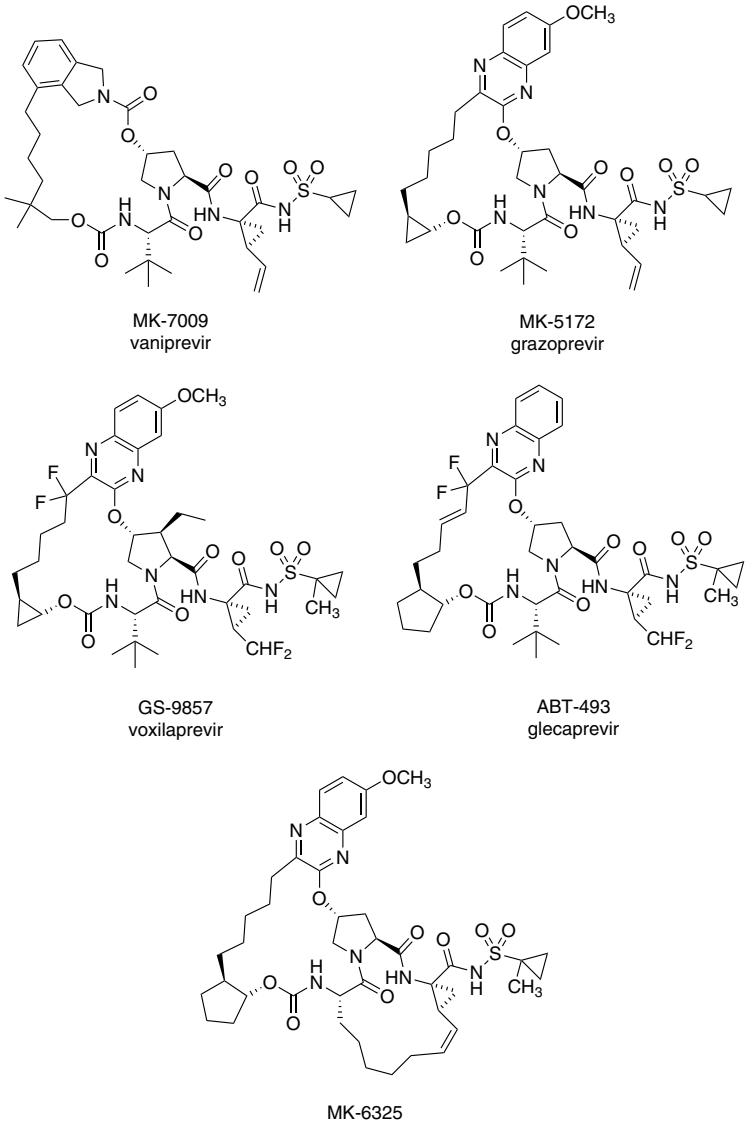


Figure 2.14 HCV NS3/4A protease P2–P4 macrocyclic reversible binding inhibitors.

Through intensive medicinal chemistry efforts, the early small molecule leads (Figure 2.16) were transformed into exceptionally potent inhibitors of viral replication (Figures 2.17 and 2.18, Tables 2.11 and 2.12). Ultimately, this work led to the approval of five agents that became integral parts of marketed curative combination regimens. The pivotal clinical study that established the excitement around this mechanism of action and prompted a frenzy of development efforts by many groups occurred with daclatasvir (Figure 2.17 and Table 2.11) [176, 177]. In a phase I study, daclatasvir delivered a 1.8, 3.2, and 3.3 $\log_{10}$ IU/mL drop in serum HCV RNA at 24 hours which persisted to 144 hours for

Table 2.10 HCV NS3/4 protease P2–P4 macrocyclic reversible binding inhibitors.

		Phase I monotherapy				
Drug	**Replicon potency GT1**	**HCV RNA reduction Log_{10} IU/mL**	**Dosage**	**Dosing period**	**GT**	**Status**
MK-7009 [158–161] Vaniprevir	EC_{50} = 4.5 nM	1.8–4.6	600 mg q.d. 700 mg b.i.d.	8 days	1	Approved Japan As combo Vanihep®
MK-5172 [162–165] Grazoprevir	EC_{50} = 7.4 nM	3	30–800 mg q.d.	7 days	1	Approved As combo Elbasvir®
GS-9857 [166–168] Voxilaprevir	EC_{50} = 1.5–6.6 nM	>3	100 mg q.d.	3 days	1	Approved As combo Vosevi®
ABT-493 [169, 170] Glecaprevir	EC_{50} = 0.85–0.94 nM	3.8–4.36	100–700 mg q.d.	3 days	1	Approved As combo Mavyret®

Replicon potency, phase I data, and development status.

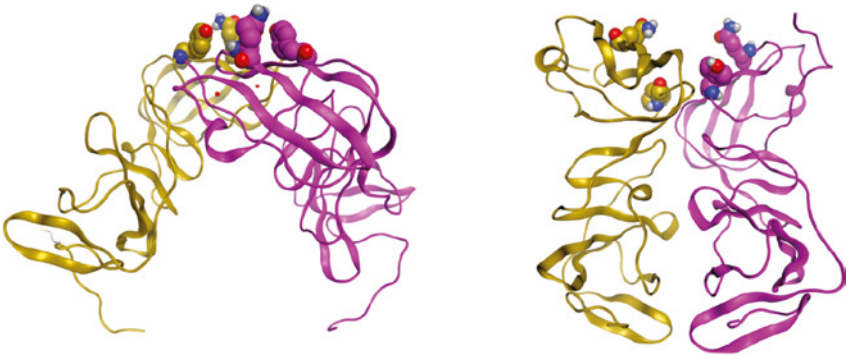


Figure 2.15 Crystal structure of HCV NS5A with potential binding residues highlighted. PDB Code 1ZH1 [171]. With permission of John Wiley & Sons.

the 100 mg dose. This exceptional efficacy was attributed to the dual effect of the drug on viral replication and virion assembly [199].

Even with exceptional clinical efficacy, first-generation NS5A inhibitors were plagued with rapid onset of resistance and limited genotype coverage. For daclatasvir, when administered as monotherapy for 14 days at doses from 1 to 100 mg q.d., most subjects experienced viral breakthrough with major mutations observed at M28T/A/V, Q30H/R/K/E, L31M/V, and Y93H/N/C in GT1a infected patients and L31M/V and Y93H/C in GT1b

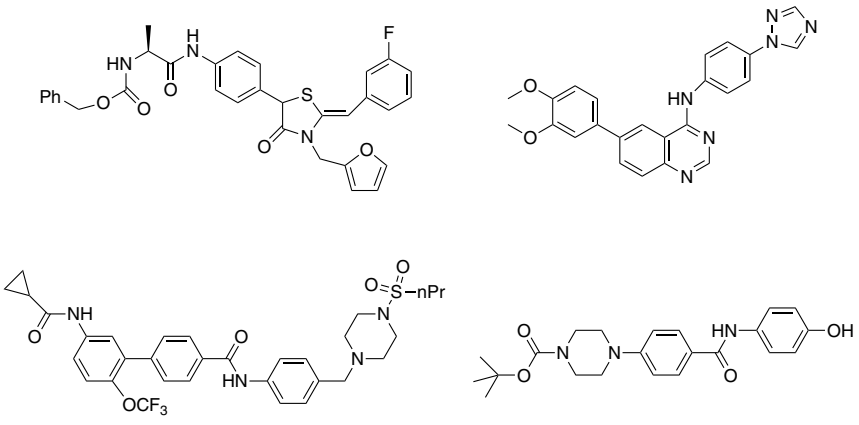
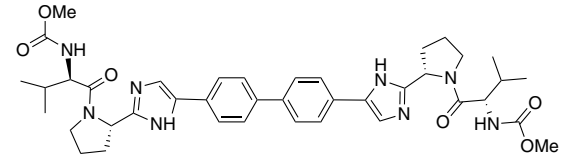


Figure 2.16 Early NS5A protein inhibitor screening leads.

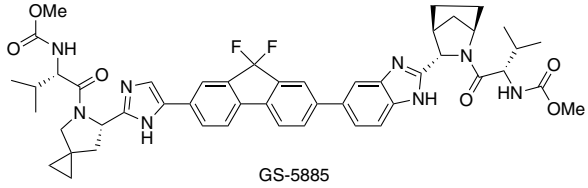
patients [172, 200, 201]. Most NS5A inhibitors suffered from these same resistance issues; however, a select group of later generation inhibitors such as pibrentasvir and valpatasvir were able to overcome many of the resistance and genotype coverage problems (Figure 2.18 and Table 2.12). NS5A inhibitors became integral components of many DAA HCV cure combinations that include Harvoni®, Epclusa®, Vosevi®, Viekira Pak®, and Mavyret® providing 95–100% SVR cure rates in only 8–12 weeks of therapy.

2.7 The Evolution of DAA Combination Therapies

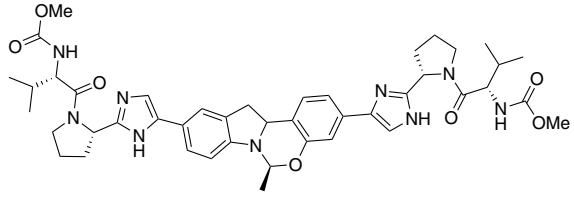
With the objective to achieve higher cure rates, early attempts to study drug combinations focused on the addition of DAAs to the standard of care PEG-IFN/RBV. Early phase clinical studies demonstrated that these combinations delivered deeper viral load reductions, faster kinetics of viral load declines, and a higher percentage of patients that reached a rapid virological response (RVR), i.e., undetectable HBV RNA levels after four weeks on therapy, than PEG-IFN/RBV alone. Triple combinations that included either protease inhibitors, nucleos(t)ide inhibitors, or non-nucleoside inhibitors with PEG-IFN/RBV were all shown to deliver additive effects clinically [18, 202]. In 2010, combinations of a NS3/4 protease inhibitor, telaprevir (Incivek®) or boceprevir (Victrelis®), with either PEG-IFN/RBV or PEG-INTRON were approved by the US FDA and European EMEA as the first DAA-containing regimens to cure HCV [202–204]. They delivered on an improved cure rate (~72%) and in some cases a shortened duration of therapy (24–28 weeks) for treatment-naïve patients. However, they became difficult regimens to administer because of the added adverse events these protease inhibitors introduced to the regimens including exacerbation of anemia and adverse cutaneous effects with telaprevir coupled with the potential for the emergence of resistance. In addition, these first DAA-containing combinations were only approved for treating GT1 patients, thus requiring patient genotyping. It remained an aspirational goal to eliminate the use of subcutaneously delivered PEG-IFN/RBV and its associated serious adverse side effects. Ultimately, with the emergence of IFN-free DAA regimens, the clinical-use lifespan of both telaprevir and boceprevir was only three years.



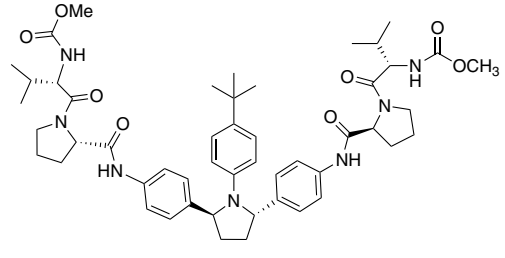
BMS-790052
daclatasivr



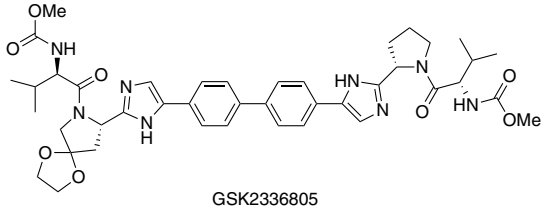
GS-5885
ledipasvir



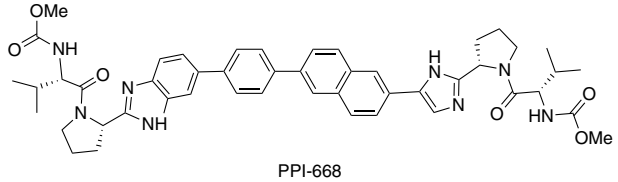
MK-8742
elbasvir



ABT-267
ombitasvir,

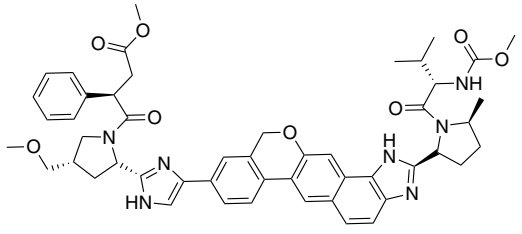


GSK2336805

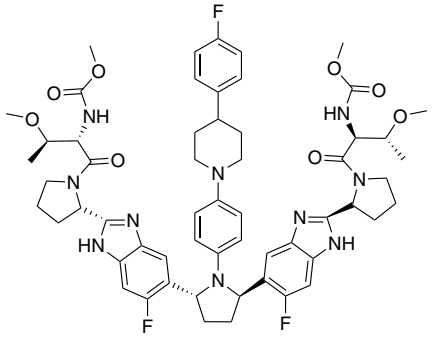


PPI-668
ravidasvir

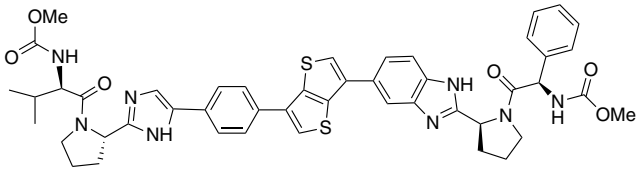
Figure 2.17 First-generation HCV NS5A inhibitors.



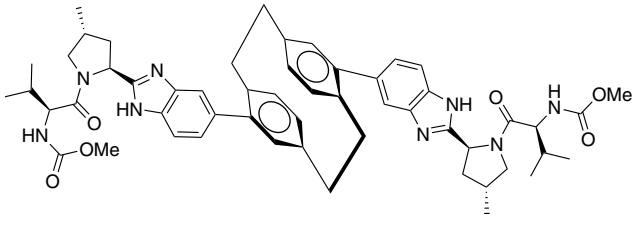
GS-5816
velpatasvir,



ABT-530
pibrentasvir



IDX-719
samatasvir



ACH-3102
odalasvir

Figure 2.18 Second-generation NS5A inhibitors.

One pivotal study that was first to investigate the possibility of eliminating PEG-IFN/RBV was called INFORM-1 [51]. This small investigational study attempted to study whether a nucleoside NS5B inhibitor, RG7128 (mericitabine, Figure 2.3), and a protease inhibitor, RG7227 (danoprevir, Figure 2.13), combination would produce a clinical response

Table 2.11 First-generation HCV NS5A inhibitors.

		Phase I monotherapy				
Drug	**Replicon potency GT1**	**HCV RNA reduction Log_{10} IU/mL**	**Dosage**	**Dosing period**	**GT**	**Status**
BMS-790052 [176–178] Daclatasvir	EC_{50} = 0.009–0.05 nM	3.6 – 4.5	100 mg q.d.	14 days	1	Approved As combo Daklinza®
GS-5885 [179, 180] Ledipasvir	EC_{50} = 0.004–0.031 nM	3.2 – 3.3	10 mg q.d.	3 days	1	Approved As combo Harvoni®
MK-8742 [181–183] Elbasvir	EC_{50} = 0.003–0.004 nM	4.2 – 5.1	50 mg q.d.	5 days	1	Approved As combo Zepatier®
GSK2336805 [184, 185]	EC_{50} = 0.008–0.06 nM	2.96	60 mg q.d.	1 day	1	Discont'd PhII
ABT-267 [102, 186, 187] Ombitasvir	EC_{50} = 0.005–0.135 nM	2.9	5, 50 mg q.d.	3 days	1	Approved As combo Viekira Pak®
PPI-668 [188–190] Ravidasvir	EC_{50} = 0.016–0.014 nM	3.5	100 mg q.d.	3 days	1	Unknown Developed in China

Replicon potency, phase I data, and development status.

similar to that seen when each of these individual agents were combined with PEG-IFN/RBV. In a 14-day study in treatment-naïve GT1-infected patients, a median change in HCV RNA from baseline of −5.1 log_{10} IU/ml was observed, thus setting the stage for future all DAA combination studies.

It was not until the results reported from two key Phase II studies that the field realized PEG-IFN/RBV-containing regimens were going to be a thing of the past. The first study examined the combination of daclatasvir (NS5A inhibitor) 60 mg q.d. and asunaprevir (NS3/4 protease inhibitor) 600 mg b.i.d. in GT1 null responder patients for 24 weeks [205]. Of 11 patients that received the dual DAA combination, 5 reached undetectable HCV RNA at end of treatment and 4 of them achieved SVR at weeks 12 and 24 post-treatment period. Of those that did not reach SVR, all six were GT1a subjects who experienced viral breakthrough and exhibited resistant mutations in both the NS3 (R155K, D168K/E/T/V/Y) and NS5A (Q30R, L31M/V, Y93C/N) proteins. The second watershed study was referred to as ELECTRON and involved a multi-arm study evaluating sofosbuvir (NS5B nucleotide inhibitor) 400 mg q.d. in combination with RBV alone or in combination with PEG-IFN/RBV for 12 weeks of therapy in GT2/3 subjects [206]. All combination arms of the study including the IFN-free sofosbuvir/RBV arm resulted in

Table 2.12 Second-generation HCV NS5A inhibitors.

		Phase I monotherapy				
Drug	**Replicon potency GT1**	**HCV RNA reduction Log_{10} IU/mL**	**Dosage**	**Dosing period**	**GT**	**Status**
GS-5816 [191, 192] velpatasvir	EC_{50} = 0.015–0.031 nM	3.6	100 mg q.d.	3 days	1	Approved As combo Epclusa® & Vosevi®
ABT-530 [169, 193, 194] pibrentasvir	EC_{50} = 0.002–0.004 nM	4.1	40 mg q.d.	3 days	1	Approved As combo Mavyret®
IDX-719 [195, 196] samatasvir	EC_{50} = 0.003–0.008 nM	3.3–4.3	25–100 mg q.d.	3 days	1	Discont'd PhII
ACH-3102 [172, 197, 198] odalasvir	EC_{50} = 0.005–0.26 nM	3.5–4.0	25–300 mg q.d.	3 days	1	Discont'd PhII

Replicon potency, phase I data, and development status.

SVR12/24 of 100% with no observed viral breakthroughs. The era of IFN-containing HCV cure regimens had come to an end.

Because of its high barrier to resistance and broad genotype coverage, sofosbuvir became the backbone agent of choice for many DAA combinations and was married in clinical studies with numerous NS5A inhibitors including daclatasvir, ledipasvir, and velpatasvir as well as protease inhibitor simeprevir [207–211]. However, other non-sofosbuvir-containing DAA combination regimens also emerged combining a NS5A inhibitor, a NS3/4 protease inhibitor with or without a non-nucleoside NS5B inhibitor. In some cases, adding RBV to the combination appeared to be beneficial.

The first IFN-free combination to be approved by the US FDA (6 December 2013) and subsequently by the European EMEA (January 2014) was 12 or 24 weeks of once-a-day sofosbuvir and RBV for the treatment of GT2, 3 patients. The sofosbuvir/RBV combination resulted in a >90% SVR rate in Phase III clinical trials with minimal side effects mostly associated with RBV [61, 62]. Subsequently, the fixed-dose combination of sofosbuvir and ledipasvir (NS5A, Figure 2.17) (Harvoni®) with or without RBV was approved in October 2014 for all GT1 patient populations after demonstrating SVR12 rates of >97% in Phase III clinical studies [212, 213]. In addition, this Phase III study showed that SVR12 rates were similar irrespective of whether the patient was non-cirrhotic or cirrhotic. Also, in 2014, it was reported that sofosbuvir was combined with the NS3/4 protease inhibitor simeprevir (Figure 2.13) with or without RBV for 12 weeks in GT1 patients [211, 214]. Here again a >97% SVR12 was observed; however, SVR12 rates for patients with the protease Q80K polymorphism were somewhat lower.

Although the marriage of sofosbuvir and the NS5A inhibitor daclatasvir was the first Phase II study to evaluate SVR rates with a nucleotide/NS5A combination demonstrating a 98% SVR24 in GT1 patients, 92% in GT2, and 89% in GT3 patients, it was not until 2015 that daclatasvir plus sofosbuvir received regulatory approval [207]. This combination became the first DAA regimen to be approved for treating GT3 patients without the use of RBV [215]. Subsequently, approval of this regimen for treating GT1 patients was achieved.

Further development of the sofosbuvir backbone franchise led to the development of the fixed-dose combinations Epclusa® and Vosevi®. Epclusa®, the combination of sofosbuvir (NS5B nucleotide) with the pangenotypic NS5A inhibitor velpatasvir (Figure 2.18, Table 2.12), provided the first true pangenotypic high barrier to resistance q.d. 12-week therapy. This regimen showed SVR12 rates of 99% in GT1 and 2 patients and 98% in hard-to-treat GT3-naïve patients [191, 216, 217]. The triple combination regimen, Vosevi®, containing sofosbuvir (NS5B nucleotide), velpatasvir (NS5A) and the NS3/4 protease inhibitor voxilaprevir, was evaluated in an 8-week q.d. regimen and delivered SVR12 of 95% and 96% in GT1 and GT3 patients, respectively [218–220]. Vosevi® has been used primarily to treat patients who have failed other HCV cure regimens.

Combination regimens lacking the nucleotide sofosbuvir backbone were also shown to be effective at delivering high cure rates. The first of these was a dual combination that included daclatasvir (NS5A inhibitor) and asunaprevir (NS3/4 protease inhibitor). This combination was studied and approved in 2014 only in Japan because of the prevalence of GT1b patients in the Japanese population and the limited genotype coverage of this combination. The Phase III clinical trial evaluated 24 weeks of dual therapy and achieved and SVR12 of 81% in IFN nonresponders and 87% in those intolerant of or ineligible for IFN use [124]. It was also shown that the T93H polymorphism in NS5A led to a reduction in clinical efficacy. Subsequently, addition of a third agent, beclabuvir (NS5B non-nucleoside inhibitor) to create a b.i.d. fixed-dose 12-week triple therapy, Ximency®, was able to capture GT1a patients in the Japanese market and deliver a 93% SVR12 in naïve patients [114].

The three-drug combination paritaprevir (NS3/4 protease inhibitor) /r (ritonavir boosting), ombitasvir (NS5A inhibitor), and dasabuvir (NS5B non-nucleoside inhibitor) branded as Viekira Pak® or Technivie® was a multi-pill regimen that delivered a 96% SVR12 after 12 weeks of therapy in GT1 patients [115, 221]. In 2016, the DAA combination of elbasvir (NS5A inhibitor) and grazoprevir (NS3/4 protease inhibitor) was launched as Zepatier® [222]. In a Phase III study, this combination delivered an overall SVR rate of 95% against a GT1, 4, and 6 patient population but suffered from lower SVR12 rates in patients with NS5A resistance-associated substitutions (RASs).

The first true pan-genotypic regimen, Mavyret®, that lacked the use of sofosbuvir combined the NS5A inhibitor pibrentasvir (Figure 2.18 and Table 2.12) and the NS3/4 protease inhibitor glecaprevir (Figure 2.14 and Table 2.10) [221]. This DAA combination delivered a 99% SVR12 in GT1 patients with either an 8- or 12-week course of therapy. In addition, SVR12 rates in GT2-7 non-cirrhotic patients ranged from 93 to 98% for an 8-week course of therapy.

Further, Phase III and post-approval studies with a number of these DAA combination regimens expanded the patient population that could benefit from these curative therapies. It became possible to now effectively cure difficult-to-treat patient populations such as cirrhotics, HIV and HBV coinfected patients, difficult-to-treat ethnicities, liver transplant patients, and pediatric patient populations [223–232].

With short duration therapies that delivered reliably high cure rates with minimal side effects, it became possible to consider the idea of transplanting organs from HCV-infected patients, thus expanding the pool of donors. Thus livers, kidneys, and hearts from HCV-infected donors can now be transplanted and the recipient successfully treated with a short course of curative therapy [229, 233, 234].

As the HCV cure field matured, the dominant therapies were characterized by q.d. dosing, pangenotypic coverage, high barrier to resistance, and fixed-dose regimens. That resulted in Harvoni®, Epclusa®, and Mavyret® being the dominant first-line cure therapies. Each of these regimens utilized a NS5A inhibitor that complements other agents in the cocktail. For Harvoni® and Epclusa®, the nucleotide sofosbuvir provided the backbone with the high barrier to resistance and pangenotypic coverage necessary to protect for NS5A resistance-associated variants (RAVs). By using both a potent pangenotypic NS5A inhibitor and a potent pangenotypic NS3/4A protease inhibitor, cross protection for RAVs is accomplished in Mavyret®.

2.8 Conclusion

In a span of 25 years, the virus that causes a debilitating and deadly liver disease was identified and oral, safe, effective, and short duration curative therapies were delivered to patients. This achievement is the first example of a chronic viral disease being cured and a story that is unparalleled in modern medicine. Today, millions of patients now live normal productive lives without HCV thanks to these new drugs. However, this accomplishment was not easy and required intense commitment from researchers and the availability of significant resources. Analysis showed that the success rate in HCV drug development was only 2%, less than half of the industry average for successful drug development, thus putting into perspective the magnitude of the challenge and the achievement [235]. It was because of this achievement that the World Health Organization (WHO) set the goal of eliminating HCV by the year 2030 [1]. Many countries around the world have established HCV elimination programs and are beginning to make measurable progress against the WHO goal.

Acknowledgment

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3

Antiviral Drugs Against Influenza Virus

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3.1 The Influenza Virus

Influenza virus is a negative sense, segmented RNA virus that causes severe respiratory disease and up to 650 000 deaths annually worldwide [1]. Two types are responsible for major disease burden: while influenza A virus causes most influenza cases and causes pandemics, influenza B virus is a major source of morbidity and mortality in influenza during interpandemic periods [2]. Seasonal influenza epidemics alone affect up to 9 million people in the United States resulting in 12 000–56 000 deaths annually and in a high economic burden with an estimated average of $11.2 billion annually [1, 3]. In addition to the seasonal epidemic influenza, worldwide pandemic outbreaks emerge at unpredictable intervals causing an enormous increase in morbidity and mortality and consequently an increase of the negative economic impact [4]. Thus, developing safe and effective strategies for prevention, prophylaxis, and treatment against influenza is crucial.

Influenza virus harbors three surface proteins, the hemagglutinin (HA), the neuraminidase (NA), and the matrix 2 (M2) protein. By phylogenic similarity, the HA proteins are divided into two groups. For cell attachment and entry, the HA binds to terminal Neu5Ac a(2,3)- and Neu5Ac a(2,6)-Gal linkages of sialic acid on the cell surface [5]. Activation of the M2 proton channel is then induced by lowering the pH of the endosome. This leads to an acid environment to the virus and triggers the release of the viral ribonucleoprotein (RNP). Replication requires the viral polymerase complex, which is highly conserved between different strains. This heterotrimer is composed of three protein subunits: polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA) [6]. The influenza A and B virions contain eight viral ribonucleoprotein (vRNP) segments, which have a double-helical hairpin structure. Each is carrying one polymerase heterotrimer and is stabilized by viral NP molecules [6–8]. Viral RNA synthesis occurs in the cell nucleus. After replication, new virions are released by budding. The NA of the ensembled virions cleaves sialic acids from the host cell receptors and from the glycosylated viral HA and NA proteins to prevent self-aggregation and to support the release of progeny virions from the cell surface [9].

New Drug Development for Known and Emerging Viruses, First Edition.
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Vaccines represent the first choice to protect against the burden of influenza [10]. However, due to antigenic drifts and occasional antigenic shifts due to exchange of RNP segments the virus is able to escape the pre-existing immunity [11]. This results in reduced efficacies of the vaccines and limits their usage. Thus, continuous adaptions of the vaccines to the current influenza virus strains and consequently annual immunization are needed. Antigenic shifts can result in influenza virus strains that cause pandemics [12]. In such a pandemic situation, the available vaccines are completely ineffective, and it takes several months after the emergence and spread of a pandemic influenza virus until a new vaccine, effective against the new emerging virus, is available [13]. Moreover, only roughly 50% of the vaccinated individuals develop an optimal immune response, but especially in younger children and the elderly, who are at risk of severe influenza, the immune response to the virus is suboptimal. Also, the influenza vaccination coverage is only about 50% of the population. This shows a second problem, which limits the prevention of influenza by vaccination [10]. Thus, because of these limitations of the current vaccines to protect during seasonal influenza, antivirals play an important role for the treatment of the disease. Furthermore, in a pandemic situation, virus-specific vaccine is unlikely to be available until after many months; thus in the early phase of a pandemic, antivirals are needed to control it.

Today, the standard of care drugs are neuraminidase inhibitors (NAIs), which show limitations regarding the treatment window for clinical effectiveness and the emergence of NAI-resistant influenza virus strains. Several attempts have been made in recent years to develop new compounds directed against influenza viruses to overcome the limitations of NAIs. Among these preparations, only baloxavir marboxil (BAM) and favipiravir (FP), both polymerase inhibitors, have reached the market (the first in Japan and the United States, and the second only in Japan). Other antiviral agents and monoclonal antibodies are in advanced stages of development, but, at present, none of these new drugs and monoclonal antibodies have satisfactory characteristics to substitute for NAIs. However, a completely new strategy in the development of antivirals has reached the clinical development stage. In contrast to target the virus directly, cellular factors were identified which are needed by the virus to ensure its propagation. Consequently, inhibiting these cellular factors leads to an inhibition of the formation of new progeny virus.

Given the inherent limitations of the currently licensed drugs against influenza, there is still an urgent need for developing new anti-influenza drugs. The focus of novel drug development should aim at the following characteristics: (i) effective, when start of treatment is late (>48 hours), (ii) low susceptibility for developing resistant virus variants, (iii) broad range of activity (influenza A and B virus), (iv) improved effectiveness compared to the standard of care (at the present: NAIs), suitable for uncomplicated as well as complicated (severe) cases of influenza [14, 15]. This chapter provides an overview of antiviral drugs against influenza that have either been licensed in at least one country or are at least under phase 2 clinical development.

3.2 The Pathogenesis of Influenza

The transmission of influenza virus occurs primarily through droplets when sneezing or coughing. In addition, it is discussed whether droplets generated during normal breathing

or speech will also contain infectious influenza virus. Furthermore, transmission occurs through direct contact, for example, by shaking hands [16].

Influenza virus infects epithelial cells of the upper respiratory tract, which represent the primary compartment of virus propagation. In a second step, the virus can spread to the distal respiratory tract to cause severe disease like pneumonia. Not the high viral load but rather an excessive persistent immune response is the determinant of a severe pneumonia with respiratory failure [17]. Lung failure or a high severity of the disease is associated with a strong activation or recruitment of monocytes/macrophages and neutrophils [18–21]. These cells are the main source for the secretion of cytokines and chemokines, which can lead to an overwhelming cytokine/chemokine response, a hypercytokinemia also known as "cytokine storm" (Figure 3.1) [22, 23].

Influenza is associated with a wide range of clinical symptoms, varying from courses with mild signs of a cold to severe pneumonia requiring ventilation. After influenza virus infection and an incubation period of one to two days, the onset of symptoms often starts rapidly with high fever and chills. Signs and symptoms like loss of appetite, dry cough, sore throat, headache, myalgia, and arthralgia are often associated with influenza. Dyspnea and pronounced exhaustion are signs for a pneumonia. Depending on the age group, the disease can develop differently. While adults often show only a slight increase in temperature after infection with a particular influenza virus strain, the same strain can lead to abdominal pain, diarrhea, and vomiting in young children [24]. Even before the onset of first clinical symptoms, the infected person can spread the virus. In immune-competent patients, this infectivity lasts for about four to five days after the onset of symptoms. In

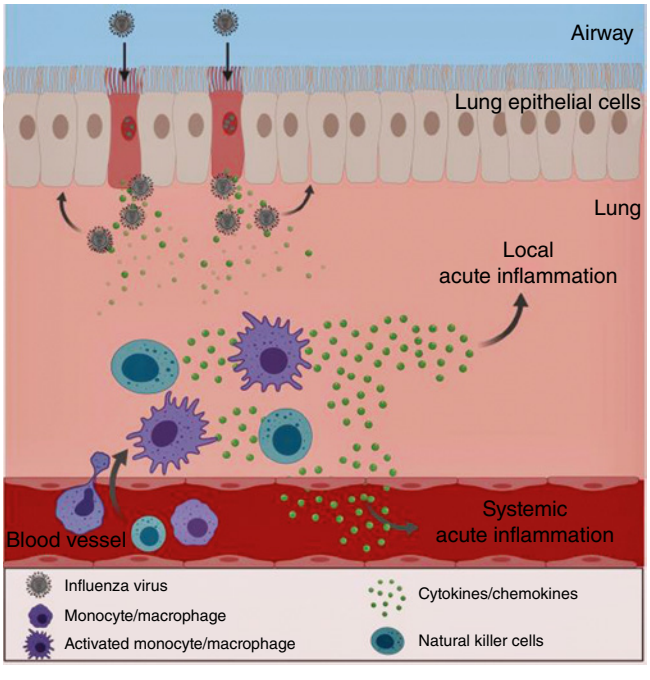


Figure 3.1 Mechanism of acute inflammation induction after influenza virus infection.

immunocompromised patients, the infectivity can last up to more than 30 days [25, 26]. Usually in an uncomplicated influenza, the symptoms disappear spontaneously within a week. The most common complication of an influenza infection is the influenza-associated pneumonia, both as primary viral pneumonia but also as a secondary bacterial coinfection [27]. The mortality risk increases with a bacterial coinfection [28]. Next to bacterial coinfections, pulmonary aspergillosis (fungal infection from the species *Aspergillus*) is another complication in influenza that is accompanied with a high mortality rate [29].

3.3 Influenza Drugs and Targets

In 2020, three major classes of antivirals were licensed for the treatment and prophylaxis of influenza: the M2 ion channel blockers (adamantanes), the NAIs, and the viral polymerase inhibitors, which have been recently progressed through late-stage clinical trials. Two of them (BAM and FP) have been licensed in Japan and BAM also in the United States. Table 3.1 provides an overview of the licensed antiviral drugs available to treat influenza.

Antiviral drugs have been developed for a long time to reduce the influenza-related risks and to overcome the challenges and problems caused by vaccines. The adamantane derivatives (rimantadine and amantadine) *a polymerase inhibitor (Baloxavir Marboxil)* and the

Table 3.1 Current licensed drugs against influenza.

Antiviral drug	Target	Route of administration	Treatment Prophylactic	Therapeutic	Effectiveness
Amantadine/ Rimantadine Symmetrel®/ Flumadine®	M2	Oral	100 mg twice daily	100 mg twice daily	Influenza A virus
Zanamivir Relenza®	Neuraminidase	Inhalative	10 mg once daily up to 28 days	10 mg twice daily	Influenza A virus Influenza B virus
Oseltamivir Tamiflu®	Neuraminidase	Oral	75 mg once daily up to six weeks	75 mg twice daily	Influenza A virus Influenza B virus
Peramivir Rapivap®	Neuraminidase	Intravenous		600 mg single dose	Influenza A virus Influenza B virus
Laninamivir Inavir®	Neuraminidase	Inhalative		20–40 mg single dose	Influenza A virus Influenza B virus
Baloxavir Marboxil Xofluza®	Polymerase	Oral	40–80 mg single dose	40–80 mg single dose	Influenza A virus Influenza B virus
Favipiravir Avigan®	Polymerase	Oral		600–1600 mg twice daily	Influenza A virus Influenza B virus

NAIs (oseltamivir, zanamivir) have been the only drugs licensed worldwide for influenza treatment and prevention. In recent years, laninamivir and peramivir were licensed in Japan, China, Japan, South Korea, and the United States. All these NAIs differ in their pharmacokinetic (PK) characteristics, routes of administration, and the age of the targeted patients [10, 30]. However, there exist limitations of NAIs in their efficacy to treat influenza. Furthermore these NAIs, mainly oseltamivir, are under discussion in terms of the risk of emergence of resistant strains and doubts regarding their efficacy in severe influenza cases, prevention of complications caused by bacterial coinfections or influenza in high-risk patients, like influenza virus-induced exacerbation of COPD [31–33]. The development of the third class of antivirals against influenza, namely the polymerase inhibitors, was aimed to overcome these limitations. BAM is the first polymerase inhibitor that was approved for uncomplicated influenza and for the treatment of influenza in high-risk patients. However, until now clinical trials have excluded hospitalized patients with severe influenza [34]. The use of BAM has already been associated with emergence of resistant influenza virus variants [35, 36].

Thus, several new approaches and strategies have been attempted to achieve influenza prevention and control. Monoclonal antibodies against the highly conserved stem region of the HA molecule of influenza A viruses and drugs that target different stages of the influenza virus life cycle in the infected human cells have been developed and tested [37]. Moreover, drugs that interfere with host cell factors are in development [38, 39]. Up to date, none of these new drugs or monoclonal antibodies that are in development has adequate characteristics to be superior over NAIs. Especially, oseltamivir remains the drug of choice for influenza treatment, even though the limitations of the drug are obvious [40].

3.4 Adamantanes and Derivatives

Influenza A virus encodes an integral membrane protein, M2 that forms a proton channel, which is required for viral replication [41]. The first anti-influenza compounds licensed were the adamantane derivatives rimantadine and amantadine, which inhibit viral propagation by blocking the proton conductivity of the M2 ion channel and preventing the migration of the vRNP complex into the nucleus of the host cell [15, 30, 42]. However, amantadine and rimantadine act only on the M2 ion channel of influenza A viruses, since the M2 protein of influenza B viruses is structurally different reducing the sensitivity to the M2 inhibitors [43, 44]. M2 inhibitors bind the viral matrix2 protein on two potential sites. One is located in the ion channel pore and has a high affinity for the inhibitor, while the second is located on the lipid face of the pore and shows a low-affinity for the inhibitor [45, 46]. M2 inhibitors have been around for almost 50 years. Their synthesis is cheap but their use for the treatment of influenza has been limited (Figure 3.2) [47].

This is partially due to the fact that amantadine- and rimantadine-resistant viruses emerge rapidly in treated patients, and in a single passage in tissue culture [48, 49]. Already in the 1980s, amantadine- and rimantadine-resistant influenza A virus strains were found [50]. Notably, the resistant virus variants showed no decline in replicative fitness and transmissibility [51, 52]. The ion channel pore harbors the two most common mutations (V27A and

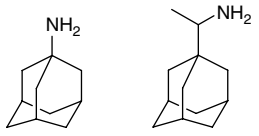


Figure 3.2 Structure of amantadine and rimantadine.

S31N), confirming this as the pharmacologically important site [46]. The pandemic A(H1N1) pdm09 virus was already resistant shortly after appearance in 2009 [53] and amantadine-resistant A(H3N2) viruses retaining a S31N substitution in the M2 protein spread globally during the 2000s [54, 55]. Many of the circulating avian Influenza A(H5N1) strains, which have led to "birdflu" in Southeast Asia, also developed resistance to M2 inhibitors [56, 57]. The levels of resistance to amantanes among circulating influenza A(H3N2) and influenza A(H1N1)pdm09 viruses is >99%. Moreover, adamantanes show poor tolerability [58]. Thus, since the 2004–2005 influenza season, the use of adamantane derivatives for antiviral treatment or chemoprophylaxis of currently circulating influenza A viruses was no longer recommended by the Centers for Disease Control and Prevention (CDC) [10, 59].

3.5 Neuraminidase Inhibitors

The recommended antiviral drugs for influenza prevention and treatment are NAIs [60]. The development of NAI started in the early 1970s with derivatives of 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (DANA) (Figure 3.3), which were known to be weak inhibitors of NA [61, 62].

A single substitution of the C4-OH with a 4-guanidino group, which led to zanamivir, enhanced binding by more than 10 000-fold over DANA [61, 63]. It was not until 1999 that zanamivir was approved as first in class (July 1999) followed by oseltamivir (October 1999) for use in humans [61, 64, 65]. The NAI bind to the NA of the virus and prevent the budding of influenza viruses from the host cell by inhibiting the enzymatic cleavage (which would facilitate the release of newly formed virions from the cell) and reduce the spread of infection in the respiratory tract [30, 40, 61, 66–68]. Based on the results of several clinical trials, NAIs have been considered efficient for treatment and for prevention of influenza virus infection [69, 70]. Four different NAIs are licensed: oseltamivir and zanamivir are approved globally for the treatment and the prevention of influenza, peramivir is licensed in various countries, while laninamivir was only approved in Japan [10].

NAIs have been found to be relatively safe and well tolerated. In particular, oseltamivir, which is the most frequently used NAI, is not only licensed for the use by otherwise healthy adults as well as subjects with severe underlying disease but also in neonates, younger infants, and pregnant women. Here, doses vary and are dependent on age and weight [69, 70].

One of the challenges of NAIs is that, in order to be effective, treatment should start within 48 hours postexposure, while first clinical symptoms will appear between 24 and 36 hours after initial infection. Since influenza virus replication is fast during the time of incubation, the effectivity of NAIs depends on the timing of the antiviral intake. Oseltamivir

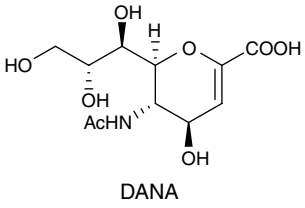


Figure 3.3 Structure of DANA.

reduced viral shedding and shortened the time to symptom alleviation by up to 1.5 days, when treatment started during the first 48 hours postinfection [71, 72].

The second challenge is the emergence of influenza virus strains that are resistant to NAIs. In this regard, most available studies were carried out with oseltamivir and zanamivir, which were approved before the other NAIs [40, 71]. The most dominant mutation which confers reduced susceptibility to both oseltamivir and peramivir is a H274Y substitution in the NA [73]. Until to date, only influenza A(H1N1) viruses harboring this mutation have circulated widely at the community level [74]. Moreover, different NA amino acid substitutions for influenza A and B viruses were found leading to a reduced sensitivity of peramivir, zanamivir, and laninamivir, but these are rarely detected and some are cell culture-only-derived substitutions [75].

The effectiveness of NAIs has been documented in meta-analyses, including those by Dobson et al. [71] and the Cochrane Report by Jefferson et al. [40]. They concluded that NAIs are suitable to limit the severity and duration of an uncomplicated influenza in patients of any age. However, the efficacy depends on the start of treatment within the first 48 hours of symptom onset. Unfortunately, there are no firm conclusions available regarding the effect of NAIs on severe influenza [10]. Here, the present results are even conflicting [40, 76]. The other NAIs, like laninamivir and peramivir, do not seem to have substantially different efficacies compared to those of oseltamivir or zanamivir. Thus, NAIs cannot be considered completely satisfactory for the treatment of influenza and the development of new drugs against influenza viruses has been considered mandatory [10].

3.5.1 Oseltamivir

Oseltamivir phosphate is a prodrug indicated for oral administration. Once in the gastrointestinal tract, the prodrug is rapidly cleaved to the active metabolite, oseltamivir carboxylate (Figure 3.4) [77].

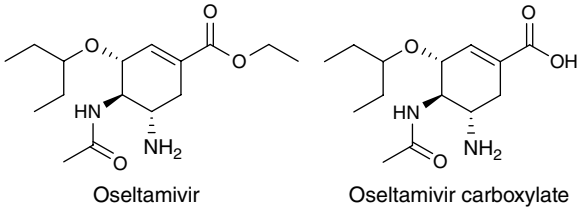


Figure 3.4 Structure of the oseltamivir phosphate (prodrug) and oseltamivir carboxylate (active metabolite).

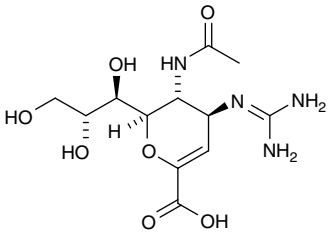
Oseltamivir was approved in 2000, shortly after zanamivir and is the most commonly prescribed NAI globally. Oseltamivir is considered the standard of care in many countries for the treatment of hospitalized influenza cases [78]. A large number of preclinical studies were conducted to investigate the efficacy of oseltamivir in cell culture and in animal models. Influenza A/H3N2 viruses are slightly more sensitive to oseltamivir than Influenza A/H1N1 virus subtypes, whereas oseltamivir demonstrated a 10–20-fold lower efficacy for Influenza B viruses compared with influenza A viruses [61, 79]. For the treatment of adults, oseltamivir is taken orally twice daily, with a dose of 75 mg (Table 3.1).

In various randomized controlled trials, which were mainly conducted in otherwise healthy adults, it was shown that oseltamivir shortens the duration of symptoms in uncomplicated influenza by approximately 1 day, if treatment was initiated within 48 hours of symptom onset [40, 71, 80]. Next to studies in uncomplicated influenza, observational studies in severely ill or hospitalized influenza patients indicated that treatment reduces the risk of pneumonia and mortality [71, 81, 82]. All of these studies demonstrated that the start of treatment is crucial and the greatest benefit is seen when start of treatment was as early as possible after symptom onset [81]. Prophylactic administration of oseltamivir, for as long as 10 days to 6 weeks demonstrated 68–90% efficacy in preventing infection during an influenza activity period [31, 83]. Nevertheless, to-date, the role of oseltamivir in reducing uncomplicated influenza symptoms and severe complications are not entirely clear, since reports with contradictory results exist. These reports were summarized in different meta-analyses like the Cochrane report in 2014. Here, it was stated that the administration of oseltamivir in adults with uncomplicated influenza decreased the time to first alleviation of symptoms by only 16.8–25.2 hours [40, 71].

Oseltamivir-resistant viruses can emerge either in the course of influenza virus evolution (naturally) or under drug-selection force (with drug intervention). The immune status of the patient and the severity of illness can also affect the frequency of resistance. Here, higher rates are often observed in hospitalized children and immunocompromised patients in whom virus replication is prolonged [84–86]. The frequency of virus resistance to oseltamivir is lower than that seen with adamantanes [74]. In the 2007–2008 and 2008–2009 influenza seasons, roughly 90% of the circulating influenza A H1N1 strains developed resistance to oseltamivir [87–89]. In contrast, the 2009 pandemic strain A H1N1pdm09, which is circulating since then, rarely (<1–3%) contained the mutations in the NA leading to resistance to oseltamivir [10, 75]. Many amino acid substitutions in the NA confer reduced susceptibility to oseltamivir [33, 75]. The most frequent amino acid substitutions in influenza A viruses of N1 NA subtypes are H274Y and N294S [90]. E119V and R292K mutations in the NA are most frequent in influenza viruses of the N2 subtypes, while influenza B viruses harbor R152K and D198N amino acid substitutions in the NA [10, 91, 92]. There is no cross-resistance among NAIs. Most of these substitutions leading to reduced susceptibility to oseltamivir do not influence the sensitivity of other NAIs. Patients infected with an oseltamivir-resistant strain can be treated successfully with zanamivir [93].

3.5.2 Zanamivir

Zanamivir was developed as GG167 (4-guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuraminic acid) in the 1990s as a novel inhibitor of the influenza virus NA (Figure 3.5) [94–96].



Zanamivir

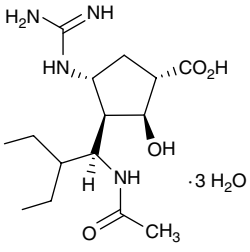
Figure 3.5 Chemical structure of zanamivir.

It exhibits a higher affinity for the NA binding site than the cellular sialic acid and therefore blocks the NA–sialic acid interaction [61]. In 1999, zanamivir was the first NAI approved for use in adults and children >7 years of age for the treatment and prevention of uncomplicated influenza. For treatment, a 10 mg dose is inhaled twice daily for 5 days, whereas for prevention, zanamivir is given once daily for 10–28 days (Table 3.1) [83]. In preclinical studies, zanamivir has demonstrated efficacy against influenza A and B virus strains including avian influenza virus strains (H5N1) in cell culture and in animal models [68, 94, 95, 97]. Various studies demonstrated that zanamivir has a higher activity against influenza B and H1N1 strains than oseltamivir, but oseltamivir is more active against H3N2 [83]. Due to its polarity, zanamivir is not well absorbed resulting in a poor oral bioavailability. Thus, zanamivir is administered as an inhaled powder [30]. Inhaled zanamivir shows a protective efficacy of 68% for seasonal prophylaxis in healthy adults [98]. Furthermore, inhaled zanamivir is effective in seasonal prophylaxis in at-risk adolescents and adults, but in older people, this effect is not significant [99]. In children, inhaled zanamivir had no significant effect on the reduction in duration of clinical symptoms [40, 100]. Inhaled zanamivir has not been found to reduce pneumonia complications during severe influenza [40]. In patients with pulmonary disease, zanamivir can precipitate bronchospasm and therefore, it is not suitable to treat patients who require mechanical ventilation [101]. Postexposure prophylaxis using inhaled zanamivir was effective in preventing transmission to adults and children [102]. Zanamivir is also available in an intravenous (IV) form for compassionate use and is currently undergoing clinical trials to determine optimal dosing [83]. IV zanamivir showed a similar safety profile as oseltamivir, but the time to clinical response was not superior to oseltamivir [103].

The frequency of resistance to zanamivir has constantly remained low, most probably due to its infrequent use and its closer homology to the natural sialic acid substrate [66]. Of note, zanamivir remains active against influenza strains that contain the H274Y NA mutation, leading to reduced susceptibility of oseltamivir and peramivir [101]. Nevertheless, since 2009, there are increased reports of oseltamivir/zanamivir-resistant A(H1N1)pdm09 and H3N2 isolates [61, 92].

3.5.3 Peramivir

Peramivir, a novel cyclopentane and structurally distinct from other NAIs, was developed as BCX-1812 and RWJ-27020 and was approved by the Food and Drug Administration in December 2014 (Figure 3.6) [104].



Peramivir

Figure 3.6 Chemical structure of peramivir.

Compared to other NAIs, peramivir shows structural substitutions, which support a tighter interaction between peramivir and the NA active site and leads to a slower dissociation rate ($t_{1/2}$ > 24 hours) when compared to oseltamivir or zanamivir ($t_{1/2}$ = 1.25 hours) [105, 106]. Peramivir is a highly selective inhibitor of influenza A and B virus NAs and an effective inhibitor of various influenza A and B virus strains including those with pandemic potential [107]. *In vitro*, the inhibitory potency for influenza B virus is higher than that for influenza A virus. It also appears to be greater than that of either zanamivir or oseltamivir due to the tighter binding of peramivir to the NA [108, 109].

In mice and ferrets infected with influenza A virus and in influenza B virus-infected mice, peramivir was highly effective in viral load reduction, preventing death and limiting disease symptoms. Peramivir was still effective in mice when therapy began 24 hours after virus infection. Preclinical pharmacology revealed that peramivir was non-toxic in mice and rats at doses of ≥1000 mg/kg/day and ≥100 mg/kg/day in ferrets [110]. Even though peramivir demonstrated a solid efficacy in human challenge studies after oral application, human clinical trials revealed a low oral bioavailability and thus low efficacy [111]. Peramivir has been approved for IV administration only. Due to high plasma and respiratory tissue concentrations, it is administered as a single-dose infusion. Thus, peramivir provides a valuable therapeutic alternative for critically ill patients or those unable to take medication via the oral or inhalative routes [112]. Several clinical trials showed that peramivir is well tolerated in adult and pediatric populations. Only mild-to-moderate adverse events were found. Common side effects include gastrointestinal disorders, electrocardiographic (ECG) abnormalities, and decreased neutrophil counts. Compared to oseltamivir, zanamivir, and other NAIs, the antiviral efficacy of peramivir is slightly lower in humans [101]. A large variety of clinical studies were performed to demonstrate the antiviral efficacy of peramivir after IV administration [112–115]. In a phase 2 trial, a significant reduction of time to alleviation of symptoms and significant viral load reduction compared to placebo was demonstrated, when peramivir was administered at doses of 300 and 600 mg, respectively [113].

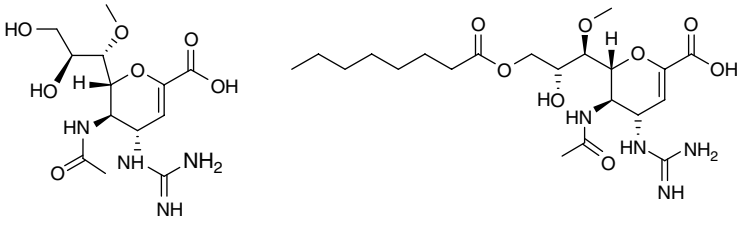
Development of resistance to peramivir among seasonal influenza A/H1N1pdm09, influenza A/H3N2 and B viruses is low (1–3%) with a higher tendency for influenza A/H1N1pdm09 viruses [116–118]. The most commonly detected H274Y framework

mutation in A/H1N1pdm09 viruses conferring resistance to oseltamivir also results in cross-resistance to peramivir but not to zanamivir. This is due to the fact that resistant variants with mutations in the enzyme framework can retain susceptibility to other NAIs, while those in the functional or catalytic residues tend to cause cross-resistance to most of the others NAIs [119]. The H274Y mutation has been shown to rapidly emerge upon IV treatment with peramivir in immunocompromised or hematopoietic cell-transplanted recipients [120].

3.5.4 Laninamivir

Laninamivir octanoate, which was developed as CS-8958, is the octanoyl ester prodrug of laninamivir (Figure 3.7).

Like zanamivir, laninamivir is an inhaled NAI, but currently approved for the treatment and prevention of influenza A and B infection only in Japan [121]. The laninamivir octanoate prodrug is converted to the active form in the respiratory tract [122]. This allows to treat influenza following a single inhaled dose. Laninamivir exhibits NA inhibitory activity against influenza A and B viruses, including the 2009 pandemic H1N1 (H1N1pdm09) viruses and has good activity against strains that contain the H274Y NA mutation [123–125]. Laninamivir octanoate has long-lasting antiviral activities, due to its long half-life and its high concentration within lung tissues [121]. Preclinical studies in mice demonstrated that the efficacy of a single administration of laninamivir octanoate was comparable to the multiple treatment regimen of zanamivir or oseltamivir [125, 126]. In humans, laninamivir showed a slow elimination lasting for up to 6 days after a single inhalation [125]. For treatment, laninamivir is approved as a single inhalation dose of 40 mg for individuals ≥10 years of age and 20 mg for children (Table 3.1). For prevention, a single inhalation of 20 mg daily for two days is recommended in adults and children and was associated with roughly 75% protective efficacy. The efficacy of laninamivir is comparable to the other approved NAIs. Like zanamivir, the structure of laninamivir is most similar to the natural target, and therefore resistance is seen less frequently, because it would lead to greater changes in the virus, which could lead to decreased fitness [61, 127]. Clinical studies found lower rates of adverse events when compared with oseltamivir. Unlike zanamivir, laninamivir was not associated with bronchospasm [128].



Laninamivir Laninamivir octanoate

Figure 3.7 Chemical structures of laninamivir (active metabolite) and laninamivir octanoate (prodrug).

3.5.5 Summary

NAIs represent a valuable drug class for treatment and prevention of influenza. They are considered as standard of care treatment against influenza. Unfortunately, the antiviral efficacy is controversially discussed due to the narrow time window for treatment and the appearance of drug-induced resistant influenza virus variants. This emphasizes the need to evaluate the potential benefits of combinations of NAIs in reducing the rates of resistance and to improve treatment efficacy. Furthermore, this also shows that there is a need to develop drugs that have different targets than the NA.

3.6 Polymerase-Inhibitors

The structure and function of the influenza virus polymerase complex has been described in detail [6, 7, 129, 130]. It is a heterotrimer composed of three protein subunits: PB1, PB2, and PA. Viral RNAs are imported into the nucleus where they are transcribed to mRNA. Here, PB2 is involved in the import of viral RNA into the nucleus and catalyzes the replication and transcription [131, 132]. A PA-dependent "capsnatching" reaction on cellular-capped RNAs is required to generate primers for the primary transcription of vRNA to mRNA [133, 134]. The elongation of the viral RNA is performed by the RNA-dependent RNA polymerase, PB1 [6, 74, 135–137].

The influenza polymerase complex is highly conserved and plays an essential role in the influenza virus replication cycle [6]. This renders the polymerase complex an attractive target for the development of small molecule inhibitors as new antiviral compounds [6, 74]. There is great hope that this group of antivirals against influenza will help to overcome the limitations found with NAIs and to increase the repertoire of antiviral options in particular for an effective treatment of severe influenza (e.g. in hospitalized patients), but also for treatment to start >48 hours post-symptom onset and with a lower tendency for resistance [6, 74]. However, as monotherapy, both licensed polymerase-inhibitors are associated with the frequent emergence of variants with reduced susceptibility. Preliminary data suggest that combination therapy with NAIs limit the emergence of antiviral-resistant strains. This represents an attractive approach for future studies:

3.6.1 Baloxavir Marboxil (BAM)

In vitro studies have demonstrated antiviral activity without cytotoxicity even in cells infected with various influenza virus strains including avian subtypes and NAI-resistant influenza viruses [138]. Mice infected with influenza virus were protected from clinical signs and mortality even when treatment started four days postinfection. Moreover, a combination of oseltamivir with a subtherapeutic dose of BAM protected mice from infection and mortality [139]. The *in vitro* susceptibility profiles are summarized by Hayden and Shindo [140].

Phase 1 clinical trials revealed that a dose escalation from 6 to 80 mg of BAM was well tolerated in healthy adults and no serious adverse events or deaths were reported [141]. Treatment-emergent adverse events included headache, increased alanine aminotransferase and

aspartate aminotransferase levels, elevated eosinophil count and white blood cell count, which were present in 8 of 55 BAM-exposed individuals. These adverse events were mild and resolved spontaneously within a few days [141]. In phase 2 and 3 clinical trials involving outpatients aged 12–64 years with symptoms of uncomplicated influenza-like illness, BAM was also well tolerated including in high-risk patients with comorbidities such as asthma or chronic lung disease [141, 142]. Adverse events were reported in ~2% of the patients and were not considered severe [10]. A recent update to BAM precautions in Japan stated that bloody stool, epistaxis, hematuria, or other forms of bleeding might occur [74]. In phase 2 and 3 clinical trials including the CAPSTONE 1 (phase 3) clinical study, different amounts of BAM treatment reduced the time to alleviation of symptoms in predominantly A(H1N1)pdm09 and A(H3N2) infected patients from 28.2 to 23.5 hours when compared to placebo [142].

BAM, also known as S-033188, was licensed in 2018 in Japan and the United States as a first-in-class polymerase inhibitor for oral treatment of uncomplicated influenza in subjects aged ≥12 years with influenza manifestations for ≤48 hours [74]. In addition, in October 2019, the FDA approved BAM for the treatment of uncomplicated influenza in people that are at high risk of developing flu-related complications. BAM is a prodrug that is hydrolyzed in the intestine, blood, and liver to form baloxavir acid (BXA), which is the active compound to inhibit cap-dependent endonuclease (Figure 3.8) [143]. This prevents the initiation of mRNA synthesis of the influenza virus [144]. The drug shows antiviral activity against several influenza A viruses, including oseltamivir-resistant viruses as well as B viruses [138].

PK data confirmed that due to a long plasma retention time (half-life of 49–91 hours), BAM could be delivered as a single dose in patients with uncomplicated influenza [141]. This avoids compliance issues for a possible combination therapy with NAIs like oseltamivir or inhaled zanamivir, which are administered twice daily for five days [74]. BXA concentrations were lower in subjects after a meal compared to the exposure measured before a meal, indicating that food influences the metabolism of the drug. Nevertheless, food uptake had no influence on BAX-mediated virus inhibition, because the BAX plasma concentration exceeded the target concentration to inhibit viral propagation, which was estimated in preclinical studies [141].

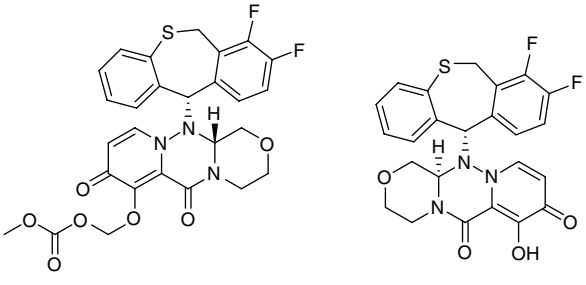


Figure 3.8 Chemical structures of baloxavir marboxil (prodrug) and its active metabolite baloxavir acid.

However, no significant difference in the clinical efficacy was observed in patients receiving oseltamivir or baloxavir although BAM administration showed greater impact on viral load reduction [142].

The CAPSTONE 2 trial (phase 3) in outpatients who were at increased risk of influenza complications due to comorbidities, again demonstrated no significant difference in the reduction of the time to improvement of influenza symptoms compared to oseltamivir treatment, although a slight difference was present [145]. However, this study demonstrated a significantly faster reduction of the time to improvement of influenza symptoms than placebo and oseltamivir, when patients were infected with influenza B viruses [145]. Another benefit of BAM in the CAPSTONE 2 study was the significantly lower use of antibiotics and the lower incidence of influenza-related complications in high-risk patients treated with baloxavir [145]. In the clinical trials, BAM resulted in a 100- to 1000-fold-reduction in viral titers [142]. Additional phase 3 clinical trials were conducted in 2019 to evaluate the efficacy of BAM treatment: in children between 1 and 12 years of age, in hospitalized patients with influenza and in a study to evaluate the prophylactic benefits of baloxavir [74].

In summary, the administration as a single oral dose and the antiviral efficacy against NAI-resistant virus strains are important advantages of BAM over other available anti-influenza virus drugs [138]. Even though oseltamivir is applied via the oral route as well, the other NAIs are either administered by inhalation (zanamivir and laninamivir) or via the IV route (peramivir). Despite these clear advantages, there are some challenges under debate for the usage of BAM in clinical practice as an alternative to NAIs. The costs of influenza treatment with BAM are roughly three times higher than the cost for oseltamivir treatment [146]. Another challenge for BAM is the emergence of variants resistant to the polymerase inhibitor due to mutations in the PA polymerase gene leading to an I38T and E23K exchange in the protein [147]. None of these mutations were detected in 2018/2019 prior to the time when BAM was licensed, thus they are likely drug-induced [35]. Both mutations, however, have been already encountered during the BAM clinical trials used to obtain the license [142]. Here, 2.2–9.7% of the patients shed mutant influenza virus and a pediatric study showed that mutant virus was found in roughly 20% of treated children [142, 144, 148]. The viruses, which were insensitive to BAM remained sensitive to NAIs and had an impaired replicative capacity *in vitro* [148]. Patients harboring mutant influenza virus shed the viruses and remained symptomatic for a longer period than patients infected with wild-type virus [142]. This clearly demonstrates a potential negative effect to BAM and up to now the routine use of BAM for treatment of uncomplicated influenza in adults is being debated [10, 35, 148–151].

Combination therapy of BAM with oseltamivir was examined in healthy subjects indicating that there was no significant drug–drug interaction in healthy subjects [141]. Studies investigating the effectiveness of oseltamivir and baloxavir combination therapy in humans, with influenza virus infections have recently been completed, results are being awaited. In influenza virus-infected mice, the combination therapy of oseltamivir/baloxavir delivered four days postinfection showed no significant clinical benefit compared to either oseltamivir or baloxavir monotherapy but showed a synergistic response with respect to reduction of viral load and lower pro-inflammatory cytokine (IL-6 and MCP-1) levels [139]. Further research and clinical studies will need to determine the potential of BAM as antiviral against uncomplicated influenza.

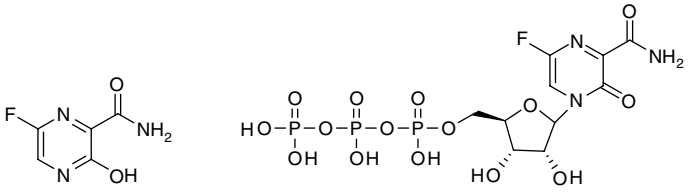
3.6.2 Favipiravir

FP also known as T-705, acts as a purine nucleoside analogue and therefore – like BAM – inhibits the activity of the RNA-dependent RNA polymerase directly [152, 153]. FP is a prodrug that after oral ingestion requires intracellular phosphoribosylation to be transformed into its active form, FP ribofuranosyl-5′-triphosphate (FRTP) (Figure 3.9) [10].

FP has a broad-spectrum antiviral activity, which is not limited to all influenza subtypes including those resistant to NA and M2 inhibitors [153]. FP is also able to inhibit virus propagation of several other RNA viruses, including West Nile virus, poliovirus, Ebola virus, and norovirus [10, 154–156]. Currently, FP has conditional marketing approval in Japan; restricted for the use to treatment of novel influenza viruses that are resistant to other available antivirals [153]. Favipiravir showed antiviral activity against SARS-CoV-2 (see Chapter 17). Favipiravir has also shown *in vitro* activity against SARS-CoV-2 and has been tested in several clinical studies. A meta-analysis revealed "a significant clinical improvement in the Favipiravir group versus the control group during seven days after hospitalization (RR = 1.24, 95% CI: 1.09–1.41; $P = 0.001$)." However, Favipiravir appeared not to exert a significant beneficial effect in terms of mortality in the general group of patients with mild to moderate COVID-19 (https://doi.org/10.1038/s41598-021-90551-6). The dosage for adults is 1600 mg administered orally twice daily on Day 1, followed by 600 mg orally twice daily from Day 2 to Day 5. The total treatment duration should be five days. A recent in vitro study showed that FP is primarily acting as a guanine and an adenine analogue; thus leading to mutations and that it consequently may harbor a risk for teratogenicity and embryotoxicity [153].

In vitro studies demonstrated a broad antiviral spectrum of FP against different influenza virus subtypes, including avian influenza viruses H5N1 and H7N9 and those that are insensitive or resistant to NAIs [84, 157, 158]. When treatment started 72 hours after infection, FP showed a significantly better outcome than oseltamivir in preventing influenza or reducing its severity in mice infected with lethal doses [158, 159]. Moreover, when used in combination with oseltamivir, protection due to the NAI was increased and the treatment efficacy window was extended to 96 hours after symptom onset [10, 160]. Combination therapy studies of FP with NAIs in mice infected with a pandemic H1N1 influenza virus or with an avian influenza virus H5N1 demonstrated that both drugs act synergistically in terms of viral load reduction, reduction of body weight loss, and in protection against a lethal challenge [161, 162].

Preclinical studies in different animal species showed that the safety and tolerability of FP was good, however, with the exception that some data revealed a teratogenicity potential of FP



Favipiravir T-705 RTP, the active form

Figure 3.9 Chemical structure of favipiravir and its active form.

[153, 163]. The dosage that caused teratogenicity in preclinical animal studies was similar to the dose of the drug that was required for influenza treatment in humans. Although *in vitro* studies have demonstrated the potential of FP to induce FP-resistant influenza virus variants, *in vivo* the use of FP was only rarely associated with mutations [153], in contrast to BAM.

A phase 1 clinical study to investigate the safety, tolerability, and pharmacokinetics (PKs) of FP showed that doses ranging from 30 to 1600 mg/kg were well tolerated and led to a rapid absorption and elimination via urine as T-705M1, the major metabolite and hydroxide of T-705. Moreover, no serious adverse incidents were reported [164]. FP-associated adverse events included diarrhea (6.3%), nausea (0.8%), vomiting (0.5%), and elevated uric acid levels in the blood (5.6%) [164].

Various phase 2 and 3 studies have been performed with FP to determine the efficacy and safety for treatment of uncomplicated influenza. The efficacy of FP to reduce the time to alleviation of symptoms varied in the different trials with patients suffering from uncomplicated influenza. A dose regimen of 1800 mg BID on day 1 and 800 mg BID on days 2–5 applied via the oral route in a phase 2 and two phase 3 trials, resulted in a reduced time to alleviation of symptoms of only 15.0, 14.2, and 6.1 hours compared to placebo-treated patients [74, 140]. FP treatment led to reduction of viral load. In patients treated with FP, a 10-fold lower viral load was measured on days 2 and 3 of the study compared to placebo-treated patients. Virus samples isolated from patients before and after treatment during the clinical trials demonstrated amino acid substitutions in proteins of the polymerase complex of post-treatment viruses. These substitutions had no influence on viral fitness. *In vitro* experiments gave no clear answers to the question on the potency of FP to induce mutations. In one study, no specific resistance mutations were found after FP treatment [165]. Another study demonstrated FP-induced mutations that led to reduced FP susceptibility [166, 167].

Preclinical combination therapy studies of FP with oseltamivir have been performed in mice. The combination therapy showed a synergistic effect of FP and oseltamivir monotherapy and also allowed a prolonged treatment window [160]. The combination therapy was also successful to protect immunocompromised mice from a lethal infection, but viruses could be detected that developed oseltamivir-resistance via the classical H274Y NA mutation. In one study, the viruses remained sensitive to oseltamivir [168], while in another study, FP did not suppress the emergence of oseltamivir-resistant variants [169].

Based on the rather modest efficacy of FP to reduce the time of disease (time to alleviation of symptoms), the ability of FP to induce resistant virus variants and the teratogenicity concerns, it appears unlikely that FP will be further developed for use in uncomplicated influenza [74].

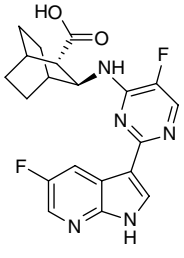
In the current COVID-19 pandemic (as of March 2021), FP has demonstrated antiviral activity against SARS-CoV-2 *in vitro* [170] and in an animal model [171] and is being investigated in several trials with different results. Benefit after FP treatment was shown in some studies in SARS-CoV-2 infected patients, while other trails demonstrated no advantage of FP treatment. Furthermore, there are contradictory statements on the dose justification, for FP use in patients infected with SARS-CoV-2 [172]. A meta-analysis revealed a significant clinical improvement in the Favipiravir group versus the control group during seven days after hospitalization (RR = 1.24, 95% CI: 1.09 –1.41; $P = 0.001$). However, Favipiravir appeared not to exert a significant beneficial effect in terms of mortality in the general group of patients with mild to moderate COVID-19 [173].

3.6.3 Pimodivir

Pimodivir, an oral drug, also known as JNJ-63623872 or VX-787, is a non-nucleoside polymerase inhibitor that inhibits viral gene transcription by preventing the PB2 subunit from binding the 7-methyl GTP cap structures on the host-capped RNA (Figure 3.10) [174, 175]. Pimodivir is metabolized by CYP 3A4, but has no effect on cytochrome P450 activity [140]. Due to structural differences in the PB2 cap-binding pocket, pimodivir is only effective against influenza A viruses. The antiviral activity can vary significantly depending on the influenza virus A subtypes [176], but it is inactive against influenza B viruses [6, 164]. Preclinical studies in cell culture and in mice have shown that pimodivir is effective against a diverse group of influenza A viruses including the H1N1pdm, H5N1, H7N9, and strains resistant to NAIs [177]. When pimodivir was given to mice as late as four days postinfection with H1N1pdm09 or H5N1, it was still able to protect against a lethal virus challenge [177]. Combination studies in mice using pimodivir and oseltamivir suggest a potential benefit of the combination [178].

In a phase 2a clinical challenge study of uncomplicated influenza, healthy volunteers were infected with an influenza A virus (H3N2). Four groups were treated once daily for a total of 5 days with different dose levels: 100 mg, 400 mg, loading dose 900/600 mg, and loading dose 1200/600 mg [178]. In the 1200/600 mg group of pimodivir-treated individuals, pimodivir significantly reduced viral shedding and clinical symptoms by 43.0 hours compared to placebo-treated individuals [178]. In this study, pimodivir was generally safe and well tolerated with no serious adverse events or adverse events [178].

A phase 2b clinical study, TOPAZ investigated the antiviral efficacy of pimodivir in influenza virus infected, but otherwise healthy patients suffering from uncomplicated influenza. Pimodivir 300 or 600 mg, or pimodivir 600 mg plus oseltamivir 75 mg was given twice daily for five consecutive days. Antiviral activity, safety, and PKs of pimodivir alone or in combination were evaluated [179]. Dose-related diarrhea (16.9%, 25/148) was the most commonly reported adverse event, with nausea (4.0%, 6/148) and vomiting (2.7%, 4/148) [179]. Viral load reduction was the primary endpoint of the study, demonstrating that 600 mg pimodivir given twice daily for five days was adequate to reduce viral load. Combination therapy (pimodivir/oseltamivir) was more effective than administration of pimodivir alone [179]. Pimodivir has recently received FDA Fast Track designation due to its potential to address an unmet medical need in those who develop influenza A infection



Pimodivir

Figure 3.10 Chemical structure of pimodivir.

complications [15]. Further clinical studies with pimodivir alone or in combination with oseltamivir in high-risk patients are ongoing [15].

The use of pimodivir in clinical studies has also been associated with the emergence of mutations in 11 of 172 patients (6.4%), resulting in virus variants with a several-fold decrease in susceptibility to the drug [179]. The mutated viruses did not affect the clinical conditions and the patients did not spread the mutated virus after treatment, still, this finding deserves attention [10]. The finding of pimodivir efficacy could not be confirmed in a hospital-based study, where pimodivir was combined with oseltamivir [140].

3.6.4 Summary

Next to NAIs, the polymerase-inhibitors represent a class of influenza antivirals, most of which show efficacy against influenza A and B virus strains. Problems and challenges found with NAIs seem to be also present with the polymerase-inhibitors: narrow treatment window, potential to resistance, and at present not approved for the treatment of severe influenza. Thus, new strategies are required.

3.7 Monoclonal Antibodies

As described above in detail, the current antivirals for influenza are suboptimal due to the limited therapeutic window and an increasing incidence of resistance [160, 180–182]. Consequently, new preventive and therapeutic strategies to tackle influenza are being sought. In this respect, monoclonal Abs (mAbs) got into focus. mAbs are highly specific and may have very limited off-target effects in line with a favorable safety profile [183–185]. They are in development for the treatment and prevention of various infectious diseases. One of these mAbs is palivizumab, which was licensed for the prevention of Respiratory Syncytial Virus (RSV) infection [186, 187]. The development of the technologies for the production of mAbs has also stimulated the use of novel mAbs-based therapies for influenza [188]. Studies of natural immune responses to influenza virus infection provided evidence that the HA is the main target of the postvaccination immune response. HA is composed of two domains: a immunodominant globular head (HA1), which varies between different strains and a stalk (HA2) that is relatively well-conserved between strains and subtypes [189]. Thus, a large number of mAbs are currently under development targeting the stem region of influenza A viruses since they should be effective against various influenza virus strains [190, 191]. Moreover, a non-neutralizing mAb, TCN032, targeting the viral matrix protein is in clinical trials as well. In a phase 2a challenge study, a single dose of TCN032 was found to be well tolerated [192]. In addition, patients with severe influenza A virus infection were treated with anti-influenza immune plasma in a phase 3 trial. However, the benefit was insufficient to justify the use of immune plasma for treating patients with severe influenza [193]. Thus, more specific antibodies are likely needed.

Three monoclonal antibodies, which have reached the phase 2 stage of development, MHAA4549A, MEDI8852, and VIS 410 will be described. Three other mAbs, CR626, CR8020, and CT-P27 [15], which are also in clinical development, will not be covered here, because of comparable results to the mAbs introduced in this chapter.

3.7.1 MHAA4549A

This is a human monoclonal IgG1 antibody targeting a highly conserved epitope on the stalk region of the HA [194]. The mAb was isolated by using an *in vivo* enrichment technique that identified neutralizing human antibodies. Antigen-specific plasma-cells (antibody-producing B cells) were identified, isolated, and enriched from human peripheral blood mononuclear cells (PBMCs). MHAA4549A was identified by screening only 840 human antibodies and has demonstrated a neutralizing activity against all tested seasonal human influenza A strains [195]. The antibody has two mechanisms of action. First, it binds to the stalk region of the HA and thus blocks the HA-mediated membrane fusion in the endosome. Second, MHAA4549A can recognize virus-infected cells and mediate antibody-dependent cell-mediated cytotoxicity. This leads to killing of the infected cells [194].

Preclinical studies demonstrated therapeutic efficacy in a mouse model against diverse influenza viruses even when administered 72 hours postinfection. Studies in ferrets demonstrated protection against an H5N1 virus challenge and synergism was observed in a combination treatment of MHAA4549A with oseltamivir at 48 hours postinfection [15, 195].

PKs of MHAA4549A were determined in mice and in cynomolgus monkeys using a single IV dose. The PK data in monkeys were used for projection of the human PK profile. Here, it was predicted that a single IV dose ranging from 15 to 45 mg/kg should achieve efficacious exposure in humans [196, 197]. Thus, clinical trials have been conducted to evaluate the efficacy of MHAA4549A but also safety and PKs. Moreover, studies were performed with MHAA4549A in combination with oseltamivir [194, 197, 198]. MHAA4549A was well tolerated with dose-proportional serum PKs, a long terminal half-life (21.9–24.6 days), and a slow clearance (152–240 ml/day) [198]. However, nasopharyngeal swab PKs were not dose proportional, which should guide future dose selection to achieve the high drug concentrations needed at the site of action for efficacy [194, 198]. MHAA4549A treatment significantly reduced viral loads in a human influenza virus challenge model [194]. In another clinical trial in adult participants hospitalized with severe influenza A, using a single IV dose of MHAA4549A in combination with oseltamivir indicated no advantage for any of the primary clinical outcomes evaluated and for the virologic outcomes evaluated when compared with the standard of care [15].

3.7.2 MEDI8852

MEDI8852 is a potent, broadly neutralizing investigational human IgG1 kappa mAb, derived from human memory B cells that also targets the HA stalk with a broad spectrum of activity by neutralizing all 16 influenza A HA subtypes [199] and lacking efficacy against influenza B. Preclinical studies in animal models have demonstrated efficacy for prophylaxis or therapy against influenza virus, including such with pandemic potential, when used alone or with oseltamivir. MEDI8852 treatment prevented death of mice and ferrets infected with a pandemic influenza virus strain, even when treatment was started up to three days postinfection. MEDI8852 treatment was also shown to be superior to oseltamivir [199]. Additionally, MEDI8852 blocked the transmission of influenza virus in a ferret model, a unique finding among influenza-specific mAbs [199, 200].

MEDI8852 was shown to be well tolerated in phase 1 clinical trials, when subjects received 250–3000 mg MEDI8852. The terminal half-life ranged from 19.4 to 22.6 days [201]. A phase 2a study was conducted to evaluate the safety of MEDI8852 in adults with acute, uncomplicated influenza. This study demonstrated an acceptable safety profile [202]. In 2016, MEDI8852 received Fast Track designation by the FDA that supports the drug for an advanced development and review [15, 203].

3.7.3 VIS410

VIS410 is a neutralizing IgG1 monoclonal antibody that is engineered like MHAA4549A/MEDI8852. Here, a novel approach called *atomic interaction network analysis* was used [204]. This method characterizes and targets functionally conserved epitopes within the influenza HA glycoprotein. In the present approach, it targets the stem region of influenza virus HA glycoprotein and has demonstrated binding to both groups HAs of influenza A viruses [205]. VIS410 is broadly active against various influenza A virus strains. In preclinical studies, VIS410 was effective at protecting mice from a challenge with a pandemic strain, when VIS410 was administered prophylactically, 12 hours prior to infection (50 mg/kg). In addition, a single therapeutic dose of 2, 10, or 50 mg/kg VIS-410 given 24 hours after infection protected almost 100% of mice, that were either infected with a NAI-susceptible or -resistant virus. Improved viral clearance and limited spread of virus in the lungs was found, when VIS410 was delivered prophylactically [206].

In a phase 1 clinical trial, the safety, tolerability, and PKs of VIS410 against Influenza A virus was investigated. The results were used to implement a mathematical modeling approach to investigate whether VIS410 could be used to treat seasonal influenza and to protect at-risk groups [207]. In this trial, VIS410 applied in the range of 2–50 mg/kg was found to be safe and well tolerated [207]. VIS410 was associated with adverse events, including diarrhea, nausea, and vomiting. In a phase 2a influenza A virus human challenge study, a single-dose IV VIS410 administration 24 hours after virus-inoculation has led to a statistically significant decrease in viral load compared with placebo. VIS410 was safe and well tolerated. This demonstrated that VIS410 treatment of patients with uncomplicated influenza may lead to favorable effects on virus replication and hopefully symptom resolution [208].

3.7.4 Summary

Monoclonal antibody preparations are a different approach compared to the NA and polymerase inhibitors harboring superior PK properties. With an average half-life of roughly three weeks, one dose would be sufficient to protect and could also control infection and clinical symptoms, even if administration occurs 48 hours after onset of clinical symptoms [199, 202, 206]. Moreover, mAb treatment did not result in mutation in the viral genome and in the appearance of resistant virus strains [196, 202, 208]. This makes these compounds potentially valuable prophylactic and therapeutic tools against influenza as monotherapy or in combination with either NA or polymerase inhibitors. However, further studies in patients with severe influenza or with patients at high risk need to be performed to finally conclude on the potential of mAbs.

3.8 Host-Targeting Candidates

In contrast to targeting the virus directly, as described above for various small molecule inhibitors and monoclonal antibodies, another strategy would be to interfere with host cell functions, which the virus needs to fulfill its replication. Small RNA viruses such as influenza viruses require extensive interactions with host-cell functions to support their life cycle. At the same time, the infected cell induces a machinery of defense mechanisms to fight the invader [209]. These processes are mediated by a variety of intracellular signaling cascades and present a great opportunity for pharmaceutical intervention. Since it is almost impossible for the virus to escape the cellular dependency, the formation of resistant viral variants should be a rare event. However, interfering with cellular functions could have an influence on cellular integrity and thus lead to adverse events. A successful antiviral will need to balance toxicity to the cell and high antiviral efficacy.

Here, three concepts that have reached phase 2 of clinical trials are summarized: (i) DAS181, a protein that cleaves the cellular receptor for the virus; (ii) nitazoxanide that interferes with the trafficking of the HA in the cell; and (iii) LASAG that functions as an NFkappaB inhibitor. Activation of the NFkappaB pathway is required by influenza virus to ensure RNP-export.

3.8.1 DAS181

DAS181, also known as Fludase, is a recombinant sialidase fusion protein composed of the catalytic domain of a bacterial sialidase (*Actinomyces viscosus*) and the epithelium-anchoring domain of the human protein amphiregulin [15, 210, 211]. DAS181 cleaves both the Neu5Ac a(2,3)- and Neu5Ac a(2,6)-Gal linkages of sialic acid, which are the primary receptors for both human and avian/equine influenza viruses to bind and enter the host cell [5]. This renders the host cells inaccessible to influenza virus infection [212]. It could be shown that DAS181 treatment removed about 90% of sialic acid receptors on human airway epithelial cells within 15 minutes after treatment [213]. At least three days were required until the amount of sialic acid receptors returned to normal [213]. DAS181 is administered by dry powder inhalation with microparticles of 5–10 μm in size. The particle size leads to a deposition of the drug to the upper and central respiratory tract, but not the lower respiratory tract [14].

Preclinical studies have demonstrated that DAS181 is functional against a broad range of human and avian influenza viruses, as well as parainfluenza viruses and human metapneumoviruses (A2, B1, and B2 strains) with no cellular toxicity [213–217]. After treatment with DAS181, mice could be protected from lethal influenza virus infection [218, 219]. In a randomized, double-blind, placebo-controlled phase 2 study, it was shown that DAS181 was effective in decreasing viral load and viral shedding in humans, with no significant adverse effects in the patients that received the treatment [220]. The active domain of the sialidase is derived from a bacterial species that is also part of the human flora. Thus, immunological reactions by the host should be unlikely [211]. Nevertheless, in a phase 1 clinical trial, it was observed that when treatment was performed for more than seven days, patients developed an immune response against the compound. Antibodies directed against DAS181 led to a reduced efficacy [15, 221]. DAS181 has also been tested in a phase

1 trial in adults with well-controlled asthma. While the generalizability of the results is limited because of a small sample size, the drug was associated with some adverse events and it was stated that caution should be employed when administering DAS181 to individuals with less stable reactive airway disease [222].

3.8.2 Nitazoxanide

Nitazoxanide (NTZ) was originally developed and licensed as an antiprotozoal/helminth drug for the treatment of enteritis caused by *Cryptosporidium* and *Giardia* infections [223]. NTZ is a compound of the thiazolide class (Figure 3.11) that is orally administered and rapidly deacetylated in the blood to tizoxanide, which represents the active metabolic form [224, 225]. In addition to its antiparasitic activity, NTZ is also effective against a broad range of bacterial and viral pathogens [226–230].

Since viruses have different replication strategies, the mode of action of NTZ against different viruses varies. The mechanism of action against influenza virus is achieved by interference of NTZ with the viral HA. NTZ inhibits the trafficking of the HA from the endoplasmic reticulum to the Golgi apparatus and thus prevents the assembly and release of viral particles from the host cell [224, 225].

Preclinical studies have demonstrated that NTZ has an antiviral activity against a range of different strains of influenza A and B virus strains from human and avian origin with IC_{50s} t.l ranging from 0.2 to 1.5 μg/mL [224, 227, 230, 231]. In addition, a combination of NTZ with oseltamivir demonstrated a synergistic effect against influenza A viruses, including oseltamivir-resistant virus [227]. The potential benefit of the combination of a compound targeting the virus directly (oseltamivir) with a host-targeting antiviral (NTZ) may be the improvement in the effectiveness in particular against oseltamivir-resistant strains and the reduction in the likelihood of selecting for resistance [14]. There are currently no published data available of the antiviral efficacy of NTZ in animal models [14, 15]. A large number of clinical studies in humans were performed with NTZ, demonstrating the potential of this repurposed drug as an antiviral against influenza [224]. Results from a phase 2b/3 human clinical trial with participants suffering of acute uncomplicated influenza demonstrated that treatment with 600 mg NTZ twice daily (started within 48 hours of symptom onset) for 5 days was associated with a reduction of the duration of symptoms of 21.2 hours. The drug was well tolerated and reduced the viral load [232]. Various phase 2 or 3 clinical trials with NTZ are currently underway, including a comparison to oseltamivir and an oseltamivir/NTZ combination therapy [14].

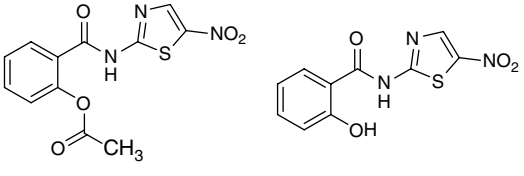


Figure 3.11 Chemical structures of nitazoxanide and its active metabolite tizoxanide.
Source: Radi et al. [244].

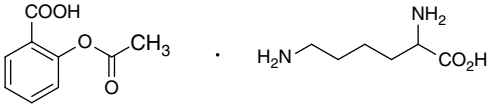
3.8.3 LASAG

LASAG (D,L-lysine acetylsalicylate · glycine) also known as BAY 81-8781 is approved as Aspirin i.v. for IV application (Figure 3.12). In addition to LASAG's function as an inhibitor of cyclooxygenases (COX), it was described that acetylsalicylic acid (ASA) and other salicylates inhibit the activation of the NF-kappaB pathway in a low millimolar range [233–235].

Newly synthesized vRNP complexes must be exported from the nucleus into the cytoplasm [129], which requires the activation of the NF-kappaB pathway by Influenza A and B viruses [236]. In cell culture, it could be demonstrated that LASAG is effective against numerous influenza A and B virus strains including highly pathogenic avian strains [237, 238]. In the mouse infection model, pharmacological studies demonstrated that the oral route of administration was not suitable to reach the sufficient concentrations of LASAG for a successfully reduction of viral load in the lung. This could only be achieved by inhalation of LASAG [239]. Inhalation of pure ASA is clinically not suitable, since it causes respiratory irritation due to its acidic properties [240, 241]. However, the D,L-lysine increases the stability and tolerability of inhaled ASA, it prevents ASA from hydrolyzing and promotes the formation of a salt. Moreover, the addition of the glycine to ASA prevents discoloration and further leads to an increase of the stability.

Both lysine and glycine are essential amino acids and are considered to have no relevant pharmacodynamic or toxic effects. They present no risk to human health. As LASAG immediately dissociates into ASA, the pharmacodynamics of LASAG are equivalent to those of ASA [242]. In mice infected with a 5× lethal dose of influenza A virus, LASAG treatment was still effective in protecting 50% of the animals from death when administration started as late as 48 hours after infection [239].

A phase 1 clinical trial demonstrated that inhalation of LASAG was well tolerated and did not lead to adverse events [243]. Patients with a reported duration of illness of less than 120 h were included in the study. Other inclusion criteria included: presence of at least one respiratory symptom (nasal congestion, sore throat, or cough) of any severity and hospital admission due to (suspected) influenza; presence of at least one constitutional symptom (aches/myalgia, fatigue, headache or feverishness/chills/sweats) of any severity and presence of fever (temperature ≥38.0 °C orally, or ≥38.5 °C rectally) at the time of screening. In a phase 2a clinical trial, hospitalized patients received inalation of LASAG for seven days, twice daily. LASAG significantly (17.2 hours) improved the time to alleviation of influenza symptoms in hospitalized patients compared to standard of care (oseltamivir) and demonstrated an influence on the viral load [243]. The LASAG group received 800 mg of LASAG/4 ml of fill dose, equivalent to 400 mg of ASA/4 ml of fill dose, resulting in an alveolar dose of 45 mg ASA. The placebo group received 4 ml saline solution (0.9% sodium chloride (NaCl))



LASAG - acetylsalicyclic acid DL lysine

Figure 3.12 Chemical structure of LASAG.

dissolved in water. This phase 2 proof-of-concept (PoC) study demonstrated that targeting an intracellular signaling pathway using aerosolized LASAG, which represents a different host cell strategy compared to DAS181 or nitazoxanide, improved influenza symptoms and reduced viral load. The mode of action of LASAG against influenza is not completely solved. Due to the fact that COX-inhibitors have anti-inflammatory properties, it might be that the influence of the disease outcome is due to an anti-inflammatory effect. However, in preclinical studies it could be demonstrated that indomethacin, a pure COX inhibitor, showed no inhibition of virus propagation and was not able to inhibit NFkappaB [237]. Also as LASAG therapy is expected to last only for seven days and the drug is inhaled, the exposure is low and typical side effects of oral ASA therapies are unlikely.

3.8.4 Summary

Host cell targets represent a potential, new strategy for the development of antivirals against influenza. One of the advantages is the reduced resistance formation due to the fact that the virus needs the particular host cell function in order to ensure its propagation. Nevertheless, targeting host cell components or host cell factors could lead to an increased risk for adverse events. This risk needs to be scrutinized in detail for each particular host cell target. However, as influenza only requires short-term interventions, it may be one of the viruses where this strategy may find a tolerable balance between attacking the virus and inhibiting essential host functions.

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4

Respiratory Syncytial Virus Immunoreactivity, Vaccine Development, and Therapeutics

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4.1 Introduction

In the past decade, respiratory syncytial virus (RSV) therapeutics and vaccine research have increased significantly but with no new therapeutics' licensing since the FDA licensed Palivizumab in 1998. However, recently, there has been some progress in the development of an RSV vaccine, with nine candidate vaccines past phase 1 clinical trials. A recent clinical trial of a nanoparticle RSV-F vaccine demonstrated at least secondary endpoint efficacy; it reduced infant hospitalizations and hypoxia due to RSV infection [1]. Clinical trials of an experimental prefusion RSV-F vaccine have also demonstrated some partial success by the generation of IgA in the airway mucosae, but with mixed results between trial participants [2]. Furthermore, there are at least eight candidate RSV antivirals in clinical trials. These range from fusion inhibitors, nucleoside analogues, antibodies, and small interfering RNA (siRNA).

This chapter will discuss the healthcare burden posed by RSV, updated morbidity, and mortality data, and evasion of adaptive immunity by RSV. We will discuss how these aspects of RSV infection have likely hampered therapeutic and vaccine development. Among the therapeutic approaches that are currently in development, we will address primarily inhibitors of the fusion process between the viral and cellular membranes as well as nucleoside analogues and the importance of their structure.

4.1.1 The Burden of RSV Infection on Human Health

In healthy adults, RSV infection ranges from common cold-like symptoms to "full-blown flu," so the vast majority of these cases are subclinical and not reported. Hospitalization is likely when RSV infects the lower respiratory tract after establishing infection in the nasopharynx. Lower respiratory tract infections are the leading cause of death in infants [3].

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RSV is a significant contributor to this healthcare burden and is the second leading cause of death worldwide in children, second to malaria [4]. These infections, however, should not be considered as mutually exclusive. The current theory on the significant lethality of RSV is that it pushes those with an already high disease load, "over the edge" [5, 6], a theory that has been outlined in two publications by Caballero et al. [7–9] accurately labeling RSV an "opportunistic killer." This is particularly true for those with congenital heart and lung defects, and those with Down's syndrome. In developing nations and those regions plagued by a massive burden on health, natural and human-caused, there is a high opportunistic-parasite and infection burden in the general population. Children in lower socioeconomic status residences are also more likely to succumb to RSV infection [7].

RSV thus is a significant infection that further complicates the health of those with other coinfections and maladies, including one of or a combination of tuberculosis, atypical mycobacterial infections, cholera, typhus, schistosomiasis, dengue virus infection, and malaria. All of the infections mentioned above do not always occur in isolation but sometimes as compounded pathogenesis. Thus, an acute RSV infection of the lower respiratory tract can be an overwhelming factor that kills those who are already suffering from other diseases.

The worldwide Pneumonia Etiology Research for Child Health (PERCH) studies on the causes of severe pneumonia showcase RSV as a critical pathogen that requires public health attention and investment. In the updated 2019 publication, the PERCH study examined children admitted to the hospital due to severe pneumonia. All hospitalized children in the study were age-matched with healthy controls from random communities around the study site. The study demonstrated that RSV was the leading pathogen causing severe pneumonia in 31% of all tested cases [10]. Overall, viruses made up 61% as the cause for severe pneumonia exceeding bacteria, which accounted for 27% of the cases. The study also noted that RSV was the most frequent virus detected in negative controls, supporting the hypothesis that RSV is an ubiquitous and opportunistic pathogen.

4.1.2 RSV Transmission

In developed nations, the virus is spread through the community and in nosocomial settings by aerosols, droplets, and by transmission from contaminated surfaces [11–14]. Standard hygienic measures, like shielding coughs and sneezes, hand washing, and social distancing play an essential role in limiting the spread of infections [15].

In 1981, Hall et al. showed the most common modes of transmission of RSV comes from close contact with large droplets or inoculation after touching contaminated surfaces [16]. Small aerosolized particles did not contribute to RSV spread and infection. These findings were then corroborated as well; more recent studies have shown that RSV can be transmitted through large droplet aerosols [11, 14].

Studies on RSV transmission in Kenya [17] and Italy [18] have identified older school-aged siblings that bring RSV home with them as a source of infant RSV.

4.1.3 The Current Therapeutic Options and Opportunity for the Development of Treatments for RSV Infection

In infected individuals, the peak of RSV viral load reaches a slow crescendo that abates slowly. This is in contrast to influenza that must be treated with direct-acting antivirals at

the onset of symptoms for therapeutics to be effective [19, 20]. For RSV, there is a sufficiently long therapeutic window in which to identify and treat RSV infection. The peak of viral load typically happens on day 6–8 after infection with RSV, in contrast to 4 days postinfection with other respiratory viruses [21].

Currently, when a patient is admitted to the hospital with RSV, the hospital can only provide supportive care such as supplemental oxygen, mechanical ventilation, and fluid replacement. There are no suitable antivirals for the treatment of RSV. Although Ribavirin is licensed for use in RSV infection, it is hardly used because of a lack of efficacy and numerous side effects. There is no vaccine for RSV but one available prophylaxis is a monoclonal antibody called Palivizumab. This drug is costly and is only partially effective at preventing hospitalization of infants with RSV (reducing hospitalizations by about 50%). Of note, Palivizumab is only effective at preventing infection; it does not provide any therapeutic relief in an already established RSV infection. Palivizumab has been licensed since 1997 [22].

4.1.4 The Unique Challenge of RSV Vaccine Development

A vaccine is needed to at least partially counter the considerable health burden posed by RSV. Any such vaccine will likely still need to be supported by using monoclonal antibody prophylaxis and antiviral therapeutics to protect in cases of vaccine breakthrough. Vaccines have all remained elusive since the discovery of RSV 70 years ago. As discussed above, only the monoclonal antibody Palivizumab has been licensed for the prevention of severe RSV infection in the most vulnerable infants—usually those born prematurely and/or with other underlying conditions.

Relative to other viral infectious diseases, RSV has posed some significant challenges, among others, due to its apparent ability to evade the immune response and immune memory. This novel aspect of RSV immunology compared with other respiratory viruses like influenza, rhinoviruses, coronaviruses, and others are complicating vaccine development. Therefore, unlike other viruses, protection from RSV infection will likely require nontraditional vaccine approaches. However, apart from a few exceptions, the bulk of the RSV vaccine candidates in clinical trials is still based on traditional vaccine strategies. In the absence of pressure from immune memory, reinfection by the same strain can occur repeatedly, and studies have shown that RSV genomic adaptation occurs [23–25]. This most likely also means that RSV will adapt through mutation to evade vaccine pressure.

4.1.5 The Virus and the Replication Cycle

Viruses are classified by the International Committee on Taxonomy of Viruses (ICTV). RSV, formally known as human orthopneumovirus, belongs to the family *Pneumoviridae*, together with human metapneumovirus (HMPV), another pediatric respiratory pathogen. These viruses are in the order *Mononegavirales*, meaning that they are within the group of viruses with a non-segmented negative strand RNA genome [26]. Two types of RSV, type-A and type-B, share 95% sequence identity. RSV virion particles are between 100 and 300 nm in diameter and can have filamentous and other polymorphic shapes (Figure 4.1). The viral particle is composed of the nucleocapsid surrounded by a matrix protein lattice,

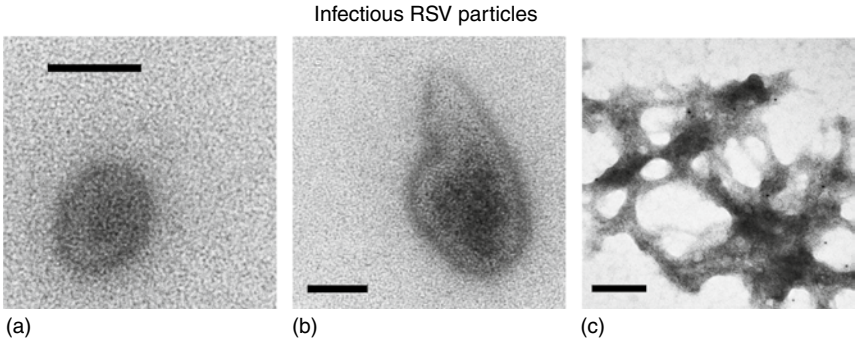


Figure 4.1 Transmission electron microscopy of RSV from tissue culture. (a and b) RSV particles imaged by transmission electron microscopy and (c) immuno-transmission electron microscopy. (a and b) Note the electron dense core. (c) Particles are in a fibrous-detritus matrix labeled with Anti-RSV-F antibody conjugated to 10 nm gold particles. The matrix confers resistance to antibody neutralization but has no effect on infectivity. Scale bar = 100 μm.

surrounded in turn by a lipid envelope [27, 28]. The nucleocapsid consists of a helical ribonucleoprotein complex of viral genome RNA and nucleoprotein (N) [29, 30]. This N-RNA complex is associated with the viral polymerase, consisting of the large polymerase subunit (L) and phosphoprotein (P). It is also associated with the viral M2-1 protein, which also forms a lattice between the nucleocapsid and the matrix protein layer in virion particles [27, 28]. Three glycosylated spike proteins, glycoprotein (G), fusion protein (F), and small-hydrophobic protein (SH), protrude from the lipid envelope [27, 28]. The nucleocapsid and glycoprotein components of the virus particle and their roles in the viral replication cycle are described in the sections below. For a more detailed description of the molecular biology of RSV, see a review by Griffiths et al. [31].

