Handbook of Experimental Pharmacology 189

Hans-Georg Kräusslich
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Editors

Antiviral Strategies

Springer

Handbook of Experimental Pharmacology

Volume 189

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ISBN 978-3-540-79085-3 e-ISBN 978-3-540-79086-0

Handbook of Experimental Pharmacology ISSN 0171-2004

Library of Congress Control Number: 2008933564

Cover Design: WMXDesign GmbH, Heidelberg

Printed on acid-free paper

springer.com

Preface

On the evolutionary scale, viruses are as old as their hosts, and reports or representations of viral diseases date back to ancient history. The awareness of viral pathogens increased in the last 500 years with deadly epidemics of smallpox and a large death toll taken by measles and influenza. A prime example for a devastating viral pandemic leaving little options for intervention is the so-called Spanish flu in 1918/1919, which demanded more victims than the entire world war I. Because of the genetic flexibility of viruses, new strains or species constantly emerge, the most prominent example being human immunodeficiency virus (HIV), the causative agent of AIDS, which was first identified in the early 80s of the twentieth century.

From the early 1900s on, hygiene measures greatly improved the containment of many infectious diseases. Furthermore, the discovery of antibiotics significantly increased the range of therapeutic options against bacterial diseases. These drugs target bacterial functions such as cell wall integrity, prokaryotic DNA replication, or protein expression. As obligate intracellular parasites, viruses heavily rely on host cell functions to achieve efficient replication and thus are not targeted by these drugs. A crucial discovery, by Enders, Weller, and Robbins in 1949, laying the ground for the development of antiviral drugs was the ability to cultivate cells in vitro and infect them with a virus (in this case poliovirus). The first antivirals were fortuitous discoveries of compounds originally developed for other purposes but inhibiting the replication of one or several viruses in tissue culture. Accordingly, their mechanisms of action were discovered significantly only later.

The advent of molecular biology greatly facilitated antiviral drug discovery in the past 30 years, with an unprecedented effort by both academic research and pharmaceutical industry, leading to approximately 25 HIV-specific drugs from various classes that are currently in clinical use. Similar drug development strategies are being pursued for treatment of hepatitis C, with first compounds reaching clinical application. Most antiviral drugs are targeting virus-encoded enzymes essential for viral replication and sufficiently different from their host counterparts to achieve acceptable toxicity (e.g., polymerases, proteases, endonucleases, sialidases, and helicases). More recent drug discovery approaches are directed against nonenzymatic viral functions (e.g., fusion inhibitors) and cellular factors required for viral

replication (e.g., coreceptor antagonists), and these are complemented by therapeutic approaches boosting the immune response against viral infection. Novel developments include small interfering (si) RNA approaches that may reach clinical use in topical applications (i.e., respiratory diseases), but may only achieve their full potential once gene therapy becomes a feasible option.

A crucial issue for antiviral therapy is the fact that all antiviral substances rapidly select for resistance; thus, monitoring and overcoming resistance has become a most important clinical paradigm of antiviral therapy. This calls for cautious use of antiviral drugs and implementation of combination therapies. In parallel, efforts in drug discovery have to be continued to develop compounds with novel mode-of-action and activity against resistant strains. This book reviews the current status of antiviral therapy, from the roads to development of new compounds to their clinical use and cost effectiveness. Individual chapters address in more detail all available drug classes and outline new approaches currently under development.

We thank the many authors for their contributions to produce this volume. As authors ourselves, we recognize the difficulties of working on a project of this nature. The fact that all authors were willing to accept changes in their chapters, especially the frequent reduction of length of drafts, facilitated our editorial efforts to a great extent. Furthermore, we thank Sandra Bühler, Antje Keppler, and Barbara Müller for critical reading of the manuscripts and their enormous help to adapt the drafts to the publisher's guidelines. We also thank Susanne Dathe, desk editor biomedicine at Springer, for her support and expert editorial help.

In conclusion, we hope that this volume will be valued by researchers in the field, and by those who are engaged in the future developments and applications of antiviral strategies.

Heidelberg, Germany

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Antiviral Strategies

Barbara Müller and Hans-Georg Kräusslich

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Abstract Viruses are obligatory intracellular parasites, whose replication depends on pathways and functions of the host cell. Consequently, it is difficult to define virus-specific functions as suitable targets for anti-infective therapy. However, significant progress has been made in the past 50 years towards the development of effective and specific antivirals. In particular, human immunodeficiency virus, hepatitis C virus, and hepatitis B virus, which cause chronic infections affecting millions of individuals world-wide, are a major focus of antiviral research. Initially, antivirals were mainly directed against virus-specific enzymes; more recently, drugs inhibiting the steps of virus entry or release have been developed. Rational approaches towards drug development, based on information about structure and function of viral proteins and molecular mechanisms of virus–host interactions, have become increasingly successful. Novel strategies currently explored in basic research or preclinical studies include approaches targeting host factors important

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H.-G. Kräusslich, R. Bartenschlager (eds.), *Antiviral Strategies,* Handbook of Experimental Pharmacology 189,

for virus replication, the exploitation of the innate immune response system as well as the use of gene silencing strategies aimed at interfering with viral gene expression. Today, a number of effective virostatics targeting various viral replication steps are approved for treatment of important viral diseases. However, the use of these drugs is limited by the rapid development of antiviral resistance, which represents a central problem of current antiviral therapy.

Abbreviations

CMV	Cytomegalovirus
dNTP	Deoxynucleotide triphosphate
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
IFN	Interferon
PI	Protease inhibitor
PR	Viral protease
RT	Reverse transcriptase
siRNA	Short interfering RNA

1 Introduction

Viruses are obligatory intracellular parasites, whose replication depends on functions of the host cell. This defining feature has a number of consequences for the development and application of antiviral drugs. The intracellular replication and the appropriation of cellular pathways for purposes of the pathogen makes it difficult to define virus-specific targets for therapeutic intervention, and inhibition strategies have to be highly specific to prevent cell toxicity. Furthermore, because of the viral dependence on suitable host cells and the fact that the pathogen is too small to be visible by light microscopy, complex systems are required for the propagation of viruses in the laboratory, the detection of virus replication, and the testing of potential inhibitors. Before tissue culture and molecular biology were established as routine methods, development of antiviral therapy depended on fortuitous discoveries, for example, the observation that thiosemicarbazones – originally employed to treat tuberculosis – could also inhibit vaccinia virus replication (Hamre et al. 1951). Based on this finding, a thiosemicarbazone derivative (marboran) active against the related smallpox virus was developed and used as the first virostatic to treat a human virus infection (Bauer et al. 1963; for review see Bauer 1985). During the past 50 years, medium to high throughput random screening of antiviral compounds

and structure-based antiviral drug design have become possible. Because of the comparably simple composition of viruses, the validation of targets by in vitro screens is often rather straightforward, while the procedure of preclinical and clinical testing does not differ from that applied in the case of other drugs. The field of antiviral research has undergone a remarkable progress in the past three decades and a number of potent antiviral drugs from several different classes active against important viral pathogens are currently approved (see Table 1). However, the selection of antivirals available for clinical use is still relatively limited compared with antibacterial drugs and new drugs are urgently required. This chapter outlines the principles and challenges of antiviral therapy and presents a brief overview on currently used antiviral drugs and future prospects. The topics touched on in the following sections will be discussed in more detail in the following chapters.

Table 1 Antiviral drugs in clinical use or in advanced stages of development (italics)

Viral enzymes	Polymerase	Acyclovir, Ganciclovir, Penciclovir, Foscarnet	Herpes viruses
		Abacavir, Didanosin, Emtricitabin, Lamivudin, Stavudin, Tenofovir, Zidovudin	HIV
		Delavirdin, Efavirenz, Nevirapin	HIV
		Lamivudin, Adefovir, Entecavir	HBV
		Valopicitabine	*HCV*
	Protease	Amprenavir, Atazanavir, Darunavir, Fosamprenavir, Indinavir, Lopinavir, Nelfinavir, Ritonavir, Tipranavir	HIV
		VX-950	*HCV*
	Neuraminidase	Oseltamivir, Zanamivir	Influenza virus
	Integrase	Raltegravir, *Elvitegravir*	HIV
Other viral targets	*Attachment proteins*	*BMS-488043*	*HIV*
	Fusion proteins	Enfuvirtide	HIV
	Disassembly/Uncoating	Amantadin, Rimantadin	Influenza virus
		Pleconaril	*Picornaviruses*
	Virion maturation	*Bevirimat, UK-201844*	HIV
Cellular targets	Receptors or co-receptors	Maraviroc, *Vicriviroc, TNX-355, Pro-140*	HIV
	Capping enzyme	Ribavirin	HCV
	Immune response	Interferons	HBV, HCV
		Actilon	*HCV*
Novel strategies	Antisense RNA	Fomivirsen	CMV retinitis
	Ribozymes		
	siRNA		
	Aptamers		

2 Principles of Viral Replication and Its Inhibition

Compared to bacteria and eukaryotic parasites, viruses are very simple pathogens. They have been described as "a piece of bad news, wrapped in protein" (Medawar and Medawar 1983) – genomes encased by a protective shell composed of protein(s) and, in the case of enveloped viruses, of lipids. In contrast to mammalian cells or bacterial, fungal, or parasitic pathogens, viruses as a group do not share the same type of genome or the principle of its replication. Viral genomes can consist of single- or double-stranded DNA or RNA, and viruses have been classified according to the type of genome and the genome replication strategy used (Fig. 1). Furthermore, they can be naked (i.e., containing only a protein shell) or enveloped by a lipid membrane that surrounds the protein shell and is derived from a host cell membrane. Important human pathogens can be found in many different virus families. This has implications for antiviral intervention. While double-stranded DNA viruses largely use cellular pathways for genome replication, RNA viruses, or viruses replicating in the cytoplasm, have to provide own enzymes to mediate their virus-specific replication strategies. These enzymes represent targets for specific inhibition (see Sect. 5.1 herein). On the contrary, viral RNA polymerases are generally more error-prone than mammalian DNA polymerases and viral replication mechanisms can favor genetic recombination; thereby promoting rapid adaptation, immune evasion, and antiviral resistance development (see Sect. 6 herein).

Although the details of the replication mechanism differ significantly between viruses, all viruses undergo the general replication steps outlined in Fig. 2. First, the

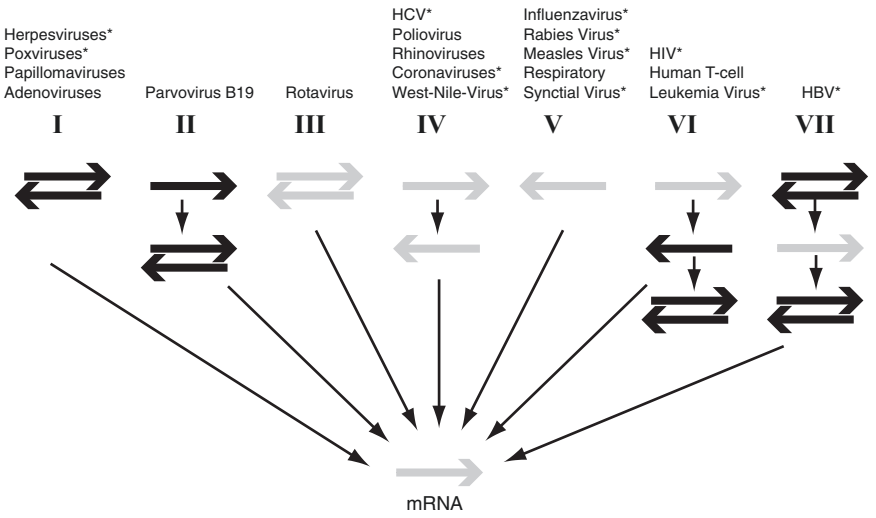

Fig. 1 Classification of viruses by their genome replication strategy according to Baltimore (Baltimore 1971). Examples for important human pathogens falling into the respective class are listed above. *Black*: DNA, *gray*: RNA; *arrows to the right*: (+) strand polarity (i.e., corresponding to mRNA); *arrows to the left*: (−)strands; *asterisk*: enveloped viruses

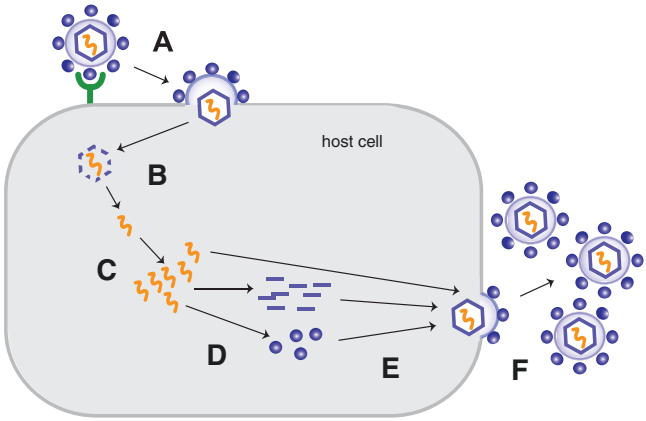

Fig. 2 Basic steps of viral replication: (**a**) binding, (**b**) entry, (**c**) genome replication, (**d**) gene expression, (**e**) assembly, and (**f**) release. Reprinted with permission from Müller and Kräusslich (2008)

virion (defined as the infectious viral particle) attaches to its host cell. Recognition of the appropriate target cell and binding is mediated by viral surface or envelope proteins interacting with one or more cellular membrane protein(s) and/or other attachment factors on the plasma membrane (e.g., heparan sulfate proteoglycans, sialic acid). Subsequently, viruses enter the cell either by uptake through a variety of endocytic pathways or directly at the plasma membrane. Enveloped viruses can enter by viral surface proteins mediating fusion of the viral lipid envelope with the plasma membrane or – often in a pH-dependent manner – with an endosomal membrane, releasing the viral core into the cytoplasm. Naked viruses cannot apply such a fusion mechanism and have to devise other strategies to cross the lipid bilayer. The following uncoating step, that is, the release of the viral genome from its protective proteinaceous shell, is currently poorly understood for most viruses. The subsequent replication of the viral genome occurs by different mechanisms for different viruses, depending on the type of genome and the site of virus replication within the cell. Transcription, post-transcriptional modification of viral mRNA, and its translation are in most cases carried out by cellular machineries, but these may be modified by virus-encoded factors. Newly generated viral proteins and genomes are transported through cellular pathways to specific assembly sites within the host cell (e.g., the plasma membrane or intracellular virus factories), where they form progeny particles. Release of these particles can occur by cell lysis as observed for most naked viruses, or, in the case of enveloped viruses, by budding from a cellular membrane, leading to the acquisition of a lipid envelope. In some cases, the generation of infectious progeny requires a subsequent step termed maturation, which involves conformational rearrangements of the virion architecture that are often triggered by proteolytic processing of viral structural proteins or changes in the environment (e.g., acidic pH).

In principle, each of these steps can be considered a target for antiviral intervention. Thus, inhibition of virus replication could be either accomplished by interfering with the specific virus–receptor interaction at the plasma membrane or the viral entry process, or by blocking viral enzymes involved in genome replication or proteolytic maturation, or by affecting virus release. Recently, the inhibition of viral gene expression and replication by use of antisense-RNA and small-interfering (si)RNA mediated silencing has been demonstrated in experimental settings, but did not yet yield new therapeutic options. Targeting steps that rely mostly on cellular factors (transcription, translation, transport of viral components) is conceptually more difficult, while cellular factors and machineries could present attractive targets due to their genetic stability if inhibited without major toxic effects. Specific interference with host cell factors or cellular machineries usurped by viruses may be achievable, provided that detailed knowledge on how the particular virus uses these pathways and on the viral and cellular factors involved is available. In addition to the direct interference with viral functions and factors, antiviral treatment can also involve immunomodulatory strategies (see Sect. 5.3 herein).

3 Development of Antivirals

Several principles, requiring different levels of knowledge and methodology, can be applied to the identification of antiviral substances (see chapter by Schinazi et al., this volume). First, a compound already in use as a therapeutic or known to inhibit another pathogen could be fortuitously discovered to inhibit the replication of a pathogenic virus. This principle is exemplified by the thiosemicarbazone derivatives (active against poxviruses), mentioned earlier. Likewise, nucleoside analogs used as inhibitors of viral polymerases (see later) are related to antiproliferative drugs targeting cellular polymerases in highly replicating tumor cells. However, fortuitous discovery does obviously not represent an ideal strategy for development of specific and effective drugs. Thiosemicarbazones, for example, were found to induce severe adverse effects, combined with very limited efficacy. In a more systematic approach, inhibitory compounds can be identified by random screening. For this purpose, a number of compound libraries compiled according to different principles (e.g., natural compounds, small molecules, peptides, drug-like molecules, ligand-based pharmacophores) are available from commercial and noncommercial sources. Beyond that, the systematic search for antivirals has not yet tapped the full potential of natural substances or mixtures, which have been used in traditional medicine (e.g., herbal extracts). Random testing for inhibition of the replication of a given virus in tissue culture is rather unbiased towards specific inhibitory mechanisms and yields some preselection for compounds that do not display significant cytotoxicity and can be taken up into the cell. However, although tissue culture of many types of mammalian cells has become a routine method, screening in this setting is relatively expensive, time consuming, and not easily automated. Furthermore, not all viruses can be easily propagated in tissue culture: in the case of the important

human pathogens hepatitis C virus (HCV) and hepatitis B virus (HBV), significant progress towards a routine tissue culture system has been accomplished only within the past 5 years (Gripon et al. 2002; Wakita et al. 2005), and only very recently have these systems been developed to allow medium to high throughput screening approaches.

Alternatively, a system for high-throughput random screening of antivirals can be set up by defining a specific viral target and establishing an in vitro assay appropriate to measure the function of this viral factor in the presence or absence of an inhibitor. Virus-encoded enzymes are particularly well suited targets for this approach, because in this case one can build on long-standing expertise from similar approaches in other areas of drug development, for example, metabolic diseases. However, in vitro systems mimicking non-enzyme mediated steps in virus replication, for example, virus assembly or protein–protein interactions between viral and cellular factors, are also being developed. Both types of random screening from compound libraries are not likely to identify a drug suited for treatment in the first round, but rather yield lead compounds that have to be validated by alternative assay procedures and subsequently improved in potency and pharmacological properties by iterative cycles of chemical modification and testing.

A fundamentally different approach is the procedure of rational drug design. Starting from detailed information on the molecular structure and function of a specific viral target, substances expected to bind to this target and to interfere with its function are identified by computer-aided design and subsequently synthesized and tested for inhibitory action. Again, suitable assay procedures to test for the inhibitory potential of in silico defined lead compounds have to be developed and the properties of the substance have to be improved by an iterative procedure. It is noteworthy that the rather limited current arsenal of antivirals already comprises several highly effective drugs resulting from structure based drug design: approved inhibitors of human immunodeficiency virus (HIV) protease as well as of influenza virus neuraminidase have been developed by rational design, highlighting the validity and feasibility of this approach. While the identification and validation of targets, development of appropriate assays, and identification of lead compounds require efforts from both academic research as well as pharmaceutical industry, the further optimization and extensive preclinical and clinical testing of candidate drugs is beyond the scope of academic institutions and can only be accomplished by pharmaceutical industry.

4 Current Status of Antiviral Therapy

When considering anti-infective therapy, one first thinks of a curative treatment, aiming at the rapid elimination of the pathogen from the human organism. This concept holds true for the treatment of most bacterial infections with antibiotics; however, in the case of antiviral therapy, a curative treatment is the exception rather than the rule. Many human virus infections are characterized by an acute, self-limiting

course. In these cases, the peak of virus replication – where therapeutic intervention would be most effective – often precedes the appearance of both clinical symptoms and virus specific antibodies detectable by routine diagnostic assays. Antiviral therapy at later time points, where symptoms and immunological markers have appeared, is of more limited clinical benefit for such acute infections, while still entailing the risk of side effects. An important acute virus infection, against which antiviral treatment is available, is influenza. Influenza virus causes severe or even life-threatening acute disease in untreated patients and is associated with devastating pandemics. For these reasons, significant efforts have been undertaken to develop effective anti-influenza virus drugs. Four substances acting against influenza virus belonging to two different drug classes (amantadine/rimantadine and neuraminidase inhibitors) are currently approved (see chapter by von Itzstein and Thomson, this volume). For other acute viral infections, therapy mostly involves symptomatic treatment or, in few cases, immunomodulatory or relatively unspecific therapy using interferon (IFN) or ribavirin (see later).

Treatment with antiviral drugs is generally more relevant in the case of persistent or chronic viral infections, and most currently available antiviral therapies are directed against such diseases. While the treatment in this case obviously also aims at eradication of the viral pathogen from the host, this is currently not achieved in many instances. In these cases, therapy aims at lowering the viral load to alleviate or prevent the clinical manifestations and long-term consequences of chronic infection (e.g., liver cirrhosis and hepatocellular carcinoma associated with chronic HBV or HCV infections), and to prevent the transmission of the pathogen. In the case of herpes viruses, which cause persistent infections, antiviral drugs are used to alleviate symptoms of primary infection or to treat recurrent infections, with the aim of forcing the virus back into a latent state. Chronic infections with HBV or HCV require prolonged virostatic treatment, which suppresses viral loads and in many, but not all, cases eventually eliminates the infection. For HIV, it is currently believed that the eradication of the virus from the organism requires decades of antiviral therapy or might not be possible at all. In this case, the indication for the initiation of therapy is based on clinical parameters and therapy aims at a sustained suppression of plasma viral load and improvement of the patient's condition. The long-term treatment of chronic infections is fraught with problems regarding adverse effects of drugs, patient compliance, therapy cost and, most importantly, resistance development (see Sect. 6 herein). Combination therapy may be required to ensure efficient reduction of viral load and prevent emergence of resistant variants (see chapter by Hofmann and Zeuzem, this volume). In the case of chronic hepatitis C, the combination therapy with ribavirin and pegylated (i.e., covalently coupled to polyethylene glycol) IFNα led to a significant improvement of success rates as compared with monotherapy (see chapter by Chevaliez and Pawlotsky, this volume; Manns et al. 2001; Fried et al. 2002)). In the case of HIV-1, the current standard is combination therapy (highly active antiretroviral therapy, HAART) using at least three drugs from more than one inhibitor class. Anti-HIV monotherapy is acceptable only in very specific settings (e.g., single dose treatment for prevention of mother-to-child-transmission) because

of rapid resistance development (see Sect. 6). For these reasons, antiviral treatment of chronic diseases requires expert knowledge, monitoring of therapeutic effects, and careful adjustment of therapy regimens.

Another fundamental difference between viruses and other pathogens which affects the development of anti-infectives concerns the fact that viruses strongly rely on host cell pathways for many of their replication steps and thus do not present many pathogen-specific targets for pharmaceutical intervention. There are no structural or metabolic features common to many viruses, which fundamentally differ from the features of the mammalian cell – comparable with, for example, the bacterial cell wall, the 70S ribosome, or the distinct metabolic pathways of parasitic pathogens. Therefore, a broad-spectrum antiviral is difficult to conceive and selection of an effective drug for antiviral treatment usually requires that the identity of the pathogen has been precisely determined by diagnostic procedures. Current genome-wide screening approaches probing, for example, the relevance of all kinases of the human genome for replication of specific viruses (see Sect. 5.3 herein) may eventually define common requirements for larger groups of viruses and thus pave the way for broader acting antivirals targeting host cell factors.

Accordingly, antiviral drugs are available against only a limited number of viruses, in contrast to the large selection of antibacterial compounds. In spite of the difficulties outlined earlier, amazing progress has been made in antiviral therapy in the past 30 years. Although HIV has only been discovered 25 years ago, more than 20 drugs targeting three different viral replication steps are approved to treat HIV infection. The number of virostatics effective against hepatitis B virus and herpes viruses is also constantly growing. Because of great efforts from basic research and pharmaceutical companies in the last decade, similar successes can be expected in the case of hepatitis C. This indicates that effective drugs can probably be developed against any pathogenic virus, provided that comprehensive and dedicated efforts from academic research and industry are undertaken. This may be more easily achieved in the case of virus diseases prevalent in the developed world, while concerted efforts including international organizations and private donors are essential to obtain drugs against viruses mostly affecting poorer countries. In view of the fact that many of the viral diseases that have great impact on public health today have arisen, or have been discovered, in the past three decades it can be assumed that important new viral pathogens will continue to emerge and the requirement for novel antiviral compounds will persist. In some cases it may be possible to build on previous accomplishments to develop drugs against novel pathogens. For example, only a few weeks after the identification of a previously unknown coronavirus as the etiological agent of the severe acute respiratory syndrome (SARS), a structural model of the viral protease as a potential target for inhibition was constructed by homology modeling based on the structure of a related coronavirus protease (Anand et al. 2003). However, even in such fortunate cases the optimization, preclinical, and clinical testing of active virostatics still requires several years of development.

5 General Antiviral Strategies

5.1 Inhibition of Viral Enzymes

The specific inhibition of enzymes, either by substrate analogs or by allosteric compounds, is a concept that is widely used in pharmacology. Thus, the inhibition of pathogen encoded enzymes in principle represents a straightforward strategy that can build on available knowledge and techniques. In the case of viral pathogens, use of this approach is restricted by the fact that virus replication strongly relies on host cell functions, and viral genomes often encode only a very limited set of enzymes. However, many viruses encode for their own nucleic acid polymerases because their genome replication is fundamentally different from that of a mammalian cell. This, together with the fact that polymerases in general are a well characterized class of enzymes made viral replication enzymes early targets for directed antiviral intervention. The classical polymerase inhibitors belong to a class of nucleoside analogs termed "chain terminators" (see chapter by Neyts and deClerq, this volume). Upon their modification to triphosphates within the cell, these compounds resemble the natural polymerase substrates, dNTPs, and are incorporated into the growing nucleic acid chain, but in contrast to the natural substrates lack the 3′OH group, which is required for the addition of the subsequent nucleotide. Many viral polymerases (RNA polymerases, Reverse Transcriptases (RT)) lack proofreading functions, preventing the removal of the incorporated inhibitor. In these cases, incorporation of a single chain terminating molecule per viral genome copy can lead to the functional inactivation of this genome molecule, making chain termination a highly effective strategy. Antiviral drugs belonging to this class are currently available against herpes viruses (e.g., acyclovir), HIV (nucleosidic RT inhibitors NRTI) and HBV (e.g., lamivudine).

One drawback of the chain termination approach is that the active site of polymerases, which is targeted by the substrate analog, displays a relatively high degree of structural conservation between enzymes of different origin. Thus, unwanted interference of the inhibitor with host cell polymerases can lead to side effects in patients treated with such drugs. An elegant way to circumvent this problem – honoured by the Nobel Prize in Physiology or Medicine to Gertrude B. Elion in 1988 – was discovered in the case of acyclovir, a potent inhibitor of the replication of herpes viruses. This acylic nucleoside analog is a poor substrate for cellular kinases, but is efficiently phosphorylated by the thymidine kinase of herpes simplex virus (HSV) to its monophosphate form, which can be further converted to the triphosphate by cellular enzymes. Thus the active form of the inhibitor is enriched specifically in infected cells, a mechanism that reduces adverse effects of the drug (Elion et al. 1977). Acyclovir displays minor efficacy against the herpes virus cytomegalovirus (CMV), which lacks thymidine kinase. However, the related compound ganciclovir is a substrate for the protein kinase UL97 of CMV and is used according to the same principle. Acyclovir and ganciclovir are not effective against viruses, which do not encode a kinase capable of mediating the initial monophosphorylation step.

However, acyclic nucleotide analogs (acyclic nucleoside phosphonates) have been developed, which carry one phosphonate moiety and require only the two subsequent phosphorylation steps (De Clercq et al. 1978). Independent of virus-encoded kinases, they display a broader spectrum of efficacy. This class comprises important drugs against HIV (tenofovir) and HBV (adefovir, tenofovir), as well as cidofovir, which is approved for use against CMV retinitis, but also displays an exceptionally broad efficacy profile against many herpesviruses, adenovirus, poxviruses, and papillomaviruses (De Clercq and Holy 2005).

Substances that do not target the active site but display inhibition by allosteric mechanisms are associated with a lower risk of unwanted interference with related cellular enzymes. Allosteric inhibition of the viral polymerase is employed in the case of HIV-1: nonnucleosidic RT inhibitors (NNRTI, see chapter by Zimmermann et al., this volume) bind outside the RT active site and act by blocking a conformational change of the enzyme essential for catalysis. A potential disadvantage of targeting regions distant from the active site is that these may be subject to a lower selective pressure for sequence conservation than the active site itself, which can lower the threshold for escape of the virus by mutation.

A second biochemically well characterized class of enzymes that is frequently found in viruses from many different families are proteases (PR; see chapter by Anderson et al., this volume). Because of their limited coding capacity, viruses often rely on the production of polyproteins, which need to be proteolytically processed into functional subunits. This is often carried out by virus-encoded proteases, which belong to the same mechanistic classes known from their cellular counterparts (e.g., thiol- or aspartic proteases), but exhibit structural differences in their active sites and substrate-binding pockets. The $\sim$10 protease inhibitors currently in clinical use against HIV are substrate analogs, which contain an uncleavable mimick of a peptide bond flanked by structural elements resembling specific features of cognate cleavage sites. Inhibitors against the PR of HCV (e.g., telaprevir) have entered the stage of clinical trials, while inhibitors of PRs of other viruses, for example, SARS coronavirus (for review, Lai et al. 2006), West Nile virus, dengue virus, pox virus, or herpes viruses, have currently been explored only in laboratory settings.

Viral integrase (IN) is an enzyme specific for retroviruses and, other than polymerases or proteases, has no closely related counterpart in human cells. It mediates the covalent integration of the viral genetic information (provirus) into the genome of the host cell by a series of concerted DNA cleavage and joining reactions. Although HIV IN has been considered an attractive target for inhibition for many years, initial studies were hindered by the difficulty of faithfully mimicking the topologically complex reaction in in vitro systems. Because of significant efforts in research and development, these obstacles have been overcome and several potent inhibitors of HIV IN have been identified (see chapter by Zimmermann et al., this volume). Clinical studies yielded highly promising results for some of these substances and the first HIV IN inhibitor (raltegravir) has been recently approved for clinical use.

Another important specific enzymatic target is neuraminidase, which is found on the envelope of influenza viruses (see chapter by von Itzstein and Thomson, this

volume). Neuraminidase function is essential for virus release. Influenza virions attach to host cells via the interaction of the viral surface protein hemagglutinin with sialic acid residues on the cell surface. While this interaction facilitates cell entry, it impedes the passage of incoming virus through the respiratory tract and keeps newly formed virions attached to the producing cell. Removal of sialic acid from the cell surface by the viral neuraminidase releases virus progeny and enables it to infect a new host cell. Based on detailed information on the structure of the enzyme and its interaction with the natural substrate, transition state analogs of sialic acid binding with high affinity to the active site of neuraminidase were designed (Bossart-Whitaker et al. 1993). Two compounds derived from this approach – oseltamivir and zanamivir – have been approved in 1999 for clinical use. Inhibitors of other respiratory viruses (e.g., parainfluenza virus) may be developed according to the same principle in the future (Alymova et al. 2005). Like viral protease inhibitors, neuraminidase inhibitors are among the still rare examples of successful structure-based rational drug design.

5.2 Other Viral Targets

On a theoretical basis, preventing a viral pathogen from entering the host cell (see chapter by Melby and Westby, this volume) represents the ideal antiviral strategy. Furthermore, the development of strategies to block viral entry can build on a considerable amount of knowledge on viral entry proteins and entry mechanisms (for review see Sieczkarski and Whittaker 2005; Kielian and Rey 2006). Surprisingly though, only few inhibitors acting at early stages of virus replication are found in the currently available antiviral arsenal. Of course, immunization leading to the development of neutralizing antibodies is effective by preventing viral entry and thus infection, but using this as a therapeutic approach would be too slow to combat acute infections and appears to be very difficult for chronic viral infections, where the virus generally replicates in the presence of a competent host immune answer.

The oldest example of a chemical targeting viral entry is amantadine, which was developed by a random screening approach approximately 40 years ago and has been in clinical use since 1976, while its mechanism of action was only unravelled in the 1990s. It acts by blocking the M2 ion channel in the envelope of influenza A viruses, which in turn inhibits conformational changes during the passage of the virus through the acidic environment of the endosome and thereby prevents release of the viral core into the cytoplasm. Unfortunately, rapid and widespread resistance development severely limits the usefulness of this drug today and neuraminidase inhibitors (see Sect. 5.1). are preferentially recommended. HIV does not enter through an endocytic pathway, but delivers its genome into the cell by fusion of the viral envelope with the plasma membrane. Fusion is mediated by the viral envelope protein gp41, which inserts into the host cell membrane. A subsequent conformational switch in gp41, where two protein helices of gp41 form a coiled–coil interaction, then draws the viral and cellular membranes into close proximity.

The peptide enfuvirtide mimicks one of the gp41 helices and binds to the cognate binding site in gp41, thereby blocking this conformational change. Enfuvirtide is the only approved example of a membrane fusion inhibitor drug and represents the fourth class of antivirals against HIV. Very recently, a natural peptide (VIRIP) that inhibits HIV fusion by a different mechanism was isolated from human blood (Münch et al. 2007). VIRIP does not block the coiled–coil formation within gp41, but interacts with the fusion peptide region of gp41 and prevents it from contacting the host cell membrane. Given promising preclinical data, the peptide may be ready to enter clinical trials within the next year.

Finally, a compound that inhibits uncoating of the nonenveloped picornaviruses (rhinoviruses, enteroviruses) has been developed: pleconaril was designed to fit into a hydrophobic canyon in the capsid protein VP1 of picornaviruses. Binding of the drug stabilizes the viral capsid and prevents the release of the viral genome into the host cell. Pleconaril has been shown to potently inhibit the human pathogens rhinoviruses, coxsackieviruses, and enteroviruses. Although the drug can shorten the clinical course of the common cold, it is not approved for therapy because the mildness of the disease requires a very rigorous risk-benefit assessment. Pleconaril is currently in clinical development for use against enteroviral meningitis.

A different approach is to target the virion structure itself by molecules interfering with capsid assembly or maturation. Viral capsid shells are assemblies of either a single or a limited number of different capsid protein(s), which are in many cases arranged in a strictly defined symmetrical architecture, or at least in arrays of local order. The integrity of the capsid depends on multiple, often rather weak, interactions between these monomers. Since capsid architecture and integrity may be disturbed by interfering with only one or a few of these interaction sites within the shell, and since interfaces between the viral capsomers are likely to be virus specific, capsid assembly represents an attractive target for antiviral therapy. However, few virus assembly inhibitors have been identified to date, mainly due to insufficient structural information or a lack of suitable assay systems. A random screening approach for nonnucleosidic inhibitors of HBV replication yielded HAP1, which was subsequently found to bind specifically to the HBV core protein and to perturb the viral capsid formation and architecture (Deres et al. 2003; Bourne et al. 2006). In the case of HIV-1, a peptide that inhibits capsid assembly in vitro by binding to a specific site on the capsid protein has been described (Sticht et al. 2005) and a cell permeable variant of this peptide has been shown to display antiviral activity in tissue culture (Zhang et al. 2008). Not only the formation of virus particles, but also a subsequent step of morphological rearrangement within the structure – capsid maturation – can be disturbed by small molecules. This principle is exemplified by the betulinic acid derivative bevirimat, which inhibits the maturation of HIV-1 particles (Li et al. 2003; for review see Allaway 2006). In contrast to HIV PR inhibitors, which block the function of the enzyme mediating the required proteolysis of the structural polyprotein Gag, bevirimat affects specifically one of several PR cleavage sites within the substrate, thereby preventing an essential processing step for capsid condensation. The virions interrupted in the process of maturation remain noninfectious. This compound represents the prototype of a novel class of HIV inhibitors and is currently in clinical development.

5.3 Interference with Cellular Factors

As outlined earlier, antiviral therapy so far is directed against viral factors, which in the ideal case are completely distinct from cellular proteins and functions. This virus-specific approach comes at a cost, however: the generally high replication rate and mutation frequency of viruses results in high rates of resistance development (see Sect. 6 herein). Alternatively, cellular factors essential for viral replication could be targeted; these would not be expected to mutate under antiviral drug pressure. This approach is much more difficult to realize than targeting of virus-specific functions, and no drugs falling into this class are yet available. Selecting an appropriate cellular target requires detailed information on the intricate network of virus–host interaction. Such information is at best only rudimentary for most viral systems. Recently, the targeted knock-down of single cellular genes by short interfering RNA (siRNA) made it possible to set up genome wide siRNA screens, which allow probing for the requirement of cellular proteins for virus replication in medium- to high-throughput screens. With the advent of this method, it can be expected that many new cellular targets will be discovered in the near future. Screens focussing on cellular kinases important for virus replication have yielded first results (Pelkmans et al. 2005; Damm and Pelkmans 2006), and very recently a genome wide siRNA screen has identified potential cellular interaction partners of HIV (Brass et al. 2008). Such approaches are likely to identify factors required for a specific virus, as well as factors, which are used by a group of viruses and therefore might in the future provide a basis for development of broad-spectrum antivirals. Once a target has been identified, the second obstacle is that inhibition has to be highly specific in molecular terms, so as not to interfere with the normal function of the respective protein. For both reasons, cellular virus receptors are conceptually promising candidates. Furthermore, since virus–receptor interactions occur on the cell surface, an inhibitor blocking this interaction does not have to be membrane permeable. For many pathogenic viruses, a receptor required for entry has been identified, and in many cases information on their molecular interactions with cognate viral proteins is available. A favorable example is represented by the human transmembrane protein CCR5, which plays a role as an HIV-1 coreceptor. A deletion in the CCR5 gene, which renders the protein nonfunctional, occurs naturally in a significant number of individuals (approximately 1% of the Caucasian population are homozygous for the Δ32deletion) without apparent pathogenic consequences; thus CCR5 appears to be functionally dispensable. Although CCR5 independent HIV entry is possible, blocking CCR5-HIV interaction is sufficient to severely affect virus replication. Inhibitors from this class (for review see Ray and Doms 2006) are under clinical development. The CCR5 antagonist maraviroc (Celsentri, Selzentry) as the first representative of this new class of antiviral drugs has been approved in 2007. In addition, antagonists of an alternative coreceptor (CXCR4) as well as of the HIV receptor CD4 are being developed. Similar concepts are also explored in the case of other viruses. For example, a peptide corresponding to the myristoylated N-terminus of the large envelope protein of HBV has been shown to block HBV replication in tissue culture (Gripon et al. 2005). Furthermore, HBV infection could be prevented

by subcutaneous application of the peptide in mice harboring transplanted HBV-susceptible hepatocytes (Petersen et al. 2008).

The interaction of a virus with its host cell often results in the activation of cellular signaling pathways. While virus-induced signaling cascades may serve to mediate an antiviral response of the host, viruses can also exploit these pathways to enhance viral replication. In these cases, cellular signaling molecules are potential targets for antiviral intervention. For influenza virus, it has been reported that virus replication in tissue culture can be impaired by inhibiting the Raf/MEK/Erk kinase pathway, and it appears that it is feasible to target this pathway without detrimental effects to the host (for review see Ludwig 2007). If realized, this approach might have the potential to target more than one virus family.

Most viruses make extensive use of the cellular transcription and translation machineries, but virus-specific inhibition of these essential pathways is conceptually difficult. One example may be the ribonucleoside derivative ribavirin (Snell 2001), identified in the 1970s by a screen searching for broad acting antivirals. Although this drug is characterized by relatively low clinical efficacy and a high probability of side effects, it can be used against severe infections with respiratory syncytial virus and has been proven particularly valuable in combination therapy against chronic hepatitis C. It is assumed that its antiviral activity is at least in part due to the inhibition of the cellular RNA capping machinery, which is also usurped by many viruses to modify their RNA. However, several other modes of action (immunomodulation, inhibition of viral RNA polymerase, incorporation into viral nucleic acids leading to hypermutation, lowering cellular GTP levels) are also discussed.

Inhibition of more complex virus–host interactions, for example, the promotion of enveloped virus budding by the cellular ESCRT machinery (Pornillos et al. 2002; Bieniasz 2006) or the intracellular transport of viral components via cellular pathways, is being discussed as a promising strategy (Li and Wild 2005). However, knowledge on how viruses use these cellular pathways and machineries and how this may differ from the normal cellular function of these elements is still very limited. Thus, it is difficult to define compounds specifically interfering with virus–cell interactions without affecting essential cellular pathways. A complementary approach to the inhibition of cellular factors facilitating virus replication would be to stimulate or enhance cellular factors that restrict virus replication. The concept of intracellular restriction against retroviral infections, that is, the existence of naturally occurring species and cell type specific inhibitors of virus replication, has been proposed several decades ago (Lilly 1967; Steeves and Lilly 1977), but respective cellular factors and their mode of action were unclear. Recently, studies from several labs identified different cellular restriction factors from the tripartite motif (TRIM; reviewed in Nisole et al. 2005; Luban 2007) and APOBEC (reviewed by Harris et al. 2004) protein families, which inhibit the replication of HIV and other retroviruses in certain host cells. These results have greatly advanced our understanding of the mechanisms of antiviral restriction, but a deeper insight into these systems is required before antiviral therapies based on these – or other yet to be identified – intrinsic restriction factors can be derived.

The interaction of viruses with the human immune system represents a level of even higher complexity than the interaction of viruses with intracellular networks. During the mutual adaptation of viruses and their natural hosts, organisms have evolved strategies to control virus infection and similarly viruses have developed strategies to counteract or evade these defence mechanisms (for review see Seth et al. 2006; Hengel et al. 2005). Stimulation of the immune system by administration of IFN has been employed as a relatively unspecific therapy against different virus infections (see chapter by Chevalier and Pawlotsky, this volume). In addition to the immunomodulatory action, IFN may also exert direct antiviral effects. As outlined earlier, pegylated INFα is a central element of the currently recommended treatment of chronic hepatitis C (for review see Hoofnagle and Seeff 2006). Pegylated IFN-α can also be used for therapy of chronic hepatitis B, in particular for adult patients. During the SARS outbreak in 2002–2003, treatment of patients with IFN was attempted, but it is unclear whether this resulted in clinical benefits (Stockman et al. 2006).

It can also be envisioned that compounds specifically disturbing the intricate relationship between a given virus and the human immune system could be employed to tip the balance in favor of the host. The innate immune response represents the front line in the defense of the organism against viral or other pathogens. It involves recognition of virus-specific structures, for example, dsRNA or uncapped RNA, by cellular receptors (toll-like receptors; retinoic acid inducible gene I, RIG-I; melanoma differentiation-associated gene 5, MDA-5), resulting in the triggering of signaling cascades, which ultimately lead to the release of type I IFN. Upregulation of this innate immune response could enable the organism to control the infection through the immunomodulatory, cell growth promoting and antiviral effects of IFN. An analogous effect could be accomplished in the opposite manner by suppressing virus-specific mechanisms, which have evolved to antagonize these cellular pathways. Recently, it has been found that the HCV encoded PR NS3/4a specifically cleaves and inactivates the cellular protein Cardif or MAVS, which is part of the RIG-I signaling cascade of the innate immune system (Meylan et al. 2005; Li et al 2005; Johnson and Gale, 2006). Thus, inhibitors of HCV PR would not only affect the essential processing of the viral polyprotein, but should also support the immune defense of the host by fending off the viral attack on the innate immune system Foy et al. 2005. An important mechanism of host defence is the elimination of virus-infected cells by apoptosis. To evade this destructive pathway, some viruses express factors exerting an anti-apoptotic effect within the infected cell (e.g., Hengel et al. 2005; Taylor et al. 2006). In particular, herpes viruses have developed numerous strategies for anti-apoptosis and often employ more than one strategy of immune evasion, since prevention of apoptosis is of importance for the establishment of latent infections. As a matter of course, interference with the highly complex network of the human immune response bears a higher risk of unforeseen complications and side effects than more conventional therapies and the successful implementation of targeted immunomodulatory strategies will require very detailed knowledge of the virus-specific aspects of the pathway.

5.4 Novel Antiviral Strategies

The antiviral strategies discussed earlier as well as all antiviral drugs available to date are based on the principles of conventional chemotherapy. However, recent discoveries and developments in molecular biology have opened perspectives for alternative approaches of intervention.

An important fraction of novel approaches involves the targeted silencing of viral gene expression through either specific degradation of a viral messenger RNA or by blocking its translation into protein. Antisense RNAs or ribozymes have been suggested and evaluated as implements for this purpose. More recently, gene silencing mediated by small interfering RNA (siRNA) has emerged as a powerful tool for molecular and cell biology. Although originally described in plants, RNA interference has also been detected in animals, including mammals, and findings in plants, *Caenorhabditis elegans*, and *Drosophila* indicate that it may have originated as an ancient intrinsic defense mechanism against viruses (Waterhouse et al. 2001; Wilkins et al. 2005; Galiana-Arnoux et al. 2006; Wang et al. 2006). Since methods for gene silencing by siRNA in experimental settings have been established and interfering RNAs can be designed against any gene with known sequence, the silencing of virus-specific genes by RNA interference appears to be an ideal method for antiviral intervention in principle (see chapter by Haasnoot and Berkhout, this volume). Successful inhibition of virus replication in tissue culture by expression of antisense RNA or siRNA has been demonstrated for a large number of viruses from many virus families, including HIV, HCV, HBV, influenza virus, measles virus, dengue virus, SARS coronavirus, and ebola virus (for review see Berkhout 2004; Haasnoot and Berkhout 2006). First clinical trials evaluating the use of siRNA against infection with respiratory synctial virus have recently been initiated. However, several obstacles have to be overcome before these results can translate into the application of siRNA as effective antiviral drugs. A crucial point is that specificity for the viral target RNA has to be ensured for any siRNA intended for use in humans. Furthermore, methods for the efficient and targeted delivery of a therapeutic RNA into the patient's cells and the maintenance of the antiviral principle in these cells have to be established. Finally, the method is particularly sensitive to resistance development. Since the inhibitory principle relies on an exact match of the inhibitory RNA with the target RNA sequence, any mutation in this target sequence can result in viral escape from the inhibition. For this reason, any successful strategy will likely have to involve more than one target sequence (e.g., ter Brake et al. 2006). Besides acting on virus encoded RNA, therapeutic RNA can also inhibit virus replication by other mechanisms. Two inhibitory principles that are being explored are "decoy RNAs," which quench viral RNA binding molecules by mimicking their natural target site and RNA aptamers (reviewed in Bunka and Stockley 2006), small RNAs selected by iterative procedures for high affinity binding to a viral enzyme, or structural protein and interference with its function. Besides being regarded as potential drugs themselves, inhibitory aptamers can also serve as tools for the selection of small molecule compounds competing for the aptamer binding site. A different kind of antisense approach has been explored in the case of

HIV: oligodeoxynucleotides targeting the polypurine tract in the viral RNA genome generate an RNA–DNA hybrid, which is prone for destruction by the viral enzyme RNaseH (Matzen et al. 2007). Finally, some viruses also express small RNAs (miRNAs) able to downregulate cellular mRNAs, presumably promoting viral replication or pathogenesis. In the case of Kaposi's sarcoma associated herpes virus, one miRNA has been shown to be functionally analogous to a cellular miRNA in downregulating a specific set of cellular mRNAs (Gottwein et al. 2007). Targeting such viral miRNAs by an antisense approach could have therapeutic benefits, while these targets – due to the similarity in specificity to their cellular analog – may be less prone to resistance mutations.

Targeted delivery of antiviral RNA molecules, as well as of genes encoding other antiviral factors, could be accomplished by gene therapy (see chapter by von Laer and Baum, this volume). Somatic gene therapy, that is, the introduction of a therapeutically effective gene within a subset of the patient's cells, can potentially ensure a sustained delivery of an antiviral principle, thereby alleviating the problem of continuous need for medication in chronic infections. Gene therapeutic approaches could be either used to selectively eliminate infected cells, to render cells of a patient resistant to virus infection ("intracellular immunization"), or to induce cells to release antiviral peptides into their environment. Because of the more complex and less understood risk potential of gene therapeutic approaches as compared to conventional chemotherapy, gene therapy is currently only considered for otherwise untreatable and potentially lethal conditions. For this reason, AIDS was among the first diseases regarded as a potential indication for gene therapeutic intervention (Baltimore 1988), and a number of potential inhibitory strategies have been suggested and evaluated. Approaches to eliminate HIV infected cells by overexpression of a CD4 T-cell receptor zeta chain fusion protein in autologous T cells, thereby generating a specific CTL-response against cells expressing the viral envelope protein, were unsuccessful. Many strategies have been designed for intracellular immunization of T-cells against HIV, acting against a number of different targets in the virus (transdominant versions of viral proteins, RNA decoys, ribozymes, membrane-bound fusion inhibitor, intracellular single chain Fv antibody fragments against viral proteins) and tested in vitro. However, in clinical studies, none of the intracellular immunization strategies tested has so far led to a sustained selective advantage and a repopulation of the immune system with the genetically modified T-cells (discussed in von Laer et al. 2006)

6 Antiviral Resistance

Short replication cycles that may be completed within a few hours, a large amount of viral progeny from one infected host-cell, as well as the general inaccuracy of viral nucleic acid polymerases result in an "evolution occurring in fast motion," allowing rapid adaptation of viruses to selective pressures (see chapter by Boucher and Nijhius, this volume). Generalizing, it can be stated that any effective antiviral therapy will lead to the occurrence of resistance mutations. A well studied example

is again HIV. The error-prone HIV RT introduces on average 10^{-4} to 10^{-5} mutations per nucleotide and per replication cycle (Mansky and Temin 1995), and the mechanism of retroviral replication favors genetic recombination. Since it is estimated that in an untreated HIV infected person up to 10^{10} new virions can be produced per day (Ho et al. 1995; Wei et al. 1995), this leads to the generation of an enormous number of mutated virus variants. While many of these random mutations will be incompatible with virus replication, others will have no or minor effects in this respect. As a consequence, the virus population in infected individuals consists not of clones of identical viruses or of a few similar variants, but rather represents a so-called quasispecies, that is, a collection of variants that all differ from each other at some positions in their genome. The situation is similar or even worse for HCV. This pool of pre-existing mutations will also comprise those that by chance confer some degree of resistance to an antiviral drug used for therapy. Since these mutations are often associated with lower viral fitness, that is, lower replication rates compared to wild-type in the absence of the drug, they generally only represent a minor fraction of the viral population before treatment is initiated; however, they will be selected by treatment with drug concentrations insufficient to completely suppress replication of moderately resistant virus. Replication under drug selection pressure can then result in the accumulation of further adaptive mutations conferring a higher degree of resistance and a higher level of fitness. Thus, resistance development is a complex stepwise process (Fig. 3) by which replicative fitness and drug resistance are balanced in response to the environmental conditions. These mechanisms are intensely investigated in the case of HIV. As an example, the first mutations observed after initiation of protease inhibitor treatment decrease the affinity to the inhibitor (primary mutations). Since primary mutations usually occur at the active site of the enzyme, substrate binding and catalysis rates are also affected and these mutations are usually associated with lower viral fitness. This can be successively compensated by secondary mutations outside the active site, which increase the resistance level

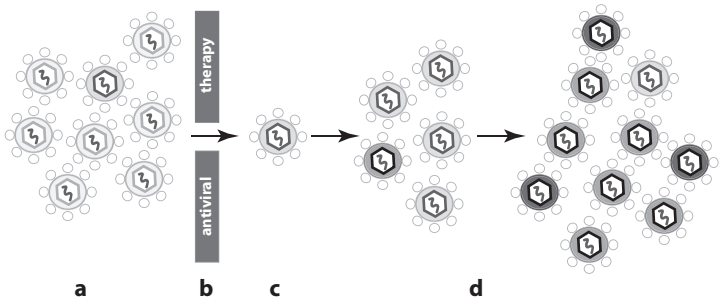

Fig. 3 Stepwise development of antiviral resistance. Because of the rapid mutation rate of viruses, the virus population before treatment (**a**) contains variants, which display by chance a low level of resistance to the drug (indicated by the darker hue). Treatment with suboptimal levels of an antiviral drug (**b**) creates a bottleneck, which selects for these variants (**c**). These can further replicate in the presence of the drug and thereby acquire additional mutations, leading to resistant variants with enhanced replicative fitness (**d**)

or restore the replicative capacity. In the case of HIV PR, tertiary mutations outside the protease gene are also observed. These affect the PR substrate Gag and can further enhance the fitness of resistant PR variants. To prevent or delay such adaptive cycles, it is essential to avoid suboptimal drug treatment regimens.