4.1.6 The Nucleoprotein–RNA Complex

As noted above, the RSV genome is a single strand of negative sense RNA. It is 15.2 kb in length and codes for 10 mRNAs and 11 known proteins [32, 33]. In addition to the proteins that comprise the viral particle, it codes for nonstructural proteins 1 and 2 (NS1 and NS2), which antagonize innate immune responses [34], and M2-2, which regulates viral polymerase activity [35]. The 3′ end of the genome contains an extragenic leader promoter region, which recruits the polymerase to begin transcription or genome replication [36, 37]. Each of the viral genes is flanked with conserved cis-acting elements, which direct the polymerase during mRNA transcription [38, 39]. The 5′ end of the genome contains an extragenic trailer region. In the replicative intermediate RNA, known as the antigenome, the complement of the trailer acts as a promoter to recruit the polymerase to begin genome RNA synthesis [40–42]. The genome is encapsidated along its length with oligomeric N protein, such that each 43 kDa N monomer contacts seven nucleotides [30]. The genome RNA remains encapsidated throughout the viral replication cycle and so presumably the polymerase transiently displaces N protein as it moves along the template during transcription and replication.

4.1.7 The L Polymerase Protein

The RSV polymerase is responsible for generating capped and polyadenylated mRNAs and encapsidated antigenome and genome RNAs [43–45]. The essential nature and distinctive features of the polymerase make it an attractive target for antiviral drug development [44, 46]. The core polymerase is a complex of the L and P proteins [41, 47–49]. L is a 250 kDa protein that possesses the enzymatic activities required for transcription and replication. It has five domains: an RNA-dependent RNA polymerase (RdRp) domain, a capping domain, a connector domain, methyltransferase domain, and carboxyl-terminal (C-terminal) domain [44, 45]. The RdRp shares a number of features with the RdRps of other RNA viruses, reflected by the fact that there are broad-spectrum RdRp inhibitors that function against the RSV polymerase [50, 51]. The capping domain functions to add a guanosine cap to the 5′ end of mRNAs [52, 53]. Although the cap structure is identical to that of cellular mRNAs, the enzymatic reaction is distinctive, occurring through a GDP polyribonucleotidyltransferase activity, rather than a guanylyltransferase activity [54–57]. By analogy with the L protein of related viruses, the capping domain might also play a structural role in initiation of RNA synthesis by providing a priming loop to stabilize the initiation complex [58–60]. The methyltransferase domain is responsible for methylating the mRNA cap, and the CTD also likely contributes to this activity [61, 62]. The role of the connector domain is not well characterized, but it is thought to aid the structural rearrangements necessary for the different enzymatic activities to come into play. The structure of the RSV polymerase and that of HMPV, its close relative, were recently solved using cryo-electron microscopy [47, 48, 63]. These studies revealed the structures of the RdRp and capping domains. These domains associate together to create the central cavity of the RdRp catalytic site. While the RdRp has features conserved with those of other RdRps, the capping domain of RSV L (and that of other non-segmented negative strand RNA viruses polymerases) is structurally unique, reflecting its distinctive enzymatic activity [45, 47, 48, 63]. The other three domains were not visible, indicating considerable structural flexibility. This is consistent with structural analysis of other non-segmented negative strand RNA virus polymerases, in which the connector, methyltransferase, and C-terminal domains have been observed juxtaposed in different positions relative to each other [58, 59, 64, 65]. These structural changes are likely to be necessary for the polymerase to be regulated between its different activities.

4.1.8 The P Phosphoprotein

The 27 kDa P protein functions as an essential cofactor for the L protein [49]. P has three domains, a central oligomerization domain that allows tetramer formation, and intrinsically disordered N- and C-terminal domains [66, 67]. P stabilizes the L protein and acts as an adaptor allowing the polymerase to associate with the other viral proteins involved in transcription and genome replication. The C-terminal domain of P binds to N-RNA, allowing the polymerase to associate with the template [68–70]. Its N-terminal domain binds to soluble N protein, presumably to enable encapsidation of the nascent RNA replication products [71, 72]. Its N-terminal domain also binds to M2-1, a transcription elongation factor [73, 74]. The cryo-electron microscopy analysis of the RSV L-P complex revealed that

the P tetramer adopts an unusual arrangement covering a relatively large surface area of the L protein [47, 48]. A similar arrangement was observed in the HMPV L-P structure [63]. P also functions independently of L to associate with N protein and form the cytoplasmic inclusion bodies that are the sites of RSV transcription and replication [75, 76]. It has recently been shown that a compound targeting these biomolecular condensate inclusion bodies has antiviral activity [77].

4.1.9 The M2-1 Protein

M2-1 is a 22 kDa protein that also forms a homo-tetramer [74, 78]. It is not required for RSV genome replication or initiation of transcription, but it is required as an elongation factor for the viral polymerase to generate full-length mRNA transcripts [79–81]. Because the RSV genes are transcribed sequentially, such that transcription of each downstream gene is dependent on transcription of all the upstream genes, M2-1 is essential for the entire viral genome to be transcribed [82]. M2-1 also binds to viral mRNAs posttranscriptionally and to select cellular mRNAs [76, 83]. However, it is not known what effect this posttranscriptional RNA binding activity has.

4.1.10 The RSV-G Glycoprotein

RSV-G is a type II transmembrane glycoprotein with the transmembrane domain at the amino-terminal end [84]. The G protein is the most variable protein of RSV, and RSV types A and B were initially differentiated, based on antibody reactivity to the G protein [20]. RSV-G serves as an attachment factor during virus entry (Figure 4.2) and is shed from infected cells as a soluble glycoprotein. The soluble form is expressed several hours before the expression of the full-length membrane-anchored RSV-G protein [85]. In the soluble form, RSV-G serves as an immune-decoy [85, 86]. RSV G protein's attachment role secures the viral particle to the host cellular membrane to prepare the fusion process to occur (Figure 4.2).

Despite its variability, RSV-G contains a central conserved domain that includes a CX3C motif [84]. Due to the highly conserved central conserved domain, which enhances infectivity and may have roles in modulating the host immune response through its interaction with CX3CR1, the G protein may emerge in the coming years as an attractive target for vaccine and therapeutic antibody development as reviewed in [84].

4.1.11 The RSV-F Fusion Glycoprotein

The fusion (RSV-F) protein is the most highly conserved glycoprotein on the surface of the RSV virion, and it is the principal neutralizing determinant on the RSV particle (Figure 4.3). Palivizumab and the most promising vaccine strategies have been developed against RSV-F. It exists on the surface of infectious virions in a metastable "prefusion" conformation that harbors the principal neutralization site, Ø [87]. The structure of RSV-F, its prefusion conformation, and the Ø neutralizing determinant were all elucidated recently [88], and some of the most promising experimental prophylactic, therapeutic, and vaccine strategies have come from these studies (Table 4.1) [87].

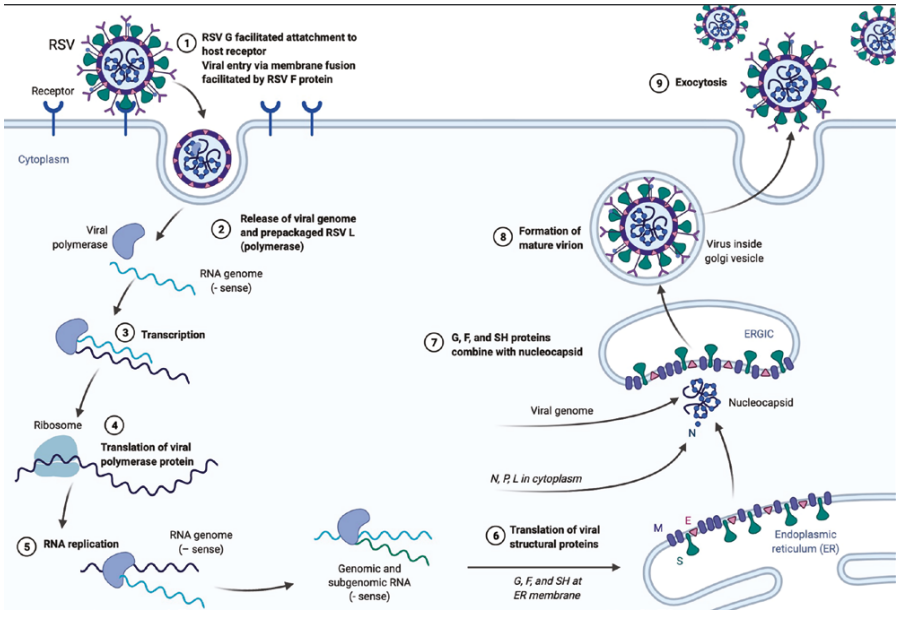


Figure 4.2 The RSV life cycle. The RSV life cycle and where potential RSV inhibitors will work to inhibit steps in the replication cycle.

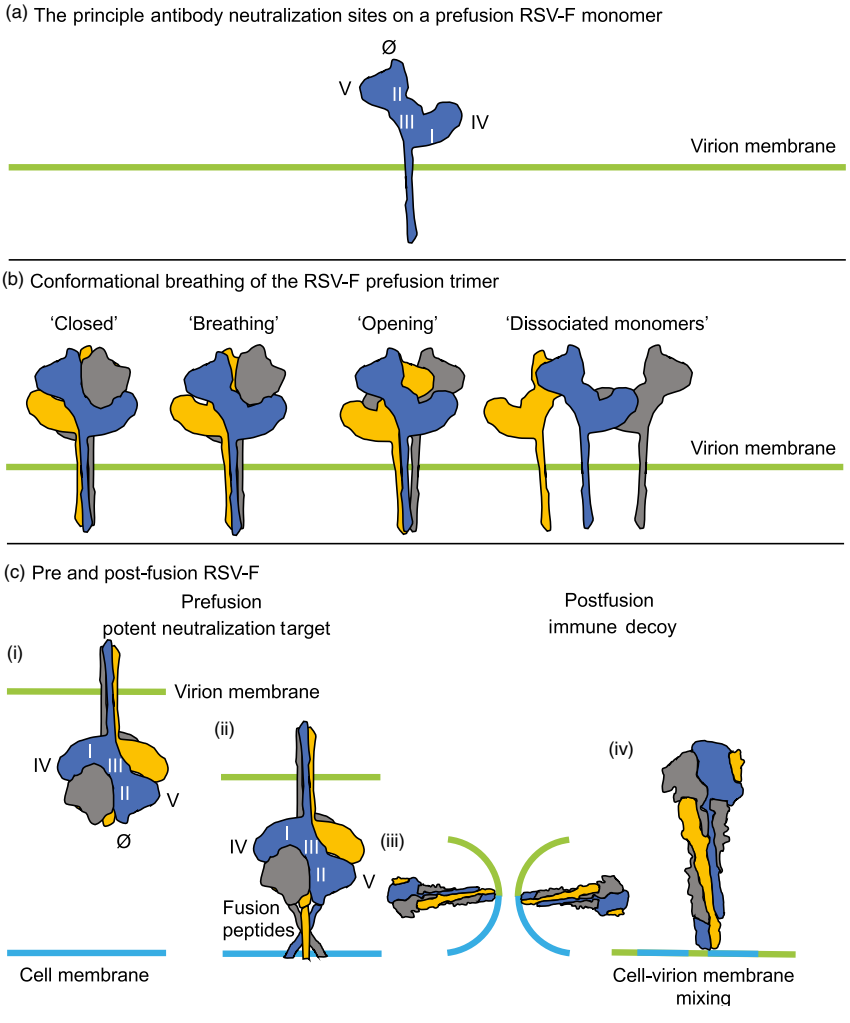
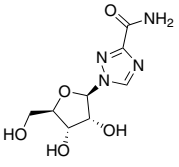
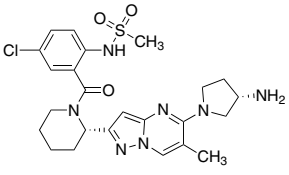


Figure 4.3 The principal neutralizing determinants of RSV-F fusion glycoprotein, conformational breathing, and the fusion reaction. (a) The neutralization sites on RSV-F are shown on an RSV-F monomer. (b) Conformational breathing of the RSV-F trimer results in transient exposure and concealment of some epitopes on RSV-F. (c) The process of the fusion reaction showing extension of the fusion peptide (i) from within the RSV-F protein into the host-cell target membrane (ii). The complementary determining region (CDR) domains are brought together that brings the virus and host cell membrane within in close apposition (iii) and promotes virus-host cell membrane fusion (iv).

4.1.12 The RSV-SH Glycoprotein

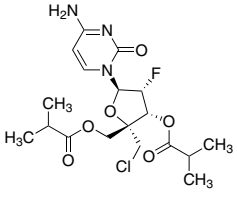
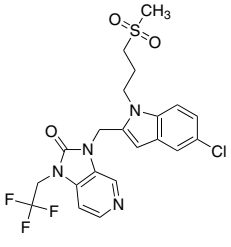
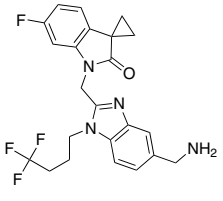
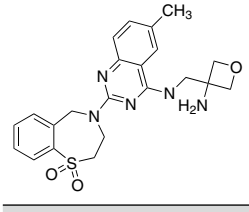
RSV-SH is the smallest of the RSV membrane glycoproteins and exists in four forms in infected cells: SH_0, SH_g, SH_p, and SH_t [89]. The predominantly expressed form is SH_0, which is 7.5 kDa and is unglycosylated. SH_g is a 13–15 kDa glycosylated form that is the precursor of the more highly glycosylated SH_p. From that is between 21 and 40 kDa. The SH_t is a truncated form with approximately 7.5 kDa [90].

Table 4.1 Antiviral compounds against RSV.

Immunotherapy under clinical development	Type	Drug target	Stage	Target population
Ribavirin 	Small-molecule inhibitor	Broad-spectrum nucleoside analogue	Marketed compound	Ribavirin is used mainly when the outcome of an RSV LRTI could be fatal, typically in the immunocom-promised
Palivizumab	Monoclonal antibody	RSV-F	Marketed compound	Prevention of serious lower respiratory tract disease requiring hospitalization in children at high risk for RSV disease
Immunotherapy under clinical development	**Type**	**Drug target**	**Stage**	**Target population**
RI-00I	IVIG	RSV proteins	Phase 2a	Immunosup-pressed RSV-infected patients at risk for LRTI
RI-002	IVIG	RSV proteins	For RSV-unknown	Primary humoral immunodeficiency disease in patients aged >12 years
Motavizumab (MEDI-524)	Monoclonal antibody	RSV-F	Failed to obtain FDA approval	High-risk infants
MEDI-8897	Monoclonal antibody	RSV-F	Phase 2b	High-risk infants
Novel antivirals undergoing clinical development	**Type**	**Drug target**	**Stage**	**Target population**
Presatovir (GS-5806)[a] 	Small-molecule inhibitor	RSV-F	Phase 2-currently on hold	RSV-infected patients

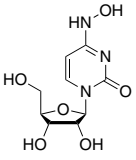
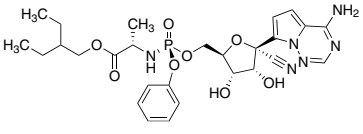
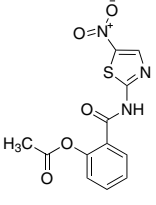
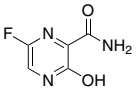
(Continued)

Table 4.1 (Continued)

Novel antivirals undergoing clinical development	Type	Drug target	Stage	Target population
Lumicitabine (ALS-008176 or JNJ-64041575)[a] 	Nucleoside analogue	RSV-L	Currently on hold	RSV-infected patients
Rilematovir (JNJ-53718678)[a] 	Small-molecule inhibitor	RSV-F	Phase 2	RSV-infected patients
Sisunatovir (RV521)[a] 	Small-molecule inhibitor	RSV-F	Phase 2a	RSV-infected patients
Ziresovir (AK0529)[a] 	Fusion inhibitor	RSV-F	Phase 2	RSV-infected patients

Other investigational treatments and broad-spectrum antivirals	Type	Drug target	Stage	Target population
RSV M2-1	Transcription anti-termination factor	RSV polymerase complex		RSV-infected patients
EDP-938	Replication inhibitor	RSV polymerase complex	Phase 2	RSV-infected patients

Table 4.1 (Continued)

Other investigational treatments and broad-spectrum antivirals	Type	Drug target	Stage	Target population
EIDD-1931[a] 	Ribonucleoside analogue inhibitor	RSV L	Preclinical	RSV-infected patients
VH244	Interfering particle		Preclinical	RSV-infected patients
Remdesivir[a] 	Nucleoside analogue	RSV L polymerase complex	Marketed for SARS-Coronavirus 2	RSV-infected patients
Nitazoxanide[a] 	Post viral entry interference	RSV L glycoprotein folding	Preclinical	RSV-infected patients
Favipiravir[a] 	Purine nucleotide analogue	RSV L	Marketed for influenza and SARS-coronavirus 2	RSV-infected patients

[a] Chemical structures (Source: Modified from DrugBank).

The function of RSV-SH is less understood; it is not necessary for viral replication *in vitro*, but it is a necessary factor for pathogenicity *in vivo*. It is known to have roles as a viroporin [91], which enhances membrane permeability [92] and potentially acts as a protein that mediates cellular homeostasis during viral infection [93]. *In vitro* studies have also shown that SH protein inhibits apoptosis by inhibiting NF-κB [94]. A recent study of Bovine RSV (BRSV) demonstrates that it shares a high level of genetic identity to Human RSV, immune-modulatory effects of SH, particularly in the inhibition of NF-κB p65 phosphorylation [95]. Since 2015, there have been advances in a vaccine derived from the SH protein [96]. Langley et al. have recently published a successful preclinical study with a vaccine formulated with a single subunit based on the SH protein of RSV. Currently, this candidate is in phase 1 clinical trials.

4.1.13 Antigenic Variation of RSV Surface Glycoproteins

Antigenic variation of the RSV surface glycoproteins is not the dominant factor that plays a role in the global persistence of RSV infection, as it does with influenza. Nevertheless, a significant mutation of RSV-G has occurred over the last 50 years and resulted in the divergence of RSV type-B as a distinct subtype. There is some evidence that genetic adaptation leads to the emergence of dominant strains in the community that replace pre-existing strains as the predominant RSV in the population [23, 97]. For example, the current dominant RSV type-A strain, called ON1 that was identified in 2010 contained, most notably, a 72 bp duplication in the RSV-G glycoprotein. This strain has overtaken other strains of RSV to become the predominant RSV type-A strain worldwide [97]. In tissue culture, these viruses replicated to the highest levels compared with other RSV-A strains from patients [23]. RSV persists in the community, and the same strain can reinfect the same population over again. Thus, it is the nature of RSV to evade or suppress memory B cell development and not necessarily genetic variation that has resulted in RSV persistence and has complicated RSV vaccine development.

4.1.14 Adaptation of RSV in the Community by Emergent High Titer Clades

RSV continues to adapt to selection pressure by mutation. In 2017, we identified emergent clades of RSV types-A and -B that were associated with higher patient titers in the 2014–2016 RSV seasons. Isolates from these clades replicated to higher titers in culture than the lower titer "resident clades" of RSV [23]. These results suggest an ongoing adaptation of RSV to naïve populations. We suggest that if a genuinely efficacious vaccine were to be realized, vaccine breakthrough and therapy resistance could occur while adapting to naïve populations.

4.2 RSV Longevity, Immune Evasion, and the Role of IgA

Environmental factors independent of viral genetics play a significant role in determining the sensitivity of RSV to antibody neutralization and longevity. Susceptibility of RSV to antibody neutralization *in vitro* varies when comparing the different methods of purification [98].

The air pollution level may also play a role in determining how long RSV can remain infectious on a dry surface. RSV is predominantly spread by aerosol and contact with contaminated surfaces [99]. RSV infectivity can be prolonged significantly, effectively preserved on a dry surface from hours to months upon complexing of RSV particles with diesel pollution [100]. This study challenges the dogma that virus infectivity will be lost upon desiccation in the environment and has implications for hygiene, sterilization procedures, and infection control during RSV seasons in urban centers.

4.2.1 Evasion or Suppression of Immune Memory

Bronchiolitis has been associated with the IgG1 subtype [101]. Secreted IgA is transported across the epithelium into the respiratory mucosa, where it confers sterilizing

protection against viral infection [2, 102, 103] (Figure 4.4). IgA, IgG, and IgM are provided in breast milk [104]. It has been suggested that breast milk regurgitation will coat the upper respiratory tract (URT), which lends protection from initial URT infection for infants.

A significant hindrance to RSV vaccine development is that RSV infection does not confer immune memory; the same virus strain can reinfect the same individual multiple times [105]. This would suggest that RSV is not immunogenic. In adult challenge studies with influenza and RSV, it was shown that although individuals generated healthy levels of IgG memory B cells, a paucity of memory IgA B cells existed after RSV infection, unlike with influenza [102]. A more recent study showed there was no enrichment of anti-RSV IgA memory B cells in adenectomies from children compared with peripheral blood [106], while there were relatively higher IgG memory B cells in the adenoids compared with peripheral blood. These results further speak to the evasion of or suppression of memory IgA B cells in the URT.

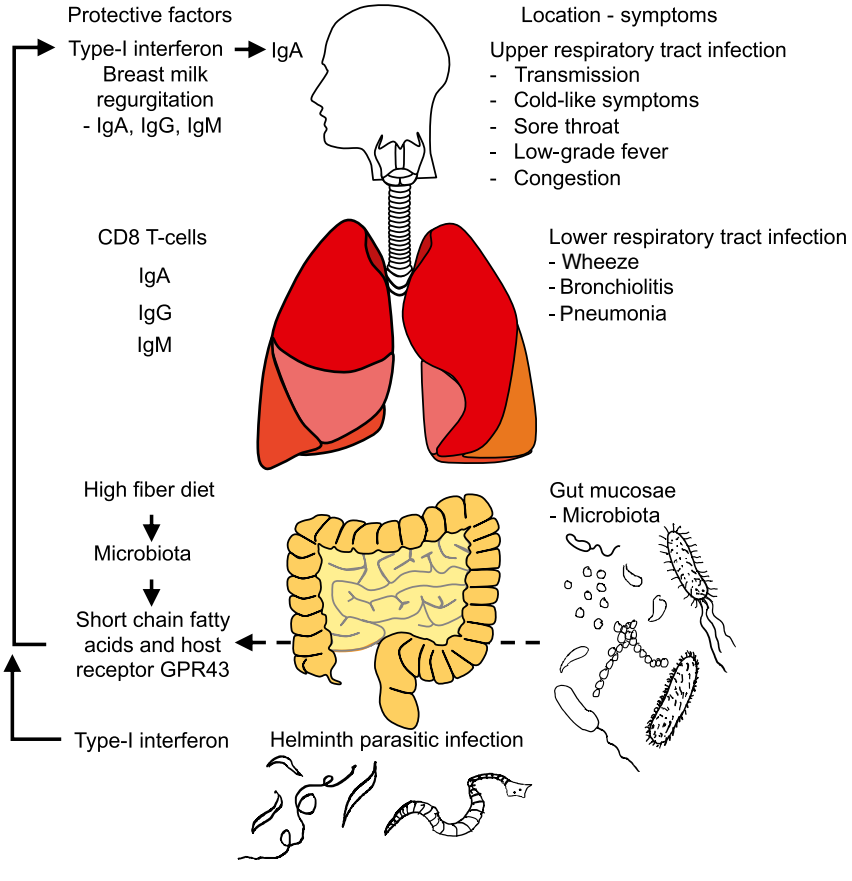


Figure 4.4 Factors that prevent and help clear RSV infection from the airway. The locations and factors protect from RSV infection in the upper, lower respiratory tracts, and in the gut.

4.3 The Impact of Immunoprophylaxis on the Health Burden of Respiratory Syncytial Virus

RSV is the most common cause of lower respiratory tract infections and hospitalizations in infants worldwide [50]. The first and only currently licensed drug to prevent RSV infection is Palivizumab. Although everyone is expected to have had an RSV infection by their second birthday [107], in certain well-recognized high-risk groups, such as preterm infants and infants born with chronic lung disease or congenital heart disease, there is a high risk of severe respiratory illness [108, 109].

4.4 Distinct RSV Symptoms

RSV causes symptoms in infants that distinguish it from other respiratory viruses. The most common is wheezing due to infant bronchiolitis. This is because RSV is highly successful at infiltrating the lower airways compared with other respiratory viruses [110]: RSV accounts for 90% of bronchiolitis in infants and 75% of all bronchiolitis cases worldwide [111]. It is also a leading cause of infant hospitalization, with an estimated 33.1 million episodes of lower respiratory tract infection (LRTI), 3.2 million hospital admissions, and as many as 118 200 deaths worldwide in 2015 [4]. Recent studies have also suggested that hospitalization due to RSV in the first three years of life is associated with long-term respiratory illness, especially asthma or reoccurring wheeze. These conditions may also persist into adulthood [112–115]. There is, however, more research needed to parse out the involvement of RSV in these longer-term chronic sequelae, like asthma. There is a relationship with the prevention of RSV in the first 12 months of life and an associated reduction in wheeze. However, this relationship is a mere association, and so mechanistic causality has not yet been demonstrated [116].

4.4.1 Wheeze

Wheeze is the result of the narrowing of the airways in the lung and is caused by inflammation due to infection, asthma, or chronic obstructive pulmonary disease. Its hallmark presentation is a whistling sound that is generated upon inspiration/expiration due to the airway narrowing [117].

The question remains as to whether preventing RSV infection with immunoprophylaxis can prevent the onset of chronic wheeze, as mixed results have been published in the literature. There are some accounts that recurrent wheeze is correlated with infants who receive Palivizumab as well as reports of a reduction in wheeze in infants that receive Palivizumab [116, 118, 119]. These results may differ, however, when confounding factors are considered. For example, those infants that receive Palivizumab are at a higher risk of recurrent wheeze because of a variety of risk factors such as prematurity, cystic fibrosis, congenital heart disease, and other factors that qualify them for Palivizumab. Studies have also only noted recurrent wheeze correlated to infants in

families with a history of atopy. Recurrent wheeze is common in infants born prematurely before 33 weeks; the risk is not consistently reduced when those infants receive Palivizumab [112, 120].

RSV also poses a significant health burden on the elderly population worldwide, especially those with pre-existing conditions like COPD and heart disease. Unlike in infants, RSV does not have distinct symptoms in adults, like a wheeze that readily distinguishes it from other respiratory pathogens [121]. RSV is also a significant contributor to pneumonia—both community and ventilator acquired. There is no treatment available for RSV in elderly populations. The prophylactic antibody, Palivizumab that prevents RSV-associated hospitalization, is only administered to high-risk infants, and Palivizumab has only been tested in clinical trials for high-risk infants. There are candidates in preclinical and clinical trials to prophylax and vaccinate the elderly; however, nothing has been approved.

4.4.2 The Effect of Immunoprophylaxis on the Development of Asthma

A recent single-blinded study published in 2018 followed the development of asthma in premature infants prophylaxed with Palivizumab in their first year of life. Their health was followed through to the age of six years [122]. After unblinding, no association of Palivizumab versus placebo on the development of asthma by six years of age was found. These results might suggest superficially that RSV infection prevention may not affect the development of asthma. However, one must remember that for the intervention with Palivizumab, efficacy hovers around a 50% reduction in hospitalizations. This is not a reduction in infections, for which, Palivizumab is even less efficacious. With this in mind, a study with an impressive participant power of 400 may still be underpowered to determine the effect of RSV infection on the development of asthma. A recent article also found that when looking at children with six years of age, there is no correlation between having received Palivizumab in infancy and the risk of decreased pulmonary function or asthma [122].

Therefore, to truly understand the link between RSV infection and asthma, considerably better interventions like those that target, e.g. the Ø neutralization site on RSV-F may have to be used. A more recent study found a significant benefit from Palivizumab prophylaxis on the development of asthma [123]. Here, there was a significant discord in the diagnosis of asthma between physicians. This may be due to a diagnosis of asthma from signs alone, which is unreliable compared with methylcholine challenge diagnosis of asthma.

4.4.3 Epidemiology and Clinical Aspects of Adult RSV Disease

Emerging data show that RSV is also a significant cause of hospitalization and deaths in adults. It has been estimated that RSV annually infects 3–10% of the adult population [124]. A recent global estimate in 2015 showed that there were about 1.5 million episodes (95%CI 0.3–6.9 million) of acute respiratory infections due to RSV in older adults in industrialized countries (data for developing countries were missing). Of these, 14.5%

or an estimated 336 000 required hospitalization (range 186 000–614 000), and there were about 14 000 deaths (range 5 000–50 000). Hospitalization rate and fatality were higher for those aged ≥65 years than for those aged 50–64 years and among those with underlying conditions (e.g. chronic lung diseases, chronic cardiovascular diseases) [125–128]. RSV has been shown to account for 5–15% of community-acquired pneumonia, 9–10% of hospital admissions for acute cardiorespiratory diseases and excessive deaths among adults during seasonal peaks. Outbreaks among nursing home residents are frequent but under-recognized [125, 127]. In hospitalized adults, the disease burden of RSV and clinical severity (as indicated by mortality 8–10%, ICU admission 10–18%, prolonged duration of hospitalization) are at least similar to, if not higher than, seasonal influenza [124, 127–129]. In particular, profoundly immunosuppressed adults, such as hematopoietic stem cell transplant (HSCT) recipients, are at high risk for progression to severe LRTI by RSV, which rapidly can be fatal (17–40%) [130]. Active pneumonic changes are evident radiographically in about 50–60% of hospitalized adults. Typically, changes include patchy consolidations and ground-glass opacities involving the lower zones, which are predictive of adverse outcomes [131]. Notably, high viral load and longer duration of viral shedding are significantly associated with clinical severity, indicating potential roles for antiviral treatment [132, 133]. Despite the availability of rapid molecular diagnostics, adult RSV infections are often not clinically suspected or are diagnosed late in the course of illness, posing challenges to potential interventions and infection control [134].

4.5 History of RSV and Vaccine Development

4.5.1 The Tragic History of RSV Vaccine Development in the First Failed RSV Vaccine Trials of the 1960s

The first vaccine clinical trials began in 1964, which is where RSV disease enhanced by the vaccine was realized. Following the success of the development of other formalin-inactivated (FI) vaccines, the RSV (FI RSV) vaccine trial in the 1960s ended in a tragedy. Most of the vaccinated children in the study had an increased risk of hospitalization due to RSV infection, and there were two deaths of vaccinees due to vaccine-enhanced RSV disease [135]. Health regulatory bodies were thus reticent to grant permission to subsequent RSV vaccine clinical trials.

It was not until 40 years after the ill-fated FI RSV vaccine trial in the 1960s that RSV research groups elucidated the reasons for the failure of the vaccine [136, 137]. Primary causes were a weak antibody avidity to the RSV F protein and inadequate Toll-Like Receptor (TLR) stimulation by the inactivated RSV vaccines, which ultimately lead to poorly neutralizing antibodies [136]. Further research identified an absence of prefusion F protein in the FI RSV particles [137]. This further explains weak antibodies that were developed in patients given the formalin-inactivated vaccine.

In the 1990s and 2000s, there were several clinical trials of RSV subunit vaccines that failed to confer robust protection from RSV infection or the preparation was not brought to market (tested in high-risk groups). However, these trials lacked the previously

noted negative consequences [138–141]. Furthermore, replication-competent attenuated vaccines proved too attenuated and not to elicit an immune response that could confer protective immune memory, even when given with an injected RSV subunit vaccine [142].

Research had led to the idea that the potential for using TLR stimulants as adjuvants will potentiate RSV vaccine reactivity. There is some evidence that stimulating with TLR agonists is a solution to an effective vaccine. TLR agonists have been shown to increase RSV and Influenza vaccine immunogenicity [143, 144]. However, such strategies must be approached with caution. In a study where TLRs were stimulated in mice, the mice responded with an increase in disease severity [145].

4.6 New Developments in RSV Vaccine Development

The *in vitro* sensitivity of RSV to antibody neutralization varies depending upon the method of virus stock purification [98]. It is, therefore, possible that the proportion of prefusion versus post-fusion RSV-F on the surface of RSV virions could be altered by different techniques and chemicals involved in the purification process.

4.6.1 Conformational Breathing of RSV-F Affects Neutralization Sensitivity

A recent study has demonstrated the conformational "breathing" of the RSV-F trimer on the surface of RSV virions (Figure 4.3) [146]. RSV-F breathing leads to conformational alterations in the principal neutralization site Ø [147]. Neutralization site Ø is recognized by two different human antibodies AM22 and RSD5, that each bind to distinct conformations of site Ø [146]. Given that the most promising vaccine strategies include prefusion RSV-F, discoveries such as these help to inform vaccine development and the mechanistic understanding of neutralization.

4.6.2 Measles Versus RSV Vaccine Development

After the failures of vaccine development for RSV in the 1960s, the development of a live attenuated measles vaccine occurred that is still used as a part of the MMR vaccine today. However, so far, live-attenuated RSV vaccines have remained elusive as they have not been immunogenic or conferred immune memory against RSV infections.

The critical difference in success between attenuated RSV and measles vaccines could be found in the mode of replication. Measles infections produce a viremia with detectable high levels of virus in the blood, whereas there is by and large no detectable RSV in the bloodstream during infection; measles virus infection is systemic whereas RSV is restricted to the airways. The lungs are an immune-privileged organ. Even alveolar macrophages are a distinct subset of white blood cells of the lung that act to moderate immune responses to microbial infections. It is, therefore, possible that attenuated measles vaccines work because there is whole-body involvement during the infection. This would include major immune organs like the spleen and the thymus.

Lastly, there is a lack of data about RSV strains and epidemiology, but presently many research groups are contributing to epidemiological data on RSV.

4.6.3 The Lack of Immunogenicity, Immune Evasion, or Immune Suppression

Research on the immune response of humans to RSV shows high levels of antibodies to RSV but no protection from infection. For reasons still unexplained, humans cannot raise the antibody titer high enough to gain complete immunity to the virus. In addition, mucosal antibody levels and memory are inadequate or nonexistent [102].

Currently, vaccine strategies use novel adjuvants, virus-like particles (VLPs), and vector vaccines. Most current vaccines use a purified F protein. Currently, there are 6 RSV vaccines in phase 2 and 14 in phase 1 with many in early development stages. The pipeline of RSV vaccine candidates ranges from whole-inactivated, live attenuated, particle- and subunit-based or different RSV glycoproteins, nucleic acid vaccines, recombinant vectors, and more immunoprophylactic monoclonal antibodies in the hope of improving the RSV treatment and prophylaxis field.

4.6.4 Recent RSV Vaccines

In the current pipeline, we have seen the failure of ResVAX-Novavax's RSV F subunit vaccine in early 2019. This preparation is a nanoparticle vaccine with recombinant RSV-F. In its prefusion conformation, the RSV-F is in a quasi-stable state so that it will rapidly and spontaneously undergo conversion to the post-fusion form. Therefore, the native RSV-F of ResVax will likely be predominantly post-fusion, thus minimizing presentation of the principal Ø neutralization epitope. The FDA has required another trial to test clinical equipoise for the reappropriation of the product as one that reduces the hospitalization of infants.

4.6.4.1 Success in Secondary Endpoints

The maternal immunization trial with ResVax concluded in Spring 2019 and achieved its secondary endpoint, which was a reduction in hospitalizations due to LRTI. Here, ResVax also protected from the serious cyanosis hypoxia complication of RSV LRTI in neonates. As discussed earlier, there was a reduction in all-cause LRTI. This is consistent with RSV as a complication of other infections and health problems, i.e. a major complication of preexisting disease burden.

4.6.5 A Resurgence in Attenuated Replication-Competent Vaccines

The development of the RSV reverse genetics system brought with it opportunities for attenuated RSV vaccine development [148–151]. Being a negative-stranded virus with relatively complex molecular biology, this system requires ectopic co-expression of numerous RSV genes with a helper virus. The helper virus is needed to provide the RNA-dependent RNA polymerase activity specific to negative-stranded virus replication. Thus, this system provides greater control over the viral genetic material that goes into making attenuated

vaccines and allows for inputting several attenuations and safety mechanisms to make reversion to fully virulent forms less likely.

One technology involves codon deoptimization by altering codons of RSV NS1 and NS2 virulence genes from a virus to a human codon bias [148]. The attenuated deoptimized virus protected mice from challenge strains and was more immunogenic than WT RSV. Currently, PATH tracks the RSV vaccine and antibody progress that occurs.

4.6.5.1 Mechanisms of Protection from RSV that Require Attention in Vaccine Development

Mice are currently the most prominent model for T cell research in humans. T cells can clear RSV and shorten the duration of viral shedding. Paradoxically, T cells can also enhance disease. There have been studies showing that when T cells are depleted, symptoms are reduced in certain conditions [152]. Also, adoptive transfer T cells can cause more disease. In conclusion, it is not clear what T cells' role in RSV infection is.

Once the lower airways become infected with RSV, CD8 T cells are essential for viral clearance [153]. However, during Th2 responses, poor outcomes may be associated, such as an enhanced respiratory disease. Enhanced respiratory disease is also partially due to an inappropriate T cell response, more specifically due to Th2 cells [136]. To assess the safety of the vaccine candidate, developers are required to study Th1 and Th2 responses.

Another issue is the duration of protective immunity: When looking at correlates of protection, Hall et al. rechallenged 15 individuals with RSV for two years [105]. Test persons were susceptible to the same strain of RSV even after a few months of clearing their infection, although antibodies were detected in their responses. There appears to be a plateau for the boosting of antibodies to a point where total immunity to the virus cannot be acquired.

4.7 Antivirals and Therapeutic Antibodies

4.7.1 Immunoprophylaxis

The only success in getting a pharmaceutical product to market.

4.7.2 RSV-IGIV

Since vaccine progress was slowing down due to the 1960s' vaccine incident with two infant deaths, RSV treatment development turned to safer options. In the 1980s, RSV researchers were testing cotton rat models for intravenous immunoglobulin [154]. In the early 1990s, the first trials for RSV hyper immunoglobin (RSV-IGIV), a polyclonal human intravenous antibody pooled preparation, took place. The pilot study showed that IGIV is safe and effective in infants for the prevention of severe RSV disease.

IVIG was the first successful prophylaxis developed for severe RSV infection in infants [155, 156]. Furthermore, this became the first time that IGIV was used to prevent a viral infection. In 1996, the American Food and Drug Administration (FDA) agency approved

RSV-IGIV [157]. IGIV became the first approved agent for RSV in high-risk infants and to treat children under the age of 2 at risk for severe RSV.

In some patient populations, there were concerns over the safety and efficacy of RSV-IGIV. The official guidelines in 1997 recommended only using the preparation in infants under 32 weeks because of data showing the highest efficacy in infants under 6 months and potential complications in children undergoing surgery [158].

In 2003, RSV-IGIV was pulled from the market after the licensing of the first monoclonal antibody for RSV-Palivizumab in 1998. However, RSV-IGIV had paved the way for humanized monoclonal antibodies to be used in treatment regimens.

4.7.3 Palivizumab

In the late 1990s, after the efficacy of RVS-IGIV against RSV severe LRTI and hospitalization was shown, pharmaceutical companies began making monoclonal antibodies against the RSV F protein. They also sought to make the injection intramuscular as opposed to IV, which is more difficult and prone to complications in infants. Three monoclonal antibodies were developed for RSV. The first by SmithKline Beecham (now GSK) [138], SB 209763, showed safety and good tolerability but lacked efficacy due to the low dosing schedule in their phase 3 trial. OraVax made the second antibody, HNK20, which displayed a lack of efficacy in a rhesus monkey trial [159]. Lastly, MedImmune created MEDI-493, a monoclonal antibody that was potent and broadly reactive to the F protein of RSV and was more potent than RSV-IGIV. MEDI-493 (Palivizumab) was later marketed for the prevention of RSV hospitalization [160].

Palivizumab (Synagis®) was approved in 1998 by the FDA; it is a humanized monoclonal antibody administered through intramuscular injections (as opposed to intravenous IGIV) at 15 mg/kg monthly for infants, who according to the guidelines have a high risk for bronchiolitis.

We are expecting a soon to be approved, new mAb from MedImmune, that is 100 times more potent than Palivizumab and would be given once a season rather than monthly. Palivizumab has a half-life of 20–30 days; therefore, high-risk infants have to be brought into hospitals and clinics every month during the RSV season to make sure they have a high-enough concentration of antibodies in their bodies to be protected from severe RSV infection. With the new mAb from MedImmune, the half-life is said to be 70–100 days, which would mean one visit to the clinic during the RSV season would be sufficient [161].

Although Palivizumab reduces hospitalization by as much as 80%, it does not prevent the URT infection [162]. Palivizumab does, however, protect against severe lower respiratory tract disease, lowering the incidence of infant hospitalization, though the cost to benefit ratio provided by widespread Palivizumab use has been questioned in the literature [163–165].

4.8 Therapeutics for Treating Active RSV Infections (Table 4.1)

Currently, only two drugs are approved by the US FDA for RSV infections: Palivizumab for prophylaxis and aerosolized Ribavirin for treatment. Ribavirin is a broad-spectrum

nucleoside analogue that was created in 1972 and found to affect infections with poliovirus, hepatitis C virus (HCV), RSV, and several other RNA viruses. Different mechanisms have been associated with the use of Ribavirin in the context of HCV treatment [166]. The triphosphate form of Ribavirin is accommodated by the RNA-dependent RNA polymerase, which can affect both efficacy and fidelity of viral genome synthesis. In 1986, the FDA approved Ribavirin for aerosol treatment of RSV infection in children in the United States.

Evidence for the clinical benefit of aerosolized Ribavirin has been inconclusive. Health care providers' perceptions of limited benefits and the cumbersome requirement for scavenging apparatus and concerns about mutagenicity, carcinogenicity, and teratogenicity have resulted in infrequent use. Currently, Ribavirin is used mainly when the outcome of an RSV LRTI could be fatal, typically in the immunocompromised. Recent studies showed similar outcomes comparing aerosolized versus oral Ribavirin, which was proposed as an alternative with better tolerance, lower cost, and easier application [130, 167].

Available data suggest that immunocompromised patients, who receive these treatments in various combinations may have a lower rate of progression to LRTI and better clinical outcomes. Treatment can be considered, though the level of evidence is weak to moderate [168, 169]. It is unknown whether these approaches can be applied to older, non-immunocompromised adults. A recent randomized placebo-controlled trial on RSV bronchiolitis in 420 infants showed that single-dose intravenous Palivizumab (15 mg/kg) did not result in faster hospital discharge or in preventing readmission [170]. Another placebo-controlled trial ($n = 429$) showed that Palivizumab for RSV prophylaxis in infants did not have a significant impact on current asthma or lung function at the age of six years [122].

4.8.1 Immunotherapy under Clinical Development

RI-001 (Table 4.1) is an IVIG prepared from plasma donors who have high titers of neutralizing anti-RSV antibodies. RI-001 was evaluated in a phase 2a placebo-controlled trial in immunosuppressed RSV-infected patients at risk of LRTI, but no data have been published. Compassionate use experience in 15 immunocompromised patients with RSV LRTI was associated with a significant increase in neutralizing antibody titers and clinical improvement, especially if given early [171].

RI-002 (Table 4.1) is an Intravenous Immune Globulin (IVIG) approved by the US FDA in April 2019 for the treatment of Primary Humoral Immunodeficiency Disease in adults and adolescents (12–17 years of age). It also has standardized elevated levels of RSV-neutralizing antibodies (from plasma donors who have high titers of neutralizing anti-RSV antibodies), and elevated antibody titers to other respiratory viruses (e.g. influenza, parainfluenza, coronavirus, metapneumovirus). In a multicenter, phase 3 trial ($n = 59$), its regular infusion over one year was associated with significantly reduced risk of severe infections, and it was well tolerated [172]. Plans on its further study for RSV prophylaxis or treatment are unknown.

Motavizumab (MEDI-524, Table 4.1), a second-generation monoclonal antibody derived from Palivizumab, was studied in a placebo-controlled trial in infants hospitalized for RSV LRTI. It was not shown to impact on viral load or clinical severity [173]. In a phase 3 prophylaxis trial in high-risk children, it was associated with hypersensitivity reactions in

some infants and consequently failed to obtain US FDA approval, although Motavizumab was shown to be more effective than Palivizumab.

MEDI-8897, Table 4.1 is a third-generation RSV-specific monoclonal antibody that targets the prefusion conformation of the RSV F protein, with an extended half-life (62.5–72.9 days). Data from a phase 1 trial in healthy adults showed a favorable safety profile [174]. In a phase 1b/2a dose-escalation study ($n = 89$), healthy preterm infants, who received a single dose showed a significant rise of serum RSV-neutralizing antibody levels compared with placebo [175]. A phase 2b trial on efficacy and safety of "once-per-season" prophylactic regimen against medically attended RSV LRTI in healthy preterm infants has been completed (NCT02878330; granted Breakthrough Therapy Designation by US FDA in February 2019).

4.9 Drug Targets

There have been decades of research on the replication and the structural components of RSV. The virus encodes 10 genes that are responsible for being translated into 11 proteins.

4.9.1 Novel Antivirals Undergoing Clinical Development

Presatovir (GS-5806, Table 4.1) is a potent small-molecule inhibitor that targets the RSV F protein by inhibiting F protein-mediated cell-to-cell fusion. Prior studies with GS-5806 in healthy adults challenged with intranasal RSV demonstrated reduced viral load and severity of clinical disease [130, 176–178]. Preliminary results from a recent phase 2 study conducted in HSCT recipients with either upper respiratory (URTI, $n = 189$) or lower respiratory tract RSV infection (LRTI, $n = 60$) were encouraging. URTI patients treated with GS-5806 demonstrated a significant decrease in time-weighted average nasal RSV RNA load over nine days compared with placebo, and reduced LRT complication progression rates (9 vs. 20%, $p = 0.04$), especially when being treated early. However, virological and clinical endpoints were insignificant in patients with established LRTI. Treatment is generally well tolerated. Several newly identified treatment-emergent RSV F substitutions (e.g. T400A/I, F140I, L138I) were found to map to interaction sites between the drug and RSV F. Resistance occurs rapidly to drugs by RNA viruses such as RSV, if the drug targets a non-conserved site on the protein. Human clinical trials were declared suspended, "on hold," in autumn of 2018 for undeclared reasons.

Lumicitabine (ALS-008176 or JNJ-64041575, Table 4.1) is an oral nucleoside analogue prodrug. The triphosphate form of the parent compound is a selective inhibitor of the RSV RNA polymerase that causes chain termination [179]. A human RSV challenge model showed rapid viral clearance and significant reduction of viral load, accompanied by improvements in the severity of clinical disease compared with negative controls [180, 181]. Human clinical trials were suspended, "on hold," in autumn of 2018 for undeclared reasons. Data on ongoing/completed clinical trials in adults and infants have not been reported (NCT02673476, NCT02935673, EudraCT number 2013-005104-33).

Rilematovir (JNJ-53718678, Table 4.1) is a small-molecule RSV fusion inhibitor. In a phase 2a adult RSV challenge study [182], oral treatment was shown to reduce viral load and mean overall symptom scores [183]. Reported treatment-emergent adverse events (grade 1 or 2 electrocardiogram change) require further study. Phase 1b and phase 2 placebo-controlled trials in adults and infants have been initiated (NCT03379675, NCT03656510).

SiSunatovir (RV521, Table 4.1) is an oral small-molecule fusion inhibitor designed to target RSV-F that mediates RSV binding to cellular receptors. Preliminary results from a randomized, placebo-controlled phase 2a study showed that oral treatment decreased RSV viral load and reduced disease severity in 66 adult participants in a virus challenge model (NCT03258502) [184].

Ziresovir (AK0529, Table 4.1) is a fusion inhibitor, active against RSV A & B. Preliminary results from a randomized, double-blind, placebo-controlled phase 2 trial in children 1–24 months and hospitalized with RSV showed a dose-dependent decrease in symptom score at 24 hours after a single dose, and no major safety signal was reported (NCT02654171). A phase 2 trial is ongoing (NCT03699202) [177].

4.9.2 Other Investigational Treatments and Broad-Spectrum Antivirals

RSV M2-1 inhibitor (Table 4.1), a transcription anti-termination factor, has been identified as a new target for RSV therapeutic intervention. Small molecules such as cyclopamine have been shown *in vitro* to inhibit the postentry phase of viral replication, reduce transcription of downstream genes in RSV replication, and disrupt IBAG (inclusion body-associated granules formation). The drug suppresses RSV lung infection in the mouse model by blocking transcription [177, 185, 186].

EDP-938 (Table 4.1) is a novel non-fusion replication inhibitor of RSV with a high barrier to resistance. Phase 1 and 2 trials are ongoing (NCT03691623).

EIDD-1931 (Table 4.1) (β-d-N^4-hydroxycytidine or NHC) is an orally available ribonucleoside analogue inhibitor with activity against RSV and several other RNA viruses. The putative mechanism of action is the induction of excessive mutations that lead to error catastrophe and, ultimately, the inability to replicate. The drug was shown to have a high resistance barrier. Animal models indicated efficacy in reducing viral load and symptoms [187].

VH244 (Table 4.1) is a novel broad-spectrum antiviral. The mechanism of action is a "Therapeutic Interfering Particle," modeled after defective, nonreplicating, influenza virus particles [188]. It has been shown *in vitro* to reduce replication of influenza viruses through genomic interference, and inhibit RSV and human rhinoviruses through host innate immunity and activation of the cell antiviral state. A surrogate murine model for RSV showed protection from the disease if given within four days postinfection [177].

Other broad-spectrum antivirals with *in vitro* activity against RSV include **Remdesivir, Favipiravir, and Nitazoxanide (Table 4.1)**. However, data specific to RSV infection is lacking [189–191]. Remdesivir is an adenosine analogue prodrug that is incorporated by the viral RNA polymerase and causes delayed chain-termination further downstream [192]. Remdesivir was developed for the treatment of Ebola Virus Disease (EVD) and is currently assessed in clinical trials for Covid-19 [177, 193]. In October 2020, the FDA approved

Remdesivir for use against SARS CoV-2 to combat Covid-19. Remdesivir is currently used worldwide with reports stating quicker recovery for patients who are hospitalized with Covid-19 [193]. Favipiravir is approved for the treatment of Influenza in Japan. The drug is structurally related to Ribavirin. Favipiravir was assessed in clinical trials against SARS-CoV-2 with minimal-to-no success. More studies are ongoing and necessary to conclude this is an effective drug against Covid 19 [194].

4.10 Conclusions

The recent partial successes in RSV vaccine development constitute a significant step forward in realizing a therapy that can confer, at least, passive immunity to the most vulnerable. However, the research community has not yet shed light on the mechanism for memory IgA B cell deficiency in the context of RSV infection. This aspect of RSV replication and biology will likely have to be elucidated before transmission-limiting vaccines can be developed. With the potential for RSV antivirals and vaccines coming along in the next decade, there may be viral factors that mutate in response to drug pressure. For example, even with the limited use of Palivizumab in high-risk infants, RSV already has shown mutations in the Palivizumab binding site that confers viral resistance to the antibody [195]. To create a vaccine: the prefusion RSV-F confirmation with a focus on anti-RSV nasal IgA levels and new live-attenuated vaccine approaches with adjuvant strategies are likely the way forward.

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5

Herpes Simplex Viruses

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5.1 Introduction

Like all members of the family of the herpesviridae, herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are enveloped, double-stranded DNA viruses [1]. Their genome size is approximately 152 kb and comprises at least 74 open reading frames. The genome consists of two unique segments, the unique long (U_L) and the unique short (U_S) regions, flanked by inverted repeat sequences. Together with the closely related varicella zoster virus (VZV), HSV-1 and -2 represent the human pathogenic members of the *alphaherpesvirinae* subfamily which share the prominent feature of latency in neurons [2]. HSV is transmitted via infectious viral particles derived from either active lesions, or (especially in the case of HSV-2) from asymptomatic shedding [3, 4]. The primary infection typically occurs during childhood in the case of HSV-1, or later during adolescence in the case of HSV-2, since the latter virus is mainly transmitted sexually [5]. HSV normally enters the body via skin or mucosa and can lead to local lesions at the site of infection, which may or may not be accompanied by clinical signs of disease. Once it has entered the host, the virus spreads to the neurons of the peripheral nervous system, where it establishes a latent and life-long infection (a notable feature of all herpesviruses) [1]. Historically, HSV-1 was associated with infection "above the belt" (i.e. mouth and eye, where the virus persists in the trigeminal ganglion), whereas HSV-2 was attributed to infection "below the belt," specifically genital herpes, by infecting the sacral ganglion [6]. However, nowadays, there is a significant overlap between the sites of HSV infection with an increasing proportion of genital herpes caused by HSV-1, which may be attributed to a decrease in childhood HSV-1 infections and an increase in the practice of oral sex [7, 8]. Once neuronal latency has been established, HSV may be reactivated by an unknown molecular trigger and spread from the ganglion to the lower epidermal layers at or near the site of the initial infection where it replicates. The severity of such a recurrence can range from asymptomatic (only recognized by the ability to detect the virus on the skin) to painful ulcers, such as the typical orofacial cold sores or genital lesions. HSV outbreaks can substantially impact patients'

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quality of life; they can be painful and stigmatizing, leading to distress and even psychosocial problems [9, 10]. Ocular HSV-1 infections can result in visual impairment [11]. Especially for genital herpes, it could be shown by quantitative polymerase chain reaction (PCR) analysis that these recurrences can occur frequently as asymptomatic episodes of viral shedding [3]. If occurring frequently, these shedding events can result in a high risk of transmission, even when no symptoms are present. At least 70% of HSV-2 transmissions take place during periods of asymptomatic shedding [12].

Normally, an HSV recurrence is self-limiting as it is rapidly cleared by the immune system, mainly via the cellular immune response [13, 14]. However, HSV reactivation can cause severe and sometimes even life-threatening infections in immunocompromised individuals (especially in those with drug-resistant infection, see Section 5.7) that can last for several months [15]. Furthermore, newborns born to an HSV-positive mother are at risk of acquiring a congenital infection that can manifest in a so-called skin, eye, and mouth infection, herpes simplex encephalitis, or a disseminated disease affecting multiple organs. Notably, even immunocompetent patients can develop herpes simplex encephalitis which mainly affects either patients between 6 months and 20 years of age or patients that are older than 50 years [16]. Especially, these severe infections, but also the stigmatizing labial or genital manifestations, represent a high medical need requiring effective treatment options to control the virus. The overall global prevalence for HSV-1 and HSV-2 infections is estimated to be 67 and 11%, respectively, with the highest rate in the age group 45–49 years (79 and 17%, respectively) [7, 8].

5.2 Overview of the Viral Replication Cycle

Like all herpesviruses, HSV is an enveloped virus, i.e. the virus particle is surrounded by a membranous lipid bilayer. The virus enters its target cell by attaching to cell surface heparan sulfate proteoglycans followed by specific and sequentially aligned interaction of viral glycoproteins with their cellular receptors and then by fusion of the viral and cellular membrane either directly at the plasma membrane or after endosomal uptake [17, 18]. The main viral proteins involved in these processes are the glycoproteins gB (responsible for attachment, together with gC), gD (required for receptor binding), and the heterodimeric complex gH/gL essential for fusion of the virion with the host cell membrane, which is triggered by a cascade of interactions with gB and gD. The cell–cell adhesion molecules nectin-1 and nectin-2, the tumor necrosis factor receptor-related molecule herpesvirus entry mediator (HVEM), and 3-*O*-sulfonated heparan sulfate have been identified as cellular receptors for HSV. After entering the host cell, the tegument-coated capsid is transported to the nuclear pores and thus into the nucleus where transcription and replication of the viral genome and assembly of progeny capsids take place. The viral particle includes several proteins essential for viral DNA replication, such as the DNA polymerase and the helicase–primase complex, both of which are involved in generation of new genomic DNA. It also includes several nonessential enzymes required for nucleotide biosynthesis and DNA metabolism, such as the viral thymidine kinase (TK) and uracil-DNA glycosylase [19]. After initiation, viral DNA synthesis follows a rolling-circle mechanism [20], producing concatemeric molecules that are later cleaved during nucleocapsid assembly by the terminase, another

essential viral protein complex, see also Chapter 6 in this edition. Assembly of the viral capsids and packaging of the viral genome occur in the nucleus and are followed by nucleocapsid egress. In the current model, readily formed capsids bud at the inner nuclear membrane into the perinuclear cleft, thus acquiring an envelope membrane. This primary envelope is then fused with the outer nuclear membrane leading to a release of naked capsids into the cytosol (summarized by [21]). Assembly of the final particle including secondary envelopment and tegumentation occurs by budding into cellular vesicles containing viral glycoproteins on the lumen side and tegument proteins on the cytosolic side. Virions are then released from the cell by fusion of the cellular vesicles with the plasma membrane.

5.3 Treatment of HSV Infections

HSV establishes persistent infections that can recur from time to time. Therefore, treatment can be required for the first infection, called primary (infection of a HSV negative person), for initial/non-primary (if an individual is already infected by one HSV type and acquires the other type), or for a recurrent infection. Since the primary or initial infection can be asymptomatic or display rather nonspecific symptoms [22], most prescribed treatments are typically used for the recurrences. Nonetheless, especially for genital herpes, the first episode may be prolonged and more painful compared to later recurrences [5]. Therefore, if recognized sufficiently early, antiviral treatment should be given to shorten these episodes. In recurrent infections, the majority of viral replication occurs during the first 24 hours of an acute outbreak. Therefore, it is important to treat a recurrent episode as early and effectively as possible ("Hit 'em early, hit 'em hard" strategy) to prevent the virus from further replication and thus to minimize tissue damage [23]. However, if the antiviral medication is not taken early enough, the course of infection can no longer be influenced by direct acting antivirals. Therefore, for labial herpes, one approved treatment option combines the antiviral drug acyclovir (see Section 5.4.3) with hydrocortisone in order to treat both the viral replication and the accompanying inflammation [24].

It is important to note that the reduced immune response in immunocompromised patients means that HSV recurrences can be longer and more severe than recurrences in the immunocompetent, and increases the likelihood of resistance development, as there is no additional defense against the virus besides antiviral treatment [15]. An alternative to the so-called "episodic" treatment described above (i.e. initiating treatment as soon as an active outbreak is recognized) is the use of suppressive treatment (i.e. to treat with an antiviral drug even in the absence of symptoms to prevent reactivation of viral replication and the associated potential recurrence from starting). For immunocompetent patients with genital herpes, especially those with frequent recurrences, suppressive treatment has been shown to be effective [25, 26]. HSV suppression is also a standard clinical practice for patients undergoing transplantation in order to prevent HSV replication under immunosuppression [27]. Furthermore, it could be shown that suppressive HSV treatment with valacyclovir is accompanied by a reduction of asymptomatic shedding leading to reduced transmission of genital HSV of about 50% [28].

5.4 Approved Anti-HSV Drugs

As described in the following section, apart from 1-docosanol, which targets the cellular uptake of the virus, almost all approved anti-HSV drugs target the viral DNA replication. This applies to previously and currently approved medications as well as to small molecule drugs currently in development. Since the virus comes with its own replicative machinery, this feature can be exploited to enhance selectivity and specificity of the treatment and thereby to reduce the risk of unwanted off-target toxicities, provided that no activity against the human enzymes can be demonstrated. An overview of the targets of antiviral drugs in the HSV replication cycle is shown in Figure 5.1.

5.4.1 5-Substituted 2-Deoxyuridine Analogues

Though not the first antiviral compound ever described, idoxuridine (5-iodo-2-deoxyuridine, Figure 5.2) was the first antiviral drug to gain marketing authorization [29]. Originally described as an antitumor agent for treatment of leukemia, it was later empirically determined that the thymidine analogue is also active against DNA viruses [30]. While it was too toxic for systemic administration, it could be shown that when administered locally at high concentrations, it was able to treat HSV keratitis, i.e. HSV infection of corneal cells [31]. Thereafter, the anti-HSV keratitis activity of trifluridine (trifluorothymidine, Viroptic™, Figure 5.2), another member of the same group, was also demonstrated [32]. However, idoxuridine and trifluridine cannot be considered as specific antiviral agents. Although there may be some specific dependence on the viral TK, both compounds can be

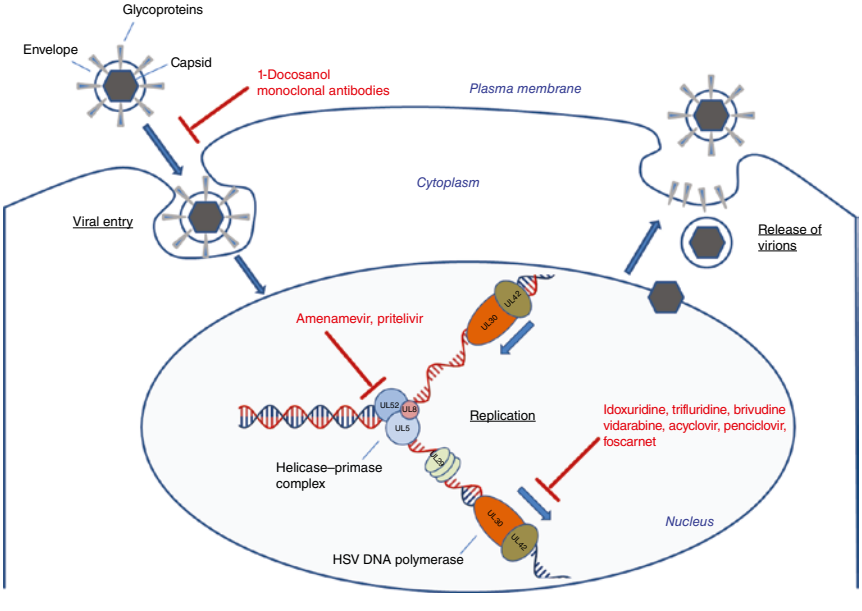


Figure 5.1 Schematic overview of the HSV replication cycle highlighting steps targeted by antiviral drugs.

phosphorylated by viral and cellular kinases to the 5-triphosphate (TP) (i.e., idoxuridine) or 5-monophosphate (i.e., trifluridine) forms, respectively. Both idoxuridine and trifluridine can thus inhibit viral as well as the cellular DNA synthesis, leading to unwanted toxicities (summarized by [33]). Trifluridine is still on the market in the United States for HSV keratitis (https://www.accessdata.fda.gov/scripts/cder/daf/ [02 March 2021]). Interestingly, in combination with the thymidine phosphorylase inhibitor tipiracil, trifluridine is also approved for oral treatment of colon cancer (Lonsurf™, [34]). Idoxuridine is no longer available due to its toxicity. In contrast to idoxuridine and trifluridine, the thymidine analogue brivudine (5-[2-bromovinyl]-2'-deoxyuridine, BVDU, Zostex™, Figure 5.2) is highly specific in its activity against HSV-1 and VZV (but not HSV-2) [35]. This specificity is derived from the fact that brivudine is specifically phosphorylated by the TK of HSV-1 and VZV, converting it to either the mono- or di-phosphate forms, which are subsequently transformed to the active TP by cellular kinases. Brivudine is approved in several European countries for treatment of VZV infections (shingles) and HSV keratitis.

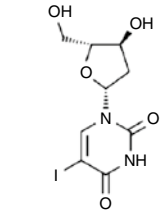
5.4.2 Vidarabine

The first drug licensed for systemic treatment of herpesvirus infections (HSV and VZV) was the adenosine analogue vidarabine (9-beta-D-arabinofuranosyladenine, Ara-A, Figure 5.2), which is phosphorylated by cellular enzymes to the TP [36, 37]. It has been shown that besides activity against VZV, vidarabine was also effective in herpes simplex encephalitis in a clinical trial [38]. The half-life of the active TP metabolite (ara-ATP) is three times longer in HSV-infected cells compared to uninfected cells, though the mechanism for this selectivity is unknown. However, vidarabine is more toxic and less metabolically stable than many of the other current antivirals such as acyclovir (see Section 5.4.3), and is therefore no longer available for systemic application. Of note, a topical preparation still remains on the market for the treatment of HSV keratitis in some countries.

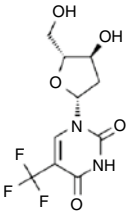
5.4.3 Acyclic Guanosine Analogues

Brivudine is not the first drug that is specifically activated by the viral TK enzyme. The breakthrough came with the discovery of the acyclic guanosine analogue acyclovir (acycloguanosine, Zovirax™, Figure 5.2), which became the gold standard for HSV therapy [39, 40]. In HSV- or VZV-infected cells, the viral TK, normally responsible for phosphorylating the pyrimidine base thymidine, surprisingly converts the purine analogue acyclovir to its monophosphate (nicely summarized in [41]). Besides mediating specificity to virus-infected cells, this conversion into a charged molecule has another beneficial effect, as it is trapped inside the cells leading to prolongation of its intracellular half-life. Acyclovir is very well tolerated and for systemic application, it can be used both orally and intravenously. The latter has the advantage that much higher systemic exposure can be reached since acyclovir has a poor oral bioavailability of only approximately 20% [42]. An intravenous formulation of acyclovir for the treatment of severe cases, e.g. immunocompromised patients, herpes simplex encephalitis, and neonatal infection, is available [43, 44].

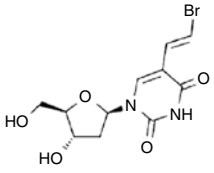
To overcome the issue of low oral bioavailability, the valine ester prodrug valacyclovir (Valtrex™; Figure 5.2) was developed, which exhibits an improved bioavailability of



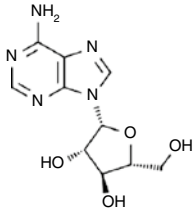
Idoxuridine



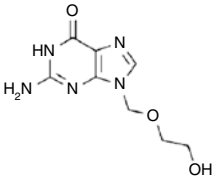
Trifluridine



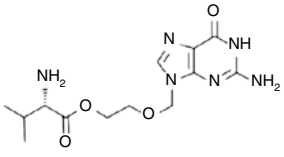
Brivudine



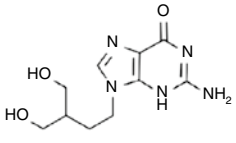
Vidarabine



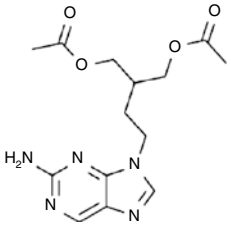
Acyclovir



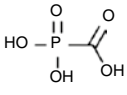
Valacyclovir



Penciclovir

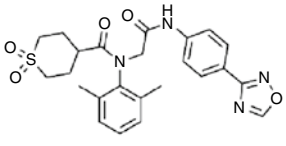


Famciclovir

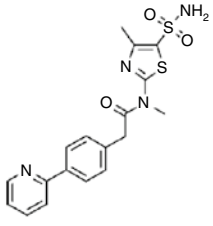


Foscarnet

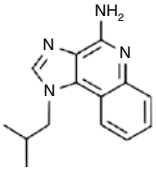
1-Docosanol



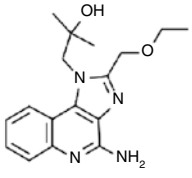
Amenamevir



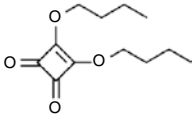
Pritelivir



Imiquimod



Resiquimod



Squaric acid dibutyl ester
(SADBE, SQX770)

Figure 5.2 Chemical structures of anti-HSV drugs.

approximately 55% [45]. Acyclovir and valacyclovir are approved for the treatment of primary and recurrent labial herpes, as well as for both episodic and suppressive treatment of genital herpes (see prescribing information for Zovirax™ and Valtrex™, https://www.accessdata.fda.gov/scripts/cder/daf/ [02 March 2021]). Moreover, based on a large trial with almost 1500 HSV-discordant couples, valacyclovir has also been approved in the United States for reduction of transmission of genital herpes [28]. Besides systemic use, acyclovir is also available in topical formulations for the treatment of cold sores (ointment) and HSV keratitis (eye drops).

Another acyclic guanosine drug with a very similar profile to acyclovir that is also specifically activated by the viral TK is penciclovir (Denavir™, Figure 5.2). Penciclovir has a somewhat lower antiviral activity against HSV in cell culture compared to acyclovir. This is compensated, however, by the higher intracellular stability of the TP ester of penciclovir, leading to an overall comparable efficacy of both nucleoside analogues [46]. Since the oral bioavailability of penciclovir is even lower than that of acyclovir, it is mainly used as topical treatment for labial herpes. Only the diacetyl 6-deoxy prodrug of penciclovir, famciclovir (Famvir™, Figure 5.2) is used for systemic treatment [47], being approved for treatment of labial herpes as well as for treatment and suppression of genital herpes (see prescribing information for Denavir™ and Famvir™, https://www.accessdata.fda.gov/scripts/cder/daf/ [02 March 2021]).

5.4.4 Foscarnet

Foscarnet (trisodium phosphonoformate, Foscavir™, Figure 5.2) was approved for clinical use in the United States in 1991 [48]. Current indications include therapy of cytomegalovirus (CMV) retinitis and acyclovir-resistant HSV infections in immunocompromised individuals (see prescribing information for Foscavir™, https://www.accessdata.fda.gov/scripts/cder/daf/ [02 March 2021]). Foscarnet selectively acts at the pyrophosphate binding site on virus-specific DNA polymerases at concentrations that do not affect cellular DNA polymerases [49]. Foscarnet does not require activation by TK or other kinases, and is therefore active against HSV strains carrying mutations in the TK gene that mediate resistance to acyclovir (see Sections 5.6.2 and 5.6.3). However, cross-resistance to acyclovir mediated by mutations within the HSV DNA polymerase itself can still occur [50]. Foscarnet use is limited by poor oral bioavailability of less than 22% necessitating intravenous administration 2–3 times a day [51]. Furthermore, foscarnet can cause severe side effects, mainly nephrotoxicity, mineral and electrolyte abnormalities, as well as seizures (see prescribing information for Foscavir™, https://www.accessdata.fda.gov/scripts/cder/daf/ [02 March 2021]). The mechanism behind the toxicities of foscarnet is not fully understood but its ability to form complexes of divalent ions such as Ca^{2+} is considered as potential reason at least for the mineral and electrolyte imbalance [52, 53].

5.4.5 Docosanol

The only approved anti-HSV drug that does not target the viral replication directly is 1-docosanol (Abreva™; Figure 5.2). The proposed mechanism of action is inhibition of viral entry by preventing cellular uptake of the virus, i.e. the fusion of the viral envelope

with the cell membrane [54]. Docosanol has been approved by the FDA for topical treatment of labial herpes even though its clinical efficacy remains controversial [55]. Interestingly, in contrast to all other approved anti-HSV drugs, it is available over-the-counter in the United States (see prescribing information for Abreva™, https://www.accessdata.fda.gov/scripts/cder/daf/ [02 March 2021]).

5.5 Anti-HSV Drugs in Advanced Development or Recently Entering the Market

Except for the helicase–primase inhibitors, the focus of new anti-HSV drugs at advanced stages of development is immunological, using either a direct (monoclonal antibodies) or indirect (therapeutic vaccines and immunomodulators) mechanism of action.

5.5.1 Helicase–Primase Inhibitors

As the name says, this class of compounds target the viral helicase–primase enzyme complex which herpesviruses need to open the DNA strands and keep them separated during DNA replication (Figure 5.1) [56]. As there is no cellular counterpart for this machinery, which herpesviruses share—at least mechanistically—with certain bacteriophages [57], it is a good target for new anti-HSV drugs. Furthermore, as for foscarnet, helicase–primase inhibitors do not require activation by TK or any other viral enzyme. Consequently, helicase–primase inhibitors can be protective to uninfected cells and are active against HSV strains with mutations in the TK or DNA polymerase genes that mediate resistance against acyclovir, penciclovir, or foscarnet [58] (see Section 5.6.3). Two helicase–primase inhibitors are currently in clinical development: amenamevir and pritelivir. Amenamevir (ASP2151, Amenalief™, Figure 5.2), which was originally discovered by Astellas, is an oxadiazolephenyl derivative active against both HSV and VZV [59]. In a Phase 2 study in patients with genital herpes, the efficacy of once-daily amenamevir in shortening the HSV episode duration was comparable to that of valacyclovir [60]. However, Astellas discontinued the development of amenamevir for HSV infections in the United States and the drug was subsequently licensed to Maruho, who conducted a Phase 3 trial in herpes zoster patients and gained approval for this indication in Japan [61]. At the time of this review, three clinical trials with amenamevir were registered in Japan, one Phase 3 trial in patients with an acute episode of genital herpes (JapicCTI-194955), one Phase 3 trial in patients with an acute episode of labial herpes (JapicCTI-194954), and one pilot trial investigating oral amenamevir against epithelial herpetic keratitis resistant to acyclovir ointment therapy (jRCTs061190001).

The second helicase–primase inhibitor is pritelivir (BAY 57-1293, AIC316, Figure 5.2), which was originally discovered at Bayer AG [62] and is currently in clinical development by AiCuris Anti-Infective Cures GmbH. In contrast to amenamevir, the thiazolylamide pritelivir specifically inhibits HSV-1 and HSV-2 and has no activity against VZV. In two Phase 2 trials in otherwise healthy adults with recurrent genital herpes, 28 days oral treatment with pritelivir once-daily demonstrated superior suppression of viral shedding and genital lesions compared to both placebo and oral valacyclovir [63, 64]. A Phase 2 trial for

the treatment of acyclovir-resistant mucocutaneous HSV infections in immunocompromised patients was ongoing in the United States at the time of writing and also included patients that are either acyclovir and foscarnet resistant or acyclovir resistant and foscarnet intolerant (NCT03073967). Furthermore, several different solid formulations are existing for potential use in various other sub-indications [65].

5.5.2 Monoclonal Antibodies

According to the Cortellis Database [Clarivate Analytics, https://www.cortellis.com/ (02 March 2021)], two monoclonal antibodies are currently in development for HSV infections, HDIT101 and UB621 (Figure 5.1). HDIT101 (Herpevizumab, Heidelberg ImmunoTherapeutics) targets an epitope on gB present on both HSV-1 and HSV-2, and is reported to neutralize cell-free HSV particles as well as to prevent cell-to-cell propagation of the virus [66]. At the time of this article two trials with HDIT101 were ongoing in Germany. The first compared one single intravenous dose of HDIT101 vs. valacyclovir in patients with anogenital HSV-2 infection with the primary endpoint "percentage of days with lesion(s)" (NCT04165122), the second compared a topical formulation applied four times over two days vs. placebo in patients with cold sores with the primary endpoint "number of recurrences after 12 months" (NCT04539483). In contrast to HDIT101, UB621 (United BioPharma) targets the viral glycoprotein gD of both HSV-1 and HSV-2 (United BioPharma company web site, http://www.unitedbiopharma.com/ [02 March 2021]). Safety has been demonstrated in a Phase 1 clinical trial. At the time of this review, a Phase 2 trial in patients with genital herpes was ongoing in the United States with the primary endpoint of viral shedding at two different doses of subcutaneously administered UB621 vs. placebo (NCT03595995).

5.5.3 Therapeutic Vaccines

Several attempts have been made to develop a therapeutic vaccine against HSV. One of the most advanced candidates so far was GEN003 by Genocea Biosciences. GEN003 comprises recombinant HSV-2 gD and ICP4 proteins together with Matrix™ M2 adjuvant [67]. The respective antigens were selected by screening of T-cell responses in asymptomatic HSV-2 positive or HSV-2-exposed but uninfected subjects to identify potential targets involved in controlling and limiting an active infection. In several clinical trials in patients with genital herpes, a moderate reduction of viral shedding vs. placebo could be shown [68–70]. However, for strategic reasons, Genocea discontinued the development of GEN003 in 2017 (Genocea press release of 25 September 2017, https://ir.genocea.com/news-releases/news-release-details/genocea-announces-strategic-shift-immuno-oncology-and [02 March 2021]).

HSV529 is a replication-defective HSV-2 vaccine that has two genes deleted, UL5 and UL29 [71]. The results of a Phase 1 trial in both HSV-negative and HSV-positive participants conducted by the National Institute of Allergy and Infectious Diseases (NIAID) and Sanofi Pasteur have been reported recently [72]. HSV529 elicited neutralizing antibodies and a modest CD4+ T-cell response in HSV-seronegative vaccines. However, no results have been reported yet from a completed trial investigating the effect of HSV529 vaccination on immunological parameters and viral shedding in HSV-2 seropositive adults after valacyclovir treatment (NCT02571166).

5.5.4 Immunomodulators

Besides antibodies and therapeutic vaccines, immunomodulators have also been developed to tackle HSV infections. Notably, the imidazoquinolinamine immunomodulators and TLR-7 antagonists imiquimod (Aldara™, Figure 5.2), and resiquimod (Figure 5.2) were tested against HSV in the clinic [73]. The impact of topical resiquimod on genital herpes has been investigated in several clinical trials with generally rather moderate outcomes but has shown a certain degree of post-treatment efficacy after intermittent administration [74, 75]. There are many reports in the literature describing imiquimod as a treatment option for acyclovir-resistant HSV infections, though no conclusive data are available from controlled clinical trials [76–79]. Another immunomodulator currently in clinical development for HSV-1 infections is SQX770 (Squarex LLC; Figure 5.2), a topical formulation of squaric acid dibutyl ester (SADBE) for prevention of recurrent cold sores [80]. At the time of this review, two randomized, double-blind, placebo-controlled clinical studies had been conducted in patients with labial herpes (https://biotuesdays.com/2019/12/03/squarex-hopes-to-begin-pivotal-testing-to-prevent-recurring-cold-sores-in-2020/ [02 March 2021]). According to company data, it could be demonstrated that SQX770 was effective in extending the time between recurrences and to reduce severity of outbreaks when a single dose is applied to the upper arm (https://www.trialsitenews.com/squarex-announces-positive-results-from-phase-2-study-of-topical-sqx770-in-prevention-of-recurrent-herpes-labialis/ [02 March 2021]). The proposed mode of action is that SQX770 shifts the immune response toward a stronger type 1 cellular immune response against the virus. Shifts in gene expression profiles were also reported, from those associated with frequent recurrences to those associated with few or no HSV recurrences [81].

5.6 HSV Resistance to Antiviral Drugs

5.6.1 Epidemiology and Manifestation

Resistance to nucleoside analogues is rare in the immunocompetent, but their prevalence is higher in immunocompromised patients [82]. In otherwise healthy individuals with labial and genital herpes, resistance to the commonly used nucleoside analogues is rare with resistant virus found in 0.1–0.7% of the cases. Notably, higher rates of acyclovir resistance have been reported for HSV keratitis after prophylactic use of acyclovir, possibly because of the immune-privileged status of the eye, i.e. a limited inflammatory response [83]. The situation is different in immunocompromised patients such as human stem cell transplant recipients or solid organ transplant recipients and in HIV patients on suboptimal antiretroviral treatment. Here, an average resistance rate to nucleoside analogues of 3.5–14% has been reported with some publications noting rates up to 36% [84]. HSV outbreaks in immunocompromised patients can manifest as atypical lesions which can be enlarged with deeper and more extensive ulceration and which may develop in atypical areas [15]. HSV may spread to other target sites in those patients to cause pneumonitis, esophagitis, hepatitis, retinal necrosis, disseminated infection, and encephalitis [85, 86].

5.6.2 Resistance Mechanisms

Resistance to the widely used nucleoside analogues is primarily mediated by mutations in the UL23 gene coding for the viral TK (ca. 95% of resistant isolates [87]) either preventing successful production of TK (TK-deficient virus) or resulting in alteration of TK substrate specificity (TK-altered virus). The remaining 5% of resistant isolates exhibit alteration of the DNA polymerase activity stemming from mutations within the UL30 gene. As mentioned earlier (see Section 5.4.4), the mutations in the DNA polymerase gene may also result in cross-resistance to foscarnet [50]. Whereas the DNA polymerase gene is an essential enzyme for the virus, TK mutants retain full replication competence in dividing cells with sufficiently high nucleoside triphosphate precursor pools such as epithelial cells; however, TK enzyme function is essential for viral replication in nondividing cells such as neurons, where nucleoside triphosphate precursors for DNA synthesis are limited [88, 89]. As a consequence, it might be assumed that acyclovir-resistance mediated by TK alterations or TK deficiency is always newly induced with every recurrence/treatment cycle. However, reports of the presence of both acyclovir-resistant and acyclovir-sensitive virus in latently infected neurons support the hypothesis that the functional TK of a sensitive virus strain might compensate for the reactivation deficiency of the mutant *in trans*, leading to successful replication of the resistant virus in epithelial cells after it spreads from the neuron [90, 91]. In which case, acyclovir resistance could derive from both newly induced mutations in the viral genome or from reactivation of already resistant HSV.

5.6.3 Management of Resistant HSV

Depending on which resistance-mediating mutations are present, the virus may remain susceptible to higher doses of nucleoside analogues. Therefore, high doses of intravenous acyclovir are frequently administered as a first step in cases of suspected clinical resistance [92]. In parallel, the resistance profile can be determined by genotypic (mainly by PCR and sequence analysis) or phenotypic resistance testing (plaque reduction assay as gold standard). In case of a confirmed acyclovir resistance that does not respond to high-dose acyclovir, the only approved treatment options in many countries including the United States is intravenous foscarnet (see Section 5.4.4). However, as mentioned above, foscarnet treatment is frequently accompanied by severe toxicities and resistance can occur through cross-resistance (in case of DNA polymerase mutations mediating the resistance to acyclovir as well, see Section 5.6.2) or by acquisition of additional mutations, leading to a situation without further treatment options for the patient. In cases of dual resistance to acyclovir and foscarnet or of acyclovir resistance with foscarnet intolerance, off-label treatments such as imiquimod (see Section 5.5.4) or cidofovir [93] are used as alternatives to foscarnet. However, efficacy of imiquimod is controversial and treatment with cidofovir can also be toxic.

A promising alternative to foscarnet for the treatment of acyclovir-resistant infections could be the helicase–primase inhibitors (see Section 5.5.1). As stated before, due to the different mode of action, these molecules are active against both TK and polymerase mutant HSV, respectively [58]. Resistance to helicase–primase inhibitors, as reported from

nonclinical studies, is mediated through changes in the UL5 (coding for the helicase) and/or UL52 (coding for the primase) genes [94, 95]. At the time of this review, a clinical trial of the helicase–primase inhibitor pritelivir in immunocompromised patients with acyclovir-resistant, mucocutaneous HSV infections was ongoing (NCT03073967).

5.7 HSV and Alzheimer's Disease

Before closing the chapter on HSV, one additional topic which is gaining increasing prominence in the scientific literature should briefly be addressed: the potential association of HSV (especially HSV-1) with Alzheimer's disease (AD) [96, 97]. Though many viruses have been connected with AD, there is an interesting line of evidence that HSV could contribute to or even be the underlying cause of AD: (i) HSV is known to trigger amyloid aggregation and its DNA is commonly found in amyloid plaques. (ii) Anti-HSV drugs reduce Aβ and p-tau accumulation in brains of infected mice. (iii) Clinical studies show cognitive impairment is associated with HSV seropositivity in various patient groups and in healthy adults. At the time of this article, a clinical trial was ongoing to assess the efficacy of 500 mg valacyclovir as a treatment for patients with mild AD (NCT03282916).

5.8 Conclusion

HSV infections are frequently found in the human population. Since it is a self-limiting infection and since treatments are available, it is not considered to be a major health issue in otherwise healthy, immunocompetent individuals. However, for immunocompromised patients such as transplant recipients or HIV patients, HSV infection can be severe and may even become life-threatening. This is especially concerning since resistance to the commonly used nucleoside analogues is more common in this patient population. Foscarnet, the only approved rescue medication in such cases, is active against acyclovir-resistant virus but is toxic, and intolerance and/or dual resistance can occur. Novel treatment options are therefore still urgently needed for HSV infections, potentially also including indications that are not yet so obvious such as AD. Helicase–primase inhibitors, such as pritelivir, may prove very useful against nucleoside analogue-resistant HSV, but also for other indications due to their superior activity compared with polymerase inhibitors [63].

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6

Antiviral Strategies Against the Human Cytomegalo Virus Inhibitors of Viral Terminase

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6.1 The Need for Novel Drugs against CMV and Attempts of the Past

Human cytomegalo virus (CMV) is a beta herpesvirus. It is widespread in the human population and prevalence ranges from about 50% to close to 100% depending on geographic location and socioeconomic status [1]. It is a large, double-stranded DNA virus harboring in its 236 kb genome at least 165 genes, 4 noncoding RNAs, and 14 miRNAs. Of the protein-coding genes, 44 are replication genes common to all herpes viruses, while about 30 are unique to beta herpes viruses. As an ancient virus, which is thought to have coevolved with its host over millions of years, it replicates systemically and has adapted to various biological niches in the host, with many genes involved in evading detection by the immune response, while others direct cell tropism [2]. Replication and molecular biology of CMV has been reviewed, e.g. in Ref. [3].

While CMV causes unapparent chronic infections lifelong, any condition of a weak or nonexistent immune system can lead to reactivation often resulting in severe disease, which may have a fatal outcome. Patients at risk include recipients of stem cells [4, 5] or solid organs [6], newborns [7], HIV/AIDS patients [8], patients in intensive care [9], or patients treated aggressively against certain autoimmune disorders and with certain cancers [10]. The cost for society, which is associated with untreated CMV infections, can be very high: Untreated congenital CMV infection alone is estimated to cost more than US$3 billion annually, adjusted for 2015 dollars [11]. This is due to the fact that congenital CMV infection may cause a number of severe disabilities, including mental retardation or hearing loss. There is a very high medical need for well-tolerated, efficacious drugs to treat the conditions, where CMV is an opportunistic pathogen.

As reviewed by Andrei et al. [12], marketed drugs against CMV, which target the viral polymerase, are limited in their use by toxic side effects, pharmacokinetic drawbacks, and resistance development. Moreover, the fact that all low molecular weight drugs licensed before 2017 share a common target (except for Fomivirsen, an antisense oligonucleotide),

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also leads to cross-resistance among the polymerase inhibitors. Furthermore, at least the nucleosidic drugs like ganciclovir are prodrugs and require a viral enzyme for their activation within cells [13]. Hence, in uninfected cells, they will only become activated after viral replicative steps have taken place and infection cannot be prevented.

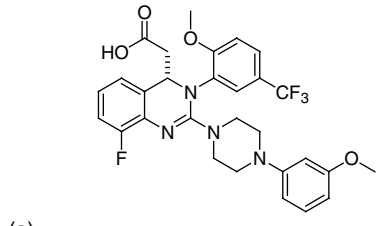
Due to the high medical need in some conditions, the existing CMV-drugs have been used for interventions against CMV outside their indication and in spite of their limitations: for example, Ganciclovir and Valganciclovir, in spite of their toxicities, have been applied to treat congenital CMV infections and are presently being investigated in clinical trials in congenitally infected children [14, 15]. However, although there obviously is a high medical need, no drug has been licensed to date for this indication, nor for any of the other conditions beyond transplantation medicine mentioned above.

Due to the urgent need to develop safer, more effective treatments, ideally also addressing different targets, a number of approaches have been taken and RNAi molecules, monoclonal antibodies, or CMV vaccines have been investigated, apart from low molecular weight drugs: synthetic siRNA against essential gene products of CMV like UL54 or UL97 or UL122/123 was shown to trigger RNAi in infected cells, leading to viral inhibition [16, 17], but clinical development of therapeutics based on this technology has not been reported. A combination of monoclonal antibodies, e.g. CSJ148, consisting of two anti-CMV human monoclonal antibodies (LJP538 and LJP539) that bind to and inhibit the function of viral CMV glycoprotein B (gB) and the pentameric complex (consisting of glycoproteins gH, gL, UL128, UL130, and UL131) have been evaluated in the clinics [18, 19]. While phase I showed a good safety and pharmacokinetic profile, there have been no positive reports on the outcome of phase II in stem cell recipients. Efforts to develop a CMV vaccine began more than 30 years ago, but so far, these efforts failed as well. Most recently, a phase 3 trial by Astellas/Vical was reported. It was designed to evaluate the efficacy of ASP0113 compared with placebo in CMV-seropositive recipients undergoing an allogeneic stem cell transplant. Efficacy was assessed using a primary composite endpoint of overall mortality and CMV end-organ disease through the first year following the transplant. Unfortunately, the endpoint was not met [20].

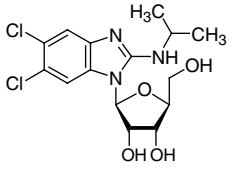
A number of small molecular weight drugs were also investigated, either by screening for anti-CMV compounds in cell culture or by screening for compounds in target-based biochemical assays.

Out of these efforts, to date, only one new inhibitor of CMV, which targets the viral terminase (BAY 73-6327, AIC246, Letermovir, Prevymis™, Figure 6.1) has been licensed for prophylaxis against CMV in patients receiving stem cell transplantations [21]. Maribavir, a benzimidazole riboside (Figure 6.1), which was shown to inhibit the CMV protein kinase UL97 (a potent inhibitor of histone phosphorylation catalyzed by wild-type pUL97), with an IC50 of 3–35 nM *in vitro* [22] was developed in the clinics up to phase III. However, in a prophylactic phase III study in patients receiving stem cells, the drug failed [23]. Similarly, great hopes were connected with CMX001 or Brincidofovir, an oral prodrug hexadecyloxypropyl-ester of Cidofovir (Figure 6.1). It was developed up to a prophylactic phase III study in stem cell transplanted patients, but failed in this study as well [24].

In an effort to repurpose drugs, the multi-targeted kinase inhibitor sorafenib has been shown to inhibit CMV in cell culture at concentrations in the one-digit μM range [25]. Development for this indication, however, has not been reported for sorafenib.



(a)



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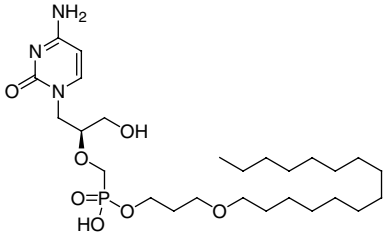

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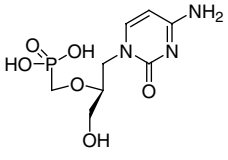

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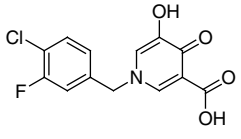


(e)

Figure 6.1 Small molecular weight inhibitors of the human cytomegalo virus. (a) Letermovir, BAY 73-6327, AIC246, trade name Prevymis, (2-[(4*S*)-8-fluoro-2-[4-(3-methoxyphenyl)piperazin-1-yl]-3-[2-methoxy-5-(trifluoromethyl)phenyl]-4*H*-quinazolin-4-yl]acetic acid). (b) Maribavir, Benzimidavir; Bzurea; Camvia; 1263W94; BW1263W94; GW257406X). (c) CMX001, Brincidofovir, prodrug of cidofovir. (d) Cidofovir, trade name Vistide, CAS Registry Number: 113852-37-2. (e) Hydroxypyridonecarboxylic acid, compound 7r from Ref. [26].

Recently, in a new approach, hydroxypyridonecarboxylic acids [26] (Figure 6.1e) have been investigated as inhibitors of CMV pUL89 endonuclease, which provides the enzymatic functions for the CMV terminase complex in viral packaging. In addition, five new hits from a cellular screen were described by Kapoor et al. [27].

The difficulty to protect patients with a very weak or missing immune system against CMV reactivation is highlighted by the failure of the multiple approaches discussed above. For Maribavir [28, 29], some clinical studies are ongoing investigating a different application, namely treatment of resistant/refractory CMV infections. Brincidofovir was licensed to SymBio and may be developed further for HHV-6 in transplanted patients. In spite of all efforts, Letermovir is the only drug that could be licensed for the prophylaxis against CMV in stem cell recipients to date. Therefore, the strategy which resulted in its discovery will be discussed.

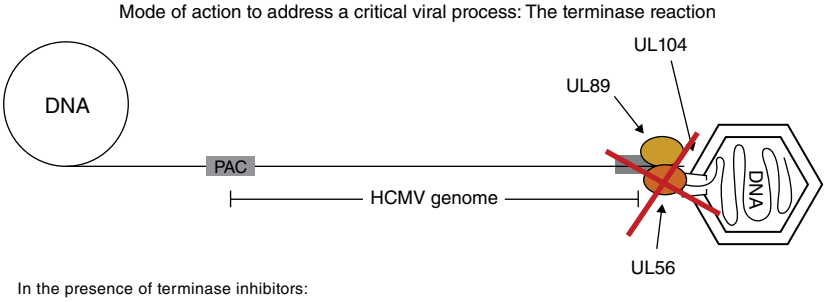
6.2 The Strategy for the Discovery of Letermovir

When researching for antiviral compounds, approaches can be to screen compounds in virus-infected cell cultures or screening compounds in target-based biochemical assays and by using molecular modeling. Target-based biochemical assays have been successful in the past for small viruses like HIV or HCV and for single enzymes like proteases. However, biochemical target-based screens bear the inherent problem that there is no guaranty that a highly active inhibitor in the biochemical assay will also pass the cell membrane and be active inside an infected cell. To date, there is no rule, which would allow medicinal chemists to make the inhibitor enter the cell by adequate rational chemical modifications. In addition, CMV, which is one of the largest viruses [2, 3], contains many more potential targets than, e.g. HIV or HCV and there is an uncertainty how to choose the "right" targets for target-based screens. Even for a small virus like HCV, inhibitors against the HCV NS5A nonstructural protein, which plays several important roles in the virus life cycle (but has no known enzymatic activity), were identified by phenotypic screening of large compound libraries and resistance mapping of early leads and might not have been discovered otherwise (see Chapter 2). Generally, for a target with many complex interactions or for multienzyme complexes, biochemical target-screens are difficult to establish and such screens may not reflect the true composition and conformation of the proteins or protein–nucleic acid complexes in a living cell. Such screens may thus yield hits, which cannot be optimized for *in vivo* antiviral activity and will not be able to deliver the relevant activity in a living cell.

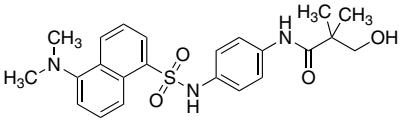
In theory, screening chemical molecules in an infected cell will allow to probe inhibition of all targets simultaneously, which are responsible for the replication of the virus, including multi-protein complexes or complexes of proteins with nucleic acid. This approach may also reveal targets, which are particularly important for the kinetics of viral replication.

Based on all these considerations, for CMV, cellular screens were performed apart from target-based screens. Interestingly, apart from several CMV core-inhibitors with distinct chemical structures (unpublished), which were discovered in cellular screens, the viral terminase complex (Figure 6.2a) was hit three times in such screens, with compounds from different chemical classes, suggesting its essential function for the replication kinetics of the virus. The terminase inhibitors inhibited neither viral DNA synthesis nor viral transcription or translation (Figure 6.2a), but particles lacking a DNA core accumulated within infected cells together with unprocessed high molecular weight DNA. Inhibition of functional cleavage at viral intergenomic transitions in inhibitor-treated cells pointed to interference with the viral DNA cleavage machinery.

The first terminase-inhibitor to be discovered was BAY38-4766 [30–32] (Figure 6.2b). In addition to inhibiting the human virus, it had strong antiviral activity *in vitro* and *in vivo* against the murine cytomegalovirus and allowed to prove its activity in a lethal challenge model in mice [30, 31]. Analysis of mutants generated *in vitro* revealed resistance mutations to BAY38-4766 which mapped to the two proteins, UL89 and UL56 [32]. UL89 and UL56 function as two subunits of the viral terminase [32]. Since human DNA does not undergo similar maturation steps and hence interaction with human enzymes was not to be expected, a good tolerability in humans was expected from targeting these structures.



(a)



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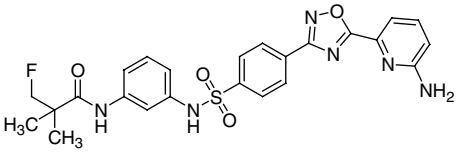

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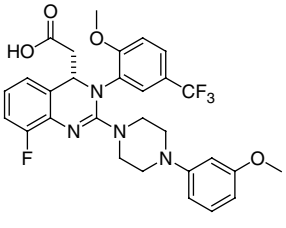

(d)

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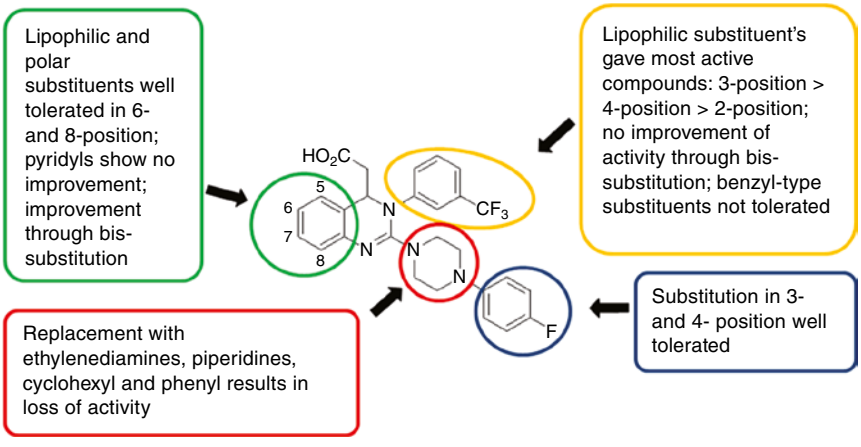

(e) IC50: (–) Entantiomer < Racemate < (+) enantiomer

Figure 6.2 Mode of action of the terminase and terminase inhibitors of CMV. (a) The mode of action of the CMV terminase. (b) The first terminase inhibitor, drug candidate Bay 38-4766, tomeglovir. (c) The second terminase inhibitor class (phenylsulfonamide aminopyridines) drug candidate: Bay 64-8030. (d) Letermovir, representing a third class (dihydroquinazoline derivatives) BAY 73-6327, AIC246, Prevymis. (e) Letermovir: Structure–activity relationship.

After demonstrating high efficacy in the murine lethal challenge model [31] and good tolerability in toxicity studies, BAY 38-4766 (tomeglovir) underwent phase I testing in humans. As expected, a good tolerability was demonstrated in man. Unfortunately, however, BAY 38-4766 was found to induce CYP enzymes leading to its own degradation. A prodrug approach was not able to improve this situation and the class was abandoned.

A second class of terminase-inhibitors to be discovered were the phenylsulfonamide aminopyridines (Figure 6.2c), with the drug candidate BAY 64-8030. Again, phase I testing in humans demonstrated good tolerability at all doses tested, but BAY 64-8030 had an unexpected high metabolic cleavage, compared with the metabolism seen in the preceding animal studies. Development was thus suspended as well and the compound was modified chemically to stabilize the position of metabolic attack.

Finally, letermovir, (BAY 73-6327, AIC 246) a dihydroquinazoline-derivative, was discovered (Figure 6.2d). It showed cross-resistance to viruses resistant to previous terminase inhibitors and hence was assumed to be a terminase-inhibitor as well. This was confirmed in dedicated studies of *in vitro* resistance-induction and marker transfer [33]. Like the other anti-terminase molecules, BAY 73-6327 was very well tolerated in animals. Figure 6.2e shows some aspects of the structure–activity relationship of letermovir.

6.3 The Link between Preclinical Models and Clinical Efficacy

As described before, three classes of terminase inhibitors were studied in phase I tests in humans and two failed due to the fact that at that time, models for predicting human pharmacokinetics by *in vitro* tests were just evolving. However, all terminase development

candidates confirmed the predictions from theoretical considerations with respect to tolerability: all three classes of terminase inhibitors were very well tolerated not only in animals, but also in humans, demonstrating that targeting an enzymatic complex which does not exist in humans is likely to confer an advantage with respect to tolerability. In addition, due to the fact that some of the marketed drugs are prodrugs (and hence dependent on cellular and viral kinase activities), it was assumed that a compound, which does not need such activation, will have a good efficacy across different cell types and across different multiplicities of infection (MOI). Table 6.1 [34, 35] shows for letermovir that the EC_{50} is in the nanomolar range (EC_{50} 4–5 nM) with a very steep dose–response curve: EC_{90} is increased only slightly compared with EC_{50} (EC_{90} 5–7 nM). In contrast, EC_{50} of ganciclovir is in the micromolar range and the compound has a much flatter dose–response curve (EC_{50} 3–4 μM, EC_{90} 11–18 μM).