Resistance development can occur extremely rapid, as illustrated by experiences with the use of single dose nevirapine. A single peripartum dose of nevirapine efficiently reduces the rate of mother-to-child-transmission of HIV, and it has therefore been part of the many regimens for HIV-infected pregnant women without access to antiviral therapy. However, in the case of nevirapine, a single point mutation in RT already confers high resistance and the pharmacokinetic properties of the drug result in suboptimal levels being sustained in the organism over days. Several studies have revealed that nevirapine resistant virus variants can be detected in blood samples of 20–69% of mothers and up to 87% of infected infants following this single dose exposure (Jackson et al. 2000; Eshleman et al. 2001, 2005a, b; Lee et al. 2005; Flys et al. 2006; Shapiro et al. 2006). Although the most detailed data are available for HIV, the resistance problem is of course not limited to this virus, but has been observed for any potent antiviral. In the course of treatment of chronic hepatitis B with lamivudine resistant virus variants emerge rapidly and rates increase over time, culminating in therapy resistant virus in approximately 65% of patients after 5 years of treatment (Lok et al. 2003). Cross-resistance against several inhibitors from one class or multi-resistance against more than one class of drugs has also been observed. Furthermore, resistant virus variants can be transmitted. For these reasons, resistance development can severely limit the usefulness of antiviral drugs. For example, sequencing of influenza virus isolates circulating in the USA at the beginning of the 2005/2006 season revealed that 92% of the isolates carried a mutation correlated with amantadine resistance and it was concluded that the drug should presently not be used for treatment or prophylaxis of influenza in this country (Bright et al. 2006). Similarly, surveillance of HIV drug resistance in Europe showed that virus variants resistant against one or more antiretroviral drugs were detectable in $\sim$10% of therapy-naïve patients (Wensing et al. 2005). These examples illustrate that, with increasing availability and use of antiviral drugs, the problem of resistance development has rapidly increased to a point where it diminishes the limited arsenal of drugs available for antiviral treatment. Resistance monitoring has become an increasingly important part of antiviral drug treatment regimens. Molecular mechanisms underlying resistance, as well as the evolution, monitoring, and prevention of antiviral resistance will thus continue to be topics of central significance in the field of antiviral research.

7 Perspectives

Viruses are important human pathogens, causing a tremendous burden of disease and death worldwide. Thus, antiviral drugs are urgently required. Although virus replication relies largely on host factors and is therefore difficult to target development of potent and specific antivirals against important pathogenic viruses, in particular HIV, herpes viruses (HSV, CMV), HBV and influenza virus have been

accomplished. Building on these experiences, a considerable expansion of the antiviral arsenal can be expected in the future. However, increasing therapeutic options and increasing accessibility of antiviral drugs is inevitably connected to increasing resistance development, which in turn creates a constant need for careful monitoring of resistance development and alternative antiviral drugs. New pathogenic viruses will continue to emerge, again creating a need for novel virostatics. Thus, antiviral drug development represents a field of growing importance in the years to come. Classical pharmacotherapy with small molecule chemicals directed against virus-specific functions will likely continue to be the major force in antiviral therapy, but this will be increasingly complemented by other approaches. The most promising alternative approaches are drugs affecting (nonessential) host factors involved in virus replication or virus-specific modifications of these factors, as well as siRNA directed at viral or cellular genes. Strategies targeting cellular factors, as well as novel immunomodulatory therapies, may hold the potential to define drugs effective against more than one class of viruses or truly broad-spectrum antivirals.

Acknowledgements Work in the author's laboratory is funded in part by the Deutsche Forschungsgemeinschaft (SFB544) and the EU (6th framework: "HIV PI resistance," LSHP-CT-2006–037693; 7th framework: "HIV-ACE," HEALTH-F3–2008–201095). We thank Paul Schnitzler, Stephan Boehm, and Manon Eckhardt for critical reading of the manuscript.

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Approaches for the Development of Antiviral Compounds: The Case of Hepatitis C Virus

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Contents

Abstract Traditional methods for general drug discovery typically include evaluating random compound libraries for activity in relevant cell-free or cell-based assays. Success in antiviral development has emerged from the discovery of more focused libraries that provide clues about structure activity relationships. Combining these with more recent approaches including structural biology and computational modeling can work efficiently to hasten discovery of active molecules, but that

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H.-G. Kräusslich, R. Bartenschlager (eds.), *Antiviral Strategies,* Handbook of Experimental Pharmacology 189,

is not enough. There are issues related to biology, toxicology, pharmacology, and metabolism that have to be addressed before a hit compound becomes nominated for clinical development. The objective of gaining early preclinical knowledge is to reduce the risk of failure in Phases 1, 2, and 3, leading to the goal of approved drugs that benefit the infected individual. This review uses hepatitis C virus (HCV), for which we still do not have an ideal therapeutic modality, as an example of the multidisciplinary efforts needed to discover new antiviral drugs for the benefit of humanity.

Abbreviations

AAG	Alpha-1 acid glycoprotein
ADK	Aryl α-γ-diketo acids
CYP450	Cytochrome P-450 isoenzymes
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
NNI	Nonnucleoside inhibitors
NTP	Nucleoside triphosphate
P-gp	P-glycoprotein
RdRp	RNA dependent RNA polymerases
SAR	Structure activity relationship

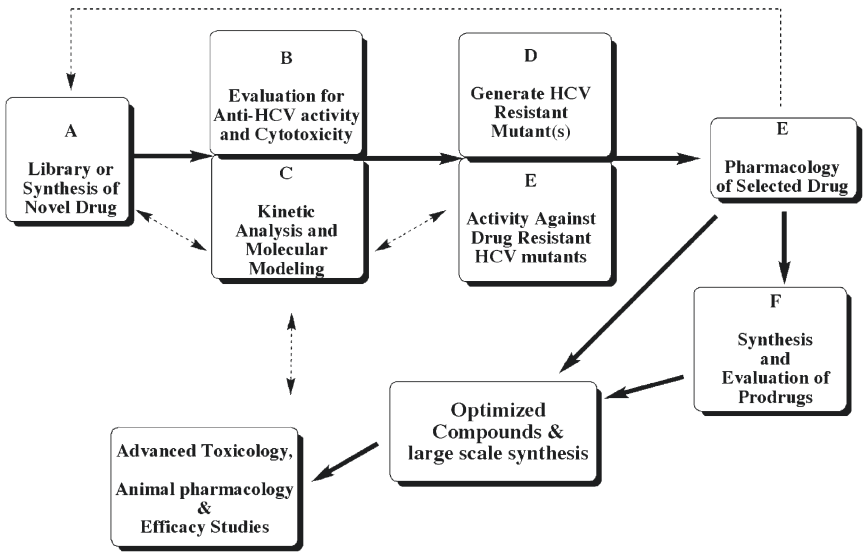
1 Introduction

The history of antiviral drug development has taken numerous circuitous routes from the discovery and development of the first US Food and Drug Administration (FDA) approved antiviral agent, 5-iodo-2′-deoxyuridine (Idoxuridine) by William H. Prusoff (Prusoff 1959; Prusoff et al. 1979), to the development of simplified but powerful triple combinations for HIV such as Atripla and Trizivir (De Clercq 2007; Gallant et al. 2006; Schinazi 1991). Many challenges are encountered during the development of antiviral agents, including adverse events and the development of drug resistant viruses, which necessitate chemists, biologists, and pharmacologists to develop improved, more potent, and less toxic medicines with "high genetic barrier." Although there are major differences among viruses, specific virological and pharmacological approaches used to develop novel antiviral agents are similar across many viral diseases. In this review, we use hepatitis C virus (HCV) as a prominent example for different strategies employed in drug discovery. Sophisticated technologies such as liquid chromatography (LC)-mass spectrometry, real-time PCR, pharmacokinetic and pharmacodynamic modeling, cryo-electron microscopy, crystallographic structure determination and modeling have advanced our capacity to develop antiviral agents in recent years, but the available tools still need to be further optimized.

HCV infection is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. It affects an estimate of 170–200 million people worldwide (Shepard et al. 2005) and there is still no vaccine available. HCV will conduce as a model in this chapter to describe the approaches to develop antiviral compounds. Although impressive progress has been made in treating HCV infection, the standard therapy of (pegylated) interferon alpha and ribavirin has low efficacy against some genotypes and is associated with important side effects. Many persons do not tolerate or respond to interferon-based therapy with or without ribavirin (Falck-Ytter et al. 2002). For example, half of the genotype 1-infected subjects are nonresponders to standard pegylated interferon–ribavirin treatment. Therefore, new therapeutic strategies are urgently needed (Manns et al. 2006), including small orally bioavailable molecules as well as combined modalities to prevent or delay the development of drug-resistant HCV.

1.1 Approaches to Discovery

Improved treatment for HCV requires the development of novel agents with unique biological profiles that are effective against a broad range of viral genotypes. While drug or target discovery represents the beginning of the process, multiple studies are required before an antiviral agent becomes a useful, and in particular, safe drug for humans. Using a modern systematic drug discovery and development process, the risk of failure in the clinic can be markedly reduced (Scheme 1).



Scheme 1 The path of least resistance in HCV drug discovery and development

The process of antiviral drug development generally consists of several steps that must be performed prior to filing an Investigational New Drug (IND). The first step is the evaluation of existing libraries of compounds against the target and/or the design and synthesis of new molecules based on knowledge of potential target site structures (Scheme 1). Then the relative potency of potential antiviral agents can be determined rapidly and reproducibly using enzymatic and cellular systems coupled with quantitative real-time polymerase chain reaction (PCR). Efficacy models include cell culture and whole animal systems, which support HCV replication and can assay antiviral molecules for their capacity to inhibit it. This can be performed in normal liver cells (including primary hepatocytes), adapted liver cells (e.g., Huh-6 or 7), and human bone marrow cells. It is critical to determine the mitochondrial toxicity of the compound, after at least 2 weeks in culture, and its effect on various human enzymes such as cellular elastases [human neutrophil elastase (HNE) and human leukocyte elastase (HLE)] and polymerases (alpha, beta, and gamma). The stability of the chemical entity and its protein binding capacity must be evaluated under various conditions (pH, temperature, whole blood, human liver homogenate, etc.). The extent of protein binding and antiviral effect in the presence of alpha-1 acid glycoprotein (AAG) is essential especially for protease inhibitors. Protease inhibitors and certain small molecules can be substrates that may inhibit or induce cytochrome P-450 isoenzymes (CYP450) (Lim et al. 2004; Yeh et al. 2006). The mechanisms of action of the compound must be explored with appropriate enzymatic tools. In vitro selection and characterization of viral variants resistant to the drug candidate is absolutely critical. These experiments may provide clues on what could happen in humans and also confirm the primary antiviral target of the agent at the genetic level. Cross-resistance studies with known site-directed mutagenized viruses having specific clinically relevant mutations are also essential. Ideally, resistance testing should include both cell culture (using viruses with defined mutations) and enzyme-based assays.

Toxicology studies must be performed in at least two animal species. If the toxicity profile of the compound is acceptable, then it joins the “hit or lead list” of compounds to proceed. The metabolism of the compound must be understood and pharmacokinetic studies must be performed in small and large animals. Efficacy studies must be performed in relevant animal models, especially in chimpanzees when more than one candidate is identified and a choice has to be made before proceeding to studies in humans. The ultimate preclinical steps include various studies testing drug combinations in vitro and in vivo, selection of resistant viruses, viral fitness, pyrophosphorolysis, and others.

2 Specific HCV Enzyme Inhibitors

HCV drugs with a direct antiviral mode of action are needed for the treatment of chronic HCV infection. The NS3 protease, NS3 helicase (which localizes to the carboxy-terminal domain of NS3 and catalyzes the unwinding of the double stranded RNA in a 3′ to 5′ direction (Tai et al. 1996)), and NS5B RNA polymerase

are all nonstructural (NS) proteins of HCV. These enzymes are essential for viral replication and are currently the key targets for the design of specific inhibitors, with the major focus on the NS3 protease and NS5B polymerase (Table 1).

The NS3 protease localizes to the amino-terminal domain of NS3 and requires the small NS4A cofactor for its function. This enzyme is a distinct serine protease that is responsible for the downstream cleavage events of the NS polypeptide at four junctions (Failla et al. 1994). It is a heterodimeric protease and a member of the chymotrypsin serine protease family. However, the substrate specificity of the NS3 protease is very distinct from that of the related host enzymes (Urbani et al. 1997). Even so, it has been a challenge to design potent and selective inhibitors against the NS3 protease, as the substrate-binding cleft of the protease is shallow and lacks cavities (Yan et al. 1998). Despite this, several groups have been successful in designing highly selective inhibitors of this enzyme. However, the relatively high sequence difference between genotypes in the protease region limited the design. For instance, many protease inhibitors in the pipeline are effective only against HCV genotype 1, and may show up to 100-fold less activity against genotypes 2 and 3 relative to genotype 1 (Reiser et al. 2005). Genotype 1 is the most common genotype in the Western world, and individuals infected with genotype 1 are less likely to respond to pegylated interferon, making this selectivity a problem. However, there is a need for more and broader acting protease inhibitors to cover all six genotypes of HCV. Specific inhibitors of NS3 protease are discussed in chapter by R. Swanstrom et al., this volume.

The NS5B RNA polymerase is highly conserved and contains a Gly-Asp-Asp motif, which is characteristic for RNA-dependent RNA polymerases (Lohmann et al. 1997). NS5B is the key enzyme responsible for the synthesis of negative strand RNA, using the genome as template and for the subsequent synthesis of genomic positive strand RNA from this template (Yamashita et al. 1998). It is an attractive target to identify selective inhibitors (e.g., nucleoside analogs), since no RNA-dependent RNA polymerase activity is present in mammalian cells. Reminiscent of HIV-1 reverse transcriptase (RT) inhibitors, two classes of HCV polymerase inhibitors, namely, nucleoside analogs and nonnucleoside inhibitors (NNI) are under development. Nucleoside analogues in their metabolically activated 5′-triphosphate form inhibit NS5B by competing with the natural 2′-nucleosides-5′-triphosphate (NTP) and/or by chain termination (see also chapter by Neyts et al., this volume). This action prevents further elongation of the nascent RNA. Intracellular phosphorylation of the nucleoside analogs to the corresponding mono-, di-, and triphosphates, respectively, is mediated by cellular kinases. For example, the phosphorylation of the C-analog PSI-6130 was demonstrated with dCK, UMP-CMP kinase, and nucleoside diphosphate kinase (Murakami et al. 2007). The highly conserved region of the polymerase catalytic site enables broad genotype specificity for nucleoside analogs in contrast to the protease inhibitors and presumably the NNIs.

It is important to note that the intracellular levels of NTP are approximately 100-fold higher than the dNTP levels. Therefore, much higher median effective

Table 1

Class of drug/compound	Mutation	Fold-resistance	Cross-resistance	Comments	Reference
Protease inhibitors	(in vitro)	(in vitro)			
BL 2021	R155Q				(Courcambeck et al. 2006)
	A156V	> 100	VX-950		(Lin et al. 2004)
	A156T	>> 100			
	D168A	400		Indirect contact with inhibitor	
	D168V	1,000		Indirect contact with inhibitor	
VX-950	A156S	10		Dominant mutation	(Lin et al. 2004)
	A156V	> 60	BL 2021		
	A156I	> 60	BL 2021		
SCH 503034	T54A	< 20			(Tong et al. 2006)
	A156S	< 20			
	A156T	> 100	BL 2021		
	V170A	< 20			
ITMN B					
Polymerase inhibitors					
NA					
NM 283	S282T	21	2′C-Methyl-Adenosine	Reduces binding with inhibitor	(Dutartre et al. 2006)
PSI 6130				Non-obligate chain-terminator	(Murakami et al. 2007)
R-1479	S96T				(Le Pogam et al. 2006a)
	N142T				
MK 0608					
A-782759 (Abbott)	H95Q	44			(Mo et al. 2005)
	N411S	28			
	M414L	70	Thiophene-2-carboxylic acid		
	M414T	> 200	Benzo-1,2,4,-thiadiazine		
	Y448H	35			
Benzo-1,2,4,-thiadiazine (GSK)	M414T	> 100	A-782759		(Nguyen et al. 2003)
NNI					
JTK-003					
Thiophene-2-carboxylic acid	M414L	10	A-782759		(Le Pogam et al. 2006b)
	L419M	20			
	M423T	10			
	I482L	20			

concentrations (EC_{50}) were expected for nucleosidic NS5B inhibitors than those observed for HIV RT inhibitors due to competition by the high concentration of natural nucleosides. However, current investigational nucleoside analogs against HCV have demonstrated reasonable potency with EC_{50} values of approximately 1 μM in replicon assays.

Resistance to nucleoside analogs has been observed using replicon assays. Biochemical studies with HIV-1 RT resistant against nucleoside analogues demonstrated two different resistance mechanisms (see also chapter by Nijhuis et al., this volume, for resistance discussion). Besides substrate discrimination, resistance could also be achieved by ATP dependent excision of nucleoside analog-monophosphates after their incorporation (Meyer et al. 1999). The same mechanism of excision (but pyrophosphate dependent) has been described for a related polymerase – bovine viral diarrhea virus RNA-dependent RNA polymerase (D'Abramo et al. 2004). Pyrimidine-based chain-terminating analogs, that is, C and U analogs, were more easily selected for excision, implying that purines (A and G analogs) are the preferred choice for further drug development. However, these compounds would then have to compete with ATP and GTP, which exhibit a much higher intracellular concentrations than at least CTP (Deval et al. 2006).

HCV polymerase, like other *flaviviridae* NS5B enzymes, is able to initiate RNA synthesis without an RNA primer, which is unique to viral RNA polymerases (Zhong et al. 2000). When designing an enzymatic NS5B assay, consideration must be given to the choice of initiation nucleotide (GTP), metal ions (Mg^{2+} and Mn^{2+}), and template design with unique hairpin loops and sequence in the 3′ end (Ranjith-Kumar et al. 2002; Kim and Kao 2001). Currently, a number of nucleosides are in clinical development, including PSI-6130 (β-D-2′-Deoxy-2′-fluoro-2′-C-methylcytidine; Pharmasset/Roche) and its prodrug R7128 as well as MK-0608 (Merck). In addition, a number of NNI of NS5B polymerase are also in development, including JTK-003 (Japan Tobacco). Some of these nucleoside analogs and NNI will be discussed further. For more information, refer to Chaps. 3 and 6.

The high rate of viral turnover in HCV infection, coupled with the absence of proofreading by the NS5B polymerase, results in a rapid accumulation of mutations (Martell et al. 1992). Therefore, one can anticipate that the use of specific enzyme inhibitors as a monotherapy will likely result in viral resistance. This suggests that, like HIV treatment, combination therapy will be necessary for HCV as well (see chapter by Hofmann, this volume). Initial regimens would be combined with pegylated interferon, as that agent is the backbone of the present drug regimens for HCV. In vitro assays have allowed investigators to identify a variety of resistance mutations against current investigational compounds. These mutations are located in the catalytic sites of HCV enzymes and result in enhanced binding discrimination over natural substrates. Therefore, as for HIV, the residue changes in the resistant viruses usually result in reduced viral fitness. For more information on resistance, refer to Chaps. 4 and 11 in this volume.

3 Drug Discovery Tools for HCV

Several excellent reviews have appeared recently (Condon et al. 2005; Huang et al. 2006; Koch and Narjes 2006; Meanwell et al. 2005; Neyts 2006; Shim et al. 2006; Wu et al. 2005), which summarize the development of agents to treat HCV infection. In this report we highlight the application of modern drug discovery tools and techniques to inhibit HCV replication by describing selected examples that have appeared recently in the scientific literature.

As illustrated in Scheme 1, antiviral discovery begins with drugs that may be used as probes of biological function. The structure-based analysis and modeling described in the next two subsections can provide a context for understanding the molecular patterns responsible for a drug's action at its receptor. To effectively evaluate structure activity relationships (SAR, focused libraries of chemically related compounds need to be developed and such methods are discussed in Sect. 4. Anti-HCV activity assays, kinetic and pharmacodynamic modeling are introduced in Sect. 5. Approaches for understanding advanced pharmacological and physiological factors that influence drug delivery are key to efficacy in humans and are described in Sect. 6. Systems for sharing and combining these different data types are the discovery tools of the future.

3.1 X-Ray Crystallographic Analysis and Structure-Based Drug Design

In a structure-based drug design program, the three-dimensional structure of a drug target interacting with small molecules is used to assist the drug discovery process. Structure-based drug design allows one to investigate the interaction of a small molecule with its target protein and to explore the possibilities to chemically modify the molecule to obtain the desired properties. The focus of this section will be on specific discovery programs where the structural information was obtained by X-ray crystallography and applied to the drug discovery process.

Crystal structures of the NS5B polymerase alone and in complexes with nucleotide substrates have been solved and applied to discovery programs (Ago et al. 1999; Bressanelli et al. 2002; Bressanelli et al. 1999; Lesburg et al. 1999; O'Farrell et al. 2003). From these studies, HCV polymerase reveals a three-dimensional structure that resembles a right hand with characteristic fingers, palm, and thumb domain, similar to the architectures of the RNA polymerases of other viruses. However, none of these experimental structures contained the ternary initiation complex with nucleotide/primer/template, as obtained with HIV RT. Accordingly, HCV initiation models have been built using data from other viral systems in efforts to explain SAR (Kozlov et al. 2006; Yan et al. 2007).

Recently, many NNI of HCV NS5B RNA polymerase have been discovered and these were recently reviewed (Condon et al. 2005). One allosteric binding site has

been identified via X-ray crystallographic analysis of NS5B complexed with several different noncovalently bound inhibitors. This predominantly hydrophobic depression is located approximately 35 Å from the polymerase active site in the thumb domain (Biswal et al. 2005; Di Marco et al. 2005; Love et al. 2003; Wang et al. 2003). A more polar area of this pocket contains Ser^{476} and Tyr^{477} residues that appear to provide critical hydrogen bonds to the carboxylate of these inhibitors. Although mechanistic details of inhibition through this allosteric site are unclear, it has been proposed that the enzyme must be forced into an inactive conformation involving the prevention of interaction between the finger-tip region and the thumb domain (Di Marco et al. 2005). A more recent crystallographic analysis of this hydrophobic allosteric binding site revealed some disruption to the integrity of the GTP binding site, thus contributing to a RNA polymerase state incapable of carrying out a polymerization cycle (Biswal et al. 2006).

Like the corresponding HIV enzyme, the HCV NS3 protease is amenable to structure-based design. Starting from the lead hexapeptide DDIVPC, the research group at Boehringer–Ingelheim rationally designed the BILN 2061 family of compounds (LaPlante and Llinas-Brunet 2005) (Fig. 1). BILN-2061 has an IC_{50} of 3 nM for the HCV NS3 protease with its shallow and relatively featureless binding pocket. When administered to HCV-infected patients for 2 days, BILN-2061 produced an unprecedented and rapid decrease in viral load, thus demonstrating the first proof-of-concept for a new class of HCV antiviral agents.

Initially, medicinal chemists undertook a systematic strategy of single amino acid changes of the DDIVPC-type hexapeptide, with the goal of improved potency and reduction in the peptidic nature of the series. Important substituents that directly contact the protease pocket were identified by a combination of differential line-broadening NMR experiments and docking of hexapeptides into the active site of an X-ray structure of the *apo* NS3 protease. The resulting model was the first liganded NS3 protease complex described (Tsantrizos et al. 2003). The most critical observation from these efforts was that only substituents of the P1–P4 regions experienced binding interactions with the protease.

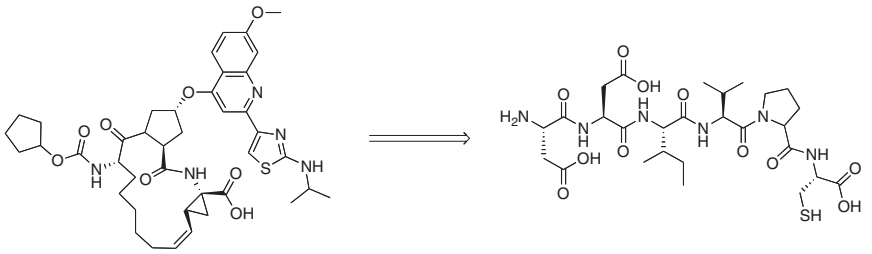


Fig. 1 Structure of BILN 2061 and initial lead peptide DDIVPC. Activities were determined in a radiometric assay against the NS3 protease domain

With the goal of reducing the entropic costs of binding, the macrocyclic nature of the BILN-2061 series was designed to rigidify the free-state conformation and to resemble the bound state observed in the peptide NS3 protease complex (see chapter by Anderson et al., this volume). The Boehringer–Ingelheim researchers were able to solve a crystal structure of NS3 protease complexed to an 11 nM macrocyclic inhibitor. The binding properties of each of the regions of the macrocyclic inhibitors could be investigated relative to the known SAR in the macrocyclic series. The *t*-butyl carbamate capping group sits in a shallow, wide, solvent exposed, and partially hydrophobic groove. The replacement of the *t*-butyl group with a variety of hydrophobic groups resulted in only minor changes in potency, which is consistent with the lack of a deep defined pocket in that region. It was also clear that the proline ring is mostly solvent-exposed and has very little direct contact with the protease; however, its role is critical in providing proper positioning of P1, P3, and the macrocycle, which are the three key binding groups (Goudreau et al. 2004).

Researchers at Schering–Plough reported an X-ray crystallographic analysis of two potent macrocyclic inhibitors bound to the NS3 protease (Chen et al. 2006). The macrocycles (Fig. 2) were prepared based on earlier modeling and X-ray crystallographic analysis, which indicated that an enhanced binding to the protease might result from a ring size of approximately 15–17 atoms. The ligand–protease complexed crystals were obtained by soaking the compound into preformed enzyme crystals. Subsequent analysis showed good binding of the macrocyclic ring with the Ala^{156} methyl group, which formed a nice doughnut-shaped crown by encircling the methyl group and strong interaction into the S4 pocket. Surprisingly, this study also revealed the formation of a covalent bond between the inhibitor P1 carboxylamide carbonyl and the Ser^{139} hydroxyl of the protease rather than by the hemiketal oxygen atom. Instead, the hemiketal oxygen was interacting with and stabilized by a catalytic histidine residue (His^{57}).

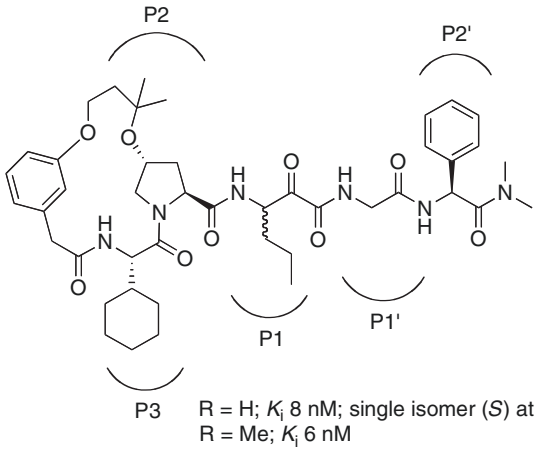


Fig. 2 Structure two Schering–Plough compounds with binding affinities for HCV

3.2 Molecular Modeling, Small Molecule Docking, and Computational Analysis

A recent molecular modeling study involving the HCV NS5B RNA-dependent RNA polymerase investigated its strong substrate specificity for RNA (Kim and Chong 2006b). Given this specificity, it may not be so surprising that HCV NS5B is not inhibited by most known inhibitors of DNA dependent DNA polymerases or reverse transcriptases. The authors state that "HCV polymerase has not been successfully inhibited by nucleoside analogs due to its strong substrate specificity for RNA." However, this conclusion fails to take into account the higher intracellular concentrations of rNTP relative to dNTP (Traut 1994) as discussed earlier.

The goal of this modeling study was to determine the specific interaction of the 2′-OH group of the RNA substrate and the active site residues to better understand the RNA-specificity. The study started from the three dimensional crystal structures of HIV-1 RT and HCV NS5B polymerase. Comparing the active site sequence alignment of HIV-1 RT and HCV NS5B showed that the "steric gate" of HIV-1 RT (Tyr^{115}) corresponds to Asp^{225} in HCV NS5B, but the small side chain of Asp^{225} provides an open space at the active site big enough to accommodate the ribose sugar moiety of a RNA substrate. In HCV NS5B, the side chain carboxylate of Asp^{225} was found to be in close proximity to the 3′-OH of a rNTP. A similar hydrogen bonding interaction occurs between the amide backbone of Tyr^{115} and the 3′-OH of NTP in HIV-1 RT. No specific interaction with an active site residue could be found close to the 2′-OH group of a rNTP. The researchers proposed an interaction with the enzyme by bridging water molecules inside the active site. The water molecules could act either as a hydrogen bond donor or acceptor to the 2′-OH group of the RNA substrate. If proven correct, the previously unknown 2′-OH binding pocket at the active site of RdRp could provide invaluable information for the development of novel anti-HCV nucleoside analogs.

Scientists at Merck constructed a binding model, based on structures of the NS5B protein, to understand the mechanism of inhibition within a series of dihydroxypyrimidines that act as pyrophosphate mimics in the NS5B active site (Fig. 3) (Koch et al. 2006). The binding of a triphosphate of a cocrystallized nucleotide with NS5B has been shown crystallographically to be mediated in the active site by two Mg ions. In other polymerases, during elongation, one Mg ion chelates the primer ribose hydroxy group and the alpha-phosphate of the incoming nucleotide. The second Mg ion is bound between the beta- and gamma-phosphate of the incoming nucleotide triphosphate and is cleaved off with the pyrophosphate after addition of this nucleotide to the primer.

The pyrimidine core of Merck's pyrophosphate mimic series is proposed to interact with the Mg ion chelating the beta- and gamma-phosphate groups of the triphosphate (Fig. 3). A binding model of the pyrimidine bound to the Mg ion in the crystal structure of NS5B was generated from the Mg–oxygen interaction geometry from crystallographic information. The model suggests the 2-thiophene of the pyrimidine to be stacked against Arg^{158} with mainly lipophilic interactions. In the crystal

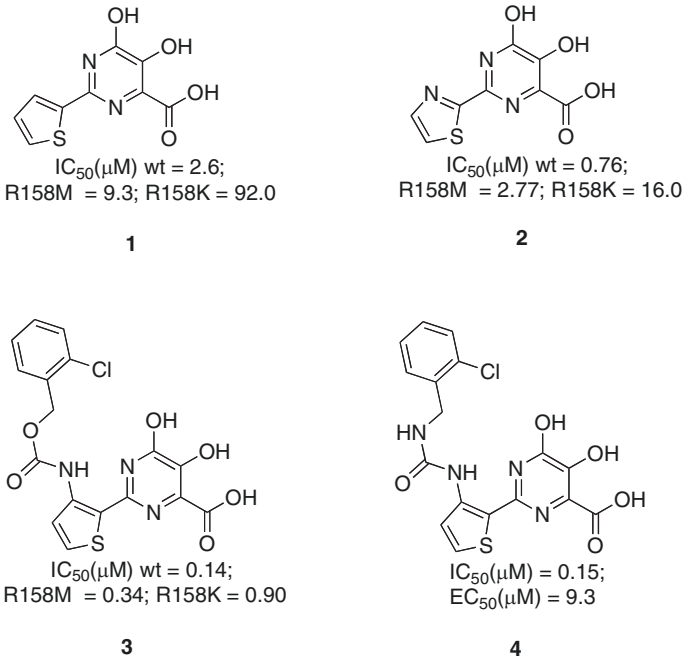


Fig. 3 Activity of **1, 2**, and **3** wild type HCV NS5B and site-directed mutations. Binding and replicon activity of **4**

structure with GTP, Arg^{158} seems to be interacting lipophilically with the base of the incoming nucleoside triphosphate and forms hydrogen bonds to the alpha-phosphate, and most likely plays a critical role in activating the alpha-phosphate for nucleophilic attack by the 2′-OH of the primer. Two mutations of Arg^{158} were prepared to test the importance of these interactions. It was suggested that mutation into Met, a more lipophilic amino acid without a charge but with similar size, should maintain the affinity of the pyrimidine. Mutation into the more polar Lys would maintain a positive charge while decreasing the capability to interact lipophilically with the thiophene. A decrease of inhibition for pyrimidines **1, 2**, and **3** for both mutations was observed upon testing (Fig. 3). The data suggested that the lipophilic component of the interaction between Arg^{158} and 2-thiophene is particularly important for thiophene **1**, as the decrease in potency is significantly larger for the Lys mutation (35-fold) than for the Met (threefold). The same kind of behavior is observed for thiazoles **2** and **3** each with a potency enhancing side-chain. Here the loss in potency on the Arg^{158}Lys mutant is less severe (sixfold), but still larger than on the Arg^{158}Met mutant (twofold).

The K_m for the substrate UTP has been measured and does not show significant differences between wt and mutant enzymes. The model shows that the space available to substituents in positions 4 and 5 of the thiophene is limited, in agreement with SAR studies. Interaction with a number of basic and lipophilic residues bound

in a cavity by some lipophilic substituents in the 3-position is responsible for the increase in potency. Urea **4** emerged from these studies as one of the most potent compounds, which also inhibited viral RNA generation in a cell-based HCV replicon assay. The dihydroxypyrimidine class of inhibitors appears to be a promising starting point for future development of drugs that could be utilized to combat HCV.

A broader docking study of the pyrophosphate mimic binding site was undertaken utilizing 88 different aryl $\alpha-\gamma$-diketo acids (ADK) (Kim and Chong 2006a). Pharmacophore-guided docking (FlexX-SYBYL 7.2) study of ADK molecules revealed two binding sites: a hydrophobic pocket and a hydrophobic groove that has excellent three-dimensional arrangements to accommodate substituted aromatic rings. The pocket is located around Leu^{159} while the hydrophobic groove is located at the opposite side of the pocket and becomes hollow at the end of a channel that provides binding sites for bulky atoms such as chlorine or bromine. Additionally, the Mg binding site in the polypeptide chain of HCV RdRp discussed earlier was also found to be critical. A nice correlation was observed in that the ADK, with potent antiviral activity and highly substituted aromatic rings, mapped well onto the hydrophobic binding sites to reinforce the hydrophobic interaction. Conversely, the lack of hydrophobic binding with ADK that were devoid of critical aromatic substitution coincided with compounds less potent toward HCV RdRp.

4 Medicinal Chemistry Approaches and the Role of Quantitative Structure Activity Relationships

4.1 One at a Time Traditional Synthesis

Because of the complexities associated with the synthesis of nucleoside analogs as active site inhibitors of NS5B polymerase, parallel and combinatorial techniques have made few inroads into these drug discovery programs. Traditional one at a time synthesis techniques are still the mainstay of nucleoside analog antiviral research. In this section, we provide one recent example of a discovery program relying on traditional synthetic methods to prepare nucleoside analog inhibitors of HCV replication. The most important nucleoside analogs currently in late development or clinical trials for treatment of HCV are shown in Fig. 4. Studies have demonstrated that the 2′-Me compounds function as chain terminators due to steric clashes with incoming nucleosides.

The Merck compound MK-0608 is a 2′-C-Me-7-deaza-adenosine analog, which has recently been reported to show a 5.7 log drop in viral load in HCV-infected chimpanzees after dosing QD at 2 mg/kg (Olsen 2006). An efficient and practical process for preparing kilogram quantities has been described (Bio et al. 2004). The 12-step synthesis provides an impressive 35% overall yield and starts from the inexpensive diacetone-D-glucose. The synthesis features a novel acyl migration in route to prepare the key crystalline furanose diol intermediate (Fig. 5). The conditions

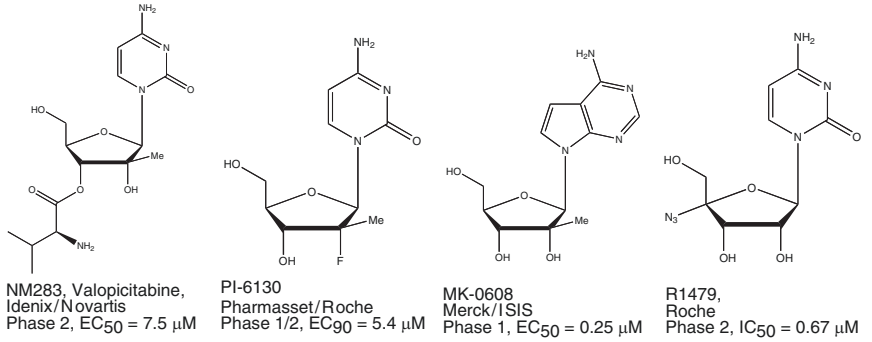


Fig. 4 Important nucleoside analogs that have undergone clinical trials for treatment of HCV. Both PSI-6130 (R7128) and R1479 were administered to humans as prodrugs

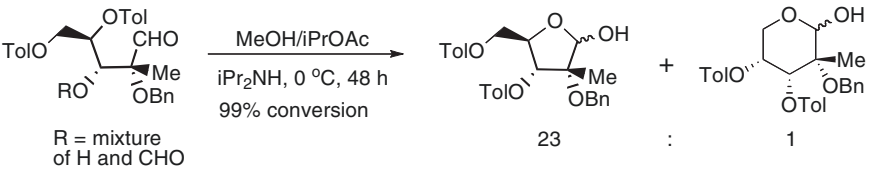


Fig. 5 Selective acyl migration and furanose formation

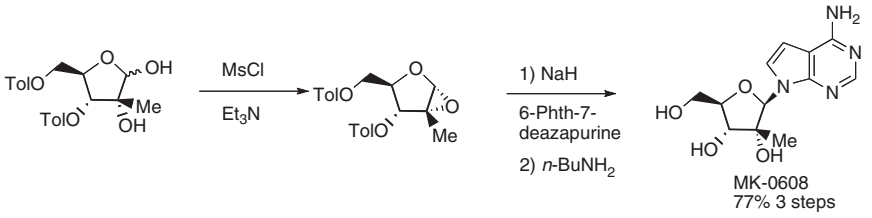


Fig. 6 Highly β-selective glycosidation to couple the C-2 branched furanose epoxide with the 7-deazapurine

required promoting acyl migration and formation of the furanose rather than the corresponding pyranose. Much experimentation resulted in the use of diisopropyl amine in methanol/isopropyl acetate to give a 23:1 ratio of the desired furanose relative to the pyranose with 99% conversion.

A second key development in the synthesis of MK-0608 was the highly β-selective glycosylation to couple the C-2 branched furanose epoxide with the 7-deazapurine. Vorbrüggen glycosylation conditions did not provide any coupled product with the 7-deazapurine presumably due to the inability of silyl migration to the 7-C. The optimized coupling conditions involved the use of sodium hydride to generate the anion of 6-phthalimido-7-deazapurine that provides a β-selective opening of an 2-β-*C*-methyl-1,2-α-anhydroribose to provide, after global deprotection, MK-0608 in 77% yield from the diol furanose (Fig. 6).

4.2 Parallel Synthesis

The use of parallel synthesis techniques in both solution and solid phase has become ubiquitous in the drug discovery process. One recent example that applies these techniques to HCV targets will be discussed. Random evaluation of compound libraries at Valient Pharmaceuticals produced a hit compound with an IC_{50} of 27 μM against HCV NS5B polymerase (Ding et al. 2006). Using the parallel synthesis strategy outlined in Fig. 7, a series of derivatives was synthesized.

A cyclo-condensation approach was used to prepare the oxopyrimidine core from substituted aldehydes, thiourea, and ethyl cyanoacetate. A subsequent sulfur alkylation, mostly with benzyl halides, provided the final compounds. After evaluating these compounds for their anti-HCV activity, it was found that all compounds containing substitutions on the oxopyrimidine ring nitrogen had $IC_{50} > 100$ μM. None of the compounds possessed a significant increase in activity toward NS5B, with the best compound active at 3.8 μM. Better success was realized with a pteridine series of compounds (Fig. 8) (Ding et al. 2005). The initial pteridine hit, again identified from screening compound libraries, had an IC_{50} of 15 μM in an NS5B polymerase assay. SAR studies focused on different substituents at the 6- and 7-positions (R groups in Fig. 4) and substitutions at the 4-position that were conducted in parallel fashion by displacement of the 4-chloropteridine. It was found that NH or OH at the 4-position is critical for the inhibitory activity. The most active compound identified (Fig. 4, $R = 4-F-\text{Ph}$) had an IC_{50} of 0.5 μM in the NS5B polymerase assay. However, this most promising compound, when tested in a replicon assay, had a disappointing EC_{50} of 90 μM – most likely due to poor cell penetration.

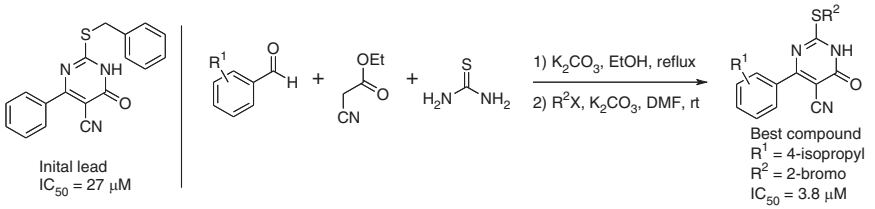


Fig. 7 An efficient cyclocondensation approach to prepare oxopyrimidines

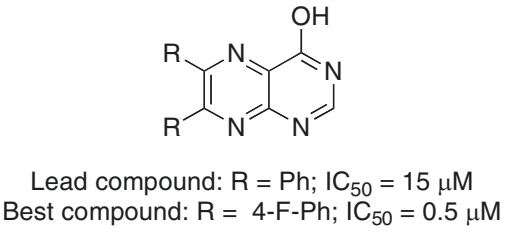


Fig. 8 Pteridine series of NS5B RdRp active compounds

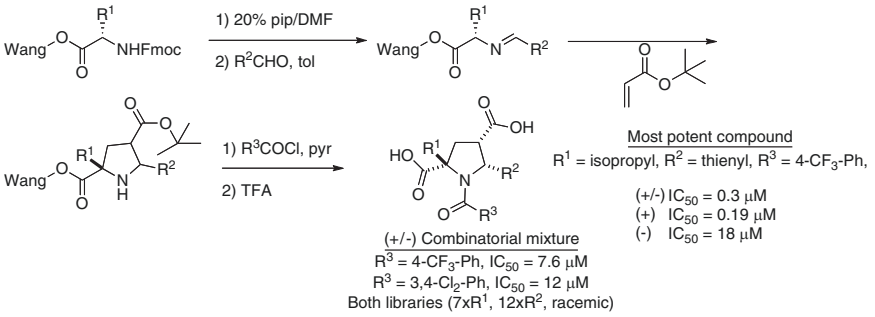


Fig. 9 A combinatorial library mixture of *N*-acylpyrrolidines with modest HCV NS5B activity

4.3 Combinatorial Chemistry

Over the last 10 years, the practice of synthesizing and screening mixtures of compounds has lost interest as a means to identify new leads in drug discovery, mainly due to problems associated with deconvolution, variable yields of components, purity, and inability to identify the source of activity.

The recent example of the identification of a small molecule inhibitor for HCV NS5B from a combinatorial mixture is an indication that these techniques still have value to the drug discovery process (Burton et al. 2005). High-throughput screening of the GlaxoSmith–Kline compound collection using a NS5B polymerase assay identified two racemic *N*-benzoyl pyrrolidine libraries. Both libraries were present as a mixture of 168 components ($7 \times R^1$, $12 \times R^2$, and racemic) and originated from a combinatorial $[3+2]$ cycloaddition reaction of *t*-butyl acrylate, a series of aromatic aldehydes, and resin bound amino acid esters followed by *N*-acylation (Fig. 9). Enumeration of the library using automated solid phase synthesis and purification techniques led to the synthesis of a 0.3 μM NS5B inhibitor. In addition, only one enantiomer of this racemate was found to possess significant NS5B activity (Fig. 9).

4.4 High-Throughput Screening for the Identification of New HCV Leads

Scientists at Ibis Therapeutics (A Division of Isis Pharmaceuticals Inc.) recently reported a new class of small molecules that bind the HCV RNA internal ribosome entry site (IRES) IIA subdomain with sub-micromolar affinity. The IRES mediates the initiation of viral-RNA translation and represents a novel drug target for inhibiting HCV replication. However, targeting RNA specifically and selectively with small molecules has met with much difficulty. The benzimidazole hit (Fig. 10) with a K_D of approximately 100 μM to a 29-mer RNA model of domain IIA was identified from a 180,000-member library using mass spectrometry-based screening

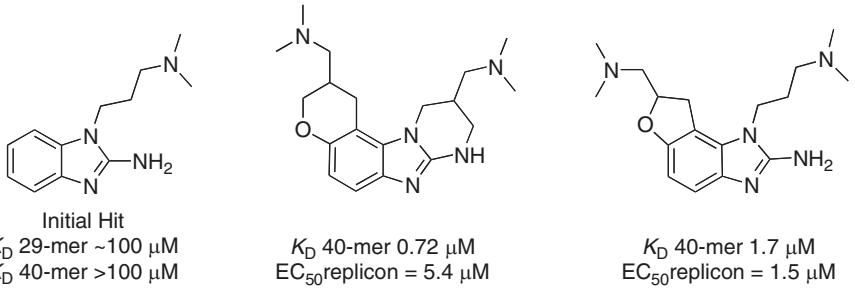


Fig. 10 Screening hit and higher affinity benzimidazoles for HCV RNA internal ribosome entry site (IRES) IIA subdomain

methods. Hits were identified via mass spectrometry as follows: (1) A mixture of the RNA substructure and the potential small-molecule ligands were introduced into the mass spectrometer by electrospray ionization; (2) Ligands that bound to the target were identified by mass shifts; (3) The observed mass equals the mass of the target plus the mass of the ligand. This technique allows one to screen multiple ligands and even multiple targets in a single assay as long as they have different masses. Benzimidazole derivatives with submicromolar binding affinity for the IIA RNA construct were discovered via MS-assisted SAR studies. The optimized benzimidazoles (Fig. 10) reduced viral RNA in a cellular HCV replicon assay and showed minimal toxicity ($CC_{50} > 100\mu M$) against Huh-7 cells in an MTT assay.

5 Cell-Based Assays to Predict Toxicity and Resistance Aspects

Before an antiviral agent becomes a drug, advanced toxicity testing, pharmacological combination, and drug-interaction studies are needed. The use of new cell-based assays that can predict mitochondrial toxicity, lactic acidosis, peripheral neuropathy, anemia, hypersensitivity, lipodystrophy, and other potential side effects can alleviate these issues (Stuyver et al. 2002).

Mitochondria-associated toxicities, such as pancreatitis, are frequently demonstrated in HIV/HCV-coinfected individuals, and may significantly influence treatment options (de Mendoza and Soriano 2005). Yet, no cell culture or animal models have been developed to predict nucleoside-induced pancreatitis. Nevertheless, an association of HCV replication and mitochondrial DNA depletion in primary human lymphocytes obtained from HIV/HCV-coinfected individuals under concomitant administration of HCV and HIV medications was demonstrated by de Mendoza and coworkers (de Mendoza et al. 2007). They claimed that the use of HCV medication together with certain antiretroviral agents seemed to enhance mitochondrial damage due to a synergistic deleterious interaction between the anti-HCV and anti-HIV drugs. In contrast, an improvement in mitochondrial content with effective

anti-HCV therapy was also confirmed in this report, and these findings may be valuable for hepatitis B infection as well (de Mendoza et al. 2007).

Testing in cell-based assays has led to the discovery of numerous anti-HCV hits and leads. For example, a base modified ribonucleoside analog called β-D-*N*-hydroxycytidine (NHC) was shown to inhibit HCV RNA replication in culture, but had a low oral bioavailability in non-human primates while metabolizing into two natural nucleosides (Stuyver et al. 2003a, b). More biologically stable nucleosides with improved pharmacological profiles are being evaluated, including a potent and highly selective nucleoside analogs [PSI-6130 (a prodrug R7128, is in clinical trials)] with anti-HCV activity in replicon systems. (Carroll and Olsen 2006; LJ et al. 2006; Murakami et al. 2007; Pierra et al. 2005; Stauber and Stadlbauer 2006, Stuyver et al. 2006a, b)

BILN-2061 (Boehringer Ingelheim) was one of the first compounds identified in the replicon system that was clinically tested (in Phase I studies, it reduced HCV-RNA 2–3 $\log_{10}$ in most patients infected with genotype 1), but trials were put indefinitely on hold due to cardiac toxicity issues. Other examples of NS3 protease inhibitors, including VX-950 (Vertex) and SCH-503034, are in Phase 2 trials (PEG-IFN combination studies) and have demonstrated potent antiviral effects (Lin et al. 2006; Neyts 2006; Reesink et al. 2006). Using the replicon system, the selection of HCV resistant mutants have been reported for BILN 2061 (at 156 and 168), VX-950 (at 156), and SCH 503034 (at 156 and 170). These results suggest that resistance studies in vitro might predict what to expect in future clinical trials (Lin et al. 2004; Tong et al. 2006) (for more details see chapter by Anderson et al., this volume).

6 Pharmacokinetic and Pharmacodynamic Aspects of Drug Development of Agents for the Treatment of HCV Infections

6.1 In Vitro Evaluation

The initial screening and early toxicity studies of novel agents to treat viral hepatitis are usually performed using in vitro assays. Use of in vitro (cell culture) and enzymatic assays to screen for and measure potency (determination of median effective concentration, or 90% effective concentration; EC_{50} and EC_{90}, respectively) at the intended site of action of the compound may include viral polymerase (nucleoside analogs, NA) protease (viral protease inhibitors) or cellular targets (interferons stimulate T and B cells of the immune system). Toxicity studies are conducted against a panel of cell lines or primary cells (e.g., human bone marrow cells) to determine cellular sensitivities (IC_{50}, IC_{90}). Compounds with desirable therapeutic index (high IC_{50}/EC_{50}) ratio are considered candidates for further in vivo studies in animal models.

6.2 Preclinical In Vivo Testing

Preclinical pharmacokinetic studies are conducted in appropriate animal species to model the relationship between plasma and target tissue concentrations vs. time and dosage regimen. Interspecies scaling of pharmacokinetic parameters may be performed to predict pharmacokinetic parameters in humans (Patel et al. 1990). However, estimates for interspecies scaling may not always be reliable, especially for compounds that are activated or metabolized by enzymes that vary significantly between species (Hurwitz et al. 2005). The pharmacokinetic model could also be used to design a dose regimen that produces the desired tissue concentration of the biologically active form of the compound (usually based on the EC_{50} and IC_{50} data derived in vitro) for the desired length of time in an appropriate animal model, for example, woodchucks for hepatitis B (Hurwitz et al. 1998, 2002) or immunocompromised mice bearing liver xenografts for hepatitis C (Feitelson and Larkin 2001). Since hepatitis B and C regimens are prolonged (months for hepatitis C, chronic for B), long-term toxicity studies are warranted to evaluate for toxicities that may occur during extended use.

7 Physiological Factors that Influence Drug Delivery for HCV Drugs

The oral bioavailability of drugs may be limited by the compound's stability in the acidic pH of the stomach, the presence of food, enzymes secreted into the lumen of the intestine, its lipophilicity, affinity for uptake proteins embedded within the villi on the surface cells of the small intestine (Patil et al. 1998), and peptide transporters (Balimane and Sinko 1999; Landowski et al. 2003). Furthermore, some compounds are actively extruded (in an ATP dependent manner) from cells lining the intestine into the lumen, by unidirectional transport proteins, such as P-glycoprotein (P-gp), or may undergo intestinal metabolism by enzymes like the cytochrome P450 3A (Cyp3A) enzymes that are located in high concentrations on the intestinal villi (Benet et al. 2004; Frassetto et al. 2003; Wacher et al. 1998). High levels of CYP3A enzymes are also present in the endoplasmic reticulum of hepatocytes (Soars et al. 2006), which are the primary substrates for HCV infection. The microstructure of the healthy liver resembles overlaying plates of hepatocytes surrounding a network of sinusoids (Marieb 2007; Han et al. 2002). The duration of exposure of drugs to the hepatocytes may be limited by susceptibility to drug metabolizing enzymes, since hepatocytes are the major site where drug metabolism occurs. Furthermore, drug exposure in hepatocytes may also be limited by the various unidirectional active transport (ATP dependent) proteins, including P-gp, BCRP\ABCG2, and MRP2 (Castell et al. 2006; Chandra and Brouwer 2004; Elferink and Groen 2002; Gomez-Lechon et al. 2004; Leslie et al. 2005; Pauli-Magnus and Meier 2006; Vermeir et al. 2005).

Although nucleoside analogs are not substrates for P-gp or CYP3A4, most protease inhibitors and NNI are substrates for both the P-gp efflux pump (Aungst 1999;

Storch et al. 2007) and CYP3A 4 metabolism (Sagir et al. 2003; Zhou et al. 2005). The HIV protease inhibitors ritonavir and atazanavir are potent inhibitors of both P-gp and CYP3A4. Therefore pharmacokinetic "boosting" has been used as a strategy in HIV therapy and may be feasible for HCV protease inhibitors for increasing peak plasma concentrations (C_{max}), reducing the rate of elimination (longer $t_{1/2}$), increasing exposure (AUC), and lowering the frequency of dosing needed to maintain sufficient drug levels (Cooper et al. 2003; King et al. 2004; Moyle and Back 2001). However, administration of other drugs with protease "boosting" regimens can result in complex drug interactions, which may require dose modification or drug replacement of either the protease inhibitor or the other drug. Various studies demonstrated the value of developing reliable assays for in vivo pharmacokinetic phenotyping of drug metabolic profiles when using drug combinations (Gerber et al. 2007). Furthermore, the reduction in the activities of drug metabolizing enzymes and active transporters may need to be considered when designing doses for individuals with severe liver disease.