Furthermore, upon increase of the multiplicity of infection (Table 6.2) [34, 35], the EC_{50} of letermovir increases by a factor of about 3 that of ganciclovir by a factor of 5 and maribavir was found to be inactive at higher viral inputs into the cell culture.

As letermovir – in contrast to BAY 38-4766 – is specific for CMV only, *in vivo* activity was assessed in an engineered mouse xenograft model, in which mice were transplanted with a gelfoam sponge carrying CMV-infected human cells. The animals were treated once daily via oral gavage (Figure 6.3) [35, 36]. Treatment lead to a dose-dependent reduction of CMV titer in the cells within the sponge transplanted to the mice, compared with a placebo-treated group. Statistical analysis revealed significant antiviral effects for the 10, 30, and 100 mg/kg/day treatment by letermovir as well as for the 100 mg/kg/day valganciclovir group ($p < 0.006$), Table 6.3.

Table 6.1 Sensitivities of different CMV laboratory strains to Letermovir and Ganciclovir in fibroblast cells.

		EC_{50}(μM)[a]		EC_{90}(μM)[a]		No. of independent expts
Assay	**CMV strain**	**Letermovir**	**Ganciclovir**	**Letermovir**	**Ganciclovir**	
CPE-RA[b]	Davis	0.0040 ± 0.0010	2.70 ± 0.70	ND[c]	ND	7
	AD169	0.0050 ± 0.0010	4.30 ± 1.80	ND	ND	13
GFP_RA[d]	AD169-GFP	0.0038 ± 0.0009	1.73 ± 0.93	0.0051 ± 0.0014	10.7 ± 2.5	18
	RV-HG	0.0049 ± 0.0009	2.33 ± 2.75	0.0071 ± 0.0025	18.3 ± 22.7	5

[a] EC_{50} and EC_{90} values were determined by the indicated antiviral assay. Nonlinear regression analysis was performed, and the resulting graphs were used to calculate the respective values. Results are expressed as means ± standard deviations.
[b] CPE reduction assay.
[c] ND, not determined.
[d] Fluorescence reduction assay.

Table 6.2 Comparison of the effect of increasing CMV viral load on EC_{50}.

Multiplicity of infection (MOI)	EC_{50} (μM) Letermovir	EC_{50}(μM) Ganciclovir	EC_{50}(μM) Maribavir
0.003	0.0013	0.99	0.29
0.01	0.0015	0.68	0.17
0.03	0.0029	1.74	0.38
0.1	0.0034	2.21	0.94
0.3	0.0036	6.51	No activity
1	0.0042	5.26	No activity

Source: From Refs. [34, 35], CMV strain AD169-GFP, EC_{50} values determined by fluorescence reduction of a GFP-labeled virus, means from 2 to 8 independent experiments.

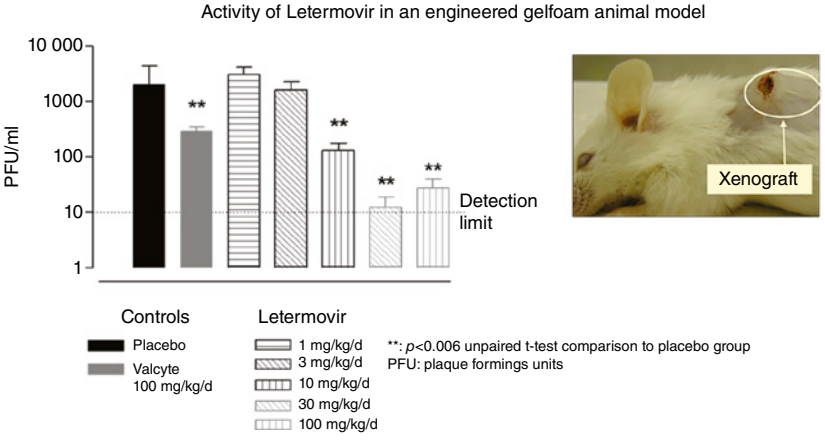

Figure 6.3 *In vivo* antiviral activity of letermovir in a mouse xenotransplant model [35, 36]. The effect of the therapy by valganciclovir (VGCV) or letermovir (AIC246) on CMV replication in a mouse xenograft model: viral titers in CMV-infected gel-sponges harvested from transplanted mice receiving antiviral treatment as indicated or placebo [35, 36]. Drugs were given once daily per os for nine days. Results are expressed as means ± standard errors of the means. Letermovir showed superior *in vivo* activity in the mouse xenograft gelfoam model. In addition, as in cell culture, *in vivo*, a remarkably steep dose–response with ED_{50} = 3 mg/kg/day and ED_{90} = 8 mg/kg/day was observed (Table 6.3) [35, 36].

Table 6.3 *In vivo* antiviral activity of letermovir in a mouse xenotransplant model [35, 36].

	EC_{50}	EC_{90} [mg/kg/d]
Letermovir	3	8
VGCV	16	>100

ED_{50} and ED_{90} values. Since ED_{90} was not reached for valganciclovir at 100 mg/kg/day, it was set to >100 mg/kg/day.

6.4 Clinical Experience

After letermovir had shown efficacy *in vitro* and in the animal model and was well tolerated in toxicological studies [37], a number of phase I studies confirmed the good tolerability and revealed a pharmacokinetic profile suggesting that a once daily application as well as a p.o. and an i.-v. formulation was possible [38–42].

The first phase II trial was conducted in kidney-transplanted patients, who had reactivated CMV [43] and who were treated orally for 14 days with letermovir either 40 mg b.i.d., 80 mg o.d., or with local standard of care. The primary endpoint was the decrease of CMV DNA copy number from baseline. This endpoint was achieved in all groups with statistical significance, proving for the first time in humans that letermovir was active against CMV. Furthermore, letermovir was well tolerated in the kidney-transplanted patients. Interestingly, as this had not been excluded by the protocol, some of the patients had a CMV infection with a proven resistance genotype against polymerase inhibitors and achieved suppression of their virus upon letermovir treatment as well [43].

While the kidney-transplanted recipients in the above study had reactivated CMV, but had not developed CMV disease yet, a single patient in whom disease due to a multiresistant virus had developed in several organs was treated under an emergency IND obtained from the FDA [44] (Figures 6.4 and 6.5). After application of letermovir (AIC246), the viral load became undetectable and all organs healed [44] (Figures 6.4 and 6.5), including the retinitis. This demonstrated for the first time that CMV disease can be treated efficiently by letermovir as well and that the drug is active against polymerase-resistant virus causing clinical disease.

As CMV-positive patients receiving stem cell transplantations have a very high medical need for suppression of CMV reactivation immediately after transplantation, a phase 2 dose-finding study was conducted to investigate the effect of letermovir on the incidence and time to onset of prophylaxis failure in CMV-seropositive patients, who had received allogeneic hematopoetic-cell transplants from matched related or unrelated donors. Patients were treated with oral letermovir at doses of 60, 120, or 240 mg/day, respectively, for 12 weeks after engraftment, or with matching placebo. The primary endpoint was all-cause prophylaxis failure, defined as discontinuation of the study drug because of CMV antigen or DNA detection, end-organ disease, or any other cause. In this study, the reduction of the incidence of all-cause prophylaxis failure was clearly dose-dependent [45] (Figure 6.6), matching the dose-dependent efficacy, which had been seen before *in vitro* and in animals. Importantly, the safety profile of letermovir was similar to placebo (Table 6.4) [45], with no indication of hematologic toxicity or nephrotoxicity. The ECGs showed no significant findings in the central laboratory readings, the vital parameters showed no statistically significant cases of predefined abnormalities between the letermovir and the placebo groups [45].

These results let to a phase III trial [46] (sponsored by MSD, Figure 6.7). In this phase 3, double-blind trial, CMV-seropositive transplant recipients, 18 years of age or older, were assigned in a 2:1 ratio to receive letermovir or placebo, administered orally or intravenously, through week 14 after transplantation. Letermovir was administered at a dose of 480 mg/day (or 240 mg/day in patients taking cyclosporine). Patients in whom clinically significant CMV infection (CMV disease or CMV viremia leading to preemptive treatment) developed, discontinued the trial regimen and received anti-CMV treatment. The primary end point was the proportion of patients, who had clinically significant CMV infection

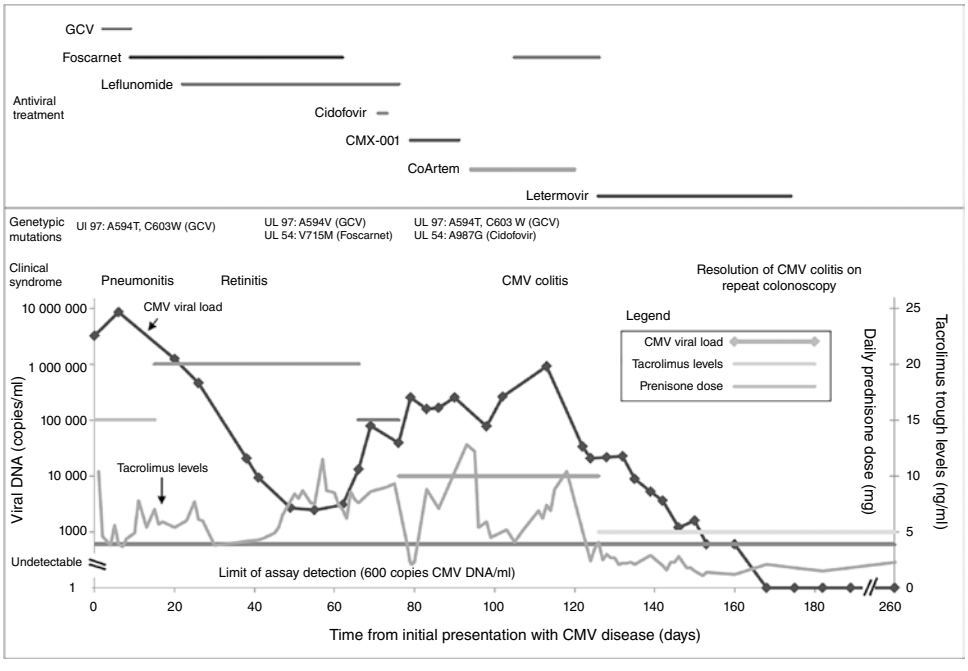

Figure 6.4 Successful treatment of a lung-transplanted patient with resistance against polymerase inhibitors and CMV-pneumonitis, retinitis, and colitis [44]. Antiviral treatment (top) and viral load and CMV disease (bottom). *Source*: Kaul et al. [44]. With permission of John Wiley & Sons.

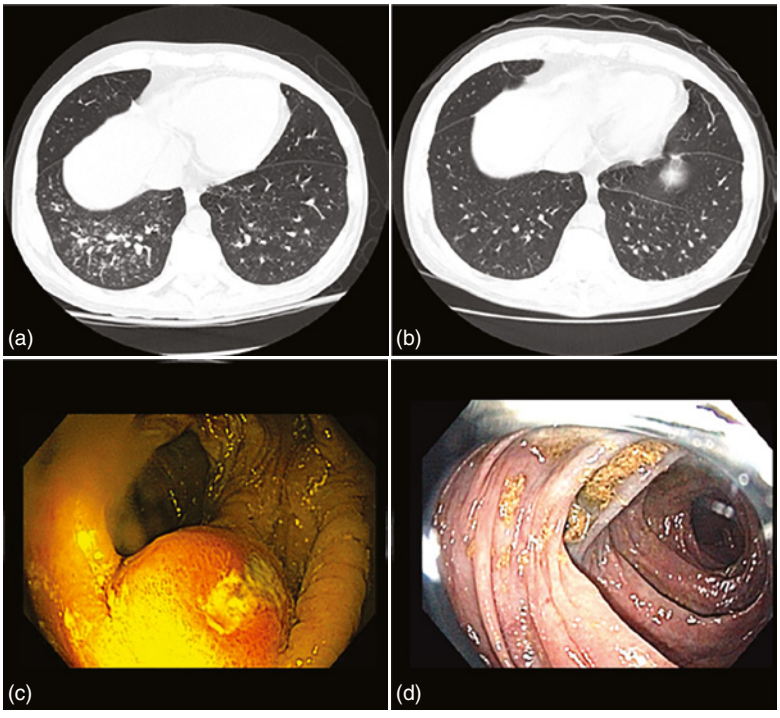

Figure 6.5 Organ disease before and after treatment with letermovir. Chest X-ray before (a) and after (b) treatment with letermovir, colonoscopy before (c) and after (d) treatment with letermovir. *Source*: Kaul et al. [44]. With permission of John Wiley & Sons.

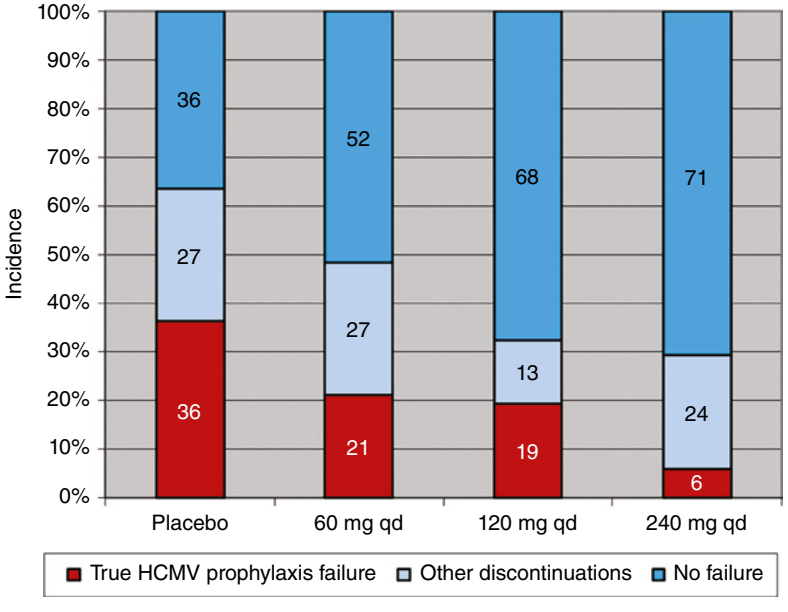

Figure 6.6 CMV prophylaxis by letermovir in patients with hematopoietic stem cell transplantation, NCT01063829: dose-dependent protection from CMV reactivation. *Source*: Based on [45].

Table 6.4 CMV prophylaxis by letermovir in patients with hematopoietic stem cell transplantation: clinical laboratory. Source: Data from [45].

N (%) Patients with at least one predefined change post baseline	Letermovir 60 mg qd *N* = 33	Letermovir 120 mg qd *N* = 31	Letermovir 240 mg qd *N* = 34	Placebo *N* = 33
Hemoglobin: PC = −2 g/dl	10 (30)	11 (35.5)	8 (23.5)	7 (21)
WBC: PC = −2000/mm^3	11 (33)	13 (42)	10 (29)	9 (27)
Eosinophils: PC = +20%	2 (6)	1 (3)	1 (3)	2 (6)
Neutrophils: PC = −20%	9 (27)	9 (29)	6 (18)	10 (30)
Platelets: PC = −100 000/mm^3	4 (12)	0	6 (18)	4 (12)

through week 24 after transplantation. Patients who discontinued the trial or had missing end-point data at week 24 were imputed as having a primary endpoint event. Patients were followed through week 48 after transplantation.

A total of 565 patients underwent randomization and received letermovir or placebo beginning a median of nine days after transplantation. Significantly fewer patients in the letermovir group than in the placebo group had clinically significant CMV infection or were imputed as having a primary end-point event by week 24 after transplantation, $P < 0.001$ (Figure 6.8). The frequency and severity of adverse events were similar in the two groups overall. The rates of myelotoxic and nephrotoxic events were similar in the letermovir group and the placebo group. In conclusion, letermovir prophylaxis resulted in a significantly lower risk of clinically significant CMV infection than placebo.

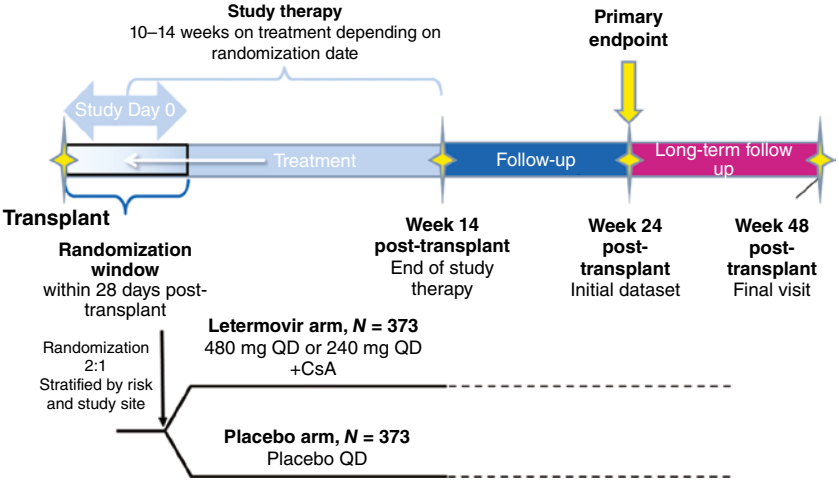

Figure 6.7 Prophylaxis by letermovir against CMV reactivation in CMV-seropositive recipients of hematopoietic stem cells: Study design. *Source*: Based on [46].

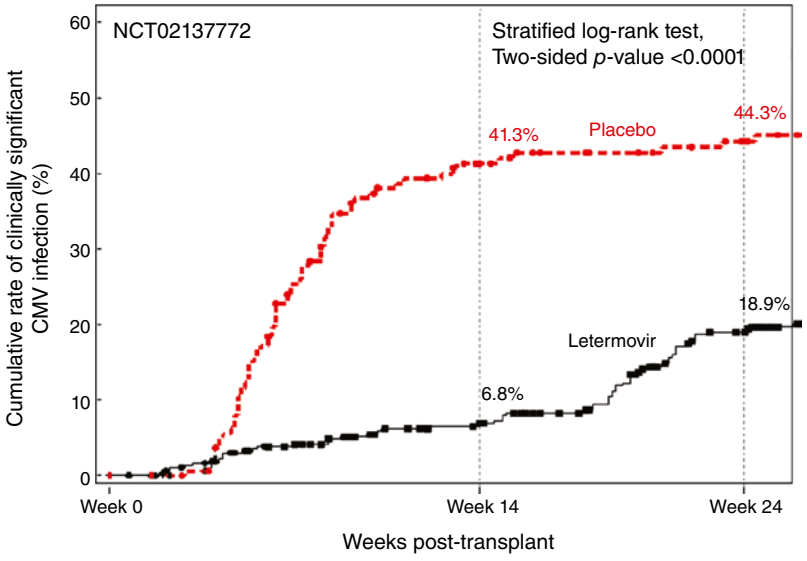

Figure 6.8 MSD phase 3 study: time to clinically significant HCMV infection through week 24 post-transplant (FAS). *Source*: Based on [46].

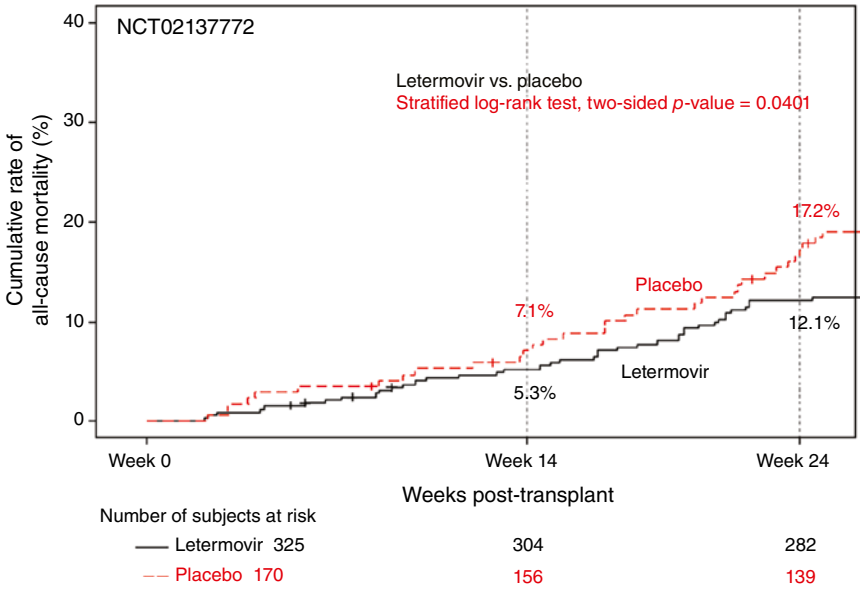

Figure 6.9 MSD phase 3 study: all-cause mortality through week 24 post-transplant (FAS, w 24 data including vital status collected post-study). *Source*: Based on [46].

It is noteworthy, that in-depth analysis of mortality in this study revealed a significant advantage for letermovir-treated patients at week 24, Figure 6.9. All cause mortality at week 24 after transplantation was significantly lower among letermovir recipients than among recipients of placebo. This is in line with earlier observations from preemptive

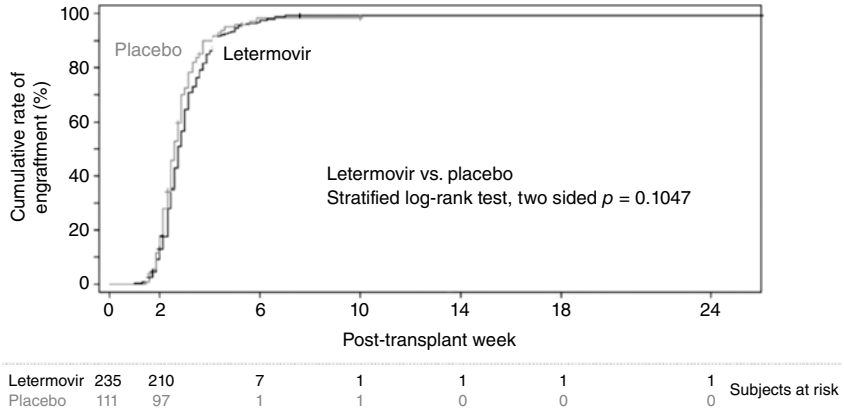

Figure 6.10 Letermovir phase 3 study (MSD): time to engraftment through week 24 post-HSCT, NCT02137772.

therapy regimens that permitting early CMV viremia – even at low copy numbers – is associated with an increased risk of overall mortality within the first year of HSCT and with a positive dose–response relationship regarding the virus, even in patients, in whom preemptive therapy has been appropriately initiated [5, 47].

Furthermore, in contrast to the toxicities seen with polymerase inhibitors, there was no negative effect on engraftment (Figure 6.10) and the hematological lab parameters were similar between letermovir and placebo [46].

This study led to the market authorization for letermovir in the United States in 2017 [21] and later in other jurisdictions as well. It is the first phase III study successfully demonstrating functional prophylaxis against reactivation of the virus for CMV carriers, who undergo stem cell transplantations.

As discussed above, all other attempts to generate prophylactic regimens in this indication have failed to date. Apart from good tolerability, letermovir has a clear dose–response relationship in *in vitro* studies, in animals and in humans and this dose–response relationship is steep (Tables 6.1–3, Figures 6.3, 6.6). Therefore, apart from carefully choosing a target not existing in humans, a clear dose–response relationship and high efficacy against increasing viral loads in cell culture as well as in animal studies appear key parameters to be optimized in the early selection of potential drug candidates.

6.4.1 Resistance Mutations and Resistance Development

The mode of action of letermovir, i.e. inhibition of CMV terminase, was proven by cross-resistance to previous terminase inhibitors, *in vitro* resistance selection, and marker-transfer studies [33]. Therefore, and due to additional studies [48, 49], the loci of terminase mutations, which are responsible for resistance against letermovir, are well known.

The terminase consists of the UL56 and UL89 proteins, associated with UL51 in the functional packaging holocomplex [32, 50, 51]. The UL56 subunit of the terminase complex is believed to play a role in DNA packaging through sequence-specific binding of DNA packaging motifs in CMV genome concatemers [32, 51].

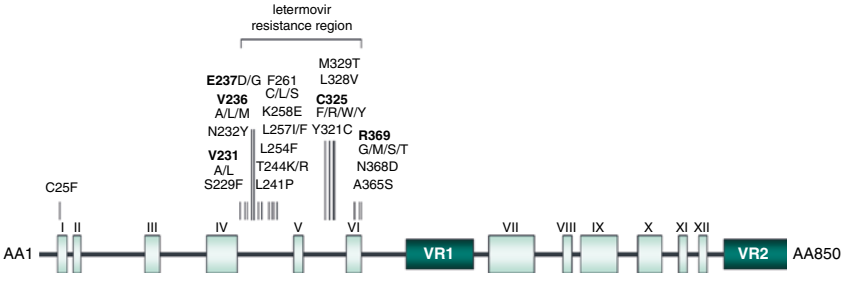

Figure 6.11 Resistance loci in UL 56 (Source: Courtesy of J. Strizki, MSD).

DNA sequencing of viruses generated in cell culture revealed resistance mutations primarily in UL56. Apart from CMV mutations associated with letermovir resistance that map to amino acids in UL56, a few mutations mapping to UL89 and UL51 were also detected. Most mutations occur in UL56 at 19 sites, two sites are mutated in UL89 and one site in UL51 [33, 52, 53]. Almost all letermovir resistance-associated variants (RAVs) map to the UL56 gene "hot spot", i.e. in the region encoding amino acids (AA) 229–369 [33, 49] (Figure 6.11). 18 of 19 UL56 mutation sites are clustered between AA 229-369 of UL56 [33, 53], the 19th mutation maps to AA 25 [49]. The UL56 mutations result in a loss of letermovir susceptibility ranging from minimal (1.8-fold) to highly resistant (>9000 fold), while the UL89 and UL51 mutations confer very modest resistance (1.6- to 5.4-fold). These letermovir mutations do not have a substantial impact on CMV fitness. Of note, common UL56 polymorphisms in wildtype virus do not impact susceptibility to letermovir nor does the subtype of glycoprotein B [54].

6.4.2 Resistance Observed in Clinical Studies

The resistance pattern emerging in clinical use may differ from the laboratory situation. Furthermore, the frequency and nature of emerging resistance determines on the long run how useful a drug proves to be in the clinics and whether it needs to be combined with other antiviral agents. Obviously, the lack of cross-resistance to CMV DNA polymerase inhibitors [33, 43, 44, 53] is a valuable feature of letermovir in this context.

Of 98 subjects receiving LET in the Phase 2b dose-finding prophylaxis trial [45], only one, who received a suboptimal 60 mg daily dose, had a mutation (pUL56 substitution V236M) that confers reduced susceptibility to LET [55].

The analysis of mutations occurring in the Phase 3 trial of CMV prophylaxis for hematopoietic stem cell transplant recipients [46] was performed using amplicon-based next-generation sequencing of UL86 and UL 56 in patients, who received 14 weeks letermovir prophylaxis or placebo and in whom clinically significant CMV infection developed through week 24 after HSCT [56]. A low incidence of resistance was detected. The RAVs that were detected, mapped to the CMV UL56 gene at positions associated with reduced susceptibility to letermovir based on resistance selection in cell culture (Figure 6.12).

Out of 373 subjects who received letermovir prophylaxis, resistance-associated mutations were identified in 3 subjects. RAVs encoding V236M and C325W were detected

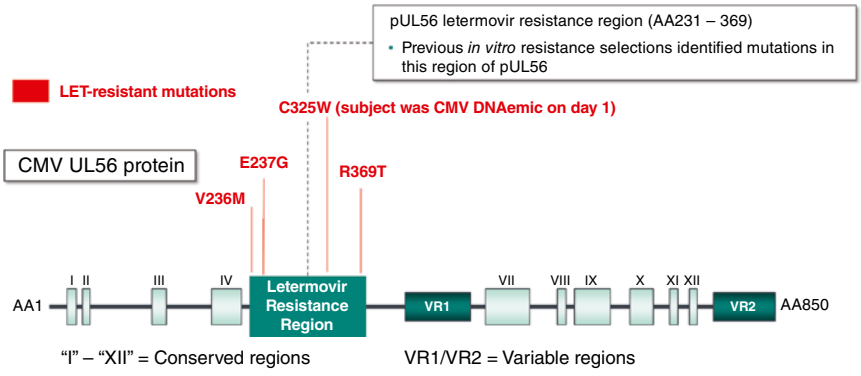

Figure 6.12 pUL56 variants observed in subjects with clinically significant CMV infection receiving prophylactic letermovir in Phase 3 Study NCT02137772 [56] (Source: Courtesy of J. Strizki, MSD).

independently in subjects 1 and 3, who experienced a clinically significant CMV infection while receiving letermovir prophylaxis. Two other variants, E237G and R369T, were detected >3 weeks after subjects 2 and 3, respectively, had discontinued letermovir prophylaxis and received preemptive therapy with ganciclovir. While V236M was seen in a patient with early noncompliance, the patient, in whom C325W was detected was viremic on day 1. The E237G variant was a minority variant (4% frequency in NGS reads) and its relevance is unclear. The mutation E237G and R369T conferred reduced susceptibility to letermovir with EC50 changes of 13- and 52-fold, respectively. Concerning reactivation of CMV during prophylaxis extended duration letermovir beyond day 100 has recently been shown to be efficacious in preventing clincally significant CMV in patients with GVHD [57].

While the above studies systematically analyzed the occurrence of letermovir resistance mutants during prophylaxis, only small patient numbers have been analyzed for resistance-development in cases, where the virus had reactivated and treatment by letermovir was initiated to achieve viral suppression.

The first trial involving 27 viremic recipients of a kidney transplant [43] met its primary endpoint with respect to viral suppression, but due to the short treatment period of 14 days, an analysis of letermovir resistance mutations was not performed. During treatment of a lung-transplanted patient who received a 49 day course with 120–240 mg letermovir daily and was suffering from CMV viremia and CMV disease in several organs due to polymerase-resistant virus [44], letermovir successfully suppressed the virus with healing of the organs, demonstrating efficacy against polymerase-resistant virus. In this case, development of resistance to letermovir was not detected. On the other hand, in another lung-transplanted patient, who was treated with 480 mg letermovir after developing ganciclovir resistance, there was only a transient reduction of viral load below detection and analysis of the rebounded virus revealed the C325Y mutation [58]. A case report of development of the C325Y mutation after treatment of CMV viremia was also described in an HSCT patient. Again, letermovir first suppressed CMV and upon relapse, the C325Y mutation was detected [59]. Four cases of letermovir use were described in patients, who received solid organ transplantations and had developed ganciclovir-resistant CMV retinitis [60]. All patients showed clinical and fundoscopical improvements with resolution of retinitis, but three patients failed to achieve sustained virologic suppression, with

one exhibiting low-grade, intermittent DNAemia, and the other two patients with high-grade DNAemia after more than one month of therapy with letermovir. While the patient with the low-grade viremia did not reveal any resistance mutation at the UL 56 site, which was sequenced, the two patients with high viremia had mutations in the 325 locus: C325F and C325Y, respectively. For one of them, a reuse of valganciclovir and subsequent virologic suppression was possible, since the virus had reverted to UL-54 wildtype. The other patient could be treated again with foscarnet and achieved virologic suppression as well. None of the patients experienced any recurrent retinitis or vision loss while on letermovir for ongoing suppression.

A first pediatric case worth mentioning here was a 14 year old girl, who was transplanted due to sickle cell disease [61]. In spite of prophylaxis against CMV and HHV6 with foscarnet and later with ganciclovir, her clinical condition worsened and on day 90, a ganciclovir-resistance mutation was found. She was transitioned back to iv foscarnet, but her clinical condition including enteric involvement remained severe and viremia rose. Iv letermovir (480 mg) was added to iv foscarnet for dual salvage therapy through day 188. During this time, her CMV levels declined to undetectable and her clinical condition improved. Iv foscarnet was stopped and the patient was managed on oral letermovir (480 mg) until discharge from the hospital. After discharge, the patient first did very well. Later, the clinical condition worsened and on day 257, a UL 56 mutation R369S was discovered. This mutation is at a locus that was seen in the prophylactic phase 3 study (R369T, see above). The authors discuss, whether while switching from iv letermovir to oral drug, the enteric absorption could have been reduced by her severe intestinal GvHD.

To date, letermovir is only licensed for the prophylaxis against CMV in stem cell recipients. Unfortunately, except for Ref. [43], there is no clinical study published for the therapy of viremia or CMV disease. While the forgoing case reports are a selection from the literature, they demonstrate clinical utility in a number of cases, but also the possibility of emergence of letermovir resistance. These case reports, however, do not allow to draw general conclusions on the frequency of resistance upon treatment of CMV reactivation, since the number of treated and nonresistant patients is not known as it is not being reported. In addition, various risk factors of the transplanted patients themselves may modify their ability to control CMV. In the absence of guidance from clinical studies, it appears very important to ensure compliance with the dose chosen and to monitor the patients for exposure to letermovir and for resistance mutations or even resistance-selection. Also, the exposure to some immunosuppressants is influenced in the presence of letermovir and needs to be monitored carefully [62] in these patients as well.

6.5 Other Potential Indications for Letermovir

As discussed above, CMV is a very widespread virus and may cause severe infections in all conditions with a weak or missing immune system. Patients at risk include recipients of stem cells, for which the drug is presently licensed. However, recipients of solid organs may also experience CMV reactivation [6] and a study investigating letermovir-prophylaxis in kidney recipients is ongoing (NCT03443869).

Table 6.5 Summarizes the conditions, where CMV may be a significant pathogen or co-pathogen beyond the transplantation field. Outside the transplantation field, newborns, who are congenitally infected by CMV, are at particular risk to develop life-long

Table 6.5 Potential indications for treating CMV infection outside transplantation medicine.

Potential indication	Reason for treatment	References
Congenital CMV infection	Congenital CMV infection represents the most common congenital viral infection, estimated incidence in developed countries: 0.6–0.7% of all live births, i.e. approximately 60 000 neonates born every year with congenital CMV infection in the United States and the European Union combined. Children infected early in pregnancy have highest risk to develop severe neurological impairment and may die	[7, 11, 63–65]
Active CMV replication in HIV patients on HAART treatment	CMV (and/or other herpesvirus) replication is a significant cause of immune activation in HIV-infected individuals with incomplete antiretroviral therapy–mediated $CD4^+$ T cell recovery. In the AIDS stage, CMV can lead to blindness, severe colitis, and death	[8, 66]
Active CMV replication in patients in intensive care	Active CMV infection is associated with a significantly higher mortality rate compared with critically ill patients without active CMV infection;	[9, 67]
	There is an independant correlation between CMV reactivation and increased morbidity in non-immunosuppressed patients with severe sepsis	[68]
CMV and glioblastoma	From [69]: "Classical and novel antiviral therapies against CMV should be revisited as they may represent a great promise for halting tumor progression and lower cancer deaths"	[10, 69]
Detection of CMV in other autoimmune diseases	Predictors of mortality with concurrent cytomegalovirus detection	[70]
CMV and inflammatory bowel disease	CMV colitis may affect up to one-third of patients with acute severe ulcerative colitis (ASUC) refractory to corticosteroid therapy (Refs. 165, 166 in Ref. [71]). Higher rate of treatment refractoriness and need for colectomy in patients with demonstrable CMV colitis. Identification of this disease should prompt treatment with antiviral therapy in the setting of refractoriness to steroids or biologic therapy	[71] Refs. 74–78 in Ref. [72]

disabilities like mental retardation or hearing loss or even to die (Table 6.5, refs. [7, 11, 63, 64, 65]). As mentioned above, apart from the significant disease burden in these children, there is a very high financial burden for society due to congenital infections with estimates of the overall economic burden exceeding US$3 billion annually [11]. A study using ganciclovir – in spite of its tolerability issues – in neonates with symptomatic disease has demonstrated that start of therapy in the neonatal period prevented hearing deterioration at six months [63]. However, two-thirds of treated infants had significant neutropenia during therapy. A small study, also using ganciclovir concluded [64]: "Asymptomatic congenital cytomegalovirus infection is likely to be a leading cause of sensorineural hearing loss in young children. Intravenous ganciclovir therapy seems to offer a medical option to prevent subsequent sensorineural hearing loss. Further studies including a greater number of children are needed. Cytomegalovirus screening models are mandatory, if medical

therapy is to be implemented in time" [64]. As screening systems are available and letermovir offers high antiviral potency together with good tolerability, time seems to be ripe for clinical trials testing the use of letermovir in CMV-infected symptomatic and asymptomatic newborns.

There are a number of other conditions where CVM is being suspected to be a pathogen with its own negative impact on the patients´ health. One example is HAART-treated HIV infection [8, 66], another patient group are individuals in intensive care with active CMV infection (Table 6.5) [9, 67, 68]. A study by Limaye et al. [67], who treated critically ill CMV seropositive adults with ganciclovir noted that treated patients had fewer days on ventilation, both, in the intention-to-treat population and in the prespecified sepsis subgroup. Similarly, CMV may be a co-pathogen in certain cancers (Table 6.5) [10, 69]. Foster et al. reported recently that CMV seropositivity is associated with decreased survival in glioblastoma patients [10]. A multicenter randomized double-blinded controlled phase 2 study evaluating the efficacy of valganciclovir as add-on therapy in glioblastoma patients is ongoing (NCT04116411).

Also in autoimmune diseases, CMV may play an important role [70]: CMV-DNA copy numbers and concurrent infections are predictors of in-hospital mortality in CMV-infected patients with autoimmune diseases (incl. SLE, RA). Therefore, serial measurements of CMV-DNA copy numbers and close observation for signs of other infections were recommended for patients with autoimmune diseases, who have concurrent CMV infection [70]. In addition, there is a strong correlation of worsening symptoms in inflammatory bowel disease, when CMV replication is present (Table 6.5) [71, 72]. Interestingly, a retrospective study of compassionate therapeutic use of letermovir for CMV infections included a patient with systemic lupus erythematosus and a patient with a multicentric form of Castleman disease. In both cases, CMV infections did not recurr [73].

The above examples for a potential benefit for patients, who might receive therapy for CMV suppression beyond the transplantation field, are not exhaustive. The existance of letermovir, a well-tolerated and efficacious drug, now allows to investigate the role of this ubiqitous virus in much more detail in these conditions, without the toxicities of the drugs that needed to be used up to now. This may open new therapeutic concepts, which may include the control of CMV among other interventions, also outside the transplantation field.

6.6 Conclusions

In conclusion, letermovir stands for a new class of CMV inhibitors, which is well tolerated and very efficacious. It is the first novel and non-nucleosidic drug for CMV since decades, which has been licensed with the exception of foscarnet and fomivirsen (for intravitreal injection), both of which have significant limitations.

The availability of letermovir allows for the first time to treat stem cell recipients prophylactically against reactivation of CMV, which translates into a survival benefit. Studies in kidney transplant recipients are ongoing. Letermovir may also be highly useful in a number of other indications outside the transplantation field.

Letermovir and its predecessors have been discovered by cellular screens. Due to the complexity of the terminase complex, a biochemical assay for the terminase function was impossible at that time. However, at least in our hands and compared with target-based screens, cellular screens have also proven to be more effective for the discovery of other drugs, such as a novel Herpes Simplex drug, Pritelivir [74] (see Birkmann, Chapter 5) or the Hepatitis B drug BAY 41-4109 (AIC 429) [75], which inhibits core formation and leads to degradation of HBV core protein. While a number of other core-inhibitors have meanwhile been discovered Bay 41-4109 and congeners remain the only drugs with the degradation mechanism of core (Chapter 9).

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7

Antiviral Targeting of the Complex Epstein Barr Virus Life Cycle

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7.1 Disease Overview

Epstein Barr virus (EBV) is surprisingly at the same time one of the most common pathogens and the most oncogenic virus that infects humans [1]. More than 95% of the human adult population carries this γ-herpesvirus as a persistent asymptomatic infection. Yet, it readily transforms human B cells in culture and is associated with a variety of lymphocyte, epithelial, and smooth muscle cell-derived malignancies that fortunately occur only at low incidence rates [2, 3]. They nevertheless are estimated to amount to around 200 000 new cancers in humans every year [4]. In addition to these tumors, EBV is also the cause of several immune pathologies. These include infectious mononucleosis (IM) as the symptomatic primary EBV infection, more frequently observed when the virus is first encountered later in life during adolescence or early adulthood [5]. Furthermore, myeloid cell activation that results in hemophagocytic lymphohistiocytosis (HLH) results from inefficient killing of EBV-infected cells, primarily B cells as the main host cell of the virus, triggering then pathogenic cytokine production by lymphocytes that stimulate myeloid cells [6]. Uncontrolled EBV infection then often also spreads from B cells to other lymphocyte compartments like natural killer (NK) and T cells [7] and might also home to the central nervous system (CNS) to stimulate autoimmunity resulting in multiple sclerosis (MS) [8]. Transition to these pathologies is fortunately avoided in most EBV carriers by immune control by cytotoxic lymphocytes, mainly NK and $CD8^+$ T cells [9]. Accordingly, the re-establishment of this immune control by antiviral therapies would be desirable for the treatment of EBV-associated diseases. In order to devise respective strategies, it is, however, necessary to understand which aspects of the viral life cycle need to be targeted.

EBV is usually transmitted via saliva exchange, although transmission by organ transplantation and maybe sexual intercourse is also possible [10]. Most likely, it enters submucosal secondary lymphoid tissues of the oropharynx, like the tonsils, via transcytosis across the mucosal epithelium [11, 12]. At these sites, it infects B cells, initiating growth transforming gene expression called EBV latency [13]. This includes at the protein level up

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to six Epstein Barr virus nuclear antigens (EBNAs) and two latent membrane proteins (LMPs). In addition, as non-translated RNAs, two Epstein–Barr virus-encoded small RNAs (EBERs) and more than 40 miRNAs are expressed [14]. B cell transformation by these latent EBV gene products was suggested to drive infected B cells into memory differentiation for long-term persistence [15], and only if T cell-mediated immune control is missing like after immune suppression following organ transplantation or due to human immune deficiency virus (HIV) coinfection, latently EBV-infected B cells emerge as lymphomas [16] (Figure 7.1). From infected memory B cells that do not express any latent EBV proteins, lytic replication for infectious virion production can be induced and seems to be stimulated after plasma cell differentiation that is stimulated after B cell receptor stimulation by the cognate antigen [17]. Thus, latent EBV proteins have been the focus of therapeutic interventions to target EBV-associated malignancies. However, it was recently noted that early, but most likely not late lytic EBV gene expression contributes to viral oncogenesis [1, 18, 19]. While late lytic gene expression and virion production lyses the respective host cells, early lytic gene products like viral IL-10 but also chemokines that are induced at this infection stage might condition the microenvironment for more efficient growth of EBV-associated malignancies (Figure 7.1). Furthermore, IM is primarily driven by the immune pathologic expansion of cytotoxic $CD8^+$ T cells that recognize early lytic antigens [20]. Thus, early, but

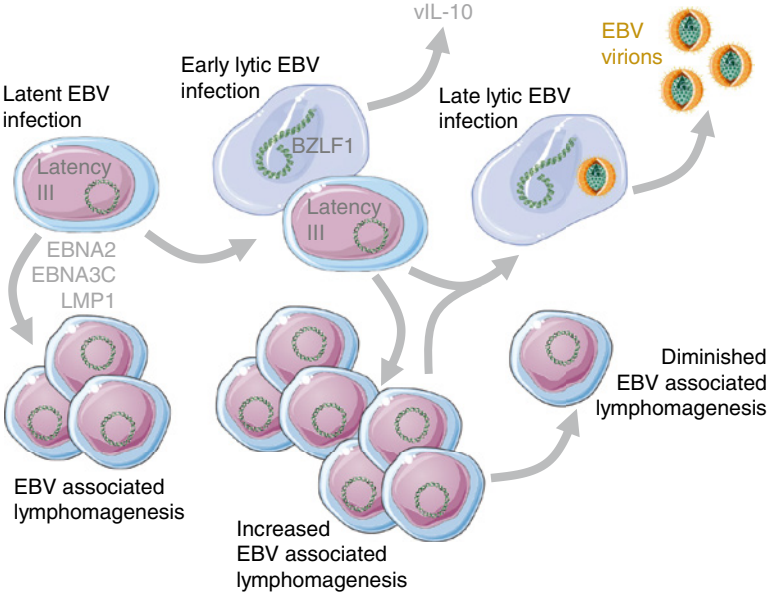

Figure 7.1 Both latent and early lytic EBV infection contribute to virus-associated tumorigenesis. EBV-associated lymphomagenesis is driven by viral oncogenes, such as EBNA2, EBNA3C, and LMP1. These are expressed during EBV latent infection with up to 6 nuclear antigens (EBNAs), 2 membrane antigens (LMPs), 2 small noncoding RNAs, and more than 40 miRNAs during latency III. Lymphomagenesis is further promoted by early lytic EBV infection, conditioning the tumor microenvironment by factors such as viral IL-10 (vIL-10). However, late lytic EBV infection with virion replication actually destroys tumor cells and thereby diminishes lymphomagenesis.
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not late lytic EBV proteins should also be targeted therapeutically in addition to latent gene products to treat EBV-associated tumors and some of the virus-associated immune pathologies.

7.2 Antiviral Strategies

7.2.1 Pharmacological Inhibition

Following the above discussed considerations, it is probably not too surprising that inhibition of EBV's DNA polymerase, required for infectious virion production, on its own has no therapeutic effects against EBV-induced malignancies like post-transplant lymphoproliferative disease (PTLD) [21]. Only in combination with B cell depleting and chemotherapy, viral DNA polymerase inhibitors like ganciclovir caused durable clinical responses in a small number of primary CNS PTLDs [22]. These combination therapies might induce lytic EBV replication and virion production, making the respective cells susceptible to viral DNA polymerase inhibition (Figure 7.2), while at the same time removing latently infected cells with B cell depleting therapy. Along these lines, histone deacetylase (HDAC) inhibitors and DNA damage-inducing drugs can be combined with ganciclovir for efficient EBV-associated tumor cell lysis [23, 24]. However, the pharmacological agents that have so far been used for this purpose in patients suffer from low specificity and efficacy for lytic EBV reactivation. Butyrates and romidepsin inhibit HDACs, but demonstrated either poor pharmacokinetics or intolerable toxicity during treatment of EBV-associated lymphomas [25–27]. Therefore, more specific inducers of lytic replication that progresses all the way to virion production need to be identified [28] and other targets of EBV infection should be explored for pharmacological inhibition of EBV-associated diseases. One such target is EBNA1 [29]. EBNA1 is required to maintain the EBV genome extra-chromosomally as a circular, multi-copy episome in infected cells by anchoring it to mitotic chromosomes during cell division and amplifying it prior to mitosis (Figure 7.2a). Without EBNA1, the virus transforms human B cells several thousand-fold less efficiently [30]. EBNA1 is therefore the only viral protein that is expressed in all EBV-associated tumors, including Burkitt lymphoma that expresses it as the sole viral protein [31]. Thus, targeting the viral genome maintenance function of EBNA1 could address an Achilles heel of EBV and be therapeutically useful against all associated malignancies. The EBNA1 domain that has mainly been explored is the C-terminal portion binding the viral DNA as a homodimer. Its structure bound to DNA had been previously solved [32] and viral DNA-derived palindromic oligonucleotide binding by recombinant versions of this domain have been explored for inhibition by small molecular compound libraries [33, 34]. Furthermore, EBNA1 phosphorylation inhibition and DNA modifications to prevent EBNA1 binding have been investigated to cause viral episome loss [35, 36]. Based on these encouraging results, four druggable pockets in the C-terminal domain of EBNA1 were predicted [37]. Two narrow sites with potential for allosteric inhibition of EBNA1 function and two wide pockets in the DNA binding or dimerization surfaces were identified. Molecular docking simulations were performed on the wide and narrow pocket in proximity to the viral DNA binding domain with a small molecular compound library. These

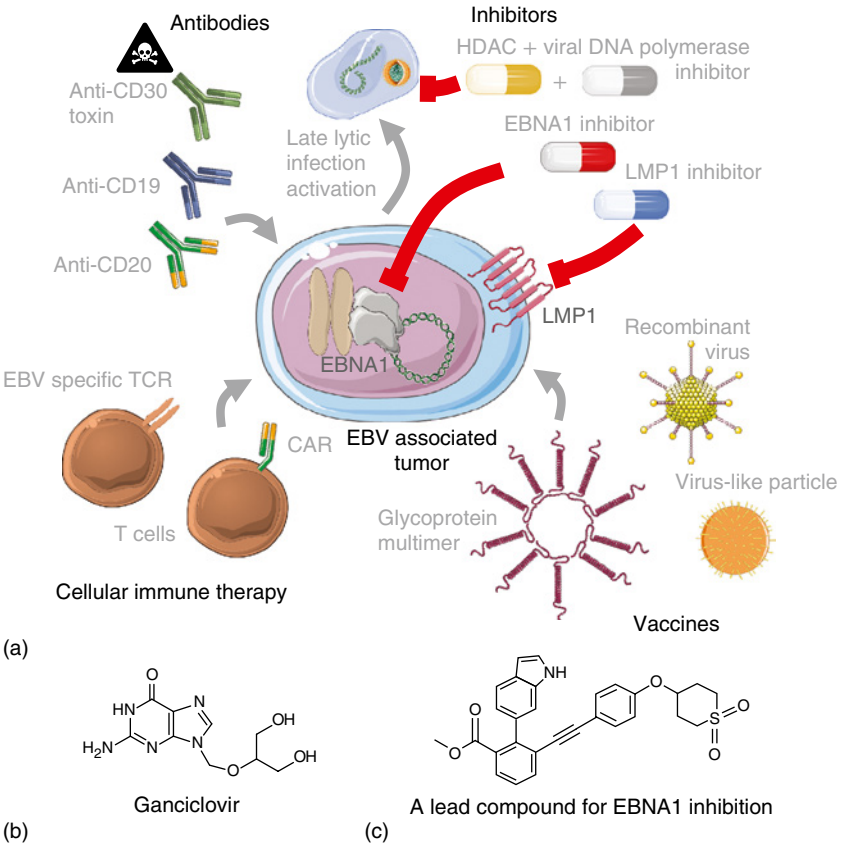

Figure 7.2 Therapeutic interventions against EBV-associated malignancies. (a) EBV-infected tumor cells express transforming latent viral proteins such as EBNA1 and LMP1. Pharmacological inhibitors against these are currently developed. Alternatively, their differentiation into late lytic EBV-infected cells has been therapeutically explored which makes these cells then susceptible to viral DNA polymerase inhibition by, for example, ganciclovir (b). Furthermore, EBV-associated lymphomas are treated via antibody-mediated depletion targeting their surface molecules CD20, CD19, and CD30. Moreover, adoptive T cell therapies have been explored against EBV-associated malignancies for more than 25 years. In addition to EBV-specific T cell receptor (TCR) specificities, also chimeric antigen receptors (CARs) targeting CD19, CD20, CD30, or LMP1 are investigated. Finally, EBV-specific vaccination is being developed with recombinant viral envelope antigen formulations like glycoprotein multimers and virus like particles (VLPs) or recombinant viral vectors expressing latent EBV antigens. (c) One of the lead compounds for an EBNA1 inhibitor [38]. *Source*: Servier Medical Art templates, Licensed under a Creative Commons Attribution 3.0 unported license: https://smart.servier.com.

studies identified lead compounds that have shown promising results in xenograft models of the EBV-associated epithelial cell cancer nasopharyngeal carcinoma (NPC) [38]. The so far most promising of these lead compounds is shown in Figure 7.2c. In addition to EBNA1, also LMP1 as the main viral oncogene [39] is being explored for pharmacological inhibition. This protein constitutively signals similar to the tumor necrosis factor (TNF)

receptor superfamily member CD40 upon auto-aggregation [40]. Indeed, homomultimerization sequences have been suggested as targets to inhibit LMP1 function and peptides that inhibit them or binding of TNF receptor-associated factors (TRAFs) are currently being explored [41] (Figure 7.2a). Thus, pharmacological targeting of latent EBV gene products, mainly EBNA1 and LMP1, or reactivation of lytic virion production are probably more suitable to target EBV-associated malignancies than viral DNA polymerase inhibition (Figure 7.2b).

7.2.2 B Cell Depleting Therapy

The greatest success in the treatment of EBV-associated lymphomas has so far been achieved with B cell depleting therapies, deploying reagents such as the anti-CD20 antibody rituximab (Figure 7.2a). Particularly for PTLD, B cell depleting therapies have reduced the cumulative incidence at least 10-fold from around 20% to slightly above 1% [42, 43]. This has currently even led to pretreatment with rituximab in transplant patients that are at risk to develop PTLD due to MHC, sex, and EBV serostatus mismatch [44, 45]. In addition to PTLD, other CD20-positive EBV-associated lymphomas, like diffuse large B cell lymphoma and Burkitt lymphoma, respond to rituximab treatment [46]. Interestingly, B cell depletions with the anti-CD20 antibodies rituximab and ocrelizumab demonstrated also clinical efficacy in MS patients [47–51] (Figure 7.3). MS risk is two-fold elevated after IM and 3.6-fold with elevated EBNA1-specific antibodies [52]. Both risk factors synergize with the main genetic risk factor for this autoimmune disease, the MHC class II molecule HLA-DRB1*1501, for odds ratios of 7 and 15, respectively. Moreover, in a longitudinal study, all EBV seronegative individuals seroconverted prior to MS onset [53]. The high viral titers during IM and the persistence of elevated EBNA1-specific antibody responses might set up and indicate, respectively, a reservoir of EBV-infected B cells in MS patients. Since LMP1 expression during latent EBV infection confers antigen presentation functions to B cells [54, 55], this reservoir in MS patients might stimulate autoimmune $CD4^+$ T cells [8] (Figure 7.3). B cell depletion might eliminate this reservoir of EBV-infected B cells and ameliorate disease. However, CD20 targeting eliminates all B cells prior to plasma cell differentiation and therefore might provoke a general loss of humoral immune control, especially after long-term application. Thus, a more selective targeting of EBV-infected cells is desirable. Along these lines, anti-CD30 antibodies like brentuximab have been explored in Hodgkin lymphoma and diffuse large B cell lymphoma, of which 10 or 40% are EBV positive [56]. Especially in its auristatin E conjugated form, inhibiting tumor cell mitosis via disruption of microtubule polymerization, CD30 targeting has resulted in promising results [57, 58] (Figure 7.2a). Enrichment of auristatin E in the tumor microenvironment might even be achieved if only a subset of the tumor cells is CD30 positive [59, 60]. In contrast to depletion of CD20 positive cells, CD30 targeted therapies show also promising results for some T cell lymphomas, like systemic anaplastic large-cell lymphoma (ALCL) [61, 62]. In individual patients, drug-conjugated brentuximab has also been reported to provide clinical benefits for EBV-associated extranodal NK/T cell lymphomas [63, 64]. Thus, CD30 targeting could deplete EBV transformed B and NK/T cells more selectively in patients with the respective lymphomas.

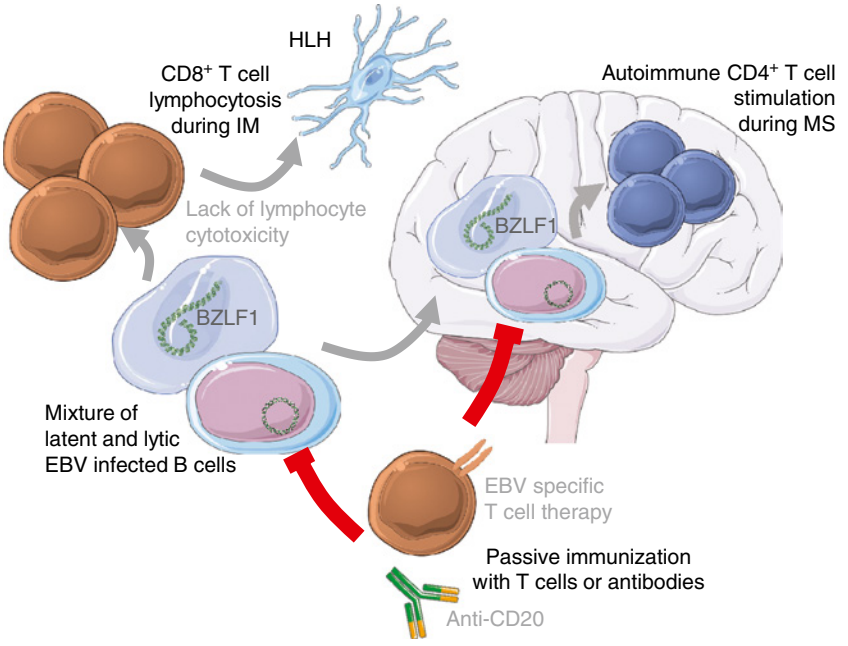

Figure 7.3 Therapeutic interventions against EBV-associated immunopathologies. Early lytic EBV antigens drive immunopathological $CD8^+$ T cell expansion during IM, the primary symptomatic EBV infection that occurs more often upon the first EBV encounter later in life. Inefficient cytotoxic immune control of EBV-infected cells allows this immune pathology then to progress to hemophagocytic lymphohistiocytosis (HLH) during which lymphocyte-derived cytokines activate myeloid cells to cause tissue damage and erythrocyte phagocytosis. IM also predisposes for multiple sclerosis (MS) by possibly establishing a reservoir of EBV-infected B cells that efficiently stimulates autoimmune $CD4^+$ T cell responses. The benefit of B cell depletion in MS and adoptive transfer of EBV-specific T cells might eliminate this detrimental reservoir. *Source*: Servier Medical Art templates, Licensed under a Creative Commons Attribution 3.0 unported license: https://smart.servier.com.

7.2.3 Adoptive T Cell Transfer and Immune Checkpoint Blockade

EBV-associated PTLD was one of the first clinical settings in which therapeutic adoptive transfer of antigen specific T cells was explored [65, 66] (Figure 7.2a). These T cell lines were originally generated by in vitro stimulation of donor-derived T cells with the autologous EBV-transformed B cell lines prior to transfer into the bone marrow recipient suffering from PTLD. However, this protocol required generation of the autologous EBV-transformed B cell line or lymphoblastoid cell line (LCL) from the bone marrow donor and expansion of EBV-specific T cells from the same donor for a month. In order to shorten the time period until transfer into the patient, EBV antigen-derived peptide pulsed DCs were explored next [67, 68]. These allowed focusing the transferred T cell product on distinct EBV antigens, some of which are also expressed in lymphomas, like Hodgkin lymphoma, with reduced latent EBV gene expression. The LMPs, primarily LMP2, were targeted in some of these approaches [69, 70]. Alternatively, EBNA1-specific T cells were selected prior to adoptive transfer with clinical responses in PTLD patients [71]. EBNA1,

LMP1, and LMP2-specific T cell lines were also explored with some clinical benefit in MS patients to improve immune control of virus-induced antigen presenting cells [72, 73] (Figure 7.3). In order to further reduce the time to treatment and choose both MHC restriction elements that are matched between bone marrow donor and recipient during allogeneic transplantation, banks of EBV-specific T cell lines and clones have been developed [74–76]. These different adoptive T cell transfer protocols achieved durable clinical responses in the majority, often two thirds, of treated patients suffering from EBV-associated lymphomas either expressing all eight latent EBV antigens or only EBNA1, LMP1, and LMP2. For further improvement of these therapies, especially to protect the adoptively transferred T cells from the immune suppressive tumor microenvironment and to endow them with additional specificities, genetic modifications were explored. For example, transgenic expression of a dominant negative receptor for the immune suppressive cytokine TGF-β seemed to confer increased clinical potency of LMP-specific T cell transfer into Hodgkin lymphoma patients [77]. Alternatively, blocking or deleting inhibitory receptors like PD-1 on these T cell products could further increase their clinical potency. Indeed, PD-1 blocking antibodies have led to significant clinical responses in Hodgkin lymphoma patients [78].

Finally, EBV-specific T cell lines might also be extremely well suitable for the expression of chimeric antigen receptors (CARs) or additional T cell receptors for mainly three reasons (Figure 7.2a). (i) For the treatment of EBV-associated lymphoma CARs that target CD19, CD20, CD30, or LMP1, and TCRs against LMP1 and 2 presented by common MHC class I molecules like HLA-A2 are explored [79–84]. The first benefit to express these additional antigen receptors on EBV-specific T cell lines is the benefit from re-stimulation upon viral antigen encounter after transfer into patients [79]. (ii) The second reason is that they can actually control reactivation of EBV infection at the same time as they mediate their antitumor effects [68]. (iii) Third, these EBV-specific T cell lines are depleted of alloreactive specificities, which minimizes cytokine release syndrome (CRS) after adoptive transfer [76]. Thus, adoptive T cell transfer is an efficient therapy of EBV-associated diseases and the use of allogeneic T cell banks and genetic modifications also reduces some of the logistic issues that were originally associated with autologous EBV-specific T cell line generation prior to transfer into patients.

7.2.4 Vaccination

Developing a vaccine against EBV to therapeutically reinstall immune control in patients with EBV-associated diseases and protect adolescents from IM is complicated due to the nature of the protective immune response against EBV. This immune control seems to nearly exclusively depend on cytotoxic lymphocytes, like $CD8^+$ T cells and NK cells, that can sufficiently expand during EBV infection [6, 9]. In contrast, type I and II interferon (IFN) and antibody deficiencies do not predispose for EBV-associated diseases [6]. Furthermore, established immune control of EBV does not protect from reinfection and/or circulation between the oropharynx and the blood [85, 86]. Thus, mucosal immunity, including neutralizing antibody responses against EBV, is not sufficient to prevent reinfection.

Nevertheless, the first vaccine that was tried against EBV aimed for the glycoprotein 350 of the EBV envelope that the virus uses to attach to B cells via CD21 [87, 88]. This

recombinant gp350 vaccine reduced IM incidence in young adults by 78%, but did not prevent EBV infection. Based on these encouraging findings multimeric gp350 vaccine formulations were developed [89] (Figure 7.2a). Furthermore, additional EBV glycoproteins were included in this vaccine formulation to elicit antibodies that block both entry into B and epithelial [90]. The neutralizing activity of the antibody responses that are raised against these vaccine candidates in mice and monkeys are quite encouraging, but any clinical effect of such a vaccination beyond IM prevention remains unclear. Therefore, additional vaccine formulations to elicit cytotoxic T cell responses have been explored. These include a recombinant modified vaccinia virus Ankara (MVA) that encodes for an EBNA1 fusion protein with LMP2 [91] (Figure 7.2a). It has been used in British and Chinese patients with the EBV-associated epithelial cell cancer NPC [92, 93]. Increased T cell responses to one or both of the vaccine antigens were observed in at least half of the treated individuals. In addition, an adenovirus encoding EBNA1 and a LMP1 and LMP2 derived polyepitope was developed [94]. It was used for expansion of EBV-specific T cell lines for adoptive transfer into NPC and MS patients [72, 73, 95]. The potency of these two recombinant vaccine formulations of adenoviral $CD8^+$ and MVA-mediated $CD4^+$ T cell priming can possibly be combined for a more potent induction of protective EBV-specific T cell responses [96]. EBNA1 as the sole vaccination antigen might be sufficient in this heterologous prime boost vaccination approach. In addition to recombinant viral vaccines, also virus-like particles (VLPs) of EBV itself have been explored to elicit both protective antibody and T cell responses, but have so far only been tested in preclinical models [97, 98] (Figure 7.2a). However, in one of these studies, a VLP containing EBNA1 in addition to the structural capsid, tegument and envelope proteins were able to elicit protective T cell responses that dampened EBV infection in mice with reconstituted human immune system components [98]. These studies with recombinant viruses and VLPs suggest that EBNA1 should be included as a vaccine component to elicit protective T cell responses [99].

7.3 Open Issues

More than 50 years after the discovery of EBV as the first human tumor virus [100, 101], investigators are starting to develop specific drugs and vaccines, encouraged by the success of virus-specific adoptive T cell transfer. While the above discussed treatments target latent transforming and late lytic structural EBV gene products, early lytic proteins that might contribute to tumorigenesis [1] are so far not explored for EBV treatments. Future treatment approaches might also want to consider this group of antigens. The implementation of the so-far developed treatments will in addition to addressing important clinical needs answer important questions of EBV immunobiology, namely if targeting of cellular transformation diminishes EBV persistence, if sterilizing immunity can be achieved with more potent antibody induction and if therapeutic reduction or maybe even elimination of persistent EBV infection has also negative effects on the human immune system. Along these lines, recent studies in preclinical models suggested that EBV persistence benefits from, but does not require cellular transformation [102].

The strong NIH program to develop an EBV vaccine that elicits envelope protein-specific antibody responses [89, 90] might elicit more potent humoral immunity than EBV infection

itself. However, the danger exists that if sterilizing immunity can only be achieved for a certain time period, primary infection is delayed and might then elicit more frequent and more severe IM symptoms. Finally, the EBV inhibitors in development could actually clear infection, but reinfection would probably occur prior to loss of EBV-specific immune memory and therefore without IM symptoms [85, 86]. However, during this time period of EBV loss, it would be interesting to investigate which overall changes occur in the human immune system and if our leucocytes benefit from having EBV as a sparring partner, as has been demonstrated for the β-herpesvirus cytomegalovirus [103].

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8

Kaposi's Sarcoma-associated Herpesvirus—Antiviral Treatment

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8.1 Introduction to Kaposi's Sarcoma-associated Herpesvirus (KSHV)

Kaposi's sarcoma-associated herpesvirus (KSHV), taxonomical name human herpesvirus 8 (HHV8), is a human γ_2-herpesvirus (rhadinovirus) and the cause of three human malignancies, a systemic inflammatory condition and rare cases of hemophagocytosis with bone marrow failure and hepatitis. The three malignant diseases are Kaposi's sarcoma (KS), in which this virus was originally discovered and from which the most commonly used name for this virus is derived [1], primary effusion lymphoma (PEL) [2], and the plasma cell variant of multicentric Castleman's disease (MCD) [3]. In addition, KSHV has been linked to rare cases of post-transplant polyclonal lymphoproliferative disorders with plasmacytic differentiation [4–6]. Among its nonmalignant clinical manifestations are KSHV-associated inflammatory cytokine syndrome (KICS) [7–10], KSHV-induced hemophagocytosis in transplant recipients [11–13], and rare cases of KSHV-associated hepatitis in transplanted patients [14, 15]. Substantial epidemiological evidence, together with a plethora of mechanistic experimental studies support the causative involvement of this virus in the pathogenesis of the three malignant tumors KS, PEL and MCD, and KSHV has consequently been classified as a class I human carcinogen by the International Agency for Research against Cancer (IARC), a WHO agency tasked with evaluating the carcinogenic risk to humans. The evidence leading to this classification has been summarized [16, 17] and has also been discussed in many recent reviews to which the reader is referred [18–21].

8.2 Epidemiological Considerations

Unlike most other human herpesviruses, KSHV shows a very uneven geographical distribution. It is common in sub-Saharan Africa, with prevalence rates of antibodies to KSHV usually higher than 50% in adult populations and particularly high seroprevalence rates in

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East and Central Africa (for a review, see [17]). In countries bordering on the Mediterranean, prevalence is intermediate, with seroprevalence rates in the 3–20% range and considerable regional variability. In contrast, the virus is very rare in Northern and Western Europe as well as most of Asia. Higher prevalence rates have also been reported in populations of African descent in the Americas, as well as in some native American populations [22, 23]; for detailed references, see [17]. Further examples of KSHV being associated with particular populations are the higher KSHV seroprevalence in Uighur populations in Xinjiang, China [24] and in the Buryat population in Southern Siberia [25], as well as the higher seroprevalence and presence of particular KSHV genotypes in Israel [26]. In addition, KSHV seroprevalence rates are often higher among men who have sex with men (MSM) than in the general population of the same country (detailed references in [17]).

In endemic countries or populations with a high KSHV seroprevalence, transmission of the virus occurs mainly in childhood before puberty, with mother–child transmission, as well as transmission among siblings with virus-containing saliva being considered an important route of spread [27–33]. In adults living in KSHV endemic countries, as well as for individuals at increased risk of sexually transmitted diseases, there is evidence of sexual transmission of KSHV [34–38]. KSHV-containing saliva is also thought to play an important role during sexual transmission [27, 37].

8.3 Disease Overview

8.3.1 Kaposi's Sarcoma

From a clinical point of view, four distinct variants of KS can be distinguished. First described by Moritz Kaposi in 1872, the "**classical**" form of KS is today defined as a mostly indolent, slowly progressing tumor on the lower extremities of elderly men from countries with a higher KSHV seroprevalence, such as countries around the Mediterranean. Patients with classic KS always have antibodies to KSHV, the virus can be detected by PCR in tumor biopsies and tumor cells latently infected with KSHV can be identified in tissue sections by immunohistochemistry staining for the KSHV latent nuclear antigen, LANA (see Figure 8.1 and Section 8.5.4.1). Classic KS is a rare tumor, with reported incidence rates ranging from less than 1 to about 3 per 100 000 population, even in regions of Southern Europe with KSHV seroprevalence rates in the range of 10–20% [17]. In these geographic regions, the development of KS in a KSHV seropositive individual is therefore a rare event and the vast majority of KSHV-infected individuals will never experience any clinical manifestations.

Clinically more aggressive and often characterized by the involvement of internal organs such as the gut or the lung is the **African endemic** form of KS in HIV-negative individuals. First described in the 1920s and characterized clinically in the 1960s, this clinical variant occurs predominantly in East and Central Africa. Environmental cofactors are thought to contribute to the more aggressive behavior of these tumors by promoting virus replication. Suggested candidates for such cofactors include other infectious agents, in particular helminths or malaria, but also certain plant chemicals: there is substantial epidemiological evidence for helminth infections and/or malaria coinfection promoting KSHV productive replication and KSHV infection [39–42]. Furthermore, there is laboratory evidence to

suggest that extracts from certain plants found in East and Central Africa may promote KSHV replication in tissue culture [43].

The third clinical KS variant is **transplant-associated** or "**iatrogenic**" **KS**. Transplant-associated KS occurs several hundred fold more frequently in transplant recipients compared to the general population of the same country [44]. It is encountered mainly in countries with increased KSHV seroprevalence rates. Estimations of its frequency vary from <1 to 11% of solid organ transplant recipients, depending on the country and the transplanted organ, with post-transplant KS more common in kidney transplant recipients than recipients of other solid organs and very rare in hematopoetic stem cell transplant recipients [45–54]. KS occurs much more frequently in transplant recipients who are KSHV seropositive at the time of transplantation: a pooled analysis of six studies on kidney, liver, and heart transplant recipients from Italy and France suggests that approximately a quarter (25.6%) of transplant recipients who were KSHV seropositive before transplantation developed transplant-associated KS [6, 55–58]; more extensive data are reviewed in [45, 59]. The

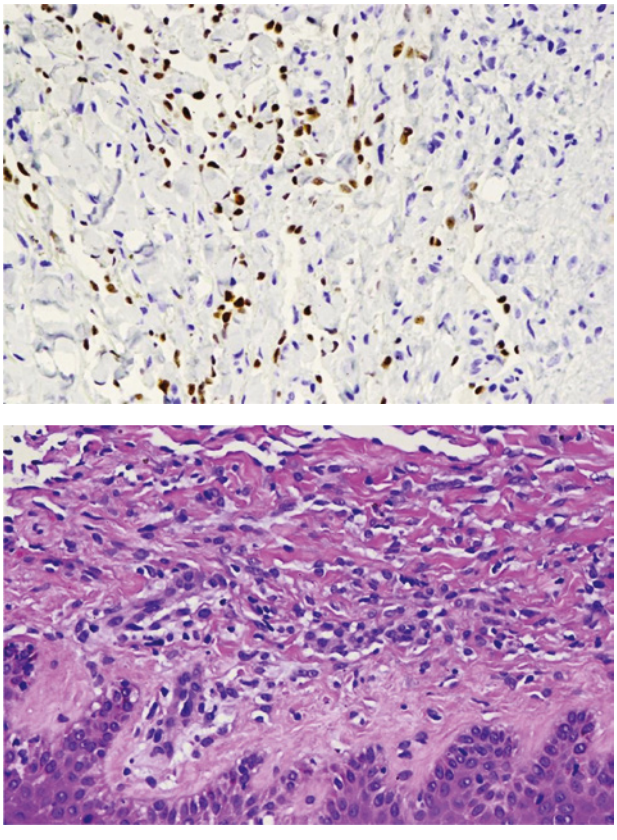


Figure 8.1 Expression of KSHV LANA in a KS biopsy. (a) Immunohistochemistry staining using an antibody to KSHV LANA. KSHV-infected, LANA-expressing endothelial spindle cells are stained in dark brown color. (b) HE stain. The panel shows the atypical endothelial KS spindle cells, which are the histological hallmark of KS lesions. *Source*: Dr. Guntram Büsche, Dept. of Pathology, Hannover Medical School.

rate of post-transplant KS may have decreased in the last decade following the switch from calcineurin inhibitors as immunosuppressive agents in solid organ transplant recipients with KS to an mTOR inhibitor-based regimen (see Section 8.4.2.1; [59]). Most cases of transplant KS are thought to result from the reactivation of KSHV in patients who were already infected with KSHV prior to transplantation [45, 56, 60]. However, cases of donor–recipient transmission of KSHV have been noted [61, 62]. These findings indicate that transplant-associated KS is more of a problem in countries with a higher KSHV seroprevalence and in transplant recipients with pre-existing KSHV infection.

The incidence of KS increased markedly with the worldwide spread of HIV in the 1980s and **AIDS-associated KS**, or **epidemic KS**, now represents the commonest KS variant worldwide. KS is several thousand times more frequent in HIV-infected individuals than in the general population of the same country [17, 44]. In East Africa (Uganda), KS was, in the early 1990s, the commonest cancer in men, and is today the second most common cancer in men and the third most frequent cancer in women in this geographic region [63]. Incidence rates of KS in Ugandan men were in the range of 30 per 100 000 in the first decade of the twenty-first century, having decreased from even higher levels after the introduction of effective antiretroviral combination therapy (ART) [63]. KS incidence rates have also decreased strongly after the introduction of ART in Western countries but remain much higher among HIV-infected persons than in the general population [64, 65].

An important therapeutic challenge therefore is the HIV-infected patient with well-controlled HIV viral load whose KS tumor does not regress or who develops new KS lesions under effective antiretroviral therapy. In the United States, AIDS-associated KS cases in patients with well-controlled HIV now account for about a third of all KS presentations [66, 67]. Together with transplant recipients with KS, these are the KS patients who are most in need of innovative treatment approaches and for whom innovative drugs with activity against KSHV-associated disease are required.

8.3.2 Primary Effusion Lymphoma (PEL)

PEL, which is also referred to as body cavity-based lymphoma, is a very rare malignant B cell non-Hodgkin lymphoma occurring mainly in AIDS patients and rarely also in transplant recipients ([2, 5, 68, 69]; further literature in [17, 59]). It accounts for 2–5% of HIV-related lymphomas and presents as pleural, peritoneal, and pericardial lymphomatous effusions, often in the absence of a clinically detectable solid tumor mass. The presence of KSHV DNA in the tumor cells is considered a diagnostic criterion. Thus, other AIDS-related lymphomas presenting with body cavity effusion but lacking KSHV in the tumor cell are not classified as PEL. PEL tumor cells are of post-germinal center B cell origin, lack many typical B cell surface markers, and show a plasmacytic differentiation pattern, as assessed by their transcriptome and the expression of CD134 [70].

8.3.3 Multicentric Castleman's Disease (MCD)

MCD is a polyclonal lymphoproliferative disease. The vast majority of HIV-associated MCD cases and about half of HIV-negative cases are infected with KSHV ([3, 71, 72]; further references in [17]). Unlike localized Castleman disease (which is often KSHV

negative), the multicentric form behaves more aggressively and is often fatal. There are several reports of non-Hodgkin lymphomas, in particular plasmablastic lymphoma and EBV-negative PEL tumors, developing out of MCD lesions [68, 73]. KSHV-infected B cells in MCD express only lambda light chains and are therefore monotypic; they express CD38, IRF4, BLIMP1 but are commonly negative for CD30, CD40, and the plasma cell marker CD138. These cells also do not exhibit somatic mutations in the hypervariable regions of their immunoglobulin genes and are therefore of a pre-germinal center differentiation stage despite displaying evidence of a plasmacytic differentiation [70].

The clinical presentation of KSHV-associated MCD cases is often characterized by symptoms and laboratory evidence of systemic inflammation. Clinical symptoms include, in addition to lymphadenopathy, fever, night sweats, weight loss, diarrhea, and fatigue. Abnormal laboratory findings consist of hypergammaglobulinemia, cytopenia, hypoalbuminemia, elevated CRP levels as well as raised serum levels of human IL6, IL10, and the viral cytokine vIL6 [72, 74–76]. There is often abundant expression of human IL6 in the germinal centers of MCD lymph nodes and expression vIL6 is regularly seen in KSHV-infected plasmacytic B cells in these lesions [75, 77, 78].

In addition to vIL6, other lytic viral proteins may also be expressed in MCD lesions [77–79], in addition to the latent viral protein LANA (see Section 8.5.4.1). KSHV viral load in peripheral blood is often high in patients with active MCD tumors [70]. There are reports of the successful treatment of MCD cases with ganciclovir, an inhibitor of the KSHV DNA polymerase and thereby of lytic (productive) replication [80] (see Section 8.4.1.1.5). More recently, a combination treatment with high-dose ganciclovir and AZT has been shown to be beneficial in MCD cases [81] (see Section 8.4.1.1.5). These observations suggest that KSHV lytic (productive) replication and viral proteins expressed during the lytic replication cycle may contribute to the pathogenesis of MCD (see Section 8.4.1.1.5). Although KSHV-infected plasmablasts in MCD are often negative for CD20, rituximab (an antibody to CD20) is very effective in controlling MCD with response rates higher than 70% [59, 82–84]. Rituximab can be applied in combination with liposomal doxorubicin to prevent flares of KS [85].

8.3.4 KSHV and Polyclonal Post-transplant Lymphoproliferative Disease

There are several case reports of polyclonal lymphoproliferative disease in transplant recipients involving KSHV-infected B cells with a plasmacytic differentiation, but which do not meet the diagnostic criteria for PEL or MCD [4–6]. Their clinical presentation may include systemic symptoms, such as fever, anemia, skin rash, arthritis, and the diffuse lymphocytic infiltration of lymph nodes and visceral organs [6].

8.3.5 Kaposi Sarcoma Herpesvirus-Associated Inflammatory Cytokine Syndrome (KICS)

KICS is defined as a clinical syndrome of systemic inflammation in KSHV- and HIV-coinfected patients in the absence of MCD. KICS is characterized by clinical signs such as fever, night sweat, fatigue, diarrhea, weight loss, thrombocytopenia, hypoalbuminemia, markedly elevated CRP levels, high KSHV viral load in peripheral blood, and raised IL6

and IL10 levels [7–10, 14, 86]. Patients with KICS frequently also present with KS or PEL and are often severely ill with 50% survival rates of around a couple of years [9]. KICS can occur in patients with well-controlled HIV load [87]. As for MCD, treatment can be attempted with rituximab to eliminate KSHV-infected B-cells [88] or with monoclonal antibodies against the IL6 receptor such as tocilizumab [86].

8.3.6 KSHV and Hemophagocytosis, Bone Marrow Failure, and Hepatitis

KSHV has also been linked to cases of bone marrow failure, sometimes associated with evidence of hemophagocytosis, and rare cases of hepatitis in transplant recipients [10–15]. These clinical manifestations may be accompanied by a high KSHV viral load in peripheral blood and respond to treatment with antivirals or rituximab (see Section 8.4.1.1 and [59]).

8.4 Antiviral Strategies

As already touched on in the preceding sections, the treatment of KSHV-associated diseases may either be directed at the virus itself, or against KSHV-infected cells, tumor cells, or cellular cytokines secreted as part of the disease process. In the following section, the focus will be on established and experimental antiviral strategies.

8.4.1 Established Antiviral Strategies

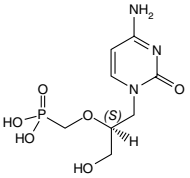
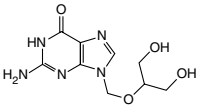
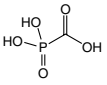
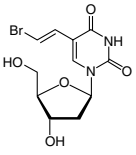
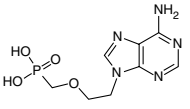
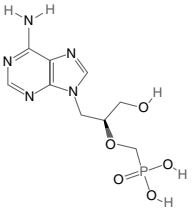
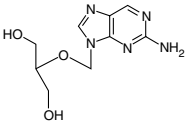
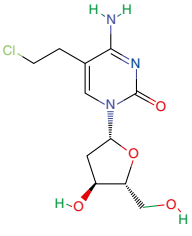
8.4.1.1 Inhibitors of the KSHV DNA Polymerase (Table 8.1)

8.4.1.1.1 Activity in Tissue Culture

Several competitive nucleoside inhibitors developed against other herpesviral DNA polymerases and approved for clinical use also show activity against KSHV. In tissue culture, several studies have found cidofovir to be the most potent drug and to inhibit viral DNA replication with IC50 values ranging from 0.05 to 6 μM, depending on the cell culture and virus detection assay used [89–92]. Ganciclovir (reported IC50 values 0.96–11 μM), foscarnet (34–177 μM), brivudine (0.6 μM), and adefovir (39 μM) are also inhibitors, while aciclovir and penciclovir are less effective [89–93]. Among newer nucleoside inhibitors that are not yet in clinical use, HPMPA ((*S*)-9-[3-hydroxy-2-(phosphonomethoxy)-propyl] adenine; 0.6 μM), S2242 ([(1,3-dihydroxy-2-propoxymethyl)purine]; 0.1 μM), (E)-CVDC ((E)-5-(2-chlorovinyl)-2'-deoxycytidine; 2.4 μM), CVDU (5-(2-Chlorovinyl)-2'-deoxyuridine; 5.5 μM), FIAC (2'-fluoro-5-iodo-aracytosine; 1 μM), N-MCT (2'-exo-methanocarbathymidine; 0.08 μM), and HDVD (1-[(2*S*,4*S*-2-(hydroxymethyl)-1,3-dioxolan-4-yl]5-vinylpyrimidine-2,4(1H,3H)-dione; 0.09 μM) showed strong activity [91, 93–95]. Additional compounds with good activity against KSHV in tissue culture include cyclopropavir and several 6-alkoxy-substituted methylene cyclopropane nucleosides (cyclopropavir derivatives; [96]). For an overview, see Table 8.1.

With the exception of foscarnet, these compounds need to be activated by phosphorylation in the infected cell by virus-encoded kinases. In the case of KSHV, the viral protein kinase (vPK) encoded by open reading frame (ORF) 36, which is the homologue of the cytomegalovirus pUL97 vPK, mediates phosphorylation of ganciclovir and acyclovir [93, 112]. The second KSHV kinase is encoded by KSHV ORF21 and represents the

Table 8.1 Directly acting antivirals (DAA) with activity against KSHV.

	Compound	Target	IC50	Chemical structure	References
Clinical trial or case report data available	Cidofovir	Viral DNA polymerase	0.05–6 µM		[89–92]
	Ganciclovir	Viral DNA polymerase	2.6–11 µM		[89–93]
	Foscarnet	Viral DNA polymerase	34–177 µM		[89–93]
	Brivudine	Viral DNA polymerase	0.6 µM		[97, 98]
	Adefovir	Viral DNA polymerase	39 µM		[98]
No clinical data available	HPMPA (*S*)-9-[3-hydroxy-2-(phosphono-methoxy)-propyl] adenine	Viral DNA polymerase	0.6 µM		[91, 93]
	S2242 (1,3-dihydroxy-2-propoxymethyl) purine	Viral DNA polymerase	0.1 µM		[91, 93]
	(*E*)-CVDC (*E*)-5-(2-chlorovinyl)-2'-deoxycytidine	Viral DNA polymerase	2.4 µM		[91, 93]

(Continued)

Table 8.1 (Continued)

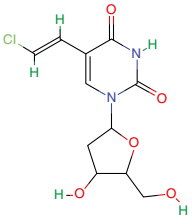
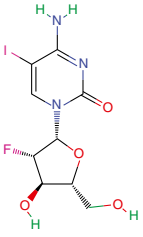
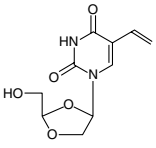
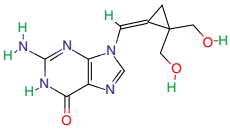
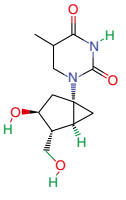
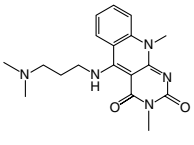
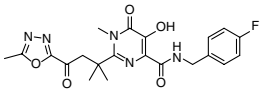
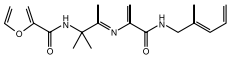
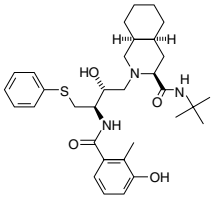
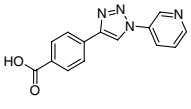
	Compound	Target	IC50	Chemical structure	References
	CVDU 5-(2-Chlorovinyl)-2'-deoxyuridine	Viral DNA polymerase	5.5 µM		[91, 93]
	FIAC 2'-fluoro-5-iodo-aracytosine	Viral DNA polymerase	1 µM		[91, 93]
	HDVD 1-[(2*S*,4*S*-2-(hydroxymethyl)-1,3-dioxolan-4-yl]5-vinylpyrimidine-2,4(1*H*,3*H*)-dione	Viral DNA polymerase	0.09 µM		[94]
	Cyclopropavir	Viral DNA polymerase	~4 µM		[96]
	2'-Exo-methanocarba-thymidine	Viral DNA polymerase	~0.1 µM		[95]
	NSC 373989	pORF9 (Pol)/ pORF59 (PAF) complex	~2 µM		[99]
	XZ45	pORF6 (ssDNA BP)? pORF29 (terminase)?	n.a.		[100]

Table 8.1 (Continued)

Compound	Target	IC50	Chemical structure	References
Raltegravir	pORF29 (terminase)			[101]
Dolutegravir	pORF29 (terminase)		 Dolutegravir (DOL)	[101]
Compound 14	KSHV protease	~24 μM		[102, 103]
Nelfinavir	KSHV protease? Cellular target?	~5 μM		[104, 105]
K8.1-scFV-immunotoxin	Glycoprotein K8.1		scFV-exotoxin A conjugate	[106]
gH-scFV-immunotoxin	Glycoprotein gH		scFV-exotoxin A conjugate	[107]
LANA inhibitor I	LANA interaction with the viral latent origin of replication	17–19 μM in *in vitro* DNA binding (fluorescence polarization) assay		[108, 109]
Cyclic IKKγ-mimetic peptide	vFLIP	Approx. 25–50 μM in apoptosis assays	Ac-LQVAYH**X** LFQ**X**YDNH IKSSC-NH_2	[110, 111]

homologue of the HSV UL23 and VZV ORF36 thymidine kinases (TK) [93, 97, 113]. Although KSHV TK is primarily a protein tyrosine kinase [113] and only a comparatively poor TK [114], it does mediate the phosphorylation and activation of brivudine and the anti-HIV nucleoside reverse transcriptase inhibitor azidothymidine (AZT) [93, 97]. In contrast to the α-herpesviral TK of HSV and VZV, KSHV TK does not efficiently phosphorylate thymidine compounds such as aciclovir [114].

8.4.1.1.2 Activity in KSHV-infected Patients

Reflecting their activity against KSHV in tissue culture, the three clinically approved nucleoside inhibitors of the herpesviral DNA polymerase (ganciclovir, cidofovir, and foscarnet) have been shown to reduce KSHV shedding or viral load in peripheral blood in patients. Oral valganciclovir has been reported to reduce KSHV shedding in oral samples in one study [115]. Valacyclovir and famciclovir treatment was associated with a moderate

reduction in the number of treated individuals shedding KSHV in the oropharynx [116]. While two groups failed to observe a reduction of the KSHV load in peripheral blood leukocytes following treatment with ganciclovir or foscarnet [117, 118], others reported that foscarnet decreased the KSHV viral load in peripheral blood and led to a clinical improvement in two transplant patients with KSHV-associated pancytopenia and hemophagocytosis [11, 119]. However, in spite of this indication of a moderate *in vivo* effect, herpesviral DNA polymerase inhibitors have only limited clinical efficacy when used to treat KSHV-associated disease.

8.4.1.1.3 Treatment of Kaposi Sarcoma

When used in patients with KS, herpesviral DNA polymerase inhibitors do not seem to be effective against this tumor. Krown and colleagues did not observe any clinical improvement in five patients with classic KS after treatment with valganciclovir [120]. Similarly, cidofovir had no beneficial impact on disease progression in five cases of AIDS-KS and two classic KS cases [121]. However, Mazzi and colleagues reported a beneficial effect of cidofovir on KSHV viremia and disease progression in two patients with AIDS KS [122]. In contrast, intralesional injection of cidofovir in one patient with classic KS was ineffective [123]. This combined experience probably reflects the fact that the viral gene expression program in KS tumors is mostly restricted to the latent viral genes LANA, vcyc, vFLIP, and a cluster of viral miRNAs, although it may in some KS tumors also extend to a few early viral genes, such as K15 [77, 78, 124, 125]. Productive viral replication—which would be susceptible to inhibitors of the viral DNA polymerase and may be required for the activation of prodrugs by KSHV vPK/ORF36 or TK/ORF21 (see Section 8.4.1.1.1)—therefore probably only occurs in a small proportion of infected cells. It is worth noting, however, that the use of herpesviral DNA polymerase inhibitors as preventive treatment, or as treatment for other herpesviral diseases, has consistently been found to lower the incidence of KS [126, 127]. This observation suggests that herpesviral DNA polymerase inhibitors may reduce viral replication and the infection of new cells in KSHV-infected patients and thereby decrease the risk of infected endothelial cells developing into KS tumors.

Valganciclovir and cidofovir have been used successfully in combination with liposomal doxorubicin/daunorubicin, the conventional cancer chemotherapy for KS [128, 129], but the contribution of the herpesviral DNA polymerase inhibitors to the treatment success is difficult to ascertain from these case studies.

8.4.1.1.4 Treatment of PEL

Since PEL is a very rare non-Hodgkin lymphoma (see Section 8.3.2), there are only case reports or case series describing the use of herpesviral DNA polymerase inhibitors in individual cases. Parenteral ganciclovir, in some cases followed by oral valganciclovir, or cidofovir, sometimes injected into the body cavities affected by malignant effusions, produced disease remissions in some, but not all cases [130–132]. Ganciclovir in combination with chemotherapy was also beneficial in one case [133]. Overall, this experience suggests that herpesviral DNA polymerase inhibitors are not the mainstay of PEL treatment, but could be tried in combination with conventional cancer therapy in particular cases.

8.4.1.1.5 Treatment of MCD

Similar to the treatment of PEL with herpesviral DNA polymerase inhibitors, the experience with these drugs, when used on their own, in patients suffering from MCD has been mixed. While there are reports of the successful treatment of KSHV- and HIV-associated MCD with ganciclovir [80], cidofovir failed in five patients [134]. Several successful treatment attempts combined chemotherapy, rituximab, or the proteasome inhibitor bortezomib with ganciclovir [135–137].

A pilot trial of combining two nucleoside inhibitors, ganciclovir and the antiretroviral reverse transcriptase inhibitor AZT,, in high doses yielded very promising results [81], with 12 of 14 treated patients showing a major clinical response and 7 experiencing a major improvement of biochemical parameters (levels of CRP, albumin, sodium, hemoglobin, and platelet count; see Section 8.3.3). Since AZT is phosphorylated and activated to a toxic moiety by KSHV TK [114], it is possible that this high-dose AZT/GCV combination therapy succeeded in eliminating KSHV-infected B-cells expressing TK, while GCV, activated by KSHV vPK/ORF36 (see Section 8.4.1.1.1), might have had an antiviral effect [81].

8.4.2 Kinase Inhibitors Against KSHV-associated Disease (Table 8.2)

8.4.2.1 Clinical Experience

Clinically approved tyrosine kinase inhibitors have been used to treat KSHV-associated diseases, in particular KS. The rationale for their use lies in the observation that several cellular receptor tyrosine kinases such as c-kit, PDGFR, and EphA2 play important roles in the KSHV life cycle (see reviews [19, 20]). Imatinib, an inhibitor of c-abl, c-kit, and PDGFR, has been used in clinical trials of KS and induced a partial tumor regression in about a third of patients [138, 139]. Similarly, sorafenib, which targets receptors of VEGF and PDGF as well as c-kit, has been tried with moderate success [140, 141].

The PI3K/AKT–mTOR pathway has emerged as an important player in the proliferation and angiogenesis of KSHV-infected endothelial cells as well as in KSHV productive replication (reviewed in [19–21]). Several viral proteins, including the nonstructural viral membrane proteins encoded by ORFs K1, ORF74 (the viral homologue of a G protein-coupled receptor; vGCR), ORF K15, the vPK encoded by ORF36, and the viral IL6 homologue (vIL6) contribute to the activation of the PI3/AKT pathway in KSHV-infected cells undergoing the early stages of viral reactivation or showing a "relaxed latency" viral transcriptional program [125, 142–146]. The mTOR kinase in the PI3K/AKT pathway is targeted by the FK506/rapamycin complex and rapamycin has proved to be successful in the treatment of transplant KS [59, 147, 148]. Rapamycin has also been tested successfully in patients with AIDS-KS [149].

8.4.2.2 Preclinical Studies

Ongoing preclinical research continues to explore cellular kinases as druggable targets to treat KSHV disease.

In addition to the PI3K/AKT/mTOR pathway (see Section 8.4.2.1 and below in this section), the ERK/MAPK pathway and its downstream effector kinases have turned out to be required for KSHV infection as well as reactivation [150, 151]. Several inhibitors targeting this pathway, including clinically approved drugs such as the MEK1/2 inhibitor trametinib,

Table 8.2 Compounds or biologicals directed at cellular targets and with activity against KSHV or KSHV disease.

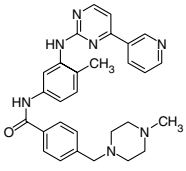
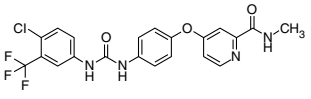
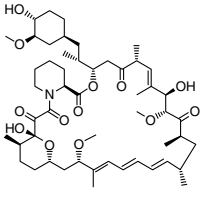
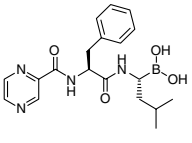
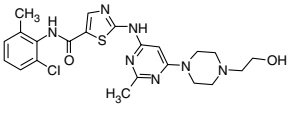
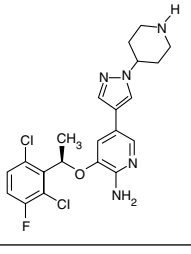
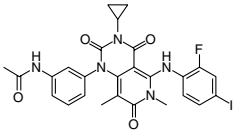
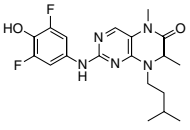
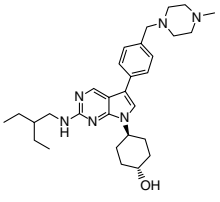
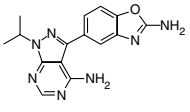
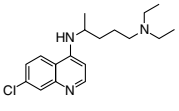
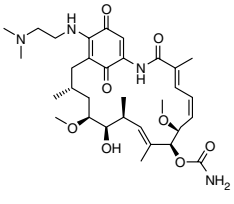
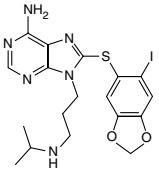
	Compound	Target	Structure	References
Clinical data available	Imatinib	c-abl, c-kit, PDGFR		[138, 139]
	Sorafenib	VEGFR, PDGFR, c-kit		[140, 141]
	Rapamycin/ Sirolimus	mTOR		[149, 166–170]
	Bortezomib	Proteasome		[184, 185, 187–189]
	Rituximab	CD20	Monoclonal antibody	
	Bevacizumab	VEGF	Monoclonal antibody	[173–175]
	Siltuximab	IL6 R	Monoclonal antibody	[176, 177]
	Tocilizumab	IL6 R	Monoclonal antibody	[176, 177]
Only preclinical experimental data available	Dasatinib	BCR-Abl, Src, Lck, Lyn, Yes, Fyn, c-Kit, EphA2, PDGFRβ		[97]
	Crizotinib (PF-2341066)	c-Met, ALK		[164]

Table 8.2 (Continued)

Compound	Target	Structure	References
Trametinib	MEK		[97]
BI-D1870	RSK-1/2		[156]
UNC3810A	Tyro 3		[165]
MLN0128/ Sapanisertib	mTOR		[171]
Chloroquine	mTOR, p38-MAPK		[172]
17-DMAG/ Alvespimycin 17-Dimethyla minoethylamino-17-demethoxygel danamycin	HSP90		[178–180]
PU-H71 8-[(6-Iodo-1,3-benzodioxol-5-yl) sulfanyl]-9-[3-(propan-2-ylamino) propyl] purin-6-amine	HSP90		[178–180]

(Continued)

Table 8.2 (Continued)

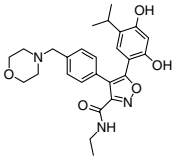
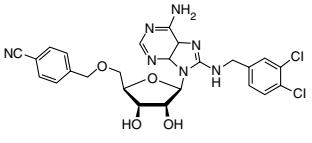
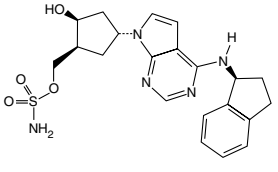
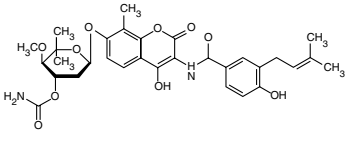
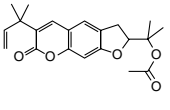
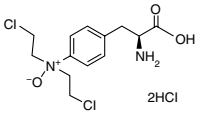
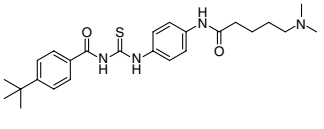
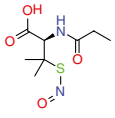
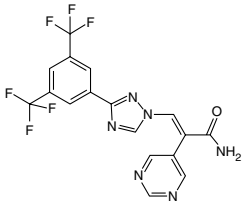
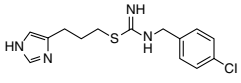
Compound	Target	Structure	References
AUY-922/ Luminespib 5-(2,4-Dihydroxy-5-isopropyl-phenyl)-*N*-ethyl-4-[4-(morpholinomethyl) phenyl] isoxazole-3-carboxamide	HSP90		[178–180]
VER-155008	HSP70		[182]
MLN4924	NEDDylation		[183]
Novobiocin	Topoisomerase II		[195]
(+)-Rutamarin	Topoisomerase II		[195]
PX-478	HIF-1α		[205]
Tenovin-6	SIRT1		[206]
C10	APE1		[207]

Table 8.2 (Continued)

Compound	Target	Structure	References
SnPP	Heme oxygenase I		[208]
KPT-8602	XPO1/Crm1		[209]
Clobenprobit	Histamine receptor 3		[210]

but also experimental compounds such as sangivamycin and capsaicin have been shown to inhibit KSHV reactivation or to induce apoptosis in PEL cells [97, 152, 153]. Several KSHV proteins such as the viral chemokine receptor homologue (vGCR) [154], the ORF36-encoded vPK [155], and the viral tegument protein encoded by ORF45 [156, 157] have been shown to induce the MEK/ERK pathway or downstream RSK1/2 kinases and with it viral replication [158]. BI-D1870, a RSK1/2 inhibitor, suppresses KSHV lytic gene expression and virus production [156]. The ORF45 tegument protein interacts directly with RSK kinases and this protein complex also contains activated ERK [157, 159] and is required to sustain late viral gene expression and virus production [156]. The pORF45/RSK complex mediates the phosphorylation of the eukaryotic translation initiation factor eIF4B; the pORF45/RSK-mediated phosphorylation of eIF4B is resistant to rapamycin (i.e. not dependent on mTOR) and U1026 (i.e. not dependent on MEK) [160]. An attempt was therefore made to target the pOR45/RSK interaction directly, resulting in the development of a small pORF45-derived peptide that inhibits the formation of this complex as well as viral lytic gene expression and virus production [161].