When drugs enter and are distributed into systemic circulation, they partition between various cellular compartments according to their relative affinities for potential binding partners. The initial binding sites of drugs are serum proteins, mainly albumin and alpha-1-acid glycoprotein (AAG) and globulins (Boffito et al. 2003; Herve et al. 1994; Sheppard and Bouska 2005). Drugs that bind protein either covalently or noncovalently with high affinity and a slow rate of dissociation (restrictive binding), usually have small distribution volumes that may be similar to that of the serum compartment. However, some agents may demonstrate nonrestrictive (permissive) binding, characterized by high protein-bound fraction at equilibrium, but accessible to other binding sites as a result of rapid dissociation rates from the protein. Permissive binding often results from several low-affinity interactions with multiple sites on albumin, rather than single high affinity interactions (Herve et al. 1994; Sheppard and Bouska 2005). Experiments conducted in cell culture demonstrated a decrease in the cellular uptake, accumulation, and antiviral potency of anti-HIV protease inhibitors in the presence of physiological concentrations of AAG. This suggests that only the unbound fraction of protease inhibitors in the extracellular fluid is capable of entering the cells by passive diffusion where they exert antiviral effects (Bilello et al. 1996; Jones et al. 2001). Certain drugs bound to certain large molecular weight moieties (e.g., albumin bound interferon) may enter cells through endocytosis. Therefore, binding affinities of drugs to serum proteins and other potential binding partners have to be taken into account when designing new antiviral strategies.

8 Conclusions

Hepatitis C infection has an unpredictable natural history with significant potential for causing severe liver disease and variable response to current therapy based on pretreatment factors. Therefore, HCV is an excellent model to describe the

approaches for the development of antiviral compounds. As we learn more about HCV molecular biology and pathobiology, newer therapies will continue to be developed. Many drugs have already failed in phase 1 or 2 because they lacked potency or showed significant toxicities (gastrointestinal, liver, bone marrow). For example, potency has been an issue with certain nonnucleoside inhibitors of HCV polymerase, as two such compounds have stumbled in the clinic because of efficacy problems. In 2004, Rigel Pharmaceuticals Inc. disclosed that its R803 did not give a significant reduction in viral levels in a Phase 1/2 trial. In June 2007, XTL Biopharmaceuticals Ltd. discontinued development of its XTL-2125 after the compound did not significantly lower viral load vs. placebo in a Phase 1 study. Not much is known about why ViroPharma Inc.'s HCV-796 caused elevations in liver enzymes in some patients who received the nonnucleoside HCV polymerase inhibitor for at least 8 weeks in a Phase 2 trial. Idenix NM-283 was recently discontinued during a phase 2 trial because of gastrointestinal problems associated with this prodrug. Similarly, Roche's R-1626 nucleoside polymerase inhibitor was also discontinued due to bone marrow effects. It is important to understand why drugs fail in the clinic and if there is a scientific or chemical modification that can be made to reduce the adverse untoward effects.

Accordingly, many lessons have been learned and extensive knowledge has been built from these failures. Newer protease and polymerase inhibitors have emerged as potent, specifically targeted therapies against HCV infection, but will need to be used in combination with interferon and probably ribavirin to minimize resistance and increase the sustained virological response. Their introduction into practice will add complexity to the treatment of HCV infection because of the potential development of resistance and drug–drug interactions with other medications. The proper timing for the introduction of these drugs relative to interferon and ribavirin therapy are factors that will need to be individualized according to patient's needs and viral kinetics, requiring specialized infectious disease physicians and gastroenterologists with intimate knowledge of how these compounds act in inhibiting viral replication to maximize their efficacy. In 2007, it became clear that add-on therapy to standard of care is the way to go. Valuable lessons have been garnered from the failures of numerous anti-HCV agents. The development of new agents will require clinical proof that they are safe and produce sustained virological responses in controlled clinical trials, leading eventually to high curative rates. We are closing the gap on this virus and with the knowledge accumulated several potential cures are within sight.

Acknowledgments This work was supported in part by NIH grants 5R37-AI-041980, 4R37-AI-025899, 1RO1-AI-071846, 1RO1-AI076535, 5P30-AI-50409 (CFAR), and the Department of Veterans Affairs. RFS is a major shareholder and founder of Idenix Pharmaceuticals, Pharmasset Inc., and RFS Pharma LLC.

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Antiviral Agents Acting as DNA or RNA Chain Terminators

E. De Clercq and J. Neyts

Contents

Abstract Nucleoside or nucleotide analogue inhibitors of viral replication almost act as chain terminators during DNA (DNA- and retroviruses) or RNA (RNA viruses) synthesis. Following intracellular phosphorylation, by viral and/or cellular kinases, the 5′-triphosphate metabolites (or 2′-diphosphate metabolites in the case of acyclic nucleoside phosphonate analogues) compete with the natural substrate in the DNA or RNA polymerization reaction. Obligatory chain terminators (e.g., acyclovir) do not offer the 3′-hydroxyl function at the riboside moiety of the molecule. Nucleoside analogues that possess a hydroxyl function at a position equivalent of the 3′-hydroxyl position may act as chain terminators if this hydroxyl group is conformationally constrained (e.g., ganciclovir) or sterically hindered to enter into a phosphodiester linkage with the incoming nucleotide. In case that the 3′-hydroxylgroup is correctly positioned, chain elongation may be hampered through steric hindrance from neighboring substituents (e.g., 2′-*C*-methyl or 4′-azido nucleoside inhibitors of HCV replication). Here, we review the molecular mechanism of action and the clinical applications of the nucleosides and nucleotides acting as chain terminators. A further discussion of clinical applications in combination therapy can be found in Chap. 12.

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H.-G. Kräusslich, R. Bartenschlager (eds.), *Antiviral Strategies,* Handbook of Experimental Pharmacology 189,

Abbreviations

TACV	Triacyclic analogue of acyclovir
TGCV	Triacyclic analogue of ganciclovir
ANP	Acyclic nucleoside phosphonate
TK	Thymidine kinase
HPMPA	(*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine
ACV	Acyclovir
GCV	Ganciclovir
TDF	Tenofovir disoproxil fumarate
HPMPC	Cidofovir, (S)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine
PMPA	Tenofovir, (*R*)-9-(2-phosphonylmethoxypropyl)adenine
PMEA	Adefovir, 9-(2-phosphonylmethoxyethyl)adenine
HDP	Hexadecyloxypropyl
ODP	Octadecyloxyethyl
ddN	2′,3′-Dideoxynucleoside
NRTIs	Nucleoside reverse transcriptase inhibitors
NtRTIs	Nucleotide reverse transcriptase inhibitors
NNRTIs	Nonnucleoside reverse transcriptase inhibitors
NRRIs	Nucleoside RNA replicase inhibitors
NNRRIs	Nonnucleoside RNA replicase inhibitors

1 Introduction

Most of the nucleoside (or nucleotide) analogues used as antiviral agents act by terminating elongation of the nucleic acid chain during DNA (DNA viruses and retroviruses) or RNA (RNA viruses) synthesis. To act as DNA or RNA chain terminators, these compounds have to be converted to their 5′-triphosphate form before they compete with the natural substrates (dNTPs for DNA synthesis and NTPs for RNA synthesis) in the DNA or RNA polymerization reaction. When incorporated in their 5′-monophosphate form, after removal of the pyrophosphate group, at the 3′-end of the growing nucleic acid chain, they terminate chain elongation. In essence, they do so if they do not offer the 3′-hydroxyl function at the (2′-deoxy)riboside moiety, which is required for attachment of the incoming nucleotide. The nucleotide analogues that act in this fashion can be termed obligatory chain terminators [typical examples are acyclovir (triphosphate) and adefovir (diphosphate)].

However, nucleotide analogues possessing an hydroxyl function at a position equivalent to the 3′-hydroxyl position may nevertheless act as chain terminator if this hydroxyl group is conformationally constrained [as in ganciclovir (triphosphate) or cidofovir (diphosphate)] or sterically hindered to enter into a phosphodiester linkage with the incoming nucleotide. But even if the 3′-hydroxyl group would be correctly positioned, further chain elongation may still be hampered through steric hindrance from neighboring substituents such as, for example, from the

2′-*C*-methyl group or 4′-*C*-azido group in nucleoside analogues targeting the hepatitis C virus (HCV) RNA polymerase NS5B.

Depending on their activity spectrum and structural characteristics, chain terminators can be divided into four categories: (1) acyclic (or carbocyclic) nucleoside analogues (prototype: acyclovir), which are primarily active against herpesviruses [HSV (herpes simplex virus), VZV (varicella-zoster virus), and CMV (cytomegalovirus)] (Fig. 1); (2) acyclic nucleoside phosphonates (prototypes: cidofovir, adefovir, and tenofovir), which are targeted at DNA viruses (cidofovir), hepadnaviruses (adefovir), and retroviruses (tenofovir) (Fig. 2); (3) 2′,3′-dideoxynucleoside analogues, which are active against retroviruses [immunodeficiency virus (HIV)] as well as hepadnaviruses [hepatitis B virus (HBV)] (Fig. 3); and (4) the anti-HBV (Fig. 4a) and anti-HCV agents (Fig. 4b). These different categories of chain terminators will be reviewed, essentially from two viewpoints: first, their clinical use, and second, their mechanism of action.

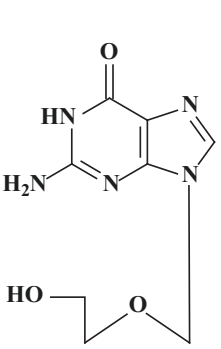
2 Acyclic (or Carbocyclic) Nucleoside Analogues

Of the acyclic nucleoside analogues (Fig. 1), acyclovir and its prodrug valaciclovir, ganciclovir and its prodrug valganciclovir, penciclovir and its prodrug famciclovir have been formally licensed for clinical use. Except for (val)ganciclovir, which can lead to bone marrow suppression (i.e., neutropenia), the acyclic nucleoside analogues are generally well tolerated with few, if any, side effects.

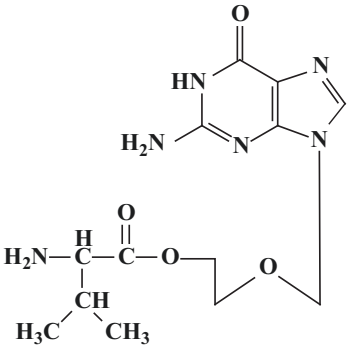
Acyclovir and valaciclovir (Fig. 1) are used in the treatment of mucosal, cutaneous and systemic HSV-1 and HSV-2 infections (including herpetic keratitis, herpetic encephalitis, genital herpes, neonatal herpes, and herpes labialis), and VZV infections (including varicella and herpes zoster). Acyclovir is administered orally at doses of 1 g $(5 \times 200\,\text{mg})$ per day for genital herpes, up to 4 g $(5 \times 800\,\text{mg})$ per day for herpes zoster; or topically as a 3% ophthalmic cream for herpetic keratitis or 5% cream for herpes labialis; or intravenously at 30 mg/kg $(3 \times 10\,\text{mg/kg})$ per day for herpetic encephalitis or other severe infections with herpesviruses. Valaciclovir is administered orally at 1 g $(2 \times 500\,\text{mg})$ per day for genital herpes and up to 3 g $(3 \times 1\,\text{g})$ per day for herpes zoster.

Ganciclovir and valganciclovir (Fig. 1) are used in the treatment of CMV infections (e.g., CMV retinitis) in immunosuppressed (e.g., AIDS) patients. Ganciclovir is administered intravenously at 10 mg/kg $(2 \times 5\,\text{mg/kg})$ per day for induction therapy. Valganciclovir is administered orally at 900 mg $(2 \times 450\,\text{mg})$ per day for maintenance therapy. Both valaciclovir and valganciclovir can also be used as prophylaxis of CMV infections via the oral route in transplant recipients.

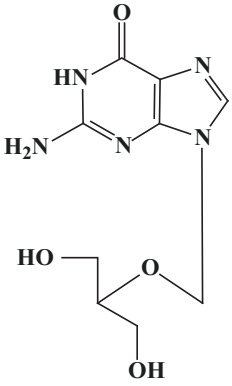
Penciclovir (Fig. 1) can be used topically (as a 1% cream) in the treatment of superficial mucocutaneous HSV infections. Its prodrug famciclovir (Fig. 1) is administered orally at 750 mg $(3 \times 250\,\text{mg})$ or 1,500 mg $(3 \times 500\,\text{mg})$ per day in the treatment of HSV-1 or HSV-2 infections and VZV infections (i.e., herpes zoster).



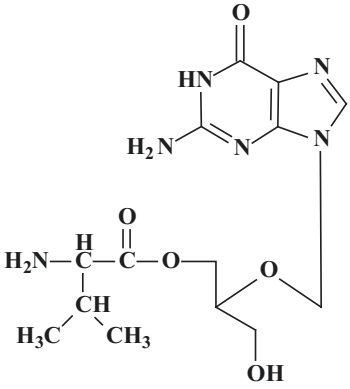
Acyclovir (ACV)
Zovirax®



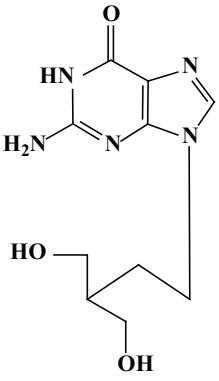
Valaciclovir (VACV)
Zelitrex®, Valtrex®



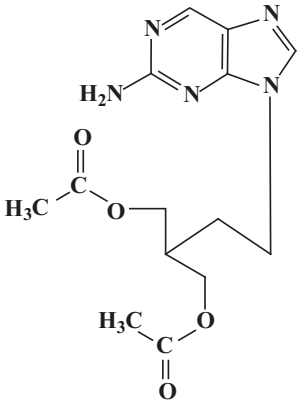
Ganciclovir
Cymevene®, Cytovene®



Valganciclovir
Valcyte®

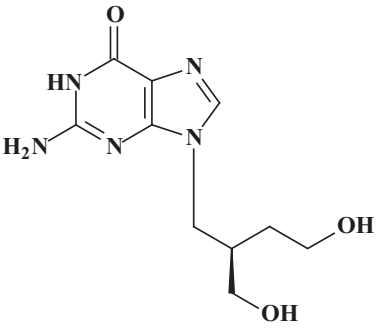


Penciclovir (PCV)
Denavir®, Vectavir®

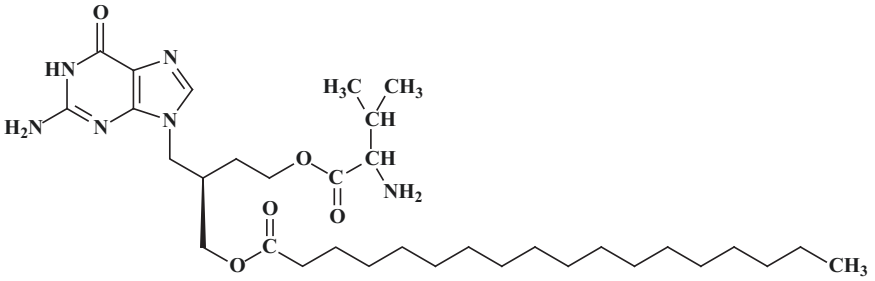


Famciclovir (FCV)
Famvir®

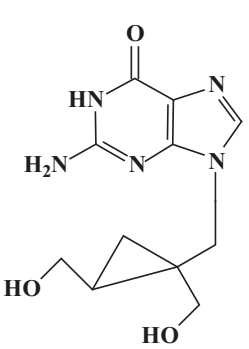
Fig. 1 Structural formulae of acyclic nucleoside analogues (anti-herpesvirus agents)



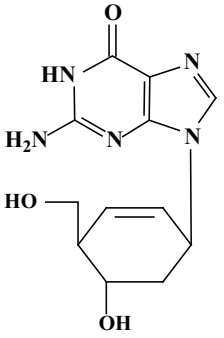
H2G
2HM-HBG



H2G prodrug
MIV-606
Valomaciclovir stearate

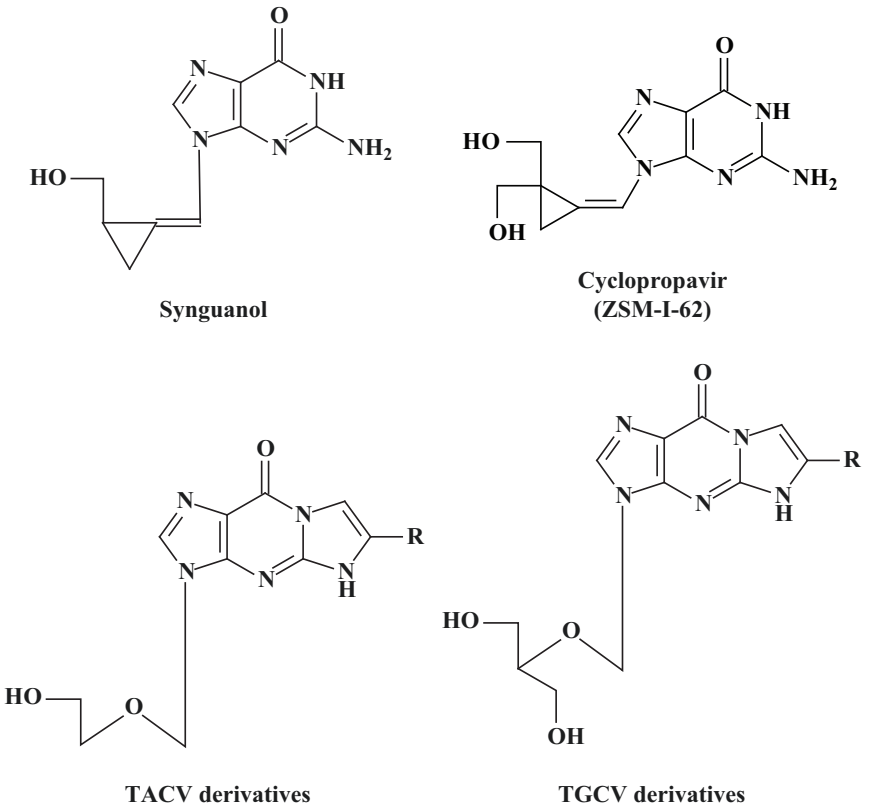


A-5021



Cyclohexenyl G

Fig. 1 (continued)



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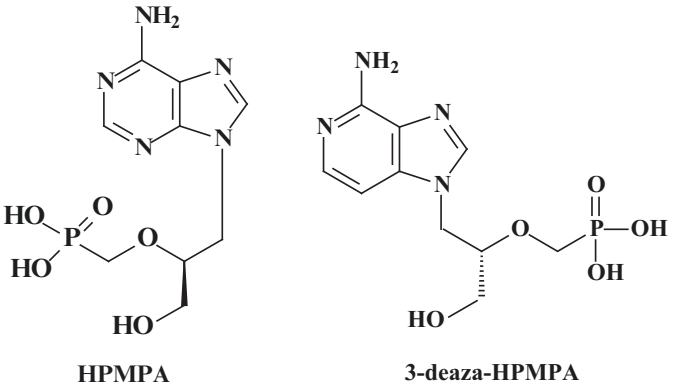
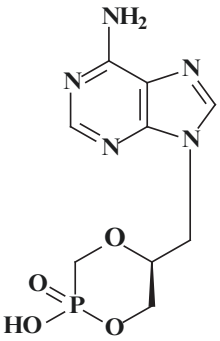
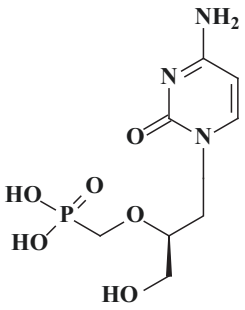


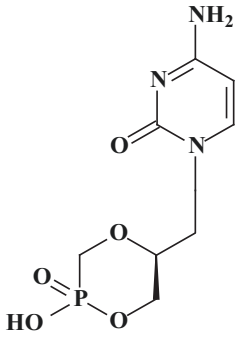
Fig. 2 Structural formulae of acyclic nucleoside phosphonates (anti-DNA virus and/or -retrovirus agents)



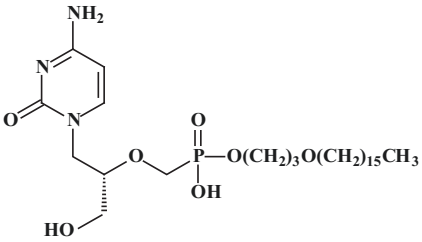
cHPMPA



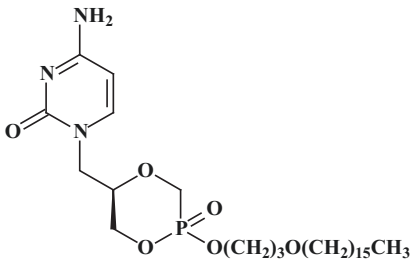
HPMPC
CDV



cHPMPC
cCDV

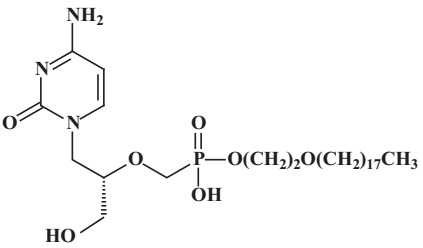


HDP-CDV
(CMX001)

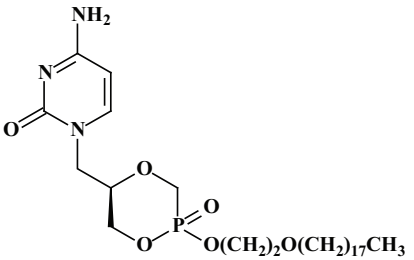


HDP-cCDV

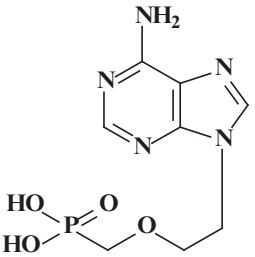
Fig. 2 (continued)



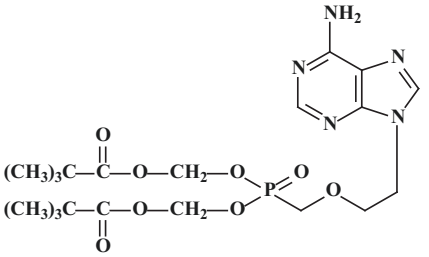
ODE-CDV



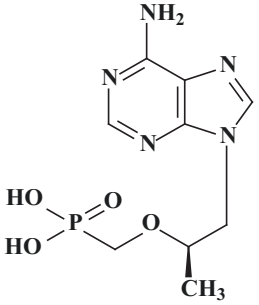
ODE-cCDV



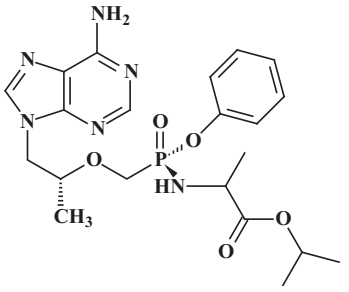
PMEA
Adefovir



Bis(POM)PMEA
Adefovir dipivoxil



PMPA
Tenofovir



GS 7340

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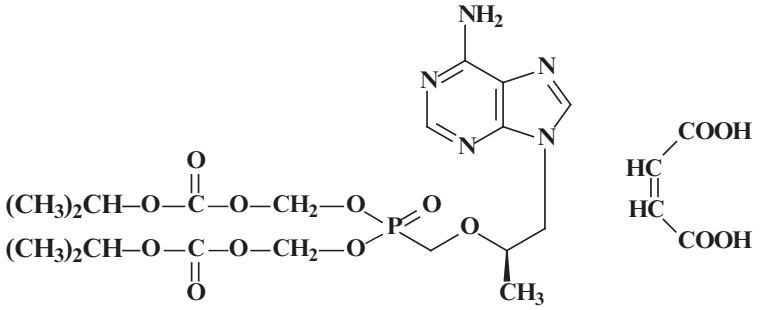
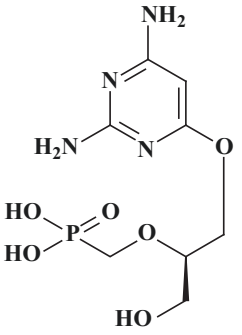
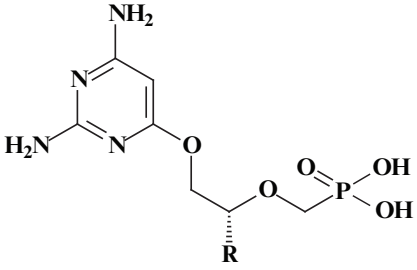


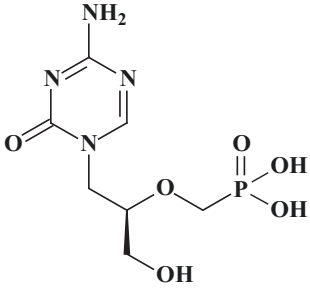
Bis(POC)PMPA fumarate
Tenofovir disoproxil fumarate



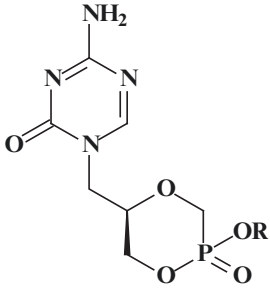
HPMPO-DAPy



R = CH3 : PMPO-DAPy
R = H : PMEO-DAPy

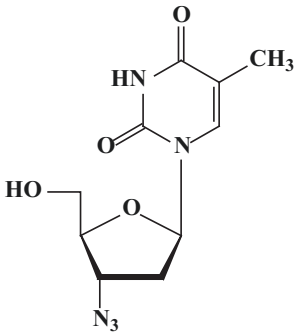


5-aza-HPMPC

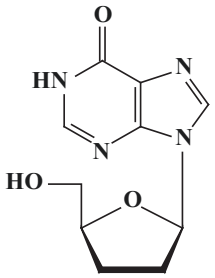


R = H: 5-aza-cHPMPC
R = Alkoxyalkyl: 5-Aza-cHPMPC prodrugs

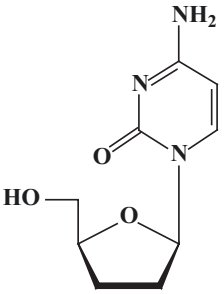
Fig. 2 (continued)



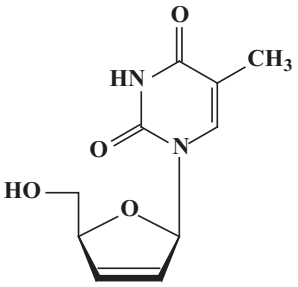
Zidovudine
3'-Azido-2',3'-dideoxythymidine,
azidothymidine (AZT)
Retrovir®



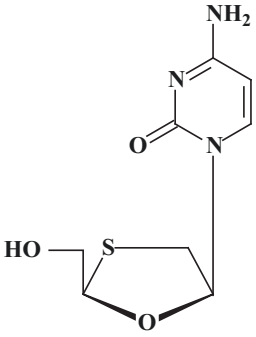
Didanosine
2',3'-Dideoxyinosine (ddI)
Videx®, Videx® EC



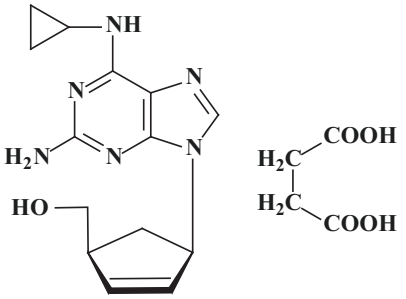
Zalcitabine
2',3'-Dideoxycytidine (ddC)
Hivid®



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

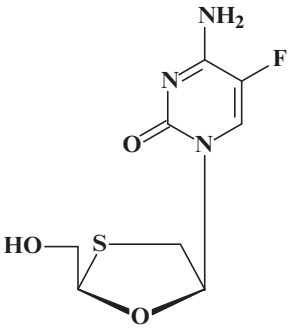


Lamivudine
2',3'-Dideoxy-3'-thiacytidine
3TC
Epivir®

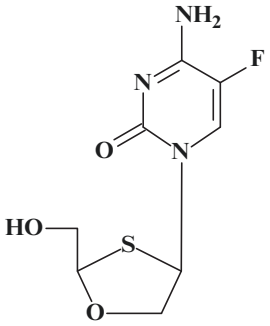


Abacavir
(1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-
9*H*-purin-9-yl]-
2-cyclopentene-1-methanol succinate
(ABC)
Ziagen®

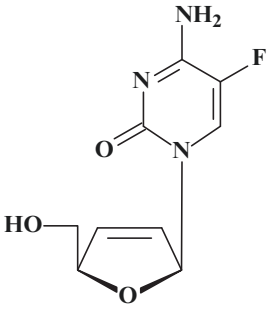
Fig. 3 Structural formulae of 2′,3′-dideoxynucleoside analogues (anti-HIV) agents



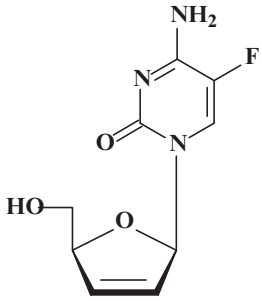
(-)-FTC
Emtricitabine
(-)-β-L-3'-thia-2',3'-dideoxy-5-fluorocytidine
Emtriva®



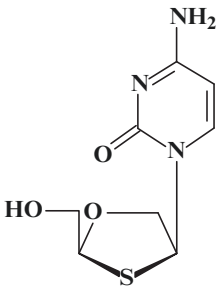
Racemic (±)FTC (FdOTC)
Racivir®



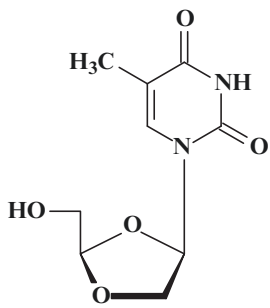
ACH-126443 (β-L-Fd4C)
Elvucitabine



DPC-817 (β-D-Fd4C)
Reverset® (RVT)
Dexelvucitabine

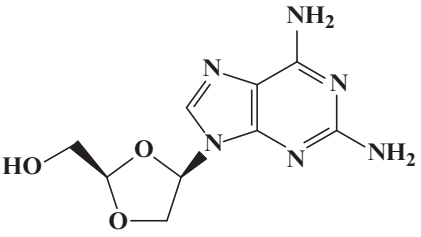


SPD-754, AVX-754 ((-)-dOTC)
Apricitabine

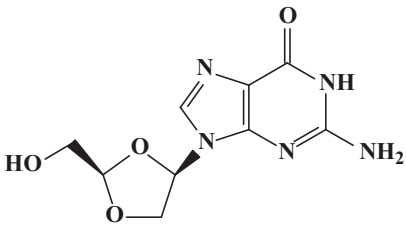


1-(β-D-dioxolane)thymine
DOT

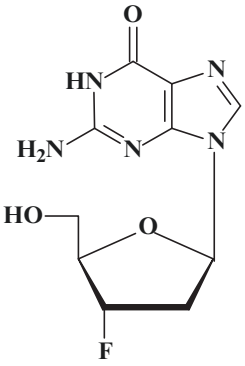
Fig. 3 (continued)



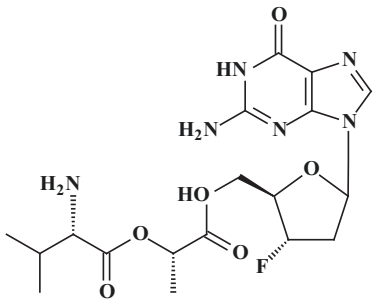
DAPD
Amdoxovir



DXG

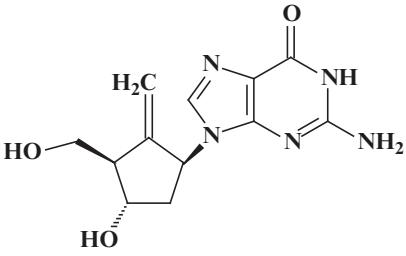


FLG
3'-Fluoro-2',3'-dideoxyguanosine

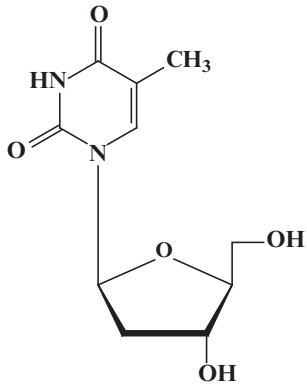


MIV-210
[FLG (3'-fluoro-2',3'-dideoxyguanosine) prodrug]

Fig. 3 (continued)

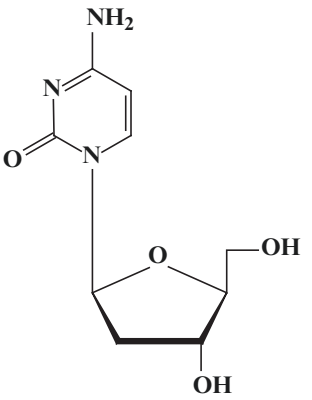


Entecavir
Baraclude®

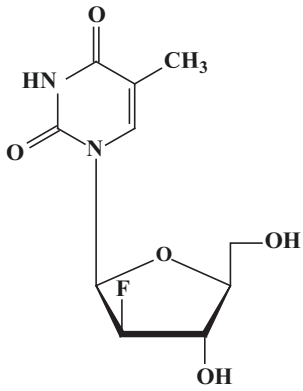


L-dT
β-L-thymidine
Telbivudine
Tyzeka®, Sebivo®

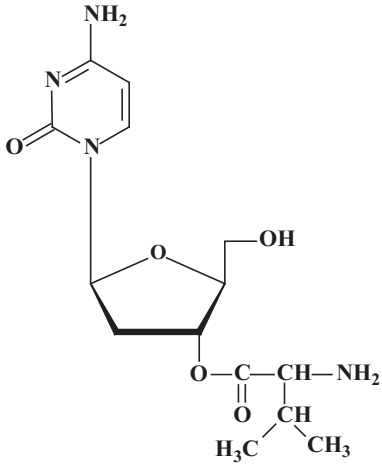
Fig. 4a Structural formulae of anti-hepatitis B agents



L-dC
β-L-2'-deoxycytidine



L-FMAU
Clevudine

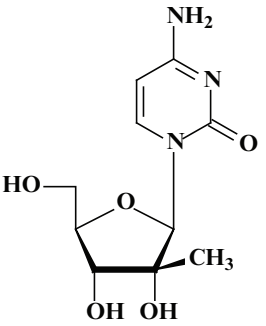


3'-Val-L-dC
3'-Valine ester of β-L-2'-deoxycytidine
Valtorcitabine

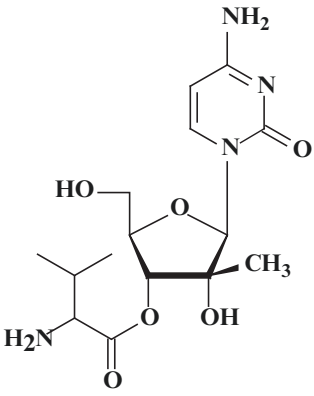
Fig. 4a (continued)

With acyclovir, valaciclovir, ganciclovir, valganciclovir, and famciclovir being on the market (De Clercq 2004a), the only other acyclic guanosine analogue under clinical development for the potential treatment of HSV and VZV infections is MIV-606 (valomaciclovir stearate), the oral prodrug of H2G (Fig. 1) (De Clercq and Field 2006). This compound is being pursued primarily for the treatment of herpes zoster.

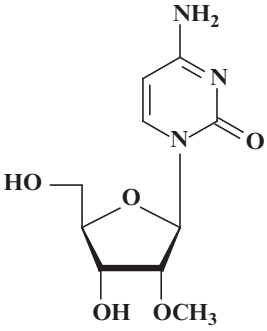
Carbocyclic guanosine analogues have been described to inhibit HSV-1, HSV-2, VZV, CMV, and/or other herpesviruses [viz. HHV-6 (human herpesvirus type 6)],



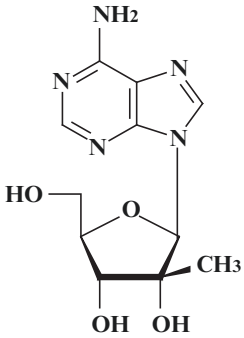
2'-*C*-methylcytidine



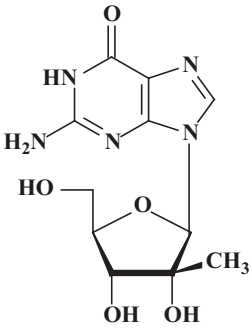
NM283
3'-Valine ester of 2'-*C*-methylcytidine
Valopicitabine



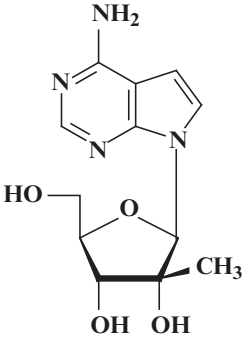
2'-*O*-methylcytidine



2'-*C*-methyladenosine

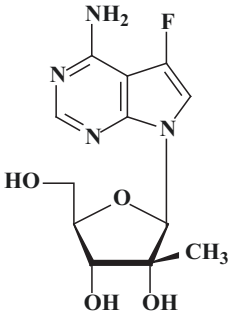


2'-*C*-methylguanosine

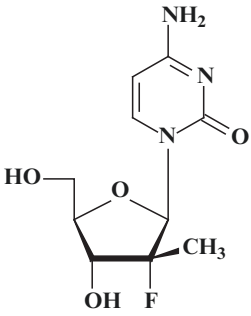


7-deaza-2'-*C*-methyladenosine

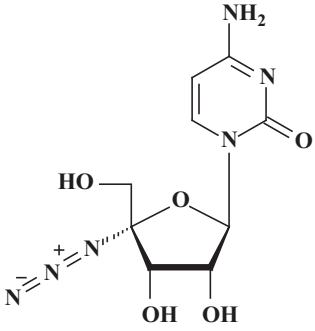
Fig. 4b Structural formulae of anti-hepatitis C agents



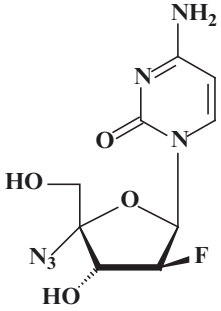
7-Deaza-7-fluoro-2'-*C*-methyladenosine



2'-Deoxy-2'-fluoro-2'-*C*-methylcytidine
PSI-6130



4'-Azidocytidine
(R1479)



RO-0622

Fig. 4b (continued)

including A-5021, cyclohexenylguanine, synguanol (De Clercq et al. 2001), and cyclopropavir (Zhou et al. 2004; Kern et al. 2005) (Fig. 1). The latter compound, cyclopropavir, has been reported to be very effective in reducing mortality in mice infected with murine CMV (Kern et al. 2004).

The acyclic guanosine analogues acyclovir (ACV) and ganciclovir (GCV) have been further modified by introduction of a 1,N^2-ethene-1,2-diyl bridge to form their tricyclic analogues TACV and TGCV (Fig. 1) (Golankiewicz et al. 1994, 2001; Ostrowski et al. 2006). These tricyclic analogues exhibited marked activity against HSV-1, HSV-2, and VZV, some of their six-substituted derivatives being intrinsically fluorescent.

All acyclic and carbocyclic guanosine analogues depicted in Fig. 1 follow the same *modus operandi* as exemplified for acyclovir (ACV) in Fig. 5, in that they need three phosphorylations to be converted to their active metabolite, the triphosphate form, which then interacts with the target enzyme, the viral DNA polymerase, as a chain terminator (De Clercq 2002). In its DNA chain-terminating

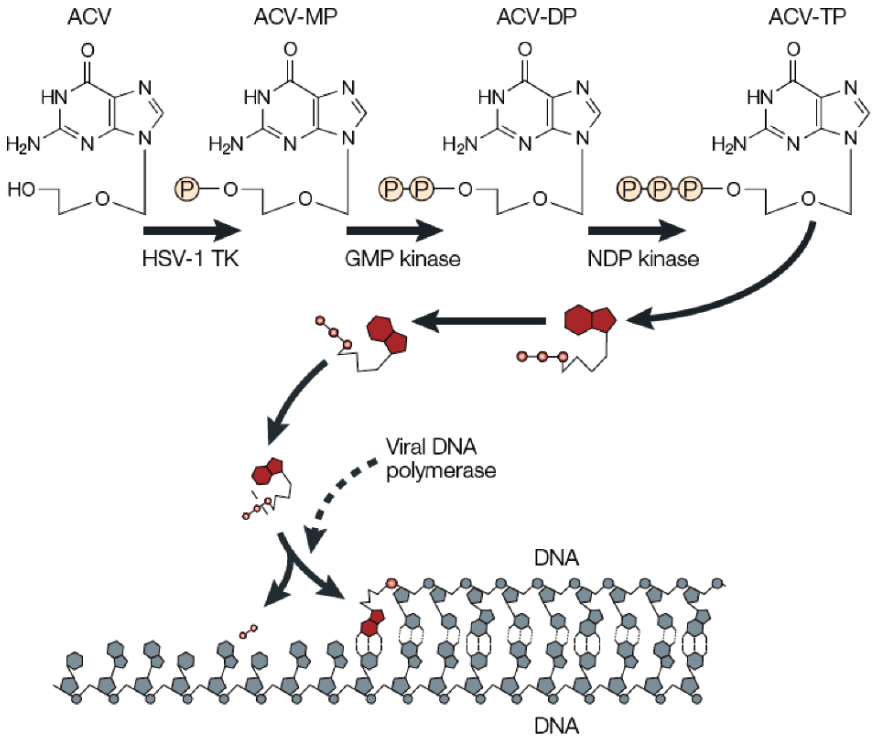


Fig. 5 Mechanism of antiviral action of acyclovir (De Clercq 2002)

action, acyclovir triphosphate (ACV-TP) directly competes with the natural substrate (dGTP). To be converted to ACV-TP, ACV is successively phosphorylated by the HSV- or VZV-encoded thymidine kinase, and two cell-derived kinases, guanosine 5′-monophosphate kinase (GMP kinase) and nucleoside 5′-diphosphate kinase (NDP kinase).

The first phosphorylation carried out by the virus-induced thymidine kinase (TK) (Elion et al. 1977) is crucial for the specific (and selective) activity of acyclovir and all the acyclic (and carbocyclic) analogues derived thereof (Fig. 1). Herpesviruses which do not encode their own TK or which lost their capacity (e.g., TK-deficient HSV-1, HSV-2, or VZV variants) no longer respond to the antiviral action of acyclovir or other acyclic guanosine analogues and vice versa; human tumor cells that have been transduced by the HSV TK become exquisitely sensitive to the cytostatic action of these acyclic guanosine analogues (i.e., ganciclovir) (Degrève et al. 1999; De Clercq et al. 2001).

In this context, it should be noted that the specific antiviral activity of ganciclovir against HSV (which is more potent than that of acyclovir) can be fully explained by the compound being specifically recognized as substrate by the HSV-encoded TK. For CMV, however, which does not encode a virus-specified TK, the activity of ganciclovir depends on the phosphorylation by a virus-encoded protein kinase, which

specifically recognizes ganciclovir as substrate for conversion to its monophosphate (Sullivan et al. 1992). The latter then follows the same pathway as illustrated for acyclovir in Fig. 5.

3 Acyclic Nucleoside Phosphonates

The acyclic nucleoside phosphonates (ANPs) can be considered as nucleotide rather than nucleoside analogues, in that, besides the purine or pyrimidine base, they contain an (acyclic) sugar moiety to which a phosphonate is attached. In these nucleotide analogues (Fig. 2), the phosphoric ester grouping $(=\mathrm{P}--\mathrm{O}--\mathrm{C}--)$ is transformed to its isomeric phosphonomethyl ether $(=\mathrm{P}--\mathrm{C}--\mathrm{O}--)$, which is able to withstand attack by cytoplasmic enzymes (De Clercq and Holý 2005). This phosphonate group contributes not only to the prolonged antiviral activity of this class of compounds and their relative resilience to antiviral drug resistance development, but is also associated with dose-limiting nephrotoxicity.

The prototype member of the ANPs is (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) (Fig. 2), first described for its broad-spectrum anti-DNA virus activity in 1986 (De Clercq et al. 1986). Then followed by the description of various other acyclic nucleoside phosphonates in 1987 (De Clercq et al. 1987). At present three acyclic nucleoside phosphonates have been licensed for clinical use: cidofovir, adefovir, and tenofovir (Fig. 2).

Cidofovir (Fig. 2) has been formally approved for the treatment of CMV retinitis in AIDS patients, where it is administered intravenously at a dose not exceeding 5 mg/kg once weekly during the first two weeks (and every other week thereafter). Cidofovir is also used "off label" for the treatment of human papilloma virus (HPV) infections (i.e., cutaneous warts, anogenital warts, laryngeal and pharyngeal papilloma), polyomavirus [i.e., progressive (i.e., multifocal leukoencephalopathy (PML)], adenovirus, herpesvirus, and poxvirus (i.e., molluscum contagiosum) infections, where it can be administered intravenously (at a dose of $\leq 5\,\mathrm{mg/kg}$ once weekly or every other week) or topically as a 1% gel or cream (De Clercq and Holý 2005). Especially in immunosuppressed patients (i.e., transplant recipients), local treatment of HPV-associated lesions has often yielded spectacular results (Bonatti et al. 2007).

Adefovir in its prodrug form, adefovir dipivoxil, is indicated in the treatment of chronic HBV infections (chronic hepatitis B), where, if administered orally as a single dose of 10 mg per day, HBV DNA load is reduced significantly $(>3\log_{10})$ over a 1- or 2-year period (Hadziyannis et al. 2005).

Tenofovir in its prodrug form tenofovir, disoproxil fumarate (TDF), is indicated in the treatment of HIV infections (AIDS). It is administered as a single oral dose of 300 mg per day. When combined with emtricitabine and efavirenz, TDF has proven to be more efficacious than the standard combination therapy of combivir (azidothymidine plus lamivudine) and efavirenz (Gallant et al. 2006) and less prone to cause adverse side effects (Pozniak et al. 2006; De Clercq 2007b).

For the treatment of AIDS, TDF is now available in three commercial preparations, as such (Viread®), in combination with emtricitabine (Truvada®) and in combination with emtricitabine and efavirenz (Atripla®). The latter represents a three-drugs-in-once combination pill, which has become available for the treatment of AIDS since July 2006. The different milestones that marked the development and ultimate commercialization of Atripla® (in 2006) since the original identification of adefovir as an antiretroviral agent (in 1986) have been previously reviewed (De Clercq 2006, 2007a–c).

In addition to cidofovir, adefovir, and tenofovir, several new acyclic nucleoside phosphonates have been recently accredited with marked antiviral effects: that is, 6-[2-phosphonomethoxyalkoxy)-2,4-diaminopyrimidines (HPMPO-DAPy, PMEO-DAPy, and PMPA-DAPy) (Fig. 2) (De Clercq et al. 2005; Balzarini et al. 2007; De Clercq 2007d) and triazine analogues of cidofovir (5-aza-HPMPC, 5-aza-cHPMPC (Fig. 2) (Krečmerová et al. 2007a, b). Further studies are needed to establish whether HPMPO-DAPy, PMEO-DAPy, PMPA-DAPy, 5-aza-cHPMPC, or alkyloxyalkyl prodrugs thereof are advantageous in terms of activity spectrum, efficacy, safety, or pharmacokinetics as compared to the first-generation ANPs cidofovir (HPMPC), adefovir (PMEA), or tenofovir (PMPA).

To increase the oral bioavailability of adefovir and tenofovir, the prodrugs adefovir dipivoxil and tenofovir disoproxil as well as alkoxyalkyl esters of cidofovir, that is, hexadecyloxypropyl (HDP) and octadecyloxyethyl (ODE) of HPMPC and HPMPA (Fig. 2), have been developed. These lipid ANP esters show indeed markedly increased oral bioavailability (Painter and Hostetler 2004) as well as enhanced in vitro and in vivo antiviral activity against a wide range of viruses [i.e., adenovirus (Hartline et al. 2005); polyoma-, herpes-, and poxviruses (Kern et al. 2002; Quenelle et al. 2004; Buller et al. 2004; Beadle et al. 2006; Quenelle et al. 2007; Hostetler et al. 2007; Dal Pozzo et al. 2007; Lebeau et al. 2007)]. Quite surprisingly, alkoxyalkyl esters of acyclic nucleoside phosphonates, that is, HDP-HPMPA and ODE-HPMPA, known for their well-established broad-spectrum activity against various DNA viruses and retroviruses, have recently been associated with anti-HCV replicon activity (Wyles et al. 2008), an observation that needs further scrutiny.

Appropriately designed prodrugs, for example, phosphonoamidates (Lee et al. 2005), may allow acyclic nucleoside phosphonates such as tenofovir to be specifically targeted at tissues, that is, lymphatic tissue, where the virus (i.e., HIV) replicates. This principle has been recently extended to another nucleotide analogue, GS-9148 (Cihlar et al. 2008) and its phosphonoamidate prodrug, GS-9131 (Ray et al. 2008).

After the ANPs (i.e., cidofovir, adefovir, and tenofovir) have been released (intra- or extracellularly) from their prodrugs through the intervention of intra- or extracellular esterases, they need only two phosphorylation steps to be converted to their active metabolites (i.e., HPMPCpp, PMEApp, and PMPApp), which will then compete with the natural substrates (dCTP for HPMPCpp, and dATP for PMEApp and PMPApp) for incorporation into the viral DNA (Fig. 6a).

If incorporated into the DNA chain, PMEApp and PMPApp obligatorily act as chain terminators (Fig. 6bb), following incorporation of a single molecule (PMEA

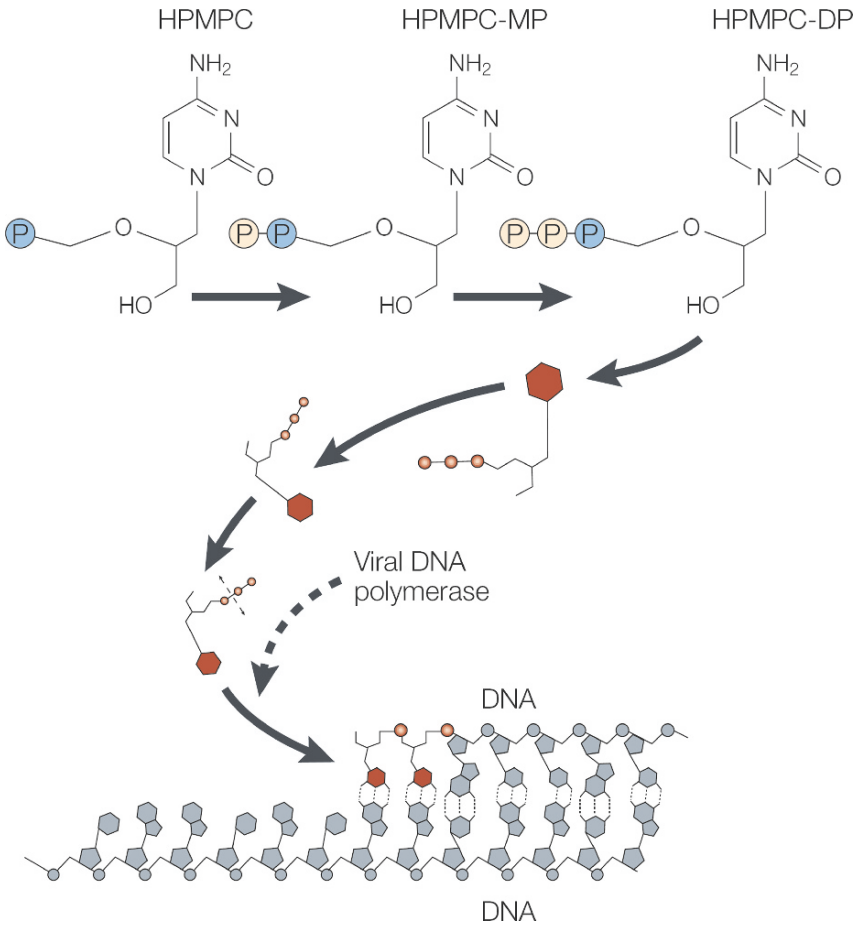


Fig. 6a Mechanism of antiviral action of cidofovir

or PMPA) at the 3′-end (De Clercq and Holý 2005). HPMPC requires two consecutive ("tandem") incorporations to efficiently terminate DNA elongation (Fig. 6ba), as has been demonstrated in the case of CMV DNA synthesis (Xiong et al. 1997). In the case of the vaccinia viral DNA polymerase, HPMPC has been shown to terminate DNA elongation after it is incorporated at the penultimate position, thus allowing one more (regular) nucleotide to be incorporated at the 3′-end (Magee et al. 2005). Recent findings indicated, however, that both HPMPA and HPMPC can be faithfully incorporated into the template strand, thereby inhibiting *trans*-lesion DNA synthesis (Magee et al. 2008).