The clinically approved tyrosine kinase inhibitors dasatinib, ponatinib and bosutinib are potent inhibitors of the protein kinase activity of KSHV TK and also of KSHV reactivation, but these two effects may not be directly linked, as dasatinib, ponatinib, and bosutinib target a range of cellular tyrosine kinases that are likely involved in KSHV reactivation [97]. Dasatinib reduced the in vivo tumor growth of KSHV-infected endothelial cells in a xenograft model to the same extent as imatinib [97]. Since these three compounds also efficiently inhibit the ability of KSHV TK to autophosphorylate tyrosine residues in its

aminoterminal end, they may be able to exert an additional inhibitory effect on KSHV replication *in vivo*, since the TK homologue of a murine gammaherpesvirus, MHV68, is required for efficient replication *in vivo* while being dispensable in tissue culture [97, 162, 163]. Ibrutinib, an inhibitor of Bruton's tyrosine kinase, also inhibited KSHV reactivation in B cells [97].

In the context of PEL, crizotinib (PF-2341066), a small molecule inhibitor of ALK and c-Met, the receptor for hepatocyte growth factor (HGF), induces PEL cell apoptosis through cell cycle arrest and DNA damage, and suppresses tumor progression in a mouse xenograft model [164]. Crizotinib also inhibited KSHV replication in KSHV-infected BJAB cells, a B-cell line [97]. The receptor tyrosine kinase Tyro 3, a member of the Tyro3/Axl/Mer TK (TAM) family that is known to promote proliferation, survival, and chemoresistance in several solid cancers, is specifically upregulated in PEL cells and essential for their survival [165]. A small molecule inhibitor, UNC3810A, inhibits PEL cell proliferation in tissue culture at nanomolar concentrations and in a mouse xenograft model [165].

As described in the preceding section, the PI3K/AKT/mTOR pathway plays an important role in the KSHV life cycle. In cultured primary endothelial cells, KSHV induces mTORC1 activation and mTOR inhibitors antagonize KSHV-induced cell proliferation and differentiation [166–168]. In tissue culture and in mouse xenograft models, mTOR inhibitors reduce the growth of PEL cell lines and the production of Il6 and IL8, in particular when combined with an AKT inhibitor [169–171]. An ATP-competitive inhibitor of mTOR, MLN0128 (Sapanisertib), which inhibits both the mTORC1 and mTORC2 complexes (unlike the allosteric inhibitor rapamycin, which only inhibits mTORC1), induces apoptosis in PEL cells and reduces growth of PEL in a xenograft model at nanomolar IC50 concentrations and is still effective against doxorubicin- or rapamycin-resistant PEL cell clones [171]. The antimalarial compound chloroquine inhibits KSHV lytic replication by suppressing the activation of mTOR and p38-MAPK signaling pathways [172].

8.4.3 Other Established and Experimental Therapies Targeting KSHV-infected Cells (Table 8.2)

8.4.3.1 Interfering with Cellular Cytokines—Clinical Experience

Vascular endothelial growth factor (VEGF) plays an important role in the pathogenesis of all three KSHV-associated neoplasms (reviewed in [19–21]). A phase 2 clinical trial of an antibody to VEGF, bevacizumab, in patients with AIDS-KS reported an overall response rate (complete and partial responses) of 31% [173]. This response rate increased, when bevacizumab was used in combination with liposomal daunorubicin, but this study did not resolve if bevacizumab provided an additional benefit over and above this standard chemotherapy treatment for AIDS-KS [174]. When injected into oropharyngeal KS lesions in addition to standard ART, bevacizumab did not increase the response rates in a small phase 2 trial [175].

Tocilizumab and siltuximab, humanized antibodies against the IL6 receptor, have been found to be effective in idiopathic (i.e. KSHV-negative) as well as KSHV-associated MCD [176, 177].

8.4.3.2 Preclinical Data on Other Cellular Targets

The cellular chaperone heat shock protein 90 (HSP90) has been shown to be involved in regulating the stability of the KSHV latency proteins LANA and vFLIP, as well as the vFLIP-interacting protein NEMO/IKKγ [178–181]. Small molecule HSP90 inhibitors such as 17-DMAG, PU-H71, AUY-922, and geldanamycin induce degradation of LANA and vFLIP, inhibit vFLIP-dependent activation of NFkB, and as a consequence induce cell death in PEL cell lines and inhibit the proliferation of KSHV-infected endothelial cell lines in vitro and in a mouse xenograft model [178–181].

Several isoforms of the HSP70 chaperone family have been reported to be involved in the formation of KSHV nuclear replication and transcription compartments (RTCs) during the early stages of viral DNA replication and a small molecule HSP70 inhibitor, VER-155008, antagonized KSHV RTC formation, and the relocalization of the RNA polymerase II to the viral genome [182]. Similarly, recruitment of the viral pre-replication complex consisting of RTA and the viral core replication proteins to the viral lytic origin of replication (ori lyt) was inhibited by MLN 4924, an inhibitor of NEDDylation, suggesting that NEDDylation is important for the formation of KSHV nuclear replication compartments [183]. MLN 4924 also inhibits NFkB activation in PEL cells and thereby induces apoptosis in PEL cells [183].

The proteasome inhibitor bortezomib has been shown to induce the reactivation of the KSHV lytic cycle and/or induce apoptosis in PEL cell lines in tissue culture [184–186] and of PEL cells in a xenograft model [187]. Bortezomib also increased the KSHV and EBV viral load in HIV-infected patients with KSHV- or EBV-associated lymphoma [188]. In a pilot trial, bortezomib induced a partial regression of KS lesions in 60% of patients with AIDS-associated KS [189].

The notch signaling pathway has also been implicated in the KSHV-induced lymphatic reprogramming and the endothelial to mesenchymal transition of KSHV-infected endothelial cells [190–192]. The γ-secretase inhibitors GSI and LY-411,575, which block the activation of the notch pathway, induced apoptosis in primary cells cultivated from KS lesions and in a KSHV-infected epithelial cell line [193]. However, to what extent these effects of γ-secretase inhibitors were specific to KSHV-infected cells remains to be clarified.

Cellular topoisomerases are recruited to the lytic replication origin in the KSHV genome and are required for lytic replication [194]. Several topoisomerase I and II inhibitors can inhibit lytic KSHV replication [195]; among these, two topoisomerase II inhibitors showed the best selectivity: novobiocin was reported to have a selectivity index around 30 [195], while a novel topoisomerase II inhibitor, (+)-rutamarin, inhibited KSHV productive replication in PEL cell lines with an IC50 concentration of around 1 μM and a selectivity index of 84 [196].

Additional cellular targets have been validated in tissue culture and small molecule inhibitors against these cellular proteins have been reported. These include HIF1α (hypoxia-inducible factor 1 alpha), which plays an important role in the KSHV life cycle by activating the expression of KSHV RTA, the main activator of the lytic replication cycle as well as other lytic viral genes; HIF1α thereby mediates the induction of the KSHV lytic replication cycle by hypoxia [197, 198]. KSHV LANA binds to and stabilizes HIF1α [199, 200], and at least two viral lytic proteins, the chemokine receptor homologue vGCR (ORF74) and the interferon regulatory factor homologue vIRF3 (ORF K10.5),

increase HIF1α expression or stabilize the HIF1α protein to thereby induce VEGF production [201, 202]. These viral proteins thus contribute to the activation of hypoxia-inducible factors in, and the metabolic reprogramming of latently infected endothelial cells [203, 204]. HIF1α has also been shown to play a role in maintaining an optimal metabolic state for the growth of PEL cells [205]. Reflecting the importance of HIF1α in the KSHV life cycle and the metabolic state of KSHV-infected cells, a small molecule inhibitor of HIF1α, PX-478, was shown to inhibit the growth of PEL cells in tissue culture at low micromolar concentrations [205].

Other metabolic targets include the metabolic sensor SIRT1, which was also found to be essential for PEL cell survival [206]. The small molecule SIRT1 inhibitor tenovin-6 was shown to inhibit PEL cell growth in tissue culture and to enhance the survival time of mice xenografted with a PEL cell line [206]. Furthermore, the multifunctional APE1 protein, which regulates the redox state of certain transcription factors and thereby their transition from an inactive to an active state, has been shown to regulate KSHV lytic replication and a small molecule APE1 inhibitor (C10) inhibits KSHV reactivation [207]. A heme oxygenase 1 (HO-1) inhibitor (SnPP) induces cell death in KSHV-infected endothelial cells and suppresses KSHV-mediated tumorigenesis in a KS nude mouse model employing KSHV-infected immortalized human endothelial cells [208].

A CRISPR-Cas9 screen of KSHV-transformed mesenchymal embryonic stem cells yielded XPO1/Crm1, a nuclear export factor, as a critical player in the proliferation of KSHV-transformed cells and a small molecule inhibitor of XPO1, KPT-8602, inhibits the proliferation of KSHV-infected mesenchymal embryonic stem cells and activates a p53-dependent cell cycle block [209].

A screen of a library of 1280 FDA-approved drugs yielded three compounds targeting histamine receptors or signaling [210]. Further analysis indicates that downstream MAPK and PI3K signaling pathways are involved in the activation of the KSHV lytic replication cycle by histamine receptors [210].

Attempts have also been made to target the interaction of the KSHV gH/gL glycoprotein complex with its receptor, EphA2. An inhibitor of the interaction of EphA2 with its physiological ligand, ephrin A5, was shown to inhibit KSHV infection of blood and lymphatic endothelial cells at two- to three-digit micromolar concentrations [211].

8.5 New Antiviral Strategies Against KSHV in Preclinical Development

The picture emerging from the experimental and clinical evidence summarized in the preceding sections is that existing antiviral drugs are mostly of moderate efficacy when used to treat KSHV-associated malignancies. Particularly in the case of KS, in which viral gene expression is mostly restricted and most tumor cells are latently infected, currently available herpesviral DNA polymerase inhibitors are largely ineffective. They may occasionally be beneficial in MCD, a KSHV-associated disease in which KSHV productive (lytic) replication plays a greater role. There is therefore a clinical need to explore new approaches to develop inhibitors against already validated as well as new targets in the KSHV life cycle.

8.5.1 New Approaches to Target Productive (Lytic) KSHV DNA Replication and/or Packaging (Table 8.1)

As is the case for other herpesviruses, the KSHV DNA polymerase, encoded by ORF9, works in concert with the processivity factor, encoded by ORF59, which promotes the elongation of newly synthesized viral DNA strands. A screen of the NCI Diversity Set library using an in vitro DNA synthesis assay based on the ORF9/ORF59 complex yielded several inhibitors with in vitro activity, of which one, NSC373989, also inhibited KSHV reactivation in a lytically induced PEL cell line (BCBL1); this observation indicates that it may be possible to develop inhibitors against the KSHV viral DNA polymerase complex with a different mode of action than the conventional nucleoside-based competitive DNA polymerase inhibitors [99].

Another approach is based on the observation that all herpesviruses contain at least two proteins with structural folds that are similar to RNAse H-like nucleotidyltransferases. These are the single-strand DNA (ssDNA) binding proteins required for viral DNA replication (ICP8/pUL29 in HSV; pUL57 in HCMV; pORF6 in KSHV) and the large terminase subunit, which contains the nuclease activity required for the trimming of the newly replicated viral DNA to the correct length and its packaging into preformed capsids (pUL15 in HSV; pUL89 in HCMV; the C-terminal domain of pORF29 in KSHV). The catalytic site of the HIV integrase is also contained within an RNAse H-like fold and HIV integrase inhibitors were therefore tested for their ability to inhibit herpesviral replication. One compound, XZ45, was found to inhibit HSV, HCMV, and KSHV replication at low micromolar concentrations [100]. This study did not resolve whether XZ45 targets primarily the KSHV ssDNA binding protein pORF6 or the KSHV large terminase subunit pORF29 [100].

In keeping with the structural similarity of the c-terminal domain of KSHV pORF29 with RNAse H-like nucleotidyltransferases, the HIV integration inhibitors raltegravir and dolutegravir inhibit the DNA nuclease activity of pORF29 in in vitro assays at 4 and <0.1 μM, respectively [101]. Derivatives of α-hydroxytropolone show a similar inhibitory activity *in vitro* and also inhibit KSHV lytic replication in tissue culture [101].

As already mentioned in Section 8.4.3.1, several cellular HSP70 isoforms are involved in the formation of KSHV nuclear RTCs and a small molecule HSP70 inhibitor, VER-155008, antagonizes RTC formation and viral gene expression [182]. This observation suggests that there may also be "druggable" cellular targets that could be harnessed to inhibit KSHV productive (lytic) replication.

8.5.2 Targeting KSHV Capsid Assembly (Table 8.1)

The KSHV protease is part of the pORF17 scaffold/protease polyprotein and a serine protease [212], which has been explored as a drug target. The KSHV protease mediates the cleavage of the pORF17 polyprotein into the protease and the scaffold (assembly) protein, which fills preformed nuclear herpesviral capsids prior to their loading with viral DNA. "Maturation cleavage" of the scaffold protein is also mediated by the herpesviral protease and is required for its release from pre-formed capsids and their loading with viral DNA [212]. The KSHV protease forms a dimer, which can be disrupted by a small α-helical peptide [213] or small molecules [102, 214]. One such compound inhibited KSHV production in tissue culture with IC50 values in the low two-digit micromolar range [103].

Although herpesviral proteases are serine proteases, inhibitors of the HIV aspartyl protease have also been explored for their activity against KSHV replication. Of several HIV protease inhibitors tested, nelfinavir inhibits KSHV reactivation in tissue culture with an IC50 of about 5 μM and also inhibits HSV and HCMV replication [104]. However, the activity of nelfinavir against KSHV may not be linked to an inhibition of the KSHV protease and could also be explained by off-target effects of nelfinavir, such as its inhibition of the PI3K/AKT or STAT3 pathways [104]. There is also clinical evidence that HIV protease inhibitors administered as part of an ART may be beneficial against AIDS-KS, but this is likely to be linked to their activity against HIV and an improved immune response against KSHV in individuals undergoing efficient HIV therapy [215, 216]. However, an ART regimen containing HIV protease inhibitors was found to be associated with lower KSHV shedding rates in HIV-infected patients and this effect was independent of CD4 count and HIV viral load [105]. Also, a favorable outcome (partial/complete remission or stabilization of tumors) was also observed in 16 out of 26 HIV-negative classic KS patients treated with indinavir [217]. Whether these observations point to a direct effect of at least some HIV protease inhibitors against KSHV or can be explained by off-target effects, or possibly a direct anti-angiogenic effect of HIV protease inhibitors [217], is uncertain.

8.5.3 Targeted Immunotoxins (Table 8.1)

In an attempt to target KSHV proteins expressed on the surface of infected cells with monoclonal antibodies conjugated to toxic moieties, such targeted immunotoxins have been generated for the K8.1 and gH glycoproteins, after coupling single-chain variable region fragments of monoclonal antibodies to the respective specificity to *Pseudomonas aeruginosa* exotoxin A. These targeted immunotoxins were capable of killing KSHV-infected cells undergoing productive replication in tissue culture and synergized in their killing potential with ganciclovir [106, 107]. However, their effect would be limited to the late stage of productive (lytic) KSHV reactivation, which is likely to be encountered in only a small proportion of KSHV-infected tumor cells in vivo.

8.5.4 Targeting the Latency Phase of the KSHV Life Cycle

The—overall only moderate—efficacy of antiviral treatments directed against the lytic (productive) phase of the KSHV life cycle raises the question if targeting earlier stages of the productive replication cycle or latent KSHV persistence might represent a better approach to treat KSHV-associated neoplasia. Experimental evidence suggests that silencing the expression of either of the three latent viral protein-encoding genes, ORF73 (LANA), ORF72 (cyclin homologue, vcyc), ORF71/K13 (vFLIP), in PEL cell lines with the help of shRNA inhibits their growth and/or induces cell death [218, 219]. Likewise, deleting or silencing the expression of ORFs K1, K15, and vGCR interferes with virus reactivation at an early stage in the productive replication cycle [125, 220, 221]. Therefore, targeting any or several of these viral proteins therapeutically *in vivo* might provide a better route to treatment success. In the following sections, the role of the above candidate targets in the viral life cycle and ongoing attempts to exploit them therapeutically are briefly summarized.

8.5.4.1 LANA (Latency-associated Nuclear Antigen) (Table 8.1)

LANA is expressed in all latently infected tumor cells (Figure 8.1). It is essential for the replication of latent viral episomes and their distribution to dividing daughter cells during mitosis (reviewed in [222]). Its C-terminal DNA binding domain (DBD) binds to the latent KSHV replication origin, which is located in the terminal repeat subunits of the viral genome (Figure 8.2) [223–225]. The structure of the C-terminal DBD has been solved, alone and in complex with an oligonucleotide representing one of the LANA-binding sites in the latent origin of replication [226–228]. A short amino acid sequence in its N-terminal domain is responsible for attaching the complex of LANA and viral DNA to histones H2A and H2B on mitotic chromosomes (Figure 8.2). Mutation of either this N-terminal histone-binding motif or the DNA-binding motifs in the DBD compromises latent replication [225, 226, 229, 230]. Silencing LANA in PEL cells with the help of shRNA induces cell death [219]. Furthermore, decreasing the expression of LANA in PEL cells by treatment with glycyrrhizic acid induces apoptosis by restoring the function of p53, which is inhibited by LANA [231], and HSP90 inhibitors can induce LANA degradation and cell death in PEL cells [178] (see Section 8.4.3.2). Together, this experimental evidence suggests that LANA could represent a promising therapeutic target.

Recently, Kirsch and colleagues used the structure of the LANA DBD in complex with the latent replication origin [227] as a starting point for a fragment-based drug discovery project that yielded a first generation of small molecule LANA inhibitors [108, 109]. The

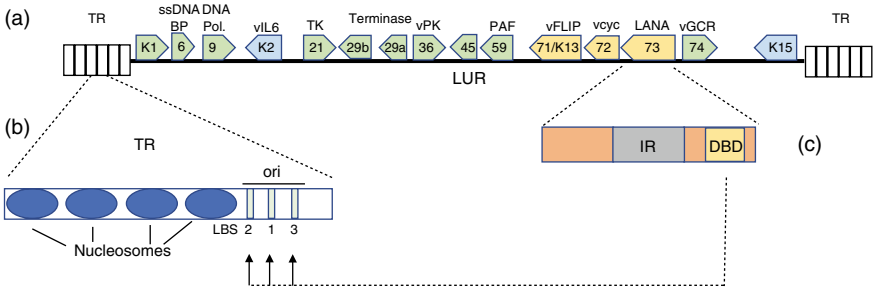


Figure 8.2 Schematic diagram of the KSHV genome and its latent origin of replication. (a) Diagram of the linear KSHV genome. The long unique coding region (LUR; 138 kbp) is shown as a dark solid line and flanked by multiple terminal repeat (TR) subunits of 801 bp each. The drawing is not to scale. Boxes represent selected KSHV genes discussed in the text and their transcriptional orientation. Numbers inside boxes denote the ORF number, the name of the corresponding viral protein is located above each box. ssDna BP, single-stranded DNA-binding protein; DNA Pol, DNA polymerase; TK, thymidine kinase; Terminase, large terminase subunit containing nuclease activity; vPK, viral serine/threonine kinase; PAF, polymerase-associated factor. Genes marked in yellow are expressed during latency, genes marked in blue show significant expression in most KS biopsies (see text), and genes marked in green are only expressed in KSHV-infected cells undergoing productive (lytic) replication. (b) A single TR subunit is enlarged to show its organization. A part of the TR subunit is chromatinized and arranged in nucleosomes, while the three LANA-binding sites (LBS) are free of histones. The position of the latent origin or replication (ori) is indicated. (c) ORF73 encodes the major latency protein LANA, which consists of an aminoterminal domain that interacts with histones H2A/B, an internal repeat region (IR), and a carboxyterminal region. The latter contains the DNA-binding domain (DBD), which binds to three specific DNA motifs (LANA-binding sites, LBS) in each TR subunit.

most promising compounds inhibited the interaction of LANA with the latent viral origin in fluorescence polarization and in electrophoretic mobility shift assays with IC50 values in the single- to double-digit micromolar range [109]. Ongoing work aims to improve the potency of these first compounds and to test them for antiviral activity in tissue culture.

8.5.4.2 vFLIP (Viral FLICE-inhibitory Protein) (Table 8.1)

Another latent protein, the KSHV homologue of cellular Fas-associated death domain (FADD) interleukin 1β-converting enzyme (FLICE)-inhibitory proteins (FLIP) inhibits Fas-induced apoptosis and is a potent NFkB activator [232–234]. Transgenic mice with vFLIP expression targeted to B cells develop B-cell lymphomas [235], while transgenic expression in endothelial cells results in elongated fusiform endothelial cells reminiscent of KS spindle cells and an inflammatory phenotype characterized by increased expression of IL6 and IL10, similar to KICS (see Section 8.3.5; [236]). The activation of the NFkB pathway by vFLIP involves its ability to interact with IKKγ/NEMO [181, 218] and the structure of a fragment of the coiled-coil domains of IKKγ/NEMO in complex with vFLIP has been solved [237]. Silencing vFLIP expression in PEL cells lines [218], or blocking the NFkB pathway with small molecule NFkB inhibitors [233, 234], induces cell death, suggesting that vFLIP-induced activation of NFkB would be a worthwhile therapeutic target. In addition to targeting other proteins in KSHV-infected cells, HSP90 inhibitors also induce vFLIP degradation and inhibit vFLIP/IKKγ-dependent NFkB activation (see Section 8.4.3.2).

Recently, two groups have reported peptide-based small molecules capable of antagonizing the vFLIP-IKKγ interaction and promoting apoptosis in PEL cell lines. Briggs and colleagues designed a conformationally constrained, stapled IKKγ peptide derived from the IKKγ–vFLIP interaction site, which could interfere with the binding of IKKγ to vFLIP and enhance apoptosis in PEL cell lines [110]. Sadek and colleagues developed a tertiary protein structure mimic of the vFLIP-interaction site in the IKKγ/NEMO helix and showed its ability to induce cell death in PEL cell lines and to delay tumor growth in a PEL xenograft mouse model [111].

8.5.4.2.1 Outlook

Although a wide range of drugs and therapies, ranging from conventional cancer chemotherapy to antiviral drugs, have been tried with some success to treat KSHV-associated neoplasia, none of them are entirely satisfactory, because of side effects or moderate efficacy. There is therefore room for the development of new therapeutic approaches. Since KSHV-associated malignancies require the continuous presence of the KSHV genome and at least latent viral gene expression in the tumor cells, targeting appropriate KSHV proteins and/or their ability to activate cellular processes involved in pathogenesis could in theory provide a more specific and effective therapeutic approach than currently used treatment strategies. KSHV-associated diseases are mostly confined to people living with HIV and transplant recipients in countries with a higher KSHV seroprevalence. Even if new specific antiviral drugs, aimed at new targets and with good activity against KSHV, could be discovered, their preclinical and clinical development will therefore present an additional challenge.

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9

Direct-Acting Antivirals for the Treatment of Chronic Hepatitis B Virus (HBV) Infection

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9.1 Nucleos(t)ide Analog Reverse Transcriptase Inhibitors

Nucleoside/nucleotide analogs (NAs) inhibit the reverse transcriptase (RT) activity of the HBV polymerase protein (Pol). Since the approval of lamivudine (3TC) for HBV treatment in 1998, several other NAs have also been approved (Figure 9.1). With their high potency and high barrier to resistance, entecavir (ETV) and tenofovir (TFV) are considered the first-line treatment NAs, while the use of 3TC, telbivudine (LdT), emtricitabine (FTC), and adefovir (ADV) in monotherapy is no longer routinely recommended due to their relatively low barrier to resistance [6, 7]. Clevudine (CLV) was approved only in South Korea and the Philippines; however, the approval was withdrawn due to drug-related side effects [8]. Besifovir (BSV) is a novel acyclic nucleotide phosphonate with a similar chemical structure to ADV and TFV. It has a good clinical safety profile and the same potent antiviral activity as ETV and TFV. In 2017, its use was approved, but only in South Korea [9, 10].

In general, NAs show good bioavailability after oral dosing, although some of them must be administered as a prodrug. They have favorable pharmacokinetics with limited drug–drug interaction potential and low pill burden. It has been demonstrated that NAs are very effective in suppressing viral load to undetectable levels in a high number of patients. In addition, it could be demonstrated that NAs are able to block mother-to-child transmission which is the main transmission path for HBV in endemic areas [11].

The mode of action of NAs has been investigated in several studies. NAs interfere with the Pol in different ways (Table 9.1). Most of them preferentially act on one activity of the Pol. 3TC, FTC, ADV, and TFV block the reverse transcription of the negative (–) strand viral DNA from the pgRNA, while LdT and CLV block the positive (+) strand viral DNA synthesis. On the other hand, ETV blocks all three major activities of the Pol: base priming, reverse transcription, and synthesis of the positive strand viral DNA [2, 3, 12, 13]. Since BSV is also a guanosine analog, it is hypothesized that it could have a mode of action

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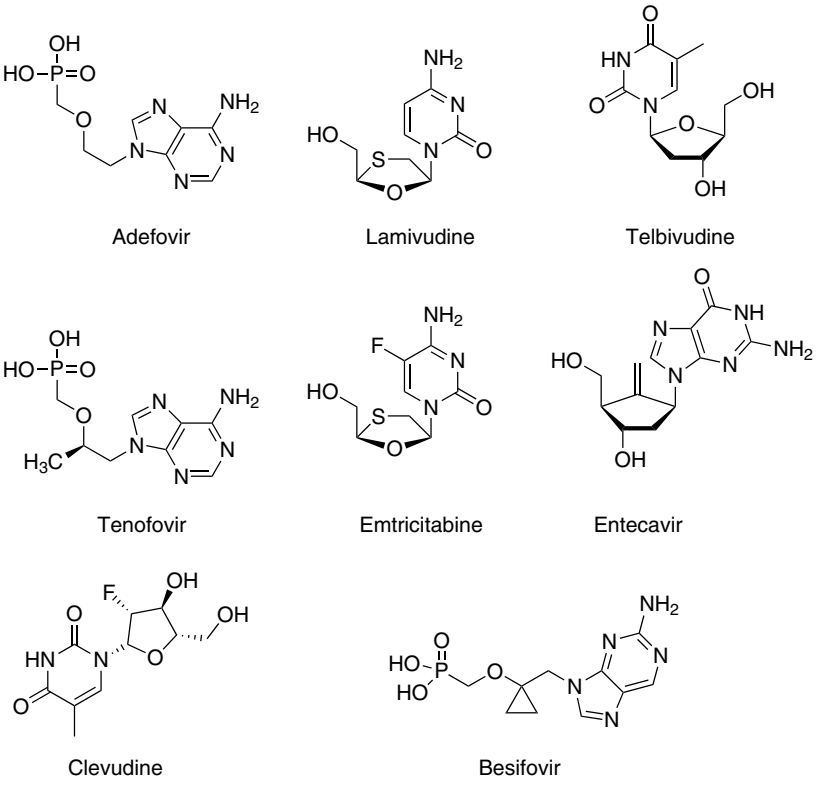

Figure 9.1 Chemical structures of NAs approved for HBV therapy. Adefovir and tenofovir are acyclic analogs of the nucleotide adenosine monophosphate, differing only slightly at the acyclic moiety. Lamivudine and emtricitabine are both analogs of cytidine with an oxathiolane ring replacing deoxyribose. Lamivudine is an L-enantiomer derivative of cytidine, while emtricitabine is a fluoro-derivative of D-cytidine [1]. Telbivudine and clevudine are both L-enantiomeric analogs of thymidine [2, 3]. Entecavir is a deoxyguanosine derivative with an all-carbon pentacyclic ring replacing deoxyribose [4], while besifovir is an acyclic analog of guanosine monophosphate [5].

similar to ETV [5]. Aside from differences in mode of action, NAs also vary in potency, safety profile, and resistance profile. The most common drug resistance mutations appear in the YMDD motif within the active site of the RT domain of the Pol. An excellent overview of resistance profiles and the advantages and limitations of currently approved NAs for HBV treatment can be found in a recent review [14].

NAs provide stronger viral suppression compared to IFN therapy; however, they do not target cccDNA transcription, and so, result in relatively low rates of HBsAg loss and/or HBeAg seroconversion. Consequently, NAs given in monotherapy are not able to cure chronic hepatitis B infection [15]; however, the discovery of new NAs with higher viral suppression potency could theoretically be an option to prevent intracellular cccDNA recycling and to increase cure rates for NA-based treatment strategies.

Combination treatment with NAs and IFN also provides another theoretical possibility for a cure because as monotherapies, they both have different but complementary effects

Table 9.1 Classification and mode of action of nucleos(t)ide analogs approved for the treatment of chronic hepatitis B [1–5, 12, 13].

Drug name and code	Classification	Mode of Action
Lamivudine (3TC)	L-cytidine analog	Acts on RT function; inhibition of (–) strand synthesis
Emtricitabine (FTC)	Fluorinated D-cytidine analog	Acts on RT function; inhibition of (–) strand synthesis
Telbivudine (LdT)	L-thymidine analog	Acts on DNA Pol function; inhibition of viral (+) strand synthesis
Clevudine (CLV)	L-thymidine analog	Preferentially acts on DNA Pol function but can also slightly interfere with priming and RT; inhibition of viral (+) strand synthesis
Adefovir (ADF)	Acyclic analog of adenosine monophosphate	Acts on RT function; inhibition of (–) strand synthesis
Tenofovir (TFV)	Acyclic analog of adenosine monophosphate	Acts on RT function; inhibition of (–) strand synthesis
Entecavir (ETV)	Deoxyguanosine analog	Acts on RT, DNA Pol and primase functions; inhibition of priming, reverse transcription and (+) strand synthesis
Besifovir (BSV)	Acyclic analog of a guanosine monophosphate	Hypothesized to be like ETV

on the immune response and viral control of HBV. PEG-IFN boosts innate immunity, while NAs boost adaptive immunity [16]. Unfortunately, current combinations only offer small improvements in the rates of HBsAg loss, the best prognostic marker for HBV control. A recent open-label, active-controlled TDF and PEG-IFN combination therapy study with 740 CHB patients has shown that 120 weeks after the start of the therapy, the group treated with TDF and PEG-IFN for 48 weeks had almost three times higher rate of HBsAg loss (10.4%) compared to the group treated with PEG-IFN alone for 48 weeks (3.5%) or the group treated with TDF and PEG-IFN for 16 weeks followed by TDF alone for 32 weeks (3.5%) [17].

Aside from its RT function, the Pol also has RNaseH activity. The RNAseH destroys the pgRNA after it has been retrotranscribed into DNA. In vitro studies have shown that inhibiting the RNaseH function leads to truncation of the (–) strand DNA and blocks (+) strand DNA synthesis, resulting in extensive accumulation of RNA/DNA heteroduplexes within viral capsids. This prevents the formation of mature HBV genomes and the production of infectious viral particles [18]. To date, there is no HBV RNaseH inhibitor in clinical development. The reasons for this are mainly technical problems in establishing a screening cascade. Firstly, it is very difficult to produce the HBV RNaseH domain in its active form for use in biochemical assays. Secondly, it is not easy to establish cell-based assays with acceptable throughput and a predictive and discriminatory readout that can detect the effects of the screening compound, such as inhibition of (+) strand DNA synthesis and the accumulation of truncated (–) strand DNA and RNA/DNA heteroduplexes [19].

9.2 HBV Entry Inhibitors

Aside from the Pol, another interesting antiviral target is viral entry. In fact, one of the most clinically advanced non-NA therapies in development is Myrcludex B (MYR Pharma/Hepatera), which currently has ongoing Phase 2b [20] and Phase 3 [21] studies in patients with chronic hepatitis delta disease. Myrcludex B [22–25] is a synthetic myristoylated peptide that mimics the binding of HBV to its cellular receptor, sodium taurocholate cotransporting polypeptide (hNTCP), and stably blocks entry of HBV and the virusoid hepatitis delta virus (HDV) into hepatocytes [26, 27]. Myrcludex B, with a median inhibitory concentration of ~80 picomolar, was developed from a consensus sequence of residues 2–48 of the preS1 domain of the large HBV surface protein [28]. In particular, studies have shown that a well-conserved motif [residues 9-NPLGF(F/L)P-15] in this preS1 domain of human and primate hepadnaviruses is required for the binding of HBV to hNTCP during viral entry, and that the surrounding residues 2–8 and 18–48 increased its viral inhibition activity, while residues 49–78 decreased it [29, 30]. Recently, Donkers et al. [31] have also shown that although Myrcludex B binding to hNTCP is very strong, Myrcludex B can also transfer to newly synthesized hNTCP molecules, possibly extending its viral blocking efficacy.

Aside from Myrcludex B, other small molecules, including the calcineurin inhibitor cyclosporine A (CsA) [32], the angiotensin II receptor blocker irbesartan [33], the cholesterol absorption inhibitor ezetimibe [34], the HIV protease inhibitor ritonavir [35], vanitaracin A [36], and the immunosuppressant macrolide compound rapamycin, and its derivatives everolimus and temsirolimus [37] have also been shown to interact with the hNTCP receptor, thereby preventing the binding of the preS1 domain to the receptor and inhibiting viral entry. All these compounds, including Myrcludex B, however, also impair the sodium-dependent bile uptake function of hNTCP, which can lead to adverse effects [32–37]. Shimura et al. [38] have recently identified CsA derivatives that have sub-micromolar activity against HBV, do not have immunosuppressive activity, and do not impair sodium-dependent bile uptake. Their findings suggest that the anti-HBV activity of entry inhibitors could be functionally separated from bile acid transport, leading to better safety profiles for this class of inhibitors.

9.3 HBV Capsid Assembly Modulators (CAMs)

Another class of direct-acting antivirals with several compounds in clinical development are the CAMs. These are small molecules that act on the HBV core protein and capsid.

9.3.1 HBV Core Protein

HBV has a partially double-stranded, relaxed circular DNA genome (rcDNA) of approximately 3200 bp. It encodes four overlapping open reading frames (ORFs) [39] including the preC/C ORF which encodes the ~25 kDa pre-core protein (HBeAg) precursor and the ~21 kDa core protein (HBc, Cp) [40]. The latter is translated from the second start codon, which is 30 codons downstream from the pre-core start codon [40, 41].

The Cp is the building block of the icosahedral viral capsid; it protects and packages the viral genome. Cp has two domains: an α-helix-rich N-terminal domain composed of

residues 1–149 (Cp149, 16.8 kDa), and an intrinsically disordered arginine-rich C-terminal domain (CTD, 4.3 kDa) composed of the remaining 34–36 residues (variations occur between different genotype sequences) [41, 42]. Cp149 forms the outer shell of the capsid and is crucial for capsid assembly. Cp149 begins with an irregular N-terminal structure, followed by α-helices α1 (residues 13–17) and α2 (residues 27–43) that has a kink from residue 37. Its structure is dominated by a long α-helical hairpin composed of α3 (residues 50–74) and α4 (residues 79–109), with a kink between residues 89 and 92. It ends with α-helix α5 (residues 113–126), an irregular proline loop (residues 128–136) and an extended strand (residues 137–142) (see Figure 9.1). The Cp149 monomer contains a hydrophobic core comprised of residues from α1, the loop connecting α1 and α2, α4b (i.e. the part of α4 after the kink) and α5, which stabilizes the monomer fold [43]. On the other hand, the CTD, located within the capsid, is important for encapsidation of the pre-genomic RNA (pgRNA) and its subsequent reverse transcription into the rcDNA genome, and for binding of the Cp to host proteins. Its function is mediated by four arginine-rich repeats punctuated with seven conserved serine residues and one threonine that can be phosphorylated [44]. In addition to its phosphorylation, the progressive dephosphorylation of the CTD as rcDNA synthesis proceeds may also contribute to the maturation signals necessary for the envelopment of the mature nucleocapsid [45, 46].

In general, capsids can assemble into two sizes: the majority (>95%) observed in natural infection and in vitro have a $T = 4$ symmetry (36 nm-diameter) composed of 120 dimers, while a small portion have a $T = 3$ symmetry (32 nm-diameter) composed of 90 dimers [41, 47–49]. The asymmetric unit of a $T = 4$ capsid has four quasi-equivalent subunits: A, B, C, and D, which form chemically identical, but structurally distinct AB and CD dimers which undergo conformational changes to reach an assembly-competent state. The intradimer interface is composed of a four-helix bundle corresponding to the highly immunogenic protruding spikes observed on the capsid [43, 44, 50, 51]. Different interactions between the AB and CD dimers form the 5-fold, 2-fold (or quasi-6-fold), 3-fold and quasi-3-fold icosahedral vertices. It is at these vertices that the capsids are fenestrated, allowing access of nucleotides and other molecules into and out of the capsid for rcDNA synthesis and CTD accessibility. The formation of the resulting interdimer interfaces is predominated by the burial of surfaces, which are approximately 75% hydrophobic, supporting an entropy-driven assembly. The resulting stable capsid is mainly held together by weak hydrophobic interactions at the interdimer contacts [43, 51, 54].

In infected cells, capsid assembly occurs in the cytoplasm and is initiated by interactions of the entire Cp with the ε signal on the pgRNA and the Pol [52]. Morphologically intact capsids can also be produced in a test tube by manipulating protein concentration, temperature, pH, and ionic strength. Capsid assembly begins with a slow nucleation step through the formation of a trimer of dimers, followed by rapid elongation by addition of one dimer at a time [41, 44, 53, 54]. Based on the property of spontaneous self-assembly, Zlotnick and his team were able to set up a target-based fluorescence quenching assay for the identification and characterization of both misdirectors and inhibitors of the HBV capsid assembly process. A mutant Cp150, in which all native cysteines dispensable for capsid assembly were mutated to alanines and an additional unique cysteine was added to the C-terminus, was used, allowing facile fluorescent labeling with cysteine-reactive agents like BODIPY-FL maleimide. The resulting dimeric Cp150-BODIPY-FL is nearly

quantitatively labeled and highly fluorescent, but a significant reduction in fluorescence is observed upon assembly of these dimers into capsids [55].

9.3.2 First-Generation CAMs

So far, several classes of inhibitors of pgRNA packaging and HBV capsid assembly have been identified, either in cell-based or target-based high-throughput screening (HTS) campaigns or in rational drug design programs (see Figure 9.2). They act by accelerating nucleocapsid assembly, by selectively inhibiting or dysregulating pgRNA encapsidation, or by both [41, 44]. For this reason, these HBV inhibitors are referred to as capsid assembly modulators (CAMs), core protein allosteric modulators (CpAMs), or capsid assembly effectors (CAEs).

The first capsid inhibitior to be identified altogether, a CAM chemotype, was the heteroaryldihydropyrimidine (HAP), BAY 41-4109 [56]. It was discovered in a cell-based HTS campaign. BAY 41-4109 (also AIC429 see compound 1 in Figure 9.3), is a first-generation HAP compound. It has been shown to inhibit HBV DNA replication with an EC_{50} of 54 nM and to reduce the intracellular amount of capsids by reducing the half-life of the Cp in the HBV-expressing cell line HepG2.2.15 from >24 hours to 3 hours [56]. In addition, as it addresses a different target, it is active against NA-resistant HBV [56], and reduces viral DNA dose-dependently in the liver and in the plasma of HBV transgenic mice by preventing capsid formation [64]. Structural studies of this series of inhibitors revealed that they induce inappropriate capsid assembly at low concentrations, leading to degradation of capsid via the proteasome pathway [56], and, when in excess, promote a misdirected assembly reaction and decreased capsid stability [55, 65]. BAY 41-4109 and related compounds were originally invented at Bayer in the late 1990s. There is still ongoing chemical evolution of this scaffold, producing new entities like GLS4 [66, 67], HAP_R10 [57], and compound 4 [68] (see compounds 2–4 in Figure 9.3), some of which are in clinical development (see Table 9.3). This enduring interest in HAPs is remarkable, and is based on the fact that among the CAM chemotypes discovered so far, this scaffold revealed a special and unique mode of action (see Section 9.3.4)

A few years before the HAP scaffold and its mode of action was published, the phenylpropenamide (PPA) derivative AT-61 was identified by Avid Therapeutics (followed up

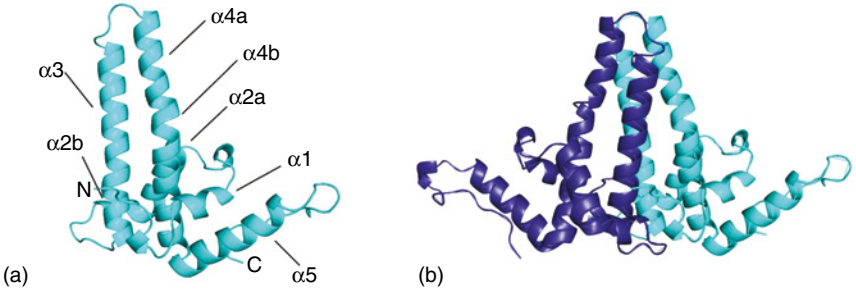

Figure 9.2 HBV core protein assembly domain. (a) Cp149 monomer viewed from the intradimer interface. The monomer is composed of five helices, indicated as α1–α5, which are connected by loops. The N- and C-terminal are indicated with the letters N and C, respectively. (b) Cp149 dimer is composed of two quasi-equivalent subunits with the majority of the contact points being at α3 from both subunits (PDB: 1QGT) [43, 50].

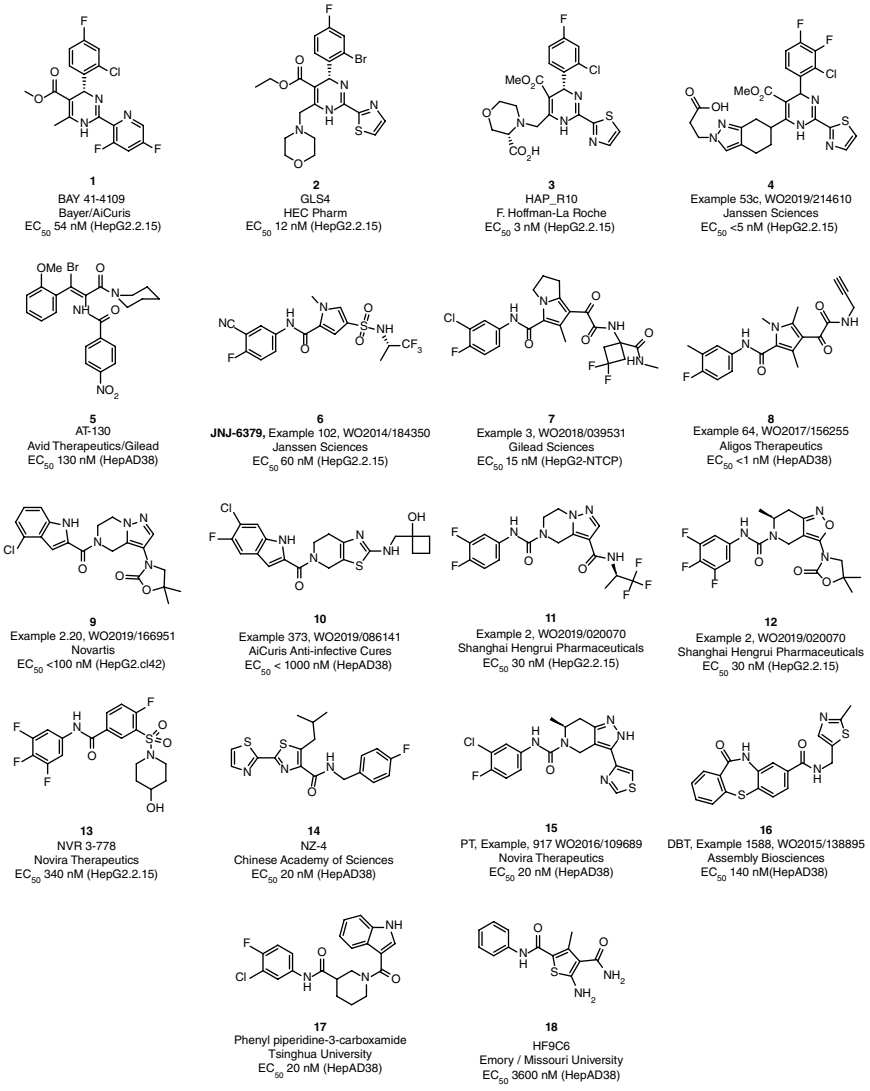

Figure 9.3 Structures of different CAMs. Antiviral activity data (EC_{50}s) were taken from the patents mentioned, except for the following references: BAY 41-4109 [56], GLS4 [42], HAP_R10 [57], AT-130 [58], NVR 3-778 [59], NZ-4 [60], PT [61], DBT [61], phenyl piperidine-3-carboxamide [62], and HF9C6 [63], respectively.

later by Gilead) as a small molecule inhibitor of HBV replication in HBV-expressing cell lines [76]. Although the mode of action was not clear at the time of its discovery, it was demonstrated in 2007 [77] that AT-130 (see compound 5 in Figure 9.3), a related analog of AT-61 [58], selectively inhibits viral pgRNA packaging and that PPAs, like HAPs, are active against both wild-type and nucleos(t)ide analog (NA)-resistant strains of HBV [77–79].

9.3.3 Recent Progress in Novel CAMs

Beyond these two CAM chemotypes, several new CAM chemical scaffolds have meanwhile been developed, some of which are also in clinical trials. Advances in the medicinal chemistry of therapeutic agents for the treatment of chronic hepatitis B, including CAMs, have been described in two review articles [80, 81]. Another recent publication of significant note is the disclosure of the structure of Janssen's sulfamoyl-pyrrolamide clinical candidate JNJ-6379 [82, 83]. Closely related pyrrolo-oxalamide analogs have also been described by Gilead Sciences, and by Aligos Therapeutics/Emory University (compounds 7 and 8 in Figure 9.3, respectively) [84–86]. Compound 8 is believed to be Aligos Therapeutics' clinical candidate ALG-001075.

Indole-2-carboxamides such as compounds 9 and 10 (Figure 9.3) have also been disclosed as a further class of CAMs [87–89]. Interestingly, the indole-2-carboxamide unit acts as an isosteric replacement for the more frequently encountered phenylurea (e.g. compounds 11 [90] and 12 [91] in Figure 9.3) or arylamide substructures (e.g. compounds 7 and 8 in Figure 9.3). The aminothiazole hydrogen bond donor-acceptor pair of compound 10 is isosteric to the oxalamide unit of compounds such as compound 7, while compound 12 shows that further core variation is also tolerated.

CAMs bind in a pocket formed at the interface of a pair of Cp dimers (Figure 9.4). The binding pocket itself is predominantly comprised of nonpolar amino acids, with a hydrophobicity ratio of 0.58. Conserved ligand pharmacophore features are typically a hydrogen bond acceptor (paired with the side chain NH of W102, shown in sticks) and an aromatic ring (typically halogenated) filling the pocket formed by V124, R127 and T128 of one Cp monomer, and P25, D29, L30, T33, W102, I105, and S106 of a second Cp monomer. Many compounds also have an H-bond donor paired with the side chain of T128. An H-bonding interaction between ligand and S141 (side chain shown in sticks) either directly or mediated via a water molecule is also frequently observed.

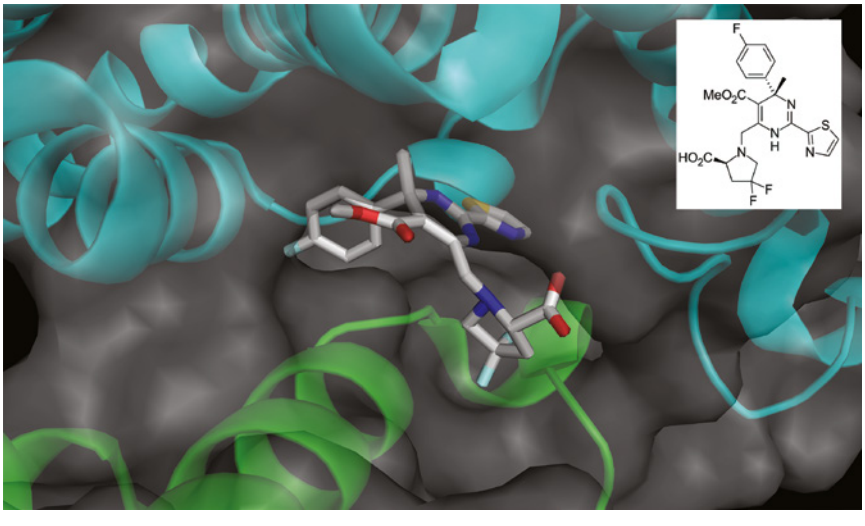

Figure 9.4 Crystal structure of a HAP molecule binding at the Cp dimer-dimer pocket found at the C-D' interface (PDB: 5GMZ). Inset: structure of the HAP molecule. [92].

9.3.4 Modes of Action of CAMs

Because of the crucial function of the Cp and capsids in different stages of the HBV replication cycle, studies suggest that CAMs, which target both Cp and capsid, can combat HBV replication and persistence on different levels.

CAMs mainly prevent HBV replication in already established infections by directly interfering with pgRNA encapsidation by increasing rate and extent of assembly (i.e. mistimed assembly), resulting in the formation of empty capsids or aberrant structures [60, 65, 70, 93–95]. Based on size exclusion chromatography and electron microscopy analyses, this effect on capsid assembly can be categorized into two types. HAPs (see compounds 1–4 in Figure 9.3), the prototypic chemical scaffold having the eponymous "HAP-like" mode of action, induce the formation of aberrant structures [61, 71, 96, 97], leading to degradation of Cp with massive reduction in the halflife of the protein [56]. They transiently stabilize the Cp assembly-active state [50, 98], and increase the thermodynamic stability of the interdimer contact points [99], resulting in a change in the quaternary structure of the capsid [71, 100]. Recently, Kang et al. [101] showed that the FDA-approved antifungal drug Ciclopirox also acts on the HBV capsid and induces the formation of aberrant structures.

On the other hand, the formation of morphologically intact but empty capsids is referred to as the "non-HAP-like" mode of action, and is exhibited by compounds, such as PPAs [93], sulfamoylbenzamides (SBAs) and benzamides [70, 96], isothiafludine (NZ-4) [60], glyoxamide-pyrrolamides (GPAs) [61], pyrazolyl-thiazoles (PTs) [61], dibenzo-thiazepin-2-ones (DBTs) [61], phenylpiperidine-3-carboxamides [62], and HF9C6 [63] (see compounds 5–8, 13–18 in Figure 9.3). Katen et al. [93] have shown that the empty capsids were not a result of direct interference with pgRNA encapsidation, but rather a consequence of the faster assembly kinetics. In particular, they suggest that PPA could indiscriminately induce assembly nucleation regardless of the presence of pgRNA and Pol. However, this may not be the case for all these CAMs as Yang et al. [60, 102] have reported that NZ-4 may directly interfere with the interaction between pgRNA and the Cp, possibly at the C-terminal, arginine-rich nucleic acid binding domain of the Cp. As a consequence of the production of empty capsids by "non-HAP-like" CAMs, Lam et al. [59] have shown that SBA can reduce the secretion of DNA- and RNA-containing viral particles, while NAs prevent production of DNA-containing viral particles but increase the production of RNA-containing particles. However, when cells are treated with SBA and an NA, better viral suppression and fewer DNA- and RNA-containing viral particles are observed [59].

As discussed, aside from their biophysical effects on capsid assembly, HAPs also cause a strong proteasome-mediated reduction in both intracellular Cp and capsid amounts [56], whereas "non-HAP-like" CAMs, such as the SBAs, do not alter the absolute amounts of intracellular Cp and capsid particles [61, 71, 103]. In addition, treatment of HBV-producing cells with HAPs result in a time- and dose-dependent localization of punctate core antigen aggregates as visualized through immunofluorescence staining. At low concentrations ($\leq$ 1.5-fold EC_{50}) and short treatment durations ($\leq$ 3 days), core antigen aggregates are mainly found in the cytoplasm, with some located near the nucleus and enclosed by invaginations of the nuclear envelope [104, 105]. With higher concentrations ($\geq$ 1.5-fold EC_{50}) and longer

treatment durations (≥ 4 days), the aggregates are mainly associated with the nucleus [61, 95, 106]. Huber et al. [106] and Lahlali et al. [95] have shown that the HAP-induced nucleus-associated core antigen aggregates are co-localized with the promyelocytic leukemia protein and 20S proteasomal subunit, which are sites of protein post-translational modification, activation, sequestration, and degradation. HAP-induced association of the Cp with these bodies may lead to different pleiotropic effects, such as induction of apoptosis or cellular senescence [106].

On the other hand, Corcuera et al. [61] and Huber et al. [63] have shown that the "non-HAP-like" CAMs induce the trapping and accumulation of the HBV core antigen signal in the cytoplasm, with virtually no apparent nuclear staining. Loss of the core antigen in the nucleus may affect maintenance of active covalently closed circular DNA (cccDNA) transcription since Cp binding to the minichromosome may alter nucleosome spacing [107], and in particular, affect histone acetylation and DNA methylation by interacting with CpG island 2 on the minichromosome [108]. Indeed, Lam et al. [59] have recently shown that treatment of primary human hepatocytes (PHHs) with SBA prior to infection resulted in inhibition of viral RNA transcription, including those for HBsAg and HBeAg, reducing their protein levels as well. Another study has shown that Cp in the nucleus may inhibit double-stranded RNA-mediated interferon response either by directly binding to the promoter regions of interferon-stimulated genes or recruitment of epigenome-modifying enzymes [109]. As a result, loss of Cp in the nucleus may remove this block on the interferon response.

Interestingly, CAMs can also act at an earlier stage of the HBV replication cycle, by inhibiting the establishment of the cccDNA pool and its maintenance via the recycling pathway. Several studies have illustrated that when CAMs were administered before in vitro infection, a reduction in cccDNA levels is observed, though this requires higher CAM concentrations and increased treatment duration compared to the primary CAM mechanism of action, i.e. interference with pgRNA encapsidation [96, 103, 110]. In particular, Guo et al. [110] have shown that for HAPs and SBAs, this effect on the cccDNA pool is possibly mediated by inducing the disassembly of nucleocapsids from de novo infection and the recycling pathway prior to delivery of the rcDNA genome to the nucleus. Their studies suggested that the intrinsic instability of mature nucleocapsids, resulting from the presence of the partially double-stranded rcDNA [111, 112], confers changes on the nucleocapsid structure which allow CAMs to bind to it and trigger its disassembly [110].

With this multi-pronged attack against HBV, CAMs have great potential to be one of the key players that can bring about HBV cure. The precise molecular (dynamic) basis for the differential effects on capsid morphology of the two CAM types remains unclear, as does the clinical relevance of this phenomenon for an eventual HBV cure [71].

9.3.5 Resistance Profile of CAMs

CAMs are generally active against the eight well-studied HBV genotypes (A–H) [59, 70, 72]. Analysis of the 10 975 deposited Cp sequences in the Hepatitis B virus database (accessed 24 October 2019) [69] has shown that all the residues at the CAM binding pocket are generally well-conserved among the eight genotypes. In addition, CAMs are also active against NA-resistant HBV strains since they interfere with a different viral target [42, 56, 70, 79, 94].

Although CAMs are hypothesized to have a higher barrier to resistance than NAs [113, 114], studies of their resistance profile are still of great interest, not just in terms of resistance emergence, but also as a tool to better understand the interactions of different CAMs in the pocket and to guide the design of next-generation CAMs. Generally, in order to study drug-resistant virus mutants, sequential passage of the virus in cells in the presence of increasing compound concentrations is performed [115]; however, such a technique has not been developed successfully for HBV. Instead, transient or stable transfection of full-length HBV PCR amplicons or plasmids containing 1.1× to 2× HBV genome units into hepatoma cell lines (e.g. HepG2 or Huh7) is used. HBV replication is then initiated through synthesis of the pgRNA by the inherent HBV promoter (in plasmids with 1.3–2 genome units) or by a strong mammalian promoter, such as CMV (in plasmids with 1.1 genome units) [116]. Several groups have made use of these transient HBV replication systems to study a plethora of amino acid substitutions at the CAM-binding pocket, some of which are listed in Table 9.2 [59, 70–75]. Because of the indispensable role of the Cp and capsid in the replication cycle, all mutants studied have replication capacities similar to, or less than, the wild-type virus [71–75]. It should be noted, however, that the in vivo selection of mutations may not be identical to the in vitro results [115], particularly if quite an artificial system has to be used, as is the case for HBV.

Among the mutations, of particular interest are the substitutions at Cp residues 25 (in loop 2), 33 (in helix 2), and 124 (in helix 5) because of their varied effects on compound activity. The P25G substitution is highly resistant to all CAMs tested, while the P25A substitution is only moderately resistant to SBA and highly resistant to the HAPs. Interestingly, a change to serine results in a high resistance against HAPs, moderate resistance against SBA, and an increased susceptibility against JNJ-6379 [71, 74]. Zhou et al. [71] suggest that the alanine and serine substitutions could possibly change the conformation of loop 2, causing a disadvantageous interaction with the thiazole group of HAPs, which is absent from SBAs. On the other hand, the smaller glycine residue may create more space in the pocket, thereby weakening the van der Waals interaction between both types of CAMs tested and the Cp, resulting in reduced activity. Similarly, a change of residue T33 to one with a bulkier side chain, such as asparagine or glutamine is suggested to cause a steric hindrance in the pocket, resulting in the observed increased resistance to all CAMs tested; this effect is reduced when the residue is replaced with the slightly smaller serine [71].

Studies have shown that position 124 is crucial to capsid assembly as both pgRNA encapsidation and plus-strand DNA synthesis were impaired when valine was substituted with alanine, leucine, phenylalanine, or tryptophan [75, 117]. In addition, as this residue is located in the wall of the pocket [71], Tan et al. [117] have shown that increasing the hydrophobic surface area with V124X substitutions increases the association energy, and fills the pocket to different extents. Changing the valine to residues with a smaller side chain, such as alanine or glycine, results in resistance to SBAs and JNJ-6379, as the pocket is opened up, possibly weakening the interactions between the compound and the protein. Meanwhile, with this substitution, susceptibility to HAPs is maintained, possibly because of its slightly larger size and ability to fill the pocket [70, 74, 75]. On the other hand, a small increase in side chain volume with an isoleucine substitution results in a more favorable fit in the pocket and an increased susceptibility for HAPs and SBA [71]. Further increasing the size of the side chain with phenylalanine renders the mutant resistant to the slightly larger HAPs, but still susceptible to SBAs [70, 71]. Filling the pocket completely with a tryptophan

Table 9.2 Effect of amino acid substitutions in the CAM binding pocket on CAM activity.

			Susceptibility to CAMs[a]					
			HAP-like CAMs				Non-HAP-like CAMs	
Position	Amino acid substitution	Frequency in Cp sequences (Genotypes A–H) [69]	BAY 41-4109 [59, 70–74]	GLS4 [75]	NVR-010-001-E2[b] [73]	HAP_R01[c] [71]	JNJ-6379 [74]	NVR 3-778 [59, 71]
24	F24L	0.082%	HR	–	–	–	S	–
	F24Y	1.084%	S	–	–	–	S	–
25	P25A	0.009%	HR	–	–	HR	–	MR
	P25G	0.000%	HR	–	–	HR	–	HR
	P25S	0.055%	HR	–	–	HR	HS	MR
29	D29G	0.146%	HR	–	–	–	MR	–
30	L30F	1.276%	HR	–	–	–	HR	–
33	T33N	0.018%	HR	–	–	HR	HR	HR
	T33P	0.009%	HR	–	–	–	HR	–
	T33Q	0.000%	HR	–	–	HR	–	HR
	T33S	1.257%	MR	–	–	S	MR	MR
37	L37Q	0.027%	MR	–	–	–	HR	–
38	Y38F	3.189%	S	–	–	–	S	–
	Y38H	1.175%	S	–	–	–	HS	–
105	I105L	0.784%	HS	–	HS	–	HS	S
	I105T	0.656%	S	–	S	–	MR	HR
	I105V	1.130%	S	–	S	–	S	S
106	S106T	0.073%	HS	–	–	–	MR	–

			Susceptibility to CAMs[a]					
			HAP-like CAMs				Non-HAP-like CAMs	
Position	Amino acid substitution	Frequency in Cp sequences (Genotypes A–H) [69]	BAY 41-4109 [59, 70–74]	GLS4 [75]	NVR-010-001-E2[b] [73]	HAP_R01[c] [71]	JNJ-6379 [74]	NVR 3-778 [59, 71]
109	T109A	0.064%	HS	–	–	–	HS	–
	T109I	1.367%	HR	HR	HR	–	HS	S
	T109M	0.738%	MR	S	MR	–	S	S
	T109S	0.155%	HS	HS	HS	–	S	MR
110	F110I	0.009%	HS	–	–	–	HR	HR
118	Y118F	0.383%	HR	–	MR	–	HR	HR
124	V124A	0.036%	S	HS	–	–	–	–
	V124F	0.009%	HR	–	–	HR	–	S
	V124G	0.064%	S	S	–	–	HR	–
	V124I	0.036%	HS	–	–	HS	–	HS
	V124W	0.219%	HR	–	–	–	–	–
125	W125F	0.000%	S	–	–	–	HS	–
127	R127H	0.064%	HR	–	–	–	MR	–
128	T128I	0.036%	HS	–	–	–	HR	–
132	Y132F	0.027%	HS	–	–	–	S	–

[a] Susceptibility to CAMs based on fold-change (FC) in activity compared to wild-type virus. HS: highly susceptible (FC ≤ 0.5), S: susceptible (0.5 < FC < 2), MR: moderately resistant (2 ≤ FC < 5), HR: highly resistant (FC ≥ 5); -: no data available

[b] NVR-010-001-E2 is a methyl analog of GLS4. It has an EC_{50} of 11 nM in HepG2.2.15 cells [73].

[c] HAP_R01 is an analog of HAP_R10, with the morpholine replaced with a 3,3-difluorpyrrolidine. It has an EC_{50} of 3 nM in HepG2.2.15 cells [57].

residue results in a mutant with faster assembly kinetics and stronger association energy, mimicking CAM-induced assembly kinetics in the absence of CAMs [99]. Because of this steric block, V124W has been shown to be resistant to both "HAP-like" and "non-HAP-like" CAMs [70, 99, 100].

The differences in the cross-resistance profiles of the "HAP-like" and "non-HAP-like" CAMs, the absence of significant cross-resistance between CAMs and NAs in the transient replication assays [59, 71, 72, 74], and the observed additive and synergistic effects with other therapies (e.g. NAs [59], an RNAseH inhibitor [118], or a TLR7 agonist [119]) provide a good basis for the use of CAMs in combination therapy, hopefully leading to a cure. Potentially, a combination of HAP-like CAMs and CAMs with the other mode of action would result in an unsurmountable barrier to replication for HBV.

9.3.6 CAMs in Clinical Trials

As discussed above, the HBV Cp and its assembly is a promising drug target to achieve HBV cure, because it is involved in several key aspects of the viral replication cycle and it has no human homologs [56, 120], in contrast to modes of action involving e.g. polymerases or proteases. Consequently, several CAMs are already being tested in clinical trials (see Table 9.3). The SBA NVR 3-778 [59] (see Figure 9.1) was the first CAM to be evaluated in HBV-infected patients. In a 28-day trial, a mean reduction in serum levels of HBV DNA of 1.43 log10 IU/ml and 1.97 $\log_{10}$ IU/ml was observed at a dose of 600 mg of NVR 3-778 given twice daily in monotherapy or in combination with pegIFN, respectively. NVR 3-778 was generally well-tolerated, and, unlike NAs, also had a clear effect on HBV RNA suppression, validating the idea of supplementing NA therapy with CAMs in order to improve viral control [121]. As of writing, no further updates have been provided regarding further development of this drug.

Table 9.3 Capsid assembly modulators in clinical development.

Compound code	Company	Phase of development	Mode of action
NVR 3-778	Novira Therapeutics/Janssen Pharmaceutica	Ph 2	Non-HAP like
GLS4 (+RTV)[a]	HEC Pharm	Ph 2	HAP like
ABI-H0731	Assembly Biosciences	Ph 2	Non-HAP like
JNJ-(5613)6379	Janssen Pharmaceutica	Ph 2	Non-HAP like
RO7049389	Hoffmann-La Roche	Ph 2	HAP like
QL-007	Qilu Pharmaceutical	Ph 2	Not disclosed
AB-506	Arbutus Biopharma	Ph 1 (discontinued)	Non-HAP like
ABI-H2158	Assembly Biosciences	Ph 1	Non-HAP like
EDP-514	Enanta Pharmaceuticals	Ph 1	Not disclosed

a) Ritonavir (RTV) was originally discovered as an HIV protease inhibitor with antiretroviral activity. Now, it is widely used as a "PK booster" of other drugs, since it inhibits cytochrome P450-3A4 (CYP3A4).

GLS4 (see Figure 9.1), a second-generation CAM of the HAP chemotype, is also under clinical investigation. It had high antiviral activity in HBV-expressing cell lines, but showed a significant reduction in efficacy when tested in metabolically active, HBV-infected PHH [66, 67]. The reason for this activity drop is its low metabolic stability. Consequently, the efficacy of the combination of 120 mg of GLS4 and 100 mg of ritonavir, a widely used CYP3A4 inhibitor, is currently being evaluated in Phase II clinical trials [122]. Before, BAY 41-4109 (AIC429) had been tested in a Phase I single dose trial with good tolerability at single doses of 2.5–300 mg, without reaching the maximum tolerated dose at the highest dose administered. However, due to preclinical data, the compound was later given up.

Since the first proof of concept study with NVR 3-778, several so-called second-generation CAMs are currently under investigation in Phase II clinical studies. However, the clinical development for some of these pipeline drugs had to be stopped due to severe side effects as it has been reported for AB-506 more recently. Nevertheless, very potent third-generation CAMs, such as ABI-H2158 and EDP-514, have just entered clinical development [123] (see Table 9.3). In 2019, Assembly Biosciences presented interim results from three ABI-H0731 and NA Phase IIa combination studies. At treatment week 24 of the study involving HBeAg-positive, treatment-naïve patients, the combination group, which received 300 mg ABI-H0731 with 0.5 mg ETV q.d., had a more pronounced HBV DNA decline (-5.30 $\log_{10}$ IU/ml) compared to the ETV monotherapy group (-4.19 $\log_{10}$ IU/ml) ($p = 0.0452$), which received only 0.5 mg ETV q.d. In addition, HBV pgRNA was reduced from baseline in the combination group by 2.34 $\log_{10}$ IU/ml, whereas in the ETV monotherapy group it was only reduced by 0.61 $\log_{10}$ IU/ml ($p < 0.001$). This long-term (24 weeks) decline in HBV pgRNA is believed to reflect the reduction in cccDNA pools. Additionally, at treatment week 48, it was observed that the reductions from baseline in mean HBV DNA and pgRNA were 6.3 and 3.0 $\log_{10}$ IU/ml, respectively, for patients treated with the ABI-H0731 + ETV combination. This second phase declines in pgRNA were strongly associated with reductions in viral antigens HBeAg and HBcrAg (surrogate markers for cccDNA) [124]. In the same year, Janssen Pharmaceuticals presented data from the first triple-combination trial, showing not only a robust HBV DNA and pgRNA decline to below the lower limit of quantification for most patients, but also an HBsAg reduction ≥ 1.0 $\log_{10}$ IU/ml for all patients who received the cocktail of JNJ-3989 together with the RNAi drug JNJ-6379 and an NA [125].

These encouraging data give hope for the concept of CAMs to be part of a future combination regimen for HBV cure. Challenges in the development of more efficacious CAMs lie in the identification of more potent compounds with superior pharmacokinetic properties in order to facilitate lower clinical dosing, anticipating the eventual necessity for a multi-component HBV cure regimen. Furthermore, there is also significant potential in targeting hepato-selective active uptake mechanisms (such as OATP1B1 and OATP1B3) to drive increased exposures in the liver and minimize toxicity and systemic side effects. Moreover, drug resistance in HBV therapy with non-NA direct acting antivirals is currently not well explored and could also be one driver for future CAM development. Further clinical studies with longer treatment durations will give more insights into the emergence of HBV drug resistance on CAMs.

9.4 Concluding Remarks

The field of targeted antiviral drug discovery for the treatment of chronic HBV infection is large and highly dynamic. After completing this manuscript in Spring 2020, improvements of existing drug programs, as well as the emergence of new direct-acting antiviral programs, have been published. The entry inhibitor Hepcludex formerly known as Myrcludex B (MYR GmbH, now part of Gilead) was conditionally approved in Europe for the treatment of chronic HDV. Several new CAM pipeline drugs have been announced, namely ABI-H3733 (Phase I, Assembly BioScience), JNJ-64530440 (Phase I, Janssen Pharmaceutica), ALG-000184 (Phase I, Aligos Therapeutics), and ZM-H1505R (Phase I, ZhiMeng Biopharma), respectively. For some of these new pipeline drugs, promising *in vitro* data showing sub-nanomolar EC_{50} values in HBV-expressing cells have already been reported. For the advanced CAM drug programs, various triple-drug combination studies (e.g., CAM plus NA plus RNAi) have been started to prove whether CAM drugs can improve the cure rates. Unfortunately, a simpler two-drug combination Phase II trial, exploring whether meaningful sustained virologic response could be achieved after discontinuing therapy in virologically suppressed patients who had received the CAM drug ABI-H0731 (vebicorvir) and an NA for 12 to 18 months, failed as 39 out of 41 patients have relapsed (Press Release from Assembly Bioscience on Nov 5, 2020). The review article [126] will give a substantial update on the recent progress in the field of targeted anti-HBV drug discovery.

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10

Hepatitis E Virus—Current Developments in Antiviral Strategies

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10.1 Introduction

The first description of Hepatitis E Virus (HEV) as a novel agent responsible for enterically transmitted non-A, non-B hepatitis by Reyes and colleagues dates back to 1991 [1]. After its first recognition during an epidemic of hepatitis in the Kashmir valley, India, in 1978 [2], similar outbreaks in the 1980s were reported in Nepal [3], Burma [4], Pakistan [5], Mexico [6], and China [7]. Nevertheless, the earliest documented epidemic outbreak retrospectively identified to have been caused by fecal–orally transmitted HEV occurred in New Delhi, India, in 1955–1956 [8, 9]. Following serological and molecular studies, HEV is today considered to be globally distributed and to be the leading cause of enterically transmitted viral hepatitis worldwide. An estimated 20 million new infections occur each year resulting in about 3.4 million symptomatic cases of Hepatitis E and 70 000 Hepatitis E-related [10]. Initially, HEV was only considered to trigger partly large outbreaks in non-developed areas. However, improved detection methods and higher awareness have also revealed substantial infection events in developed areas [11]. In parts of Europe, a prevalence of anti-HEV-IgG up to 50% has been observed [12]. Being usually an uncomplicated and self-limiting infection, in individuals with underlying liver diseases, it induces progression to rapid liver failure. Most strikingly, and still not fully understood, HEV causes an acute symptomatology with death rates in up to 25% in pregnant women [13]. Furthermore, patients with hereditary or acquired immunodeficiency can become chronically infected [11]. Here, HEV shows similar escape mechanism as the Hepatitis C Virus (HCV). However, in contrast to HCV, treatments have not been well established yet. Standard of care for chronic infections involves either treatment with ribavirin or

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interferon, both having considerable side effects. Overall, despite the widespread distribution of this virus with approximately 3 million people affected worldwide HEV has been greatly neglected in the past. Direct-acting antivirals against HEV have not been described so far. However, for patients at risk to develop acute liver failure as well as chronically infected individuals, new antivirals need to be developed in the near future. In this chapter, we summarize the current knowledge about HEV and its therapy and provide an overview of the current developments in antiviral strategies.

10.2 Genetic Diversity and Molecular Virology of HEV

The human pathogenic HEV has recently been newly taxonomically assigned to the genus *Orthohepevirus A* in the family of *Hepeviridae*. The other members of this family being *Orthohepevirus B* (avian hepatitis E virus), *Orthohepevirus C* (rat hepatitis E virus), and *Orthohepevirus D* (bat hepatitis E virus) [14]. HEV infections in humans can be caused by five different genotypes (gt) which differ in their worldwide distribution, hosts, and route of transmission. Genotype 1 and 2 viruses are described mainly in developing countries and are transmitted via the fecal–oral route by waterborne outbreaks. They solely infect humans and show high epidemic potential in India, North Africa, and Asia, causing up to 120 000 symptomatic cases in a single outbreak [15]. In contrast, HEV genotype 3 and genotype 4 infections are primarily diagnosed in industrialized nations. There have been many animals described, that serve as reservoir, including pigs [16, 17], deer and mongoose [18], wild boars [19], shellfish [20], rodents, bison, cattle, and dogs [16]. In 2014, a new genotype 7 was identified in dromedaries in the Middle East. HEV genotypes 3, 4, and 7 can cross the species barrier, as several reports indicate, where individuals became infected with HEV after eating infected raw meat from deer [21, 22], sausages containing pig liver [23, 24], or drinking camel milk [25]. Of special note, there have also been reports from Canada and Hong Kong on rat HEV, a member of the *Orthohepevirus C* genus that shares 50–60% nucleotide identity with the HEV A, to cause chronic infections in immunocompromised humans [26, 27].

HEV is an icosahedral-shaped virus with about 27–34 nm in diameter [28, 29]. The virion contains a ~7.2 kb long single-stranded RNA genome in positive orientation, which encodes for three open reading frames (ORF1-3) and is shaped like eukaryotic messenger RNA (mRNA) with a 5′ 7-methylguanylate ($7^{m}G$) cap and a 3′ poly(A) tail [29]. During the viral replication in the host cell, at least two RNA species are generated. In addition to the full-length RNA, also a subgenomic RNA of ~2.2 kb is transcribed, allowing expression of ORF2 and ORF3 [30, 31]. HEV is a quasi-enveloped virus. It can exist in a non-enveloped state, which is shed in the feces or it can be coated with a lipid-derived membrane, which circulates in the blood and might protect the virus from neutralizing antibodies [32]. ORF1 is the largest viral gene product and compromises a methyltransferase (MeT), a macro domain, a papain-like cysteine protease (PCP), a helicase (Hel), and an RNA-dependent RNA polymerase (RdRp), connected by a Y-domain and a hypervariable region (HVR) (Figure 10.1). The maturation of the ORF1 polyprotein and potential processing by the encoded protease are not fully understood and have been under discussion [33–38]. It has recently been suggested that the PCP domain, instead of having protease activity, rather exerts broad

de-ubiquitination activity preventing the proteosomal degradation of viral or selected host proteins [39]. The MeT, Hel, and RdRp have also been functionally analyzed, while the functions of the Y, the variable (V), and the macro domain remain uncertain [40]. An additional ORF4 has been identified in the coding sequence of ORF1 exclusively in HEV genotype 1 and is reported to increase the RdRp activity [41].

ORF2 encodes for the 660 amino acid (aa) virus capsid protein. Its transcription is driven by an intragenomic promoter in the junction region at the ORF1 3′ end (Figure 10.1) [42]. It is relatively conserved among the viral genotypes and harbors a typical signal peptide sequence and three potential glycosylation sites [43]. This capsid protein plays a crucial role during the viral attachment process to the host cell and is the major target for neutralizing antibodies. The ORF3 protein is only 360 bp in length and is a multifunctional 13-kDa protein of 113 (genotype 3) or 114 aa (genotypes 1, 2, and 4), respectively (Figure 10.1). This small protein is implicated as part of quasi-enveloped virions and plays an important role during viral egress [44]. Palmitoylation of the ORF3 protein drives its membrane association [45]. Recently, for ORF3 an ion channel activity has been reported, which is critical for release of infectious particles [46].

With respect to the viral life cycle, many steps are only partially understood [47]. Similar to other viruses, heparan sulfate proteoglycans on the host cell surface are required for attachment [48]. Cellular surface receptors that trigger virus entry are still

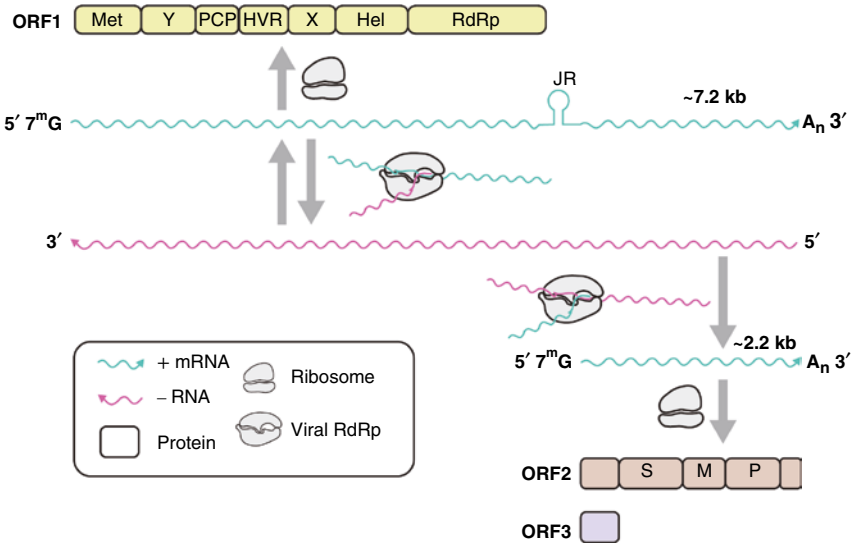


Figure 10.1 Hepatitis E virus genome organization. Upon entering host cells, the viral ss(+)-RNA (blue) ORF1 is initially translated by the host's ribosomes to produce the nonstructural viral proteins methyltransferase (MeT), papain-like cysteine protease (PCP), helicase (Hel), and RNA-dependent RNA polymerase (RdRp). The functions of a Y-domain, X-domain, and hypervariable region (HVR) are not fully understood yet. The newly produced RdRp subsequently transcribes the +mRNA into a −RNA (pink). This −RNA is then used as a template to amplify new +RNA, full length (7.2 kb) as well as a 2.2 kb long subgenomic RNA which encodes for the ORF2 capsid proteins S (small), M (middle), and P (protruding) and for the ORF3. An intragenomic promoter (JR) controls subgenomic RNA transcription. *Source*: Based on [42].

to be identified. Very recently, integrin α3 was suggested as key molecule for cellular attachment and entry of naked HEV virions [49]. Following clathrin-dependent endocytosis [50] and cytoskeleton remodeling, the viral genome is uncoated and released, probably in a pH-independent mechanism [51]. Similar to all positively orientated single-stranded RNA viruses that do not bring any accessory proteins, it requires translation of the viral replicase by the host ribosome machinery. The HEV RdRp then produces complementary negative-strand RNA and subsequently synthesizes genomic positive-strand RNA, full length as well as subgenomic RNA, from this negative-strand RNA template in an alternating manner [52]. The subcellular site of RNA replication has not been identified yet. The ORF1 protein has been shown to be membrane-associated and to localize to an intermediate compartment between the endoplasmic reticulum and the Golgi, suggesting localization within the early secretory pathway [35]. For virus particle assembly and release, HEV hijacks the host cell's endosomal sorting complexes required for transport (ESCRT) machinery by the interaction of ORF3 with the tumor susceptibility gene 101 (TSG101) [53]. ORF2 is reported to bind to the 5′ end of the HEV genome, implicating an encapsidation mechanism. It exists in several capsid forms, from which only a minority assembles into infectious viral particles [54]. After the release from hepatocytes, ORF3 plays an essential role in envelope formation. This envelope is stripped in the biliary tract, so that fecal HEV is found in high-density, non-enveloped structures [55].

10.3 Clinical Course of HEV Infections

The clinical course of HEV and Hepatitis A Virus (HAV) are in major parts indistinguishable. Many infected individuals may have subclinical symptoms and clinicians will only observe a seroconversion over time. In a large double-blinded vaccine trial from China, 56 000 individuals were followed. Only 5% of the individuals of the control arm, who seroconverted developed symptoms of an acute HEV infection [56]. In these clinically apparent cases, overall mortality rates of 0.2–4% were observed [57]. It is estimated that HEV causes 70 000 deaths per year [10], but this number could be largely underestimated due to assay variability. Of note, outcome may vary among infected individuals depending on patient-specific characteristics. Pregnant women are more likely to develop acute hepatitis (60 vs. 30% control group) [58] with higher mortality rates (up to 25%) [59] and increased numbers of stillbirths [10]. This might be explained by the different hormonal and immunological features during pregnancy. In particular, reduced expression of progesterone-receptor expression is associated with severe HEV-infection outcome. Of note, until now this has been only shown for genotype 1-infected pregnant women. Furthermore, higher viral load serves as an indicator for a severe outcome of the infection as well as increased maternal and fetal mortality [13].

Additionally, in individuals with pre-existing liver disease, HEV infection is associated with increased morbidity and mortality due to liver failure [60–63]. Also, prolonged alcohol

consumption serves as a risk factor for fulminant liver disease in the course of acute HEV infection [64] and intergenotypic differences have been described, causing more severe courses in genotype 1 compared to genotype 3-infected individuals [65]. In clinically apparent cases, the incubation period ranges from two to eight weeks, with a mean of 40 days [66]. The initial symptoms are typically unspecific and include flu-like symptoms like myalgia, arthralgia, weakness, loss of appetite, abdominal pain, and vomiting. This is usually accompanied by highly elevated liver enzymes (aminotransferases, alkaline phosphatase, γ-glutamyltransferase, and bilirubin) and the development of liver signs like jaundice, pruritus, uncolored stool, and darkened urine. Usually these symptoms are self-limiting and resolve within four to six weeks [67–70].

Of special note, despite HEV being a primary hepatotropic pathogen, there have been numerous extrahepatic manifestations described [71]. Besides renal, hematologic, and pancreatic manifestations, neurological symptoms are the most prominent, including neuralgic amyotrophy (brachial neuritis, Parsonage-Turner syndrome), and Guillain–Barré syndrome [72–79]. In addition to this, HEV RNA has been identified in the cerebrospinal fluid of infected patients [75, 76, 78, 80]. Whether these symptoms are caused by active replication of the virus in the respective tissues is still unknown, but *in vitro* data prove HEV to be capable of replicating in neuron-derived [81], as well as in placenta-derived cell lines [82].

Despite the different genotypes of HEV, only one serotype is described which has major implications for vaccine approaches as well as clearance of HEV-infection. With the onset of elevated transaminases, IgM against HEV can be detected [83]. Shortly thereafter, highly specific IgG is observed, which indicates control of infection and confers resistance to reinfection in immune-competent individuals for at least a certain time.

Besides these humoral responses, cellular immunity plays a critical role in virus control. Potent HEV-specific T-cell responses can be observed in individuals, who spontaneously or treatment-induced cleared HEV infection [84]. Additionally, these strong T-cell responses can be detected in patients, who previously cleared a subclinical HEV infection. In general, every circumstance that negatively influences important mechanisms of viral clearance can cause chronic infection, which is defined as persistent infection over a time-period of six months. Individuals, who are not able to clear the virus, display impaired T-cell responses [84]. Furthermore, at least in case of transplanted patients that were investigated, IgG could be detected although persistent infection took place. This might be due to low titers or reduced neutralizing potency of these antibodies. Theoretically, similar to what is known for HCV, the virus could potentially escape humoral responses due to its high genetic variability [85, 86]. Controversially, in chronically infected HEV patients, well-known interferon-stimulated genes like IFIT1, IFI44L, RSAD2, EPSTI1, and ISG15 are over-expressed compared to healthy individuals [87]. Interestingly, this is again in line with findings in chronically HCV-infected patients, where it causes nonresponse to interferon-based treatment [88].

Chronic infection with HEV is solely described in the context of genotype 3. However, this observation could be biased as chronic infection is almost exclusively observed in areas, where occurrence of other genotypes is unlikely.

10.4 HEV Therapy

HEV-specific antiviral drugs are still lacking and treatment for patients with hepatitis E currently is only supportive. The current treatment algorithm for chronic infections by the European Association for the Study of the Liver (EASL) was published in 2018 [89]. First of all, the possibility of reduction of immunosuppressive medication needs to be evaluated [11]. Previous studies reported clearance rates of up to 25% employing this strategy [90]. However, the risk of rejection of the allograft is constituted by reduction of immunosuppression beyond a certain level [67, 91].

The treatment of choice for HEV infections is the off-label use of RBV. Its efficacy in reducing viral loads in patients has been evaluated in large studies for acute as well as chronic infections [65, 92, 93]. Also, in immunocompromised, SOT recipients or HIV-infected individuals, it can be safely applied and results in clearance of the viral RNA [94–96]. Furthermore, *in vitro* data suggest a moderate synergistic effect when combined with pegIFN-α [94]. However, because of its teratogenic characteristics, RBV cannot be administered to pregnant women and there are reports on RBV treatment failure in risk group patients. In the latter cases, RBV doses had to be reduced because of severe anemia and one patient died after experiencing a virological breakthrough associated with RBV dose reduction [65, 97]. Recently, investigations suggest that these treatment failures were possibly linked to the selection of a distinct HEV polymerase variant (G1634R) resulting in increased replication fitness [98, 99]. In a subsequent study, 63 SOT patients with chronic hepatitis E were screened for the existence of this variant. The authors found that the presence of the G1634R variant at RBV initiation does not lead to absolute RBV resistance and although its proportion was increased in patients whose treatment failed, the presence of the 1634R variant did not compromise the response to a second RBV treatment [100].

There are several modes of action proposed for RBV and its antiviral effect against RNA viruses [101, 102] and reviewed in the context of HEV [103, 104]. Among the indirect mechanisms, a T-cell-mediated effect is described, switching the balance of T helper cells from a T helper type 2 phenotype to a T helper type 1. RBV restores the T helper 1 phenotype needed for balanced expression and secretion of cytokines produced from type 1 and 2 T helper cells. A second indirect mode of action is the inhibition of the cellular inosine-5′-monophosphate dehydrogenase (IMPDH). In mammalian cells, RBV is phosphorylated and RBV monophosphate (RMP) perfectly mimics inosine monophosphate (IMP) and thereby inhibits the synthesis of IMP to xanthosine monophosphate (XMP) by IMPDH. Consequently, no guanosine triphosphate (GTP), necessary for production of nascent (viral) RNA, is synthesized. This depletion of GTP pools has been proven to play a role in the anti-HEV effect of RBV in vitro [94]. Further mechanisms described for RBV are a direct influence on the expression if IFN stimulated genes (ISGs), the inhibition of (viral) MeTs, thereby interfering with the catalysis of the formation of 5′ 7-methylguanosine cap structure, and the direct inhibition of viral polymerases [104]. Here, RBV triphosphate (RTP) is recognized by the RdRp leading to chain termination or preventing the binding of other nucleotides important for elongation [105]. In recent years, an additional mode of action for RBV was described, i.e. a mutagenic effect via incorporation into newly synthesized RNA genomes,

leading to viral extinction. This effect has been observed in several RNA viral populations [106–110], as well as for HEV [99, 103, 104, 111].

10.5 Development of Novel Antivirals Against HEV

Both antiviral drugs—interferon as well as ribavirin—are not highly selectively targeting the viral life cycle. Therefore, both display considerable side effects, in particular flu-like symptoms, depressions, (interferon) or severe anemia (ribavirin). Also, treatment of transplanted patients with interferon might induce graft rejection and therefore should be used with caution. In particular, treatment of pregnant women is difficult, as both drugs are contraindicated under these circumstances. Therefore, novel antiviral against HEV are urgently needed [112].

The most advanced drug candidate in development as novel antiviral molecule against HEV is the highly potent HCV RdRp inhibitor sofosbuvir (Figure 10.2). Sofosbuvir is a nucleotide prodrug (Table 10.1) that acts as a chain terminator of the replicase after activation and incorporation into the viral genome [127]. Different studies reported an antiviral activity of sofosbuvir against HEV using different HEV replicons and human liver cell lines [113, 117, 128, 129]. Dao Thi and colleagues were the first to show that the drug-inhibited HEV replication in a low micmolar range and was additive with RBV [117]. However, the antiviral activity was 10–100-fold lower compared to HCV replicons and could not be confirmed by Wang et al. with GT1 and GT3 replicons in different cell lines [130]. The drug with or without RBV has been used to treat patients, who failed previous RBV or were ineligible for RBV therapy. However, the results of the published case reports are inconclusive and provide no clear evidence of efficiency [131]. While in one case successful treatment with sofosbuvir/RBV was reported [132], other studies showed only weak or moderate effects of sofosbuvir [133–137]. For these reasons, a multicenter study (SofE), an investigator-initiated, phase 2 pilot trial (NCT03282474), recently investigated the efficacy and safety of 400 mg sofosbuvir daily for 24 weeks in nine immunocompromised patients with chronic hepatitis E, who were ineligible or failed prior ribavirin therapy [138]. Sofosbuvir treatment showed only weak antiviral activities without curing the infection and HEV RNA decline was associated with ALT improvements [138]. These results suggest that sofosbuvir is not suited as monotherapy treatment and requires further investigations for possible combination treatments.

The reuse of an already existing compound is referred as drug repurposing [139]. The advantage of drug repurposing is that pharmacological information is already available and that *in vivo* testing has already been performed, although for another primary indication. Repurposing approaches in the field of HEV were reported for the compounds 2′-C-methylcytidine (2-CMC), NITD008, and GPCN114 (Figure 10.2; Table 10.1). The nucleoside analogue 2-CMC, which is also known as NM107, was originally developed against HCV but has a low oral bioavailability. However, the development of a prodrug, NM283, resulted in adverse toxic effect as the drug can serve as a substrate for the mitochondrial DNA polymerase [119]. An inhibitory effect of 2-CMC was demonstrated against HEV GT3 with a half maximum inhibitory concentration (IC_{50}) of 22 μM [117]. Qu et al. confirmed these anti-HEV effects and showed antagonistic effect when combined with RBV [118]. NITD008 is another nucleoside analogue with described antiviral effects against different Flaviviruses

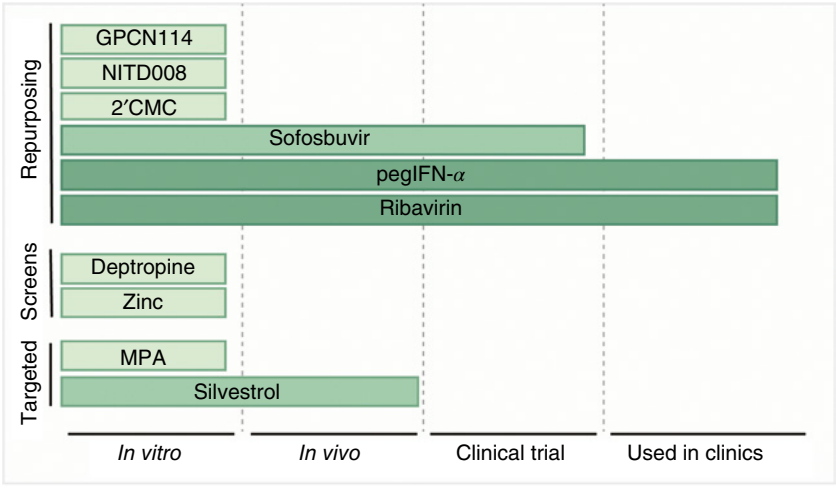


Figure 10.2 Summary of the discussed anti-HEV compounds. The depicted molecules/extracts are classified according to the strategy that was used to identify them. So far, the antiviral activity against HEV of only four drugs (sofosbuvir, pegIFN-α, ribavirin, and silvestrol) was approved in experimental settings beyond *in vitro* cell culture systems. Source: [112]. CC BY 4.0.

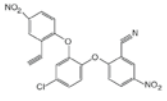
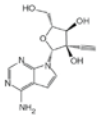
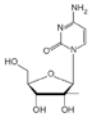
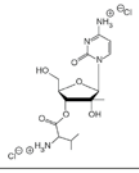
[114–116] and showed a potency against HEV GT1 replicons with a half maximum effective concentration (EC_{50}) of 0.03 μM [113]. GPCN114 as non-nucleoside inhibitor with known activity against Picornaviruses was also identified by Netzler and colleagues to inhibit HEV GT1 replicons [113]. For both drugs, no clinical trials have been filed so far (Figure 10.2) [112].

Another molecule discussed as therapeutic agent for HEV is zinc (Figure 10.2; Table 10.1). Zinc treatment inhibited viral replication in the human hepatoma cell culture models of genotype-1 and genotype-3 strains in vitro at a concentration of $IC_{50} = 10\,\mu M$ and $IC_{95} = 200\,\mu M$ for ZN sulfate and ZN acetate [123]. These data suggested that the antiviral activity of zinc on HEV is likely mediated through its effect on the viral RdRp. However, the observed antiviral effect could be attributed to direct and indirect action(s) of zinc on multiple distinct virus/host targets/processes, as shown for other viruses [140]. A clinical study has not been conducted so far (Figure 10.2), but a case study evaluated the influence of zinc levels on the outcome of RBV treatment. Here, clearance of infection was not associated with higher zinc levels [141].

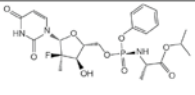
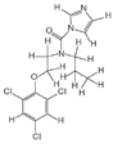
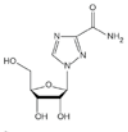
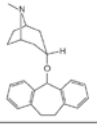
A recently reported high-throughput screening of a library containing over 1,000 FDA-approved drugs for anti-HEV activity identified deptropine, a histamine H1 receptor antagonist (Table 10.1) used in clinics to treat asthmatic symptoms (Figure 10.2) [122]. Although the exact mode of action remains to be elucidated, the anti-HEV activity of deptropine seems to involve the NF-κB-RIPK1-caspase axis. Interestingly, the authors find a pronounced antagonistic effect of the identified drug with IFNα, while the effects combined with RBV add up [122]. No clinical data on deptropine in the context of HEV infections are published so far (Figure 10.2).

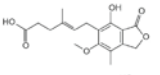
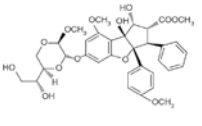
Besides tackling down the virus by direct-acting drugs, compounds interfering with the host cell biology needed for the obligate intracellular pathogens is another promising

Table 10.1 Compounds tested for anti-HEV effect and discussed in this chapter.

Name	Structure	Function	CAS	References
GPCN114 2,2'-[(4-Chloro-1,2-phenylene)bis(oxy)] bis(5-nitrobenzonitrile)		Non-nucleoside inhibitor of the HEV RdRp		[113]
NITD008 7-(2-C-Ethynyl-β-D-ribofuranosyl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine		Adenosine analogue	1044589-82-3	[114–116]
2′CMC 2′-C-methylcytidine		Nucleoside analogue	20724-73-6	[117, 118]
NM283 3′-O-valinyl ester of 2′-C-methylcytidine (dihydrochloride salt)		Nucleoside analog prodrug	640281-90-9	[119]

(Continued)

Name	Structure	Function	CAS	References
Sofosbuvir Isopropyl (2*S*)-2-{[(*S*)-{[(2*R*,3*R*,4*R*,5*R*)-5-(2,4-dioxo-3,4-dihydro-1(2H)-pyrimidinyl)-4-fluoro-3-hydroxy-4-methyltetrahydro-2-furanyl]methoxy}(phenoxy)phosphoryl]amino}propanoate		Nucleotide conjugate	1190307-88-0	[117]
pegIFNα		Triggers interferon-alpha receptor 1 and 2 (IFNAR1/2)	99210-65-8	[120, 121]
Ribavirin 1-(*β*-D-Ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide		Nucleoside analogue	36791-04-5	[92, 93, 95]
Deptropine Dibenzheptropine		Antihistamine	604-51-3	[122]

Name	Structure	Function	CAS	References
Zinc	Zn^{++}	Not fully understood		[123]
MPA Mycophenolic acid		Immuno-suppressant	24280-93-1	[124]
Silvestrol Methyl (1*R*,2*R*,3*S*,3a*R*,8b*S*)-6-({(2*S*,3*R*,6*R*)-6-[(1*R*)-1,2-dihydroxyethyl]-3-methoxy-1,4-dioxan-2-yl} oxy)-1,8b-dihydroxy-8-methoxy-3a-(4-methoxyphenyl)-3-phenyl-2,3,3a,8b-tetrahydro-1H-benzo[b]cyclopenta[d] furan-2-carboxylate		eIF4A inhibitor	697235-38-4	[125, 126]

approach [112]. Due to the lack of knowledge in the molecular virology of HEV and its possible host interaction partners, only a few targets with antiviral properties have been evaluated though [47]. They are summarized in the following:

Silvestrol is a structurally unique cyclopenta[b]benzofuran (Table 10.1) that can be isolated along with epi-silvestrol from the plant *Aglaia foveolata* and other *Aglaia s*pecies belonging to the family of *Meliacea* [142]. It is described as a highly efficient, nontoxic, and specific inhibitor of the DEAD-box RNA helicase eIF4A [143], which is part of the eIF4F complex that drives cap-dependent translation initiation in eukaryotes [144]. The molecule has been analyzed mainly in the context of cancer treatments, but has been found in independent studies to exert an antiviral effect against HEV (Figure 10.2) [125, 126]. It was shown that silvestrol was able to reduce viral titers in A549 as well as HepG2 cells and block viral protein synthesis at concentrations of IC_{50} = 2.9–6.7 nM, IC_{90} = 27.3–64.7 nM [125, 126]. This anti-HEV activity was further observed *in vitro* using laboratory and primary isolates to infect human liver cells and *in vivo* using humanized mice [126]. Of note, silvestrol was additionally effective against HEV harboring a fitness mutation [99], which renders the virus resistant to RBV *in vivo* [103]. At the same time, several other studies reported an antiviral effect of silvestrol against Ebola virus at concentrations of IC_{50} = 96 nM, Coronovirus at concentrations of IC_{50} = 1.3–100 nM, IC_{90} = 12–900 nM depending on cell line and virus, and Zika virus at concentrations of 5–50 nM (no dose-response relationship) infections using *in vitro* cell culture model systems [145–147]. These results identified silvestrol as a novel natural compound blocking replication of several RNA viruses and may provide a basis for a chemical, biological, and preclinical development of silvestrol as broad-acting novel antiviral agent including HEV. As the antiviral target is a host factor, the emergence of resistance should be lower compared to viral targets; however, blocking essential cellular targets may also come with a risk of side effects.

The inosine-5′-monophosphate dehydrogenase (IMPDH) is another host target that was pursued as an antiviral approach. IMPDH is an essential enzyme in the purine nucleotide synthesis and is part of immunosuppressive regimens in organ transplant recipients. Next to RBV, mycophenolic acid (MPA) (Table 10.1), which targets an enzyme in the nucleotide synthesis pathway, was shown to inhibit HEV replication of GT3 replicons in Huh7 cells (Figure 10.2) [124]. This effect could be reverted by the addition of guanosine. However, these antiviral activities could not be confirmed *in vivo* and no additive inhibition of HEV RNA with RBV and MPA was observed in French patients (Figure 10.2) [90].

In summary, drug design and development for HEV infections are at their beginnings and have focused mainly on drug repurposing approaches with limited success [112]. A better understanding of the replication cycle of HEV is necessary to specifically develop direct-acting antivirals [47].

10.6 Prevention of Infection and Vaccination Strategies

HEV genotypes 1 and 2 are waterborne viruses. Therefore, similar to HAV, clean water facilities can efficiently prevent outbreaks of HEV. It is presumed that HEV, being a nonenveloped virus in the fecal–oral route of transmission, is relatively robust and stable upon environmental harms. The risk of foodborne HEV can be reduced significantly by cooking

meat. *In vitro* assays suggest that cooking meat for one minute at 70 °C leads to a 0.48 log reduction of infectivity and heating to 95 °C lead to pronounced inactivation of 3.67 log of infectivity [148–150]. Blood products are screened for HEV in some countries, but it is unclear whether common inactivation procedures cause elimination of infectious HEV-particles. While direct human-to human transmission of HEV is usually uncommon, patients should be advised to perform cleaning of used sanitary facilities. HAV is partially resistant to 80% ethanol-based disinfectants. Assuming similar environmental stability of HAV and HEV, disinfectants based on 80% ethanol only, should not be recommended for decontamination of HEV either [151]. Overall, intra-family or direct person-to person transmission seems to be seldom events, but have been described and suggested in a few cases [152]. Therefore, testing of partners/family-members should be considered if risk factors for development of severe infection are present in these individuals, in particular having an underlying liver disease, prolonged alcohol abuse, or being pregnant.

In early neutralization studies *in vitro*, infection of hepatoma cells (HepG2/C3A) could be efficiently inhibited with serum of vaccinated or previous infected rhesus monkeys. Of note, the neutralization was cross-reactive: sera of animals, which were infected with genotypes 1, 2, 3, and 4, potently inhibited infectivity of genotype 1 HEV-virus [153]. These findings give hope for the development of a potent, pangenotypic vaccine. In a large-scale vaccine campaign in China from 2011, the vaccine HEV 239 (Hecolin®) has been tested in over 50 000 individuals. Indeed, it displayed a strong potency by preventing acute HEV in 94–100% of the cases [56]. No major side effects have been documented and the vaccine got licensed in China in December 2011.

Nevertheless, it has not been approved in other countries yet and long-term prevention has not yet been demonstrated. This vaccine was evaluated by the NIH in a phase 1 clinical trial in US adults and the primary completion date was reached in August 2020 (NCT03827395, https://clinicaltrials.gov/). So far, results from this study have not been published yet. Additionally, the Norwegian Institute of Public Health is testing its efficacy in women with childbearing potential in Bangladesh (Phase 4) (NCT02759991, https://clinicaltrials.gov/). In a different vaccine study from Nepal, anti-HEV-IgG-titer of 120 Walter Reed units/ml (2–6 WHO units/ml) were protective, but lower levels could lead to risk of reinfection [154]. Although this vaccine completed phase 2 trials and showed potent inhibition of infection in 2000 tested individuals, further development was stopped and it has not been approved so far.

10.7 Conclusions

Hepatitis E is not affecting only developing nations, but is a globally existing health threat. Especially, several subgroups—pregnant women, persons with underlying liver diseases, immune-compromised patients—have a risk of high morbidity and mortality by infection with HEV and "standard" experimental treatment with interferon or ribavirin is often not eligible in this cohort due to various side effects.

Therefore, HEV should be excluded by differential diagnostics in any case of disease, associated with elevated liver enzymes. However, until now direct-acting antivirals have not been established, mainly due to lack of efficient in vitro systems. Recent achievements

in HEV cell culture systems have been made [155, 156] that hopefully facilitate the identification of new drug targets and novel antiviral compounds. The first high-throughput screening of compound libraries has been performed and may lead to attractive drugs. Additionally, two vaccine approaches have already proven good safety and potency in protection against HEV infection, although until now, there is no vaccine on the market, which can be applied globally and the duration of protection still needs to be evaluated. This would not only be beneficial and desirable for endemic areas. Patients in developed countries which display certain risk factors would also be candidates in need for a protective vaccine.

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11

Antiviral Therapy of Adenovirus Infections

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11.1 Human Adenovirus

Human adenoviruses (HAdVs) are ubiquitous, non-enveloped, linear double-stranded DNA viruses first isolated over 65 years ago from human adenoid tissue [1]. Since then, more than 80 different serotypes have been identified, classified into seven subgroups (A–G) primarily on the basis of genomic sequence homology, oncogenicity in rodents, and the ability to agglutinate erythrocytes of different species [2, 3]. Virus transmission in the general population occurs through close contact with infected bodily fluids, aerosol droplets, water, contaminated fomites, or medical instruments. In contrast to enveloped viruses, HAdVs remain infectious outside of the body for extended periods of time (e.g. up to three weeks on surfaces) and are highly resistant to physical and chemical reagents; these properties render the virus highly contagious [4].

The infectious virus particles have a diameter of 70–100 nm and are composed of two major structural elements: (i) the nucleoprotein core that contains the genomic DNA (~36 kb, encoding ~35 genes) and (ii) the outer icosahedral capsid which is largely made up of the major virus protein, hexon. The virus capsid contains 12 projecting spikes at the vertices of the icosahedra consisting of thin fibers attached to the penton base [3, 5, 6]. These spikes, together with the hypervariable regions of the capsid hexons, mediate attachment (and ultimate entry) to one of several specific cellular surface receptors such as CAR (coxsackievirus and adenovirus receptor), CD46, sialic acid, etc., and thus confer tissue specificity (Figure 11.2) [8, 9]. Interestingly, cellular receptors for HAdV appear to vary between distinct virus subgroups which might contribute to the varying tissue tropism and the different disease associations observed for distinct HAdV serotypes [4, 5, 10, 11].

In fact, HAdVs are common human pathogens responsible for a wide range of human diseases including respiratory tract infections, gastrointestinal tract disorders, genitourinary diseases, or ocular infections (Table 11.1). While HAdV infections mostly cause mild and self-limiting diseases in immunocompetent adults and children, there are at least two

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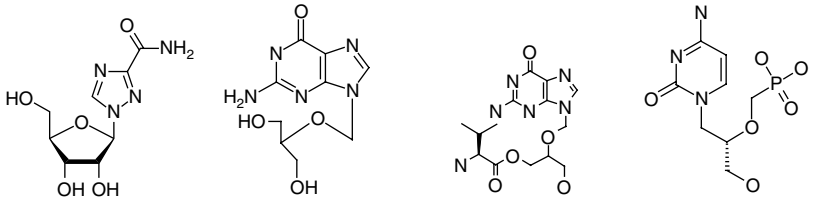
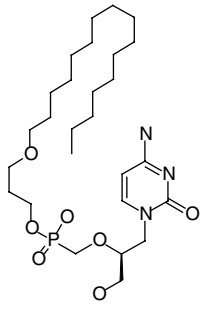
Table 11.1 Human diseases caused by adenoviruses.

	Adenovirus serotypes associated with disease (sorted in subgroups)						
Syndrome	**A**[a]	**B**	**C**	**D**	**E**	**F**	**G**
Gastroenteritis	12[b], 18, 31					40, 41	52
Hemmorrhagic cysitis		11, 21					
Hepatitis			1, 2, 5				
Upper respiratory disease		3, 7, 21	1, 2, 5, 6				
Lower respiratory disease		3, 7, 14, 21	1, 2		4		
Pertussis syndrome			5				
Pharyngoconjunctival fever		3, 7, 14	2		4		
Follicular conjunctivitis		3, 7, 11			4		
Epidemic keratokonjunctivitis				8, 37, 53, 54, 56, 64			

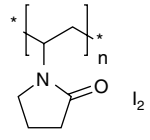
a) Adenovirus subgroup.
b) Adenovirus serotype.



(a)



(b) Brincidofovir (BCV; CMX001)



(c) Povidone iodine (PVP-I; IVIEW-1201)

Figure 11.1 Chemical structures of antiviral drugs used for therapy of HAdV infections. (a) Drugs currently marketed for an unrelated indication and used "off label" for systemic treatment of HAdV infections in HSCT patients. (b) Novel antiviral drug in clinical development for systemic HAdV treatment. (c) Povidone-iodine, an extended release formulation of this disinfectant is currently in clinical development for the topical treatment of acute HAdV conjunctivitis.

common manifestations of major clinical concern: (i) severe ocular infections, i.e. epidemic keratoconjunctivitis (EKC) and (ii) infections of immunocompromised individuals, in particular transplant recipients undergoing an hematopoietic stem cell transplantation (HSCT). A HAdV infection in the latter patient population is often associated with serious and even life-threatening disease, whereas a severe HAdV eye infection carries the risk of potential long-term consequences for visual acuity [3, 4].

Despite the significant clinical impact of HAdV, there are currently no approved drugs available to combat these infections. A live oral vaccine against serotypes 4 and 7 was developed exclusively for the US military to limit HAdV infections in recruits [12]. However, since this vaccine is not available to the general population, there remains no vaccine available for general use in children or adults in any part of the world [5]. Given this, current antiviral therapies are entirely dependent upon suboptimal "off-label" therapeutic options or palliative care. Hence, there is a high unmet medical need for potent and specific anti-adenovirus drugs, particularly for severely immunocompromised HSCT patients and patients suffering from severe eye infections like EKC.

11.2 Adenovirus in Human Stem Cell Transplantation

11.2.1 Incidence, Transmission, and Clinical Manifestation

Worldwide, more than 140 000 solid organ- and 90 000 bone marrow transplants are performed annually and viral infections continue to be a major cause of morbidity and mortality following both solid organ transplantation (SOT) and hematopoietic stem cell transplantation (HSCT) due to a combination of direct cytopathic effects of replication and indirect, host-dependent immunopathological mechanisms. Besides herpesviruses like human cytomegalovirus (HCMV; see also chapter 6), herpes simplex virus (HSV; see also chapter 5), varicella zoster virus (VZV), and Epstein Barr virus (EBV), human polyomaviruses (e.g. BK-Virus), and in particular HAdV infections have increasingly been recognized as significant causes of transplant-related morbidity and mortality in the context of HSCT [13, 14]. Clinical manifestations following a HAdV infection in HSCT recipients include respiratory tract disease, gastroenteritis, hepatitis, cystitis, and multi-organ disseminated disease which can ultimately cause lethal organ damage.

In the setting of pediatric allogeneic HSCT, an overall incidence of HAdV infection from 6 to 42% has been observed, with a mortality rate of up to 80% for disseminated disease. The estimated incidence range in adult allogeneic HSCT is significantly lower (3–15%) but the clinical manifestations can be equally severe [4, 15–18]. Why HAdV is so much more problematic in pediatric HSCT patients than in adults is not fully understood but may be attributable to the permanent circulation of the virus among children, the higher persistence rate of the virus in early childhood, and/or the site of virus reactivation (see below) [15, 16].

The vast majority of HAdV serotypes associated with infection and disease in transplant recipients belong to subgroup C (e.g. C1, C2, C5), though certain serotypes of subgroup A and B have also been observed in these patients (Table 11.1). Coinfections with different

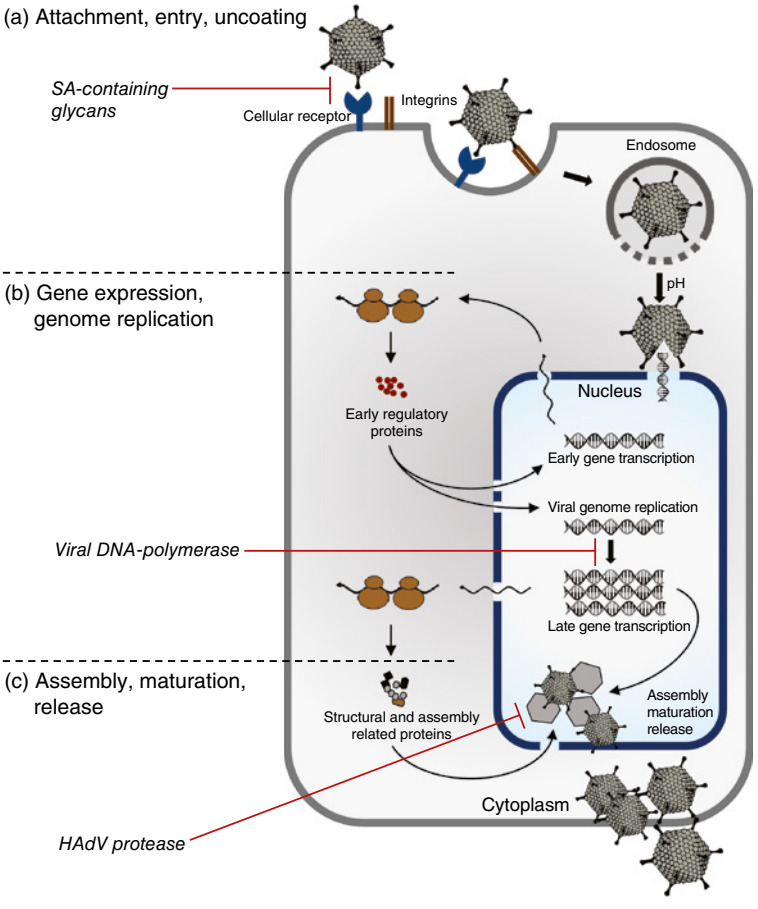


Figure 11.2 Adenovirus replication cycle. (a) Attachment, entry, uncoating: infection starts with binding of fiber to its specific cellular receptor and binding of penton base to αβ-integrins followed by endocytosis. The virion is released into the cytoplasm via acidification of endosomes and travels to the nuclear pore while undergoing a stepwise uncoating process. After final dismantling, viral DNA is delivered into the nucleus. (b) Gene expression and genome replication: the HAdV replication cycle is divided in two stages, early and late phase. During the early phase, mainly nonstructural regulatory proteins are expressed which function to (i) activate additional virus genes, (ii) modulate expression of host genes necessary for DNA synthesis, (iii) avoid premature death of infected cells, and (iv) initiate virus genome replication. DNA replication is followed by the transcription of the late virus genes encoding mainly structural proteins and proteins necessary for particle maturation. (c) Assembly, maturation, release: newly synthesized structural components are imported into the nucleus and assemble into new, immature viral particles. This process is accompanied by packaging of progeny viral DNA. After particles have undergone a maturation process, infectious virus is released from the cell as a result of virally induced cell lysis. Proteins studied as potential antiviral targets in the different steps of the HAdV replication cycle are indicated (for details, see text). *Source*: Modified from [7].

serotypes are common and have been frequently reported [4, 15, 19]. It should be noted that all disease manifestations listed in Table 11.1 are seen in transplant recipients. However, transplant recipients are at risk to develop a disseminated infection originating from one of these local infections.

Although nosocomial outbreaks of adenovirus infections in transplant centers have been reported [20–22], it is believed that donor stem cell product-associated virus transmission and/or reactivation of latent virus are the major sources of early infection in HSCT patients. This hypothesis is underpinned by the following facts and observations: (i) following a primary infection in early childhood, HAdV can persist in a latent state in a variety of tissues of the infected host (tonsils, adenoids, lung, and intestines) from which the virus can reactivate upon severe immunosuppression (ii) a HAdV infection/disease in heavily immunosuppressed HSCT patients stereotypically tends to occur within the first few months after transplantation while patients are still subject to strict protective isolation, and (iii) HAdV strains detected in patients after HSCT are usually identical to those identified prior to transplantation [4, 23].

Interestingly, although virus reactivation could generally occur at different sites and tissues, a clinically significant HAdV replication preceding an invasive infection or disseminated disease in pediatric HSCT recipients almost always starts in the GI tract. It goes without saying that this recent finding has major implications for diagnosis, monitoring, and therapy of a HAdV infection at least in the pediatric allogeneic HSCT setting. Unfortunately, there are insufficient data available on adult HSCT to date [4, 15, 24].

11.2.2 Current Therapy Strategies and Antiviral Agents

HAdV infections in immunocompromised individuals arising from reactivation of latent viruses are difficult to treat and sometimes overwhelm the patient and result in death. In principle, there are three strategies to limit the impact of HAdV in patients undergoing HSCT: prophylaxis, pre-emptive therapy, and therapeutic treatment. In prophylaxis, the drug is given to all patients from the time of transplantation onward, irrespective of the risk for HAdV infection or reactivation. Thus, prophylaxis follows the rationale of preventing virus replication during the period of greatest immunosuppression after HSCT and prior to immune reconstitution. In pre-emptive therapy, patients are monitored by means of laboratory tests for active infection and are treated once a certain viral threshold has been detected. The goal of this intervention strategy is to prevent the progression of an active but asymptomatic infection toward disseminated disease. The treatment approach finally provides antiviral therapy only to symptomatic patients with overt adenovirus disease.

Unfortunately, recent data suggest that all drugs that are currently available for pharmacologic intervention against HAdV infections (i) have no or only limited efficacy when started as treatments for active, symptomatic HAdV disease and (ii) suffer from inadequate overall efficacy and/or are associated with toxicities when applied in a prophylactic regimen. As a consequence, major guidelines do not recommend either strategy with the currently available antivirals, thus leaving clinicians with only pre-emptive therapy as the mainstay for prevention of HAdV-associated morbidity and mortality [4, 15, 23, 25]. However, once better-tolerated and highly potent drugs become available, treatment

guidelines will most likely switch to universal prophylaxis, since this strategy is less dependent on the availability and cost issues of diagnostic procedures required for pre-emptive therapy and might even be associated with health and survival benefits for the transplant patient similar to those recently observed from prophylactic therapy of HCMV infections in HSCT with letermovir (see chapter 6, [26–28].

In recent years, *in vitro* activity against HAdV was reported for many different drugs and molecules; however, as outlined in Section 11.1, there is still no specifically approved antiviral therapy available for HAdV infections in the transplant setting [13, 15, 18]. Among those compounds with substantial antiviral activity in cell culture, case reports and case series describing clinical use in the immunocompromised host are available for only three drugs: ribavirin, (Val)ganciclovir, and cidofovir (Figure 11.1a) [4, 15, 23, 24].

11.2.2.1 Ribavirin

Ribavirin (Figure 11.1a) is a purine nucleoside analogue with broad-spectrum *in vitro* activity against many RNA and DNA viruses. Accordingly, the drug was approved for the treatment of chronic hepatitis C virus in combination with peginterferon alpha (Copegus™; Rebetol™) or as a treatment for respiratory syncytial virus in infants and young children (Virazole™). Although different mechanisms of action have been proposed for this drug, including inhibition of viral polymerases, inhibition of viral RNA capping, or interference with the GTP synthesis pathway, the exact mechanism explaining its anti-HAdV activity remains unknown [29]. *In vitro*, anti-HAdV activity of ribavirin appears to be serotype-dependent with highest antiviral activity seen against subgroup C viruses [30, 31]. However, depending on the assay used, the cells and the readouts as well as on the virus genotypes, EC_{50} values vary widely from 0.5 to 34 μM. Clinical data for ribavirin are sparse and inconsistent, with some case reports suggesting a therapeutic benefit for some patients while others do not see an antiviral response on ribavirin treatment [32–34]. Consequently, ribavirin is not generally recommended for treatment of HAdV infections but can be considered in cases with serotype C infections, especially in patients with decreased renal function (see cidofovir side effects).

11.2.2.2 (Val)Ganciclovir

Another more specific antiviral drug that was assessed for efficacy against HAdV is the approved anti-CMV drug ganciclovir (GCV; Cymeven®, Roche) and its oral prodrug valganciclovir (VGCV; Valcyte®; Roche) (Figure 11.1a) [35]. GCV/VGCV is a deoxyguanosine analogue that requires specific intracellular phosphorylation for full activation. In a first step, GCV is selectively monophosphorylated in infected cells by virus-encoded protein kinases like the HSV thymidine kinase or the HCMV protein kinase pUL97. Subsequently, GCV-P is converted to a triphosphate by cellular enzymes. The active form of GCV preferentially inhibits viral DNA polymerases and is also incorporated into progeny viral DNA which drastically slows down chain elongation [29]. However, there is also off-target activity in the form of cellular polymerase inhibition. Neutropenia, anemia, thrombocytopenia, and a putative long-term reproductive toxicity are the most common serious side effects associated with GCV treatment [36]. Importantly, adenoviruses, in contrast to herpesviruses, lack a viral kinase gene resulting in inefficient activation of GCV in HAdV-infected cells. Accordingly, GCV has demonstrated only a modest anti-HAdV efficacy

in vitro (EC50 values in the upper two digit μM range) and the rare reports on systemic (V)GCV administration in the clinics do not support its use as an anti-HAdV drug in the HSCT setting [35, 37].

11.2.2.3 Cidofovir

Most evidence for the *in vivo* efficacy of antiviral HAdV therapy in the pre-emptive setting is available for cidofovir (CDV; Vistide®, Gilead, Figure 11.1a), a drug that was initially approved for the treatment of HCMV retinitis [29]. CDV is a phosphonomethoxy analogue of cytosine that has demonstrated broad-spectrum activity against double-stranded DNA viruses including herpesviruses and adenoviruses. In contrast to GCV, CDV does not require initial modification by a viral enzyme, since its conversion to a triphosphate is catalyzed by cellular enzymes. The active form of CDV acts as a competitive inhibitor of the viral DNA polymerase and causes premature chain termination during viral DNA synthesis [36, 38]. CDV is sufficiently active against all HAdV subtypes (EC50 values ranging from 0.5 to 62 μM) and is characterized by a long intracellular half-life allowing infrequent administration of the drug [13]. Given, that a pre-emptive CDV therapy of HAdV-infected HSCT patients is associated with clinical improvement and a survival benefit, this drug is the current standard of care (SOC) for controlling HAdV infections and preventing disseminated HAdV disease in immunocompromised patients undergoing an allogeneic stem cell transplantation [15, 23, 25, 39]. Although encouraging outcomes are seen with CDV therapy, the widespread clinical use of CDV is limited since the drug is slowly absorbed, poorly bioavailable, myelosuppressive, and importantly, causes severe nephrotoxicity [18, 29, 40].

In summary, current antiviral therapy of HAdV infections in HSCT is suboptimal and available drugs suffer from low efficacy and/or problems with associated toxicities which are particularly detrimental after HSCT. Accordingly, there is a high unmet medical need for new, safe, and effective anti-HAdV agents that can be used in pre-emptive but hopefully also prophylactic strategies in HSCT.

11.2.3 Novel Antiviral Approaches in Clinical Development

11.2.3.1 Brincidofovir

As outlined under Section 11.2.2.3, the current standard drug for therapy of HAdV-related disease in HSCT is the broad-spectrum antiviral CDV, but its use is limited due to poor oral bioavailability and inherent nephrotoxicity. Attempts to overcome these drawbacks led to the synthesis of the CDV derivative brincidofovir (BCV; CMX001; hexadecyloxypropyl-CDV; Cidofovir-HDP) (Figure 11.1b; Table 11.2). BCV is a lipid ester prodrug of CDV which is highly efficiently delivered into target cells. Within cells, the lipid side chain of BCV is cleaved off by phospholipases and CDV is released (see Section 11.2.2.3) [41, 42]. Initial studies evaluating the antiviral activity of BCV have demonstrated an increased *in vitro* potency relative to CDV against a wide range of double-stranded DNA viruses including cytomegaloviruses (~400× higher efficacy) and adenoviruses (~65× higher efficacy) presumably due to the higher intracellular concentrations of the active drug CDV [43]. Subsequent preclinical and early clinical studies indicated that the lipid conjugation of CDV also results (i) in improved oral bioavailability of the drug and (ii) in a favorable

Table 11.2 HAdV antiviral agents in clinical development.

Product	Sponsor	Phase	Indication	Delivery	Status
CMX001 (Brincidofovir)	Chimerix	Phase 2/3 (NCT03749317[a], NCT02087306)	Adenovirus infections in pediatric HSCT recipients	Oral delivery (tablet and pediatric liquid suspension)	Terminated due to low enrollment rate
SyB V-1901 (Brincidofovir)	SymBio	Phase 2a (NCT04706923)	Adult and pediatric subjects with adenovirus viremia	Intravenous infusion	Not yet recruiting
OKG-0301 (Ranpirnase/ Onconase)	Okogen	Phase 2 (NCT03856645)	Acute adenoviral conjunctivitis	Ophthalmic drops	Recruiting
IVIEW-1201 (Povidone iodine 1%)	IVIEW Therapeutics	Phase 2 (NCT03749317)	Acute adenoviral conjunctivitis	Ophthalmic sustained release gel	Recruiting

a) ClinicalTrials.gov identifier.

safety profile with little to no evidence of associated nephrotoxicity [18, 44]. These findings paved the way for BCV to move into full clinical development. Initially, *Chimerix Inc.* (Durham, North Carolina, USA) sought to develop BCV as an oral therapeutic for the prophylaxis of CMV infections in immunocompromised HSCT recipients and for the potential treatment of smallpox infections in case of a bioterrorism attack. However, development in the CMV indication was discontinued based on negative results of the phase 3 SUPPRESS trial [45].

Clinical development for HAdV was started after a series of case studies reported favorable clinical outcomes following BCV administration in immunocompromised patients with disseminated HAdV infection [43, 46, 47]. Several clinical trials investigating the efficacy of BCV in patients with HAdV were initiated by *Chimerix Inc.*, including a phase 3 open-label study in HSCT (AdVise Trial; NCT02087306). Although rapid declines in HAdV-DNA levels were seen in the majority of treated subjects, no meaningful difference in overall survival was observed between BCV-treated patients and historic controls [48]. One reason for this could have been that ultimately the identification of a valid comparator cohort for use as historic controls had not been possible [48]. Based on this outcome and due to the observation that diarrhea was the main dose-limiting adverse effect associated with prolonged BCV administration, a phase 2 study was initiated to assess safety, overall tolerability, and antiviral activity of a "short-course" BCV therapy for the treatment of HAdV infections in pediatric HSCT recipients (AdAPT Trial, NCT03339401). Since previous studies have shown that clearance of HAdV from the blood of patients has a positive impact on overall survival after HSCT, the primary endpoint chosen for this study was a comparison of the viral burden (average HAdV DNA levels) in blood over 16 weeks in subjects treated with short-course oral BCV versus those who receive local SOC. However, in the second half of 2019, all active trials evaluating the use of BCV in humans were halted due to low patient

recruitment (Table 11.2). At the same time, *Chimerix Inc.* announced the out-licensing of BCV to *SymBio Pharmaceuticals Limited* for all human indications excluding smallpox (http://www.chimerix.com). Given this, the future of BCV as novel HAdV therapy is currently unclear though a new phase 2a dose ranging study study in adult and pediatric subjects with HAdV viremia was recently innitiated by SymBio (NCT04706923; Table 11.2). [18, 24, 27].

11.3 Ocular Adenovirus Infections

11.3.1 Incidence, Transmission, and Clinical Manifestation

Acute infectious conjunctivitis is an extremely common condition that, in principle, can be caused by either bacterial or viral pathogens. However, it is estimated that more than 50% of all infectious conjunctivitis cases have a viral etiology and that up to 90% of these cases are caused by HAdV, making this virus the single-most common cause of ocular infections worldwide. Accordingly, HAdV presents a serious public health risk with around 25 million HAdV-associated conjunctivitis cases per year worldwide including ~6 million cases in the United States [8, 49, 50]. Virus outbreaks occur globally on a regular basis but are more frequently seen in densely populated countries or regions like Southeast Asia. Accordingly, Japan alone has an incidence of more than one million cases of ocular HAdV infections per year [51, 52].

Ocular HAdV infections affect all ages and socioeconomic classes with no specific gender affinity [53]. Virus transmission occurs primarily through respiratory droplets or direct contact with ocular secretions, e.g. via finger-to-eye infection. Importantly, patients start to shed infectious virus several days prior to the onset of symptoms and remain infectious during the entire course of the acute phase of the disease (~two to three weeks) thus giving the virus time to spread among people in close proximity, e.g. family members, coworkers, etc. Due to the highly contagious nature of HAdV in connection with its extreme extracellular stability on common fomites like towels, doorknobs, soap, eyeglasses, etc., epidemic HAdV outbreaks are frequently seen in closed settings with high population densities such as schools, day care institutions, hospitals, and the military and—unfortunately—are also spread in ophthalmologic units via patients, healthcare workers, or contaminated medical instruments [9].

Manifestations of ocular HAdV infections can be categorized into three major clinical syndromes: follicular conjunctivitis (FC), pharyngeal conjunctival fever (PCF), and EKC. The first two conditions (FC and PCF) are relatively mild, self-limiting infections of the conjunctiva that last for 3–7 days and importantly do not result in long-term sequelae. FC symptoms include bulbar conjunctival injection, chemosis, and eyelid edema. PCF appears similar, though in addition to the ocular manifestations, PCF is associated with cold-like symptoms including fever, sore throat, and rhinitis. The most common HAdV serotypes causing FC and PCF are members of the subgroups B (e.g. B3, B7, B11, B14) and E (e.g. E4) (Table 11.1) [3, 8, 54].

EKC, however, is a more frequent and far more serious condition that, as indicated by its name, involves both the conjunctiva and the cornea. EKC is caused largely by a subset

of subgroup D viruses namely D8, 37, 53, 54, 56, and 64 (previously described as HAdV-D19a) (Table 11.1) [3, 53]. A correlation between receptor usage and cell tropism of EKC-causing HAdVs has been suggested, given that almost all EKC-causing HAdVs are capable of using sialic acid glycans on the surface of corneal epithelial cells for primary attachment, preceding (co-)receptor-mediated entry into the cornea [9, 10, 55]. EKC typically starts unilaterally, but due to the contagious nature of the virus, both of the patient's eyes usually become involved in the course of the disease. Clinical signs of EKC appear after an incubation period of 5–12 days and patients present with red eye, ocular irritation, foreign body sensation, photophobia, watery discharge, FC, edema, and pain. Corneal involvement, which is a hallmark of EKC, typically starts three to four days after the onset of symptoms with diffuse epithelial keratitis accompanied by preauricular lymphadenopathy followed by the formation of subepithelial corneal infiltrates (SEIs) or "nummuli" [3, 8, 53]. SEIs are punctate multifocal areas of epithelial opacity that form in the corneal stroma due to the infiltration of various immune cells. This leukocyte infiltration is a response to cytokines secreted from productively HAdV-infected keratocytes located in the corneal stroma [8–10, 55]. Approximately 30–50% of patients with EKC will develop stromal infiltrates that cause photophobia and disturbances in vision. Although the acute phase of EKC is self-limiting and usually lasts up to three weeks, corneal opacities due to SEIs may persist for months to years after the initial infection has resolved. Moreover, a chronic subepithelial infiltration of the corneal stroma by leucocytes can damage the superficial stroma of the eye and may lead to corneal scarring causing permanent vision loss and photophobia. EKC may be further complicated by persistent dry eye syndrome requiring long-term treatment [8, 9, 54, 56].

11.3.2 Current Therapy Strategies and Antiviral Agents

Although EKC (without knowing the underlying pathogen) was initially clinically described in Austria more than 100 years ago, to date there is still no specific approved antiviral therapy to alleviate the clinical symptoms, shorten the course of infection, prevent the formation of corneal opacities, or to block virus spread [8, 57]. Given this unmet medical need, several drugs were evaluated in the past for their potential clinical efficacy as topical treatments for EKC, albeit with moderate success.

11.3.2.1 Corticosteroids

The first example is topical corticosteroids that were prescribed frequently during the acute phase of the infection to reduce inflammation-related discomfort and pain and to prevent the formation of SEIs. Although steroid treatment can provide transient symptomatic relief, its use remains controversial since steroid treatment (i) could enhance HAdV replication, (ii) prolongs HAdV shedding and disease, and (iii) may cause a rebound increase in SEIs upon cessation of drug [8, 53, 58]. These findings, in addition to the known steroid-associated side effects (e.g. glaucoma, cataract formation) restrict the use of steroid treatment to complicated cases with severe keratitis, photophobia, and SEIs, significantly impairing visual acuity. Nonsteroidal anti-inflammatory agents do not increase HAdV replication but have been shown to be ineffective against SEIs [54].

11.3.2.2 Povidone-iodine and SHP640

Concerns about prolonged viral shedding upon steroid usage in EKC patients lead to the idea of combining a potent topical corticosteroid with a suitable antiviral agent in order to treat both the inflammatory and the infectious components of EKC [59]. SHP649 (FST-100, TAK-640; *Shire, Foresight Biotherpeutics, Takeda*) is a combination of 0.1% dexamethasone with 0.6% povidone-iodine (PVP-I; Figure 11.1c). PVP-I is a powerful disinfectant routinely used in ocular and general surgery. It is highly effective against viruses, bacteria, fungi, and other parasites, immune to the development of bacterial/viral resistance and does not cause irritation on skin [8, 60]. Several concentrations of PVP-I were tested as monotherapy for potential clinical use in EKC, albeit with inconclusive outcomes [60, 61]. A combination of PVP-I and dexamethasone, however, should have the potential to treat the viral component of EKC as well as immune-related sequelae such as SEIs. Moreover, since PVP-I basically kills bacteria and viruses, this medication could potentially be used to treat patients suffering from any kind of infectious conjunctivitis without the need for identification of the underlying pathogen [59]. However, concerns regarding the potential efficacy of PVP-I in EKC have also been raised given that PVP-I (i) does not accumulate in ocular tissue and thus has a very short residence time on the ocular surface, (ii) is not active against intracellular adenoviral particles, and (iii) has only a reduced virucidal activity against EKC-causing HAdVs of subgroup D [60–63]. Following the acquisition of *Foresight Biotherapeutics*, *Shire* (a wholly owned subsidiary of *Takeda*) was developing SHP640 for the potential treatment of adenoviral and bacterial conjunctivitis. Results of a phase 2 proof-of-concept trial were recently published comparing SHP640 against PVP-I and vehicle in patients with HAdV conjunctivitis [64]. Patients treated with SHP640 showed significantly better outcomes in terms of clinical resolution and virus eradication than vehicle, but only a trend toward clinical significance for SHP640 versus PVP-I alone. Moreover, SHP640 treatment had no effect on SEI formation or on virus spread from the primary infected eye to the fellow eye [64]. Based on these initial efficacy data, SHP640 advanced to phase 3 clinical trials. However, in May 2019, two ongoing phase 3 studies in adenoviral conjunctivitis (NCT02998541, NCT02998554) were terminated by the sponsor, suggesting that the program has been discontinued by *Takeda*.

11.3.2.3 Nucleoside Analogues

Additional treatment avenues have been pursued beyond these, but none have led to approval of a drug for ocular HAdV infections [8, 53, 60]. Among others, these approaches included ophthalmologic formulations of the classic antiviral nucleoside analogues GCV (Zirgan, *Bausch & Lomb*) and CDV (*Gilead*, *Bausch & Lomb*) (for details about the drugs, see Section 11.2.2; Figure 11.1a). GCV demonstrated only limited efficacy against ocular HAdV serotypes *in vitro* (EC50 values in the upper two digit μM range) and produced variable results when tested as an ophthalmic formulation in ocular adenoviral animal models or in small clinical trials [8, 60, 65]. Data from large-scale controlled clinical trials are not available but the controversial efficacy results of GCV for treating HAdV conjunctivitis might be explained by insufficient GCV activation in certain cell types due to a lack of a viral kinase in HAdV (see Section 11.2.2.2). In contrast, topical CDV has demonstrated significant antiviral activity both *in vitro* and in animal models and has

successfully completed a small randomized controlled phase 3 trial [66]. In this trial, the administration of 1% CDV eye drops significantly reduced the frequency of severe corneal opacities. Unfortunately, administration of 1% CDV was associated with substantial local toxicities and a lower, less toxic dose (0.2%) was clinically ineffective [66, 67]. Consequently, the clinical development of topical CDV for ocular HAdV infections was discontinued [53, 65].

11.3.2.4 Palliative Treatment

Since there is little evidence for a beneficial effect on ocular HAdV infections from any of the agents discussed above (see Sections 11.3.2.1–11.3.2.3), current treatment strategies still rely entirely upon supportive therapy directed toward limiting the severity of symptoms such as cold compresses and artificial tears. However, palliative approaches do not (i) prevent the spread of disease, (ii) interfere with virus replication/shedding, or (iii) reduce the risk of long-term sequelae or vision loss, nor the spread in the community. Accordingly, there remains an unmet need for a specific, safe, and effective therapy or prophylaxis that is well tolerated as an antiviral for the treatment of EKC.

11.3.3 Novel Antiviral Approaches in Clinical Development

In spite of the increasing need for a novel HAdV antiviral, only two agents are currently in active clinical development for ocular HAdV infections. Interestingly, neither agent is a classical direct acting antiviral (DAA); one drug is an ophthalmic formulation of a repurposed biological anticancer compound, and the other agent is a novel formulation of the broad range antiseptic PVP-I (see Section 11.3.2.2).

11.3.3.1 OKG-0301

OKG-0301 (Ranpirnase, Onconase; *Okogen* under the license of *Tamir Biotechnology,* Table 11.2) is an ophthalmic formulation of the amphibian endoribonuclease ranpirnase that previously advanced under the name Onconase as an intravenous formulation to phase 3 clinical trials in oncology [68]. *Okogen* licensed ranpirnase for ocular use from *Tamir Biotechnology* after preclinical studies found that ranpirnase demonstrates a broad-spectrum antiviral activity in cell culture, including HAdV-infected cells. OKG-0301 is thought to preferentially enter virally infected cells and to inhibit viral replication via degradation of tRNAs resulting in the inhibition of protein synthesis [69]. Further studies showed that OKG-0301 significantly reduced viral titers in adenovirus infected eyes in addition to shortening the duration of viral shedding in a clinically predictive rabbit ocular model [70]. In January 2019, a multisite, randomized, placebo-controlled, double-blinded phase 2 proof-of-concept trial (RUBY) was initiated in patients with acute adenoviral conjunctivitis in Australia. The goal of the study is to evaluate the safety and efficacy of two different doses of OKG-0301 versus placebo (NCT03856645). By February 2021 the study is still ongoing and recruiting patients.

11.3.3.2 iVIEW-1201

The second agent currently in clinical development for the potential treatment of adenoviral conjunctivitis is iVIEW-1201 (*iVIEW Therapeutics*), a long-acting, extended-release, gel

formulation of PVP-I that retains the broad *in vitro* antimicrobial and antiviral properties of "classical" PVP-I preparations (http://www.iviewtherapeutics.com). Given its long-acting sustained-release formulation, iVIEW-1201 addresses one major difficulty in topical treatment of ocular diseases namely, provision and maintenance of an optimal ocular drug concentration over an extended period of time. By prolonging the corneal retention time of PVP-I, it is hoped that the ocular bioavailability of iVIEW-1201 is improved leading to an enhanced virucidal activity on the surface of the infected eye [71]. However, despite this apparent improvement, it remains to be seen whether topically administrated iVIEW-1201 will significantly reduce titers of EKC-causing HAdV serotypes and, even more importantly, whether it will limit the development of corneal infiltrates given that the drug does not permeate into infected corneal cells. iVIEW obtained an approval for clinical phase 2 trials in patients with HAdV conjunctivitis in China, India, and the United States (http://www.iviewtherapeutics.com). With a first patient treated December 2019 in India, this trial should address the question of efficacy of iVIEW-1201 in the intended opthalmologic use (NCT03749317). By February 2021 no interim results were reported and the trial continues to recruite patients.

11.4 Drug Targets for Direct Acting Antivirals

As outlined above (Sections 11.2 and 11.3), current treatment options for combating HAdV infections are either ineffective and/or cause severe toxicities. Despite the clear medical need, HAdV drug discovery has received limited attention in recent years and only very few candidate compounds are currently in clinical development for HAdV-associated diseases. To overcome this problem, additional research is needed to discover new antiviral drugs that are potent, safe, and well tolerated and ideally possess a new mode of action. In theory, HAdV is a good target for direct acting antivirals as its DNA genome encodes a series of proteins that (i) are highly conserved among disease-related serotypes, (ii) are dissimilar to- or even lack a human analogue, and most importantly (iii) fulfill essential functions at multiple stages in the virus life cycle [7]. However, in reality, the number of viral proteins that have been exploited as potential anti-HAdV targets in the past is very low. Even so, those proteins already studied extensively as potential anti-HAdV targets cover at least three different steps of the HAdV replication cycle: (i) virus attachment/entry, (ii) viral genome replication, and (iii) particle maturation (Figure 11.2).

11.4.1 Virus Attachment and Entry

Blocking the attachment of viral pathogens to its specific cellular receptors is, in principle, an attractive approach for antiviral drug discovery since potential drugs interfere with the very first step in the viral replication cycle and thus prevent cell penetration and the subsequent expression of potentially toxic viral genes. Several agents inhibiting HAdV attachment and/or entry *in vitro* have been identified ([7], Figure 11.2a) and at least one, APD-209 (*Adenovir Pharma*), has been evaluated as a topical drug in clinical trials for EKC. APD-209 is based on small soluble multivalent sialic acids (SAs), that prevent EKC causing HAdV serotypes from binding to its receptor on ocular cells: SA-containing glycans [10, 72]. Based on favorable preclinical safety and efficacy data, a phase 2 proof-of-concept trial was initiated in EKC

patients (NCT0197743). Unfortunately, ADP-209 failed to demonstrate statistical significance in this study leading to discontinuation of the program. Currently, it is unclear whether the negative outcome of this trial is due to the specific properties of ADP-209 (e.g. insufficient efficacy, strain coverage, drug stability, frequency of dosing, etc.) or whether the overall concept of combating HAdV eye infections with molecules that act solely on the surface of an infected eye is inadequate. Potential limitations of topically applied entry inhibitors are (i) no penetration of the drug into infected cells, (ii) no activity against intracellular virus, (iii) no accumulation in infected tissue, and (iv) rapid clearing from the ocular surface, in particular by lacrimal flow and eye blinking. Since these limitations also apply to topically administered antiseptic antivirals such as iVIEW-1201 (see Section 11.3.3.2), the outcome of the ongoing iVIEW-1201 trial will shed more light on this conceptual question.

11.4.2 Virus Genome Replication

Not least due to the success of the anti-herpesviral drugs acyclovir, ganciclovir, and cidofovir (see Section 11.2.2), targeting the viral DNA-polymerase with e.g. classical nucleoside analogues was a popular antiviral approach to interfere with viral genome replication (Figure 11.2b). HAdVs encode a DNA-polymerase that is structurally divergent from host polymerases but utilizes the same intracellular pool of nucleotide substrates. This is important since in contrast to herpesviruses, HAdVs lack a viral "nucleotide kinase" and thus, the principle of activating nucleoside drugs only in infected cells by phosphorylation cannot be applied to HAdV. Accordingly, the main challenge for the design of novel nucleoside inhibitors is to identify molecules that preferentially inhibit the viral polymerase over the host's polymerases since this is a prerequisite for preventing off-target effects and to ensure that a triphosphate is generated with high efficacy by the cell or by offering a nucleotide. An alternative approach would be the design of drugs addressing unique sites on the HAdV polymerase, distinct from those targeted by the nucleoside analogues. Successful antiviral agents following the latter principle are exemplified by the anti-HIV non-nucleoside reverse transcriptase inhibitors (NNRTIs) [73]. Today, many publications have reported on various molecules targeting the HAdV DNA-polymerase, including nucleoside and nucleotide analogues [35, 65, 74]. However, with the exception of the drugs discussed earlier (GCV, CDV, and BCV), all remain to be evaluated in clinical trials. Interestingly, the majority of these molecules originated from drug repurposing approaches performed by academic research groups and not from industry-driven drug discovery programs specifically designed to identify HAdV-polymerase inhibitors which again demonstrates the recent lack of apparent interest in anti-HAdV drug discovery. Given that a natural consequence of drug repurposing is an intrinsic lack of target specificity, the chance that molecules identified by these approaches will be advanced to clinical development is low.

11.4.3 Particle Maturation

A key enzyme in HAdV maturation and in the generation of infectious virus particles is the virus-encoded cysteine protease AVP (adenain). AVP plays an essential role in several steps of the virus life cycle including uncoating of the incoming virus particle and proteolytic maturation of newly assembled, noninfectious particles giving rise to mature, infectious

progeny virus (Figure 11.2c) [75, 76]. Viral proteases are proven targets of efficacious antiviral therapies, including human immunodeficiency virus (HIV) and hepatitis C virus (HCV) [77]. Accordingly, specific inhibition of the essential adenovirus protease AVP may also represent an effective treatment strategy for HAdV infections [78]. Proteases constitute a large family of enzymes, frequently with binding pockets which are difficult to target, e.g. because of their topology or lipophilic nature. Given that many naturally occurring protease inhibitors are proteins, a recent two-pronged Hit-discovery campaign identified a tetrapeptide nitrile and a pyrimidine nitrile as two independent starting points for structure-guided medical chemistry [79]. A subsequent Hit-to-Lead approach resulted in potent AVP inhibitors with *in vitro* IC_{50} values in the picomolar range. Unfortunately, despite high solubility and good cell permeability, the exceptional anti-AVP potency of these inhibitors could not be translated into adequate antiviral activity in cell culture infection experiments and the program was discontinued [80]. In conclusion, despite the identification of highly potent and specific inhibitors of AVP, the final validation of AVP as a valid anti-HAdV drug target thus remains elusive.

The approaches discussed in this review describe the key antiviral strategies currently being pursued. However, there are a multitude of additional highly specific viral processes presenting opportunities for the development of novel antiviral targets and strategies (extensively reviewed in [7]), but these will require more intensive and concerted effort if they are to yield effective novel treatments. Ideally, a multipronged approach addressing several targets should be pursued to allow suppression of the virus at different steps in the replication cycle. This would offer the added benefit of counteracting the potential emergence of drug-resistant virus strains, an issue of particular importance given the lack of alternative treatment options.

11.5 Conclusion

HAdV infections remain a significant cause of transplant-related morbidity and mortality in the context of pediatric HSCT and ocular HAdV infections represent a serious public health burden due to their rapid spread and the potential severity of ocular symptoms. To date, there is no systemic or topical treatment approved in the United States or the EU. Current antiviral therapies of HAdV infections are inadequate and rely solely on suboptimal "off-label" therapeutic options or palliative care. Despite this high unmet medical need, only very few candidate compounds are currently in clinical development for HAdV-associated diseases. Accordingly, additional research is urgently needed to find specific, safe, and effective antiviral agents for systemic use in immunocompromised individuals and/or for the topical or systemic treatment of adenovirus ocular infections.

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12

ssDNA-Viruses: Human Parvovirus Infection

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12.1 Introduction

Single-stranded DNA-viruses are classified into the families of *Parvoviridae*, *Circoviridae*, and *Anelloviridae*. All these viruses are characterized as small, non-enveloped particles with genomes of 1.7–2.1 kilo bases (1000 bases) (kb) (*Circoviridae*), 2.0–3.9 kb (*Anelloviridae*), and 4.0–6.0 kb (*Parvoviridae*) [1–3]. With respect to anelloviruses, various species of Torque-teno-, Torque-teno-midi- and Torque-teno-mini-viruses (TTV, TTMDV, TTMV) have been identified to persist in vertebrates including humans, but diseases have been associated neither with acute infection nor with persistence [4–6]. Circoviruses are well-known pathogens of livestock and animals: human-associated circoviral DNA-sequences could be amplified from various samples and excretions from patients as well as from healthy humans [7]. With respect to parvoviruses, several species are well-known risk factors for fetal health both in livestock and pets, e.g. porcine parvovirus, canine minute virus, and feline panleukopenia virus. Two parvoviral species, human parvovirus B19 (B19V) and human bocavirus (HBoV), are recognized as human pathogens and will be discussed in this chapter.

12.2 Classification

The family of *Parvoviridae* comprises viruses characterized by small (lat. *parvus* = small), non-enveloped particles with a diameter of 20–28 nm containing a linear single-stranded DNA (ssDNA) molecule of about 5000–6000 nucleotides. Parvovirus B19 (primate erythroparvovirus 1, B19V) and human bocavirus (primate bocaparvovirus HBoV 1, 2) occur within the subfamily *Parvovirinae,* genera *Erythroparvo-* and *Bocaparvovirus,* respectively. During the past years, several other parvoviruses have been isolated from humans, including parvovirus 4 (PARV4), human bufavirus (BuV), cutavirus (CutaV), and tusavirus

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(TusaV) [8, 9]. Until now, their clinical significance and association with human diseases remain unclear. Furthermore, most humans are infected with adeno-associated virus (AAV), members of the genus Dependoparvovirus, without developing symptoms. Whereas AAV-replication is dependent on concurrent infection of the cells by adeno- or herpesviruses, all other human parvoviruses are not dependent on helper-viruses and replicate autonomously. Parvovirus B19 displays a preference to infect erythroid precursor cells; the tropism of human bocavirus is targeted to cells of the respiratory and/or gastrointestinal tract [10–12].

12.3 Molecular Biology

Parvoviral particles (Figure 12.1) are very stable. The non-enveloped icosahedral capsids consist of 60 capsomers formed by two (B19V: VP1, VP2) or three [HBoV: viral protein 1, viral protein 2, viral protein 3 (VP1, VP2, VP3)] structural proteins [8]. Approximately 90–95% of the particles consist of the major capsid protein VP2 (B19V: 58 kDa, HBoV: 60 kDa) [13]. Minor capsid proteins VP1 and VP3 are incorporated into the structure in non-stochiometrical relations [14, 15]. VP2s are identical to the carboxyterminal regions of VP1. Aminoterminal extensions of 227 and 129 amino acids represent the so-called "VP1-unique region" of VP1-proteins of B19V (83 kDa) and HBoV (74 kDa), respectively. VP3 (64 kDa) is present as a third component in HBoV-capsids displaying an aminoterminal extension of 39 residues as compared to VP2. Phospholipase A2-like activities are associated with the VP1-unique region [16, 17]. This enzyme activity is necessary for parvoviral infectivity and responsible for particle release from endosomes following receptor-mediated uptake. It may

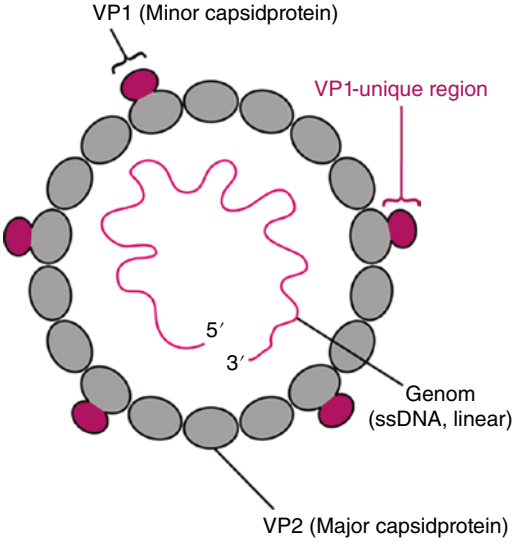

Figure 12.1 Schematic structure and composition of a parvovirus particle. *Source*: Modrow, Falke, Truyen, Schatzl: Molekulare Virologie, 3.Aufl. © 2010, Springer Nature.

further contribute to inflammatory processes mediated by the production of leukotrienes and prostaglandins, the natural products of phospholipase A2 (PLA2)-enzymatic activity.

B19V- and HBoV-genomes span over 5596 and 5543 nucleotides of single-stranded DNA, respectively. Sequencing of several isolates of both parvoviral species led to the identification of distinct genotypes with sequence divergence of approximately 11–14% [18–20]. The internal coding sequences of about 4800 (B19V) and 5200 (HBoV) bases are flanked by terminal repeats that are necessary for genome replication [8, 10]. The coding regions contain two large open reading frames (ORFs), whose expression is controlled by only one promoter element at the 3′end of the genome. Viral transcripts are processed by alternative splicing to mRNAs and used for the synthesis of the respective viral proteins [21, 22]. The ORF adjacent to the promoter encodes the nonstructural proteins while capsid proteins are encoded by the ORF localized further downstream (Figure 12.2). Nonstructural proteins 1 (NS1, B19V: 77 kDa; HBoV: 100 kDa) are multifunctional and possess site-specific DNA-binding, endonuclease, and helicase/ATPase activities, which might be used for the development of specific antiviral drugs. The NS1 of B19V has been shown to transactivate the viral and various cellular promoters [23–26]. Its cytotoxicity is related to cell cycle arrest and apoptosis involving the caspase 3-pathway [27–30]. As similar NS1-activities are known

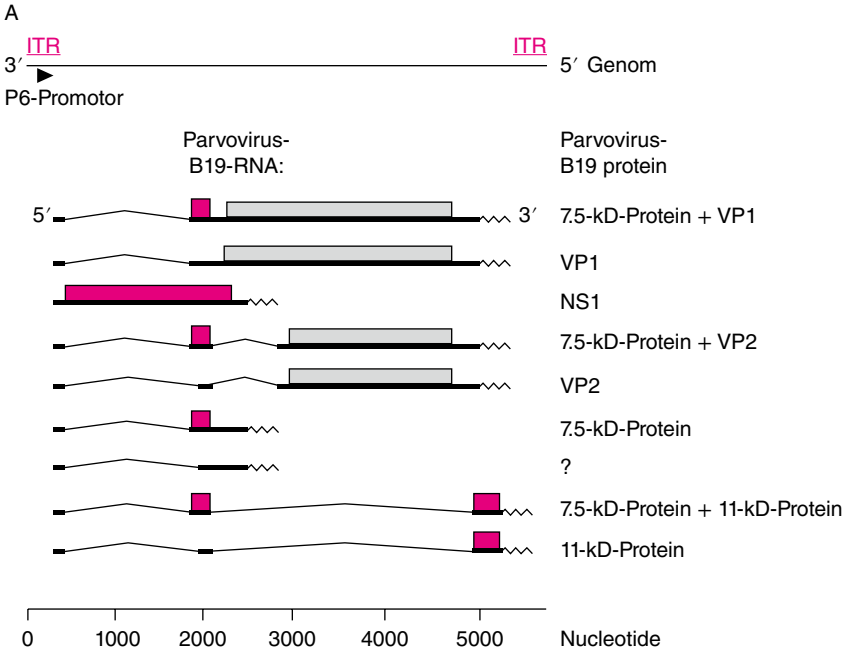

Figure 12.2 Genome, transcription and translation map of parvovirus B19. The line at the top of the figure represents the parvovirus B19 genome with its ITR-elements at the ends and the location of the p6-promoter. The lines underneath represent the various transcripts which are used for the translation of the respective viral proteins indicated by bars. Exon sequences are indicated by thick lines, the introns which are removed by RNA-splicing by thin lines. Polyadenylation sites are represented by the jagged symbols. *Source*: Modrow, Falke, Truyen, Schatzl: Molekulare Virologie, 3.Aufl. © 2010, Springer Nature.

for other parvoviruses, analogous functions are assumedly to be associated with NS1 of HBoV. The B19V-genome encodes at least two additional small nonstructural polypeptides of 11 and 7.5 kDa. During HBoV-infection, some smaller NS1-variants (NS2, NS3, and NS4) are produced by alternative RNA-splicing sharing the carboxyterminal domains of NS1. The function of these small nonstructural proteins is poorly understood. HBoV encodes a further nonstructural protein NP1 (25 kDa) with functions in genome replication and RNA-processing [31, 32].

12.4 Parvovirus Replication

A common feature of all parvoviruses is the dependence for infection and replication on diving cells. In contrast to other DNA-viruses, polyoma-, papilloma-, adeno-, and herpesviruses parvoviruses do not encode factors that induce the entry of the host cells into the S-phase. At least in part, this feature may explain the tropism of parvovirus to infect special cell types with distinct states of cellular differentiation. However, the analysis of the replication cycle of both B19V and HBoV is hampered by the lack of cell culture systems that allow efficient and reproducible virus propagation in vitro.

Following airborne transmission via droplets, first cycles of both B19V- and HBoV-replication are presumed to occur in the nasopharyngeal lymphoid or epithelial tissues resulting in viremia. B19V subsequently infects erythroid precursors [burst-forming units – erythroid (BFU-E), colony forming units – erythroid (CFU-E), and erythroblasts] in the bone marrow by first binding to blood group antigen P (globoside, Gb4) as cellular receptor [33–35]. Receptor-mediated endocytosis is mediated by binding to a co-receptor, $\alpha 5\beta 1$ integrin, in high affinity conformation [36] and/or to the autoantigen Ku80 [37].

The cellular receptor used for HBoV entry has not yet been identified. From the nasopharyngeal tissue, HBoV is transferred to the tracheobronchial (HBoV1, 3) or intestinal epithelial cells (HBoV2, 4) that become infected and support efficient replication [8]. These first events determine the different cell and organ tropism of B19V and HBoV. During the next steps, PLA2-like activity as part of the VP1-unique region catalyzes the release of the viral capsid from the endosome, followed by the transport into the nucleus, where the ssDNA genome is assumed to be completed to a dsDNA molecule by cellular DNA-polymerases using the 3′end of the genome as primer. During the following steps, the transcription of the viral genome is catalyzed by cellular RNA-polymerase II. The primary transcripts are spliced, thereby generating mRNA-species that are transported into the cytoplasma and translated by ribosomes. The respective viral nonstructural and structural proteins are retransported into the nucleus. NS1 is active as transcriptional activator of the viral promoter. The endonuclease and helicase activities of NS1 are involved in the replication of the genome. Viral DNA-synthesis is performed and catalyzed by cellular DNA-polymerases δ and α [38]. In the nucleus, newly generated ssDNA-genomes assemble with VP1, VP2, and—in case of HBoV—VP3 into viral particles that are released by NS1-mediated apoptosis.

12.5 Diseases Associated with Parvovirus Infection

12.5.1 Parvovirus B19

The virus was first discovered in 1975 as a cause of systemic infections of adults who either were asymptomatic or had mild nonspecific symptoms such as headache, pyrexia, malaise, fatigue, and myalgia [39–41]. In 1983, B19V was identified as the etiological agent of erythema infectiosum (fifth disease) [42, 43]. B19V-infection is ubiquitous and frequent in both children and adults. In Europe, about 70% of adults (age 25–40 years) and more than 80% of the elderly (age > 80 years) are seropositive, although these values may vary slightly by country [44–48]. In general, B19V is transmitted by respiratory aerosol spread or by hand-to-mouth contact from individuals with acute infection [49]. In immunocompetent individuals, acute B19V-infection is asymptomatic or may induce a flu-like disease. Viremia is extremely high before the onset of detectable immune responses and up to 10^{13} particles and/or virus genomes may be present per milliliter blood [50]. Similar amounts may be detected in respiratory secretions [51] and other body fluids (S. Modrow, personal communication). Due to its tropism for erythroid precursor cells, B19V infects and destroys erythroid precursors (BFU-E, CFU-E, and erythroblasts) in the bone marrow, thereby causing transient anemia. Initiating immune responses and B19V-specific antibodies are thought to be responsible for development of rash and arthritis. Whereas arthritis is generally transient in children, symptoms may last for several weeks and months in adults. Neutralizing antibodies directed against epitopes present in the VP1-unique region and against particulate structures of VP2 persist life-long [8, 52, 53].

Besides generally mild leucocytopenia or thrombocytopenia, severe hematologic sequelae presenting as pure red cell aplasia or pancytopenia, hepatitis, myocarditis, myositis, acute lung injury, meningoencephalitis, and neurological disease may occur in rare cases [54–59]. In some patients, acute B19V-infection may induce or trigger autoimmune disorders ranging from mild arthralgias, Hashimoto thyroiditis, to severe necrotizing vasculitis [60, 61]. Furthermore, B19V-infection may be associated with a wide spectrum of additional diseases:

After acute infection and elimination of B19V from peripheral blood, low amounts of viral DNA (10^2–10^3 geq/10^6 cells) can be detected in various tissues of healthy adults, e.g. myocardium, skin, bone marrow, tonsils, and synovia, probably life-long [62–68]. Since similar concentrations of viral DNA are present in myocardial tissues from healthy individuals and patients suffering from dilated cardiomyopathy, the causal association between B19V-DNA detection and heart disease has been discussed, but appears rather questionable [69]. Whether this B19V-DNA latency in tissue may be a source for virus reactivation in immunocompetent individuals is currently unknown.

Severe disease manifestations, however, are associated with special situations or immunological and genetic features of individual patients. In pregnancy, B19V may be transmitted to the unborn child. Depending on the stage of gestation and fetal development, B19V-infection may cause spontaneous abortion, miscarriage, nonimmune hydrops fetalis, and fetal death. Due to the lack of erythroid precursor cells that are only present in fetal blood after weeks 10–12 of gestation, B19V-infection in very early pregnancy is not

associated with fetal anemia. However, the risk of B19V-associated fetal anemia resulting in nonimmune hydrops fetalis is particularly high during the second trimester, between weeks of gestation 11–23 when the fetus is growing rapidly and the fetal red cell mass increases 30-fold [70–73]. Fetal death has been largely confined to maternal B19V-infection within the first 20 weeks of gestation [74–79]. Another, but rare manifestation is fetal myocarditis. After birth, some of these children need heart transplantation [80, 81]. Epidemiological data in developed countries indicate that 200–400 of 10 000 pregnant women may suffer from acute B19V-infection, potentially endangering fetal life and causing 2–3 cases of fetal death per 10 000 live births [82–85].

Besides pregnant women, patients with shortened half-live of red blood cells are at high risk to establish a transient aplastic crisis (TAC). The development of this severe and life-threatening disease may occur in acutely B19V-infected patients with underlying hereditary or acquired hematologic disorders. This problem is a well-known complication in patients with sickle-cell anemia. Due to chronic hematolytic anemia, these patients display increased red blood cell destruction, which is considerably worsened by the viral infection. Although less frequently, similar complications are observed in B19V-infected individuals with hereditary somatocytosis, Fanconi-anemia, thalassemia, hemoglobin C disease, red cell enzyme deficiencies, iron deficiency anemia, immune hemolytic anemia, and in patients suffering from high blood loss due to trauma or surgery [8, 86–93].

In immunocompromised B19V-infected patients, viremia due to ongoing viral replication may persist over long time periods and cause continuous or recurrent symptoms [91–94]. In the absence of antiviral immune response, symptoms induced by immunocomplex formation (rash, arthritis) do not occur. Due to their inability to produce neutralizing antibodies, immunocompromised individuals are particularly at risk to establish persistent B19V-infection, resulting in pure red-cell aplasia and severe chronic anemia [59, 95–97]. In transplant recipients, the virus may be either transmitted by contact to acutely infected individuals or via the donor organ from seropositive individuals. Whereas donor-transmitted B19V-infection is associated with transient, low-level viremia in seropositive transplant recipients, persistent high-level viremia may be observed when initially seronegative recipients, e.g. children, are infected during the phase of intensive immune suppression [98, 99].

12.5.2 HBoV

HBoV1-infection is ubiquitous and widespread, especially in children. At an age of six years, more than 95% of children are seropositive. HBoV1 has been detected in nasopharyngeal samples of patients with upper and lower respiratory tract disease, frequently in combination with other viral or bacterial pathogens [8, 100, 101]. As HBoV-DNA could also be amplified from healthy individuals, asymptomatic infection seems to be common, but some severe courses of severe airway constriction, e.g. pneumothorax and pneumomediastinum, have been reported, mostly in immunosuppressed patients [102, 103]. Following HBoV1-infection, viral genomes are detected in nasopharyngeal tissue, possibly life-long [104]. Genomes of HBoV2 have been detected preferentially in stool of patients with diarrhea, frequently combined with noro- or rotavirus [105]. As HBoV2 can also be found in stool of asymptomatic children, the causal association with disease is unclear. HBoV3 and HBoV4 infections have been diagnosed rarely, therefore the association with distinct

symptoms remains unclear [105, 106]. In general, the infection is mild and self-limited, so there is no antiviral therapy available. In case of severe HBoV-infection in immunocompromised patients, immunoglobulin therapy might be considered,

12.6 Antiviral Chemotherapy of Parvovirus B19-infection

In vitro, some inhibitory effects on B19V-replication have been shown by hydroxyurea, cidofovir, brincidofovir, and coumarin derivatives, but the results were not convincing [107–111]. Since parvoviruses do not encode respective DNA-polymerases, the antiviral effects of these compounds might be associated with inhibitory effects on cellular processes and impairment of cell growth and viability. Similarly, unspecific inhibitory effects have to be discussed for telbivudine. Telbivudine is known as nucleotide inhibitor of the hepatitis B virus reverse transcriptase, an enzyme not encoded by parvoviruses. In vitro, telbivudine did not display direct effects on B19V-replication or gene expression, but may inhibit virus-induced apoptosis [112, 113]. Another approach intended to address the NS1-endonuclease activity by various flavonoid molecules similarly showed only limited effects [114].

In general, acute B19V-infection is mild and self-limited. In these uncomplicated cases, antiviral therapy is not necessary. The situation is different in immunosuppressed patients and in individuals with underlying disorders in red blood cell differentiation, which develop severe aplastic anemia. Similarly, in acutely infected pregnant women, measures to control virus propagation are not available. Respective antiparvoviral chemotherapy would be helpful to avoid transuterine B19V-transmission and infection of the fetus. The development of antiparvoviral chemotherapy would also be desirable for treatment of fetal anemia and hydrops fetalis. At present, only supportive, symptomatic, or unspecific treatment is recommended, such as erythrocyte donation and immunoglobulin therapy. The development of antiviral chemotherapy is hampered by the fact that good viral targets are scarce. Due to the restricted information encoded by parvoviral ssDNA-genomes, almost all steps of viral gene expression and genome replication are dependent on cellular enzymes. The only targets may represent the viral phospholipase A2-like activity of the VP1-unique regions and endonuclease and helicase associated with the NS1-protein. Since these viral enzymes display catalytic activities comparable to cellular functions, severe side effects have to be expected by antivirals addressing VP1- and NS1-proteins and good counterscreens with the cellular enzymes will be necessary to achieve a sufficient therapeutic window. Furthermore, testing of respective compounds is impeded as neither animal models or reliable cell culture systems are available for B19V-infection.

12.7 Therapeutic Options and Recommendations

12.7.1 Acute B19V-infection Associated with Transient Aplastic Crisis (TAC)

TAC in patients with underlying hereditary or acquired hematologic is a severe and potentially life-threatening disease that has to be treated by erythrocyte transfusion.

12.7.2 Prolonged B19V-symptoms in Immunocompetents

In patients with prolonged medium-/high-level viremia, the donation of immunoglobulins may be considered. Some benefit has been shown in patients with persistent arthritis and arthropathy [115–118].

12.7.3 Fetal Disease

Intrauterine transfusion of erythrocytes is the treatment of choice in fetal anemia usually diagnosed by peak systolic velocity (PSV)-middle cerebral arterial (MCA) (PSV-MCA). The actual hemoglobin level, reticulo- and thrombocyte counts can only be measured invasively by puncture of the umbilical vein, i.e. when it is punctured for transfusion. A recent report on the management of B19V-infection in pregnancy describes pretransfusion hemoglobin concentrations ranging between 1.6 and 7.4 g/dl [119]. It may be concluded that hemoglobin values below 8 g/dl require intrauterine erythrocyte transfusion [120]. In most cases, a single erythrocyte transfusion of some milliliters is sufficient to resolve anemia and hydrops. The proportion of fetuses with severe hydrops that survive after intrauterine transfusion is 83–85% [118, 119, 121]. The outcome of the treated fetuses/children is uneventful, especially regarding neurological development [122, 123]. The intravenous administration of immunoglobulin is not recommended for treatment of acute B19V-infection in pregnancy. There is only one report which describes resolution of fetal symptoms after immunoglobulin administered to the pregnant woman [124].

12.7.4 Immunocompromised Patients

High-dose intravenous immunoglobulin therapy is recommended in immunocompromised patients and transplant recipients with chronic anemia and pure red cell aplasia. Actually, 0.4 g of immunoglobulins are used per kilogram body weight and day over a period of five days [94, 97, 125–128]. Following treatment, symptoms may recur at intervals and repeated application of immunoglobulin therapy may be needed.

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13

Antiviral Targets and Strategies to Treat and Prevent Human Norovirus Infections

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13.1 Introduction

13.1.1 Disease Burden and Pathogenesis

Human norovirus (HuNoV), previously known as Norwalk virus, was first discovered after an outbreak of gastroenteritis in Norwalk, Ohio [1], and was the first viral agent shown to cause gastroenteritis. HuNoV is the most common cause of viral gastroenteritis, causing annually ~700 million infections and resulting in ~200 000 deaths worldwide [2]. Norovirus gastroenteritis affects all age groups, with symptomatic infections being characterized by an acute onset of nausea, abdominal pain, vomiting, and non-bloody diarrhea. The incubation period is 24–48 hours, with the symptoms lasting 2–3 days in healthy adults. A norovirus infection can be particularly severe for young children, the elderly, and immunocompromised, lasting weeks to months and with higher risk of hospitalization than for the general population. Diarrhea caused by various agents is still one of the most important causes of childhood mortality in low- and middle-income countries; one million young children die annually due to diarrhea before they reach the age of 5 [3–5]. Around 70% of these cases have a viral etiology [6]. Besides HuNoV, other human viruses cause diarrhea, mostly in young children. These include human rotaviruses, human enteric adenoviruses (*Adenoviridae*, dsDNA), human sapovirus (HuSaV, also belonging to the *Caliciviridae*, with (+)ssRNA) and human astroviruses (HAstVs, *Astroviridae*, (+)ssRNA). In young children, rota- and norovirus are the two most important gastro enteric viruses. Noroviruses cause an estimate of 200 000 deaths yearly and are currently the most important pathogen causing severe childhood diarrhea in countries where vaccination against rotavirus is routinely implemented [7, 8]. Among the elderly, norovirus accounts for the majority of gastroenteritis-associated hospitalizations [9, 10]. In patients with an immunocompromised state, norovirus gastroenteritis can become chronic and may persist for months to years [11–14]. This can result in dramatic weight loss due to prolonged diarrhea,

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malnutrition, and dehydration [15]; all of these will contribute to an altered intestinal mucosal barrier, increased morbidity, and will highly likely worsen the underlying condition [16]. Reduction of immunosuppressive therapy is, when feasible, the strategy of choice in transplant patients.

Norovirus outbreaks are very common due to a plethora of reasons. The virus particles remain infectious for up to two weeks on surfaces, are resistant to many disinfectants, heating up to 60 °C and freezing. Moreover, infected persons can shed the virus for 20–40 days in their feces (also in asymptomatic infections) and it is estimated that only ~18 particles are necessary to cause an infection [17–20]. Norovirus is acquired via the fecal–oral route, most often via the consumption of contaminated food or water, or person-to-person contact due to the aerosolized particles from vomit or stool. Transmission occurs mostly in communal, semi-closed environments [21–23]. Long-term care facilities are the most common setting for norovirus outbreaks, followed by restaurants, schools, hospitals, and cruise ships [21]. Noroviruses are a major culprit for the closure of hospital wards. Every winter in the United Kingdom, an average of up to 113 000 beds are unavailable due to gastroenteritis, resulting in a final cost in the United Kingdom of £6.9–£10.0 million when including staff absence costs due to illness [24, 25]. The closure of hospital wards, may be cost-effective if done efficient and particularly if targeted to high-throughput units [25].

Currently there is no approved antiviral available to treat and/or prevent a norovirus infection. The current treatment is merely supportive through rehydration and supplementation of electrolytes. In some patients, approved drugs have been used off-label to treat norovirus infections with varied success, such as ribavirin [26], favipiravir [27], and nitazoxanide [28]. The strategy for the development of a HuNoV antiviral could go in two different directions. Firstly, an antiviral could be developed to treat ongoing acute and chronic infections. This could have a tremendous impact on the morbidity and mortality of populations at risk for a prolonged and severe disease (young children, the elderly, and immunocompromised), while also reducing the shedding of the virus and therefore reducing the risk of further transmission. The latter has been shown to be successful in the treatment of HIV, as antiretroviral drugs can reduce the concentration of virus in the blood and genital secretions of the person with HIV and therefore reduce transmission of the virus. Second, a prophylactic approach should be considered as HuNoV outbreaks are very impactful and antivirals could also play a major role in the prevention and control of outbreaks that occur in hospitals or cruise ships, for example. One could also consider household prophylaxis to prevent the virus to spread from a child to the parents and siblings, as the probability of this happening is high. Furthermore, HuNoV outbreaks mostly occur during winter. Since nursing homes are the most common setting and comprise a high-risk population, strategies for seasonal prophylactic use of an antiviral could be designed. Antivirals as prophylaxis are successfully being used against other human viruses such as influenza A virus [29]. In addition, it has been put forth that antiviral strategies should not per se target merely a single virus, but also yield options for syndrome-based treatment, including the development of broad-spectrum antivirals. This would be particularly relevant for acute viral gastroenteritis, as the clinical symptoms are similar for every viral agent and treatment should be started as early as possible. As multiple viruses can cause diarrhea, an antiviral that could target multiple viral agents of diarrhea, by focusing on highly conserved viral proteins such as the RNA-dependent RNA polymerase (RdRp), could be the way forward [30, 31].

13.1.2 Viral Genome

The *Caliciviridae* are a family of small (27–40 nm), non-enveloped viruses with a linear (+) ssRNA genome and an icosahedral capsid, containing 11 different genera. *Noro-* and *Sapovirus* are the only genera that comprise human viruses. The norovirus genome is organized into three open reading frames (ORF1–3). The ORF1 of norovirus encodes the six nonstructural (NS) proteins in the following order: the p48/N-terminal protein (or NS1/2), the NTPase (NS3), the p22 (NS4), the VPg (NS5), the viral protease (Pro, NS6), and the viral RNA-dependent RNA polymerase (RdRp, NS7). ORF2 and ORF3 encode for the major and minor structural capsid proteins VP1 and VP2, respectively (Figure 13.1). In the mouse norovirus (MNV), a fourth ORF is described, this is encoded by the subgenomic RNA, in an alternative reading frame overlapping with the VP1 coding region [32]. The ORF4 is translated during virus infection, encoding for virulence factor 1 (VF1) which localizes mostly to the mitochondria and has a role in infection and virulence. Due to an increase in reports of new noroviruses, the classification was recently updated [33]. The genus *Norovirus* is now organized in 10 genogroups (GI–GX), which can be further divided into 60 P-types based on the sequence of the RdRp. The addition of the RdRp sequence in the classification is due to the high recombination rate in the junction of ORF 1 and 2 [34]. Norovirus from the genogroups GI, GII, GIV, GVIII, and GIX contain strains that can infect humans. GIII comprises bovine and ovine strains and GV holds the mouse and rat noroviruses. GIV, GVI, and GVII include canine strains and GX contains bat strains [33]. There are no reports of zoonotic transmission, although this could potentially occur since antibodies against HuNoV have been found in swine's and HuNoV can infect gnotobiotic piglets and calves [35, 36]. The GII.4 noroviruses are responsible for the vast majority of outbreaks in humans (80%) and are linked to the highest mortality and hospitalization rates over the last years [37]. Since 2014, the GII.17 has also emerged in eastern Asia and has spread globally [38, 39].

13.1.3 Replication Cycle

The current knowledge on HuNoV replication still derives partly from studies with related caliciviruses and is based on the analogy with other (+)ssRNA viruses. HuNoV replication occurs in the cytoplasm of the cell. In order to attach to the cell surface, the P2 subdomain of the VP1 capsid protein interacts with the histo-blood group antigens (HBGAs), heparan sulphate or sialic acid [40–43]. HBGAs are carbohydrates that contain saccharide moieties that are expressed on red blood cells and mucosal epithelia. They can also be found as free oligosaccharides in saliva, blood, and intestinal contents [44]. The different HBGAs are

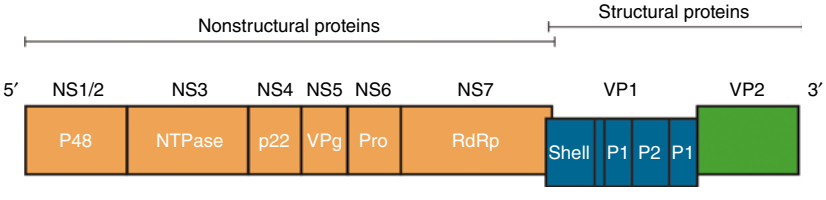

Figure 13.1 The norovirus genome. *Source:* Created with www.biorender.com.

formed through sequential addition of a monosaccharide to the terminal disaccharide of a precursor glycan: this involves multiple fucosyltransferases (FUT). Susceptibility to HuNoV depends on FUT2 and FUT3; these are responsible for the biosynthesis of secretor and Lewis antigens, respectively. Individuals who lack both alleles of the FUT2 gene (nonsecretors) are less susceptible to GII.4 HuNoV infection [45]. Although HBGAs are considered the main attachment factor for HuNoV, there are HuNoV strains that do not interact with any of the available synthetic HBGAs [46–48]. This suggests that it is possible that another (still unidentified) protein receptor or additional cofactors may be required [48–51]. For HuNoV GII.3, it was shown that bile acid was necessary for replication in the human intestinal enteroids (HIE) [52]. It was recently reported that bile acids promote replication by enhanced endosomal uptake, endosomal acidification and subsequent activity of endosomal/lysosomal enzyme acid sphingomyelinase (ASM), and enhanced ceramide levels on the apical membrane [53]. The role of bile acid for other HuNoV genotypes remains to be investigated. In contrast to HuNoV, the entry process of MNV is better understood. MNV entry is clathrin/caveolin-independent and mediated by dynamin II and cholesterol [54]. Entry is pH-independent, which is expected for an enteric virus; acidic pH does not cause any conformational changes in the capsid [55]. Proteinaceous receptors (CD34, CD300lf, CD300ld) were found to modulate and facilitate MNV entry and infection [56, 57]. But CD300lf was shown not to be a receptor for HuNoV [58]. Although the process of virus uncoating is not known, recent work showed that the minor capsid protein of feline calicivirus (FCV) forms a pore in the capsid upon receptor engagement, hypothetically playing an important role in viral genome release [59]. Once the viral genome is released into the cytoplasm, translation starts by the recruitment of the cell translation machinery through interactions with the VPg. Translation of the ORF1 results in a large polyprotein which is cleaved co- and post-translationally by the viral NS6 protease. This results in the release of the NS proteins, ready for replication complex formation. The replication complex is formed by recruitment of cellular membranes to the perinuclear region of the cell, through interactions with NS1/2 and NS4 [60–62]. After the replication complex is fully assembled, the negative RNA strand (antigenomic RNA) is synthesized from the mRNA template. This antigenomic RNA is then used as a template for production of new genomic and subgenomic RNA (ORF2/3) by the viral RdRp. Due to the large amount of VP1 particles required for the capsid formation, the VP1 and VP2 proteins are translated from the subgenomic RNA as this is present at higher levels than the genomic RNA. Finally, the replicated genomes are translated (within the replication complex) or packaged into the capsid for virion assembly and exit. It was recently shown that noroviruses can exit in a non-lytic manner, as viral clusters enclosed within vesicles [63]. These vesicles provide a protective environment and significantly contribute to viral infectivity and transmission.

13.1.4 *In Vitro* Models

A first HuNoV replication model using B cells was described in 2014 [64]. However, the replication observed is modest and not readily reproducible [65]. Still, it was remarkable to discover that B cells are a target cell of norovirus (further demonstrated in [66]) and that the use of unfiltered feces as inoculum rendered a higher yield of replication, highlighting the role of the gut microbiota in enhancing HuNoV infectivity (see Section 13.2). In 2016,

an ex vivo model using stem cell-derived HIEs was developed [52]. HuNoV was shown to replicate in non-transformed HIE monolayer cultures, which contain multiple cell types such as paneth cells, goblet cells, and enterocytes. Both HuNoV GII.4 and HuNoV GII.3 were able to replicate, although addition of bile to the culture was necessary to achieve HuNoV GII.3 replication [52]. As the use of HIEs in virology becomes more standardized and further optimization is carried out, this system offers an important opportunity to study HuNoV replication and inhibition thereof. In both models, antiviral testing has been attempted using 2'-*C*-methylcytidine, an experimental broad-spectrum polymerase inhibitor [67–69]. Before these models were available, the only possible approach to study the HuNoV RNA replication were HuNoV GI.1 replicon-bearing cell lines, in which HuNoV GI·I NS proteins are expressed [70, 71]. Since the structural proteins are not expressed in these HuNoV GI.1 replicon cell lines, early (attachment, entry, uncoating) or late events (assembly, genome packaging, exit) of the virus life cycle cannot be studied. However, the study of the activity (and inhibition) of all the replicative enzymes/NS proteins, replication complex formation, and replication of the viral genome are possible. Since models that support HuNoV replication have only been developed/reported recently, most antiviral studies have used MNV as a cultivable alternative. MNV is similar to its human counterparts in terms of the fundamental mechanisms of replication, genetic similarity, and environmental stability [72–74]. MNV has a tropism for macrophages, B cells, and dendritic cells and can be grown routinely in the murine macrophage cell line RAW 264.7 and the murine microglial BV-2 cell line [72].

13.1.5 *In Vivo* Models

13.1.5.1 Human Norovirus

Multiple larger animal models were used to test HuNoV such as chimpanzees, gnotobiotic pigs, and calves [35, 36, 75]. After intravenous (iv) infection in chimpanzees, no diarrhea was observed, but the infection induced a week-lasting shedding in stool and a serum antibody response. Virus was detected in the intestinal and liver biopsies of the chimpanzees. In gnotobiotic pigs and calves, diarrhea and mild lesions in the intestine were observed after an oral infection with filtered feces from a HuNoV GII.4-infected patient. Such large animal models are valuable but are economically and practically not useful in large-scale antiviral studies.

In 2013, the first mouse model of HuNoV infection was reported, using $Rag^{-/-}yc^{-/-}$ BALB/c mice [76]. Infection was successful after simultaneous intraperitoneal (ip) injection and oral administration of a human stool suspension containing HuNoV; however, the oral route was not sufficient to cause infection [77]. Furthermore, the infected mice did not develop clinical symptoms and the virus was cleared by day three post infection (pi). Viral antigens were detected in the intestines (stomach, ileum, caecum, jejunum, etc.). In 2019, our group established zebrafish larvae (*Danio rerio*) as a new small animal model to study replication of clinically relevant HuNoV strains [78]. After injecting a HuNoV-containing (HuNoV GI or GII) human stool suspension into the yolk of three-day-old zebrafish larvae, the virus replicates to high titers in the intestine and in cells of the hematopoietic lineage. A virus-specific innate immune response was observed after inoculation of the virus and importantly, viral replication could be reduced upon an antiviral treatment. This model

serves as an excellent platform to aid in the search for norovirus antivirals, as zebrafish larvae are small, easy to breed, have a low-cost maintenance, rapid life cycle, and are optically transparent. Moreover, the zebrafish larvae fit in 96- and 384-well plates, compounds can be simply added to their swimming water and very little quantities of compound are needed for testing, in contrast to what is needed for studies in mice [79].

13.1.5.2 The Murine Norovirus as a Surrogate for *In Vivo* Studies

There are around 30 known strains of MNV, the majority of which cause a persistent asymptomatic infection in immunocompetent mice. When infected with the MNV-1.CW3, innate immune-deficient mice [$STAT1^{-/-}$, AG129 (*deficient* in the interferon alpha/beta and gamma receptor)] can develop an acute infection with severe diarrhea and weight loss, and almost 100% mortality in four to nine days after infection [80]. In these immune-deficient mice, MNV largely recapitulates the pathogenesis of the HuNoV in humans namely: infection via the oral route, resulting in diarrhea, gastric bloating, and high levels of viral RNA detected in feces. Infection of mice with the MNV-1.CW3 strain allows to study the antiviral effect of small-molecule inhibitors on an acute infection and the virus transmission [81, 82]. Infection of mice with the MNV.CR6 strain results in an asymptomatic persistent infection; it persists asymptomatically in both wild-type and innate immune-deficient mice [83, 84]. The MNV.CR6 strain has been used to study the antiviral effect of small-molecule inhibitors on a persistent infection [85].

13.2 Antiviral Targets

Multiple antiviral targets can be considered in the many steps in the replication cycle of norovirus; these are highlighted in Figure 13.2.

13.2.1 Binding and Entry

Since the binding of HuNoV to HBGAs is the better characterized initial step of viral entry, targeting the HBGAs could be an interesting strategy to develop antivirals that prevent the start of viral replication [86, 87]. By X-ray crystallography and saturation transfer difference nuclear magnetic resonance (STD NMR), citrate was shown to compete with HBGA to bind the norovirus capsid protruding domain [88]. The latter suggests that citrate (and chemicals that mimic certain carbohydrates) could be used as anti-norovirus therapy and that the P2 subdomain of the VP1 capsid protein could be targeted as this is the region that interacts with the HBGAs.

After binding, the viral genome must be released from the capsid and enter the cytoplasm where replication occurs. For HuNoV GII.3, it was shown that bile acid promotes entry into jejunal HIEs [53]. However, more information on entry, release, and pH-dependence of the HuNoV is still lacking. The VP2 could also be considered an antiviral target as it was shown (for FCV) to form a pore in the capsid upon receptor engagement and it was hypothesized that this pore could allow the release of the genome, through the endosomal membrane, into the cytoplasm [59]. When targeting the viral capsid protein, one has to

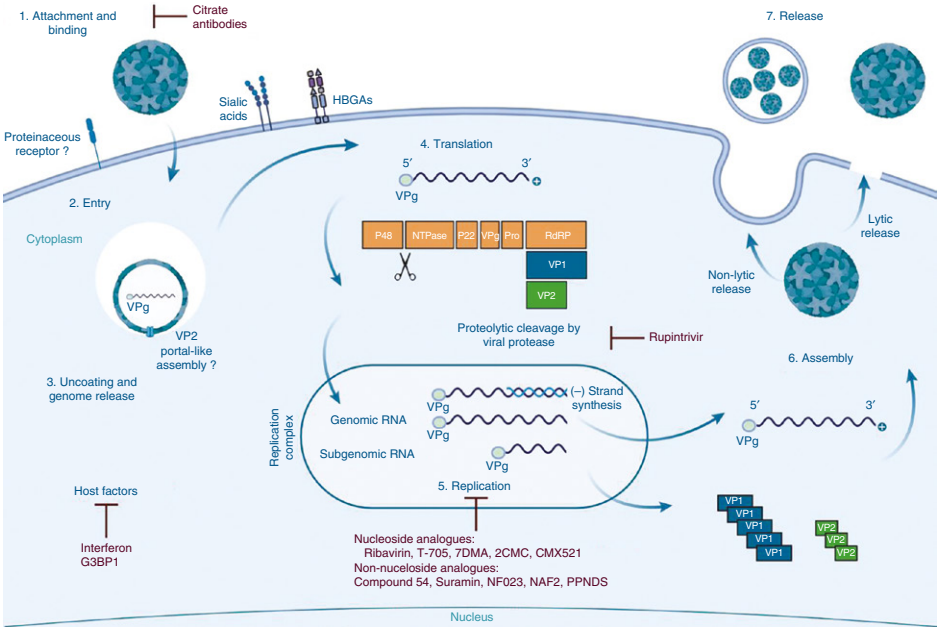

Figure 13.2 Targeting the various steps of the replication of human norovirus. *Source*: Created with www.biorender.com.

take into account that the capsid region is highly variable and RNA viruses have a high mutation rate with mutations arising mostly within the capsid region. Therefore, the challenges of rapid occurrence of drug-resistant virus variants should be considered. For example, resistance developed rapidly against pleconaril, which is a capsid-binding antiviral for human rhinoviruses (HRV) [89]. Thus, the use of a capsid inhibitor as a single antiviral therapy would not be advised, although such capsid binders may have potential in combination with antiviral molecules targeting other steps in the viral replication.

13.2.2 Nonstructural Proteins

Out of the six NS proteins, the protease and the RdRp are the proteins with a better-characterized function and structure. These are also very similar to the picornavirus homologs; therefore, the knowledge gained from picornaviruses could aid in the search for HuNoV antivirals. Below we describe each NS protein including the protease and RdRp and how they could serve as a potential target for antiviral therapy.

13.2.2.1 NS1/2 (p48)

The NS1/2 has no significant sequence similarity to any viral or cellular proteins, and is the least understood protein of the norovirus genome. The latter could imply that this protein has a role that is unique for noroviruses. Thus, it could be a very interesting target for specific anti-norovirus drug development. The protein consists of two parts: an N-terminal and a C-terminal part. When studying different caliciviruses, the NS1/2 protein has been detected in different organelles, but overall it is shown that this protein encodes for mechanisms to impede normal trafficking within secretory pathways in a way that promotes replication via membrane reorganization [61, 90–93]. The N-terminal part (NS1) is a secretory protein which is required to overcome epithelial host defense barriers mediated by IFN-λ [94]. Moreover, RNAseq analysis showed that the NS1/2 protein can affect the immune system (chemokine, cytokine, and Toll-like receptor signaling) and intracellular pathways (NFκB, MAPK, PI3K-Akt signaling) in murine monocytes [95]. The C-terminal part contains a transmembrane domain and is more conserved within the *Caliciviridae* family and contains a highly conserved transmembrane domain. This domain potentially acts as an anchor involved in intracellular membrane rearrangements; this conserved region could potentially be targeted [96]. When using the Tulane virus (a rhesus Calicivirus), it was shown that the virus uses Ca^{2+} signaling during infection and that NS1/2 has a viroporin activity that disrupts Ca^{2+} homeostasis [97]. Since viroporins are viral proteins that modify cellular membranes, targeting this protein could result in the inhibition of virus release from infected cells.

13.2.2.2 NS3 (NTPase/Helicase)

The NS3 has high sequence similarities with the NTPase/helicases of picornaviruses (2C protein) and is classified within the superfamily 3 of RNA helicases [98, 99]. The NTPase domain catalyzes the hydrolysis of nucleoside triphosphates, using the released energy (NTP) to unwind the viral nucleic acids (helicase function) [100]. It was recently confirmed that the NS3 of norovirus indeed has both the NTPase and helicase activity [101]. This study also showed that NS3 stimulates the NS7/RdRp-mediated RNA synthesis *in vitro*,

suggesting that NS3 plays an important role in norovirus RNA replication [101]. Moreover, the authors showed that NS3 could be a relevant antiviral target because guanidinium chloride (GuHCl, Figure 13.3a) can inhibit the RNA helicase activity of NS3 *in vitro* and can inhibit the replication of the HuNoV GI.1 replicon [101]. The NS3 of HuNoV GII.4 was detected on different membrane compartments of the secretory pathway and was closely associated to intracellular lipid storage compartments [93].

13.2.2.3 NS4 (p22)

The NS4 has some sequence similarity to the 3A protein in *Picornaviridae,* which inhibits the protein trafficking from the ER to the Golgi. A similar role has been described for the norovirus NS4 [62, 98, 102] that is identified as a key factor in inducing membrane alterations [93]. Targeting this mechanism for the inhibition of the normal ER transport or the formation of the membranous viral replication complex could be an attractive target for future antiviral therapies.

13.2.2.4 NS5 (VPg)

The VPg is linked to the 5′ end of the genome and acts as a cap substitute, serving as a primer and recruits translation initiation factors from the host cell [103]. The initiation of antigenomic RNA synthesis by the RdRp is dependent upon uridylylation of VPg. Blocking the uridylylation step or the interaction between the uridylylated VPg and the poly(A) tail of the viral genome could be a good antiviral strategy [104]. Norovirus translation is dependent on the interaction of VPg with the eIF4F complex (eukaryotic initiation factor

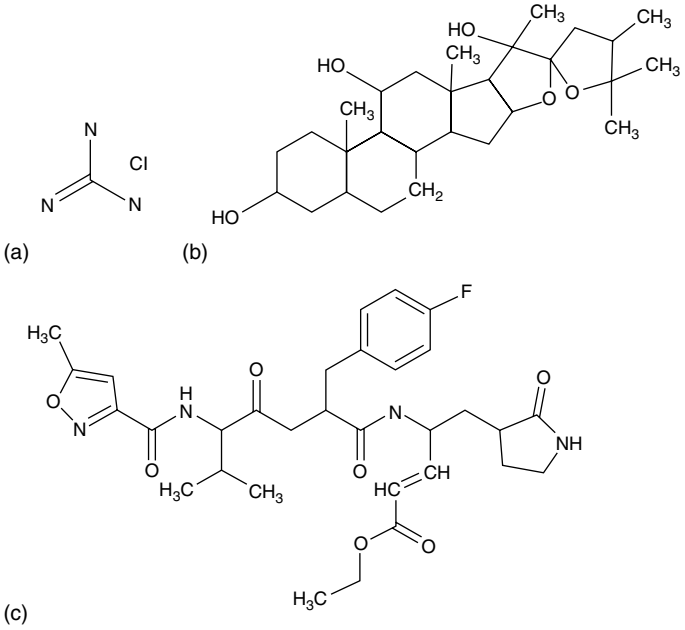

Figure 13.3 The chemical structure of (a) Guanidinium chloride, (b) Hippuristanol, (c) Rupintrivir. *Source:* Created with ChemDraw.

4F) [105]. The HuNoV GI.1 VPg interacts with the eIF4E (cap binding protein) [106, 107], while FCV and MNV interact with the complex via another component, namely eIF4A (RNA helicase) [108]. The eIF4A inhibitor, hippuristanol (Figure 13.3b), was able to block the *in vitro* translation of both FCV and MNV [108]. However, this compound has a cytotoxic effect as it can also inhibit cellular protein synthesis. The VPg structures of FCV and MNV are available, revealing that VPg is involved in multiple protein–protein interactions [109]. There is no crystal structure available to study the VPg–RdRp interaction; however, this interaction should be further investigated as a potential antiviral target. While targeting the VPg would likely yield a strong inhibition of norovirus replication due to its critical role, the challenge is to do so by directly disrupting a virus-specific interaction and not by interfering with normal cellular processes that could result in toxic side effects.

13.2.2.5 NS6 (Protease)

The NS6 is a cysteine protease with high structural and sequence similarities with the protease of picornaviruses (3C protein) and coronaviruses [110, 111]. The protease cleaves the NS polyprotein into the single NS proteins. This enzyme is one of the few HuNoV proteins for which the structural information is available, so there is a good basis for in silico drug design [112, 113]. An enzymatic assay to study the HuNoV protease using fluorescence resonance energy transfer (FRET) is also available [114]. It has been put forth that the role of the norovirus protease extends to the prevention of eliciting an immune response upon infection by cleavage of the mitochondrial antiviral-signaling protein (MAVS) [61]. Protease-targeting antivirals that were developed against other RNA viruses have been studied for potential anti-norovirus activity, for example, rupintrivir (AG-7088, Figure 13.3c).

Rupintrivir is a protease inhibitor designed for the treatment of HRV. After successful treatment of volunteers that were experimentally induced with a rhinovirus infection, a second phase II trial was conducted in which rupintrivir was administered intranasally to patients with a natural HRV infection within 36 hours of the appearance of the first symptoms. In the latter study it was shown that rupintrivir was not able to efficiently inhibit natural HRV infections, and further development was stopped. Even the orally available analogue could not reduce disease severity or viral load in naturally infected HRV patients sufficiently [115–117]. However, this compound was shown to have broad-spectrum antiviral activity against, for example, other picornaviruses, coronaviruses, and caliciviruses [118]. Rupintrivir inhibited the HuNoV GI.1 replicon with an EC_{50} of $<1.5\,\mu M$ [71, 119].

13.2.2.6 NS7 (RNA-Dependent RNA Polymerase)

The RdRp is a critical enzyme of viral replication. It is responsible for the synthesis of the genomic, subgenomic, and antigenomic norovirus RNA. The RdRp targeting compounds are divided into the nucleoside and non-nucleoside analogues.

13.2.2.6.1 Nucleoside Analogues Nucleoside analogues with antiviral activity (such as the anti-herpes drug acyclovir or the anti-HCV drug sofosbuvir) mimic as their 5'-*O*-triphosphate metabolite an dATP, dCTP, dGTP, or dTTP when it concerns DNA viruses or either ATP, GTP, CTP, or UTP when it concerns RNA viruses. Nucleoside analogues are

incorporated by the viral polymerases into nascent nucleic acid chains, preventing the incorporation of the next nucleotide, which results in the formation of an incomplete and nonfunctional RNA strand. In addition, they may increase the error frequency of the RdRP. The active site of the RdRp is highly conserved among viral families. This implies that nucleoside analogues are highly likely to have a broad-spectrum activity [31]. Chimerix reported that CMX521 (Figure 13.4a) (a purine nucleoside analogue) inhibits norovirus replication in mice. This is the first norovirus inhibitor that progressed to phase 1 clinical studies. Further development has been halted because of a poor pharmacokinetic profile [120]. A number of other nucleoside analogues that were developed against other (+)ssRNA viruses have been studied for their potential anti-norovirus activity, such as 2'-*C*-methylcytidine (2CMC, Figure 13.4b), 7-deaza-2′-*C*-methyladenosine (7DMA Figure 13.4c), ribavirin (Figure 13.4d), and T-705 (Favipiravir, Figure 13.4e).

2CMC is a cytidine analogue that was initially developed (as its prodrug valopicitabine) as an inhibitor of hepatitis C virus (HCV) replication, but further development was stopped due to gastrointestinal side effects [121, 122]. 2CMC is the most active and most thoroughly studied anti-norovirus molecule *in vitro* and *in vivo* and is therefore considered as the

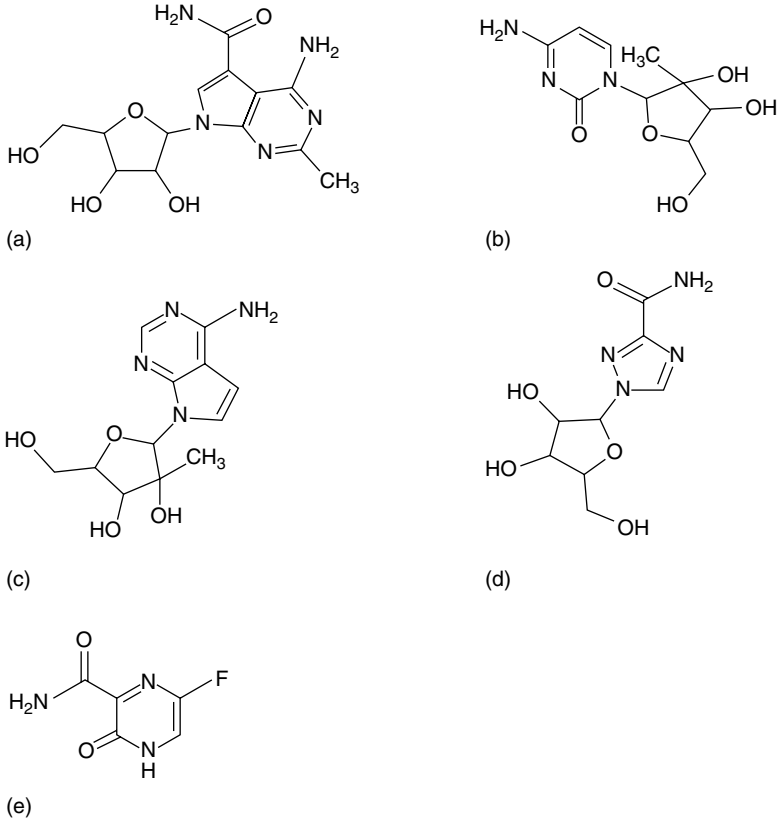

Figure 13.4 The chemical structure of (a) CMX521, (b) 2'-*C*-Methylcytidine, (c) 7-Deaza-2'-*C*-methyladenosine, (d) Ribavirin, (e) Favipiravir. (*Source*: Created with ChemDraw.

benchmark compound in antiviral studies against HuNoV [67, 69, 81, 82]. *In vitro*, 2CMC inhibits MNV replication, the HuNoV GI.1 replicon as well as HuNoV GII.4 replication in infected B cells [69, 81] at an EC_{50} of ~5 μM or ~0.3 μM, respectively. 2CMC rescued MNV-1. CW3-infected AG129 mice from virus-induced death upon prophylactic or postexposure treatment. Moreover, viral transmission was blocked when AG129 mice (sentinels) were treated prophylactically [81, 82, 123]. In mice persistently infected with MNV.CR6, 2CMC reduced viral shedding to undetectable levels [85]. 2CMC could also significantly reduce HuNoV GII.4 viral loads in $Rag^{-/-}yc^{-/-}$ BALB/c mice and HuNoV GII.6 viral loads in zebrafish larvae by ~1 $\log_{10}$ or 2.4 $\log_{10}$, respectively [67, 78].