As has been demonstrated for tenofovir, when incorporated at the 3′-end of reverse transcriptase (RT)-driven DNA chain allows the PMPA residue to adopt multiple conformations [in contrast with the more rigid conformation of the 2′,3′-dideoxynucleosides (see infra) (Tuske et al. 2004). This greater flexibility in conformation may impede development of resistance to tenofovir.

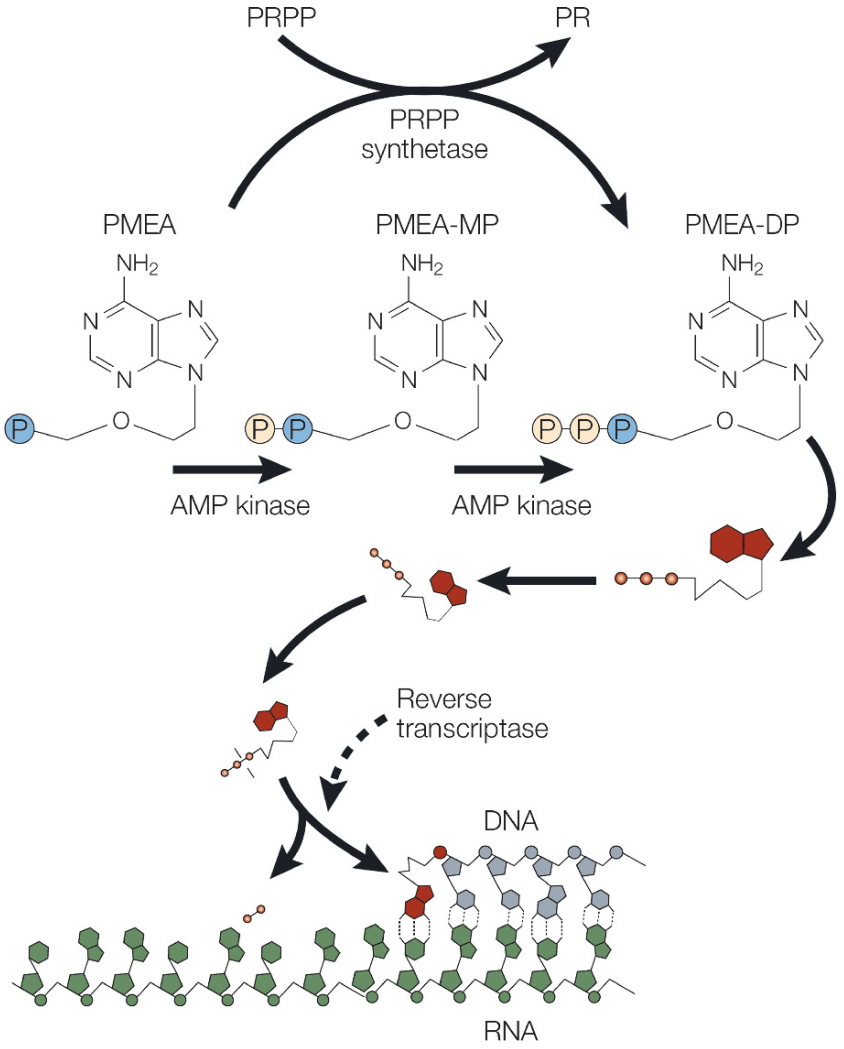


Fig. 6b Mechanism of antiviral action of adefovir

4 Dideoxynucleoside Analogues

The 2′,3′-dideoxynucleoside (ddN) analogues (Fig. 3) encompass a vast group of compounds that have been found active against HIV and HBV, although they have been primarily pursued for the treatment of HIV infections (AIDS). They are targeted at the HIV-associated reverse transcriptase (RT) and therefore also referred to as nucleoside reverse transcriptase inhibitors (NRTIs). They have to be distinguished from the nucleotide reverse transcriptase inhibitors (NtRTIs) such as adefovir (PMEA) and tenofovir (PMPA) (see above) which, like the NRTIs, act as chain

terminators, and the NNRTIs (nonnucleoside reverse transcriptase inhibitors), which directly bind to, and thereby inactivate, the functioning of the HIV-1 RT.

At present there are seven NRTIs, which have been formally approved for the treatment of AIDS: 3′-azido-2′,3′-dideoxythymidine (AZT, zidovudine), 2′,3′-dideoxyinosine (ddI, didanosine), 2′,3′-dideoxycytidine (ddC, zalcitabine), 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T, stavudine), (−)-L-3′-thia-2′,3′-dideoxycytidine (3TC, lamivudine), cyclopentenyl N^6-cyclopropylaminopurine (abacavir, ABC), and (−)-L-5-fluoro-3′-thia-2′,3′-dideoxycytidine ((−)FTC, emtricitabine) (De Clercq 2004a) (Fig. 3).

Depending on the nature of the compound, the ddN analogues have been associated with varying toxic side effects such as bone marrow suppression (AZT), pancreatitis (ddI), hypersensitivity reactions (ABC), and neurologic complications consequently to mitochondrial toxicity (ddC), while others, such as 3TC and (-)FTC, have few, if any, side effects.

These compounds are used in the treatment of HIV-1 and HIV-2 infections (lamivudine is also used in the treatment of HBV infections). All the NRTIs are administered orally; AZT at a dose of 600 mg $(2 \times 300\,\text{mg})$ per day, ddI at a dose of 400 mg $(2 \times 200\,\text{mg})$ per day, ddC at a dose of 2.25 mg $(3 \times 0.75\,\text{mg})$ per day, d4T at a dose of 80 mg $(2 \times 40\,\text{mg})$ per day, 3TC at a dose of 300 mg $(2 \times 150\,\text{mg})$ per day (for the treatment of HBV infections at a daily dose of 100 mg), ABC at a dose of 600 mg $(2 \times 300\,\text{mg})$ per day, and (−)FTC at a dose of 200 mg per day. Commercially available are also fixed dose combinations of AZT (300 mg) with 3TC (150 mg) (Combivir®, 2 pills daily) and of AZT (300 mg) with 3TC (150 mg) and ABC (300 mg) (Trizivir®, 2 pills daily), and, as mentioned earlier, of (−)FTC (200 mg) with TDF (300 mg) (Truvada®, 1 pill daily), and of (−)FTC (200 mg) with TDF (300 mg) and the NNRTI efavirenz (600 mg) (Atripla®, 1 pill daily).

All NRTIs, as exemplified for AZT (Fig. 7), act in a similar fashion: following their uptake by the cells, they are phosphorylated successively to their 5′-monophosphate, 5′-diphosphate, and 5′-triphosphate form (De Clercq 2002). Unlike the first phosphorylation step in the metabolic pathway of the acyclic guanosine analogues (see above), which is carried out by a virus-encoded enzyme (thymidine kinase), the first as well as the subsequent phosphorylations of the 2′,3′-dideoxynucleosides are carried out by cellular enzymes, that is, a 2′-deoxynucleoside (e.g., dThd) kinase, a 2′-deoxynucleotide (e.g., dTMP) kinase, and a (2′-deoxy)nucleoside 5′-diphosphate (NDP) kinase.

After the ddNs have been converted to their 5′-triphosphate form (ddN-TP), they shut off RNA-dependent DNA synthesis as obligate chain terminators: AZT-TP and d4T-TP in competition with dTTP; ddC-TP, 3TC-TP and (−)FTC-TP in competition with dCTP; ddA-TP (originating from ddI via the following pathway: ddI → ddIMP → succinyl − ddAMP → ddAMP → ddADP → ddATP) in competition with dATP; and carbovir-TP (formed from ABC upon its phosphorylation to ABC-MP, deamination to carbovir-MP, and phosphorylation to carbovir-DP and -TP) in competition with dGTP. Thus, for all natural substrates of DNA synthesis, competing ddN analogues have been developed that eventually act as chain terminators of the HIV RT-driven DNA polymerization (De Clercq 2004b).

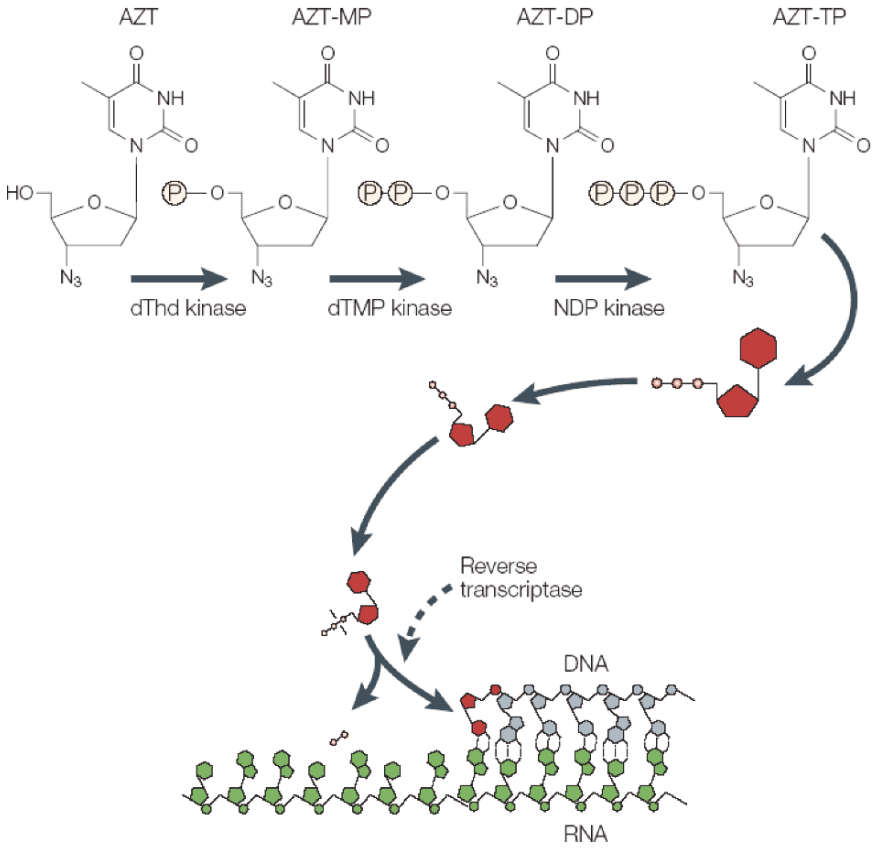


Fig. 7 Mechanism of antiviral action of AZT

In addition to the seven ddNs (or NRTIs) that have been formally approved for the treatment of HIV infection, several other NRTIs acting as chain terminators are still (or have been) under (pre)clinical development: that is, amdoxovir, racivir, dexelvucitabine [previously called reverset (D-d4FC)], elvucitabine (L-d4FC), and D-DOT (dioxolane thymine) (Fig. 3), and starting from D-d4FC and L-d4FC, D- and L-2′,3′-didehydro-2′,3′-dideoxy-3′-fluoro-carbocyclic nucleosides have been synthesized (Wang et al. 2005, 2007).

In principle, resistance to ddNs can be attributed to reduced binding affinity of the ddN-TP to the HIV RT or increased excision velocity of the chain terminating ddN-MP from the 3′-end (see chapter by Nijhuis et al, this volume, for a discussion of resistance mechanisms). The new ddN analogues (i.e., D-d4FC and D-DOT) were designed to circumvent the resistance problem. As compared to other ddN analogues, D-DOT is more resilient to TAM (thymidine analogue-associated mutations), and, also as compared to AZT-MP, DOT-MP is less efficiently removed from the 3′ -end through ATP-mediated pyrophosphorolysis (Lennerstrand et al.

2007). The potent activity of D-DOT against AZT- and 3TC-resistant HIV-1 strains together with its excellent pharmacokinetic profile in rhesus monkeys suggest that further development of D-DOT towards HIV-1 chemotherapy is warranted (Asif et al. 2007).

5 Nucleoside analogues active against HBV or HCV

All the nucleoside (and nucleotide) analogues that have entered the clinic for the treatment of HBV infections (i.e., nucleoside analogues: lamivudine, entecavir, telbivudine; nucleotide analogues adefovir and tenofovir) are fairly well tolerated without side effects that would preclude their long-term usage. The nucleoside analogues in (pre)clinical development for the treatment of HCV infections are not yet sufficiently advanced to assess their tolerability and/or safety.

5.1 Anti-HBV Agents

In addition to the NRTI lamivudine (3TC) and the NtRTI adefovir dipivoxil and tenofovir disoproxil fumarate (which has been recently licensed for the treatment of chronic hepatitis B), two other nucleoside analogues, that is, entecavir and L-dT (telbivudine) (Fig. 4aa), have been licensed for the treatment of HBV infections. Two other compounds 3′-Val-L-dC (valtorcitabine) and L-FMAU (clevudine) (Fig. 4aa) are in clinical development for the treatment of HBV infections, and yet two other compounds, that is, racivir and elvucitabine (Fig. 3), yield potential for the treatment of both HBV and HIV infections.

Thus, entecavir (Zoulim 2006) and telbivudine (Ruiz-Sancho et al. 2007) represent new treatment options for chronic hepatitis B, although, surprisingly (Hirsch 2007), entecavir led to the emergence of HIV-1 variants with the lamivudine-resistant mutation M184V in patients with HIV-1 and HBV coinfection (McMahon et al. 2007). This argues against the use of entecavir in persons coinfected with HIV-1 and HBV, who are not receiving fully suppressive anti-HIV drug regimens.

Entecavir, telbivudine, clevudine, and the other nucleoside analogues (Fig. 4aa) need to be phosphorylated to their 5′-triphosphate form to be antivirally active (Fig. 8). This again implies three phosphorylation steps based successively on a nucleoside kinase, nucleoside 5′-monophosphate kinase, and nucleoside 5′-diphosphate kinase. These reactions have been characterized only in a few cases, that is, thymidylate kinase in the metabolism of clevudine (Hu et al. 2005).

The 5′-triphosphate metabolite of entecavir has been shown to accumulate intracellularly at concentrations that are inhibitory to 3TC-resistant HBV DNA polymerase (Levine et al. 2002). This would imply that entecavir should be active against HBV infections that have become resistant to treatment with lamivudine. Yet, it should be taken into account that treatment with lamivudine leads to the same

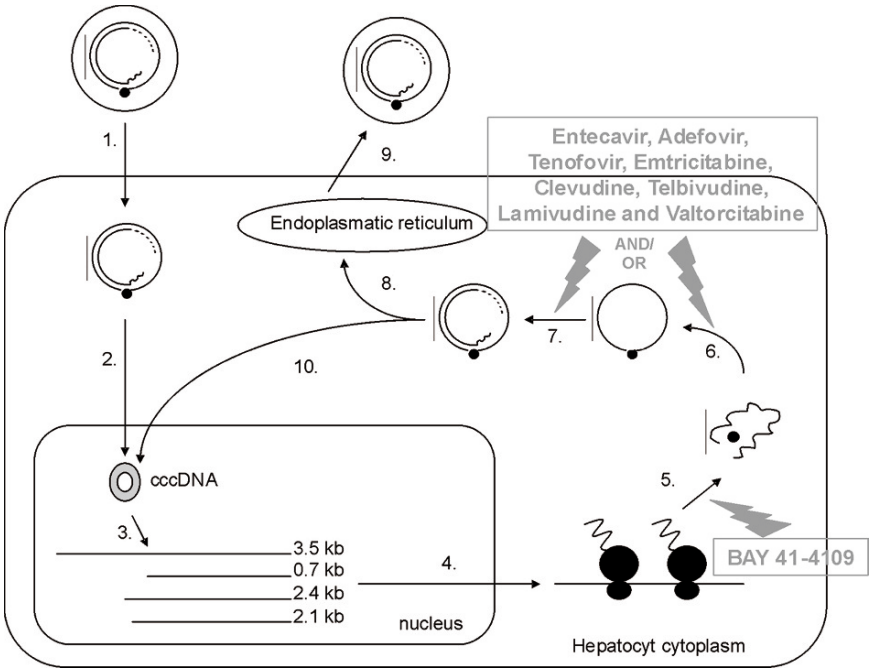


Fig. 8 HBV replication cycle and site of action of several anti-HBV agents

resistance mutations (M204I/V) that may also appear following entecavir treatment (Locarnini et al. 2004). Adefovir resistance, however, is based on mutations (A181V, N236T) that do not give cross-resistance with either lamivudine or entecavir (Locarnini et al. 2004).

The mechanism of action of entecavir, L-dT, L-dC, and L-FMAU at the HBV DNA polymerase level remains to be established. Since these compounds contain a 3′-hydroxyl group, they should, theoretically, be able to permit DNA chain elongation. If, nevertheless, they act as chain terminators, they may do so through an altered conformation of the sugar ring, resulting in an inadequate positioning of the 3′-hydroxyl group prohibiting DNA chain elongation.

5.2 Anti-HCV Agents

In analogy with the designation of NRTIs and NNRTIs for the "nucleoside" and "nonnucleoside" type of reverse transcriptase (RT) inhibitors to target HIV, the corresponding inhibitors to target HCV may be termed NRRIs (for nucleoside RNA replicase inhibitors) and NNRRIs (for nonnucleoside RNA replicase inhibitors).

The NRRI 2′-*C*-methylcytidine (Fig. 4ab) has in its oral prodrug form (NM 283, valopicitabine) acceded into phase II clinical trials in chronic HCV-infected patients.

Preclinical data suggest antiviral synergy for the combination of NM 283 with interferon (IFN) (Afdhal et al. 2004). In vitro ribavirin [a nucleoside analogue that acts as an IMP dehydrogenase inhibitor (not a chain terminator)] was shown to antagonize the anti-HCV activity of 2-*C*-methylcytidine (Coelmont et al. 2006). Treatment with NM 283 at optimal dosing produced consistent HCV RNA reduction averaging >1.2 $\log_{10}$ in a difficult-to-treat cohort, that is, patients that had previously failed pegylated IFN and ribavirin combination therapy (Toniutto et al. 2007). Valopicitabine combined with standard therapy of pegylated interferon and ribavirin was shown to clear HCV in 72% of patients who completed 12 weeks of treatment (www.idenix.com).

Following 2′-*C*-methylcytidine, various other nucleoside analogues targeting the HCV NS5B polymerase have been reported to inhibit HCV replication in vitro (HCV replicon system): 2′-*O*-methylcytidine (Carroll et al. 2003), 2′-*C*-methyladenosine (Carroll et al. 2003; Tomassini et al. 2005), 2′-*C*-methylguanosine (Migliaccio et al. 2003; Eldrup et al. 2004), 7-deaza-2′-*C*-methyladenosine (Olsen et al. 2004), 7-deaza-7-fluoro-2′-*C*-methyladenosine (Carroll et al. 2007), 2′-deoxy-2′-fluoro-2′-*C*-methylcytidine (PSI-6130) (Stuyver et al. 2006), 4′-azidocytidine (R1479) (Klumpp et al. 2006) (and an oral prodrug thereof, termed R1626) (Klumpp et al. 2007) (Fig. 4ab).

How do these NRRIs interact with their final target, the HCV RNA replicase? They are phosphorylated to their 5′-triphosphate form, and then inhibit the HCV replicase. As they possess a 3′-hydroxyl function, they may not be considered as obligate chain terminators, but they may act as virtual chain terminators, viz. by steric hindrance exerted by the neighboring 2′-*C*-methyl and/or 4′-C-azido groups. Similar to their NRTI and NNRTI counterparts in the case of HIV reverse transcriptase, the NRRIs (2′-*C*-methylnucleosides) interact, upon their phosphorylation to the corresponding 5′-triphosphates, with a region of the HCV RNA replicase (or NS5B RNA-dependent RNA polymerase) that is clearly distinct from the site(s) of interaction of the NNRRIs (Tomei et al. 2005).

For the 2′-*C*-methyladenosine triphosphate, significantly higher concentrations were detected than that for 2′-*O*-methylcytidine triphosphate, which is consistent with the greater potency of 2′-*C*-methyladenosine over 2′-*O*-methylcytidine in the HCV replicon assay (Carroll et al. 2003). The relative inactivity of 2′-*O*-methylcytidine in inhibiting HCV replication could be ascribed to its poor intracellular conversion to the 5′-triphosphate; its activity could be restored when using a monophosphate prodrug (Tomassini et al. 2005). The 2′-*C*-methyl ribonucleosides 2′-*C*-methyladenosine and 2′-*C*-methylguanosine were identified as potent inhibitors of HCV RNA replication and the corresponding triphosphates were found to be potent inhibitors of the HCV NS5B-mediated RNA synthesis (Eldrup et al. 2004). The 2′-*C*-methyl ribonucleosides were shown to be efficient chain-terminating inhibitors of HCV genome replication (Migliaccio et al. 2003). Characterization of drug-resistant HCV replicons defined a single S282T mutation within the active site of the viral RNA polymerase that conferred loss of sensitivity to 2′-*C*-methyl ribonucleosides in both replicon and isolated polymerase assays (Migliaccio et al. 2003). It has been suggested that the 2′-*C*-methyl entity

is able to sterically block the next incoming ribonucleotide 5′-triphosphate (NTP) (Migliaccio et al. 2003). Additional modifications, as, for example, the 7-deaza modification, may further disrupt the alignment of the 3′-OH for nucleophilic attack on the α-phosphate of this incoming NTP, so as to more efficiently terminate chain elongation (Olsen et al. 2004).

The 2′-*C*-methyl-substituted ribonucleosides 2′-*C*-methyladenosine and -guanosine were also found to inhibit the replication of flaviviruses other than HCV, such as bovine viral diarrhea virus (BVDV), yellow fever virus, and West Nile virus (Migliaccio et al. 2003). Other 2′-*C*-methylribonucleosides such as β-D-2′-deoxy-2′-fluoro-2′-*C*-methylcytidine (PSI-6130), however, showed little if any activity against BVDV, West Nile virus, or yellow fever virus (Stuyver et al. 2006).

PSI-6130, upon phosphorylation to its 5′-triphosphate, is incorporated (as PSI-6130-MP) as a nonobligate chain terminator (Murakami et al. 2008) into RNA catalyzed by purified RNA-dependent RNA polymerase (NS5B). PSI-6130 is metabolized intracellularly to the 5′-triphosphate of β-D-2′-deoxy-2′-fluoro-2′-*C*-methyluridine (PSI-6206) (Ma et al. 2007), but, as compared to the 5′-triphosphate of PSI-6130, the 5′-triphosphate of PSI-6206 is less efficient an inhibitor of HCV RNA-dependent RNA polymerase than PSI-6130 5′-triphosphate (Murakami et al. 2007).

R1479 (4′-azidocytidine) inhibits HCV replication in the replicon system with similar potency compared with 2′-*C*-methylcytidine (Klumpp et al. 2006). R1479 5′-triphosphate elicits similar potency compared with 3′-dCTP in inhibiting the NS5B-dependent RNA synthesis. R1479-TP was incorporated into nascent RNA by the HCV polymerase and reduced further elongation with similar efficiency compared with 3′-dCTP, an obligate chain terminator. The S282T mutation in the NS5B polymerase, which has been shown to confer resistance to 2′-*C*-methylnucleosides (see above), did not confer cross-resistance to R1479 (Klumpp et al. 2006). In vitro studies mapped resistance to R1479 to amino acid substitutions S96T and S96T/N142T of NS5B. These mutations, in turn, did not confer resistance to 2′-*C*-methylcytidine (Le Pogam et al. 2006). These findings would argue for combination therapy of R1479 with 2′-*C*-methylribonucleosides.

HCV polymerase that carried the S282T mutation did no longer incorporate 2′-*C*-methyl-CTP during the initiation step of RNA synthesis (Dutartre et al. 2006). The presence of the S282T mutation induces a general reduction (5–20-fold) in terms of polymerase efficiency (Dutartre et al. 2006), which may translate to decreased viral fitness (Ludmerer et al. 2005).

Nonobligate chain terminators, such as 2′-*C*-methylated nucleosides, following incorporation in their 5′-monophosphate (nucleotide) form, can be excised again through pyrophosphorolytic excision in the presence of pyrophosphate (PPi) (Deval et al. 2007). Previous studies have suggested mechanisms of action of removal of (obligate) chain terminators by both BVDV RNA-dependent RNA polymerase (D'Abramo et al. 2004) and HIV-1 reverse transcriptase (Götte 2006).

6 Perspective

Chain terminators acting at the DNA level such as the acyclic guanosine analogues (following phosphorylation to their triphosphate form) have gained wide acceptance as antiviral drugs, that is, (val)acyclovir in the treatment of HSV infections and (val)ganciclovir in the treatment of CMV infections (in immunosuppressed patients). The acyclic nucleoside phosphonates (or nucleotide analogues), also acting at the DNA level (following phosphorylation to their diphosphate form), have proven to be key antiviral drugs for the treatment of HIV infections (tenofovir) and HBV infections (adefovir and tenofovir). The 2′,3′-dideoxynucleoside analogues, which following their phosphorylation to the 5′-triphosphate form, act as chain terminators of the HIV reverse transcription reaction, are inherent part of various antiviral drug cocktails used in the treatment of AIDS. Whether RNA chain terminators may ultimately evolve to useful drugs in the treatment of HCV infections is anticipated, but not yet demonstrated. An additional, albeit yet to be ascertained, application for the RNA chain terminators is their potential use for the treatment of flavivirus infections (other than HCV) and RNA virus infections other than flavi-, such as a picornavirus infections.

Acknowledgments This is part of the activities of the VIRGIL European Network of Excellence on Antiviral Drug Resistance supported by a grant (LSHM-CT-2004-503359) from the Priority 1 "Life Sciences, Genomics and Biotechnology for Health" and is supported by a grant from the FWO (no. G.0267.04).

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Viral Protease Inhibitors

Jeffrey Anderson, Celia Schiffer, Sook-Kyung Lee, and Ronald Swanstrom

Contents

Abstract This review provides an overview of the development of viral protease inhibitors as antiviral drugs. We concentrate on HIV-1 protease inhibitors, as these have made the most significant advances in the recent past. Thus, we discuss the biochemistry of HIV-1 protease, inhibitor development, clinical use of inhibitors, and evolution of resistance. Since many different viruses encode essential proteases, it is possible to envision the development of a potent protease inhibitor for other viruses if the processing site sequence and the catalytic mechanism are known. At this time, interest in developing inhibitors is limited to viruses that cause chronic disease, viruses that have the potential to cause large-scale epidemics, or viruses that are sufficiently ubiquitous that treating an acute infection would be

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H.-G. Kräusslich, R. Bartenschlager (eds.), *Antiviral Strategies,* Handbook of Experimental Pharmacology 189,

beneficial even if the infection was ultimately self-limiting. Protease inhibitor development is most advanced for hepatitis C virus (HCV), and we also provide a review of HCV NS3/4A serine protease inhibitor development, including combination therapy and resistance. Finally, we discuss other viral proteases as potential drug targets, including those from Dengue virus, cytomegalovirus, rhinovirus, and coronavirus.