7DMA is an adenosine analogue that was also initially developed as an inhibitor of HCV replication, (it was shown to reduce the HCV loads in infected chimpanzees [124, 125]). 7DMA inhibits MNV replication with an EC_{50} of ~14 μM, with no toxic side effect in the RAW cells up to 100 μM, the highest concentration tested and inhibits the HuNoV GI.1 replicon with an EC_{50} ~ 3 μM [30].

Ribavirin is a guanosine analogue with broad-spectrum activity against both RNA and DNA viruses. It is approved to treat chronic HCV infections (in combination with pegylated interferon) [126], and also to treat respiratory syncytial virus (RSV) infections in young children [127, 128]. It is also active against several other viruses such as Lassa fever virus, hepatitis E virus, and hantaviruses [129–133]. By co-crystallization it was shown, as expected, that the 5′-*o*-triphosphate of ribavirin binds to the active site of the MNV RdRp [134]. Ribavirin has an inhibitory effect against MNV *in vitro* and inhibits the HuNoV GI.1 replicon with an EC_{50} of ~40 μM [135]. However, for norovirus it was shown that ribavirin likely exerts its antiviral effect by depletion of intracellular GTP pools and not via a direct interaction with the RdRp [135, 136].

T-705 is a pyrazine analogue; it needs to be phosphoribosylated intracellularly to become active. The active form is recognized as a substrate by RdRp and inhibits the RNA polymerase activity. Its antiviral effect is attenuated by the addition of purine nucleic acids, indicating the viral RdRp mistakenly recognizes favipiravir as a purine analogue [137]. Favipiravir (T-705) has broad-spectrum activity against multiple RNA viruses such as several flaviviruses, hantaviruses, arenaviruses, and Ebola virus [138, 139]. T-705 is approved in Japan to treat influenza infections [140]. *In vitro,* T-705 exerts some antiviral activity against MNV and the HuNoV GI.1 replicon, with an EC_{50} of ~250 μM and an EC_{50} of ~21 μM, respectively [141, 142]. T-705 treatment of MNV-infected mice showed variable efficacy in the reduction of viral loads [85, 143]. T-705 treatment of an immune-compromised patient with a chronic norovirus infection was reported to reduce some clinical symptoms. Sequencing revealed that the patient was infected with different noroviruses of the same monophyletic clade and that upon treatment, there was a selection for a distinct viral variant [27]. Overall, the RdRp is one of the most important NS proteins for many viruses. However, we still lack important information on the progress of norovirus RNA synthesis [144] and compared with picornaviruses, the norovirus RdRp is much understudied. A better understanding of the exact mechanism of action of this viral protein would aid in the development of HuNoV antivirals.

13.2.2.6.2 Non-Nucleoside Analogues Non-nucleoside analogues will inhibit the RdRp by an allosteric mechanism that may result in a more specific antiviral effect as compared

with (certain) nucleoside analogues [145]. There are three known binding sites for non-nucleoside analogues in the norovirus RdRp [146–148]. In silico approaches and RdRp enzyme activity assays allowed for high-throughput screenings to find new non-nucleoside RdRp inhibitors [149]. Recently, compound **54** (Figure 13.5a) was selected as the best candidate in a large in silico screen with an IC_{50} of 5.6 μM (HuNoV RdRp) and an IC_{50} of 12.1 μM (MNV RdRp) [150]. Other molecules such as NAF2 (Figure 13.5b), suramin (Figure 13.5c), PPNDS (Figure 13.5d), and NF023 (Figure 13.5e) have also been described as inhibitors of the RdRp [146–148, 151, 152]. However, due to their poor cell permeability, the *in vitro* activity could not be confirmed or the efficacy is much lower in cell culture [146, 149]. For example, suramin needed liposomes to be delivered before it could inhibit MNV *in vitro* [153]. These molecules can be considered as tool compounds which cannot be further developed; however, they can be used as a starting point to find new molecules that can bind the same pockets. To progress in the development of non-nucleoside inhibitors for norovirus, more research is needed to prevent off-target effects, better bioavailability, and cell permeability.

13.2.3 Host Factors

Antivirals may also be directed at host factors that are essential for the viral replication cycle. Targeting host factors could result in higher barrier to resistance, but these could also affect the host and result in adverse effects [154]. An interesting antiviral approach is to stimulate the immune response of the host. Interferons (IFN) play an important role in the antiviral mechanisms and in the innate immune response. Interferon has been used in the past for the treatment of infections with HBV and HCV. A number of studies have shown the impact of interferon type I and II on norovirus infections [72, 80, 135, 155–157]. Also, type III interferon plays an important role in blocking norovirus replication. Interferon λ was shown to protect mice against MNV challenge; therefore, IFN-λ may possibly be explored for the prophylaxis of norovirus infections [158–160]. Toll-like receptor (TLR) 7 agonists that stimulate interferon production were shown to block MNV replication, these include resiquimod (R-848, Figure 13.6a), Vesatolimod (GS-9620, Figure 13.6b), Gardiquimod (Figure 13.6c), and imiquimod (R-837, Figure 13.6d) [161]. Furthermore, R-848 also reduced replication of the HuNoV GI.1 replicon by 50%; however, at the very high concentration of 100 μM [161]. The TLR4 agonist, poly-γ-glutamic acid (γ-PGA, Figure 13.6e) inhibits MNV replication very efficiently *in vitro* ($EC_{90} < 100$ nM) and *in vivo* when administered orally (50 mg/kg) [162]. As TLR agonists have been used for many years as vaccine adjuvants, they may thus possibly be repurposed for the treatment of norovirus infections. Another host factor that interacts and plays a role in norovirus replication and can therefore be targeted is the heat shock protein 90 (Hsp90), a chaperone protein that assists in the maturation of multiple proteins [163]. The inhibition of Hsp90 activity by 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG, Figure 13.6f) resulted in the inhibition of MNV replication in BV-2 cells and reduced MNV replication in the distal ileum of MNV-1.CW3-infected BALB/c mice by ~1 $\log_{10}$ [163] at a dose of 30 mg/kg. Recently, using a combination of a CRISPR screen and a proteomic analysis of the viral translation and replication complexes, the core stress granule protein G3BP1 was identified as a host factor essential for norovirus infection and may thus be a potential antiviral target [164].

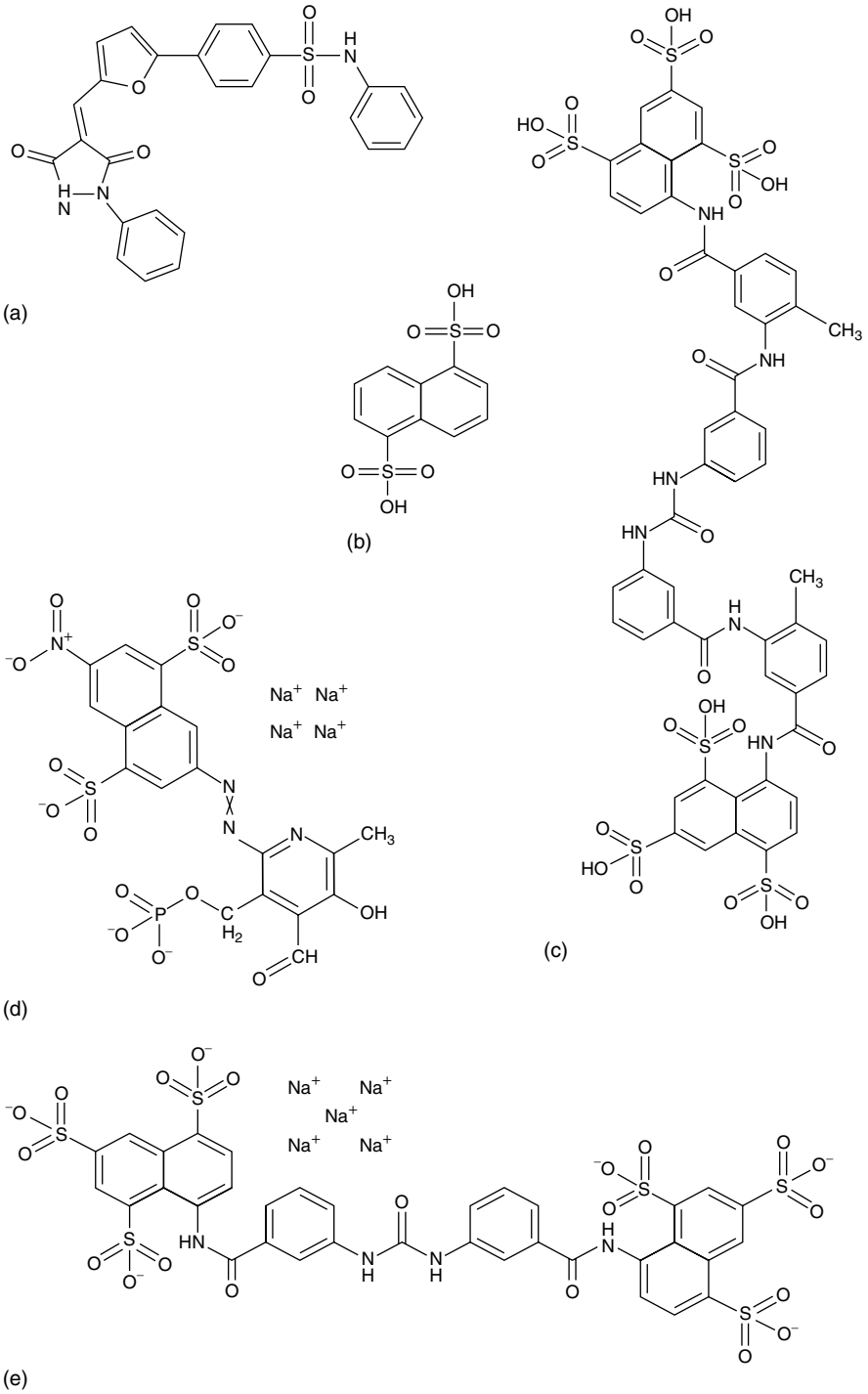

Figure 13.5 The chemical structure of (a) Compound 54, (b) NAF2, (c) Suramin, (d) PPNDS, (e) NF023. *Source:* Created with ChemDraw.

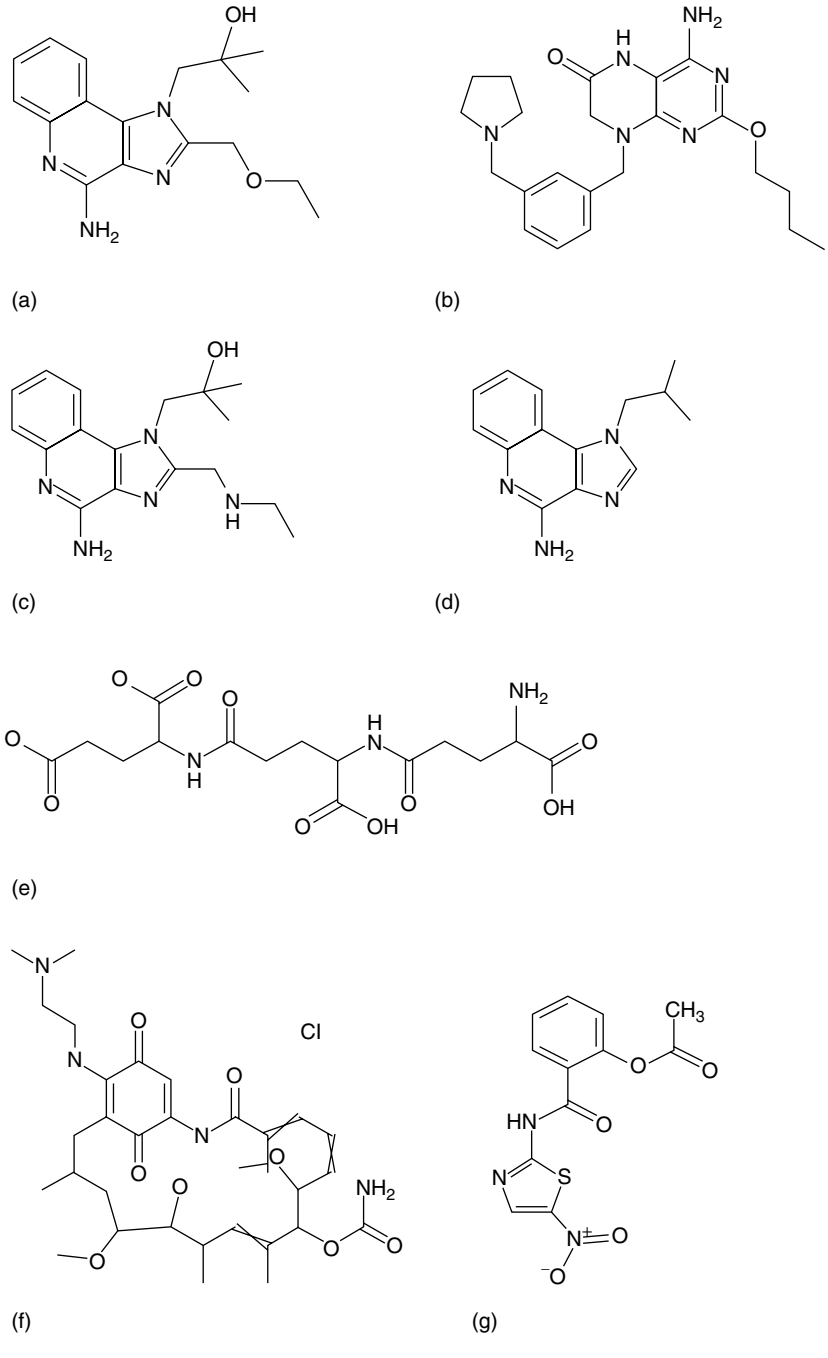

Figure 13.6 The chemical structure of (a) Resiquimod (R-848), (b) Vesatolimod (GS-9620), (c) Gardiquimod, (d) Imiquimod (R-837), (e) Poly-γ-glutamic acid (γ-PGA), (f) 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), (g) Nitazoxanide. *Source:* Created with ChemDraw.

13.2.4 Antibodies

Monoclonal antibodies (mAbs) are frequently being used to treat cancer and inflammatory diseases, and have shown (some) promise in the treatment for HIV, HCV, and Ebola virus [165–167]. The application of antibodies as prophylaxis or as therapy for HuNoV may be useful for vulnerable individuals. The use of orally administered human immunoglobulins or specific immunoglobulin (Ig)A purified from sera collected from GI.1-infected participants has also been shown to reduce disease symptoms [168–171]. The latter results have been from limited individual cases and more studies are required to determine if this treatment results in a specific immune response or it merely helps the boosting of the immune system and therefore clearing the virus. Treatment with antibodies retrieved from human rotavirus immunized chicken eggs or immunized bovine colostrum resulted in modest antiviral activity against human rotavirus in young children [172, 173]. mAbs against HuNoV GI.1 from human and chimpanzee B cells were shown to block carbohydrate binding by HuNoV GI.1 virus-like particles (VLPs) in a neutralization assay [174]. Two mAbs (D8 and D7) mixed with HuNoV GI.1 were able to neutralize the virus and therefore prevented infection in chimpanzees [174]. The binding spectrum of the antibodies was limited to the HuNoV GI.1, so the immune response is genotype-specific. The latter points out that much more research is needed and that antibodies need to be able to recognize diverse HuNoV genotypes in order to be used successfully as an antiviral strategy for HuNoV infections. Llama-derived nanobodies are fully functional antibodies that only consist of heavy chains and are able to neutralize HuNoV [175, 176]. Nanobody 85 has shown binding affinity with multiple norovirus VLPs (GI.11, GII.1, GII.2, GII.4, GII.12, GII.17). As nanobody 85 has shown to be less HuNoV genotype-specific than mAbs, these could have a higher potential to be used as an antiviral strategy [177, 178]. However, these studies are performed only using VLPs, structural analysis, and surrogate assays for HuNoV GI.1 neutralization. The recent positive developments in model systems for HuNoV will allow us to better study and understand the use of mAbs and nanobodies as antiviral strategies.

13.2.5 Gut Microbiota

Norovirus, being an enteric virus, naturally encounters another import player in human health and disease: the gut microbiota. Recent studies have shown that norovirus, but also poliovirus, mouse mammary tumor virus (MMTV), rotavirus, and reovirus T3SA+ can bind commensal gut bacteria and use this interaction to their advantage [179]. For example, incubation of poliovirus in the presence of bacterial LPS stabilizes the virus capsid making it more resistant against heat and disinfectants. Moreover, preincubation of poliovirus with LPS resulted in a 200 times more efficient binding to its receptor in HeLa cells [180, 181]. A GII.4 HuNoV-positive stool sample used to infect human B cells resulted in a hundredfold increase in viral genomes after five days, while a much less efficient replication was observed when the fecal sample was first filtered [64]. However, HuNoV replication was restored in a dose-dependent way when the commensal bacteria *Enterobacter cloacae,* which expresses H-type HBGA on its outer surface, was added. The importance of the HBGA-like structures on the bacteria was confirmed when the addition of synthetic H-type HBGA resulted in a similar effect [64]. HuNoV GII.4 is not the only genotype and

E. cloacae not the only bacteria that show these norovirus–bacteria interactions. A strong binding was observed between HuNoV GI.7, HuNoV GII.6, and HuNoV GII.3 VLPs and the *Enterobacter sp* SENG-6 (closely related to *E. cloacae*) [182]. Another study showed that HuNoV GII.4 and HuNoV GI.6 could efficiently bind other common gut bacteria such as *Enterococcus faecium*, *Lactobacillus gasseri*, *Bacteroides thetaiotamicron,* and *Lactobacillus plantarum,* which all express HBGAs [183]. For MNV, a similar relationship with the gut microbiota is observed: an antibiotic pretreatment of two weeks to deplete the gut microbiota of mice prevented the establishment of a persistent infection by MNV.CR6 virus. This effect was reverted when the gut microbiota of the mice was restored with fecal microbiota transplantation (FMT) [184]. In gnotobiotic pigs that received a FMT, with a human fecal sample, an enhanced replication of HuNoV GII.4 was observed [185]. This intricate relationship between norovirus and the gut microbiota could be the next target for new antiviral drugs or therapeutic strategies. For example, preventing the binding between the viral capsid and the HBGA-like structures (although their exact role during HuNoV infection is still unknown) is still an unexplored route to treat or prevent HuNoV infections. Structural analysis suggests that HBGA-blocking IgA antibodies can prevent the attachment of norovirus by sterically masking the HBGA binding site on the P-domain of the virus [186]. Alternatively to steric hindrance, HBGA-blocking antibodies could also work through directly competing with HBGA binding site or by inducing conformational changes in the P-domain and thus allosterically disrupting the binding site [186].

Another option is to cleave the terminal sugar moiety of the glycans using an enzyme. This could be used both against the glycans on the outer surface or on the epithelial cells, depending on which structures are more crucial during HuNoV infection. This concept led to the development of compound DAS181 (fludase, Ansun Biopharma) [187]. This drug, currently in phase 3 clinical trials (NCT03808922), is a sialidase that cleaves sialic acid and so prevents the entry of para-influenza viruses, which use sialic acid as their receptor, into respiratory epithelial cells [188]. A similar drug could be developed for HuNoV, potentially both by targeting fucose [189] and sialic acids [41], which have been found to interact with the HuNoV capsid.

Alternatively, there is evidence that bacteria in the form of probiotics could be used to help fight off norovirus infections. Supplementation of retinoic acid (the metabolite of vitamin A) during MNV-1 infection in mice reduced MNV persistence while a simultaneous upregulation of the levels of *Lactobacillus* sp., a known probiotic, was seen in the gut microbiome [190]. In gnotobiotic pigs, by pre-colonization with a cocktail formulation of *Lactobacillus rhamnosus GG* and *Escherichia coli,* both bacteria that can bind norovirus, viral shedding was completely inhibited during GII.4 infection and stronger antiviral T-cell responses were observed [191]. Similar symptom-reducing effects like reduced viral shedding, diarrhea, or viral replication were also seen in other viral diseases such as rotavirus (supplementation with *Bifidobacterium lactis*) and influenza virus (supplementation with *Lactococcus lactis*) [192].

Furthermore, the gut microbiota also shapes the antiviral immune response by maintaining intestinal epithelial cell integrity [193] and regulating Treg and Th17 cells [194, 195]. Interestingly, the persistent MNV.CR6 infection observed in WT C57BL/6 mice is suggested to be facilitated by commensal bacteria skewing the antiviral IFN-λ response, as gut microbiota-depleted WT C57BL/6 mice were no longer susceptible to a persistent MNV.

CR6 infection. On the other hand, microbiota-depleted *Ifnlr1*$^{-/-}$ mice, *Stat1*$^{-/-}$ mice, and *Irf3*$^{-/-}$ mice (all lacking important genes involved in IFN-λ signaling) were still susceptible to MNV.CR6 [184]. Also, commensal gut bacteria in mice were shown to prevent acute MNV-1 infection in the proximal small intestine but also enhanced infection in the distal parts of the gut (caecum, colon). This prevention of infection in the proximal gut was lost in Ifnlr1$^{-/-}$ mice, showing that type III interferon (interferon class to which IFN-λ belongs) is involved [196]. Interestingly, mice treated with clindamycin to deplete their bile acid composition and mice treated with antibiotics to deplete their gut microbiota both showed a similar increase of viral titers in the proximal small intestine, suggesting that the bile acids metabolized by the gut bacteria are responsible for priming the IFN response in the proximal intestine [196]. This theory was strengthened when after colonizing the antibiotics-treated mice with Clostridium scindens, a known producer of secondary bile acids by 7α-dehydroxylation and oxidation, the viral inhibition was restored [196]. Moreover, IFN-λ was able to cure WT C57BL/6 mice of their persistent MNV.CR6 infection [158] and transmission of MNV-1.CW3 from mice to mice could be prevented by injection (10 μg in the left and right tibialis anterior muscles) of an IFN-λ expressing plasmid in sentinel mice that were co-housed with MNV-infected untreated mice (see also Section 13.3) [159]. There is no evidence yet that a similar skewing is happening during HuNoV infection, but using the newly developed models, this could be elucidated in the near future and allows us to target this process during antiviral treatment.

13.2.6 Unknown Targets

There are compounds that exhibit anti-norovirus activity but for which the mechanism of action is unknown, for example, nitazoxanide (Figure 13.6g) and zinc.

Nitazoxanide is an antiprotozoal drug that has a broad-spectrum activity against parasites (*Giardia*, *Cryptosporidium parvum*), but also against multiple bacteria and viruses, including norovirus and FCV [197, 198]. No antiviral effect in MNV-infected cells has been observed [30, 199]; however, nitazoxanide reduced viral RNA levels in the HuNoV GI.1 replicon system at a concentration of 5 μg/ml [199]. Despite the fact that the exact mechanism of action is still lacking, nitazoxanide is one of the few molecules that has gone to clinical trials for the treatment of HuNoV infection [200]; however, the antiviral effect of nitazoxanide in norovirus-infected patients is controversial [28, 200, 201]. In clinical trials and patient records, significant reduction of the duration of symptoms was noted [200, 201]; however, many cases in which nitazoxanide was unsuccessful have been reported as well [28]. Moreover, in most reports, the focus is merely on the duration of symptoms and no reduction in viral RNA data is stated [202–204]. Although several clinical trials have been set-up to test the use of nitazoxanide for HuNoV infections, there is no consensus on the beneficial results. These contradictory results ask for caution when prescribing nitazoxanide for the treatment of HuNoV infections.

Zinc is a vital mineral that is ubiquitous within cells and has multiple important biological functions. Zinc is normally absorbed in the small intestine from the consumption of various foods, such as animal products, shellfish, seeds, and nuts. The WHO recommends zinc supplementation in young children, as it reduces the duration and severity of diarrhea [205–209]. However, the exact mechanism of action is still unclear [210, 211].

In a study using rat enterocytes from the ileum, it was shown that zinc inhibits cAMP-stimulated Cl secretion by selectively inhibiting a cAMP-activated basolateral potassium channel [212]. Because zinc has multiple functions in the human body, it is highly likely that there are many mechanisms of action that all contribute to a better clearance of pathogens. Zinc is not pathogen-specific and it has also shown as to reduce the susceptibility and duration of acute lower respiratory tract infections [205, 213, 214]. Many studies suggest that zinc supplementation during acute diarrhea in young children could have a significant effect; however, also a positive effect on the growth of *Campylobacter jejuni* has been observed upon zinc supplementation [215]. Therefore, this treatment strategy needs to be studied in more detail and appropriate fine-tuning based on the specific causative pathogen is needed.

13.3 Vaccine Development

The approval of a HuNoV vaccine could have a tremendous impact on the large extent of HuNoV outbreaks and HuNoV-related deaths. For example, the introduction of two human rotavirus vaccines, Rotarix (GSK Biologicals) and Rotateq (Merck & CO), resulted in a significant reduction of rotavirus-related childhood deaths. The numbers declined globally from almost 600 000 in 2000 to 215 000 in 2013 [216, 217]. Until today, there is no vaccine on the market to prevent HuNoV infections, but significant efforts have been made and are ongoing. One of the vaccine candidates is an oral vaccine based on a non-replicating adenovirus vector expressing the HuNoV GI.1 VP1 gene, containing a double-stranded RNA adjuvant (Vaxart). Phase I clinical trials have been successfully completed in which the vaccine showed to be immunogenic with no reports of any safety concerns [218]. Currently, the most advanced vaccine candidate is a GI.1/GII.4 bivalent VLP-based vaccine which is administered intramuscular (Takeda); it has finished phase II of clinical development [219, 220]. However, after the last clinical trial, no significant reduction of illness was reported after a rechallenge with a GII.4 norovirus [221]. Additional studies are ongoing to evaluate the long-term immunogenicity after a single vaccine administration (NCT03039790) and results from a phase IIb field efficacy study in recruits of the US military (NCT02669121) are pending. The vaccine studies are promising, but we still need to understand more about the immune response during and after a HuNoV infection. Information is lacking due to the fact that potential neutralizing antibodies could not be studied until very recently as there were no HuNoV cultivation systems. From volunteer studies in the 1970s, we know that after a norovirus infection a rather short-term (<five years) immunity develops; however, without cross-protection against other norovirus strains [222–224]. This means that HuNoV infections occur repeatedly during a lifetime.

As seen with the rotavirus vaccines, some factors can condition vaccine effectiveness. First, there is a great diversity in HuNoV genotypes with continuous antigenic evolution and a new pandemic strain emerges every two to three years. Just a few genotypes are covered by the currently studied vaccine candidates [225, 226]. Especially after the emergence of the HuNoV GII.17, it needs to be considered if a GII.4 vaccine would provide sufficient coverage for the future. Second, the immune response upon vaccination of young children

in developing countries can be influenced by malnutrition, immune status, and co-pathogens. Third, the risk of intussusception in young children upon vaccination must be taken into account, although if rotavirus vaccination causes intussusception in young children is still under discussion [227]. The latest clinical trial using the HuNoV GI.1/GII.4 bivalent VLP-based vaccine reported only one child with intussusception upon vaccination (NCT02153112). Taking all of these into consideration, the development of a HuNoV vaccine is very challenging and could likely require annual vaccination [228].

13.4 Conclusion and Perspectives

In this chapter, we reviewed and suggested potential targets and antiviral strategies for HuNoV infections. Despite the fact that it has been more than 50 years since HuNoV was first discovered, no antivirals are available. However, in the last five years, important progress in HuNoV *in vitro* and *in vivo* systems has been made, which will contribute significantly to a better understanding of the HuNoV biology and possible targets for antiviral strategies.

Theoretically, many targets could be explored; however, we believe that the main focus should be toward essential viral replication enzymes, such as the protease and the RdRp. The active sites within these enzymes are conserved across multiple diarrhea-causing viruses, potentially allowing for broad-spectrum antivirals. Targeting viral-specific enzymes will likely also reduce cellular toxicity. Moving toward the development of a pan-diarrheal antiviral, using a combination therapy of antivirals that act on different critical steps in the viral life cycle is likely the way forward.

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14

Antiviral Strategies Against (Non-polio) Picornaviruses

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14.1 Classification and Clinical Impact

Enteroviruses (EV) are non-enveloped positive single-stranded RNA [(+)ssRNA] viruses belonging to the family of the *Picornaviridae*, and are mainly transmitted by the fecal–oral route [1]. The human EV are subdivided into different species according to molecular classification (Table 14.1). EV of group A (EV-A) include Coxsackievirus group A (CVA) and the main causative agents of hand, foot, and mouth disease CVA6, CVA16, and EV-A71 [2]. Recently, EV-A71 has been reported to cause serious epidemics in Asia that are accompanied by severe neurological complications similar to poliomyelitis [3]. EV-B contains six serotypes of Coxsackievirus B (CVB1-6) viruses as well as echoviruses and can cause various pathologies ranging from mild illness to myocarditis, encephalitis, aseptic meningitis, and pancreatitis [4]. In particular, CVB3 is considered to be a major cause of viral myocarditis that can be fatal for neonates. Echoviruses can also cause severe infections in infants that may lead to sepsis [4]. EV-C includes the nearly eradicated polioviruses (PV) (etiological agent of paralytic poliomyelitis) and Coxsackievirus responsible for hemorrhagic conjunctivitis, such as CV-A24. Lastly, enterovirus D comprises the re-emergent EV-D68, recently responsible for epidemics of respiratory diseases and polio-like paralysis [5]. The rhinoviruses (hRV) A–C also belong to the genus of the human EV. Rhinoviruses are the causative agents of the common cold and are the most important factors in the exacerbations of asthma and chronic obstructive pulmonary disease (COPD) (Table 14.1) [6].

14.2 The Enterovirus Replication Cycle

14.2.1 Virion Structure

EV are non-enveloped viruses with a single-stranded positive-sense RNA genome that is contained within a ~30 nm capsid. Although the external features of the capsid can differ

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Table 14.1 Classification and clinical manifestations of human enteroviruses [4, 7].

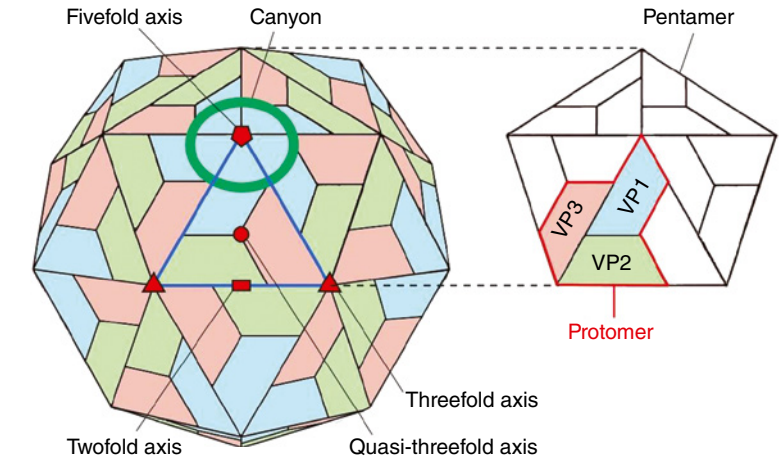
Enterovirus species	Types	Clinical manifestations (non-exhaustive list)
Enterovirus A	Coxsackievirus A [1-8, 10, 12, 14, 16], Enterovirus A [71, 76, 89, 90, 114, 119-121]	• Hand, foot, and mouth disease (HFMD) • Herpangina • Encephalitis
Enterovirus B	Coxsackievirus B 1–6, Coxsackievirus A9, Echovirus [1-7, 9, 11-21, 24-27, 29-33], Enterovirus B [6, 69, 73-75, 77–84, 86-88, 93, 97, 98, 100, 101, 106, 107]	• Viral myopericarditis • Aseptic meningitis • Encephalitis
Enterovirus C	Poliovirus 1-3 Coxsackievirus A [1, 11, 13, 17, 19-22, 24], Enterovirus C [95, 96, 99, 102, 104, 105, 109, 113, 116-118]	• Paralytic disease • Hemorrhagic conjunctivitis (Coxsackievirus A24)
Enterovirus D	Enterovirus D [68, 70, 94, 111]	• Acute respiratory infections • Acute flaccid paralysis • Hemorrhagic conjunctivitis (EV-D70)
Rhinovirus A	~83 types	• Common cold • Exacerbations of asthma and chronic obstructive pulmonary disease (COPD)
Rhinovirus B	~32 types	
Rhinovirus C	~55 types	

Sources: From D. Lugo, P. Krogstad, Enteroviruses in the early 21st century: new manifestations and challenges, *Curr. Opin. Pediatr.* 28 (2016) 107–113 and O.S. Nikonov, E.S. Chernykh, M.B. Garber, E.Y. Nikonova, Enteroviruses: classification, diseases they cause, and approaches to development of antiviral drugs, *Biochemist* 82 (2017) 1615–1631.

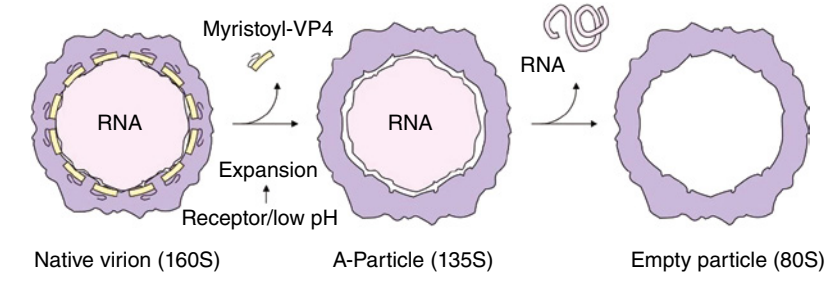
between enterovirus types, their virions have overall a similar architecture. The capsid has an icosahedral symmetry with a pseudo $T = 3$ arrangement and is composed of 60 identical protomers, each protomer consisting of the capsid proteins VP1, VP2, VP3, and VP4 (Figure 14.1a). VP1, VP2, and VP3 constitute the outer surface, whereas the small myristylated protein VP4 resides at the interior of the capsid. The virion has several surface-exposed loops that differ strongly between serotypes and determine their antigenicity. Most EV have a circular depression around the five-fold symmetry axis that is termed the *canyon* and serves as the binding site for many enterovirus receptors [8]. At the floor of the canyon, there is a hydrophobic cavity (the *pocket*) that contains a lipid molecule, the *pocket factor*, which regulates virion stability.

14.2.2 Genome Structure

The 5′-end of the viral single-stranded RNA genome is covalently linked to the peptide VPg (3B), which serves as a primer for RNA replication. The genome consists of a highly structured 5′ untranslated region (UTR), a single open reading frame encoding the viral



(a)



(b)

Figure 14.1 Enterovirus structure. (a) Schematic structure of an enterovirus particle, which is composed of 60 protomers that contain each of the surface proteins VP1, VP2, VP3, and the internal protein VP4. Shown are the asymmetric unit (outlined in blue), the canyon (green), and the different symmetry axes (red). (b) Particle types observed during in vitro uncoating. Sedimentation coefficients are shown between brackets. Uncoating cues (receptor binding or low pH) induce structural changes in the native virion, including particle expansion and release of VP4. The resulting intermediate particle is the A-particle (altered particle). Further stimuli induce release of the RNA genome, resulting in the empty particle. *Source*: From [9]. © 2018, Springer Nature.

polyprotein, and a short 3′ UTR that is terminated by a poly(A) tail. RNA structures in the 5′ UTR form an internal ribosome entry site (IRES), which recruits the translation machinery and is required to initiate polyprotein translation.

14.2.3 Replication Cycle Stages

14.2.3.1 Entry

The life cycle of an enterovirus starts with the attachment to a host cell via receptor binding (Figure 14.2). Some enterovirus serotypes require a single receptor for

infection, whereas other serotypes need to engage multiple receptors [9]. Attachment receptors facilitate binding to the cell surface and may promote virus internalization, whereas uncoating receptors also induce structural changes in the virion that promote genome release. Receptor binding induces endocytic uptake of the virus, after which cellular cues (receptor binding or a low endosomal pH) promote virus uncoating. The uncoating process usually begins with release of the pocket factor, followed by structural changes that result in particle expansion [10], exposure of the VP1 N-terminus [11], and release of VP4. *In vitro*, these events lead to the formation of an uncoating intermediate termed A-particle (altered particle) [12] (Figure 14.1b). Finally, the viral genome exits the virion and enters the cytoplasm via a proteinaceaous transmembrane pore [13].

14.2.3.2 Translation and Replication

After cytoplasmic delivery, the genome is translated into a polyprotein, which is proteolytically processed by viral proteases ($2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$) into capsid proteins (VP0, VP1, and VP3), replication proteins (2A–C and 3A–D), and several functional cleavage intermediates. The proteases $2A^{pro}$ and $3C^{pro}$ also cleave several host proteins in order to suppress the cellular antiviral responses and to promote viral protein expression via *host shut-off*, the arrest of transcription and translation of host mRNA. Genome replication takes place at the surface of virus-induced tubulovesicular membrane structures termed replication organelles, which are mainly organized by the viral proteins 3A and 2BC together with several host proteins, such as ACBD3, PI4KB, and OSBP [14–16] (Figure 14.2). These factors enrich the membranes in cholesterol and phosphatidylinositol-4-phosphate lipids, which are required for efficient replication. The viral genome is replicated by the viral RNA-dependent RNA polymerase $3D^{pol}$, aided by the viral helicase 2C. First, a negative-strand copy of the incoming positive-strand genome is synthesized, yielding a double-stranded replication intermediate. Subsequently, the negative-strand RNA is replicated to form new positive-strand RNA genomes, which can either serve as templates for additional rounds of translation/replication or may be packaged into new virions [9].

14.2.3.3 Assembly and Release

The encapsidation of newly synthesized enterovirus genomes into progeny virions is a stepwise process that is thought to be closely associated with the replication process [13]. The formation of new virions begins with the self-assembly of protomers (consisting of VP0, VP1, and VP3) into pentamers (Figures 14.1 and 14.2). These pentamers assemble around a nascent viral genome to form an immature particle, the provirion. Finally, the genomic RNA catalyzes the cleavage of VP0 into VP2 and VP4, which yields the mature virus particle [17]. The release of progeny virions from an infected cell was long thought to occur exclusively via cell lysis. However, it was shown more recently that enterovirus spread occurs from cell to cell in a non-lytic manner, via extracellular vesicles that contain multiple virions [18, 19]. These may be formed through the engulfment of virions by double-membrane autophagosomes, which subsequently fuse with the plasma membrane to release single-membrane vesicles (Figure 14.2).

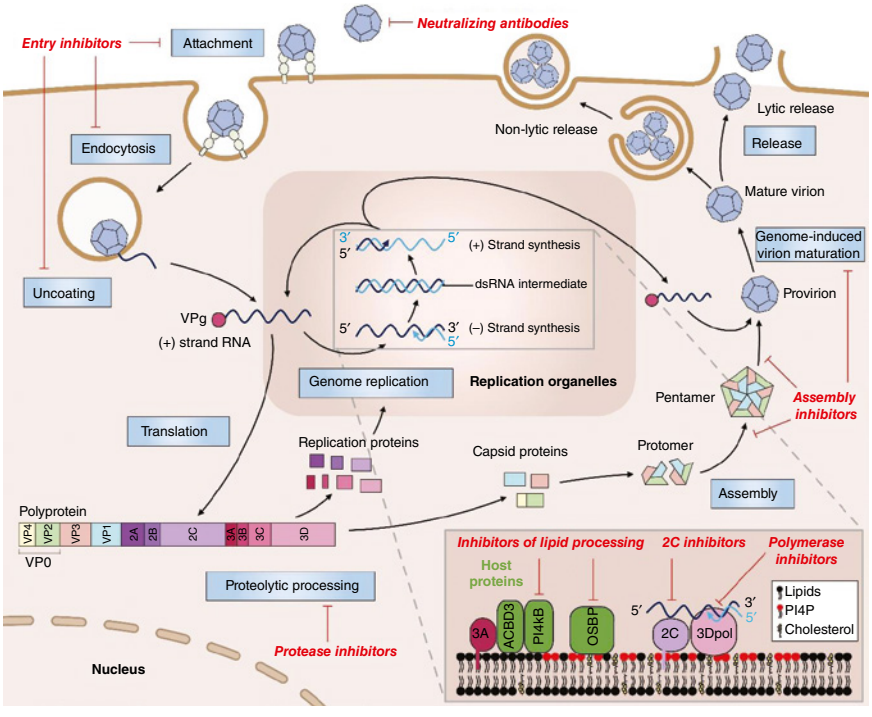


Figure 14.2 The enterovirus life cycle and strategies for its inhibition. After receptor binding, a virion is internalized into the cell via endocytosis and delivers its positive(+)-strand RNA genome across the endosomal membrane into the cytoplasm. The genome is covalently bound to viral protein VPg (3B), which serves as a primer for replication. The genome is translated into a single polyprotein, which is subsequently cleaved into replication proteins (2A–2C and 3A–3D) and capsid proteins (VP0, VP1, and VP3). Genome replication takes place on virus-induced replication organelles, in which a favorable lipid environment is created by viral proteins 2BC and 3A, together with the host factors acyl-CoA-binding domain-containing protein (ACBD3), phosphatidylinositol 4-kinase-β (PI4KB) (which synthesizes phosphatidylinositol-4-phosphate (PI4P)), and oxysterol-binding protein (OSBP) (which recruits cholesterol). The first step of replication is the synthesis of a negative(–)-strand RNA by the viral RNA-dependent RNA polymerase 3Dpol. This strand serves as the template for the synthesis of new (+) strand RNA molecules, which can either enter another round of replication or be incorporated into progeny virions. The capsid proteins can self-assemble into protomers and pentamers, after which the pentamers assemble around a nascent genomic RNA to form the provirion. Finally, the genomic RNA catalyzes the cleavage of VP0 into VP4 and VP2 to yield the mature, infectious virion. Mature virions are released from the host cell via cell lysis or via non-lytic release in extracellular vesicles. *Source*: From [9]. © 2018, Springer Nature.

14.3 Prevention

For the human EV, approved vaccines are available only for PV and EV-A71 (two EV-A71 vaccines were approved in China) [20]. There are two types of poliovirus vaccines. The inactivated poliovirus vaccine (IPV) was developed by Jonas Salk in the 1950s and it is

currently used in most developed countries because of its safety. The live-attenuated oral polio vaccine (OPV) was developed by Albert Sabin and was first licensed in the United States in 1963 [21]. OPV has been widely used because of its lower cost, higher ability to induce mucosal immunity than IPV, and ease of administration. However, the use of OPV is associated with the risks of vaccine-associated paralytic poliomyelitis (VAPP) and development of mutants with the neurovirulence and transmission characteristics of the wild-type virus. These are either vaccine-derived polioviruses (VDPV) or recombinants in the nonstructural region of the genome with co-circulating members of enterovirus of species-C [21]. In 1988, the WHO launched the Global Polio Eradication Initiative (GPEI). These efforts reduced the number of PV cases by >99% [22]. However, in the last years, wild-type PVs are still circulating in parts of sub-Saharan Africa, the horn of Africa, Pakistan, and Afghanistan, whereas many VDPV strains are reported throughout the central African region [22, 23]. Some immunodeficient individuals also excrete PV, including VDPV. Efficient and safe antiviral drugs are needed to clear the excretion of the virus in such individuals. Both EV71 vaccines are formalin-inactivated and based on the C4 genotype virus strain [20].

14.4 Antiviral Strategies Against Enteroviruses

Currently, there is no approved antiviral drug for the treatment or prophylaxis of enterovirus infections. Here, we will review the potential targets for the development of antivirals against EV and provide examples for molecules that act on these targets (Figures 14.3 and 14.4).

14.4.1 Directly Acting Antivirals

14.4.1.1 Early-Stage Inhibitors

The first enterovirus inhibitors in clinical development were the WIN compounds (developed by Sterling Winthrop). These molecules are capsid binders that interact with the hydrophobic pocket underneath the canyon floor, which results in capsid rigidity. As a consequence, these molecules prevent interaction of the virion with its receptor and/or the resulting destabilization of the capsid (A-particle formation) and uncoating [24, 25]. Among these compounds, pleconaril (WIN63843) showed efficient and broad-spectrum activity against EV, Figure 14.3 [26]. Although the compound resulted in some antiviral effects in clinical trials [27, 28], its application as a drug for common cold treatment was rejected by the FDA in 2002 because of safety reasons [29]. Other capsid binders (Figure 14.3), with a similar mechanism of action, include pirodavir (by Janssen Pharmaceutica) [30], BTA-798 (vapendavir, by Biota Pharmaceuticals) [31], ca603 [32], and pocapavir (V-073) [33]. Although BTA-798 successfully passed Phase 1 and the first stage of Phase 2 clinical trials, the compound failed to prove effective *versus* placebo in the second stage of the Phase 2 trial for the treatment of rhinovirus infections in patients with asthma [34].

In addition, two selective poliovirus entry inhibitors were reported (Figure 14.3), i.e. pocapavir (V-073) [33] and H1PVAT [35], with poor or no antiviral activity against other EV. The efficacy of pocapavir has been evaluated in healthy volunteers challenged with the

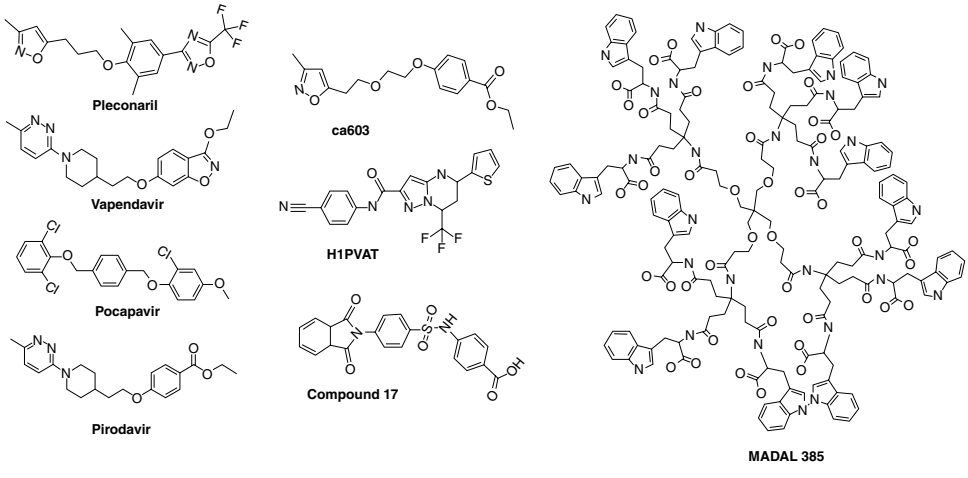


Figure 14.3 Structural formulae of molecules targeting the entry stages of the enterovirus replication cycle. *Source*: Based on [26].

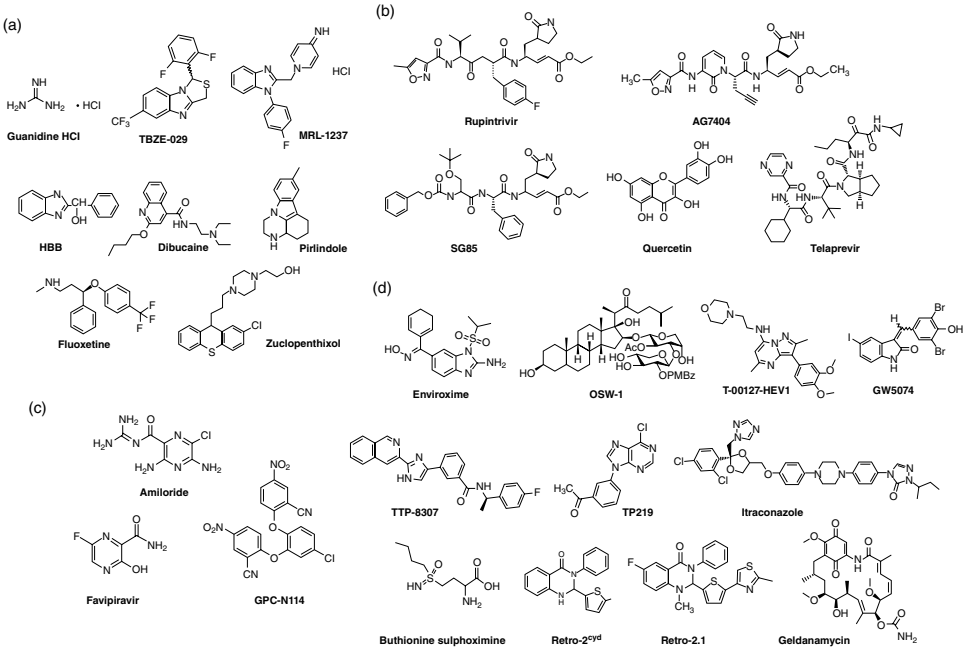


Figure 14.4 Structural formulae of molecules targeting the post-entry stages of the enterovirus replication cycle. (a) Molecules targeting the enterovirus 2C protein. (b) Molecules targeting the enterovirus proteases. (c) Molecules targeting the enterovirus RdRp. (d) Molecules targeting host factors.

monovalent oral PV type 1 vaccine [36]. The drug was well tolerated and markedly enhanced the clearance of virus but drug-resistant variants emerged rapidly in the treated volunteers [36]. Unfortunately, most of the developed capsid binders share the same resistance profile where the virus rapidly acquires resistance mutations in the capsid, mainly in the pocket region, that reduce the binding of the compounds. In addition, hRV-C (associated with severe respiratory infections and childhood asthma exacerbations) are completely resistant to these classical capsid binders because the hydrophobic pocket is collapsed in the particle [37].

A class of tryptophan dendrimers (MADAL compounds) has been reported by our team (Figure 14.3), recently. It potently inhibits the entry of different EV-A71 clinical isolates (IC_{50} values in the nM range) [38]. Unlike classical capsid binders, these compounds bind to the five-fold axis of the viral capsid, thereby preventing attachment of the virus to its cellular receptors PSGL1 and heparan sulfate [38]. Since MADAL compounds have high molecular weights (that may result in low bioavailability *in vivo*), medicinal chemistry efforts are currently ongoing to simplify and reduce the backbone of this series without affecting the antiviral activity [38]. The anti-trypanosomiasis drug suramin was reported as a selective inhibitor of EV-A entry and attachment through interaction with the positively charged region surrounding the five-fold axis of the capsid [39]. Interestingly there is no cross-resistance between suramin and MADAL compounds [38].

Recently, we identified a new druggable pocket in the entero-/rhinovirus particle [40]. The newly discovered pocket is formed by viral proteins VP1 and VP3 and appears to be present in most, if not all entero- and rhinoviruses, including the pleconaril-resistant RV-C species [40]. The compounds targeting this pocket (i.e. compound 17 and its analogues, Figure 14.3) exert activity against CVBs (IC_{50} of 0.7 μM against CVB3) and other EV of groups C and D and even against rhinoviruses A and B [40]. Interestingly, these molecules are not cross-resistant with the classical capsid binders. Binding of the inhibitors to the pocket is believed to stabilize the conformation of a key region of the virion and hence to prevent rearrangements that allow the transition to the A-particle. Therefore, this new pocket can be considered as a promising target for the design of broad-spectrum antivirals against different EV [40].

14.4.1.2 Viral Protease Inhibitors

The protease activity of EV is encoded by the 2A and 3C proteins. Both proteins are chymotrypsin-like cysteine proteases and are necessary for proteolytic cleavage of the viral polyprotein into mature and functional proteins [41]. The cleavage site for the 2A protease is located at the VP1-2A junction whereas the 3C protease cleaves the viral polyprotein at all the other junctions [41].

The 3C protease is an attractive target for drug development due to its highly conserved catalytic domain among EV and its limited similarity to host cell proteases [42]. The most potent and extensively studied 3C inhibitor is rupintrivir (Figure 14.4).

Rupintrivir is a Michael-acceptor peptidomimetic that binds irreversibly to the 3C protease leading to formation of a stable covalent complex [43]. The compound exerts potent and broad-spectrum activity against EV and was able to reduce symptoms and viral load in rhinovirus human challenge trials [44, 45]. However, the molecule failed to result in a significant reduction in disease severity and viral load in naturally infected patients [46].

AG7404 (also named compound 1, Figure 14.4) is a rupintrivir analogue with improved oral bioavailability [47]. AG7404 elicits potent antiviral activity against rhino- and enteroviruses (mean IC_{50} values of 50 and 75 nm, respectively) and was also well tolerated in a phase 1-ascending, single-dose study but was not further developed because of marginal pharmacokinetics [46]. A novel 3C inhibitor was reported (SG85) with broad-spectrum anti-rhinovirus activity (mean IC_{50} of 0.04 μM) that is not cross-resistant with rupintrivir, Figure 14.4 [48, 49]. Structural modeling and the mutations detected in the drug-resistant variants suggested that a hydrogen bond is formed between the backbone amide of the P2 residue of the inhibitor and the side chain oxygen of the serine residue at position 127 of the RHV14 3C protease [49]. In addition, the natural flavonoid quercetin (Figure 14.4) has been reported to inhibit the *in vitro* replication of EV-A71 (mean IC_{50} value of 10 μM) by blocking the purified EV-A71 3C protease [50]. Molecular modeling suggests that the compound blocks the substrate-binding pocket of the enzyme [50].

The HCV protease inhibitor telaprevir (Figure 14.4) was recently shown to inhibit EV-D68 replication in RD cells with an IC_{50} below 1 μM [41]. The molecule was found to be an irreversible inhibitor of the EV-D68 2A protease [41]. Docking of telaprevir in the EV-D68 2Apro model revealed that the α-ketoamide of telaprevir is in close proximity to the catalytic C107 residue of the enzyme, suggesting a covalent complex formation. These results also highlight the importance of the enterovirus 2A protease as a potential drug target and the promise for potentially repurposing of HCV protease inhibitors.

14.4.1.3 2C Targeting Molecules

The enterovirus 2C protein is a multifunctional protein, which is highly conserved among EV. 2C possesses ATPase, GTPase, and helicase activity [51] and has also been reported to be involved in RNA binding, in the interaction with several viral and host proteins, morphogenesis, and uncoating [42]. Guanidine hydrochloride (GuaHCl, Figure 14.4) is a 2C targeting compound with antiviral activity against several EV including PV and Coxsackieviruses [52]. The IC50 of Guanidine HCL is 320-390 μM against Polio and ~2 mM against Coxsackieviruses. GuaHCl inhibits the 2C ATPase activity and its interaction with host cell membranes [53]. The compound also inhibits the initiation of negative-strand RNA synthesis during the poliovirus replication cycle [54]. Other reported 2C-targeting molecules include HBB, MRL-1237 [55], and TBZE-029 [56], which share a benzimidazole scaffold, Figure 14.4a. However, TBZE-029 does not inhibit the 2C ATPase activity [56]. The FDA-approved drugs dibucaine (local anesthetic), pirlindole (antidepressant), and zuclopenthixol (antidepressant) (Figure 14.4a) were identified as *in vitro* CVB3 replication inhibitors [57]. The molecules also elicit antiviral activity against other EV and proved cross-resistant with TBZE-029 and GuHCl, suggesting that 2C is the potential target for these compounds [57]. Similarly, the serotonin reuptake inhibitor fluoxetine (Prozac, Figure 14.4) inhibits enterovirus replication *in vitro* (IC_{50} values 0.4–8 μM) and has been proposed to target the viral 2C protein as well [58].

14.4.1.4 RNA-dependent RNA Polymerase (RdRp) "(3D) Inhibitors"

3D polymerase inhibitors can be classified into nucleoside and non-nucleoside analogues. The nucleoside analogues bind to the active site of the polymerase causing chain termination and/or increasing the error rate when incorporated into the growing RNA chain. On the other hand, non-nucleoside analogues bind to allosteric sites of the enzyme, which

inhibit conformational changes necessary for the efficiency of the polymerase catalytic activity.

The polymerase targeting drug favipiravir (Figure 14.4c) exerts, although weak, activity against some EV such as CVB3 [59] and EV-A71 [60]. Favipiravir-resistant EV-A71 variants were selected that carry a serine to asparagine mutation at position 121 in the fingers subdomain of the viral RdRp [60]. In addition, the conserved lysine residue (at position 159) in the F1 RdRp motif (which has also been reported to be linked to resistance of the chikungunya virus to favipiravir) was shown to be involved in the antiviral activity of favipiravir against CVB3 [59]. Favipiravir appears also to increase the mutation rates during CVB3 replication [59]. Amiloride (anti-hypertensive drug) and its derivatives were shown to inhibit the replication of rhinovirus 2 (IC_{50} values 7–120 μM) and CVB3 (IC_{50} values 2–60 μM) [61] by targeting the viral polymerase and by exerting a mutagenic effect on viral RNA replication [62]. GPC-N114 (Figure 14.4c) is a non-nucleoside inhibitor that exerts broad-spectrum activity against EV (IC_{50} values 0.1–1.7 μM) by targeting the RNA-binding channel of the viral polymerase [63]. The broad-spectrum activity of GPC-N114 makes it a promising RdRp inhibitor for EV.

14.4.2 Host-targeting Antivirals

14.4.2.1 Inhibitors of Lipid Processing

During enterovirus replication, the viral 3A protein recruits phospatidylinositol-4-kinase IIIβ (PI4KIIIβ) to secretory organelle membranes, which generates a phosphatidylinositol 4-phosphate (PI4P) lipids-enriched membrane microenvironment (Figure 14.2). Consequently, PI4P recruits the cellular oxysterol binding protein (OSBP) into these organelles that results in a cholesterol-rich environment which is favorable for viral RNA replication [15]. Molecules that target the PI4KIIIβ-PI4P-OSBP pathway were shown to inhibit enterovirus replication. These molecules include PI4KIIIβ inhibitors (Figure 14.4d) such as enviroxime [64], GW5074 (IC_{50} values 2–6 μM) [65] and T-00127-HEV1 (IC_{50} of 0.8 μM against PV pseudovirus) [66] as well as the OSBP inhibitors (Figure 14.4d), e.g. OSW-1 (IC_{50} values 2.4–9.4 nM) [67], itraconazole (IC_{50} values 0.3–1.6 μM) [68], and TTP-8307 (IC_{50} values 0.27–5 μM) [69].

14.4.2.2 Assembly Inhibitors

Glutathione (GSH) is an essential host factor for stabilization of EV during viral morphogenesis [70, 71]. Molecules that rapidly deplete the intracellular GSH levels such as TP219 (inhibits CVA16 and CVB3 replication with IC_{50} values of 2.7 and 7 μM, respectively) [70] (Figure 14.4d), or which block the GSH biosynthesis, e.g. buthionine sulphoximine (BSO, Figure 14.4d), can inhibit enterovirus replication [71, 72]. Intracellular vesicular transport is involved in several processes during the viral replication cycle including virus assembly and release. Retro-2^{cycl} (Figure 14.4d) and Retro-2.1 are modulators of vesicular trafficking through intracellular redistribution of Syntaxins, which is a family of proteins involved in intracellular vesicle transport [73]. Both compounds inhibited the *in vitro* replication of EV-A71 by blocking the vesicle transport steps required for progeny virus release (IC_{50} values of 12.5 and 0.05 μM, respectively) [73]. Dosing of Retro-2^{cycl} (10 mg/kg) to EV-A71-infected mice resulted in 90% protection from a lethal challenge [73]. Geldanamycin

(Figure 14.4d) is an inhibitor of HSP-90 (heat shock protein 90) that was shown to interfere with the correct folding and maturation of enterovirus capsid proteins [74]. The compound inhibited poliovirus replication in HeLa cells with an IC_{50} of 0.11 μM [74].

14.4.3 Monoclonal Antibodies

Two monoclonal antibodies (mAbs) against EV-A71 (named A9 and D6) were obtained by immunizing mice with a mixture of mature and pro-mature virus particles. Both antibodies potently inhibit EV-A71 replication in cell culture [75]. Mechanistic studies and cryo-EM structures of these mAbs bound with EV-A71 revealed that A9 and D6 block the interaction between the virus and its cellular receptor SCARB2 [75]. In addition, these mAbs, especially A9, resulted in destabilization of the viral capsid structure [75]. In another study, a bispecific mAb against both EV-A71 and CVA16 (named Bs(scFv)4-IgG-1) was developed and was shown to result in 100% protection against lethal disease in mice infected with either EV-A71, CVA16, or both when dosed (10 or 3 μg/g) at 24 hours postinfection [76].

14.4.4 Alternative Strategies

14.4.4.1 Direct Targeting of the RNA Genome

RNA viruses, such as enterovirus, are purely replicating in the cytoplasm. Blockage of their viral cycle via genome degradation could in theory constitute a strategy to block infection at early stages. Two main approaches have been or are being explored to that end: RNA interference (RNAi) and CRISPR-Cas13. Whereas the RNAi strategy exploits a physiological mammalian response and cellular machinery (via Dicer/RISC), the CRISPR-Cas13 requires adaptation of a physiological bacterial response to the mammalian host. siRNA approaches have been developed for several members of the enterovirus genus, mostly EV-A71, EV-D68, and CV-B3 [77–79]. On the other hand, a proof-of-concept study for the CRISPR-Cas13 strategy has been published in the context of other RNA virus infection but not yet for EV [80]. The main limitations of these strategies are the need for pan-enterovirus activity as well as the issue of delivery. Both siRNA and CRISPR strategies rely on RNA sequences or guides that are complementary to the target. Given the molecular diversity of enterovirus, the design of pan-enterovirus RNA sequences/guides that will provide a good coverage is challenging. In addition, these strategies need to be coupled with innovative delivery system to limit off-target effects or systemic toxicity.

14.4.4.2 Dual Target Therapeutics

Although enteroviruses are mostly known as viruses inducing acute infections, there is an increasing body of evidence that associates enterovirus with chronic inflammatory syndromes. Rhinovirus association with asthma and COPD is probably the most relevant example. The development of therapeutic approaches that simultaneously relieve the symptoms of the chronic syndromes being also active against the viral infection would be the most ideal scenario [6]. Interferon signaling could be an important target to alleviate symptoms but also to prime the cells and subsequently prevent viral infection. As an example, azithromycin is a macrolide antibiotic used for the treatment of COPD since it shows a

reduction in exacerbation frequency together with an improvement in quality of life of patients with COPD. Azithromycin transiently induced an increase of IFNβ, IFNλ, and RIG-I expression in bronchial epithelial cells that had been infected with rhinovirus. As a consequence, viral infection and release were reduced [81].

14.5 Conclusions

Recent re-emergence of some non-polio EV and the severe complications associated with their infections indicate the urgent need for potent antivirals against these viruses. Despite the diversity of EV, the development of potent and broad-spectrum rhino-/enterovirus inhibitors should be very well feasible. Potent directly acting antivirals (DAA) are available for the treatment of infections with herpesviruses, HIV, HBV, HCV, and influenza viruses [82]. Most of these drugs target the viral polymerases and/or reverse transcriptase and viral proteases, but also factors such as the HCV NS5A protein, the HIV-co-receptors, and the influenza neuraminidase. Recently, a drug targeting a viral terminase has been approved for treatment of the human cytomegalovirus [83] and capsid binders [84] are presently intensively being researched for hepatitis B [84]. Excellent targets for the development of (broad-spectrum) rhino-/enterovirus inhibitors include the viral 3C, and possibly also the 2A, protease, the 2C ATPase/helicase, and the 3D RdRp. Capsid binders have since long been studied. For various reasons (which may also include the rapid development of resistance to such inhibitors and the fact that hRV-C have a collapsed pocket and is thus not sensitive to capsid binders) none has yet been approved. Recent discoveries in rhino- and enteroviruses biology may also provide new potential targets for drug discovery. For example, the recently discovered VP1–VP3 interprotomer druggable pocket in the capsid of rhino-/enteroviruses [40]. Finally, an important aspect to be studied is whether combination therapy of potent entero-/rhinovirus inhibitors may be needed to avoid/prevent the development of drug-resistance, a strategy which was very successful for HIV and HCV.

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15

Novel Antiviral Strategies Against Emerging Arbovirus Infections

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15.1 Introduction

Arthropod-borne viruses (arboviruses) are distributed worldwide and over 500 species grouped into 8 virus families have been described so far [1]. Several of these viruses constitute a significant global health burden as they can cause a variety of symptoms in humans upon infection [2]. Generally, arboviruses require a minimum of two hosts, a vertebrate and an arthropod host, for successful proliferation and dissemination [3]. In most cases, humans are considered as dead-end or incidental hosts since they usually do not contribute to the transmission cycle by developing viremia which would be high enough to infect arthropod hosts [4].

Even though arboviruses are distributed globally, their majority can be found in tropical areas where they can be transmitted permanently by cold-blooded arthropods [5]. However, several factors facilitate the (re-) emergence of these viruses toward previously naïve populations. An increase in human population densities, combined with urbanization and globalization as well as other demographic and societal changes have significantly influenced transmission dynamics of arboviral diseases [6]. Consequently, exposure frequencies of humans, domestic animals, and livestock to mosquitos have increased and combined with international travel and globalization accelerate the geographic redistribution and outbreak of more frequent and larger arbovirus epidemics. During recent centuries, five human epidemic mosquito-borne arboviruses namely Zika virus (ZIKV), yellow fever virus (YFV), dengue virus (DENV), West Nile virus (WNV), and chikungunya virus (CHIKV) have emerged in both hemispheres [7]. The first four viruses are grouped into the genus of flaviviruses of the family of *Flaviviridae*, which also includes other clinically relevant members (e.g. Japanese encephalitis virus [JEV], tick-borne encephalitis virus [TBEV], and Usutu virus [USUV]) [8]. In contrast, CHIKV is classified as an alphavirus belonging to the family *Togaviridae* [9]. All are transmitted in zoonotic cycles involving nonhuman primates (i.e. prosimans, monkeys, apes), and in case of WNV vertebrate hosts, and arboreal

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mosquitoes. They have entered human-to-human cycles involving *Aedes* and *Culex* spp. mosquito transmission.

15.1.1 Zika Virus

ZIKV infection is the most recent example of a (re-)emerging arbovirus and was declared as a Public Health Emergency of International Concern by the World Health Organization in 2016. ZIKV was first isolated in 1947 from a sentinel monkey during studies of enzootic yellow fever in the Zika forest in Uganda, East Africa [10]. The first potential infections of human individuals were reported during the 1950s in Africa and later in Asia and in the Pacific regions. The early perception ZKIV infection was that of a relatively mild, self-limiting illness, occasionally accompanied by fever, red eyes, joint pain, headache, and a maculopapular rash. Hence due to its mild clinical symptoms, ZIKV infections were rarely investigated and further potentially misdiagnosed due to serological presentation and/or clinical signs resembling mild forms of chikungunya or dengue infection [11]. Therefore, the virus circulated silently for several decades between Africa and Southeast Asia without causing any detectable epidemics and with only 13 reported cases of natural human infections, until in 2007 the first known epidemic outbreak of ZIKV on Yap Island, Micronesia, was reported [11, 12]. Since then several outbreaks of ZIKV spawned large regions of the South Pacific and in 2015/16, large outbreaks occurred in the Americas. Importantly, during the recent epidemics, ZIKV infections were unexpectedly linked to birth defects like severe neurological complications such as microcephaly, brain malformations, and other birth defects of the fetuses of infected pregnant women [13]. Furthermore, in adults, ZIKV infections were found to be associated with the autoimmune disorder Guillain–Barré syndrome [14]. As of September 2019, a total of 86 countries have reported evidence of mosquito-transmitted ZIKV infections.

15.1.2 Yellow Fever Virus

YFV and its associated human disease yellow fever have been known in Africa for centuries. The virus circulates in an enzootic cycle between nonhuman primates and *Aedes* mosquitos in Sub-Saharan rain forests [15]. Most YFV infections are asymptomatic and self-limiting within one week . Patients experience fever, headache, chills, muscle pain, and nausea. However, about 10–25% of patients further develop hemorrhagic fever, a pansystemic viral sepsis with viremia, fever, kidney, and liver damage and with a case-fatality rate of 20–50% in symptomatic cases [16]. Historical records implied that YFV and its mosquito vector were introduced into the Western hemisphere via the slave trade from the fifteenth century onward [17]. Changes in the transmission pattern from a forest or jungle cycle to an urban cycle are characterized by a rapid human to mosquito to human transmission [18]. The virus has been known to cause major epidemics in the Americas and in Africa from the seventeenth century to present times. Within Africa, YFV epidemics arise irregularly and have caused major outbreaks in the past (e.g. in Gambia in 1978–1979, Nigeria in 1969, and Ethiopia in 1962) [19]. In the Western hemisphere, major outbreaks occurred as early as 1737 in coastal Virginia, a major outbreak in Philadelphia with 5000 deaths in the summer of 1793, and during the next few years repeated outbreaks in New York City and north to Boston [20]. Furthermore, several outbreaks were identified in

European ports. In December 2015, a yellow fever epidemic broke out in Angola and the Republic of Congo and quickly spread within densely populated urban areas. International travel facilitated disease spread to Kenya, the Democratic Republic of the Congo, and China [19]. Consequently, in 2016, the WHO declared the current yellow fever epidemic as a global threat [21]. Several YFV outbreaks are continuously being reported within Africa and 32 African countries are being considered at risk of yellow fever, including a total population of 610 million people, among which more than 219 million live in urban settings. The virus is by now considered to be endemic in in tropical Africa, ten South- and Central American countries, and in several Caribbean islands [21]. Although an effective vaccine has been available since the 1930s [15], effective implementation is frequently hampered during outbreaks in resource-limited countries due to limited vaccine supply.

15.1.2.1 Dengue Virus

Dengue viruses are one of the geographically most widespread arboviruses. It is estimated that 390 million people are being infected annually, of which 96 million show a disease manifestation [22]. There are four antigenically distinct DENV serotypes (DENV 1–4) that display comparable epidemiology and cause similar diseases in humans [23]. In contrast to other arboviruses, DENVs are independent of a primitive enzootic forest cycle and have fully adapted to the human host, resulting in direct circulation of the virus between humans and their arboviral *Aedes* vector [24]. It is speculated that DENV has emerged during the fifteenth through nineteenth century in Africa, before it was globally transmitted during the eighteenth and nineteenth century due to the expansion of commercial shipping [25]. For the first time isolated in the 1940s, the virus has by then already spread globally and is considered a major public health problem in most tropical countries [26]. Asian countries are mostly affected and account for 75% of the dengue disease burden, followed by Latin America and African countries [22]. Infections result in a wide variety of clinical manifestations ranging from mild febrile illness to severe and fatal disease [27]. The initial phase can be accompanied by high temperature, headache, vomiting, myalgia, and joint pain, as well as mild hemorrhagic manifestations [23]. Critical phases can include increased vascular permeability (systemic vascular leak syndrome) and shock, with case-fatality rates in patients with dengue shock syndrome as high as 44% [23, 27]. In general, infections with DENV can have different outcomes. A primary infection can provide lifelong protection, however only against the infecting serotype. Secondary infections after infection with a different serotype as well as infections of infants born to dengue-immune mothers often result in more severe cases with hemorrhagic fever/dengue shock syndrome [28]. In late 2015, the world's first dengue vaccine by Sanofi Pasteur, CYD-TDV or Dengvaxia, was licensed and was approved 2019 by the FDA [29, 30]. However, given the increased severity of secondary DENV infections, the vaccine is not approved for use in individuals not previously infected by any DENV serotype or for whom this information is unknown [30].

15.1.3 West Nile Virus

WNV is second to DENV based on the extent of its global distribution [7]. WNV has a broad vector and host tropism and infects next to a variety of *Culex* spp. mosquitos also a broad range of vertebrates including humans, birds, horses, and pigs, with wild birds being

suspected to be the optimal hosts for viral amplification [31]. WNV causes disease in humans with a wide variety of presentations. In 75% of cases, the infection remains asymptomatic; however, it can also lead to fever and malaise as well as to neuroinvasive disease cases (less than 1%) with a case-fatality rate of approximately 10% [32]. The virus was originally isolated in Uganda in 1937 and since then has been shown to be widespread in the Middle East and India. It caused infrequent outbreaks in Israel, Egypt, France, and South Africa [33]. Until the early 1990s, human outbreaks were mainly associated with mild febrile illnesses; however, this changed dramatically with the emergence of new viral strains with likely African origin and increased human disease incidence in parts of Russia and Southern and Eastern Europe and with large outbreaks of increased clinical severity occurring in several countries [32]. In 1999, WNV expanded to the Western hemisphere resulting in increasing reports of deaths and encephalitis in New York. It spread within three years to most parts of the United States and the neighboring countries of Canada and Mexico [34]. As of today, WNV is found in Africa, Europe, Asia, North America, Australia, and the Middle East and is now considered the most important causative agent of viral encephalitis worldwide [33].

15.1.4 Chikungunya Virus

The arthropod-borne CHIKV (CHIKV) is an alphavirus grouped into the family of *Togaviridae* and is the causative agent of the so-called Chikungunya fever (CHIKF). CHIKV was first isolated in 1953 in Tanzania and re-emerged in an unprecedented outbreak between 2005 and 2006 in the Indian Ocean islands [35–37]. In October 2013, the first known autochthonous CHIKV cases in the Western hemisphere have been reported on the island of Saint Martin [38]. Since then, several autochthonous cases were reported from numerous other Caribbean islands leading to the assumption that virus spread to other island countries and expansion into mainland areas of South, Central, and North America are inevitable [38, 39]. Initially rated as a nonfatal disease, CHIKV infection developed as a global health burden. The acute phase of an infection is characterized by symptoms of high fever, severe joint pain, and rash, which improve after 7–10 days. Some patients suffer from severe outcomes by developing neurological complications including encephalopathy and Guillain–Barré syndrome (reviewed in [40, 41]). Moreover, approximately 50% of infected patients develop chronic chikungunya arthritis (CCA) persisting for month or years [42–44]. In some individuals, mostly neonates, the elderly and patients with underlying medical comorbidities, CHIKV infections can lead to death [45, 46]. CHIKV has been classified into three different genotypes: East-Central-South-African (ECSA), West African, and Asian. The virus is mainly transmitted via two species of infected mosquitoes, namely *Aedes aegypti* and *Aedes albopictus*. Furthermore, maternal-to-child transmission with neonates acquiring the infection during birth has been reported [47, 48]. Until the massive outbreak and viral spread in 2005–2006, CHIKV has been primarily found to be transmitted by *Aedes aegypti*. The adaption to the vector *Aedes albopictus* is the result of a single mutation A226V within the E1 glycoprotein [49]. In addition, co-circulation of different CHIKV genotypes enhances the risk of coinfections and recombination. Of note, multiple coinfections with DENV, ZIKV, and CHIKV have also been reported [50]. For example, a simultaneous transmission of CHIKV and ZIKV as result of a single bite of a mosquito is conceivable [51]. These scenarios pose additional challenges to the identification of potent antivirals.

15.2 Intervention Strategies

So far, no specific antiviral therapeutics to prevent or treat arbovirus infection have been licensed. In most cases, infected patients are treated symptomatically using antipyretics and nonsteroidal anti-inflammatory drugs (NSAIDs). Therefore, currently the best strategy to prevent arbovirus infection and viral spread remains vector control and avoiding mosquito bites. However, extensive global efforts to understand the biology and pathogenesis of various (re-)emerging arboviruses are carried out to identify potential molecular targets for antiviral compounds. Furthermore, by screening large compound libraries or repurposing of clinically approved drugs, great efforts have been made to identify drug candidates directed against viral proteins (DAA; direct-acting antivirals) and against host targets (host-targeting antivirals). Potential broad-spectrum antiviral activity has been observed for various compounds during target-specific or phenotypic *in vitro* and *in vivo* assays (Table 15.1).

15.3 Genome Organization and Viral Replication Cycle

All flaviviruses share a similar genome organization: one long open reading frame (ORF) which is translated as a polyprotein and cleaved by virus-encoded and host proteases into three structural proteins (the capsid [C], precursor membrane [prM], and envelope glycoprotein [E]) that form the viral particle and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) that fulfill several roles during viral replication (Figure 15.1) [56]. Both E and prM form the surface structure of the virion. Whereas the viral E protein mediates virus adsorption, internalization, and fusion with the host cell membranes, prM acts as a chaperone to facilitate conformational folding of E [57]. The surface structural protein E is the primary viral protein against which neutralizing antibodies are produced [58]. The capsid protein mediates assembly and packaging of the viral RNA genome to form the viral nucleocapsid. Furthermore, it has a crucial role during viral particle assembly where it associates with other proteins on lipid droplets and the endoplasmic reticulum (ER) [59]. The nonstructural proteins coordinate viral replication, assembly, proteolysis, maturation, and host immunity regulation [60]. NS1 has several roles; the intracellular dimer form contributes to genome replication and virion maturation, whereas the secreted hexamer plays a role in immune evasion [60]. The NS2 region encodes two proteins, NS2A and NS2B [61]. NS2A is involved in the proteolytic processing of the C terminus of NS1, which forms a part of the replication complex and modulates host antiviral responses, while NS2B acts as a cofactor to the N-terminal region of NS3 and its associated protease activity [61]. Together they are responsible for the cleavage and post-translational modification of the viral polyprotein [62]. Next to its protease function, the NS3 C-terminal domain possesses RNA helicase,

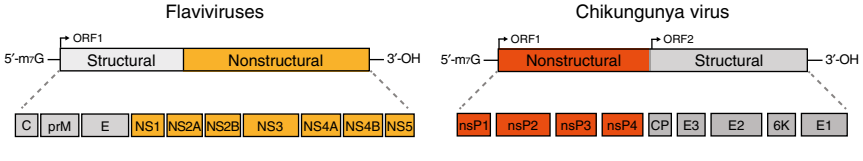

Figure 15.1 Schematic representation of the flavivirus (left) and CHIKV genome organization (right).

Table 15.1 Antiviral drugs and vaccines for emerging arboviruses.

Family	Genus	Virus(es)	Vaccine	Antivirals	Discovery of virus
Flaviviridae	Flavivirus	Yellow fever virus	Yes	No	1927 [52]
		Dengue viruses	Yes	No	1960s [53]
		West Nile virus	No	No	1937 [54]
		Zika viruses	No	No	1947 [55]
Togaviridae	Alphavirus	Chikungunya virus	No	No	1953 [36]

nucleoside, and RNA triphosphatase activities and contributes to viral RNA replication and virus particle formation. In addition, NS2B-NS3 is involved in the assembly of the flavivirus replication complex and modulates viral pathogenesis and the host immune response [63]. The NS4A protein associates with the virus replication complex and induces ER membrane rearrangements and autophagy to promote viral replication [64–67], while the NS4B protein modulates the host innate immune response and the helicase activity of NS3 [68–70]. The NS5 protein is composed of an N-terminal methyltransferase (MTase) domain and a C-terminal RNA-dependent-RNA polymerase (RdRp) domain [67].

In contrast, the genome of CHIKV contains two ORFs which encode for five structural proteins (including the glycoproteins E1, E2, and E3 as well as 6k and capsid protein) and four nonstructural proteins (nsPs1–4) which mediate genome replication, RNA capping, polyprotein cleavage, and other functions required for viral replication (Figure 15.1) [71].

The replication cycle of flaviviruses along with the most relevant sites of pharmacological intervention are schematically outlined in Figure 15.2a. In short, following binding to a host cell through interaction with specific attachment factors, viral particles are internalized by clathrin-mediated endocytosis. Endosomal acidification triggers fusion of viral and endosomal membranes and thereby releases the viral nucleocapsid into the cytosol [72]. The viral genome is then translated resulting in the synthesis of a polyprotein that is co- and post-translationally cleaved by cellular and viral proteases [56, 73].

Flaviviruses subsequently initiate the formation of a replication complex on modified ER membranes which mediates the replication of the genomic RNA (+) template into RNA (−), serving as a template for new RNA (+) synthesis. Viral assembly then begins through budding of immature virions containing a newly synthesized RNA genome into the ER lumen. Various protease and pH-dependent maturation steps in the ER and Golgi complex including different post-translational modifications generate mature infectious particles, which are released through the secretory pathway [56, 74–76].

In contrast, upon release into the cytosol, the CHIKV genome is only partially translated into a polyprotein, which consists of the four nsP1–4 [74]. NsPs subsequently assemble and modify the plasma membrane to form viral replication compartments (spherules) to protect viral RNA intermediates from degradation and recognition by cellular pattern-recognition receptors [71]. As infection progresses, spherules are internalized to the surfaces of modified endo- and lysosomal compartments to form so-called cytopathic vesicles (CPV) [77]. Subsequent RNA translation produces the structural polyprotein (C-E3-E2-6K-E1) and autoproteolysis releases free capsid protein (C) into the cytoplasm which assembles with the viral RNA genome to form nucleocapsids. The remaining polyprotein is then

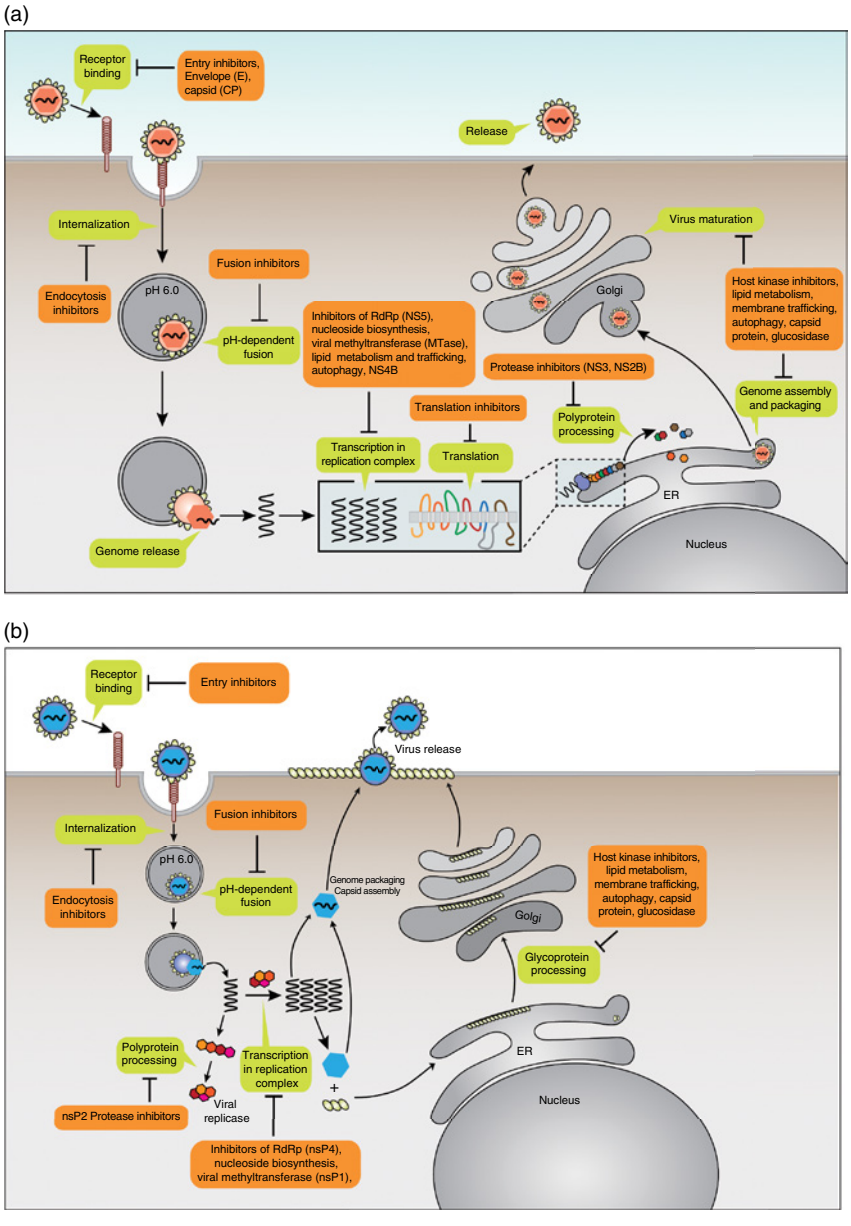

Figure 15.2 Schematic representation of the replication cycle and polyprotein organization for flaviviruses (a) and CHIKV (b) and potential sites of pharmacological intervention. C, capsid protein; E, envelope glycoprotein; NS (Flavivirus) or nsPs (CHIKV), nonstructural protein; prM, pre-membrane glycoprotein; RdRp, RNA-dependent-RNA polymerase; MTase, methyltransferase.

directed to the ER where it undergoes cleavage into individual proteins. The E2/E1 proteins are further post-translationally modified and transit through the secretory system to the plasma membrane where they assemble with previously formed nucleocapsids to release mature progeny viruses [71, 78] [Figure 15.2b].

15.4 Targets For Antiviral Therapy

A viral target protein should ideally combine two features: it should be essential for the viral replication cycle and have a low frequency of "tolerated" (nonlethal, but resistance-conferring) mutations [79]. Direct-acting antivirals (DAAs) often facilitate the emergence of resistance in RNA viruses, as the extraordinarily high mutation rates paired with short generation times promotes rapid generation of viral variants, which escape treatment [80]. RNA viruses thus do not exist as a homogenous clonal population within an infected host, but rather diversify into a heterogenous population (a so-called "quasispecies"), which hovers around a most fit "master sequence" [81]. In contrast, host-targeting antivirals are less likely to result in the selection of resistant viruses as host factors are genetically more stable than viral factors [79]. Nevertheless, essential structural or functional features within viral proteins also often have a high genetic barrier to mutations and thus show a high degree of evolutionary conservation. A high degree of structural conservation across different viruses can further indicate targets with potential for broad-spectrum relevance and further enables to estimate the likelihood of mutations conferring drug resistance [79].

Several of the enzymatic motifs involved in protein and RNA processing such as the catalytic motifs of the viral protease (NS3 flavivirus; nsP2 CHIKV), the ATP- and RNA-binding regions of the RNA helicase, and the substrate or metal recognition motifs of the RdRp (flavivirus: NS5, CHIKV nsP4) are highly conserved among different RNA viruses [82–84]. Both NS5 and NS3 proteins of different flaviviruses, including HCV, have thus emerged as key targets for antiviral drug development. The pangenotypic prodrug sofosbuvir is an example of an efficient direct-acting antiviral drug that interferes with the NS5B polymerase of HCV [85]. On the other hand, the nonstructural proteins NS2 and NS4 display remarkably high interspecies variability and therefore are more prone to drug resistance-conferring mutations and further provide fewer promising targets for antiviral drugs with broad activity. Notably, the treatment of chronic hepatitis C virus infection has been revolutionized by the development of several DAAs, including nonstructural proteins 3/4A (NS3/4A) protease inhibitors (PIs), NS5B nucleoside polymerase inhibitors (NPIs), NS5B non-nucleoside polymerase inhibitors (NNPIs), and NS5A inhibitors [86]. Table 15.2 provides an overview of selected DAAs and their current stage of development, mostly repurposed drugs from e.g. HCV.

15.5 Direct-acting Antivirals (DAAs)

See Table 15.2.

15.6 RNA-dependent RNA Polymerase Inhibitors

The RNA-dependent RNA polymerase (RdRp) (flavivirus: NS5, CHIKV nsP4) is the most conserved gene among the members of the *Flaviviridae* family [114]. Since human cells lack the RdRp domain, this viral class of enzymes provides a highly attractive target for the

Table 15.2 Selected direct-acting antivirals for different arboviral viral proteins and the respective viruses/biological systems which have been used to evaluate the respective drugs.

Target	Compound	Virus	Biological system	Comment
RdRp	7DMA	ZIKVV WNV DENV	Cell culture (EC_{50} 0.6 μM), mice [79, 87] Cell culture (EC_{50} 0.3 μM), mice [88] Cell culture (EC_{50} 15 μM) [89], mice [79]	Nucleoside analogue originally developed as a treatment for HCV but failed during clinical trials [90].
	BCX4430	ZIKV DENV YFV WNV JEV CHIKV	Cell culture (EC_{50} ~5 μg/ml) [91], mice [91] Cell culture (EC_{50} 32.8 μM) [92] Cell culture (EC_{50} 14.1 μM) [92], Hamster [93] Cell culture (EC_{50} 1.5 μM) [94] Cell culture (EC_{50} 43.6 μM) [92] Cell culture (EC_{50} >100 μM) [92]	Nucleoside analogue with potential broad-spectrum activity. A phase 1 trial of clinical safety and pharmacokinetics has been successfully completed [79].
	Sofosbuvir	ZIKV DENV YFV CHIKV	Cell culture (EC_{50} 1–5 μM), mice [95–98] Cell culture (EC_{50} 1.4 μM) [99, 100] Cell culture (EC_{50} 4.2 μM), mice [101] Cell culture (EC_{50} 17 μM) [102], mice [102]	FDA-approved nucleoside analogue inhibitor for the treatment of chronic HCV [103, 104].
Protease	Temoporfin	ZIKV	Cell culture (EC_{50} 0.02 μM), mice [105]	Photosensitizer drug approved by the European Union for the treatment of squamous cell carcinoma of the head and neck.
	Erythrosin B	ZIKV DENV YFV WNV	Cell culture (EC_{50} 0.6 μM) [106] Cell culture (EC_{50} 1.2 μM) [106] Cell culture (EC_{50} 0.4 μM) [105] Cell culture (EC_{50} 0.7 μM) [106]	FDA-approved food additive.
NS4B	Lycorine	WNV DENV YFV	Cell culture (EC_{50} 0.2 μM) [107] Cell culture (1.2 μM) [107] Cell culture (1.2 μM) [107]	Natural alkaloid found in various *Amaryllidaceae* species.
MTase	4-HPR	ZIKV DENV	Cell culture (EC_{50}.2.3 μM) [108], mice [109] Cell culture (EC_{50} ~1 μM), mice [110]	Potential chemotherapeutic agent with different phase 1/2 clinical trials for breast cancer treatment completed [111].
NS2B	NSC135618	DENV ZIKV WNV YFV	Cell culture (EC_{50} 0.81 μM) Cell culture (EC_{50} 1 μM) Cell culture (EC_{50} 1.27 μM) Cell culture (EC_{50} 0.28 μM)	[134] [134] [134] [134]

(Continued)

Table 15.2 (Continued)

Target	Compound	Virus	Biological system	Comment
NS3 helicase	ST-610	DENV	Cell culture (EC_{50} 0.272 μM)	[135]
			AG129 mice	[135]
Nonspecific	Ivermectin	DENV	Cell culture (EC_{50} > 1 μM) [112]	FDA-approved antiparasitic agent.
		JEV	Cell culture (EC_{50} 0.3 μM) [112]	
		YFV	Cell culture (EC_{50} 3.1 μM) [112]	
		CHIKV	Cell culture (EC_{50} 0.6 μM) [113]	
	Chloroquine	CHIKV	Cell culture [158]	Phase 3 clinical trial (ClinicalTrials.gov Identifier: NCT00391313) showed no difference in infection outcome.
		ZIKV	Cell culture (EC_{50} 9.82–14.2 μM) [157]	

development of broad-spectrum antivirals. RdRp inhibitors can be classified into two groups: nucleoside analogues and non-nucleoside inhibitors (NAI and NNI, respectively) [115]. Nucleoside analogues are among the largest class of small molecule-based viral inhibitors. Current efforts to identify novel compounds targeting the RdRp of different emerging arboviruses have generated encouraging results in cell culture and animal model systems. Whereas NNI directly bind to the RdRp to inhibit RNA synthesis, NAIs are substrate analogues which either act as chain terminators upon incorporation into the nascent RNA chain or promote accumulation of mutations in viral genomes beyond an error threshold that eventually results in the loss of viral infectivity [116–119]. Numerous nucleoside inhibitors based on the structure of the four natural nucleotides have been designed and evaluated regarding their antiviral activity *in vitro* [115]. 7-Deaza-2′-C-methyladenosine (7DMA) was originally developed as a treatment for HCV, but despite promising results in animal studies, it ultimately failed in clinical trials [90], potentially due to mitochondrial toxicity [96, 120]. Subsequent studies further showed broad-spectrum antiviral activity against ZIKV, WNV, DENV *in vitro* and *in vivo* [79]. Since 7DMA could potentially be applied during short-term therapy of acute arboviral diseases, it may still represent a promising antiviral candidate to date [96]. The adenosine analogue BCX4430, originally also intended as a treatment for HCV and Filovirus infections was shown to exert broad-spectrum antiviral effectiveness *in vitro* against mosquito-transmitted viruses such as YFV, DENV-2, JEV, CHIKV, WNV, and ZIKV [92–94]. An antiviral activity and a favorable pharmacokinetic profile against YFV and ZIKV were further observed during *in vivo* animal studies and a phase 1 clinical trial (ClinicalTrials.gov identifier: NCT02319772) has been completed [91, 93, 121].

The conversion of NAI into a biologically active triphosphate by cellular kinases is an essential and often limiting step for the function of all NAI but mostly not assessed during initial biochemical assays. The efficiency of this step can vary greatly among individual host cellular types due to differences in expression/activity levels of nucleoside kinases and other components involved in nucleoside metabolism and transport, and therefore result in

cell-type-dependent antiviral activity [115]. Using a monophosphate prodrug approach based on the introduction of the phosphorylated group into the 5′ nucleoside position, this limitation can be partially bypassed and has been successfully used during the development of the phosphoramidate prodrug sofosbuvir [122]. Sofosbuvir is a commercially available, FDA-approved RdRp inhibitor for the treatment of Hepatitis C [103, 104]. Within cells, the prodrug is converted to the pharmacological active compound GS-461203 (2′-deoxy-2′-α-fluoro-β-C-methyluridine-5′-triphosphate), which acts as a defective substrate of the RdRp to inhibit viral RNA synthesis [123]. Beyond HCV, the antiviral activity of sofosbuvir was also confirmed during cell-based assays for different emerging arboviruses, including ZIKV, DENV, YFV, and CHIKV [95, 99, 100, 102, 124, 125]. Antiviral activity against ZIKV varied greatly among cell lines with different origin [96]. This cell-line-dependent antiviral activity of sofosbuvir was later shown to correlate with the intracellular concentration of its active triphosphate, implying a link to cell-specific metabolism [126]. Additionally, sequence analysis of ZIKV isolated from sofosbuvir-treated cells further revealed an increase of mutations in the viral genome, suggesting a secondary mode of action by increasing error-prone replication [127]. Oral treatment with sofosbuvir was shown to reduce ZIKV-induced mortality in various mouse model systems and further reduce ZIKV levels from 60 to 90% in different anatomical compartments [95–98]. Likewise, sofosbuvir treatment of YFV- or CHIKV-infected mice reduced virus-induced mortality [101, 102]. Due to its safety profile in humans and antiviral effects *in vitro* and in mice, sofosbuvir may represent a novel therapeutic option for the treatment of different emerging arboviruses. However, since sofosbuvir was designed to target HCV within liver cells, it remains to be determined if sufficiently high exposure levels are also reached within the respective viral replicating sites to achieve antiviral effects in humans. Overall, the development of specific nucleoside inhibitors is often complicated due to efficacy and toxicity issues and in the case of NAI repurposed from HCV/HIV treatment, differences in the cellular tropism.

15.7 Protease/Helicase Inhibitors

Following the RdRp, the NS3 protein (nsP2 for CHIKV) is the second-most conserved viral protein among the family of *Flaviviridae* [74, 128]. NS3/nsP2 perform a variety of essential functions within the viral life cycles rendering them attractive drug targets. The N-terminal (C-terminal for CHIKV) protease domain mediates proteolytic processing of the viral polyprotein, while the C-terminal (N-terminal for CHIKV) domain possesses ATPase/helicase activity to unwind the double-stranded RNA (dsRNA) intermediates [78, 128]. Considering its structural conservation and multifunctional roles during viral replication, targeting the protease/helicase is widely considered as an attractive strategy for the development of broad-spectrum antivirals [129].

Targeting viral proteases has been successfully applied for the treatment of HCV and HIV with various peptidic and pseudopeptidic compounds currently in clinical use [130], see also the article by Stephan in this book. However, examination of selected HIV/HCV PIs against DENV and CHIKV revealed only weak antiviral activity and a lowered selectivity index ($SI = CC_{50}/EC_{50}$) for the individual drugs [131]. Nonetheless, given the encouraging results from HCV and HIV, and due to straightforward enzymatic assays and availability of structural information, various potential inhibitors of the NS3 protease domain have been

evaluated during both high-throughput screens (HTS) and structure-based drug design approaches [79]. Unfortunately, so far, most attempts to develop potent flavivirus PIs which focused on the NS3 active site had limited success, potentially complicated by two features: First, due to a rather shallow substrate binding site, potential inhibitors likely rely on only a few binding sides, creating a low barrier of resistance-conferring mutations as observed for HCV PIs [132]. Second, the active site displays a strong preference for substrates with polybasic recognition sites. However, the incorporation of basic or polar functional groups in potential inhibitors frequently limits bioavailability and therefore antiviral efficiency in vivo [79]. Hence, inhibitors derived from substrate-mimicking peptides often display highly potent activities during initial *in vitro* studies but may display poor *in vivo* efficacy due to poor penetration of charged peptides across cellular membranes [133, 134]. Notably, the flavivirus NS3 protease relies on NS2B as a cofactor to perform its function. Targeting the NS3–NS2B interaction thus provides a different strategy to inhibit flavivirus NS3 protease function [129, 135]. Unlike the flat and featureless active site, the NS3 pockets containing the NS2B interaction residues are deep and hydrophobic [136]. A HTS assay to identify inhibitors of the NS2B–NS3 interaction revealed three compounds (temoporfin, niclosamide, and nitazoxanide) with broad-spectrum anti-flavivirus activities (Table 15.2). The most potent drug, temoporfin, not only inhibited ZIKV infection in human placental and neural progenitor cells, but also prevented ZIKV-induced viremia and mortality in mouse models [105]. Likewise, erythrosin B (EB), an FDA-approved food additive was shown to interfere with the NS3–NS2B interaction and to significantly reduce the titers of DENV2, ZIKV, YFV, JEV, and WNV *in vitro* [106]. Due to its excellent safety profile, EB might represent a promising candidate for future pharmacological studies. Furthermore, the previously described structure of the NS2B/NS3 protease complex opens the possibility to perform "structure-guided" virtual screens to identify potential small molecule inhibitors [137, 138]. For example, a virtual screen of a library from the National Cancer Institute Developmental Therapeutics Program (NCI DTP) to identify inhibitors of the viral protease cofactor NS2B revealed the compound NSC135618 as a potent inhibitor of several flaviviruses including DENV, ZIKV, WNV, and YFV [139]. The development of compounds targeting the NS3–NS2B interaction thus seems to provide a promising approach without suffering from the previously discussed drawbacks of active-site inhibitors.

So far, only limited progress has been made toward the development of specific inhibitors against the flavivirus helicase and currently no helicase inhibitors have been approved for clinical trials or usage. The benzoxazole analogue, ST-610, was reported to display low cytotoxicity and inhibit viral replication of DENV-1–4 and YFV (but not WNV or JEV) *in vitro* and to lower viral load in a mouse model upon DENV infection [140]. Further compounds such as the pyrrolone derivative (compound 25) and suramin were found to lower WNV, DENV, and CHIKV replication in cell culture [141–143]. Nonetheless, the development of specific inhibitors is frequently complicated by the need to achieve selectivity against host enzymes with similar enzymatic functions.

15.8 Envelope Protein Inhibitors

Viral envelope proteins mediate virus entry to initiate an infection. Unlike viral proteases and polymerases, envelope proteins lack a conserved active site providing a major barrier in the development of effective antivirals. The flavivirus envelope protein is composed of

three ectodomains; a conserved pocket (βOG pocket) located between domains I and II mediates conformational changes in the E protein necessary for membrane fusion and viral entry [144]. The βOG pocket has therefore been suggested as potential target to develop broad-spectrum antivirals against multiple, mosquito-borne flaviviruses [145]. In agreement with this, different pyrimidine and cyanohydrazone inhibitors were recently shown to efficiently block ZIKV, WNV, and JEV replication *in vitro* and inhibit DENV *in vivo* [146–148]. A virtual docking screening approach further identified different potential compounds, including NITD-448, compound 6 (with a quinazoline nucleus), P02, D02, D04, D05, A5, and 1662G07 [149]. Some of these compounds such as A5 were further tested in cell culture and showed inhibition of WNV, DENV, and YFV [117]. Likewise, various potential inhibitory compounds including phenothiazine and bafilomycin for CHIKV were identified *in silico* and could be further evaluated during future studies [150, 151].

15.9 Capsid Protein Inhibitors

Compared to other viral proteins, little efforts have been carried out to identify potential capsid protein inhibitors. A single small molecule inhibitor, ST-148, has been found to inhibit DENV replication in multiple cell types and to reduce viral load in a mouse model [140]. On the other hand, ST-148 did not affect JEV replication and displayed only weak activity against YFV. Targeting a hydrophobic pocket of the CP of CHIKV using picolinic acid blocked its interaction with the E2 protein and inhibited CHIKV replication *in vitro* [152].

15.10 NS4B Inhibitors

The flavivirus NS4B protein forms an integral membrane protein which localizes to intracellular membranes and functions as an essential component of the viral replication complex. Since the NS4B proteins of flaviviruses are orthologous genes (genes encoded at the same position of the viral ORF) but not homologous (evolutionarily related) and therefore frequently differ in their structure and/or function in the viral life cycle inhibitory effects of specific compounds often remain limited to a single virus [68, 99]. The natural alkaloid lycorine (Table 15.2) potently inhibited viral replication of WNV, DENV, and YFV in cell culture and so far is the only identified compound with potential broad-spectrum antiviral activity [153].

15.11 Methyltransferase Inhibitors

Besides its function as RdRp, the N-terminal domain of the NS5 protein of flaviviruses and the nsP1 protein of CHIKV mediate capping of the 5′ end of newly synthesized viral RNAs, which is critical for viral genome replication. Structural conservation between the core domains of various MTases harbors the potential to identify inhibitors with broad-spectrum antiviral activity, but also complicates the design of virus-selective inhibitors without affecting host MTases [154]. Various inhibitors of viral MTases for CHIKV and flaviviruses

have been identified by cell-based assays, virtual screening, and structure-based design [117, 155–158]. Despite the identification of specific viral MTase inhibitors during structure-based design approaches, the respective compounds such as the natural nucleoside sinefungin frequently lack efficient membrane permeability and therefore robust antiviral activity [117, 159, 160]. The synthetic retinoid, N-(4-hydroxyphenyl)-retinamide (4-HPR, Table 15.2), has been extensively examined in humans for treatment or prevention of a variety of cancers [111]. Recent studies further reported antiviral activity of 4-HPR against DENV and ZIKV in cell culture and different mouse models [108–110, 161]. Although the exact inhibitory mechanism remains unclear, 4-HPR has been proposed as a candidate for drug repurposing due to its demonstrated activity against multiple flaviviruses and its well-established safety and tolerability in humans [109].

15.12 Inhibitors with Nonspecific Action

Various compounds with antiviral activity against different arboviruses but unknown mechanism have further been identified during different phenotypic assays. Repurposing strategies are a particularly attractive option due to the established pharmacokinetics and safety profiles in humans. This strategy further holds the potential to yield "emergency" antivirals, for which a higher incidence of side effects and limited broad-spectrum activity can be tolerated [79]. Ivermectin (Table 15.2), a broadly used antihelminthic drug, was shown to selectively inhibit the replication of JEV, DENV, CHIKV, and YFV in cell culture [112, 113]. Given that ivermectin has been used for the treatment of a variety of parasitic diseases for over two decades, assessing its potential for the treatment of arboviruses infections in clinical trials maybe feasible. Chloroquine is used as an antimalarial drug since 1934 and is suggested to inhibit viral entry or assembly and has further been shown to exert antiviral effects against various flaviviruses as wells as CHIKV (Table 15.2) [162–165]. Of note, chloroquine has been tested in the phase 3 clinical trial "CuraChik" (ClinicalTrials.gov Identifier: NCT00391313) in patients with clinical chikungunya disease diagnosed within less than 48 hours; however, treatment showed no significant difference in outcome regarding the duration of febrile arthralgia or decrease in viral load. Consequently, administration of chloroquine to treat acute CHIKV infection was abandoned [166]. Nevertheless, considering the extensive clinical experience with this drug in the context of malaria, chloroquine may be an attractive candidate for prophylactic use. An overview for more information about potential candidates can be obtained from the following reviews [79, 165, 167–169].

15.13 Host Targeting Antivirals

Targeting of host factors to combat viral infections can provide an attractive antiviral strategy, given that host cell processes are often employed by multiple viruses and are less prone to the emergence of drug-resistant variants [170]. However, it is important to consider that cellular proteins do function in a complex network of interactions which could facilitate potential off-target effects and toxicity. Nevertheless, it has been shown previously that interference with host cell molecules or pathways that are required for viral replication can be a promising approach [171].

15.14 Host Cell Nucleoside Biosynthesis Inhibitors

The availability of nucleosides is one of the main requirements for virus replication. Host enzymes that are involved in host cell nucleoside biosynthesis such as inosine monophosphate dehydrogenase (IMPDH) and dihydroorotate dehydrogenase (DHODH) offer attractive targets for a broad-spectrum antiviral therapy. One of the most prominent drugs interfering with viral RNA synthesis is the guanosine analogue ribavirin (RBV, Table 15.3). Next to its direct inhibition of IMPDH, it is believed that RBV exerts in addition immunomodulatory effects as well as direct effects on the virus replication, including interference with RNA capping, polymerase inhibition, and lethal mutagenesis [216]. RBV has been used in combination therapies against HCV [217] and it has been shown to act as an antiviral *in vitro* against various flaviviruses as well as CHIKV; however, antiviral efficiency was often dependent on the cell line [175, 218, 219]. Despite its potent antiviral activity *in vitro* and *in vivo*, clinical studies often failed to show a potent antiviral effect against flaviviruses [175, 176, 220], indicating that it is not a suitable flaviviral therapeutic. There are only few studies testing the efficacy of RBV *in vivo* against CHIKV; however, some studies suggest that in mouse models, a combination therapy of RBV together with non-steroidal anti-inflammatory drugs or doxycycline can reduce CHIKV replication and attenuate its infectivity [177, 178]. In addition, RBV was tested in an observational study with 20 CHIKV-infected patients. Seven out of ten patients treated with RBV for seven days completely recovered in the monitored time period of four weeks follow-up [179]. Due to the lack of a placebo control group and the limited number of patients, this study gives a hint for the potential of RBV to treat CHIKV infection and further research should be conducted to confirm its efficacy.

In addition to RBV, several other IMPDH inhibitors have been developed, including 5-ethynyl-1-*β*-*d*-ribofuranosylimidazole-4-carboxamide (EICAR; 5-ethynylribavirin, Table 15.3) or the non-nucleoside mycophenolic acid, which both showed increased antiviral activity against selected flaviviruses [79]. However, whereas the former resulted in higher cytotoxicity [221], the latter exhibited immunosuppressive activity [222], rendering both not suitable for clinical implementation. Other IMPHD inhibitors that have been tested against various emerging viruses include merimepodib and azathioprine, the former showing an antiviral activity against several viruses including ZIKV and CHIKV [174] and the latter against ZIKV *in vitro* [172] (Table 15.3).

Next to IMPDH inhibitors, interference with the pyrimidine biosynthesis via inhibition of the DHODH has been exploited. Brequinar has been shown to be potently antiviral against selected flaviviruses *in vitro* (Table 15.3); however, due to its low therapeutic index, it was not approved for clinical use [223]. Other DHOD inhibitors include the indole derivative compounds A3 and 2-(4-benzyl-3-ethoxy-5-methyl-1H-pyrazol-1-yl)pyrimidine, of which the compound A3 showed antiviral activity against certain flaviviruses *in vitro* [180, 224]. Further studies will be needed to evaluate their potential as broad-spectrum antiviral compounds against emerging arboviruses *in vivo*.

Despite their potential as broad-acting antiviral compounds, nucleoside biosynthesis inhibitors often have severe limitations due to their often narrow therapeutic window as well as a potential for immunosuppressive effects which render them not suitable for treatment with expected coinfections, pregnancy, or extended (prophylactic) dosage regimens. Furthermore, resistance-associated mutations can occur due to various mechanisms requiring combination regimens with other antiviral compounds [79].

15.15 Host Cell Lipid Biosynthesis Inhibitors

Several studies have implicated a crucial role of the host cell lipid metabolism for flavivirus infection by an altered lipid homeostasis as well expansive membrane rearrangements within the infected cell [225]. Key processes that might be influenced by differential lipid biosynthesis could involve virion biogenesis, membrane remodeling, genome replication, as well as autophagy and apoptosis processes. Several host enzymes offer potential as antiviral targets as they regulate different aspects of the lipid metabolism which also benefits viral replication (extensively reviewed in [226]).

The sterol regulatory element-binding proteins (SREBP) have been analyzed as targets for antiviral intervention. Different SREBP inhibitors, namely nordihydroguaiaretic acid (NDGA), its derivate M_4N, as well as PF-429242 and fatostatin (Table 15.3) have been successfully used to reduce ZIKV, DENV, or WNV infection *in vitro* [181–183]. Another class of inhibitors targets activators of the adenosine monophosphate-activated protein kinase (AMPK), which constitutes one of the main cellular energy sensors responsible for glycolysis and lipid metabolism. Specific inhibitors of these activators (Table 15.3), e.g. the small molecule compound PF-06409577 as well as the licensed drug metformin or 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), have been shown to inhibit ZIKV, DENV, and WNV *in vitro* [184–186].

One of the key enzymes of the fatty acid metabolism, acetyl-CoA carboxylase (ACC), offers another target for antiviral interference. Several compounds that target this enzyme have been shown to affect replication of ZIKV, DENV, and WNV [187, 227]. Of note, one of these compounds, PF-05175157 (Table 15.3), has already been evaluated in clinical trials in healthy volunteers (ClinicalTrials.gov Identifier: NCT01433380) and for the treatment of diabetes mellitus (ClinicalTrials.gov Identifier: NCT0179263). PF-05175157 has further been tested against WNV in a mouse model, which resulted in a reduction of viral loads in serum and kidney [187]. Additionally, therapeutic intervention with the fatty acid synthase (FASN) has been shown to inhibit DNEV and WNV replication [228–230].

Other potential approaches could target the sphingolipid metabolism which has been suggested to play a functional role in flavivirus infection [231]. Indeed, treatment with an inhibitor of neutral sphingomyelinase-2, GW4869, was able to inhibit infection with WNV, USUV, and ZIKV in cell lines as well as primary human fetal astrocytes [232–234]. Of note, the same compound seems to have the opposite effect on alphavirus infection, as Sindbis virus infectivity was increased after GW4869 treatment [233].

Given the crucial role of host cholesterol during flavivirus infection [235], direct interference with the cholesterol metabolism offers another promising antiviral approach (Table 15.3). Statins (e.g. lovastatin, fluvastatin), a class of cholesterol biosynthesis inhibitors that are a safe and widely used class of drug for the treatment of hypercholesterolemia, have been shown to efficiently inhibit WNV [194, 236] and DNEV replication [191, 192, 237]. However, treatment with lovastatin in a randomized, double-blind, placebo-controlled trial found no evidence of a beneficial effect on any of the clinical manifestations or on dengue viremia [193]. Furthermore, lovastatin increased replication of Semliki Forest Virus in an *in vitro* model, another alphavirus demonstrating the virus-specificity of these inhibitors [194]. In contrast, intracellular cholesterol transport inhibitors, e.g. treatment with the FDA-approved antidepressant drug imipramine, have been shown to successfully reduce not only flavivirus infectivity, but also CHIKV in cell culture [195], demonstrating the

Table 15.3 Overview of different classes of host-targeting antivirals.

Inhibitor class	Target[a]	Compound[b]	Biological system	Viruses
Nucleoside biosynthesis inhibitors	IMPDH	Ribavirin EICAR Mycophenolic acid Merimepodib Azathioprine	Cell culture Mice Clinical trial	ZIKV [172–174], DENV [175], WNV [176], CHIKV [174, 177–179]
	DHODH	A3 (indole derivate)	Cell culture	DENV [180], WNV [180]
Lipid biosynthesis inhibitors	SREBP	NDGA M_4N PF-429242 Fatostatin	Cell culture	ZIKV [181], DENV [182, 183], WNV [181]
	AMPK	PF-06409577 Metformin AICAR	Cell culture	ZIKV [184, 185], DENV [185, 186], WNV [185]
	ACC	PF-05175157	Cell culture Mice Clinical trial	ZIKV [187], DENV [187], WNV [181, 187]
	Cholesterol metabolism	Fluvastatin Lovastatin Imipramine Cholesterol derivates	Cell culture Mice	ZIKV [188–190], DENV [191–193], WNV [194], CHIKV [195]
	Sphingomyelinase-2	GW4869	Cell culture	WNV [228], ZIKV [227, 229]
Kinase inhibitors	NAK family of serine/threonine kinases	Sunitinib Erlotinib	Cell culture Mice	ZIKV [196], DENV [196], WNV [196], CHIKV [196]
	Src family kinases	Dasatinib Sarcatinib	Cell culture	DENV [197–199], WNV [200], CHIKV [17]
	CDK	PHA-690509	Cell culture	ZIKV [201, 202]
	Receptor tyrosine kinases (c-Abl)	Imatinib, GNF-2	Cell culture	DENV [140, 242, 243]
	mTORC1/2	Dasatinib, Torin 1	Cell culture	CHIKV [244]
Protein metabolism inhibitors	α-Glucosidase	Iminosugars	Cell culture Mice Clinical trial	DENV [203–206]

(Continued)

Table 15.3 (Continued)

Inhibitor class	Target[a]	Compound[b]	Biological system	Viruses
	α-glucosidase	Castanospermine, Celgosivir	Cell culture, mice, Clinical trial	DENV [199, 200]
	UPP	Bortezomib	Cell culture Mice	DENV [209], ZIKV [172, 210]
	Cyclophilins (CyPs)	Cyclosporine A (CsA)	Cell culture	WNV [207], DENV [207], YFV [207, 208], JEV [254]
Endocytosis and membrane fusion inhibitors	Membrane fusion	Arbidol	Cell culture	ZIKV [211, 212], WNV [211], CHIKV [213]
	Endosomal acidification	Chloroquine Ammonium chloride Bafilomycin A1 Niclosamide	Cell culture	DENV [214], ZIKV [95], CHIKV [215]

Summarized are tested compounds for different targets, the biological system used, and the viruses.

a) IMPDH, inosine monophosphate dehydrogenase; DHODH, dihydroorotate dehydrogenase; SREBP, sterol regulatory element-binding protein; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA-carboxylase; CDK, cyclin-dependent kinases; UPP, ubiquitin-proteasome pathway.

b) EICAR, 5-ethynyl-1-beta-D-ribofuranoysylimidazole-4; NDGA, nordihydroguaiaretic acid; M4N, tetra-*o*-methyl nordihydroguaiaretic acid; AICAR, 5-aminoimidazole-4-carboxamide-1-*β*-D-ribofuranoside.

potential of such drug candidates for a broad anti-arboviral treatment. Finally, cholesterol derivatives including 25-hydroxycholesterol (25-HC) and 7-ketocholesterol (7-KC) have shown promising antiviral properties against various viruses, including ZIKV *in vitro* and in a mouse model [188–190].

Importantly, even though most of the studies mentioned above are still in the initial stages of experimental validation and have mainly been performed *in vitro*, several of the compounds target key processes that are involved in a variety of human diseases, including obesity, metabolic syndrome, type II diabetes, and cancer. In consequence, some of the drugs mentioned above have already undergone diverse phases of clinical trials or are licensed for use in humans, facilitating drug repurposing [238–242].

15.16 Host Kinase Inhibitors

Phosphorylation of viral proteins mediated by cellular kinases is an important post-translational modification to regulate and ensure viral replication. Therefore, known host kinase inhibitors are potential candidates to be used as broad-spectrum antivirals in the future [243]. For example, two approved anticancer drugs, sunitinib and erlotinib (Table 15.3),

have been tested for their antiviral activity against flaviviruses as well as the alphavirus CHIKV [196]. These drugs inhibit the host kinase adaptor protein 2 (AP2)-associated protein kinase 1 (AAK1) and cyclin G-associated kinase (GAK), both kinases belonging to the Num-associated kinases (NAK) family of serine/threonine kinases. Since these kinases are involved in clathrin-mediated trafficking, they might affect intracellular viral trafficking and thus lower viral infectivity [244, 245]. Indeed, antiviral activity has been shown *in vitro* against DENV, WNV, ZIKV, and CHIKV at micromolar concentrations (EC_{50} values between 0.51 and 6.28 μM). Furthermore, the combination of both drugs was beneficial in reducing viremia and the overall outcome of DENV infection in a mouse model at a dose of 30 mg/kg [196]. Another host kinase family known to play a role during viral infections are the Src family kinases (SFKs) [246]. DENV replication has been found to depend on SFKs such as Fyn, Lyn, c-Src, and CSK [197–199], whereas for WNV, the kinase c-Yes seems to be important [200]. Particularly for DENV infection, two approved drugs, namely dasatinib and sarcatinib (Table 15.3), inhibited viral assembly in an *in vitro* study (EC_{90} values 12.2 and 4.7 μM, respectively) [199]. In addition, both inhibitors target the cellular Abelson tyrosine kinase (c-Abl), which belongs to the Abl family of nonreceptor kinases, a kinase involved in the replication of many viruses including DENV. In cell culture assays at a concentration of 15 μM, the approved anticancer drug imatinib showed anti-DENV activity and GNF-2, an allosteric inhibitor of c-Abl, developed as anticancer drug was even more effective against DENV infection in this study [145, 247, 248]. Recently, the effect of SFK inhibitors has been also tested against alphaviruses including CHIKV infection *in vitro*. Both dasatinib and Torin 1, an inhibitor of mTORC1/2, successfully blocked CHIKV replication during translation of structural proteins at concentrations of 100 nM [249]. Cyclin-dependent kinases (CDKs) are broadly used by many viruses to manipulate the host cell cycle [250]. Since flaviviruses do not express any CDKs, CDK inhibitors seem to impair host functions essential for viral replication. For example, the compound PHA-690509 (Table 15.3) was shown to inhibit ZIKV infection ($IC_{50} = 0.9$ μM) by analyzing NS1 expression *in vitro* [201, 202]. Overall, host kinase inhibitors may be promising in their capability to be used as antivirals. There is a broad potency in repurposing already approved host kinase inhibitors and the effort to balance effective antiviral activity with reduced cell toxicity to speed up the evaluation for clinical use should be made.

15.17 Protein Metabolism Inhibitors

Viruses hijack the host protein metabolism for a successful replication and translation of viral proteins. Key components include α-glucosidase, cyclophilins, and the ubiquitin-proteasome pathway (UPP). α-Glucosidase catalyzes the removal of glucose units from N-linked oligosaccharides and has been shown to participate in the folding and maturation of flaviviral glycoproteins [251]. Indeed, several α-glucosidase inhibitors have been shown to act as antivirals against several viruses, including flaviviruses [252]. Importantly, they have been shown to display a high genetic resistance barrier toward the emergence of escape mutations in a mouse model during DENV infection [203]. Among the most promising inhibitors are iminosugars and their derivates (Table 15.3).

The iminosugar castanospermine and its derivate celgosivir have been shown to efficiently inhibit DENV at a low micromolar IC_{50} *in vitro* and *in vivo* at doses of 10–250 mg/kg and 7.5 or 75 mg/kg, respectively (reviewed in [204]). In a randomized, double-blind, placebo-controlled trial (CELADEN; ClinicalTrials.gov Identifier: NCT01619969), celgosivir led to a mean virological log reduction (VLR = −0.22) of DENV from baseline for days 2, 3, and 4 compared to the placebo group; however, the difference was not statistically significant [205], and the study failed in reaching its primary endpoints of lowering viremia or fever [206]. A second phase 2 clinical trial with a revised dosing regimen is currently ongoing (ClinicalTrials.gov identifier: NCT02569827). One of the main disadvantages of iminosugars are the generally high doses, which are required and which can be associated with a high toxicity. Furthermore, such compounds seem to have a weak activity during the postinfection period. Whether these issues can be overcome at all by derivatization or combination with other antivirals will need to be shown in further studies. In addition, they might be suitable candidates for an early or even prophylactic treatment [79].

Cyclophilins (CyPs) facilitate proper protein folding and have been shown to play a crucial role in the replication cycle of various viruses [253]. Cyclosporine A (CsA), an 11-amino-acid cyclic peptide inhibitor of CyPs, has been shown to act as antiviral against WNV (20 μM), DENV (8 μM), YFV (8 μM), and JEV (~5 μM) *in vitro* [207, 208, 254]. However, no antiviral activity of CsA against CHIKV could be observed [255]. Besides, CsA other CyPs inhibitors might offer potential as anti-flaviviral compounds. Alisporivir, a cyclophilin inhibitor with pan-genotypic anti-HCV activity and a high barrier to viral resistance, has been shown to be effective as IFN-free option for the treatment of HCV in phase 2 (ClinicalTrials.gov Identifier: NCT01215643) [256]. *In vitro* studies did show an antiviral activity against selected tick-borne flaviviruses, including tick-borne encephalitis virus [TBEV] at low μM concentrations (0.5–10 μM); however, no effect on WNV replication [257]. Nevertheless, it is important to keep in mind that the dependency of CypA during virus replication can be cell-type specific, thereby resulting in inconclusive results *in vitro* which should be validated *in vivo*.

The host UPP has been implicated as an important host factor during positive-stranded RNA viral infections [258]. It is speculated that viruses from several families are able to reprogram the UPP and thereby alter the cellular environment toward viral replication, whereas inhibition of the UPP can interfere with viral propagation. Importantly, several drugs that inhibit the function of the proteasome, a major player of the UPP, have been licensed for therapeutic use. Bortezomib (Table 15.3), a licensed proteasome inhibitor to treat multiple myeloma and mantle cell lymphoma, has been shown to reduce DENV titers of all four serotypes at low nanomolar drug concentrations in primary monocytes [209]. In addition, *in vitro* assays revealed bortezomib as an antiviral drug against ZIKV [172] and against alphaviruses [259]. Despite its moderate toxicity *in vitro* [172], it has been shown to reduce viral load and signs of pathology in ZIKV-infected mice [210]. Finally, the drug has been shown to reduce JEV-induced lethality in mice, to alleviate suffering in JEV-infected mice and reduce the damage in brains caused by JEV infection [260]. Other inhibitors of the proteasome, including MG132 and USP14, have shown to have an antiviral activity against flaviviruses and alphaviruses *in vitro* [261–265], highlighting proteasome inhibitors as potential anti-arboviral compounds.

15.18 Endocytosis and Membrane Fusion Inhibitors

Flaviviruses and CHIKV enter host cells through clathrin-mediated endocytosis [266–269]. Conformational changes in viral envelope proteins triggered during endosome acidification mediate membrane fusion and subsequent release of the viral genome from the capsid [71, 270, 271]. Each individual step during the virus entry/membrane fusion process is critical to successfully establish an infection and thus represents a potential druggable target. One example is arbidol (ARB, also known as umifenovir, Table 15.3), a broad-spectrum antiviral compound and clinically approved in Russia and China for prophylaxis and treatment of influenza virus infections [272, 273]. It intercalates with membrane lipids to prevent fusion events between virus particles and host membranes and thereby blocks virus entry into host cells [274]. *In vitro* studies revealed antiviral activities against ZIKV (IC_{50} 11–15 μM), CHIKV (IC_{50} ~25 μM), and WNV (EC_{50} ~18 μM), indicating that ARB may represent a potential candidate for treatment and prophylaxis of emerging arbovirus infections with a well-established safety profile [211–213]. Various lysosomotropic agents, such as chloroquine, ammonium chloride, bafilomycin A1, and niclosamide (Table 15.3), have been shown to exert direct antiviral effects on several emerging arbovirus infections *in vitro* [96, 214, 215, 275], implying that the blockade of endosomal acidification during endocytosis can be an effective strategy to block viral entry. However, although inhibitors perturbing cellular processes such as endocytosis and endosomal acidification have been valuable tools to study various aspects of the viral life cycle, translating these findings into clinical use remains challenging due to frequently observed significant cytotoxic effects. Nonetheless, results from genome wide screens may identify novel targets in the endosomal pathway without deleterious effects on the host cell [268, 276].

15.19 Conclusion and Future Perspectives

Arbovirus infections constitute a global health burden and have a long history of infecting humans. They result in regular pandemics as well as in an increasing frequency of autochthonous transmission. However, they are mostly considered as neglected tropical diseases. That means they are outside the purview of the Global Fund and its related programs which mostly concentrate on HIV, tuberculosis (TB), and malaria [277]. Consequently, only two candidate vaccines for DENV and YFV have been approved, while no specific antiviral therapeutics to prevent or treat arbovirus infections have been licensed yet. Notwithstanding, several approaches have been employed in recent years, including testing of drugs with known antiviral activity against other viruses or screening of compound libraries comprising hundreds to thousands of bioactive compounds (see Table 15.4 for selected compounds). In this context, repurposing of drugs that have already gone through several steps of drug approval by regulatory agencies may offer potential as fast intervention strategies in cases of virus (re-)emergence. In addition, novel approaches including structure-based drug design, computational approaches using docking algorithms and virtual screening, as well as traditional techniques of HTS with combinatorial chemistry can increase the efficiency and speed of drug discovery. Along this line, future research should

Table 15.4 Chemical names and formula of selected potential antiviral drugs.

Name	Chemical name	Formula	CAS number
7DMA	7-Deaza-2′-C-methyladenosine	$C_{12}H_{16}N_4O_4$	443642-29-3
BCX4430	(2*S*,3*S*,4*R*,5*R*)-2-(4-amino-5H-pyrrolo[3,2-d]pyrimidin-7-yl)-5-(hydroxymethyl)-3,4-pyrrolidinediol	$C_{11}H_{15}N_5O_3$	222631-44-9
Sofosbuvir	Isopropyl-(2*S*)-2-({[(2*R*,3*R*,4*R*,5*R*)-5-(2,4-dioxopyrimidin-1-yl)-4-fluor-3-hydroxy-4-methyl-tetrahydrofuran-2-yl]methoxy-phenoxy-phosphoryl}amino)propanoat	$C_{22}H_{29}FN_3O_9P$	1190307-88-0
Temoporfin	3,3′,3′′,3′′′-(2,3-Dihydroporphyrin-5,10,15,20-tetrayl)tetraphenol	$C_{44}H_{32}N_4O_4$	122341-38-2
Erythrosin B	Dinatrium-2-(2,4,5,7-tetraiodo-6-oxido-3-oxo-xanthen-9-yl)benzoate	$C_{20}H_6I_4Na_2O_5$	16423-68-0
Lycorine	1,2,4,5,12b,12c-Hexahydro-7H-[1,3]dioxolo[4,5-j]pyrrolo[3,2,1-de]phenanthridine-1,2-diol	$C_{16}H_{17}NO_4$	476-28-8
4-HPR	15-[(4-Hydroxyphenyl)amino]retinal	$C_{26}H_{33}NO_2$	65646-68-6
Ivermectin	22,23-Dihydroavermectin B1	$C_{48}H_{74}O_{14}$	70161-11-4
Ribavirin	1-[(2*R*,3*R*,4*R*,5*R*)-3,4-Dihydroxy-5-hydroxymethyl-oxolan-2-yl]-1,2,4-triazol-3-carboxamid	$C_8H_{12}N_4O_5$	36791-04-5
EICAR	5-Ethynyl-1-beta-D-ribofuranosylimidazole-4-carboxamide	$C_{11}H_{16}N_3O_{14}P_3$	214827-88-0
Mycophenolic acid	6-(4-Hydroxy-6-methoxy-7-methyl-3-oxo-1H-2-benzofuran-5-yl)-4-methylhex-4-enoic acid	$C_{17}H_{20}O_6$	24280-93-1
Merimepodib	[(3*S*)-Oxolan-3-yl] *N*-[[3-[[3-methoxy-4-(1,3-oxazol-5-yl)phenyl]carbamoylamino]phenyl]methyl] carbamate	$C_{23}H_{24}N_4O_6$	198821-22-6
Azathioprine	6-(3-Methyl-5-nitroimidazol-4-yl)sulfanyl-7H-purine	$C_9H_7N_7O_2S$	446-86-6
NDGA	4-[4-(3,4-Dihydroxyphenyl)-2,3-dimethylbutyl]benzene-1,2-diol	$C_{18}H_{22}O_4$	500-38-9
M4N	4-[(2*S*,3*R*)-4-(3,4-Dimethoxyphenyl)-2,3-dimethylbutyl]-1,2-dimethoxybenzene	$C_{22}H_{30}O_4$	24150-24-1
PF-429242	4-(Diethylaminomethyl)-*N*-[2-(2-methoxyphenyl)ethyl]-*N*-[(3R)-pyrrolidin-3-yl]benzamide	$C_{25}H_{35}N_3O_2$	947303-87-9
Fatostatin	4-(4-Methylphenyl)-2-(2-propylpyridin-4-yl)-1,3-thiazole	$C_{18}H_{18}N_2S$	298197-04-3
PF-06409577	6-Chloro-5-[4-(1-hydroxycyclobutyl)phenyl]-1H-indole-3-carboxylic acid	$C_{19}H_{16}ClNO_3$	1467057-23-3
Metformin	3-(Diaminomethylidene)-1,1-dimethylguanidine	$C_4H_{11}N_5$	657-24-9
AICAR	5-Amino-1-[(2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]imidazole-4-carboxamide	$C_9H_{14}N_4O_5$	2627-69-2

Name	Chemical name	Formula	CAS number
PF-05175157	1'-(2-Methyl-3H-benzimidazole-5-carbonyl)-1-propan-2-ylspiro[4,6-dihydroindazole-5,4'-piperidine]-7-one	$C_{23}H_{27}N_5O_2$	1301214-47-0
Fluvastatin	(*E*,3*R*,5*S*)-7-[3-(4-Fluorophenyl)-1-propan-2-ylindol-2-yl]-3,5-dihydroxyhept-6-enoic acid	$C_{24}H_{26}FNO_4$	93957-54-1
Lovastatin	[(1*S*,3*R*,7*S*,8*S*,8a*R*)-8-[2-[(2*R*,4*R*)-4-Hydroxy-6-oxooxan-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl] (2*S*)-2-methylbutanoate	$C_{24}H_{36}O_5$	75330-75-5
Imipramine	3-(5,6-Dihydrobenzo[b][1]benzazepin-11-yl)-*N*,*N*-dimethylpropan-1-amine	$C_{19}H_{24}N_2$	50-49-7
Sunitinib	*N*-[2-(Diethylamino)ethyl]-5-[(*Z*)-(5-fluoro-2-oxo-1H-indol-3-ylidene) methyl]-2,4-dimethyl-1H-pyrrole-3-carboxamide	$C_{22}H_{27}FN_4O_2$	557795-19-4
Erlotinib	*N*-(3-Ethynylphenyl)-6,7-bis(2-methoxyethoxy)quinazolin-4-amine	$C_{22}H_{23}N_3O_4$	183321-74-6
Dasatinib	*N*-(2-Chloro-6-methylphenyl)-2-[[6-[4-(2-hydroxyethyl)piperazin-1-yl]-2-methylpyrimidin-4-yl] amino]-1,3-thiazole-5-carboxamide	$C_{22}H_{26}ClN_7O_2S$	302962-49-8
Sarcatinib	*N*-(5-Chloro-1,3-benzodioxol-4-yl)-7-[2-(4-methylpiperazin-1-yl)ethoxy]-5-(oxan-4-yloxy) quinazolin-4-amine	$C_{27}H_{32}ClN_5O_5$	379231-04-6
PHA-690509	(2*S*)-2-(4-Acetamidophenyl)-*N*-(5-propan-2-yl-1,3-thiazol-2-yl)propanamide	$C_{17}H_{21}N_3O_2S$	492445-28-0
Cyclosporine A	(3*S*,6*S*,9*S*,12*R*,15*S*,18*S*,21*S*,24*S*,30*S*,3 3*S*)-30-Ethyl-33-[(*E*,1*R*,2*R*)-1-hydroxy-2-methylhex-4-enyl]-1,4,7,10,12,15,19,25,28-nonamethyl-6,9,18,24-tetrakis(2-methylpropyl)-3,21-di(propan-2-yl)-1,4,7,10,13,16,19,22,25,28,31-undecazacyclotritriacontane-2,5,8,11,14,17,20,23,26,29,32-undecone	$C_{62}H_{111}N_{11}O_{12}$	59865-13-3
Bortezomib	[(1*R*)-3-Methyl-1-[[(2*S*)-3-phenyl-2-(pyrazine-2-carbonylamino)propanoyl]amino]butyl]boronic acid	$C_{19}H_{25}BN_4O_4$	179324-69-7
Arbidol	Ethyl 6-bromo-4-[(dimethylamino)methyl]-5-hydroxy-1-methyl-2-(phenylsulfanylmethyl) indole-3-carboxylate	$C_{22}H_{25}BrN_2O_3S$	131707-25-0
Chloroquine	4-*N*-(7-Chloroquinolin-4-yl)-1-*N*,1-*N*-diethylpentane-1,4-diamine	$C_{18}H_{26}ClN_3$	56598-66-4
Ammonium chloride	Ammonium chloride	NH_4Cl	12125-02-9
Bafilomycin A1	(3*Z*,5*E*,7*R*,8*S*,9*S*,11*E*,13*E*,15*S*,16*R*)-16-[(2*S*,3*R*,4*S*)-4-[(2*R*,4*R*,5*S*,6*R*)-2,4-Dihydroxy-5-methyl-6-propan-2-yloxan-2-yl]-3-hydroxypentan-2-yl]-8-hydroxy-3,15-dimethoxy-5,7,9,11-tetramethyl-1-oxacyclohexadeca-3,5,11,13-tetraen-2-one	$C_{35}H_{58}O_9$	88899-55-2
Niclosamide	5-Chloro-*N*-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide	$C_{13}H_8Cl_2N_2O_4$	50-65-7

focus on understanding the replication mechanisms and identifying virus–host interactions to facilitate the different novel approaches regarding drug design. In order to develop successful therapeutic approaches to treat emerging arbovirus infections, a combination approach utilizing antivirals and host response-directed countermeasures could be employed. So far, most of the antiviral candidates have been evaluated *in vitro* only, some of them have also been tested in animal models. However, only few candidates have advanced into clinical trials (i.e. BCX4430, chloroquine, PF-05175157, celgosivir, alisporivir), highlighting the need for further research and efforts toward developing an effective treatment of these neglected diseases.

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16

Current Therapies for Biosafety Level 4 Pathogens

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16.1 Introduction

Members of several virus families are classified as Biosafety Level 4 (BSL4) pathogens based on their ability to cause life-threatening human disease with a significant risk of spread, thus posing a high risk both to the individual and the community. While the list of viruses included in this category varies somewhat by country (often taking into account whether a particular pathogen is endemic to that region), members of four virus families are almost universally included: filoviruses, henipaviruses, arenaviruses, and bunyaviruses.

The exotic nature of BSL4 viruses and the frequently fatal outcome of these diseases, combined with the sporadic occurrence of outbreaks and limited case numbers, set them apart from other infectious diseases, and poses special challenges for the development of treatment or prophylactic strategies. Indeed, clinical trials are very difficult to conduct in many instances due to the lack of a well-developed medical infrastructure in the affected areas and/or limited patient numbers. Further, there is often only a very limited commercial interest in the development of treatments for these diseases since they affect relatively small numbers of patients, often in some of the world's poorest countries. Consequently, the perceived importance of therapeutic development for these agents has traditionally been limited and, therefore, treatment options are sparse. Even fewer have been approved by any kind of regulatory body.

More recently, however, the development of novel treatments and vaccines for many of these agents has been recognized as international priority, for instance, in WHO R&D Roadmaps [1–4]. This has led to a drastic increase in early-stage research for such therapies; however, few have so far progressed to testing in highly stringent and predictive animal models, usually nonhuman primates (NHPs), or even to human clinical testing. Nonetheless, there have clearly been successes toward the treatment of BSL4 virus infections in these more advanced phases of testing, and it is these strategies that are summarized here.

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16.2 Filoviruses

16.2.1 Virus and Disease Overview

Filoviruses are negative-sense, single-stranded RNA viruses of the family *Filoviridae*, which includes six genera. Those known to be relevant for human disease belong to the *Ebolavirus* and *Marburgvirus* genera, and cause viral hemorrhagic fever (VHF) outbreaks. Consequently, filoviruses are classified as BSL4 agents.

The first member of the filovirus family, Marburg virus (MARV, genus *Marburgvirus*), was identified in 1967 as the causative agent of a VHF outbreak primarily affecting the city of Marburg, Germany, and specifically individuals who had contact to tissues derived from African green monkeys [5, 6]. Of the 32 cases recognized during this outbreak, 7 died, resulting in a case fatality rate (CFR) of 23% [7]. Members of a second filovirus genus, *Ebolavirus*, emerged in 1976 concurrently in South Sudan (formerly Sudan) and the Democratic Republic of Congo (formerly Zaire), resulting in the infection of 284 (CFR 53%) and 318 (CFR 88%) individuals, respectively. Since then, outbreaks of filovirus disease (FVD) of variable extent have occurred sporadically throughout Western and Central Africa. The most devastating outbreak was caused by Ebola virus (EBOV) and lasted from late 2013 until 2016. It mainly affected the West African countries of Guinea, Sierra Leone, and Liberia, resulting in 28 652 confirmed cases, of which 11 325 patients died (CFR 40%) [8]. Together with another large outbreak in DRC from 2018 to 2020 with almost 3 500 cases [9] that defied control efforts for two years despite public health intervention by the global community (including vaccination), these episodes highlight the previously neglected potential for filoviruses to cause large and even multinational outbreaks.

Whereas both marburgviruses, MARV and Ravn virus (RAVV), cause FVD [10], of the six ebolaviruses known to date, only four are associated with human disease (EBOV, Sudan virus (SUDV), Bundibugyo virus (BDBV), and Taï Forest virus (TAFV)). In contrast, Reston virus (RESTV) appears to cause asymptomatic infections in humans [8, 11], while for several novel filoviruses, which were recently identified in diverse geographical regions [12–14], the pathogenic potential remains unknown. This raises the possibility of future emergence of filovirus-induced diseases, also outside known filovirus endemic regions.

FVD is a zoonotic infection, but the exact transmission route remains unclear in many cases. Spillover into the human population is believed to occur from infected animals through exposure to body fluids, i.e. blood or excreta (for instance, as a result of bites, during handling of carcasses, or through environmental contamination), with further spread from human-to-human via blood and other body fluids. Both MARV and RAVV have been isolated from fruit bats (*Rousettus aegypticus*), which seem to be a key reservoir for these viruses. While no ebolavirus has yet been isolated from bats (reviewed in [15]), based on serological analyses they are also proposed to be the reservoir for members of this genus.

Symptoms of FVD infection begin suddenly after an incubation period of 3–13 days and are initially unspecific [16–19]. During the first 10 days, fever is the most frequently reported symptom, followed by fatigue, headache, myalgia, and renal dysfunction. In some

cases, a rash also develops early during infection. Gastrointestinal symptoms such as diarrhea, nausea, and vomiting lead to fluid loss and further weaken the patient. On days 7–12 after onset of symptoms, patients either show signs of recovery or progress to exhibit symptoms of shock including diminished consciousness and coma [16]. Hemorrhages and overt bleeding are not universally observed, even among fatal cases, but if they do occur, they typically affect the gastrointestinal tract [16–18]. Higher viral loads in the blood of patients correlate with a poor prognosis, and an influence of patient age on outcome has also been reported [20–22]. Additionally, respiratory symptoms such as dyspnea, tachypnea, and hiccups are indicative of a poor prognosis [20]. Sequelae of FVD include arthralgia, temporary hair loss, and ocular complications such as blurred vision, conjunctivitis, uveitis, or vision loss [23]. Data on neurological sequelae suggest increased rates of fatigue, insomnia, and depression in FVD survivors; however, these could also reflect the psychological trauma and effects of stigmatization by the community [24].

16.2.2 Antiviral Strategies

In response to the dramatic West African EBOV outbreak, tremendous effort has been devoted recently to the development of antiviral approaches against filovirus infection. However, to date, most of them have not yet shown efficacy in highly predictive models of filovirus infection (i.e. NHPs) and/or advanced to clinical testing. Nonetheless, there are a few different strategies that have shown promise. Currently, the approaches that have advanced the furthest are immunotherapies using specific monoclonal antibodies and postexposure vaccination with a recombinant vesicular stomatitis virus (rVSV) encoding the EBOV glycoprotein GP (rVSV-EBOV, also known as Ervebo, rVSVΔG-EBOV GP, or rVSV-ZEBOV). However, small molecule, antisense, and host-directed therapies have also shown promise in NHPs and could potentially fill important treatment gaps.

16.2.2.1 Immunotherapies

Antibodies can counter filovirus infection in two ways: (i) through direct virus neutralization by targeting domains of GP necessary for entry, or (ii) through non-neutralizing antibodies that direct the immune-mediated clearance of infected cells via antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity [25]. During the West African EBOV outbreak, antibody-containing convalescent sera were tested for treatment in a nonrandomized historically controlled clinical trial [26]. No serious adverse reactions were detected; however, no benefit for patient survival was observed either. Later analysis of antibody titers in these sera revealed no significant correlation between the amount of IgG or neutralizing antibody (nAb) titers and survival [27].

Monoclonal antibodies (or mixtures of several monoclonal antibodies) have also been developed as potential treatments of FVD, and these show more clinical success than convalescent sera. The three antibody preparations that are the most advanced in terms of testing are ZMapp, REGN-EB3, and the monoclonal antibody (mAb) mAb114. ZMapp is a humanized antibody cocktail based on antibodies originally isolated from mice immunized with Venezuelan equine encephalitis virus replicons encoding EBOV GP or with a rVSV expressing EBOV GP [28, 29]. It consists of two nAbs binding the EBOV GP

base (2G4 and 4G7) and one non-neutralizing antibody targeting the glycan cap of EBOV GP (13C6) [30]. This antibody cocktail was tested in a randomized, controlled clinical phase 1/2 study [31]. While this study suggested a benefit of treatment with ZMapp, it failed the prespecified statistical threshold, possibly as a result of lower than anticipated enrollment numbers due to the end of the outbreak. The second antibody cocktail, REGN-EB3 (also known as Regeneron), also contains three antibodies (REGN 3470, 3471, and 3479), which were isolated from immunized mice encoding fully human antibody variable region gene segments [32]. REGN 3479 and REGN 3470 show neutralizing activity by targeting the fusion loop and the head region of the GP subunit GP1, respectively, whereas REGN 3471 is non-neutralizing and binds the outer glycan cap of this protein. Tolerability and safety of this cocktail was shown during a randomized phase 1 clinical trial [33]. Finally, mAb114 is a single nAb isolated from an FVD survivor [34] that binds the glycan cap and the GP1 core, thereby preventing binding of GP to its receptor, Niemann-Pick C1, after endosomal cleavage of the glycan cap [35]. Safety and tolerability of mAb114 were also confirmed during an open-label phase 1 study [36]. Each of these three mAb preparations, in addition to the nucleoside analogue remdesivir (described in more detail in Section 16.2.2.3), have recently been tested for treatment of FVD in a clinical trial (registration number NCT03719586) in the Democratic Republic of Congo, where a prolonged FVD outbreak occured from 2018 to 2020. Data from this trial showed that patients receiving either REGN-EB3 or mAb114 had a higher chance of survival compared to the other treatments, making them the only treatment options to date for which significant effectivity against FVD has been demonstrated in a clinical trial [37]. Based on this success, both compounds have recently obtained U.S. Food and Drug Administration approval.

16.2.2.2 Postexposure Prophylaxis

While primarily used for preventive purposes, the rVSV-EBOV vaccine also has potential for use in postexposure prophylaxis (PEP). It is based on an attenuated, replication competent vesicular stomatitis virus, which encodes the EBOV GP instead of the VSV glycoprotein (G). Vaccination results in viral protein expression and virus replication; however, virus particles express the EBOV GP on their surface instead of the VSV G and facilitate the induction of humoral immune responses directed against EBOV GP, as shown in NHPs [38]. Several clinical trials have shown safety and efficacy of the vaccine, which recently obtained approval by both the European Medicines Agency as well as the U.S. Food and Drug Administration, and it has been used for ring vaccinations with more than 250 000 recipients to date [9] (see [39] for an overview of EBOV vaccine candidates in clinical trials). As a PEP treatment, rVSV-EBOV vaccination was analyzed in NHPs 20–30 minutes after challenge with an uniformly lethal dose of EBOV [40]. While 50% of animals survived, all showed signs of illness by day 6, and the severity of these signs correlated with death. When NHPs were treated with rVSV-encoding MARV GP instead of VSV G (rVSV-MARV) 20–30 minutes after lethal infection with MARV, all animals survived [41, 42]. Initiation of treatment with rVSV-MARV 24 hours after infection still resulted in the survival of five out of six animals, while treatment after 48 hours saved two out of six animals [42]. In 2009, following a potential EBOV infection due to an accidental needle stick injury during an animal experiment in a

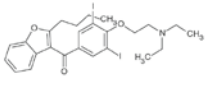
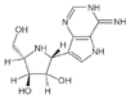
BSL4 facility, a virologist received rVSV-EBOV 48 hours after the accident as PEP [43]. Similarly, in 2014 and 2015, five people involved in the West African EBOV outbreak, including nurses and physicians, were treated with rVSV-EBOV between 24 and 72 hours after potential EBOV exposure [44]. None of the patients developed signs or symptoms of FVD, and no laboratory evidence for EBOV infection was detectable. Indeed, while four out of the six patients showed seroconversion to EBOV GP, which is encoded by the rVSV-EBOV vaccine, no evidence for antibodies directed against other EBOV proteins could be found [43, 44], suggesting not only that rVSV-EBOV can function as PEP in humans, but may induce sterile immunity (provided that these individuals were, indeed, infected).

16.2.2.3 Small Molecules

Where their mechanism of action is known, most small molecules (Table 16.1) that have been tested in NHPs or in clinical trials against filovirus infection target either entry of the virus or viral RNA synthesis. For example, amiodarone, which is approved for anti-arrhythmic therapy, also inhibits filovirus entry, with IC_{50} values of 16 μM in Vero E6 cells and 7 μM in human monocyte-derived macrophages, by targeting virus entry in the late endosomal compartment. As such, it was administered to 65 patients during the West African outbreak following compassionate use protocols [48, 63] based solely on in vitro data [47]. Unfortunately, in patients the treatment did not show any beneficial effect, and indeed an in vivo study in guinea pigs conducted afterward also showed no protection after challenge with EBOV in this animal model [46].

A more frequently pursued approach involves the use of nucleoside analogues such as BCX4430, favipiravir (also known as T-705) and remdesivir (also known as GS-5734), which are incorporated into the viral RNA as purine analogues during replication, thereby leading to mismatch mutations and/or early chain termination (Table 16.1). Such strategies would be particularly promising as these drugs show broad efficacy against several RNA viruses. While BCX4430 (an adenosine analogue) has been tested in a phase 1 clinical study confirming its safety and tolerability following intramuscular application [51], its efficacy for the treatment of FVD remains to be evaluated in phase 2 and 3 clinical trials. The purine analogue favipiravir shows antiviral activity against several RNA viruses in vitro and in various animal models [64]. It is currently licensed for the treatment of Influenza virus infections in Japan and is in clinical trials in the United States for the same indication [65]. With respect to its ability to treat EBOV infection in humans, two retrospective analyses of compassionate use in Guinea [58] and Sierra Leone [60] showed no significant improvement in CFR, although in one study prolonged survival times and reduced viral loads were noted. A historically controlled, non-randomized, single-arm proof-of-concept study in Guinea did not show any clinical benefit as well, possibly due to low target serum concentrations of favipiravir, which might have had an influence on the outcome [59, 66]. Thus, further studies remain necessary to evaluate the potential benefits of favipiravir against FVD. A third nucleotide analogue, i.e. the adenosine analogue remdesivir (Table 16.1), showed reduced viral titers and survival benefit when delivered intravenously in NHPs at two or three days post infection with EBOV [61]. The efficiency of remdesivir in humans was tested in a randomized clinical trial (also discussed in Section 16.2.2.1); however, its effect was

Table 16.1 Overview of small molecules tested against filovirus infection.

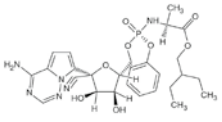
Name	Structure	Mechanism of action	In vitro effect	In vivo effect in NHPs	Clinical regimen
Amiodarone		• Inhibitor of viral fusion activity in the late endosomal compartment [45]	**EBOV:** • IC_{50} of 15.9 µM in Vero cells [46] • IC_{50} of 5.5 µM in Huh7 cells [46] • IC_{50} of 6.6 µM in human monocyte-derived macrophages [46] • IC_{50} of 0.4 µM in EAhy cells [47]	Not determined	**EBOV:** • Compassionate use in 65 patients during the West African EBOV outbreak, details regarding regimen not available [48]
BCX4430		• Polymerase inhibitor • Mutagenic agent	**EBOV:** • IC_{50} of 11.8 µM in HeLa cells [49] **SUDV:** • IC_{50} of 3.4 µM in HeLa cells [49] **MARV:** • IC_{50} of 4.4–6.7 µM in HeLa cells [49]	**EBOV:** • 16 or 25 mg/kg twice daily [50] • 100 mg/kg twice as loading dose, then 25 mg/kg twice daily [51]	**EBOV:** • Up to 10 mg/kg daily for seven days [51]

Name	Structure	Mechanism of action	In vitro effect	In vivo effect in NHPs	Clinical regimen
Brincidofovir		• Unknown	**EBOV:** • IC_{50} of 0.6–4.2 μM in Huh7 cells [52] • IC_{50} of 0.2 μM in A549 cells [52] • IC_{50} of 0.6 μM in HT-1080 cells [52] • IC_{50} of 6.7–8.1 μM in Vero E6 cells [52] • IC_{50} of 0.3 μM in HeLa cells [52] • IC_{50} of 0.2 μM in HUVEC cells [52] • IC_{50} of 9.6–11.5 μM in monocyte-derived macrophages [52]	Not determined	**EBOV:** • 200 mg as loading dose, 100 mg on days 3, 7, 10, 14 [53]

(Continued)

Table 16.1 (Continued)

Name	Structure	Mechanism of action	In vitro effect	In vivo effect in NHPs	Clinical regimen
Favipiravir (T-705)		• Polymerase inhibitor • Mutagenic agent	**EBOV:** • IC_{25} of 30.9 μM in HEK 293T cells [54] • IC_{50} of 67.0 μM in Vero E6 cells [55] **MARV:** • IC_{50} of 43.3 μM in Vero cells [50]	**EBOV:** • 400 mg/kg as loading dose on day 3, 200 mg/kg on days 2–10 [56] • 250 mg/kg twice or 125 mg/kg twice as loading dose on day 0, then 150 or 75 mg/kg twice daily on days 1–13 [56] • 200 mg/kg twice as loading dose on day 2, then 100 mg/kg twice daily until day 12 [57] • 150 or 250 mg/kg twice daily until day 12 [57] **MARV:** • 250 mg/kg twice as loading dose on day 0, then 150 mg/kg twice daily on days 1–13 [56]	**EBOV:** • 6000 mg as loading dose, then 2400 mg daily until day 9 [58, 59] • 800 mg twice as loading dose, then 600 mg twice daily [60]

Name	Structure	Mechanism of action	In vitro effect	In vivo effect in NHPs	Clinical regimen
Remdesivir (GS-5734)		• Polymerase inhibitor • Delayed chain termination	**EBOV:** • IC_{50} of 0.1 μM in HeLa cells [61] • IC_{50} of 0.003–0.1 μM in Huh-7 cells [61, 62] • IC_{50} of 0.1 μM in HFF-1 cells [61] • IC_{50} of 0.1 μM in HMVEC-TERT cells [61] • IC_{50} of 0.1 μM in human monocyte derived macrophages [61] **MARV:** • IC_{50} of 0.01–0.02 μM in Huh-7 cells [62]	**EBOV:** • 3 mg/kg on days 0–11 or days 2–13 after infection [61] • 10 mg/kg as loading dose on day 2 or 3, then 3 mg/kg for 11 days [61] • 10 mg/kg on days 3–14 after infection [61]	**EBOV:** • 200 mg as loading dose, then 100 mg daily for 9–12 days [37]

limited compared to two other treatment options under investigation, i.e. the antibodies mAB114 and REGN-EB3 [37].

Finally, brincidofovir (Table 16.1), which was originally developed as an antiviral drug against double-stranded DNA viruses, also shows efficacy against EBOV in vitro at concentrations of 0.3–8 μM (IC_{50} values in HeLa and Vero E6 cells, respectively), although the mechanism is unknown [52]. Based on these data, a single-arm phase 2 clinical trial was started in January 2015 in Liberia but was stopped after the enrolment of only four patients (all of whom died of FVD), due to the decision of the manufacturer to stop development of brincidofovir for treatment of filovirus infections [53].

16.2.2.4 Antisense Therapy

To date, two nucleic acid-based antisense therapies have shown efficacy in NHPs [67–70]. The first one uses uncharged DNA analogues based on phosphorodiamidate morpholino oligomers (PMOs), which block protein translation of complementary mRNAs by steric hindrance. The tested PMOs were directed against the EBOV viral proteins VP35 or VP24 (AVI-6002) or MARV VP24 and the MARV nucleoprotein (NP; AVI-6003) [67, 68]. Tolerability of AVI-6002 and AVI-6003 were analyzed in phase 1 single-ascending-dose studies, which showed safety and tolerability of the tested products in humans, independent of the target sequences used [71]. However, despite these initially promising results, development has been discontinued by the manufacturer.

The second strategy is based on antisense therapy using small interfering RNAs (siRNAs), which bind and direct degradation of complementary RNA sequences, thereby allowing silencing of gene expression [72]. Here, a combination of siRNAs targeting the EBOV proteins L, VP24, and VP35 and prepared in a lipid nanoparticle formulation was analyzed in a historically controlled, single-arm phase 2 clinical trial. However, the trial was terminated early after interim analyses showed a lack of patient benefit with respect to survival [73]. After the trial was concluded, the manufacturer discontinued development of filovirus-specific siRNA products.

16.2.2.5 Host-directed Therapies

To date, only one strategy aimed at modulating the host response has reached advanced stages of testing for treatment of FVD. It is based on the observation that filovirus infection of macrophages leads to secretion of high levels of an interferon (IFN)-α subset, whereas only low levels of IFN-β secretion can be observed [74]. To balance this dysregulation, IFN β-1a treatment was tested in a historically controlled, single-arm proof-of-concept trial with nine patients. A possible survival benefit was suggested, but due to the low patient numbers, no firm conclusions could be drawn [75].

16.3 Henipaviruses

Henipaviruses are part of the family *Paramyxoviridae*, and as such are negative-sense single-stranded RNA viruses. Two species within the genus *Henipavirus*, Hendra virus (HeV) and Nipah virus (NiV), are classified as BSL4 agents due to their ability to cause severe and frequently fatal human disease.

16.3.1 Disease Overview

HeV was first identified in 1994 in Hendra (a suburb of Brisbane), Australia, when 14 out of 21 horses suffering from a severe respiratory illness, as well as one out of two patients with close contact to the infected horses, succumbed to infection [76]. Indeed, transmission for all human cases reported to date, of which four out of seven have been fatal (CFR 57%), occurred through close contact with infected horses [77]. The natural reservoirs of HeV are flying foxes of the genus *Pteropus*, from which HeV has been successfully isolated [78]. Since 2015, a HeV vaccine for use in horses containing a soluble version of HeV G is available in Australia, thereby reducing the risk of human spillover [79]. Symptoms of HeV disease begin after an incubation period of 7 to 21 days, and in 6 of the 7 reported cases symptoms were initially unspecific, including fever, myalgia, and respiratory disease, while the seventh case developed encephalitis without any initial unspecific symptoms [80]. In severe cases, encephalitis is common, with three patients (of which only one recovered) developing acute encephalitis and one patient developing a fatal encephalitis 13 months following initial recovery from the infection. To date, there is no evidence for human-to-human transmission of HeV [81].

In 1998, NiV, a second human pathogenic *Henipavirus*, emerged in Malaysia [82]. Here, transmission could be traced back to infected pigs that probably fed on fruit contaminated by infected *Pteropus* bats [83, 84], which were identified as the natural reservoir host for NiV. The outbreak in Malaysia was eventually contained in 1999, but during this time 265 human cases were reported, of which 105 died (CFR 40%). Interestingly, however, 8% of infections were reported to be asymptomatic [85]. The outbreak also spread to Singapore through the import of infected pigs, resulting in illness in 11 slaughterhouse workers [86]. While neither of these countries has reported additional NiV cases since this time, Bangladesh and India have faced repeated outbreaks with CFRs of approximately 75% [87]. Other than transmission from infected pigs, the consumption of raw date palm sap contaminated by NiV shedding bats is believed to be a major route of transmission to the human population. Human-to-human transmission of NiV also occurs through shedding of virus in urine and respiratory secretions [88, 89]. Symptoms of NiV infection start after an incubation period of between four days to two months with unspecific symptoms similar to those observed for HeV infection, including fever, headache, unconsciousness, weakness, and respiratory difficulties, and in severe cases infection can also result in encephalitis [90, 91]. Similar to HeV, encephalitis in NiV-infected patients can also develop months to years after the initial infection. Furthermore, other neurologic sequelae have been described, e.g. personality changes and deficits in attention [90, 92].

16.3.2 Antiviral Strategies

16.3.2.1 Small Molecules

To date, several purine analogues (Table 16.2) have been tested for the treatment of HeV or NiV in clinical trials and/or shown promise in NHP studies. Already in 2008 and 2009, ribavirin was used to treat three individuals with confirmed HeV infection, which had close contact with infected horses [80, 95]. Of these patients, two died. While this suggests a possible lack of efficacy, it has also been suggested that the ribavirin serum

concentration was too low for efficient reduction of viral replication, when compared to in vitro efficacy data where an inhibitory effect was observable at concentrations of 49 μM and above [95, 100]. In contrast, NHP data showed a prolonged survival time in HeV-infected animals when ribavirin was given, although no benefit was observed with respect to CFR [97]. Ribavirin was also tested for NiV treatment in an open-label study during the Malaysian outbreak. In this study, 140 patients received ribavirin (compared to 54 patients who did not), resulting in a 36% reduction in mortality and a tendency toward a reduction in neurological sequelae in survivors [98]. Similarly, aciclovir (Table 16.2) was given empirically to nine patients with encephalitis during a NiV outbreak in Singapore. Eight of these patients survived [93]. Finally, remdesivir (Table 16.2) has recently been shown to protect NHPs from lethal NiV challenge [94]; however, clinical data in humans are still lacking.

16.3.2.2 Immunotherapies

Another promising approach for the treatment of HeV and NiV infection is based on mAb therapy. A mAb with cross-reactivity to both HeV and NiV (m102) was produced through recombinant antibody technology [101]. Modification of m102 through light-chain shuffling and heavy-chain variable domain random mutagenesis enhanced binding and potency further leading to mAb m102.4 [102]. m102.4 binds the site on the Henipavirus G protein responsible for interaction with its entry receptors Ephrin-B2 and Ephrin-B3. In lethal NHP models of HeV and NiV infection, all animals treated with m102.4 survived, even if treatment was initiated as late as five days after infection [103]. Furthermore, 10 human patients have received m102.4 after high-risk exposure to HeV infection, and one due to a potential NiV infection, none of which subsequently showed any signs of disease [104]. Thus, while data remain limited, m102.4 appears to be a promising treatment option, despite the need for larger and controlled clinical trials.

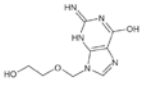
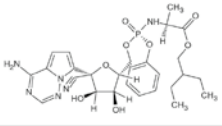
16.4 Arenaviruses

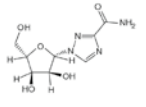
Arenaviruses are bi-segmented single-stranded negative-sense RNA viruses that compose the *Arenaviridae* family. Arenaviruses that infect mammals are divided into two serocomplexes correlating with their endemicity: the Old World Arenaviruses, found for the most part in Africa, and the New World Arenaviruses, which are mainly restricted to South America. Both groups include a number of viruses for which infection results in severe human disease, as well as others with little or no ability to cause human disease.

16.4.1 Old World Arenaviruses

Old World Arenaviruses mostly originate from Africa, with the notable exceptions of Lymphocytic Choriomeningitis Virus (LCMV), which has been found to circulate across the globe, as well as Dandenong virus, which was responsible for a small cluster of transplant-related deaths in Australia. The latter is believed to have been acquired by the donor during travel in rural areas of former Yugoslavia [105]. Among the Old World Arenaviruses, significant disease is known to be caused in humans following natural infection by LCMV,

Table 16.2 Overview of small molecules tested against henipavirus infection.

Name	Structure	Mechanism of action	In vitro effect	In vivo effect in NHPs	Clinical regimen
Aciclovir		• Unknown for henipaviruses	Not determined	Not determined	**NiV:** • Empirical therapy with aciclovir IV in nine patients, details regarding regimen not available [93]
Remdesivir (GS-5734)		• Polymerase inhibitor • Delayed chain termination	**HeV:** • IC_{50} of 0.06 μM in HeLa cells [62] **NiV:** • IC_{50} of 0.03–0.05 μM in HeLa cells [62]	**NiV:** • 10 mg/kg daily for 12 days [94]	**HeV:** • 30 mg/kg loading dose, then 15 mg/kg four times a day for four days, then 8 mg/kg three times a day [95]

Name	Structure	Mechanism of action	In vitro effect	In vivo effect in NHPs	Clinical regimen
Ribavirin		• Polymerase inhibitor • Mutagenic agent • Increases antiviral immune response • Increases cell survival	**NiV:** • IC_{50} of 1.3 µM in A549 cells [96] • IC_{50} of 0.7 µM in BAEC cells [96] • IC_{50} of 1.8 µM in BHK21 cells [96] • IC_{50} of 1.0 µM in BSR cells [96] • IC_{50} of 0.6 µM in ECV304 cells [96] • IC_{50} of 1.0 µM in HeLa cells [96] • IC_{50} of 0.9 µM in Hep2 cells [96] • IC_{50} of 1.9 µM in M17 cells [96] • IC_{50} of 1.1 µM in MMEC cells [96] • IC_{50} of 2.5 µM in Vero cells [96]	**HeV:** • Loading dose of 50 mg/kg, 10 mg/kg three times a day for 14 days starting 1 day before infection, 12 hours or 2 days after infection [97]	**NiV:** • 2 g loading dose, 1.2 g three times a day on days 2–4, then 1.2 g twice a day on days 5 and 6, then 0.6 g twice a day for additional 1–4 days [98] • 30 mg/kg loading dose, then 16 mg/kg four times a day for four days, then 8 mg/kg three times a day for three days [98] • 2 g loading dose, then 1 g four times a day for four days, then 0.5 g four times a day for six days [99]

Lassa virus (LASV) and Lujo virus (LUJV), with the last two causing VHF and being classified as BSL4 agents. Interestingly, however, LCMV has also been reported to cause severe illness, including features similar to that of VHF in patients who become infected through organ transplantation [106].

16.4.1.1 Disease Overview

LASV is the etiological agent of Lassa fever disease (LFD), which was first recognized in 1969 in Nigeria, following the infection of three missionary nurses, of whom two died [107]. An estimated 100 000–300 000 humans are infected each year, mainly in Nigeria, Guinea, Sierra Leone, Benin, and Liberia, resulting in about 5000 fatalities annually [108]. Further, the seroprevalence for LASV can be very high, ranging from 14 to 58% in highly endemic areas of Liberia and Nigeria, respectively [109, 110]. Based on such data it appears that up to 80% of LASV infections are in fact asymptomatic, with an overall fatality rate of 1–2%, although this may reach 15–20% in hospitalized patients [108, 111].

LASV is a zoonotic pathogen and is typically transmitted from its reservoir host, the multimammate mouse (*Mastomys natalensis*), through urine and feces. LASV can also be transmitted from human-to-human via direct contact with body fluids, albeit with limited efficiency. Nosocomial transmission is, however, a significant concern [112]. After a 7- to 21-day incubation period, patients develop a nonspecific febrile illness and may further develop joint pain, diarrhea, vomiting, abdominal pain, and headaches. In the most severe cases, patients can show facial edema and bleeding, as well as pleural and pericardial effusions, the latter being associated with a poor prognosis. Death usually occurs within 14 days after the onset of symptoms due to multiple organ failure and terminal shock (reviewed in [113]). While most infections are nonfatal, one common and severe consequence is acute sensoneural deafness, which affects 29% of patients [114].

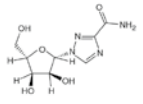
More recently, a novel and apparently highly pathogenic Old World Arenavirus named LUJV was isolated after a nosocomial outbreak in South Africa in 2008. The index patient was a tour operator evacuated from Zambia who presented symptoms very similar to severe LFD. Four of the five infected patients died, resulting in an apparent CFR of 80%. This remains the only occurrence of LUJV described to date. Attempts to identify the reservoir host of LUJV based on sampling of rodent species in Zambia have so far been unsuccessful [115, 116].

16.4.1.2 Antiviral Strategies

16.4.1.2.1 Small Molecules

There is currently no specific approved antiviral treatment available for either LASV or LUJV. The current standard of care for LFD is limited to supportive care and an off-label use of ribavirin (Table 16.3), generally as a 10-day course administered intravenously [125, 132]. Two regimen are currently used: the first regimen involves a loading dose of 33 mg/kg followed by 16 mg/kg every six hours for four days and then 8 mg/kg every eight hours for six days [132]. The second regimen starts with a loading dose of 100 mg/kg divided in two doses, followed by 25 mg/kg daily for six days and then 12.5 mg/kg daily for three days [125]. The mechanism of action of ribavirin against LASV is unknown, but may involve the induction of error catastrophe during viral replication, as has been suggested for LCMV [133]. Early work, already in the late 1970s and early 1980, in LASV-infected NHPs showed

Table 16.3 Overview of small molecules tested against infection with BSL4 arenaviruses.

Name	Structure	Mechanism of action	In vitro effect	In vivo effect in NHPs	Clinical regimen
Ribavirin		• Polymerase inhibitor • Mutagenic agent • Increases antiviral immune response • Increases cell survival	**LASV:** • IC_{50} or ED_{50} from 26.5 to 82 µM in Vero cells [117, 118] **JUNV:** • IC_{50} of 4.5 to 13 µM in Vero cells [117] **MACV:** • ED_{50} of 131 µM in Vero cells [119]	**LASV:** • 50 mg/kg loading dose, 10 mg/kg every eight hours [120, 121] **JUNV:** • 60 mg/kg loading dose, 15 mg/kg daily [122] • 15 mg/kg every 12 hours [123] • 75 mg/kg loading dose, 25 mg/kg every 12 hours [123] **MACV:** • 10 or 20 mg/kg every 12 hours [124]	**LASV:** • 33 mg/kg loading dose, 16 mg/kg every six hours for four days, then 8 mg/kg every eight hours for six days IV [111] • 100 mg/kg loading dose, 25 mg/kg daily for six days, then 12.5 mg/kg daily for six days IV [125] **LUJV:** • The only surviving patient received 2 g loading dose orally, then 1 g every six hours orally until IV was available, then 20 mg/kg IV every six hours[126] **JUNV:** • 34 mg/kg loading dose, 17 mg/kg every six hours for four days, then 8 mg/kg every eight hours for six days [127] **SABV:** • 30 mg/kg loading dose, 15 mg/kg every six hours for four days, then 7.5 mg/kg every eight hours for six days [128]
Favipiravir (T-705)		• Polymerase inhibitor • Mutagenic agent	**LASV:** • IC_{90} from 10.8 to 70.7 µM in Vero cells [129] • IC_{50} of 29.3 µM in Vero cells [118]	**LASV:** • 300 mg/kg daily [130]	**LASV:** • 16 mg/kg ribavirin IV every 6 hours, additionally 2 g favipiravir orally as loading dose followed by 1 g every 12 hours [131] • 1 g ribavirin every six hours, additionally 2 g favipiravir orally as loading dose followed by 1.2 g every 12 hours [131]

a beneficial effect of ribavirin treatment when treated with a 50 mg/kg loading dose followed by 10 mg/kg every eight hours [120, 121, 124]. Additionally, a number of case reports and retrospective cohort studies showed a beneficial effect of ribavirin in hospitalized patients [134–139]. However, it is important to recognize that many retrospective and prospective cohort studies evaluating the efficacy of ribavirin against LFD present a heavy risk of bias. This can arise, for instance, due to a lack of transparency and randomization regarding patient assignment to "no treatment" versus "treatment" arms (which may lead to inclusion of severely ill patients that experience rapid death after hospitalization, thereby preventing the start of ribavirin treatment) or the lack of appropriate control groups [140]. Indeed, a recent meta-analysis indicates the possibility of ribavirin being more detrimental than beneficial in patients presenting with a mild infection [140]. Doubts regarding the efficiency of ribavirin were also raised by an early clinical trial performed by the US Centers for Disease Control and Prevention in Sierra Leone that found little evidence of efficacy of ribavirin, but an increased risk of death for patients with aspartate transaminase (AST) levels below 150 IU/l [141]. Additional concerns have been raised by the fact that orally delivered ribavirin may not reach concentrations shown to inhibit LASV (26.5–82 μM in vitro IC_{50}) [117], and that some in vivo studies have failed to show an effect of ribavirin on viremia [118, 129]. These observations clearly need to be taken into consideration when reassessing recommended guidelines for treating LFD. With regard to LUJV, among the two of the five patients who received ribavirin, one subsequently died. While it is interesting to note that the patient who survived was the only one to receive intravenous ribavirin [126], these extremely limited case numbers currently preclude evaluation of the efficacy of ribavirin against LUJV.

More recently, favipiravir (Table 16.2) has been shown to be effective against LASV infection in vitro with an IC_{50} of 29.3 μM [118], and in guinea pigs and NHPs at a dosage of 300 mg/kg [129, 130]. Further, the possibility of combination therapies between favipiravir and ribavirin are being pursued with promising results in a lethal mouse model [118]. Moreover, this combination was also used in the field for two patients infected from the same index case [131]. Both patients survived and showed a decrease in viremia after administration of combination treatment. However, as the authors emphasize, there remains a lack of evidence to definitively attribute this decrease in viremia to (co-)treatment with favipiravir, and further clinical studies are clearly required to assess its efficacy against human LASV infection.

16.4.1.2.2 Immunotherapies

Early in vivo work in NHPs showed the potential of a combination of plasma from convalescent animals that survived LASV infection together with ribavirin to increase survival when administered up to seven days postinfection [142]. Further, nAb concentration in the immune plasma used for treatment was found to be crucial for treatment efficacy [143–145]. However, the utility of this approach is hampered by the limited cross-reactivity of sera against different LASV strains [143, 145], which are genetically highly diverse [146]. Additionally, nAb titers are low in convalescent plasma from LASV-infected patients. Perhaps as a result, a prospective cohort study failed to see a significant benefit of human convalescent plasma in patients when administered as monotherapy [132], while mixed results have been obtained from other clinical studies and case reports. These include the

successful treatment of a laboratory-acquired infection [147], variable results when immune plasma was administered early after onset of symptoms [148, 149], or even lack of any beneficial effect [150]. As an alternative to convalescent immune plasma, the use of human mAbs appears to be a viable option. In a recent study performed in NHPs, B cells isolated from convalescent donors were used to produce human mAbs. Treatment with a cocktail of these human mAbs led to 100% survival when administered immediately post-challenge [151]. However, while this suggests a possible utility for treatment of known accidental exposures (i.e. in a hospital or laboratory setting), further studies are clearly also still needed to assess the efficacy of these mAbs when given at a more clinically relevant stage of the infection.

16.4.2 New World Arenaviruses

New World Arenaviruses are divided into three clades A, B, and C, with all of those known to cause VHF belonging to Clade B. These include Junín virus (JUNV), Machupo virus (MACV), Guanarito virus (GTOV), Sabiá virus (SABV), and Chapare virus (CHPV), which are the etiological agents for Argentine, Bolivian, Venezuelan, Brazilian, and Chapare hemorrhagic fever, respectively. They are typically classified as BSL4 agents, although in some countries work with JUNV is possible under enhanced BSL-3 conditions and/or in restricted access areas by vaccinated personnel [152–154]. While other New World Arenaviruses are also able to infect humans, and can in some cases cause disease, they are normally nonfatal and infection is primarily laboratory acquired or identified by serological investigations. However, Whitewater Arroyo virus (a recombinant clade A/B virus) was suggested to be responsible for three fatal infections in California in 1999/2000 [155].

16.4.2.1 Disease Overview

Among the highly pathogenic New World Arenaviruses associated with VHF, JUNV infection is by far the most prevalent. Outbreaks in the pampas areas of Argentina have been recorded since the 1950s, with the virus being isolated in 1958. Worryingly, the endemic area has been growing constantly, correlating with the intensification of agricultural practices in this area, and likely resulting in increased contact between human populations and the rodent reservoir host *Calomys musculus* (reviewed in [156]). The incidence of Argentine hemorrhagic fever was up to 3500 cases per year prior to the introduction of the Candid#1 live-attenuated vaccine, which became available in the early 1990s, and with a CFR that ranged from 15 to 30% in untreated patients [156]. Similarly, in 1959, an outbreak of VHF in the rural area of Beni Department in Bolivia led to the identification of the closely related MACV (reviewed in [157]). Since then, sporadic outbreaks have been recorded in the same area, which corresponds to the region where a specific lineage of *Calomys callosus*, the reservoir host, is found [157]. While the number of cases is limited, the average CFR ranges from 25 to 35%, and a recent increase in disease incidence has been observed [157]. Three decades later, in 1989, GTOV was recognized and isolated in Venezuela, where several hundred cases have been documented to date [158]. The virus reservoir is the cotton rat *Sigmodon alstoni* [159], which thrives in rural areas of the country. Also here, infection seems to be seasonally associated with agricultural work [160].

While the aforementioned New World Arenaviruses are highly endemic and human infections occur on a regular basis, SABV and CHPV infections are considerably scarcer. SABV was first reported in 1990 in Brazil [161], and only three subsequent cases have been documented, two of which were laboratory-acquired infections (reviewed in [162]). Both naturally acquired infections were fatal. The reservoir for this virus has so far not been identified despite an attempt to survey rodents in the area [163]. Finally, CHPV was identified in Bolivia in 2008, after several cases of VHF were reported in a single province (Chapare province, Cochabamba Department), with one fatal case [164]. In June 2019, more than 15 years after the first outbreak, five new cases including three deaths were reported [165]. The reservoir host for this virus is not known.

Zoonotic transmission of New World Arenaviruses generally occurs via rodent urine and feces. Based on the greater case numbers, disease progression has been best described for JUNV. Here, as for other VHF diseases, early symptoms are rather unspecific, with patients showing signs of febrile illness after a 6- to 14-day incubation period. In severe cases, which represent 30% of patients, the second phase of the disease is characterized by signs of hemorrhage in about half of the cases (bleeding from mucosa, hematemesis, and petechia), and/or neurological symptoms (encephalopathy, delirium, tremor coma, and seizures). About eight days after onset of symptoms, surviving patients enter the convalescent phase, which can last for up to three months, and in which they display general weakness, hair loss, and tachycardia [166–168]. A similar disease progression is also observed for other VHFs caused by New World Arenaviruses [160, 161, 164, 169].

16.4.2.2 Antiviral Strategies

JUNV is the only New World Arenavirus, and indeed currently the only BSL4 virus for which both an effective human vaccine [170–172] and a highly effective specific treatment (based on plasma from convalescent patients) [173, 174] are available and are being used routinely. However, decreasing case numbers (due to the success of the vaccination program) threaten the long-term viability of convalescent plasma banking, while funding limitations make continued vaccine availability uncertain. Further, the lack of similar vaccine and treatment options for other New World Arenaviruses emphasizes the continued need to develop additional treatment options.

16.4.2.2.1 Immunotherapies

JUNV is the only arenavirus for which treatment with plasma from convalescent patients has been developed into a clearly defined therapeutic regimen with demonstrated clinical benefit. An initial double-blind randomized study in the late 1970s already showed that such treatment was able to decrease mortality from 16.5 to 1.1% [174]. A prospective cohort study further confirmed these results and showed a dose-dependent response of patients to the treatment [173]. In this study, all patients treated with a dose of nAbs over 3000 therapeutic units (TU)/kg survived and survival gradually decreased with lower doses [173]. The currently defined standard of care for JUNV infection involves plasma infusions corresponding to at least 3500 TU/kg of neutralizing antibody, an approach that is highly effective if started within eight days after onset of symptoms [167]. While this treatment approach is highly efficient at controlling the acute infection and preventing death, a significant complication is the occurrence of a nonfatal late neurological syndrome in 10% of

treated patients following initial convalescence [174]. The mechanism underlying the development of this syndrome remains unknown; however, similar observations have also been made in various animal models [175–177].

The relatively low number of cases for other New World Arenaviruses coupled with their often sporadic occurrence has so far limited the possibility to evaluate the efficacy of similar treatments. However, in principle, such an approach is feasible since passive immunotherapy using plasma from human donors previously infected with MACV decreased mortality in NHPs when given early after onset of symptoms, albeit with the occurrence of late neurological syndrome [178]. Although such treatments in human patients have so far shown only limited success [179, 180], an immune plasma bank was created in Bolivia in 2010, in collaboration with the Pan American Health Organization (PAHO) and World Health Organization (WHO), to face future outbreaks. Unfortunately, even if such treatments were proven to be effective for other New World Arenaviruses, there would likely be significant difficulties in obtaining sufficient amounts of high-titer plasma. Nonetheless, the possibility to use plasma from patients that have recovered from infection with a different New World Arenavirus has been suggested due to the existence of cross-neutralizing antibodies. However, it remains unclear whether such sera would have sufficiently potent cross-neutralizing activity to be clinically effective. Alternatively, research aiming at the identification of mAbs able to efficiently cross-neutralize multiple species of New World Arenaviruses is a promising approach, and some preliminary studies in this direction have already been performed [181, 182].

16.4.2.2.2 Small Molecules

Based on its use in the treatment of the related LASV, ribavirin (Table 16.3) was one of the first treatment options investigated for New World Arenaviruses. It was successful in controlling infection with JUNV in vitro at IC_{50}s ranging from 5 to 13 μM [183] as well as in vivo in both rats and NHPs at dosages above 15 mg/kg daily [122, 123, 184]. More recently, however, more mixed results were obtained in guinea pigs, with ribavirin increasing survival time, but without always improving CFR [185]. Similarly, a prospective study performed on six patients treated with ribavirin between 9 and 11 days after onset of symptoms using a loading dose of 34 mg/kg followed by 17 mg/kg every six hours for four days and then 8 mg/kg every eight hours for six days showed an increased survival time but no impact on CFR. However, this may have been due to the very late onset of treatment [127] due to ethical limitations regarding the use of alternative therapies during the window within which the standard of care (i.e. plasma therapy) is known to be highly effective. Ribavirin is currently not recommended for use in JUNV-infected patients, but rather the use of immunotherapy is advised. However, it is considered a viable treatment option for VHFs caused by other New World Arenaviruses, or in cases where immunotherapy is not accessible (i.e. imported infections), even if data examining the efficacy of its use against those specific pathogens are scarce [186]. In this regard, one in vivo study has shown that ribavirin was able to induce a dose-dependent decrease of viremia in NHPs infected with MACV at dosages above 10 mg/kg every 12 hours [124]. Further, intravenous ribavirin was used experimentally in two of three suspected cases of MACV infection. Both patients who received ribavirin survived, but laboratory confirmation of MACV infection was only conducted for one of them [187]. Intravenous ribavirin was also successfully used as PEP

in a case of occupational exposure to SABV with a 30 mg/kg loading dose followed by 15 mg/kg every six hours for four days and then 7.5 mg/kg every eight hours for six days [128]. Overall, while these data are clearly too limited to draw strong conclusions, they at least suggest a possible benefit of ribavirin treatment. Consistent with this, during the June 2019 outbreak of CHPV, PAHO and WHO emphasized that there are currently no clinical trials demonstrating ribavirin efficacy against this virus and advised caution regarding its use [165].

16.5 Bunyaviruses

Bunyaviruses are tri-segmented single-stranded negative-sense RNA viruses of the order *Bunyavirales*. Those that are recognized as presenting a significant risk to human health are members of the families *Nairoviridae* (genus *Orthonairovirus*), *Phenuiviridae* (genus *Phlebovirus*), *Peribunyaviridae* (genus *Orthobunyavirus*), and *Hantaviridae* (genus *Orthohantavirus*). While many bunyaviruses can cause human disease of varying severity, including Rift Valley Fever virus, La Crosse virus, Hantaan virus, and Oropouche virus, only Crimean Congo Hemorrhagic Fever Virus (CCHFV) is widely classified as a BSL4 agent.

16.5.1 Disease Overview

CCHFV was first recognized in 1944 after an outbreak in the Crimean peninsula, and an identical virus was isolated from a patient in the former Belgian Congo in 1956. However, it was only more than a decade later that the identity of both viruses was recognized. CCHFV is endemic to a vast region spanning Europe, Asia, and Africa, where it circulates in a variety of wild and domestic mammals, including cattle, goat, and sheep. Transmission of CCHFV is primarily facilitated by ticks of the genus *Hyalomma*. However, infection of humans also occurs as a result of direct contact with blood from infected animals (primarily agricultural animals), and via human-to-human transmission through contact with body fluids. Indeed, the seroprevalence in farm animals can be as high as 80% in endemic areas [188], while in humans in these areas, it is on average around 4.5%, with a higher risk in specific populations such as farmers, butchers, and slaughterhouse workers [188]. While most infections are believed to be asymptomatic, in symptomatic cases the disease starts with a nonspecific febrile illness after an incubation time ranging from one to seven days. Three to five days after onset of symptoms, severe cases may show signs of hemorrhage. High viremia and a lack of virus-specific IgG production, as well as clinical signs including ecchymoses and gingival bleeding, are associated with a poor prognosis [189, 190]. Reported CFRs in outbreaks vary widely from 3 to 80% and high CFRs are usually reported in small outbreaks, suggesting a bias in case detection (reviewed in [191]). There also appear to be strain-specific differences in disease severity [192]. In fatal cases, death usually occurs between 5 and 14 days after the onset of symptoms due to shock and multi-organ failure. The convalescent phase in CCHF survivors can last up to a year and is characterized by general weakness, tachycardia, and sometimes other symptoms like memory loss [193].

16.5.2 Antiviral Strategies

As for most BSL4 agents, there is no specific treatment for CCHFV. An inactivated vaccine produced in neonatal mouse brain was developed in the former Soviet Union and is still in use in Bulgaria [194, 195]. However, due to safety concerns and limitations in the scale of production, as well as a lack of data regarding its efficacy against most strains of CCHFV, it appears unlikely that its use will be extended. Thus, the development of effective antiviral strategies is clearly needed. Unfortunately, the lack of adequate animal models has for decades impaired the preclinical evaluation of treatments against this disease (reviewed in [196]). Indeed, it was only in 2018 that a cynomolgus macaque model that recapitulates CCHFV infection in humans was first reported [197], However, issues with reproducibility, possibly due to the outbred nature of the animals [198], indicate that there are still significant challenges that have to be overcome in this area.

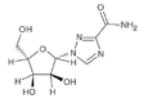
16.5.2.1 Immunotherapies

Immunotherapy has only been used in a limited number of CCHFV-infected patients. A study described the benefit of treating severe cases of CCHFV with an intravenous preparation of immunoglobulin from the plasma of donors vaccinated with the CCHFV Bulgarian vaccine. All patients recovered, but there was no control group [199, 200]. Two other studies used sera from convalescent patients to treat CCHFV during outbreaks in South Africa and Dubai [201, 202]. In the South African outbreak, all patients treated by passive immunotherapy survived and the two patients who died did not receive immune serum due to delayed diagnosis [201]. During the outbreak in Dubai, only one of the seven patients received immunotherapy, and that individual subsequently survived the infection [202]. Similarly, hyperimmune globulin against CCHFV was prepared from pooled plasma of multiple CCHFV survivors and used to treat patients with viral loads above 10^8 copies/ml. Almost 90% survival was observed, while historically without treatment the CFR in such cases is close to 90% [203]. However, while certainly encouraging, none of these studies have included a proper control arm or had a significant number of patients based on which to draw firm conclusions. The WHO is currently advocating additional studies on the development of mAbs for CCHF treatment [4], and while to date only a few such studies have been conducted, they show encouraging results both in vitro and in animal models [204–206].

16.5.2.2 Small Molecules

Currently, CCHFV treatment relies mostly on supportive care, together with the use of ribavirin, which, though controversial, is generally recommended. In vitro data have shown an inhibitory effect of ribavirin (Table 16.4) on CCHFV with IC_{50}s ranging from 11.5 to 56.6 μM, as have in vivo studies in neonatal mice using dosages above 50 mg/kg [207–209, 227]. Deep sequencing analysis in a single patient after treatment suggests that ribavirin has a mutagenic effect on CCHFV, thereby driving the virus into error catastrophe [222]. Numerous retrospective cohort studies [210–213, 215, 219–221, 228, 229], case-control studies [218], and case reports [216, 217, 222–224, 230, 231] on the efficacy of ribavirin have been conducted and most of them have suggested a beneficial effect. However, the only randomized clinical trial performed to date failed to show a beneficial effect of ribavirin [214]. Additionally, three meta-analyses concluded that there are neither beneficial nor

Table 16.4 Overview of small molecules tested against infection with CCHFV.

Name	Structure	Mechanism of action	In vitro effect	In vivo effect	Clinical regimen
Ribavirin		• Polymerase inhibitor • Mutagenic agent • Increases antiviral immune response • Increases cell survival	• IC_{50} or ED_{50} of 11.5–65.5 µM in Vero cells [207, 208]	• 50 or 100 mg/kg, single or daily dose for four days in neonatal mice [209]	• 30 mg/kg initial loading dose, then 15 mg/kg every six hours for four days, then 7.5 mg/kg every eight hours for six days orally [210–214] • 4 g daily for four days, then 2.4 g daily for six days [215–217] • 17 mg/kg loading dose, then 17 mg/kg every six hours for four days, then 8 mg/kg every eight hours for six days IV [218] • 4 g daily for four days, then 2.4 g daily for six days [219] • 2 g loading dose, then 1 g every six hours for four days, then 500 mg every eight hours for six days [220, 221] • 1 g every six hours for one day, then 1 g every six hours for one day IV, then 500 mg every eight h for four days IV [222] • 2 g loading dose, 4 g daily for four days, then 2 g daily for six days [223, 224]

Name	Structure	Mechanism of action	In vitro effect	In vivo effect	Clinical regimen
Favipiravir (T-705)		• Polymerase inhibitor • Mutagenic agent	• IC_{50} of 1.03 µM in Huh7 cells [225] • IC_{50} of 7 µM in Vero cells [207]	• 15 mg/kg, 30 or 300 mg/kg daily in $IFNAR^{-/-}$ mice [55] • 7.5 or 30 mg/kg daily of favipiravir + 100 mg/kg of ribavirin in $IFNAR^{-/-}$ mice [55] • 300 mg/kg daily in $IFNAR^{-/-}$ mice [226]	Not determined

detrimental effects of ribavirin treatment [232–234]. This is consistent with the results of the largest cohort study to date, which showed no beneficial effect of ribavirin in patients with a mild infection, and a potential detrimental effect in patients with a severe infection displaying impaired renal and liver functions [212]. Additionally, two of the meta-analyses evaluated the benefit of early versus late administration of ribavirin (i.e. before or after five days after onset of symptoms), and both concluded that there is no beneficial effect of an early administration of the drug [233, 234]. One of these meta-analyses featured a particularly thorough analysis of both included and excluded studies [234], noting a number of limitations in existing studies. In particular, many studies use historical records as their control arm, resulting in the risk that patients received a significantly different quality of supportive care. Also, the dosage and route of administration of ribavirin, while in the same range, varies between available studies. Thus, a proper clinical trial, in the context of good supportive care, remains necessary to fully evaluate the efficacy of ribavirin for the treatment of CCHFV.

Similar to other VHF, the use of favipiravir (Table 16.4) could also be beneficial. To date, it has been shown to exert antiviral effects alone or in combination with ribavirin in vitro, with an IC_{50} between 1 and 7 μM, and in vivo against multiple strains of CCHFV in preliminary studies at dosages above 15 mg/kg daily, or as low as 7.5 mg/kg if used in combination with 100 mg/kg ribavirin daily [207, 225, 226]. Further, a recent study has shown decreased viremia in NHPs treated with a 300 mg/kg dosage of favipiravir starting 24 hours post infection [235], and future clinical trials with this molecule against CCHFV are on the WHO priority list [4]. A major concern in the treatment of CCHFV is the finding that the virus can persist in treated animals and cause a delayed fatal infection [226], highlighting the possible need for combination therapy against CCHFV, as well as a close follow-up of patients during and after treatment.

16.6 Considerations for the Development of Treatment Strategies Against Viral Hemorrhagic Fever Viruses

The ability to conduct high-quality trials for BSL4 agents is often hampered by the epidemic nature of most of these diseases, coupled with limited numbers of patients and/or poor infrastructure in affected areas [236]. Combined with the lack of economic incentives for commercial development of such treatments, this has been a major hurdle to the development and testing of therapies. Further, even where such therapies exist, they may face significant logistical problems with respect to production and/or availability. This advocates for multiple treatment options against a given pathogen in order to protect against changes in the availability of a single treatment or shortages during outbreaks, thereby ensuring sustainable management of these diseases. Indeed, this also would help to ensure that products are available that are suitable for specific applications, for instance, in patients with severe vomiting where oral treatment may be difficult, or in locations where the medical infrastructure needed for antibody delivery may be unavailable, an issue that can be particularly important in the treatment of these exotic infections. A multipronged treatment approach would also increase the chances to have effective treatment options available in the event of newly emerging virus species/strains, which—depending on their makeup—may not be adequately managed with all approaches.

Despite the fact that the major limitation in the treatment of infections with BSL4 pathogens is the lack of available therapies with demonstrated efficacy, the testing of potentially novel treatments for viruses where a standard of care (whether truly effective or not) has already been established presents special challenges. This is most notable for infection with JUNV, where the testing of antiviral compounds has so far been restricted to patients already outside the treatment window for convalescent plasma therapy. Thus, while these treatment attempts have been relatively unsuccessful [127], it is clear that treatment of these patients represents a major challenge, and that such approaches might be much more successful if initiated earlier. In particular, it would appear that testing of novel therapies in combination with the existing standard of care might offer a way forward. Indeed, this approach is being pursued for treatment of LASV (where there is conflicting evidence about the efficacy of the standard therapy alone) with apparent success at the level of small animal model testing [118].

At the same time, however, the high CFRs associated with many of these infections make it very tempting to use potential treatments even if only limited information regarding their efficiency in animal models is available. An example is the development of countermeasures for filovirus infection, which was highly promoted during the West African Ebola outbreak in 2013–2016. In this case, soon after the outbreak was declared a public health emergency of international concern, the WHO decided that it was ethically acceptable to start clinical trials for EBOV countermeasures showing promising results in laboratory and animal models, but for which safety and efficacy had not yet been tested in humans [237]. However, most of the treatments failed to show efficacy, and for some of these studies considerable ethical concerns have been raised [238]. This experience clearly demonstrates the need for sufficient in vivo data using predictive animal models before conducting clinical trials in humans, even in outbreak scenarios. Further, careful study planning, although challenging, remains critical to obtain high-quality data to support the establishment of well-supported treatment guidelines that will truly be effective.

Finally, with respect to the use of treatments for which only limited data may be available, the research communities working with BSL4 agents present an additional dilemma. For most of these agents no vaccines or treatment options have been licensed (and even where this has been achieved, there remain major hurdles regarding their availability in practise), posing the difficult question what to do in the event of a laboratory-acquired infection. While in specific cases access to experimental therapies has been made available in the past, the management of such cases is currently left to be dealt with on an emergency case-by-case basis with little or no formal planning or coordination. While there are considerable legal obstacles that may prevent the import and stockpiling of experimental medications for use under such circumstances, the consequences of not doing so are potentially dramatic and raise significant ethical issues per se. Additionally, there are logistical issues that surround acquiring and coordinating the access to these medications, and in some cases also maintenance of the expertise needed to deliver them correctly.

It is important to note that the same issues arise in relation to our ability to treat imported infections with these agents, something that is increasingly likely in a highly connected and mobile world. To address this problem, conditions have to be generated that facilitate rapid and reliable access to both licensed therapies (where they exist) as well as the use of experimental treatment options, and particularly to allow stockpiling of the necessary therapeutics or PEP for use in emergencies in countries with BSL4 laboratories.

Nonetheless, while there are many challenges inherent in the development and testing of therapeutic options for BSL4 viruses, past and recent successes exist and demonstrate that treating these diseases is clearly possible. What is critically needed, and unfortunately often lacking, is sustained political interest and continuous support at the national and international level. On the other hand, increased public awareness of the potential for even these relatively rare and exotic viruses to cause large outbreaks, including the potential for significant spread to non-endemic areas, may be a reason for cautious optimism regarding the development and availability of treatment options with proven efficacy against BSL4 agents in the future.

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17

A Focus on Severe Acute Respiratory Syndrome (SARS) Coronavirus (SARS-CoVs) 1 and 2

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17.1 Overview on Coronavirus (CoV)

17.1.1 CoV Epidemiology

Coronaviruses (CoVs) are pleomorphic, large, enveloped, positive-sense single-stranded RNA ((+)ssRNA) viruses belonging to the order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae*, classified into four genera: *α-, β-, γ-, and δ-CoVs*. CoVs are globally distributed in a large number of species including bats, camels, pigs, bovines, felines, poultry, mice, dogs, rabbits, whales, humans, and many others. The *α-* and *β-CoVs* infect only mammals, while *γ- and δ-CoVs* infect birds, even if some of them may also infect mammals [1]. The ~30 kb RNA genome of CoV contains at least six open reading frames (ORFs, see later for details), with an overall 54% identity at the whole genome level. However, each virus lineage can be distinguished by its ORF structure.

The 5′ genomic region encodes the first ORFs, making about two-thirds of the whole genome length and scoding 16 nonstructural proteins (nsps) (nsp1–16), except *γ-CoVs* that lacks nsp1, and is mostly conserved with a 58% nsps identity. Differently, the 3′ genomic region varies among genera, and even from strain to strain within a viral group, with a 43% identity on the structural protein-coding region [2, 3]. CoVs encode four main structural proteins: the spike surface glycoprotein (S) protein, the nucleocapsid (N) protein, the membrane (M) protein, and the envelope (E) protein. While most CoVs require all of them for a mature virion, some do not, indicating that some structural proteins might be dispensable, and their functions can be taken over by additional proteins [4]. Most often, the CoV envelope contains three to four viral proteins. The most abundant structural proteins are the S and M proteins, while the E protein constitutes a minor but critical structural component. In addition, in some CoVs, a hemagglutinin esterase is also found. The last structural protein, N, binds the viral RNA genome to form the nucleoprotein.

Viral entry is initiated by the specific engagement of CoVs S proteins with the corresponding cell host surface receptors, a key step to establish infection. In recent years, a number of

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studies have proposed that changes in S proteins can foster the expansion to CoV from one species to another [5, 6] and hence the wide range of animal hosts has been proposed to be mainly due to both high mutation rates and recombination events of CoVs [7, 8]. In particular, cross-species transmission has been proposed to take place between different CoVs infecting a common host. In this respect, it is worth to note that CoVs infecting single animal species belongs to different genera. As an example, the six CoVs infecting pigs belong to *α-CoVs* (porcine epidemic diarrhea virus, PEDV; porcine respiratory coronavirus, PRCV; acute diarrhea syndrome virus, SADS-CoV; porcine transmissible gastroenteritis coronavirus, TGEV), *β-CoVs* (porcine hemagglutinating encephalomyelitis virus, PHEV), and *δ-CoVs* (porcine *δ-CoV*, PDCoV). A similar picture is also present in circulating human CoVs that can also be divided into low and high pathogenic viruses. Among the former are the *α-CoVs* HCoV-229E, HCoV-NL63, and the *β-CoVs* HCoV-OC43 and HCoV-HKU1 that generally cause mild upper respiratory illness and collectively are associated with 10–30% of common cold cases [9]. Among the latter are the highly similar Severe Acute Respiratory Syndrome CoV (SARS-CoV-1) and SARS-CoV-2, and the Middle East respiratory syndrome CoV (MERS-CoV) that caused three outbreaks in 2002 (SARS-CoV-1), in 2011 (MERS-CoV), and in 2019 (SARS-CoV-2). While the 2002 epidemic has been declared to be over in 2003 [10], the 2011 is still ongoing [11] and has involved >25 countries so far, and the 2019 is currently determining the worse pandemic event of this century. As in the case of other CoVs and also many human viruses causing burdening diseases, existing sometimes since centuries, such as measles virus, influenza virus, smallpox virus, or more recently dengue virus, Ebola virus, HIV, Chikungunya virus, Zika virus, and others, also these highly pathogenic CoVs originated by animal-to-human host-switching. In particular, it is likely that SARS-CoV-1 originated from bats through sequential recombination of bat CoV (SARS-CoVs) and that masked palm civets (*Paguma larvata*) were intermediate hosts [2]. MERS-CoV is also believed to have originated in bats, and dromedary camels were suggested to play roles as intermediate hosts [11]. In the case of SARS-CoV-2, also bats have been suggested as likely reservoir hosts [12] and pangolins have been suggested as possible hosts in the emergence of the SARS-CoV-2 [13], even if the scientific debate seems to be not concluded yet.

17.1.2 HCoV Replication

In the present book chapter, we present the CoV replication cycle taking into account mostly the etiological agent of the current pandemic, SARS-CoV-2. The considered steps are mostly shared among the three human highly pathogenic viruses that, otherwise, differ for their accessory proteins that probably modulate virus–host interactions processes such as in cell proliferation, innate immune evasion, programmed cell death, and others. While these viral functions could be indeed exploited as drug targets, they are not the specific focus of the present chapter.

SARS-CoV-2 has a single-stranded, 5′-capped, (+)ssRNA genome, and has been annotated to contain 14 ORFs, coding for 27 proteins (Figure 17.1a) [14]. The first two ORFs (ORF1a and -b) encompassing roughly the 5′-terminal two-thirds of the viral genome, encode for the 16 viral nsps. In particular, the ORF1ab encodes the pp1ab polyprotein that contains 15 nsps (nsp1–nsp10 and nsp12–nsp16), while the ORF1a encodes the pp1a polyprotein that contains 10 nsps (nsp1–nsp10). The ORFs at the 3′-terminus of the genome encode for four

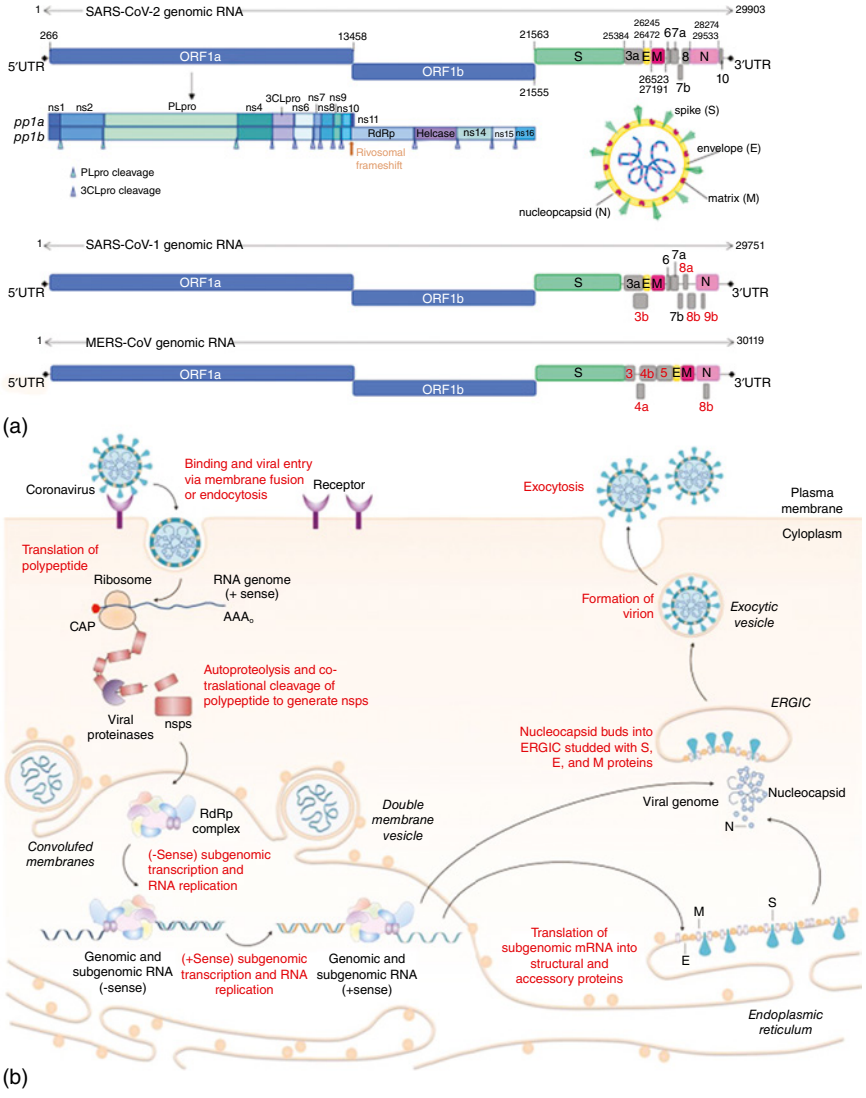


Figure 17.1 (a) Genomic structure of SARS-CoV-2 and comparison with respect to SARS-CoV-1 and MERS-CoV. ORFs encoding for polyproteins (pp) including the various nonstructural (ns) elements are represented in blue, while genes encoding for accessory proteins are colored in gray. For SARS-CoV-2, the various structural proteins are represented in a schematic virion structure as well. Proteins that differ from SARS-CoV-2 in SARS-CoV-1 and MERS-CoV are indicated in red. Nucleotide positions are referred to the representative genomic sequences for SARS-CoV-2 (NC_045512), SARS-CoV-1 (NC_004718), and MERS-CoV (NC_019843). (b) Schematic representation of CoV life cycle.

structural proteins, namely the S surface glycoprotein, the small E protein, the M protein, and the N protein, and eight accessory proteins (3a, 3b, p6, 7a, 7b, 8b, 9b, and orf14).

The SARS-CoV-2 replication cycle starts with the interaction of the viral S protein with the human angiotensin-converting enzyme 2 (ACE2), found in lung, heart, kidneys, and

intestine cell membranes (Figure 17.1b) [15]. The S protein is a large glycosylated transmembrane protein forming homotrimers and, in addition to playing a crucial role in mediating virus attachment to the host cell receptor, is also the main antigen exposed at the surface of the virion, acting as a major inducer for the host immune response. The S protein, mostly extending outside the virion, belongs to the class I viral fusion proteins and it can be divided into a S1 subunit, critical for receptor recognition, and the S2 subunit, important for membrane fusion. In fact, following ACE-2 binding, S is cleaved into the two S1 and S2 subunits by a cellular protease triggering an efficient entry into the cytoplasm by endocytosis. In the acidic endosome, S is further cleaved by lysosomal proteases for exposing the fusion peptide (FP), leading to fusion of the virus envelope with the endosome membrane, and ending in the viral RNA release into the cytosol. Different from SARS-CoV-1 and SARS-CoV-2, the receptor for MERS is the dipeptidyl peptidase 4 (DPP4) also known as CD26, a multifunctional type-II transmembrane glycoprotein endowed with exopeptidase activity that preferentially cleaves dipeptides from hormones and chemokines at a site following a proline amino acid residue, important for controlling their bioactivity [11]. Of note, it has been shown that CoVs can enter cells also by membrane fusion [16], and which of the two pathways (endocytosis or membrane fusion) is most relevant needs to be clarified yet. Once entered into the cytoplasm, the 5′-capped, (+)ssRNA genome is recognized by the cellular translation machinery leading to the first viral polyproteins production that are subsequently processed into individual proteins (Figure 17.1b). Of note, the shift between the shorter ORF1a and the longer ORF1b is due to a -1 ribosomal frameshift in the overlapping region between ORF1a and -1b just upstream the stop codon, hence enabling the production of the larger pp1ab polyprotein. The frameshifting process occurs with a ~20–50% efficiency and is triggered by a slippery sequence, UUUAAAC, followed by an RNA pseudoknot structure [17]. The pp1a and pp1ab polyproteins are cleaved into single proteins by two viral proteases, a papain-like protein (PL^{pro}) encoded by nsp3, and a chymotrypsin-like cysteine protease ($3CL^{pro}$) encoded by nsp5 [18]. While $3CL^{pro}$ catalyzes the proteolytic cleavage of all nsps downstream of nsp4, and is thus referred to as the main viral protease, PL^{pro}, in addition to being responsible for the few other cleavage events, has also additional roles in the evasion of the immune system. Liberation of the single nsps allows formation of the replication complex, whose main element is the RNA-dependent RNA polymerase (RdRp). It is encoded by the nsp12 gene, and copies the (+)ssRNA into (−)ssRNA that, in turn, serves as template for more (+)ssRNA genomic copies and subgenomic mRNAs. The replication complex is also formed by processivity factors (nsp7–8), a helicase (nsp13), single-strand binding proteins (nsp9), a proofreading exonuclease (nsp14), other cofactors (e.g. nsp10), and capping enzymes (e.g. nsp16). Such multifactorial replication complex is not common among (+)ssRNA viruses and has also the peculiarity of a proofreading system leading to high-fidelity viral replication. In fact, due to the big size of SARS-CoV-2, avoiding the accumulation of mutations is essential for the viral population, since it has been shown that 15 kb is the living threshold for viral genomes without proofreading and that bigger genomes require proofreading to avoid a high mutational burden leading the viral population into catastrophic events [19]. Indeed, the similar SARS-CoV-1 mutation rates (10^{-6}) are some order of magnitude lower than the ones of most RNA viruses [20]. The SARS-CoV-2 replication, as the one of other CoVs and (+)RNA viruses, has been proposed to take place in double membrane vesicles to which the replication complex is anchored and that spatially

divide the site of viral RNA replication from intrinsic immune sensors and from downstream virion assembly taking place in the endoplasmic reticulum–Golgi intermediate compartment (ERGIC). Of note, nsps with integral transmembrane domains such as nsp3, nsp4, and nsp6 are involved in membrane manipulation allowing vesicle formation, probably by introducing curvatures in the ER membranes [21]. While the copies of the (+)ssRNA genome are produced by the replication complex, the structural glycoproteins (M, E, and S) incorporated into the envelope are translated into the ER and accumulate in a ERGIC budding site different from the one producing viral genomes. The M protein is important for incorporating essential viral components into the new virions during morphogenesis. The E protein participates in viral maturation and forms ion channels in the viral membrane whose role is still unclear, while the S protein is decorating the external portion of the virion in view of the next viral replication cycle. The last structural protein, the N protein, has the role of associating the viral genome and M and hence directs genome packaging into new viral particles. Finally, progeny virions in vesicles are transported to the cell surface and released to the outside environments through the exocytic pathway or cell lysis.

17.1.3 Human Corona Virus (HCoV) Diseases Natural History

HCoVs are known to cause a large proportion of minor upper respiratory tract infections; however, SARS-CoV-1, MERS, and SARS-CoV-2 cause severe diseases that can have a fatality of up to 10% for SARS-CoV-1 and 36% for MERS [10, 22, 23]. Both SARS-CoV-1 and MERS had a limited number of cases as compared with SARS-CoV-2, and so their pathogenesis is poorly understood. Both viruses were reported to have around 5 days of incubation and then 95% of patients developed symptoms within 13 days of exposure. Common early symptoms were fever, coughing, myalgia, and headache, then lung disease takes place leading to abnormal chest X rays in 60–100% SARS-CoV-1 and 90–100% MERS patients. Immunopathogenic events seem to contribute to disease progression into acute respiratory distress syndrome (ARDS) that is associated with high upregulation of pro-inflammatory cytokines production.

The current pandemic events have put in place enormous efforts in understanding the SARS-CoV-2-induced infection events, and the close examination of the COVID-19 disease progression in patients has identified three phases of the disease (Table 17.1). There is general agreement to consider an individualized treatment approach for patients based on their symptoms corresponding to these stages of infection. The three phases have a different type of biological interaction with the virus.

The first phase (phase 1) starts through the contact with the infected droplet from individual to individual. SARS-CoV-2 binds to epithelial cells presenting the ACE2 receptor and begins to replicate (Table 17.1). The virus diffuses locally activating a well-coordinated innate immune response that is the first-line physiological response to infection. This phase can cause an asymptomatic stage, in which the infected individual is positive to RT-PCR tests, can spread the virus but does not clinically manifest disease symptoms, or it can cause mild symptoms that are generally confused with a common cold or flu such as cough, fatigue, headache, sore throat, runny nose, diarrhea, and others.

The second phase (phase 2) occurs during a persistent replication of the virus in the upper and lower airway and at the pulmonary level. In this phase, the immune system can be severely affected by the infection leading to primarily respiratory symptoms such as

Table 17.1 Phases of COVID-19 disease.

Phase	Virus location	Host response	Symptoms
1	Early infection in epithelial cells of upper airway	Innate immunity	Asymptomatic or mild symptoms similar to common cold or flu (cough, fatigue, headache, sore throat, runny nose, diarrhea, and others)
2	Persistent replication at the pulmonary level	Immune system severely affected	Respiratory symptoms (shortness of breath, persistent cough, up to low oxygen levels) and formation of blood clots
3	Gas exchange units of the lungs	Hyperactivation of the immune system with hyperinflammation	Serious respiratory symptoms, alveolar damage, multiorgan injury

shortness of breath, persistent cough, up to low oxygen levels. Another severe complication in this phase can be the formation of blood clots.

The third phase (phase 3) corresponds to hyperactivation of the immune system and hyperinflammatory progression. The virus reaches the gas exchange units of the lungs leading to diffuse alveolar damage and at the same time induces dysregulation of immune response. The hyperinflammatory profile consistent with a cytokine storm, in which the body attaches its own tissue, is robustly associated with COVID-19 severity and is also accompanied by injuries to other organs. In fact, COVID-19 infection has been reported to impact on the cardiovascular system, the renal system, the hepatic and gastrointestinal system, and to lead to neurological complications. A number of COVID-19 sequelae have been also reported and are currently under scrutiny.

The three phases clearly overlap and, to have an effective therapy, it is very important to recognize each stage and tailor the general treatment toward a specifically personalized treatment.

Looking at the physiopathology of the disease, the antiviral drugs should be more efficacious in the phase 1 and 2 of the disease. This also opens up the possibility to use potent selective and safe antiviral for prophylaxis when an individual gets in contact with a positive patient and still does not know if it has been infected. The scenario clearly indicates that there is space for antiviral drugs for prevention and treatment in the presence of vaccines and therapeutic antibodies that will be developed.

In the following sections, the antiviral approaches exploited to fight CoV infection, mainly SARS-CoVs and to a lesser extent MERS-CoV, are described focusing on licensed and investigational drugs. Moreover, the most promising and characterized viral targets, such as the $3CL^{pro}$, the nsp12 RdRp, and the S fusion protein, and the S/ACE2 interaction as the viral target to inhibit entry, and the inhibitors, reported so far, are analyzed.

17.2 Licensed and Clinical Investigational Drugs Against CoVs

The $3CL^{pro}$ and the nsp12 RdRp are the best characterized SARS-CoV targets and show the highest degree of conservation across CoVs, fostering the identification of broad-spectrum inhibitors. Indeed, the most advanced antiviral agents approved for COVID-19 or in clinical development are inhibitors of RdRp and of $3CL^{pro}$.

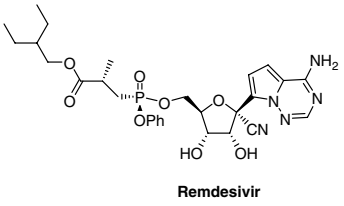
17.2.1 Licensed Drugs Against CoVs

The first and currently only approved drug against a CoV infection and licensed for COVID-19 treatment is Remdesivir (GS-5734), developed by Gilead Sciences (Figure 17.2). Remdesivir received FDA and EUA [28] approval following the encouraging results from the National Institute of Allergy and Diseases (NIAID), Gilead clinical trials, and from the compassionate use programs [29–31]. The fast approval was the consequence of a drug repurposing approach applied for the SARS-CoV-2 pandemia. Remdesivir is the Sp isomer ester monophosphoramidate prodrug of 1′-cyano-substituted adenine C-ribonucleoside analogue known as GS-441524 [32–34]. Originally identified within an antiviral research program against HCV and respiratory syncytial virus (RSV), Remdesivir showed a broad antiviral activity against other RNA viruses, including Ebola virus and SARS-CoV [32, 35, 36]. Although Remdesivir performed well in preclinical studies, it did not meet efficacy endpoints in a randomized trial conducted during an Ebola outbreak [37].

Remdesivir has shown [38, 39] potent antiviral activity against SARS-CoV-1 and MERS-CoV (EC_{50} = 0.069 and 0.074 μM, respectively) in primary human airway epithelial cell (HAE) cultures [38]. More recently, Remdesivir also showed antiviral activity against SARS-CoV-2 infections, with a potency varying from the nM to the μM range depending on cell lines and evaluation method (Figure 17.2) [24–27].

Remdesivir treatment significantly ameliorated loss of pulmonary function in female C57Bl/6 carboxyl esterase 1c deficient (Ces1c-/-) mice administered (25 mg/kg sc) 1 day postinfection by SARS-CoV-2 and BID thereafter. The transgenic mouse model better recapitulates the DMPK profile of Remdesivir in humans, that lack Ces1c, a mouse serum esterase dramatically reducing the half-life of Remdesivir [38]. Remdesivir in vivo efficacy against SARS-CoV-2 was also observed in rhesus macaques (12 h p.i with 10 mg/kg and once-daily for 6 days with 5 mg/kg) [40] reducing virus titers, clinical disease, and damage to the lungs. The treatment resulted in reduced virus replication in the lower respiratory tract, but not in the upper tract, suggesting the need to explore drug delivery strategies to improve Remdesivir distribution.

Regarding the use of Remdesivir in humans, the optimal duration of treatment is still under evaluation in clinical trials. Both 5-day and 10-day treatment durations are suggested, based on the severity of the disease, with 200 mg iv on the first day, followed by 100 mg on each consecutive day. The approval was granted on the basis of the available data from the Gilead's SIMPLE trials (NCT04292899), the Adaptive COVID-19 Treatment Trial (ACTT), sponsored by NIAID (NCT04280705), in patients with severe manifestations of COVID-19, and from the compassionate use program.



$EC_{50\ SARS\text{-}CoV\text{-}1\ (HAE)}$ = 0.069 μM
$EC_{50\ MERS\text{-}CoV\ (HAE)}$ = 0.074 μM
$EC_{50\ SARS\text{-}CoV\text{-}2\ (Vero)}$ = 0.77[a] or 29.9[b] or 1.49[c] μM (RNA quantification)
$EC_{50\ SARS\text{-}CoV\text{-}2\ (Vero)}$ = 1.65[c] μM (Plaque assay)
$EC_{50\ SARS\text{-}CoV\text{-}2\ (Vero)}$ = 0.65[d] μM (CPE)
$EC_{50\ SARS\text{-}CoV\text{-}2\ (Calu3)}$ = 0.60[c] μM (RNA quantification)
$EC_{50\ SARS\text{-}CoV\text{-}2\ (Calu3)}$ = 0.28[c] μM (Plaque assay)
$EC_{50\ SARS\text{-}CoV\text{-}2\ (HAE)}$ = 0.01[c] μM (Plaque assay)

Figure 17.2 Remdesivir and its antiviral activity against SARS-CoVs and MERS-CoV. *Source*: [a]From Ref. [24], [b]from Ref. [25], [c]from Ref. [26], and [d]from Ref. [27].

The two SIMPLE trials evaluated the safety and efficacy of 5-day and 10-day duration of Remdesivir in hospitalized patients with severe (first study) and moderate (second study) COVID-19, showing similar results for both regimes [29]. Thus, priority should be given to a five-day regimen for patients at the early stages of severe disease [30]. During the trial, the side effects were mild and, however, the absence of a placebo control group and the open-label design of this study did not permit an overall assessment of the benefit of Remdesivir. Another limitation is the lack of SARS-CoV-2 viral-load data. The recently published results of the second SIMPLE trial study showed that among patients with moderate COVID-19, those randomized to a five-day course of Remdesivir plus standard of care had higher odds of having an improvement in clinical status compared with those randomized to standard of care alone [31].

In contrast to Gilead's trials, interim results from another large trial, the WHO's SOLIDARITY, seem to indicate that none of the four repurposed antiviral drugs tested including Remdesivir have a significant effect in reducing COVID-19 mortality, the need for ventilators, or the duration of hospitalization [41]. However, this was an open-label trial as well.

The ACTT trial is a randomized, double-blind, placebo-controlled multicenter trial conducted in around 100 sites globally, evaluating the time to recovery of hospitalized adults diagnosed with COVID-19 up to day 29. Remdesivir was superior to placebo in shortening the time to recovery and lowering mortality even though the difference was not significant [31].

Further data will be needed, including the full statistical analysis of the entire population involved in the ACTT trial, in order to fully understand the optimal use of Remdesivir [42]. Moreover, there is room for further studies involving potential combination therapies with other antivirals and anti-inflammatory agents in appropriate regimens. In this regard, the ACTT3 trial (NCT04492475) will evaluate the combination of IFN-β1a and Remdesivir compared with Remdesivir alone.

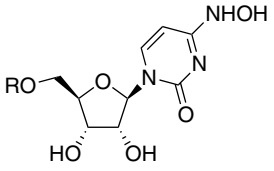
Significant efforts have been made to find an alternative route of administration. Clinical trials (GS-US-553-9018 and NCT04539262) have been initiated to evaluate an inhaled nebulized formulation of Remdesivir, which can be particularly suited for early-stage COVID-19 patients since the upper respiratory tract is the initial site of SARS-CoV-2 infection and spread.

The interaction of Remdesivir with the SARS-CoV-2 RdRp has been structurally characterized and the details are described in Section 17.3.1.

17.2.2 Clinical Investigational Drugs Against CoVs

Along investigational drugs targeting the CoV RdRp, the isobutyrate ester derivative Molnupiravir (EIDD-2801), the orally bioavailable prodrug of the ribonucleoside β-D-N^4-hydroxycytidine analog **1** (NHC, EIDD-1931) (Figure 17.3), represents a promising candidate which has advanced into clinical trials.

Compound **1** is a broad-spectrum antiviral agent with in vitro activity against multiple unrelated viruses [43–47] (including CoVs). In particular, **1** was able to inhibit SARS-CoV-2 (EC_{50} = 0.3 and 0.08 μM in Vero and Calu3 cells, respectively), SARS-CoV-1 (EC_{50} = 0.14 μM in HAE cells), and MERS-CoV (average EC_{50} = 0.15 μM in Calu3 cells) with low toxicity (CC_{50}s > 10 μM), showing also remarkable potency against a model CoV mouse hepatitis virus (MHV), bearing resistance mutations to Remdesivir [43]. DMPK profiling for **1** in rodents demonstrated dose-dependent oral bioavailability (56, 43, and 36%, $T_{1/2}$ of 5.2, 3.2, and 2.7 hours following oral administration of 50, 150, and 500 mg/kg, respectively).



Molnupiravir R = *i*BuCO

1 (NHC, EIDD-1931) R = H

$EC_{50\ SARS\text{-}CoV\ (HAE)}$ = 0.14 μM

$EC_{50\ MERS\text{-}CoV\ (Calu3)}$ = 0.15 μM

$EC_{50\ SARS\text{-}CoV\text{-}2\ (Vero)}$ = 0.3 μM

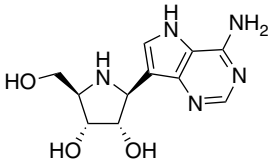
$EC_{50\ SARS\text{-}CoV\text{-}2\ (Calu3)}$ = 0.08 μM

CC_{50s} > 10 μM

Figure 17.3 Chemical structures of the prodrug Molnupiravir and parent compound **1** with its biological activity.

Conversely, its oral bioavailability in cynomolgus macaques was limited, thus prompting the development of the 5′-isobutyrate ester prodrug Molnupiravir, which demonstrated good oral bioavailability in nonhuman primates and quick release of free parent drug **1** [45]. In prophylactic in vivo efficacy studies, Molnupiravir has shown a significant reduction of the virus titer and ameliorated the pulmonary function of mice infected with SARS- and MERS-CoVs (500 mg/kg p.o. administration 2 hours before infection and every 12 hours) [43]. The decrease of MERS-CoV yields both in vitro and in vivo were correlated with an increase of the frequency of mutation in the viral RNA, highlighting a putative mechanism of lethal mutagenesis. The interesting preclinical results prompted the FDA and the UK Medicines and Healthcare Products Regulatory Agency to allow for the human safety testing of Molnupiravir. Phase I trial recently has been successfully completed (NCT04392219) and currently un number of additional Phase II/III studies are ongoing. In particular, two Phase IIa randomized, double-blind, placebo-controlled trials were activated to evaluate the antiviral efficacy of Molnupiravir in adults diagnosed with COVID-19 (NCT04405570, NCT04405739). In the late October 2020, two Phase II/III studies have started to evaluate the efficacy, safety, and PK of in non-hospitalized (NCT04575597) and hospitalized (NCT04575584) adult participants with COVID-19, and results are expected for the end of 2021. To this aim, the Drug Innovation Ventures at Emory (DRIVE) LLC, which developed the drug, partnered with the biotech company Ridgeback Biotherapeutics LP and with Merck, to advance Molnupiravir through clinical development and to optimize the drug's availability during the pandemic.

Galidesivir (BCX4430) is a *C*-adenosine analogue acting as a non-obligate RNA chain terminator, initially investigated as antifilovirus agent by BioCryst Pharmaceuticals (Figure 17.4) [48]. Galidesivir was shown to possess weak antiviral activities against several RNA viruses, including SARS-CoV-1 (EC_{50} = 57.7 μM, measured by neutral red uptake assay) and MERS-CoV (EC_{50} = 68.4 μM assessed by high-content image analysis) [48], whereas it does not



$EC_{50\ SARS\text{-}CoV\ (HAE)}$ = 57.7 μM

$EC_{50\ MERS\text{-}CoV\ (Calu3)}$ = 68.4 μM

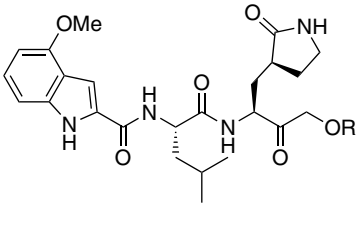
$EC_{50\ SARS\text{-}CoV\text{-}2\ (Vero)}$ > 100 μM

Galidesivir

Figure 17.4 Structure and biological activity of Galidesivir.

inhibit SARS-CoV-2 up to 100 μM [25]. Notably, Galidesivir can be administered by both parenteral (im and ip) and oral routes [49]. In mouse, rat (both treated with 2 mg/kg), guinea pig (50 mg/kg), and cynomolgus macaque (20 mg/kg), Galidesivir DMPK is characterized by rapid clearance from the plasma with a $T_{1/2} < 5$ min. Conversely, the $T_{1/2}$ of the active triphosphate form in the liver in rats (administered 30 mg/kg) is substantially longer at 6.2 hours [48]. Despite the apparently weak anti-CoV activity, in April 2020, the company BioCryst Pharmaceuticals, Inc. started a randomized, double-blind, placebo-controlled Phase 1 trial to evaluate the PK, safety, and antiviral effects of Galidesivir (IV administered) vs. placebo in hospitalized adult subjects with either Yellow Fever or COVID-19 in Brazil (NCT03891420).

Other classes of antivirals that exhibit single agent efficacy or that are complementary to RdRp inhibitors for use in combination regimens are essential to meet the unmet medical need of a cure against for COVID-19 and potentially emerging CoVs. PF-07304814 is the phosphate ester prodrug of PF-00835231, a peptidomimetic reversible covalent inhibitor of CoVs $3CL^{pro}$, which entered into a Phase Ib clinical trial. Following the SARS outbreak in 2002–2003, researchers at Pfizer identified SARS-CoV-1 $3CL^{pro}$ covalent reversible/irreversible inhibitors [50, 51] having hydroxymethylketone-based warheads, but the project was discontinued thanks to the control of the first SARS pandemic. Following the pandemic SARS-CoV-2, PF-00835231 was rescued from the shelf and has been identified as an SARS-CoV-2 $3CL^{pro}$ inhibitor exerting very potent activity against a wide panel of $3CL^{pro}$ from α-, β-, and γ-CoVs, with K_i/IC_{50} ranging from the low-nM to pM range (K_i = 0.27 nM and IC_{50} = 6.9 nM against SARS-CoV-2 $3CL^{pro}$; K_i = 4 nM against SARS-CoV-1 $3CL^{pro}$), whereas inactive on HIV, HCV, and a panel of human proteases (Figure 17.5), proving its selectivity for CoVs [52, 53]. The efficacy of PF-00835231 against SARS-CoV-1 and SARS-CoV-2 was evaluated in Vero cells, showing EC_{50} values of 4.8 and 39.7 μM, respectively. However, PF-00835231 has been shown to be a substrate of P-gp highly expressed in Vero cells. In fact, in the presence of a P-gp inhibitor, the antiviral activity of PF-00835231 was significantly increased, with EC_{50} values of 0.23 and 0.76 μM against SARS-CoV-1 and SARS-CoV-2,



PF-00835231 R = H

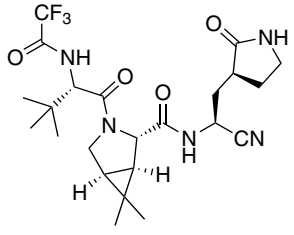
$K_{i\ SARS\text{-}CoV\text{-}1\ 3CL}$ = 4 nM

$K_{i\ SARS\text{-}CoV\text{-}2\ 3CL}$ = 0.27 nM, IC_{50} = 6.9 nM

$EC_{50\ SARS\text{-}CoV\text{-}1\ (Vero)}$ = 4.8 (0.23) μM[a]

$EC_{50\ SARS\text{-}CoV\text{-}2\ (Vero)}$ = 39.7 (0.76) μM[a]

PF-07304814 R = PO_3H_2



PF-07321332

Figure 17.5 SARS-CoVs 3CLpro inhibitors: PF-00835231 with biological activity and its phosphate iv prodrug PF-07304814; oral candidate PF-07321332; [a]EC_{50} value, in brackets data in the presence of P-gp inhibitor.

coupled to low toxicity ($CC_{50} > 100\,\mu M$). Interestingly, PF-00835231 exhibits additive/synergistic effect in combination with Remdesivir against SARS-CoV-2 in cell-based assays [53]. Compound PF-00835231 has been co-crystallized with both $3CL^{pro}$ of SARS CoV-1 and CoV-2 (PDB Codes 6XHL and 6XHM) [52], showing – as expected – almost identical binding modes. Structural details of $3CL^{pro}$/PF-00835231 interaction are discussed in Section 17.3.2 (Figure 17.14a). PF-00835231 has been extensively characterized for its PK properties following iv administration to rats (2 mg/kg), dogs, and monkeys (1 mg/kg) resulting in moderate plasma clearance (rat, 27, dog, 18, and monkey, 29 ml/min/kg), short half-life (rat, 0.7, dog, 1.5 and monkey, 1.2 hours), low volumes of distribution (rat, 0.75, dog, 1.1, and monkey, 1.4 l/kg), and good AUC (rat, 1250, dog, 932, and monkey, 583 ng.h/ml) [53]. Unfortunately, PF-00835231 shows very low oral bioavailability in rats and monkeys (<2%). Further preclinical characterization demonstrated that PF-00835231 did not inhibit CYP450 enzymes and membrane transporters, thus indicating a low risk to cause drug–drug interactions. A prodrug strategy has been applied to PF-00835231 in order to improve the aqueous solubility and its phosphate ester PF-07304814 has been reported [53]. After iv administration, PF-07304814 rapidly released 68, 81, and 76% active form PF-00835231 in rats, dogs, and monkeys, respectively. The safety profiles of PF-07304814 and PF-00835231 were individually evaluated in vitro and in vivo. In vitro, the compounds were negative in the AMES test, did not induce micronuclei formation, did not inhibit hERG, and had no effect in human blood hemocompatibility tests. Following continuous iv infusion (24 hours) of PF-07304814 in rats, no toxic signs were observed until 1000 mg/kg. On the basis of these promising preclinical results, on 15 September 2020, Pfizer announced the initiation of a double-blind, placebo-controlled Phase Ib clinical trial (NCT04535167) to evaluate the safety, tolerability, and pharmacokinetics of PF-07304814 [54]. Moreover, Pfizer presented its oral COVID-19 antiviral clinical candidate PF-07321332 (Figure 17.5) on April 6th at ACS Spring 2021 Meeting. The compound, which is currently in Phase 1 clinical trials (NCT04756531) in healthy patients, is the first orally administered inhibitor of SARS-CoV-2 3CLpro in the clinic. The compound is structurally related to PF-00835231, which was modified on order to retain the excellent enzymatic inhibition and antiviral activity and gaining oral bioavailability. Importantly, the P2 residue was modified replacing the leucine with a less polar bicycloproline moiety. The indole was substituted with a tertbutylglycine with a trifluoroacetyl capping group and a nitrile was inserted as warhead instead of the hydroxyketone moiety. Unfortunately, data on biological activity and on PK properties of PF-07321332 have not been published yet.

17.3 Medicinal Chemistry Approaches Toward the Identification of New Drugs

Different strategies targeting both viral and host factors essential for the CoVs replication have been pursued [55, 56]. The most promising are described in the following paragraphs. The $3CL^{pro}$ and the nsp12 RdRp are the best characterized SARS-CoV targets and show the highest degree of conservation across CoVs, fostering the identification of broad-spectrum inhibitors [57, 58]. Besides targeting intracellular steps of viral replication, virus entry into host cells represents another way to prevent/treat viral infections. The main target for this approach are the CoV S fusion protein and the S/ACE2 interaction as viral factors to inhibit entry [16, 59].

To date, the most promising and investigated approach to fight SARS-CoVs infection relies on targeting these three proteins. The analysis of the targets and the inhibitors discovered until now are reported below.

17.3.1 SARS-CoVs RdRp: Structure, Function, and Inhibition

The RdRp encoded by the nsp12 gene catalyzes the formation of a phosphodiester bond between NTPs in a primer-dependent manner and requires the formation of a complex with nsp7 and nsp8 cofactors to acquire processivity [60]. In addition, nsp8 exerts RNA primase activity enabling *de novo* RNA synthesis [61]. The structural and functional features of the RdRps among the CoV family are highly conserved with the RdRps of SARS-CoVs sharing 96% amino acid identity [43, 62].

Structures of SARS-CoV-2 full-length RdRp, in complex with nsp7 and nsp8, were determined by cryo-EM (PDB ID: 6M71 [63], 7BV1, [64] 7BW4 [65]) showing the typical right-hand architecture (Figure 17.6), constituted by the finger, palm, and thumb subdomains [66]. The nsp12 RdRp domain is connected through an interface to the N-terminal region possessing nucleotidyltransferase activity (NiRAN), whose exact role in the viral life cycle remains elusive [67]. SARS-CoV-2 RdRp cryo-EM structure revealed also an additional, unique N-terminal β-hairpin, which was previously not observed in SARS-CoV-1 RdRp [63].

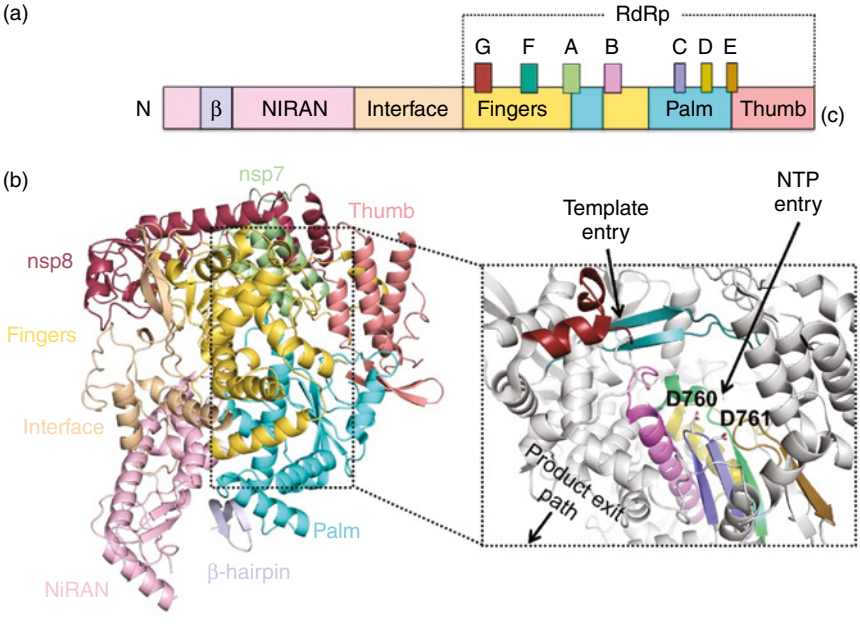


Figure 17.6 Architecture of SARS-CoV-2 nsp12. (a) Schematic diagram outlining the domain organization of SARS-CoV-2 nsp12. (b) Structure of SARS-CoV-2 PDB ID: 6M71. Left: Ribbon diagram of nsp12 showing palm, fingers, and thumb domains, colored as in (a). Nsp7 and nsp8 cofactors are shown as pale green and raspberry ribbons. Right: overview of nsp12 active site, shown as white ribbons, whereas the conserved motifs A–G are colored as in (a). The catalytic Asp760 and Asp761 are shown as white sticks. The template entry, NTP entry, product hybrid exits paths are indicated by orange arrows.

The RdRp active site is composed by seven conserved motifs (A–G) lining a central cavity where the template-directed RNA synthesis takes place (Figure 17.6). In particular, the incoming NTP binds within motif F, whereas the RNA template enters the active site through a channel formed by motifs F and G; motif E and the thumb subdomain sustain the primer strand. The product of RNA synthesis leaves the active site through an RNA exit path, situated at the front side of the polymerase [63].

Moreover, the replicating nsp12–nsp7–nsp8 complex has been solved bound to RNA template-product duplex (PDB ID: 6YYT; Figure 17.7) [68]. The active site cleft of nsp12 binds the first turn of RNA, whereas two nsp8 subunits bind to opposite sides of the cleft, flanking the exiting RNA duplex with long α-helical extensions, called "sliding poles." These nsp8 extensions are rich in positively charged residues and form multiple RNA backbone interactions. The mutation of one of these positively charged residues, namely Arg58, with Ala, was previously reported as lethal in SARS-CoV-1, because of the strong decrease of polymerase activity, leading to a nonviable phenotype [60].

Despite the large amount of structural data produced for the SARS-CoVs RdRp, there was no clear identification of putative allosteric binding pockets for allosteric inhibitors. Furthermore, there are no reports of medicinal chemistry programs aimed at the identification of specifically designed inhibitors of CoVs RdRp, both active site or allosteric inhibitors. Indeed, the only inhibitors identified and characterized derive from repurposing of known broad-spectrum nucleoside inhibitors (NIs) acting as non-obligate chain terminators or mutagens [69]. The first possess a natural base and a 3′-hydroxyl on the sugar and an additional substituent at the C-1′ or the C-2′ positions of the ribose ring blocking the formation of the phosphodiester linkage with the incoming NTP [69]. The mechanism of lethal mutagenesis involves the inability to recognize the nucleoside analogues as regular

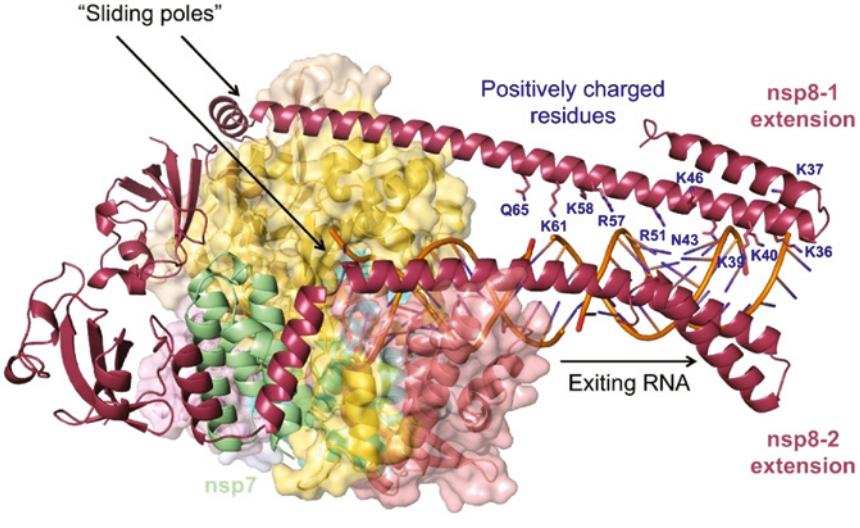


Figure 17.7 Structure of SARS-CoV-2 replicating RdRp–RNA complex (PDB ID: 6YYT). Nsp12 is shown as a molecular surface (color code as in Figure 17.6), the cofactors nsp7 and nsps8 (1 and 2) are shown as pale green and raspberry ribbons. RNA turns are shown as orange ribbons. The positively charged nsp8 residues, proposed to interact with RNA, are shown as sticks.

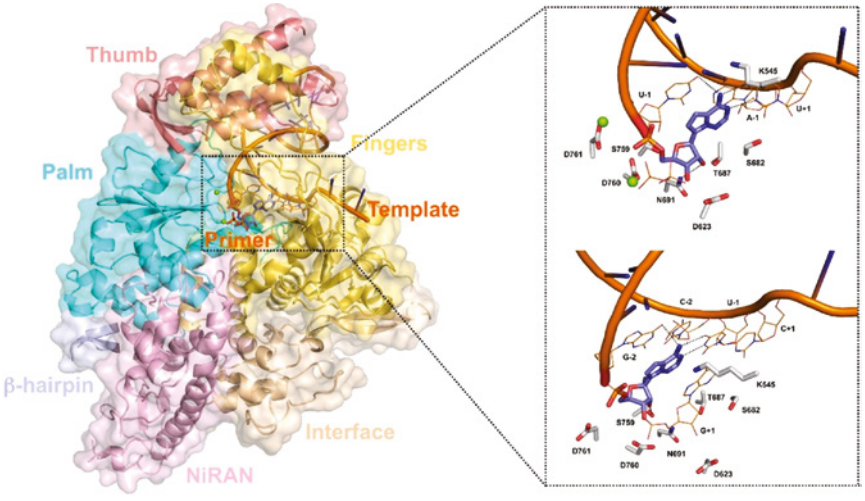


Figure 17.8 Binding mode of Remdesivir into the SARS-CoV-2 nsp12 active site (PDB ID: 7BV2). Left: nsp12 is shown as a molecular surface, colored according to the schematic diagram in Figure 17.6. For clarity, nsp7 and nsp8 cofactor have been removed. The template and primer RNA are shown as ribbon models and labeled. Right: zoom-in of the nsp12 active site. The covalently bound monophosphate form of Remdesivir and the pyrophosphate group are shown as sticks. Mg^{2+} ions are shown as green spheres. The RNA bases interacting with Remdesivir are shown as orange thin sticks, while protein residues are shown as white thick sticks. Hydrogen bonds are shown as black dashed lines.

nucleobases, thus inducing a mismatch in base pairing and an increase in mutations, ultimately leading to nonviable genomes.

Remdesivir has been deeply characterized for its mode of action and interaction with SARS-CoV-2 RdRp. The cryo-EM structure of Remdesivir monophosphate in complex with SARS-CoV-2 RdRp and a 50-base template-primer RNA (PDB ID: 7BV2) [64] shows the covalent bond to the primer strand at the 3′-end (Figure 17.8) to terminate chain elongation.

Remdesivir is positioned at the center of the catalytic site, with the adenine stacking with the upstream bases U-1 and A-1 of the primer and template strands. In addition, Remdesivir engages three strong H-bonds with the uridine bases U−1 and U+1, whereas the sugar 2′-OH group forms a further H-bond with Asn691. A "delayed" chain termination has been reported for Remdesivir, according to which the molecule blocks CoVs RdRps after the addition of three more nucleotides [70–73]. This hypothesis is consistent with a recently released cryo-EM structure of the SARS-CoV-2 RdRp in complex with RNA, before and after RNA translocation (PDB IDs: 7C2K and 7BZF, respectively) [74]. In the pre-translocated catalytic complex structure, the incorporated Remdesivir has been translocated to the −1 position, whereas the 3′-guanosine occupies the +1 position (Figure 17.8). Remdesivir engages four H-bonds, with the upstream G-2 base, the uridine base U−1, and with Ser759. The primer strand with the incorporated Remdesivir may translocate without obstruction to positions i+1, i+2, or i+3, allowing the incorporation of three subsequent nucleotides. However, at position i+4, a putative steric clash was postulated between the 1′-CN substituent of Remdesivir and Ser861 along the RNA exit tunnel [72]. Ser861 is highly conserved among CoV RdRps and its important role in Remdesivir-induced RdRp inhibition

was ultimately supported by mutagenesis studies, revealing that the Ser861Ala RdRp mutant yields a smaller fraction of i+3 termination compared with the wild-type RdRp, possibly supporting the steric clash hypothesis [74].

Two amino acid substitutions were found in the nsp12 polymerase providing low-level resistance to Remdesivir, corresponding to the SARS-CoV residues Phe480Leu and Val557Leu. Such residues are identical across CoVs, and have been found to cause an impairing of fitness and virulence [75].

Non-nucleoside inhibitors (NNIs) have not yet been reported for CoVs RdRp. The NNIs are characterized by drug-like heterocyclic scaffolds and thus offer the possible advantage to handle molecules with higher optimization potential, despite generally showing lower resistance barrier with respect to NIs. However, no allosteric sites have been mapped on the RdRp protein surface for CoVs. Since viral RdRp shows the typical 3D right hand shape, organized in palm, thumb, and fingers subdomains, the knowledge acquired on other RNA virus polymerase may allow similar considerations also for CoVs RdRp. In this regard, the best characterized viral RdRp is HCV NS5B that, despite working in the absence of a primer in a cellular environment, shows four distinct binding sites for different classes of NNIs that are able to allosterically inhibit the enzyme [76]. A very large number of inhibitors, specifically binding one of these sites, have been reported, with some of them reaching late stages of clinical trials and also approval (for instance, Dasabuvir).

The integrated computational and experimental analysis of the nsp12 surface may point toward the discovery of potential allosteric sites. Moreover, the disruption of the polymerase complex integrity, for example, targeting the interaction between nsp8 and nsp12 would be an interesting avenue to be explored. Unfortunately, also for this approach, there are no molecules reported acting with this mechanism.

17.3.2 The CoVs 3CL^{pro} (or Main Protease): Structure, Function, and Inhibitors

The 3CL^{pro}, known also as main protease or M^{pro}, is a 33.8 kDa cysteine protease able to process the polyprotein at no less than 11 conserved sites (starting with the autolytic cleavage from pp1a and pp1ab) for the release of most of the nsps CoV functional proteins. Due to its key role in viral replication and the lack of human counterparts, over the years, the discovery efforts have focused on 3CL^{pro} to identify antiviral agents against human CoVs. Indeed, a large number of crystal structures of 3CL^{pro} from CoVs have been solved either in their apo forms or in complex with inhibitors. Analysis of crystal structures highlight as SARS-CoV-1 3CL^{pro} (PDB ID: 2BX4) [77] and SARS-CoV-2 3CL^{pro} (PDB ID: 6Y2E) [78] shows almost identical tridimensional spatial organization, and binding pockets, consistent with the 96% sequence identity between the two proteins. The active form of 3CL^{pro} is a dimer, with each protomer formed by three domains [77, 78]: six antiparallel β-barrels form domains I and II (residues 10–99 and 100–182, respectively) and allocate the substrate-binding site, while domain III (residues 198–303) is a globular cluster of five helices that regulates protein dimerization. Arg4 and Glu290 from each protomer establish an ionic bond driving the formation of the dimer, which has a contact interface between the perpendicularly oriented domain II of protomer A and domain III of protomer B (Figure 17.9). The N-terminal tail, called the "N-finger," of molecule B is squeezed in between domains II and III of the parent monomer and domain II of the other one. This

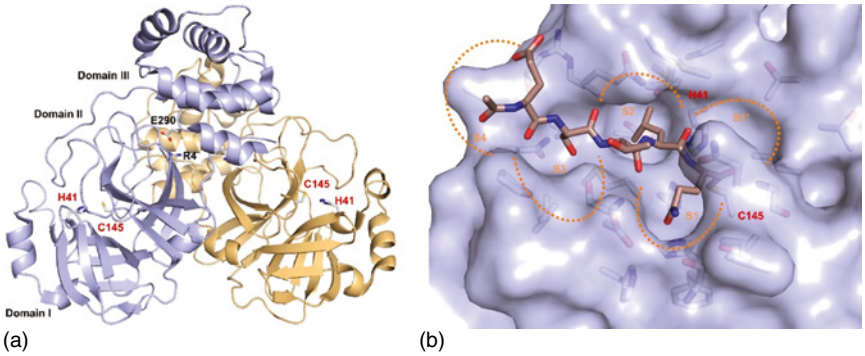


Figure 17.9 (a) The X-ray structure of SARS-CoV-2 $3CL^{pro}$ (PDB ID: 6Y2G), selected as a representative example, is shown as ribbon model. For clarity, the bound inhibitor has been removed. Protomers A (light blue) and B (light orange) associate into a dimer stabilized by a salt bridge between Glu290 and Arg4, while the substrate binding site resides at the interface of domains I and II. The catalytic Cys145 and His41 are highlighted. (b) Surface representation of the active site pocket of SARS-CoV-1 $3CL^{pro}$ bound to a peptide aldehyde inhibitor (dark salmon sticks, PDB ID: 3SNE), chosen as a representative substrate-like inhibitor. The S1–S4 and S1′subsites are indicated with red lines and labeled. The key residues forming the active site pocket are displayed as white sticks; the catalytic Cys145 and His41 are labeled.

peculiar arrangement is stabilized by some key H-bonds, particularly those formed by Ser1 and Glu166 from each unit. The substrate binding site contains the catalytic Cys145-His41 flanked by the subpockets S4, S3, S2, S1, and S1′ for the binding of the substrate P4, P3, P2, P1, and P1′ amino acids. The substrates of CoVs $3CL^{pro}$ display almost identical recognition motifs, which is consistent with the high structural similarity of the catalytic domains of these enzymes [79]. The $3CL^{pro}$ of several α- and β-CoVs have the Leu-Gln-Ser (Ala, Gly) as preferred P2–P1–P1′ sequence, suggesting the possibility to identify very effective broad-spectrum inhibitors. In particular, the requirement for the P1 Gln is almost a unique feature that is common only to enteroviruses (EV) $3C^{pro}$ and is unknown in human proteases, thus increasing the appeal of these viral proteins as targets for safe and selective inhibitors.

The SARS-CoVs $3CL^{pro}$ substrate affinity and specificity can be reasonably explained by the shape and amino acid composition of the different sub-pockets of the cleavage site. In both SARS-CoVs $3CL^{pro}$, the S1 pocket is formed by the side chains of residues Phe140, Asn142, His163, Glu166, and His172 and the main chains of Phe140 and Leu141. Interestingly, the imidazole of His163 is located at the very bottom of the cleft, suitably positioned to donate a H-bond to the side chain carbonyl of substrate/inhibitor P1 Gln. The S2 subsite of SARS-CoVs $3CL^{pro}$ is a buried hydrophobic pocket that can host bulky alkyl/aryl substituents as the substrate P2 Leu side chain. This cage is defined by a "lid" comprising the 3_{10} helix residues 46—51, particularly Met49, three walls defined by the main chain of residues 186–188 and by the side chains of His41, Asp187 and Gln189, and a floor lined by Met165. Notably, the shape and the size of the S2 subsite of SARS-CoVs $3CL^{pro}$ are highly similar to that of the MERS-CoV homologue. Indeed, only two conservative mutations can be found, specifically the replacement of Met49 and Arg188 in SARS-CoVs with Leu49 and Lys191 in MERS-CoV, respectively. On the other hand, the volume of the S2

subsite in SARS-CoVs 3CL^{pro} (252 Å^3) is significantly larger than in other CoVs' homologues of the α-genus, such as the HCoV-NL63 3CL^{pro} (45 Å^3) [80]. Compared with S1 and S2, the S4, S3, and the S1′ pockets of the 3CL^{pro} are more shallow and exposed to the solvent and could accommodate groups of various size and nature. In particular, S3 and S4 are defined by the flexible loops connecting residues 165–168 and 189–192, which can rearrange upon ligand binding, while the S1′ cleft is characterized by a number of threonine residues (24–26) which can potentially form either hydrogen bonds or lipophilic contacts with the substrate/inhibitor P1′ group.

The targeting of proteases represents a solid route for antiviral drug discovery as demonstrated by the therapeutic success of HIV and HCV proteases inhibitors. The more attractive and time-saving approach to identify protease inhibitors is the design of peptidomimetics endowed with an electrophilic warhead thus acting as covalent irreversible/reversible inhibitors, that are designed based on the substrate sequences. Thus, it is important to gain specificity for target proteins, which reduces their potential side toxicity.

In 2003, the first X-ray structure of the SARS-CoV-1 3CL^{pro} in complex with the peptide inhibitor **2** (Figure 17.10), functionalized with a chloromethyl ketone as warhead, was released (PDB ID 1UK4) [81]. Moreover, the ER 3C^{pro} inhibitor Rupintrivir, that successfully completed Phase II trials for human rhinovirus (HRV) infection in 1999 without showing toxicity in common cold patients [82], in the complex with HRV2 3C^{pro} (PDB ID 1CQQ) showed an orientation comparable to that of peptide **2** bound to 3CL^{pro} [58, 83]. Although Rupintrivir is not active against CoVs protease [84, 85] likely due to the presence of a P2-*p*-fluorobenzyl group that is too large for the 3CL^{pro} S2-pocket [86], this investigational drug has inspired design of CoV 3CL^{pro} inhibitors [58]. Indeed, the Gln mimetic γ-lactam, that replaces the substrate P1 Gln in Rupintrivir, constitutes a key structural moiety kept constant in the large majority of the CoVs 3CL^{pro} inhibitors. Because of the homology between the CoVs proteases, known inhibitors of 3CL^{pro} from SARS-CoV-1 and other CoVs have proven to be active also against the SARS-CoV-2 3CL^{pro} and/or have been optimized to develop new inhibitors. This supports the hypothesis to develop a pan-CoVs inhibitor.

In the peptidomimetic covalent reversible/irreversible inhibitors of SARS-CoVs 3CL^{pro} reported so far, the explored chemical warheads include Michael acceptors, aldehydes, (halomethyl-, aryl-, hydroxymethyl-, acyloxymethyl-) ketones, and α-ketoamides [57, 58] . In order to efficiently compete with the natural substrates at the catalytic site, most of the inhibitors span from P1 to P4 to establish a considerable number of specific interactions with the binding pockets (namely, the enzyme S1–S4 subsites), to facilitate

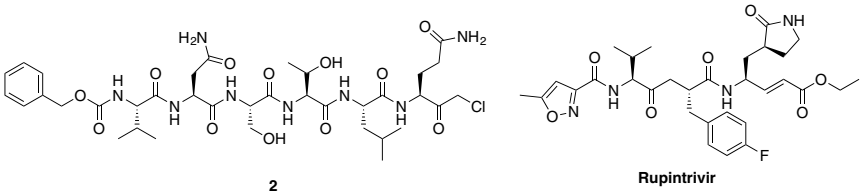


Figure 17.10 Chloromethylketone peptide **2** and Rupintrivir as starting point for the design of 3CL^{pro} inhibitors.

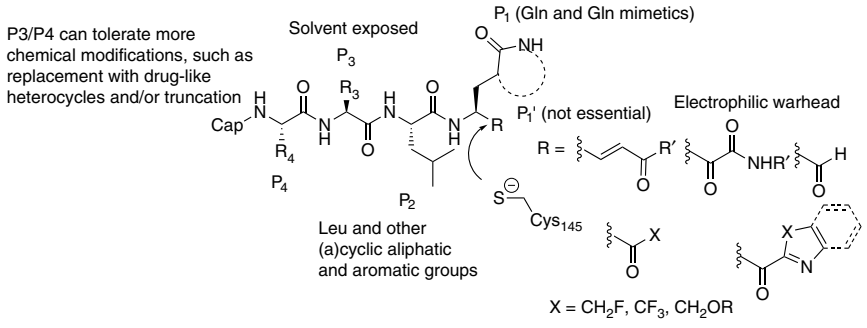


Figure 17.11 Schematic representation of the main features of covalent reversible/irreversible 3CL^{pro} inhibitors.

the covalent reaction between the Cys145 and the warhead (Figure 17.11), contributing to the selectivity, affinity, potency, and to the binding specificity. In addition, the reported inhibitors share similar peptide sequence organization in P1–P2 residues, while there are remarkable differences at P3/P4 positions and in the overall compound molecular size (Figure 17.11).

The first publication on compounds specifically designed to target the SARS-CoV-1 3CL^{pro} described a small series of Rupintrivir analogues endowed with a vinyl ester acting as the Michael acceptor warhead, but showing differences in the P2 [83]. Indeed, compound **3** has a smaller benzyl instead of the *p*-F-benzyl of Rupintrivir, enough to equip compound **3** with inhibitory activity against the enzyme ($K_{inact} = 0.014\,min^{-1}$), showing also a moderate antiviral activity in a cell-based assay ($EC_{50\,SARS\text{-}CoV} = 45\,\mu M$, $CC_{50} > 100\,\mu M$) (Figure 17.12).

Subsequently, the tripeptide derivative **4** endowed with an aldehyde warhead and resembling the sequence of compound **2** was reported as potent inhibitor of SARS-CoV-1 3CL^{pro} (Figure 17.12) [87]. In particular, the presence of the cyclohexyl alanine in P2 and of a *tert*-butyl threonine in P3 provided potent enzymatic and antiviral activity ($K_{i\ SARS\text{-}CoV\text{-}1\ 3CLpro} = 53\,nM$, $EC_{50\ SARS\text{-}CoV\text{-}1} = 0.6\,\mu M$). The X-ray structures of compound **4** bound to SARS-CoV-1 3CL^{pro} (PDB ID 2GX4) or SARS-CoV-2 3CL^{pro} (PDB ID 7C8T) showed the formation of the thiohemiacetal derived from the nucleophilic attack of Cys145 to the C-term aldehyde of the inhibitor, with the oxyanion stabilized by H-bond of Gly143 and Cys145.

A small series of *N*-benzyloxy capped dipeptides functionalized with a fluoromethylketone warhead were reported as inhibitors of SARS-CoV-1 replication in cell-based assays, with compound **5** showing $EC_{50} = 2.4\,\mu M$ and low cytotoxicity in Vero cells ($CC_{50} > 100\,\mu M$, $SI > 40$) while no data on the enzymatic assay are available (Figure 17.12) [88]. Nonetheless, compound **5** was not active against HRV thus hinting to a specific inhibition against CoVs. The main difference with most of the 3CL^{pro} inhibitors is the presence of a *N,N*-dimethylglutamine residue in P1, instead of the γ-lactam.

A series of dipeptides having a ketobenzothiazole warhead was reported as potent inhibitors of SARS-CoV-1 3CL^{pro} (Figure 17.12) [89]. Similar to inhibitors reported by Pfizer (Section 17.2.2), these compounds were characterized by the presence of an heterocyclic drug-like N-cap, with the 4-methoxyindolyl derivative **6** showing the best activity in the nM range ($K_i = 6\,nM$, $IC_{50} = 0.74\,\mu M$, $K_D = 16\,nM$), while no data on antiviral activity in cell-based assays have been described.

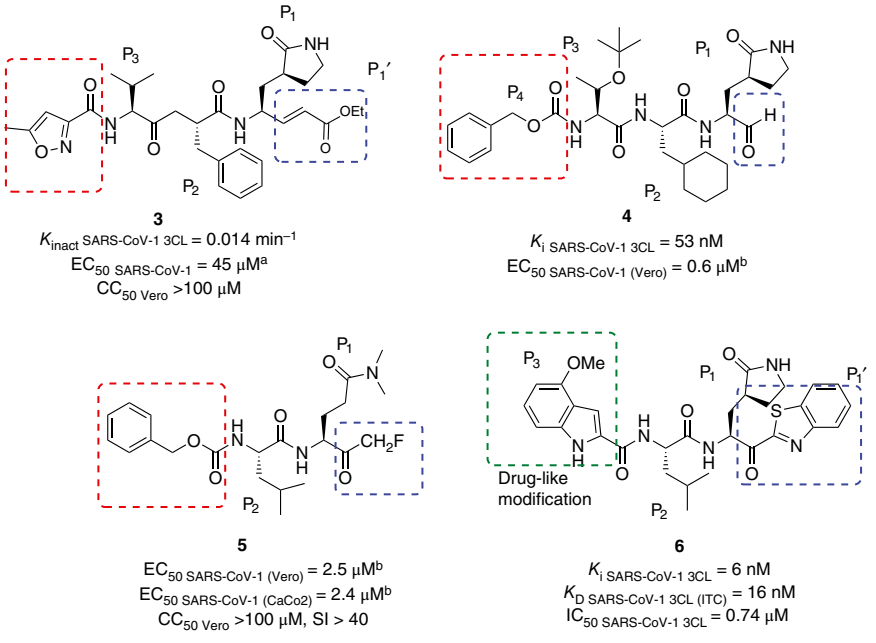


Figure 17.12 Compounds **3–6** reported as SARS-CoV-1 3CL^{pro} inhibitors; [a]antiviral activity determined by NR assay; [b]antiviral activity determined by CPE reduction.

In the first released X-ray structure of SARS-CoV-2 3CL^{pro}, the protein was in complex with the N-term isoxazole capped tetrapeptide **7** endowed with a vinyl carboxyl ester acting as the Michael acceptor warhead (PDB ID: 6LU7, superseded by 7BQY; Figures 17.13 and 17.14) [79]. Compound **7** was previously reported as an inhibitor of 3CL proteases of other human CoVs [90, 91], including SARS-CoV-1 (K_i = 9 μM [92]) and MERS-CoV (IC_{50} = 0.3 μM) [93], while no data in an enzymatic assay were reported against SARS-CoV-2 3CL^{pro}. Nonetheless, the high structural identity between SARS-CoVs 3CL proteases would suggest that **7** may reasonably inhibit also SARS-CoV-2 3CL^{pro}. In a cell-based assay, compound **7** inhibits SARS-CoV-2 replication with an EC_{50} of 16.77 μM, while it has modest selective index with respect to other CoVs (EC_{50} = 4.0, 8.8, 2.7, and 3.4 μM against HCoV-229E, FIPV, MHV-A59, and MHV, respectively), and most likely the variability depends on subtle differences in amino acid sequences in the different CoV proteases and cellular assay conditions.

Peptidomimetic α-ketoamides have been reported as broad-spectrum inhibitors of 3CL and 3C proteases effective in cell lines against different CoVs and EVs, with derivative **8** showing the most promising activity (Figure 17.13) [80]. Preliminary chemical optimization of **8** by structure-based drug design (SBDD) led to derivative **9**, the first published SARS-CoV-2 3CL^{pro} inhibitors designed *ad-hoc* (Figure 17.13) [78]. The X-ray structure of the complex **9**/SARS-CoV-2 3CL^{pro} (PDB ID: 6Y2F) (Figure 17.14) confirmed the formation of a thiohemiketal intermediate by the nucleophilic attack of the Cys145 over the ketoamide α-carbonyl [78]. The oxyanion group is stabilized by a H-bond with His41, while the amide oxygen of **9** accepts a H-bond from the main-chain amides of Gly143 and Cys145 which, together with Ser144, form

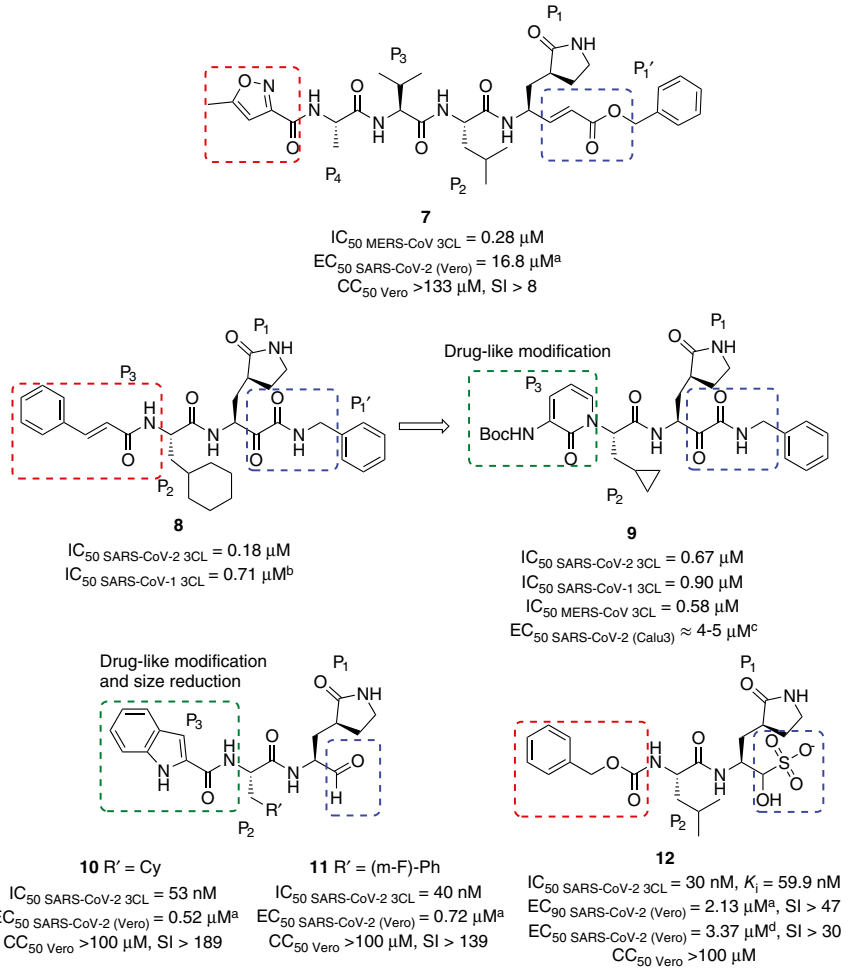


Figure 17.13 Compounds **7–12** with their biological activities; [a]antiviral activity evaluated by plaque assay; [b]data from Ref. [80]; [c]antiviral activity evaluated by viral RNA qRT-PCR quantification; [d]antiviral activity evaluated by CPE reduction. The main structural differences, significant modifications, and warheads are highlighted.

the so-called "oxyanion hole" typical of cysteine proteases. Ketoamide derivative **9** inhibits SARS-CoV-2 3CL^{pro} with an $IC_{50} = 0.67\,\mu M$, showing similar potency against SARS-CoV-1 and MERS-CoV M^{pro}. **9** showed EC_{50} ~4–5 μM against SARS-CoV-2 replication in human lung (Calu3) cells but no data on cytotoxicity were provided (Figure 17.13).

In another study, a SBDD strategy led to the identification of dipeptides **10** and **11** having an aldehyde as warhead and exhibiting excellent inhibitory activity against SARS-CoV-2 3CL^{pro} in enzymatic assays ($IC_{50} = 53$ and 40 nM, respectively; Figures 17.13 and 17.14) and SARS-CoV-2 infection in cell culture ($EC_{50} = 0.53$ and 0.72 μM, respectively), coupled with low toxicity (CC_{50}s > 100 μM, SIs > 139) [94]. Both compounds have an indolyl acid in P3 and are reminiscent of 3CL^{pro} inhibitors reported by Pfizer,

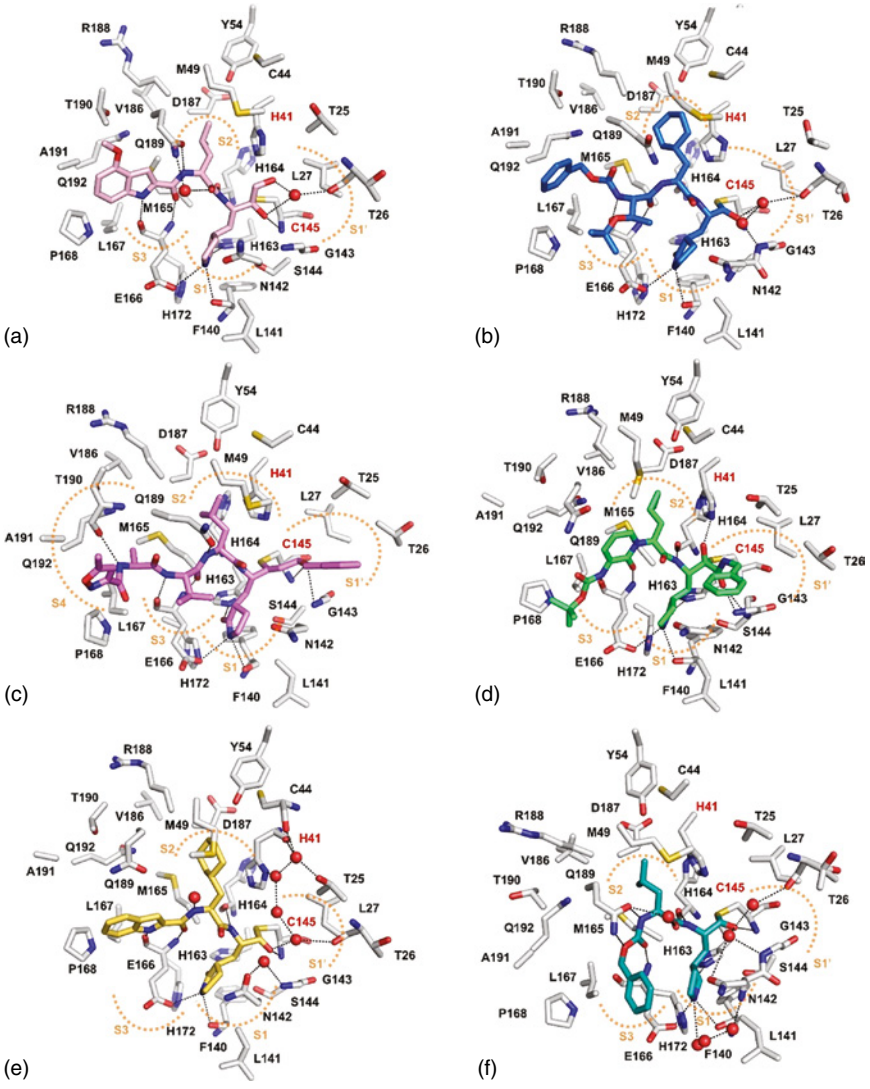


Figure 17.14 Co-crystallographic pose of compounds PF-00835231, **4**, **7**, **9**, **10**, and **12** in complex with SARS-CoV-2 $3CL^{pro}$. The compounds' co-crystallographic poses are shown only into the active site of SARS-CoV-2, even for those co-crystallized in SARS-CoV-1, given the high degree of structural similarity between SARS-CoV-1 and SARS-CoV-2 $3CL^{pro}$ enzymes. The key residues forming the active site pocket are displayed as white sticks, water molecules are shown as red spheres, H-bonds are depicted as dashed black lines. (a) PF-00835231 (pink sticks, PDB ID: 6XHM). (b) **4** (blue sticks, PDB ID: 7C87). (c) **7** (violet sticks, PDB ID: 7BQY). (d) **9** (green sticks, PDB ID: 6Y2F). (e) **10** (yellow-orange sticks, PDB ID: 6LZE), (f) **12** (teal sticks, PDB ID: 7BRR).

such as PF-00835231. As expected, compounds **10** and **11** bind to the protein with similar poses, where the carbon of the aldehyde and the sulphur of Cys145 form the thiohemiacetal, the oxygen of the resulting tetrahedral adduct is stabilized by interaction with backbone of residue Cys145 and through a water bridge with the Thr26 side chain.

We report only the co-crystal structure of **10**/SARS-CoV-2 $3CL^{pro}$ as representative example (PDB IDs: 6LZE; Figure 17.14) [94].

The α-hydroxy-bisulfite dipeptide **12** (GC376), originally developed as investigational veterinary drug (usually administered via s.c. route) for feline infectious peritonitis (FIP) [95, 96], is able to potently inhibit SARS-CoV-2 $3CL^{pro}$ (IC_{50} = 30 nM) and viral replication, even though with almost two orders lower potency (EC_{50} = 3.37 μM in CPE assay; CC_{50} > 100 μM; Figure 5) (Figure 17.13). [85] This inhibitor is a prodrug, converted into the aldehyde form by the removal of the bisulfite group to alkylate the Cys145 of the $3CL^{pro}$. Derivative **12** shows activity against multiple 3CL proteases ($IC_{50\,FIPV\,3CL}$ = 0.72 μM, $IC_{50\,TGEV\,3CL}$ = 0.82 μM, $IC_{50\,SARS\text{-}CoV\text{-}1\,3CL}$ = 4.35 μM, $IC_{50\,MERS\text{-}CoV\,3CL}$ = 1.56 μM) and against CoVs (TGEV, FIPV, MHV, 229E, and BCV) in cell lines with high nM potency [95–97]. **12** resulted also in potent inhibition of EV 3C and norovirus 3CL cysteine proteases and of the replication of several of these viruses, including HRVs [97]. Notably, **12** has been co-crystallized with SARS-CoV-2 $3CL^{pro}$ (PDB IDs: 7BRR [98], 6WTJ [99], 6WTT [85], 7C8U, 7C6U, 7CBT) (Figure 17.14). Recently, a small set of new analogues of compound **12**, differing for the N-terminal capping moieties, has been reported as inhibitors of $3CL^{pro}$ from SARS-CoVs and MERS-CoV [100]. The new compounds showed biological activities in the same range of parent compound **12** and have proven in vivo activity in mice infected with MERS-CoV.

The anti-HCV drug Boceprevir [101] showed low μM potency against the isolated SARS-CoV-2 $3CL^{pro}$ and the viral replication with no host cell toxicity up to 100 μM. Despite deviating from 3CL substrate sequence specificity, Boceprevir has been recently co-crystallized with SARS-CoV-2 $3CL^{pro}$ (PDB IDs: 7BRP [98], 6WNP, 7C6S, 6ZRU) providing hints to improve its anti-enzymatic activity (Figure 17.15). To be noted, none of the HIV protease inhibitors, including lopinavir, have shown activity in these studies, thus providing an experimental explanation to the failing of these drugs in clinical trials [41].

In spite of different warheads, most of the reported $3CL^{pro}$ inhibitors share in P1 a Gln mimetic γ-lactam. As shown in the co-crystal structures of compounds PF-00835231, **4**, **7**, **9**, **10**, and **12**, the P1 γ-lactam specifically fills the S1 subsite establishing key H-bonds with the Phe140 main chain and with the side chains of His163 and Glu166 (Figure 17.14). Notably, Glu166 is involved not only in substrate recognition but also in the substrate-induced dimerization of SARS-CoVs 3CL proteases through the interaction with Ser1 from the other monomer [78, 102]. Therefore, the interaction of the inhibitor γ-lactam with this residue could stabilize the monomeric and inactive form of the enzyme. Literature data on CoVs $3CL^{pro}$ inhibitors clearly indicate that the P1 γ-lactam enhances the inhibitory potency up to 10-fold, probably because the higher rigidity reduces the loss of entropy upon binding, if compared with the flexible Gln [58]. Paradoxically though, Boceprevir having a cyclobutyl ring in place of the γ-lactam, cannot form any hydrogen bond at the S1 subsite, but nevertheless was able to inhibit the SARS-CoV-2 $3CL^{pro}$. The presence of a modified proline in the Boceprevir at the P2 position would suggest the possibility to synthesize proline-based analogues with higher affinity and specificity toward the target enzyme. Indeed, in the first trimester of 2021, new P2 bicycloproline-containing compounds derived from Boceprevir have been reported as potent inhibitors of SARS-CoV-2 $3CL^{pro}$ with IC_{50} in the nM range [103]. Some of the new compounds showed also antiviral activity in cell-based assays and reduced lung viral loads and lung lesions in SARS-CoV-2 infection transgenic mouse model, after oral or ip treatment. A similar design strategy has been exploited for the oral clinical candidate PF-07321332

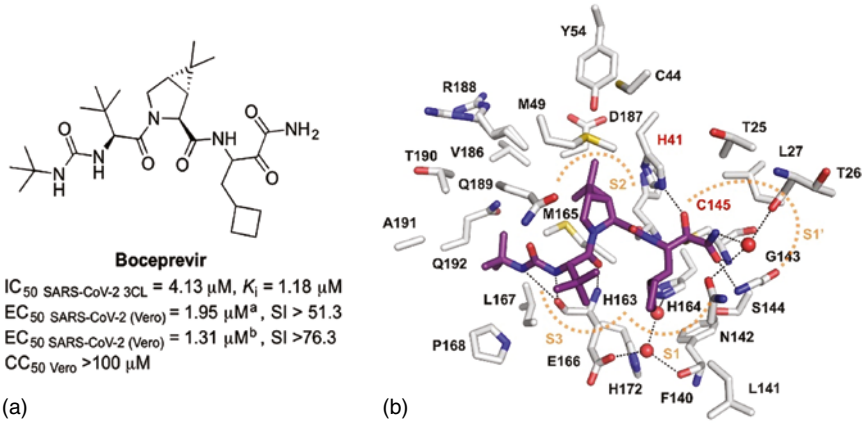


Figure 17.15 (a) Boceprevir and its biological activity against SARS-CoV-2. [a]Antiviral activity evaluated by viral plaque assay; [b]antiviral activity evaluated by CPE reduction. (b) Co-crystallographic pose of Boceprevir (purple sticks, PDB ID: 6WNP) in complex with SARS-CoV-2 $3CL^{pro}$. The key residues forming the active site pocket are displayed as white sticks; water molecules are displayed as red spheres. H-bonds are depicted as dashed black lines.

(Figure 17.5), containing the druglike bicycloproline in P2 similarly to Boceprevir, as described in Section 17.2.2. Indeed, the P2 amino acids of the other reported inhibitors show that this position tolerates a wide variety of aliphatic or aromatic residues characterized by similar lipophilicity and size to the substrate Leu and that it is important to modulate activity. The S3 and S4 pockets of SARS-CoVs $3CL^{pro}$ are less structured and can rearrange upon the binding of distinct P3/P4 residues that are generally modified to modulate both potency and drug-like properties of the inhibitors. For instance, in peptide **7**, the P3 lipophilic Val is solvent exposed, although it can establish van der Waals contacts with P1 γ-lactam to stabilize the inhibitor binding conformation, while the P4 N-capped Ala can form some lipophilic contacts with residues Met165, Leu167, and Gln192 in S4 (Figure 17.14). Remarkably, the P3 backbone of the inhibitors **4** and **7** establishes two key hydrogen bonds with the main chain of Glu166. These interactions can also be formed by derivative **9** (Figure 17.14) in which the P2–P3 amide bond is masked by a *N*-Boc-aminopyridone, likely to prevent cellular proteases cleavage. Indeed, the pyridone CO donates a H-bond to Glu166, which in turn accepts another H-bond from the NH of the ligand carbamate. Similar to **9**, the N-terminal P3 indole of derivatives PF-00835321 and **10** does not protrude into the S3 but it is exposed to solvent and stabilized by H-bonds with the amide backbone Glu166 (Figure 17.14). Also, in derivative **12,** the P2 Leu is capped by a benzyloxycarbonyl group which can alternatively extend toward the S3/S4 pockets or downward to form intramolecular contacts with the P1 γ-lactam; in both cases, however, this moiety can form only one hydrogen bond with Glu166 with respect to the two formed by the other inhibitors. On the other hand, the role of the P1′ substituent when present needs to be further investigated. Worth noting, some of the co-crystallized inhibitors, namely derivatives **4**, **7**, **12**, and Boceprevir, show that the inhibitors fold in such conformation that bring in closer proximity the P3 residue (compounds **3**, **7**, and Boceprevir) or the *N*-benzyloxycarbonyl cap (compound **12**) and the P1 substituent, thus suggesting that P1–P3 macrocyclization could be explored as optimization strategy. Indeed,

macrocyclization has been widely employed as strategy to preorganize HCV NS3 protease inhibitors in their bioactive conformation leading to BILN-2061 [104, 105] and the approved pan-genotypic drug Grazoprevir [106, 107]. Moreover, macrocyclization could lead to additional advantages such as greater stability and improved PK properties.

Beside PF-00835231 and its prodrug PF-07304814 in clinical Phase 1b, also the new inhibitors **9–11** have been evaluated for their in vivo PK properties in mice. Pyridone **9** upon sc administration (3 mg/kg) showed modest half-life ($T_{1/2} < 2$ hours) and a rather low C_{max} (around 126 ng/ml), indicating a suboptimal PK profile and the need of further optimization [78]. More interestingly, indole derivatives **10** and **11** were dosed via iv administrations (single dose) at (5 mg/kg), and ip (5 and 20 mg/kg) resulting in reasonable profiles with $T_{1/2}$ ranging from ~2 to ~5 hours, depending on the administration route and the compounds, CL = 17 and 21 ml/min/kg, respectively, via iv, high C_{max} (>2390 ng/ml) and availability >85% [94]. Overall, comparing PK profiles of both compounds after iv administration at 5 mg/kg, **10** appeared metabolically more stable than **11,** thus compound **10** was further evaluated in rats (10 mg/kg, iv) and dogs (5 mg/kg, iv) showing good half-life values (rat, 7.6 hours and dog, 5.5 hours), low clearance (rat, 4.01 ml/min/kg and dogs, 5.8 ml/min/kg), and high AUCs (rat, 41 500 h*ng/ml and dog, 14 900 h*ng/ml). The PK profile was considered good enough to proceed to acute and seven-day toxicity studies in rats and dogs. In particular, compound **10** was evaluated for acute toxicity in rats by iv administration at 24 mg/kg (1 rat), 40 mg/kg (10 rats), and 60 mg/kg (4 rats), one rat from the last group died. The seven-day studies were carried out in rats and dogs by via IV administration at 2, 6, 18 mg/kg of the compound to four rats per study. Four dogs were dosed via IV at 10 mg/kg (the first day), 15 mg/kg (the second day), 20 mg/kg (the third day), 25 mg/kg (the fourth day), 25 mg/kg (the fifth to seventh days, randomly two dogs), and 40 mg/kg (the fifth to seventh days, other two dogs). All animals were clinically observed during seven days for toxic signs, which include bodyweight, food intake, and hematology, and no anomalies were observed. Overall, derivative **10** shows a PK profile comparable to PF-00835231. Therefore, the most promising compounds identified as CoVs $3CL^{pro}$ inhibitors are reversible covalent inhibitors (ketone of aldehyde) relying on small dipeptide sequences N-capped with drug-like indolyl acid, which establish key interaction in the active site of the enzyme and likely provide suitable PK properties. Indeed, as described in Section 17.2.2, the phosphate prodrug of PF-00835231, namely PF-07304814 has entered testing in humans in a Phase Ib clinical trial (NCT04535167). Moreover, a further very promising direction is the design of small peptidomimetic inhibitors based on the bicycloproline moiety as P2 fragment, as in the oral clinical candidate PF-07321332.

17.3.3 The S Protein: Structure, Function, and Inhibition

In the known human CoVs, the S glycoproteins mediate attachment to host cell surface and entry. The S protein is a 1200 aa long homotrimeric class I fusion protein, synthesized in the secretory pathway of the host cells, comprising a large ectodomain with a receptor-binding subunit S1 and a membrane-fusion subunit S2, a single-pass transmembrane anchor, and a short intracellular tail [108]. The N-linked glycans protruding from the trimer surface are responsible for the S folding and for the recognition by neutralizing antibodies [109].

Both SARS-CoVs enter into cells through the binding of S1 RBDs to the host ACE2, even though RBDs show moderate sequence similarity (64% identity) [15]. Conversely, the S2

domain is highly conserved and contains the FP, heptad repeat 1 (HR1), heptad repeat 2 (HR2), transmembrane region (TM), and cytoplasmatic tail (CT) (90% identity, with HR1 and HR2 identity of 92.6 and 100%, respectively) [110].

Cryo–EM structures of SARS-CoV-1 (PDB IDs: 3JCL [111], 5X58 [112]) and SARS-CoV-2 (PDB IDs: 6VXX [113], 6VSB [110]) S-trimer revealed a metastable prefusion ("down") conformation inaccessible to the host receptor, that changes into a less stable receptor-accessible ("up") conformation, exposing the determinants for receptor binding (Figure 17.16). The RBD undergoes a hinge-like movement to bind the peptidase domain of ACE2, resulting in a shedding in S1 and a cleavage in S2′ site, with a refolding in S2 to adopt a stable post-fusion hairpin conformation [114, 115]. This six-helix bundle (6-HB) drives viral and cell membranes in close proximity for the fusion event [110].

The X-ray crystal structures of SARS-CoV-1 and SARS-CoV-2 RBD domains in complex with ACE2 (PDB ID: 2AJF and 6M0J, respectively) show the RBD presenting a loop-dominated concave surface with at the basis two-stranded antiparallel β-sheets and two ridges formed by loop interactions (Figure 17.17) [116, 117]. The α1-helix of the ACE2 protease domain shows the main polar interactions with RBD, which contains nine cysteine residues with eight of them forming four disulfide bridges, in particular three in the RBD core and one in distal loops. Eight identical amino acids (Tyr 449/Tyr436, Tyr453/Tyr440, Asn487/Asn473, Tyr489/Tyr475, Gly496/Gly482, Thr500/Thr486, Gly502/Gly488 and Tyr505/Tyr491) have been found in SARS-CoV-2 and SARS-CoV-1 RBDs, respectively. On the other hand, key amino acids of SARS-CoV-1 RDB, i.e. Tyr442, Leu472, Asn479, Thr487, are replaced by Leu455, Phe486, Gln493, Asn501 in SARS-CoV-2 RDB, establish more interactions with the host receptor, along with a unique salt bridge between S Lys417 and ACE2 Asp30. Altogether, these differences may explain the about 20-fold higher affinity of SARS-CoV-2 S to ACE2 compared with SARS-CoV-1 and this likely is at the base of higher transmissibility of SARS-CoV-2 infection (Figure 17.17) [117, 118].

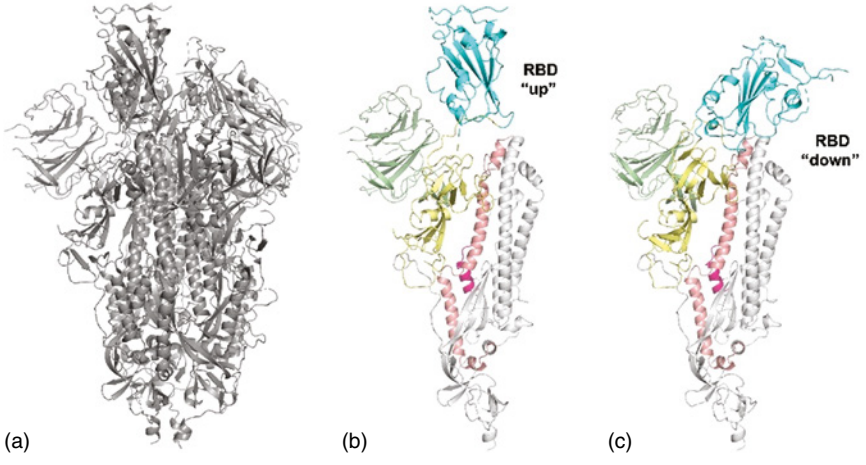


Figure 17.16 Structure of the SARS-CoV-2 S protein in its pre-fusion conformation, displayed as cartoon (gray, PDB ID: 6VSB) (a) and of a single protomer in the RBD up conformation (b) next to the RBD in the down conformation (c). RBD is colored cyan, the N-terminal domain (NTD) is pale green, subdomains 1 and 2 (SD1 and SD2) are yellow, the S2 domain is white, with HR1 colored salmon and FP hotpink.

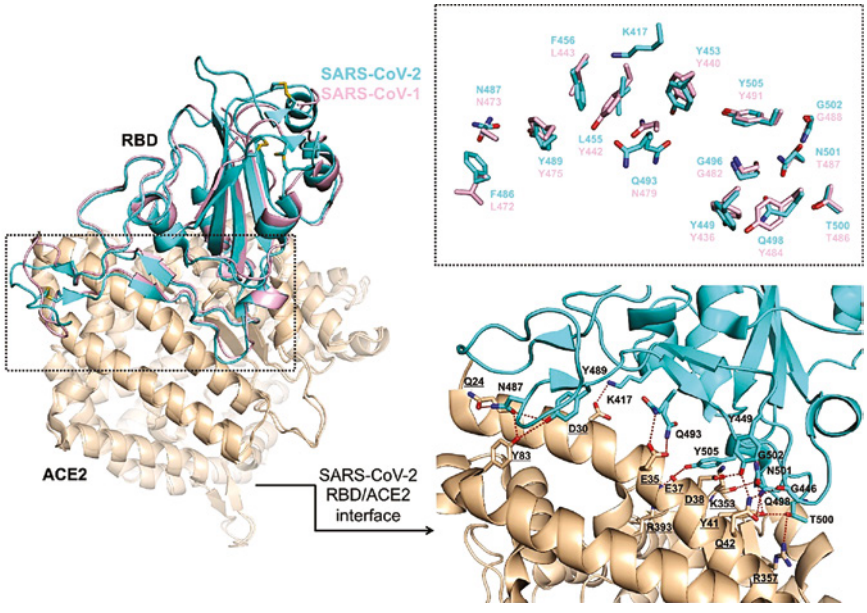


Figure 17.17 Structural details of the interface between SARS-CoV-1 (pink, PDB ID: 2AJF) or SARS-CoV-2 RBDs and ACE2 (wheat), displayed as cartoon. The region enclosed by the black dashed lines, encompassing the interface between RBD and ACE2 is illustrated in detail in panel (bottom right). Overlay of the RBD interface residues of SARS-CoV-1 (pink sticks) and SARS-CoV-2 (cyan sticks) and Q493 is shown in two alternate positions (top right). Hydrogen bonds and salt bridges are displayed as dashed red lines.

A comparison between the 6-HB fusion core structure of SARS-CoV-2 and SARS-CoV-1 S proteins, in order to investigate the structural basis for S-mediated membrane fusion, has been carried out analyzing recombinant fusion proteins containing the major parts of HR1 (residues 910–988) and HR2 (residues 1162–1206) merged with a linker (L6, SGGRGG). Comparing the X-ray crystal structures of post-fusion hairpin conformation of SARS-CoV-1 S2 (PDB ID: 1WYY) [119] with the recently solved post-fusion core of SARS-CoV-2 S2 subunit (PDB ID: 6LXT) [120], the overall 6-HB structure of SARS-CoV-2 appears highly conserved with respect to those of other HCoVs, including SARS-CoV-1 and MERS-CoV, presenting hydrophobic residues (Val1164, Leu1166, Ile1169, Ile1172, Ala1174, Val1176, Val1177, Ile1179, Ile1183, Leu1186, Val1189, Leu1193, Leu1197 and Ile1198) in the central fusion core region (Figure 17.18). Fusion core of the SARS-CoV-2 S2 reveals eight different amino acids in the HR1 domain which provide stronger interactions between HR1 and HR2, whereas HR2 domain results fully identical; in particular, Ser929, Arg933, Asp936, Ser943, and Lys947 provide new strong H-bonds and stronger salt-bridge interactions, explaining the higher fusion activity compared with SARS-CoV-1 (Figure 17.17) [120].

The considerable amount of structural data on SARS CoVs RBD/ACE2 interaction provides useful structural information to drive the development of disruptors of the SARS-CoVs/ACE2 interaction. However, considering the high variability of RBD regions within S protein across different CoVs, the RBD motif is not an ideal target for the design of

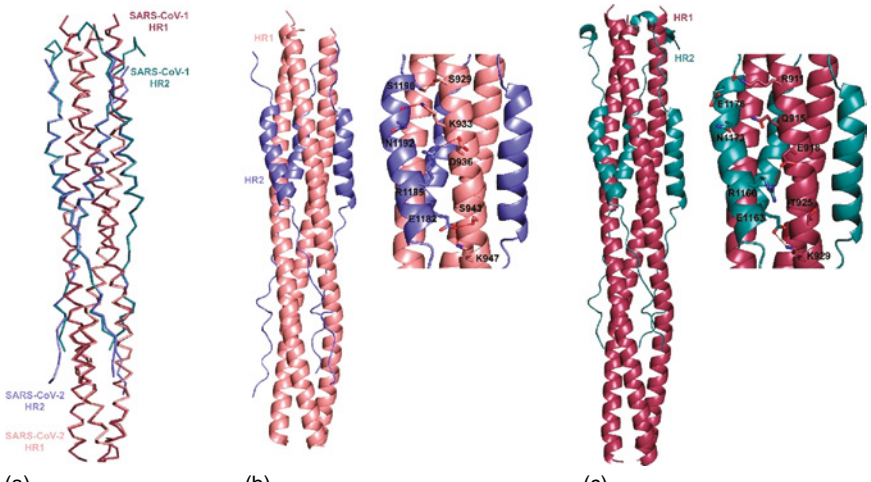


Figure 17.18 The 6-HB fusion core structures of SARS-CoV-1 and SARS-CoV-2. (a) Overlay of 6-HB structures of SARS-CoV-1 (PDB ID: 1WYY) and SARS-CoV-2 (PDB ID: 6LXT) displayed as ribbon. The HR1 and HR2 domains are colored salmon and slate for SARS-CoV-2 raspberry, and deep teal for SARS-CoV-1, respectively. (b) Side view of SARS-CoV-2 and (c) SARS-CoV-1 6-HB displayed as cartoon. A close-up view of the interactions between HR1 and HR2 is shown on the right side. Key residues are displayed as sticks and labeled; hydrogen bonds and salt bridges are displayed as dashed red lines.

broad-spectrum inhibitors. Otherwise, HR1 and HR2 conserve their sequences and play a key role in viral fusion by forming the 6-HB [121]. Thus, the identification of peptide fusion inhibitors may represent a more advantageous strategy. Based on X-ray crystal structures of MERS-CoV and SARS-CoV-1 S protein fusion cores, several peptides derived from HR1 and HR2 sequences have been reported [120, 121]. However, SARS-CoV-1 and MERS-CoV HR1 peptides did not display antiviral activity likely due to their tendency of aggregation in the absence of HR2 sequences [16]. Interestingly, peptides derived from the HR2 region of SARS-CoV-1 [122] (namely, CP-1: GINASVVNIQKEIDRLNEVAKNLNESLIDLQELGKYE) and MERS-CoV [123] (namely HR2P: SLTQINTTLLDLTYEMLSLQQVVKALNESYIDLKEL) have been previously reported to inhibit HCoV S-mediated cell–cell fusion, using effector cells carrying S-GFP fluorescent fusion protein. Unfortunately, these HR2-derived peptides do not cross-inhibit MERS-CoV and SARS-CoV-1, thus lacking broad-spectrum antiviral activity against heterologous HCoVs. The insertion of some charged amino acids like Glu and Lys, as reported for HIV-1 fusion inhibitors, may increase the solubility and stability of these peptides as well as the introduction of mutations at specific sites not involved in HR1 binding, may enhance the antiviral activity [124]. By applying this strategy to the HR2P peptide derived from the short 6-HB fusion core of OC43-HCoV, EK1 has been identified (Figure 17.19) as a broad-spectrum inhibitor. It shows an IC_{50} ranging from 0.19 to 0.62 μM in the cell fusion assays for SARS-CoV-1 and MERS-, 229E-, NL63-, OC43-, Rs3367-, WIV1-, and SHC014-CoVs [125], and dose-dependent antiviral activity against live HCoV infections. Preliminary data showed that EK1 peptide was effective against SARS-CoV-2 S protein-mediated membrane fusion and PsV infection in a dose-dependent manner [121].

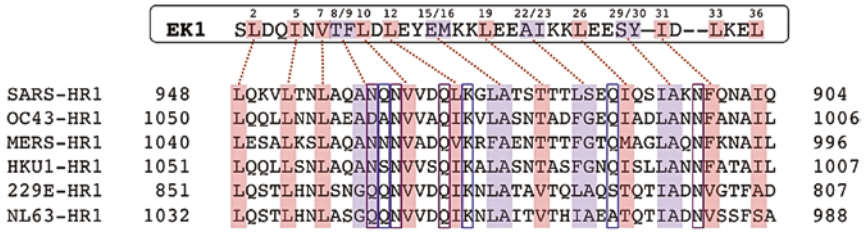


Figure 17.19 The interactions of the broad-spectrum peptide inhibitor EK1 with HR1 residues of different HCoVs, including SARS-CoV-1. EK1 and HR1 residues connected with dashed gray lines locate to the same layers on the 3HR1 triple helix. Burying EK1 residues are highlighted in dark pink, and ridge-packing EK1 residues are highlighted in violet. HR1 residues forming conserved side-to-side and side-to-main chain hydrophilic interactions with EK1 residues are indicated with boxes colored blue and purple, respectively. *Source*: Adapted from Ref. [125].

A lipidation strategy was undertaken for optimization of the EK-1 peptide which was derivatized with cholesterol and palmitic acid covalently bound to the C-terminus with a spacer of polyethylene glycol, yielding lipopeptides EK1C and EK1P (IC_{50} = 48.1 and 69.2 nM, respectively, in the cell fusion assay using SARS-CoV-2 S) (Figure 17.20) [120]. Moreover, the introduction of a GSGSG linker between EK1 and cholesterol, as in the EK1C4 peptide, increases potency (IC_{50} = 1.3 nM) in the cell–cell fusion assay. Thermal shift experiments confirmed the specific binding of EK1C4 to S-HR1s of SARS-CoVs and MERS-CoV. Interestingly, EK1C4 exerted very potent activity in cell-based assays against SARS-CoV-2, MERS-CoV, HCoV-OC43, HCoV-229E, and HCoV-NL63 (EC_{50}s ≤ 180 nM), with low cell toxicity (CC_{50} > 5 μM, SIs > 136). In particular, EK1C4 potently inhibits SARS-CoV-2 replication, with EC_{50} = 36.5 nM, resulting 67-fold higher potency compared with EK1 (EC_{50} = 2.47 μM) in the same assay. EK1C4 was also tested in mice infected with HCoV-OC43 (intranasal administration, 0.5 mg/kg) at different time points pre- (0.5, 2, 4, 12, and 24 hours) and post- (0.5 and 2 hours) infection. Pretreatment from 0.5 to 4 hours resulted in the 100% survival rate, at lower dosage than parent EK1 (20 mg/kg), while the protecting effect decreased to 83 and 0% at 12 and 24 hours, respectively. Post-treatment was evaluated at 0.5 and 2 hours postinfection, EK1C4 was highly effective (100%) if administered 0.5 hour postinfection while after 2 hours the effect was much lower (17%).

Other HR2 sequence-based fusion inhibitors have been reported as C-terminally cholesterol-linked IPB01 lipopeptides (Figure 17.21) [126]. IPB01–IPB04 peptides potently inhibit cell fusion in a dual-split protein (DSP)-based cell fusion assay of SARS-CoV-2,

Figure 17.20 Amino acid sequences of EK-1 and its lipopeptide derivatives.

Figure 17.21 SARS-CoV-2 HR2-derived peptides.

showing IC_{50} values between 15 and 33 nM. However, only IPB02 maintained comparable activity in cell-based assays against SARS-CoV-2 PV with $EC_{50} = 0.08\,\mu M$ while truncation at the N-terminus as in IPB03 and IPB04 resulted in 12- and 3-fold reduction in activity, respectively. IPB01 lacking the cholesterol unit is instead only weakly active ($EC_{50} = 33.7\,\mu M$) in cell-based assay, thus the lipidation strategy is very important to improve activity in cells. In the early 2021, additional HR C-terminus derived lipopeptides have been reported as potent fusion inhibitors of SARS-CoV-2 in vitro and ex vivo. In particular, a dimeric 36-aminoacid lipopeptide showed the highest potency in inhibiting viral entry of SARS-CoV-2, including several emerging variants, of SARS-CoV-1 and of MERS-CoV, and prevented direct-contact SARS-CoV-2 transmission in ferret animal models. [127].

17.4 Conclusions

CoVs are important human pathogens that cause infections with symptoms that range from mild symptoms to a serious or even lethal pathology, with SARS-CoVs and MERS-CoV associated with the most serious diseases. Because of the outbreak of SARS-CoV-1 in 2002 and MERS-CoV in 2011, the scientific community has been involved in characterization of these viruses and exploration of antiviral strategies. However, even if associated with fairy high mortality, the control of SARS-CoV-1 infection in 2003 and the limitation of MERS-CoV spreading to an area of the Middle East have somehow decreased the interest to find drugs against these viruses. The pandemic outbreak of SARS-CoV-2 has now pushed the scientific community in collaboration with private and public funding organization to find effective antiviral agents to stop the COVID-19 crisis.

Drug repurposing has allowed the identification of RdRp NIs as first anti-CoVs agents. Hence, Remdesivir is the first and, at the time of writing this chapter, the only licensed drug to treat the COVID-19 disease and a SARS-CoV infection. However, its efficacy is modest and clinical data are still controversial, while iv administration route represents a limitation for its wide use. Another promising clinical candidate is the oral NI Molnupiravir currently evaluated in a Phase II trial. However, RdRp is still underexplored as antiviral target. Indeed, no specific inhibitors derived from focused medicinal chemistry programs have been reported, although the protein has been deeply characterized from a functional and structural point of view.

The most exploited strategy to identify antiviral agents against CoVs is the inhibition of the $3CL^{pro}$, the main protease of these viruses. Indeed, many structural information and several inhibitors, mostly peptidomimetic covalent reversible/irreversible inhibitors, have

been reported for SARS-CoV-1, MERS-CoV, and other HCoVs, thus accelerating drug discovery also against SARS-CoV-2. Indeed, PF-00835231 is a very potent broad-spectrum inhibitor of CoVs $3CL^{pro}$ originally developed within a SARS-CoV-1 drug discovery campaign that recently has been exploited as precursor for the design of its prodrug PF-07304814 that is being evaluated in a Phase 1b trial in hospitalized participants with COVID-19. A further follow-up is represented by the related clinical candidate PF-07321332, that is the first orally administered inhibitor of SARS-CoV-2 $3CL^{pro}$ and is currently in a Phase 1 clinical trial.

Additionally, the inhibition of the entry of CoVs into host cells is another deeply investigated approach to prevent/treat these infections. In this scenario, fusion inhibitors targeting the conserved HRs represent an approach. In particular, HR2 sequence-based peptides could pave the way for the development of new therapeutics with broad-spectrum anti-CoVs activity.

Finally, another putative emerging target is the papain-like protease, which is nevertheless a more challenging and underexplored protein for the identification of inhibitors. Furthermore, this protease is less conserved across the CoVs family, and for this reason it may be less attractive in view of possible future CoV outbreaks.

The COVID-19 pandemia has emphasized that control of the CoV infection will benefit from broad-spectrum antivirals, which today is still an unmet need. Therefore, stimulating research efforts toward this direction is of pivotal importance in order to face the present pandemic, but also potential future pandemics from new emerging CoVs or other zoonotic RNA viruses.

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