1 Introduction

This chapter reviews the development of inhibitors of viral proteases. A major focus will be on inhibitors of the HIV-1 protease, because this target has seen the greatest amount of work, and many of the biological lessons learned are directly applicable to other viral systems. A major limitation in antiviral therapy is the development of inhibitor resistance, and this topic will also be considered, while a more general review on antiviral resistance also referring to protease inhibitors is provided in Chap. 11 of this book. Space does not permit comprehensive referencing; therefore, we cite seminal initial reports, refer to comprehensive review articles, and discuss the more recently published literature. Finally, although many viruses encode essential proteases, we focus on viruses where drug development either has or is likely to progress to the clinic.

Viral proteases are part of a common strategy of the synthesis of large precursor proteins that are cleaved to generate mature active proteins. Most viral proteases are derived from well-known eukaryotic protease families, but have evolved their own specificities. Thus, the catalytic mechanisms of peptide bond cleavage follow well known chemistry catalyzed by canonical active site residues, but the specificity of the site of cleavage is determined by interactions between the protease and the amino acid side chains that flank the site of cleavage (i.e., the scissile bond). Drug design takes advantage of the known features of the catalytic mechanism and adds specificity based on the flanking interactions, guided in part by structural information. Given this starting point, the design of protease inhibitors has typically proceeded through the large-scale screening of substrate-like molecules.

The requirements of protease inhibitors as drugs in terms of potency, pharmacokinetics, and toxicity will vary depending on the nature of the infection and the goals of therapy. At one extreme is treatment of HIV-1, a chronic infection that requires life-long therapy and full suppression of viral replication. At the other extreme is the treatment of human rhinovirus (i.e., the cold virus), where short-term treatment to blunt viremia will likely be sufficient to reduce the unwanted symptoms of a cold. In all cases, viral proteases represent very attractive targets with familiar mechanisms of catalysis that frequently allow for the design of transition state analogs and with distinct specificities from host proteases.

2 Biochemistry of HIV-1 Protease and Development of Inhibitors

The main features of HIV-1 protease expression and processing have been reviewed (Swanstrom and Wills 1997). The protease is encoded in the viral *pro* gene, located downstream of the *gag* gene and upstream of the *pol* gene, which encodes the viral polymerase and integrase. It is expressed as part of a large polyprotein precursor, the Gag–Pro–Pol precursor. Given the dimeric nature of the viral protease, it is clear that this Gag–Pro–Pol precursor must dimerize to allow the protease to become active. The details of how the protease excises itself from the precursor are poorly understood, although the initial cleavage appears to occur in *cis* at a novel cleavage site (Pettit et al. 2004).

The HIV-1 protease, like other retroviral proteases, is a homodimeric aspartyl protease (see Fig. 1). The active site is formed at the dimer interface, with the two aspartic acids located at the base of the active site. The enzymatic mechanism is thought to be a classic acid–base catalysis involving a water molecule and what is called a "push–pull" mechanism. The water molecule is thought to transfer a proton to the dyad of the carboxyl groups of the aspartic acids, and then a proton from the dyad is transferred to the peptide bond that is being cleaved. In this mechanism, a tetrahedral intermediate transiently exists, which is nonconvalent and which is mimicked in most of the currently used FDA approved inhibitors.

A distinctive feature of the protease is the presence of a mobile beta turn in each subunit, which serves as a flap covering the active site. For substrate to get access to the active site, the flaps have to move away in what must be an ongoing dynamic

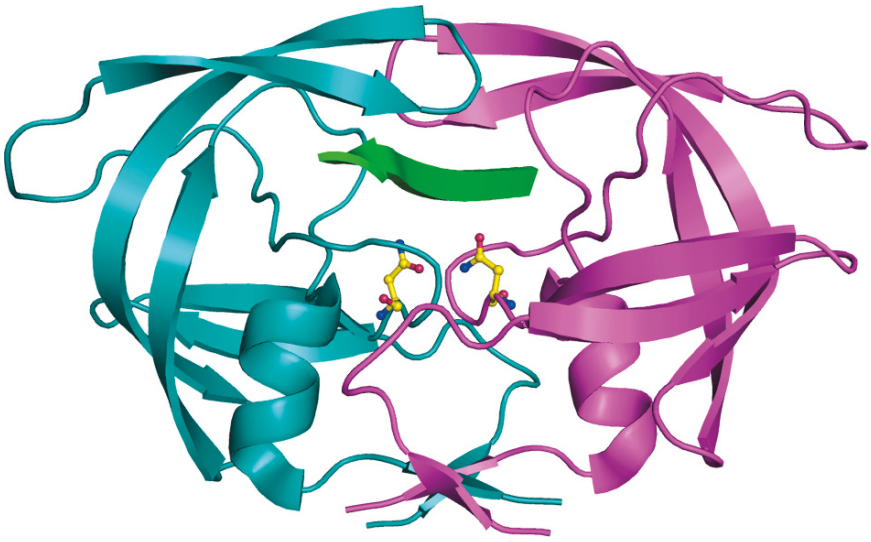

Fig. 1 A ribbon diagram of the crystal structure of a substrate complex of the homo-dimer HIV-1 protease (1kj7) (Prabu-Jeyabalan et al. 2002). Each monomer is shown in *cyan* and *pink*; the substrate is shown in *green*, and the catalytic aspartic acids are highlighted in *yellow*

process. Once the substrate is recognized and bound, the flaps move back over the active site and lock down over the bound substrate, completing the active site cavity and permitting substrate cleavage.

The protease cleaves the Gag and Gag–Pro–Pol polyproteins at ten sites. The enzyme recognizes a series of eight residues as a substrate cleavage site, ranging from P4–P4′. Nearly $1,000$ Å^2 of the substrate is buried within the active site (Prabu-Jeyabalan et al. 2002). The sequences of these ten substrate sites are quite diverse and are cleaved with different efficiencies. Most of the substrate sites have a branched amino acid residue at the P2 site, a hydrophobic residues at P1, and an aromatic or proline at P1′. However, there are exceptions to each of these patterns: the nucleocapsid-p1 cleavage site (which is the last and slowest site to be cleaved in the processing of Gag) has an alanine at P2 and an asparagine at P1. The different substrate sequences result in cleavage rates, with nearly 400-fold differences in cleavage efficiencies. These differences in rates coupled together with the three-dimensional topology of the polyproteins likely contribute to the sequential order in which the substrates are cleaved.

Crystallographic studies imply that although little sequence homology exists between the different protease cleavage sites, what is conserved is the shape that they adopt within the active site of the enzyme (Prabu-Jeyabalan et al. 2002). This shape has been termed the "substrate envelope" and represents the consensus volume overlapping the majority of the substrates. Most likely, HIV-1 protease recognizes a particular peptide sequence as being a substrate by a combination of accessibility and the shape the sequence can adopt.

The development of HIV-1 protease inhibitors (PI) represents a highly successful effort in drug discovery, with nine PI currently approved by FDA and several more in clinical trials. The initial leads for HIV-1 PI were found within the pharmaceutical companies' libraries, which had been developed for another pharmaceutically interesting aspartyl protease, renin. These initial lead compounds, knowledge of the cleavage site sequences, a known catalytic mechanism with a generalizable strategy to generate a transition state analog, and the ability to readily crystallize the protease with a bound inhibitor have provided all the tools necessary for this successful and ongoing drug development effort.

HIV-1 protease was the first therapeutic target where "structure-based drug design" was broadly used to optimize inhibitor design (Wlodawer and Erickson 1993). Potential lead compounds were cocrystallized in complex with the enzyme and new compounds were designed to better fit and complement interactions within the active site. In the race to develop HIV-1 PI, competing laboratories would cocrystallize compounds that their competitors patented in order to figure out alternative chemical scaffolds that would preserve the same contacts but with better pharmacokinetics or bioavailability. This resulted in many of the PI occupying and filling the active site in a highly overlapping manner. These inhibitors are fairly hydrophobic and generally fit into the P2–P2′ region of the active site, often with a hydrophobic cyclical side chain at P1 and a fairly bulky functional group at P1′. All nine FDA-approved PI are competitive active site inhibitors that bind with affinities to the purified enzyme ranging from low nanomolar to low picomolar (Fig. 2) (Chen et al. 1994; Kaldor

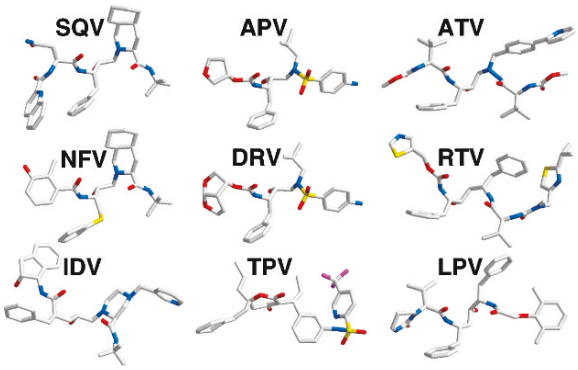

Fig. 2 The three-dimensional structures of the nine currently FDA-approved HIV-1 Protease inhibitors, colored by atom-type, *white*-carbon, *blue*-nitrogen, *red*-oxygen, *yellow*-sulfur, *purple*-florine. (Figure made in Pymol; (de-Lano 2002). *SQV* saquinavir, *APV* amprenavir, *ATV* atazanavir, *NFV* nelifinavir, *DRV* darunavir, *RTV* ritonavir, *IDV* indinavir, *TPV* tipranavir, *LPV* lopinavir

et al. 1997; Kempf et al. 1995; Krohn et al. 1991; Stoll et al. 2002; Thaisrivongs and Strohbach 1999). Seven of the nine inhibitors (except Tipranavir and Darunavir) are peptidomimetics that mimic the enzymatic transition state, containing a variety of noncleavable dipeptide isosteres as core scaffolds to mimic the transition state of substrate cleavage. Thus, although chemically different, many of the PI have very closely overlapping structures and interactions.

2.1 Clinical Use of HIV-1 Protease Inhibitors and Evolution of Resistance

The introduction of PI and the beginning of highly active antiretroviral therapy (HAART) in 1995 has had a profound impact on the paradigm of the treatment of HIV-1 infection: the potential to change an often progressive and fatal infection to a chronic and manageable disease. The continuing evolution of PI-based antiviral therapy has been due to numerous factors, including the ongoing need for more potent inhibitors with improved pharmacokinetics, decreased side effect profiles, and higher genetic barriers of resistance to combat multidrug resistant HIV. The nine PI compounds currently approved by the United States Food and Drug Administration for treatment of HIV-1 infection (Fig. 2) are as follows: saquinavir (SQV), ritonavir (RTV), indinavir (IDV), nelfinavir (NFV), lopinavir (LPV), atazanavir (ATV), fosamprenavir (FPV), tipranavir (TPV), and darunavir (DRV). We discuss the clinical use of each of the PIs in chronological order of FDA approval.

Saquinavir. SQV was first shown in vitro to have potent HIV-1 inhibition in acutely infected cells with an IC_{50} in the subnanomolar range and to inhibit viral maturation in chronically infected cells at 10 nM (Craig et al. 1991). Subsequently, clinical trials with SQV monotherapy in HIV-1 infected men at concentrations up to 600 mg three times a day for 16 weeks resulted in a decrease in HIV-1 RNA of 80% $(0.7\log_{10})$ (Kitchen et al. 1995). These and other data facilitated SQV in becoming the first FDA-approved PI in December 1995. In its original formulation,

SQV had a low bioavailability of 4%, due to extensive first-pass metabolism in the intestine and in the liver by cytochrome P450. Characterization of HIV-1 mutants with decreased sensitivity to SQV revealed that amino acid substitutions G48V and L90M significantly increased IC_{50} concentrations by approximately 40-fold (Jacobsen et al. 1995; Turriziani et al. 1994). Thus, the need for more potent PIs with improved pharmacokinetics and a higher genetic barrier to resistance quickly became apparent.

Ritonavir. RTV was derived by optimization of the pharmacokinetic properties of a series of C2 symmetry-based PIs, and in vitro studies demonstrated RTV to be a potent antiviral agent with activity against a variety of HIV-1 strains with an IC_{50} of 10–40 nM (Kempf et al. 1995). Antiretroviral activity in vivo was demonstrated over a broad range of doses, with a peak decline in HIV-1 plasma viremia of 1.2 $\log_{10}$ (Danner et al. 1995; Markowitz et al. 1995b). Unfortunately, despite its inhibition of HIV-1, RTV often resulted in dose-dependent GI side effects that were frequently intolerable. These side effects are secondary to many unique features of RTV: high oral bioavailability of 80% (Kempf et al. 1995) and potent inhibition of cytochrome P450–3A4 (the most potent of all PIs) and 2D6 (Hoetelmans 1999). The fortuitous strong inhibition of the P450–3A4 metabolism pathway resulted in a strikingly long half-life for RTV. However, another limitation of RTV monotherapy was its low genetic barrier to resistance, with mutations at codons 82 or 84 substantially reducing viral susceptibility (Markowitz et al. 1995a). The selection of protease variants with reduced affinity for RTV is consistent with the hydrophobic interaction between RTV and the isopropyl side chain of Val 82 as observed by X-ray crystallography. Because of the ability of RTV to inhibit P450–3A4, the paradigm of PI-based therapies changed from using RTV as a single PI to "boosting" of other PIs with RTV to increase their half-lives (Kempf et al. 1997). This has become the standard and maintains prolonged blood levels of the boosted PI, increases potency, decreases the required dosage, and minimizes PI side effects. In a similar fashion, RTV boosting impacts the dosing requirements of other classes of antiviral agents, including the CCR5 coreceptor antagonist, Maraviroc.

Indinavir. The final design of IDV included elements to improve absorption and bioavailability by the addition of a basic amine into the backbone of a hydroxyethylene transition-state mimic compound. IDV demonstrated in vitro inhibition of a variety of HIV-1 and HIV-2 isolates with an IC_{95} in the 12–100 nM range (Vacca et al. 1994). Monotherapy trials demonstrated up to a 3.1 $\log_{10}$ decline in HIV-1 RNA levels (Stein et al. 1996). The genetic correlates of in vivo resistance revealed variable patterns of multiple substitutions, indicating that resistance evolved through multiple pathways during ongoing replication; however, resistant isolates typically had substitutions at codons 46 and/or 82 (Condra et al. 1996).

Nelfinavir. Using structure-based design in conjunction with predicted oral pharmacokinetics, NFV was identified and found to have potent inhibition of HIV-1 in vitro with an IC_{50} in the 2 nM range (Kaldor et al. 1997). Clinical trials of NFV revealed robust and sustained reductions in HIV-1 RNA with over half of all subjects attaining a persistent 1.6 $\log_{10}$ reduction at 12 months, in conjunction with a mean increase in CD4 cells of 180–200 per mm^3 (Markowitz et al. 1998). In subjects

with viral rebound, the active site mutation allowing for NFV resistance was often mediated by a unique D30N substitution; of note, this mutation does not confer phenotypic cross-resistance to other PIs.

Lopinavir. The many limitations of the first generation PIs (modest oral bioavailability, short plasma half-life, high binding to plasma proteins, strict dietary restrictions, and a low genetic barrier to resistance) led to the eventual discovery of LPV (Sham et al. 1998). In vitro studies of LPV demonstrated inhibition of wild type and mutant proteases with an IC_{50} in the subnanomolar range. Furthermore, LPV, like most PIs, is exquisitely sensitive to pharmacokinetic enhancement by codosing with ritonavir, producing sustained concentrations in the plasma that are $>$ 50-fold over the EC_{50}, which has led to the coformulation LPV/RTV (Kaletra). In vivo, potent and durable viral suppression has been examined in antiretroviral naïve patients receiving LPV/RTV in conjunction with two nucleoside reverse transcriptase inhibitors (NRTI), with 85% of patients having HIV-1 viral loads ≤ 400 copies/ml at 48 weeks (Murphy et al. 2001). In contrast to first generation PIs, Kaletra has been shown to have a much higher genetic barrier of resistance. Selection of highly resistant viral variants is due to the sequential appearance of multiple mutations, such as I84V-L10F-M46I-T91S-V32I-I47V (Carrillo et al. 1998). Moreover, mutations at 11 amino acid positions in protease (L10F/I/R/V, K20M/R, L24I, M46I/L, F53L, I54L/T/V, L63P, A71I/L/T/V, V82A/F/T, I84V, and L90M) are associated with reduced susceptibility, but these must appear in combinations. The median IC_{50} of LPV against isolates with 0–3, 4 or 5, 6 or 7, and 8–10 of the 11 mutations is 0.8-, 2.7-, 13.5-, and 44.0-fold higher, respectively, than the IC_{50} against wild-type (Kempf et al. 2001). The genotypic inhibitory quotient (relationship between LPV trough levels and the number of *pro* mutations) is the main independent predictor of response to LPV/RTV (Gonzalez de Requena et al. 2004). To further reinforce the potency and high resistance barrier, small-scale clinical trials have shown that two-thirds of patients on Kaletra alone are capable of maintaining suppression to below the limit of detection (50 HIV-1 RNA copies/ml) after 4 years of follow-up (Pulido et al. 2008).

Atazanavir. Designed to simplify drug dosing regimens, ATV was approved in 2003 and became the first once daily PI. In vitro, ATV has robust anti-HIV activity with an IC_{50} less than 5 nM and an IC_{90} of 9–15 nM in cell culture (Robinson et al. 2000). A phase 2 clinical trial showed efficacy of ATV in combination with didanosine and stavudine in ART-naïve patients after 48 weeks, with a mean reduction from baseline HIV-1 RNA (> 2.33 $\log_{10}$) and over half of all patients with HIV-1 viral loads < 400 copies/ml (Sanne et al. 2003). In contrast to other PIs, especially when boosted with RTV, ATV has not been associated with clinically relevant dyslipidemia or insulin resistance. Other large scale, phase 3 trials have confirmed these findings and revealed noninferiority between ATV vs. a nonnucleoside reverse transcriptase inhibitor (NNRTI) (efavirenz) in conjunction with two NRTIs (combivir) (Squires et al. 2004) or ATV/RTV vs. LPV/RTV (Johnson 2006). ATV has a distinct resistance profile relative to other PIs, with susceptibility maintained against 86% of isolates resistant to 1–2 PIs (Colonno et al. 2004); however, a unique I50L substitution has been shown to be the signature mutation for resistance to ATV.

Fosamprenavir. FPV is an inactive, highly water soluble phosphate ester prodrug of amprenavir (APV), a PI that includes a sulfonamide moiety. FPV was synthesized to allow decreased pill burden and improve patient compliance, and is completely hydrolyzed to APV in the gut endothelium. Although APV was FDA-approved in April 1999, production was discontinued after FPV became available in October 2003. Initial in vitro studies of APV demonstrated potent HIV-1 inhibition, with a mean IC_{50} of 12 nM against six HIV clinical isolates (St Clair et al. 1996). Two pivotal large scale clinical trials demonstrated superior of FPV vs. NFV to contribute to the suppression of viral RNA load are the NEAT (Rodriguez-French et al. 2004) and SOLO trials (MacManus et al. 2004). Like other second generation PIs, FPV can select for an unusual in vitro resistance profile, with multiple substitutions at codons 46, 47, and 50 needed to confer high-level resistance (Partaledis et al. 1995).

Tipranavir. In contrast to the previously approved PIs that are classified as peptidomimetics and transition state analogs, that is, structurally similar to the tetrahedral intermediate that forms during hydrolytic cleavage of a peptide bond of the natural substrate (Randolph and DeGoey 2004), TPV was the first of the nonpeptidic PIs of the dihydropyrone sulfonafamide class that are structurally similar to coumadin. Initial in vitro data demonstrated that TPV was a promising candidate for further development (Poppe et al. 1997). Further in vitro studies demonstrated potent inhibition by TPV against clinical isolates resistant to multiple PIs, due to its molecular flexibility, allowing TPV to fit into the active site of PR that has become resistant to other PIs (Plosker and Figgitt 2003; Rusconi et al. 2000). Phase II clinical trials have shown as many as 16–20 *pro* mutations, including at least three PI resistance-associated mutations are needed to confer decreased susceptibility (Plosker and Figgitt 2003). The Randomized Evaluation of Strategic Intervention in Multi-Drug Resistant Patients [RESIST 1 AND RESIST 2 trials (Cahn et al. 2006; Gathe et al. 2006)] assessed the safety and efficacy of boosted TPV vs. other boosted PIs (LPV, SQV, APV, and IDV) in the setting of optimized background regimens. At week 24, half of the patients with fewer than 12 PI mutations vs. 30% in the comparator PI-containing arm responded to therapy (≥ 1 $\log_{10}$ decline in baseline HIV-1 viral RNA) ($p = 0.025$). At week 48, the proportion of patients with undetectable viral loads (≤ 400 copies/ml) was significantly greater in the TPV arm vs. the comparator arm (30.4% vs. 13.8%, respectively, $p < 0.001$).

Darunavir. DRV is the second nonpeptidic PI and was synthesized by replacing the tetrahydrofuranyl (THF) urethane moiety of amprenavir with a bis-THF component (Koh et al. 2003). Additional hydrogen bonding between the bis-THF ring and the PI backbone results in activity against a broad range of clinical isolates with an IC_{50} in the nanomolar range, including multidrug-resistant HIV-1. Phase IIb clinical trials [Performance of TMC114/r when evaluated in treatment experienced patients with PI resistance (POWER1 and 2)] have examined highly treatment-experienced patients randomly assigned to either DRV/RTV or comparator boosted PIs (SQV, APV, SQV, or ATV). The proportion of patients achieving viral suppression (HIV-1 RNA ≤ 400 copies/ml) was 63% vs. 19% in the DRV vs. comparator arm, respectively. Subset analyses revealed a significantly greater proportion of patients receiving DRV, who had up to eight PI resistance mutations, achieved HIV-1 viral loads

≤ 50 copies/ml at 24 weeks vs. patients receiving a comparator PI (47% vs. 25%, respectively). Viral load suppression was sustained until at least week 48, with 61% of DRV/RTV patients vs. 15% of comparator patients having viral load reductions ≥ 1 $\log_{10}$ copies/ml (Clotet et al. 2007). Surface plasmon resonance technology has been used to analyze the unique binding kinetics of DRV and its substrate to explain the potent antiviral activity; the dissociative half-life of DRV was found to be significantly greater than other analyzed PIs (FPV, ATV, LPV, and TPV) and binding affinity of DRV was >2 orders of magnitude higher vs. other PIs (Dierynck et al. 2007).

2.2 The Structural and Mechanistic Basis of HIV-1 Protease Inhibitor Resistance

Nearly 70% of the 99 residues in HIV-1 protease are known to mutate (Hoffman et al. 2003; Johnston et al. 2004; Rhee et al. 2005; Wu et al. 2003). Many of these mutate with a higher frequency in patients undergoing antiretroviral therapy. Although some sites such as D30N, G48V, I50V/L, V82A, V84I, and L90M are considered primary drug-resistant mutations, they occur in complex interdependent combinations with many other sites throughout the enzyme. These sites of mutation are both within and outside the active site. In addition, specific coevolution of the sequences of particular cleavage sites has become evident. The residues that are conserved appear to be primarily for enzymatic activity, or structural reasons, that is, the dimer interface, flexibility, or key glycine residues. The extensiveness of the mutational patterns in both the enzyme and the substrates indicates that this is a very plastic and adaptable target. Chapter 11 provides more detailed information about resistance development in HIV-1 antiviral therapy.

At a fundamental level, mutations can confer only drug resistance if they maintain function. For HIV-1 protease, this implies that the enzyme continues to recognize and cleave its diverse set of substrate sequences permitting viral maturation. Since all the protease inhibitors are competitive active site inhibitors with the substrates, how resistance can occur without impacting substrate recognition was somewhat of a dilemma. In comparing the crystal structures of the substrate complexes with the crystal structures of the inhibitor complexes, it was found (King et al. 2004a) that the inhibitors protruded from the "substrate envelope" at specific locations. The positions at which each inhibitor protruded away from the substrates and contacted HIV-1 protease corresponded very closely with the site of drug resistant mutations (King et al. 2004a; Prabu-Jeyabalan et al. 2006). In fact, inhibitors that fit more tightly within the substrate envelope have a higher threshold for drug resistance such as APV and DRV (King et al. 2004b). Thus, drug resistance can potentially be greatly limited by restricting the inhibitors to fit within the substrate envelope region. Testing of this as an inhibitor design strategy has been recently utilized, resulting in a number of high affinity, novel HIV-1 protease inhibitors that retain a flat binding profile to drug-resistant protease variants (Altman et al.

2008; Chellappan et al. 2007). However, other resistance-associated mutations exist throughout the enzyme and these likely have a role in maintaining the activity or fitness of the enzyme.

3 The Flavivirus NS3/4A Serine Protease

Several viruses in the Flaviviridae family are targets for antiviral development. Hepatitis C Virus (HCV), of the *Hepacivirus* genus, has the greatest impact on the human population. Dengue Virus of the *Flavivirus* genus also represents an important human pathogen for which no vaccine is available. These are enveloped viruses with a plus-stranded RNA genome of approximately 10 kb. The genome encodes a single large precursor protein of approximately 3,000 amino acids, which includes all the structural and nonstructural viral proteins. Viral replication takes place in the cytoplasm of the infected cell.

3.1 Discovery and Structure of the HCV NS3/4A Serine Protease

The early characterization of HCV polyprotein processing and the enzymes involved has been reviewed (Bartenschlager 1999). The presence of a chymotrypsin-like serine protease was first inferred based on sequence comparisons, with its presence placed within the amino-terminal third of the NS3 protein and the C-terminal two-thirds of NS3 encoding a helicase activity. This protease activity is responsible for four of the ten cleavages that occur in the large precursor protein. The immediate downstream product, NS4A, is a subunit of the protease that greatly increases the level of catalytic activity, giving rise to its name of NS3/4A.

The extensive work understanding the nature of the NS3/4A protease culminated with the determination of the structure at atomic resolution (Kim et al. 1996; Love et al. 1996). The structure confirmed the relationship to members of the chymotrypsin family, with the overall structure being a double β-barrel. One of the structures (Kim et al. 1996) included a peptide representing the NS4A cofactor. The NS4A peptide forms one of the eight β-strands that make up the N-terminal β-barrel of the NS3/4A protease whose presence helps to form the expected catalytic triad (His57, Asp81, Ser139) juxtaposed in the structure to form the active site; the structure also revealed a relatively shallow substrate binding cleft (Fig. 3).

Comparison of the NS3/4A protease cleavage sites shows only three positions that are conserved: E/DXXXXC/T-S/A (Bartenschlager 1999). The availability of a robust recombinant enzyme assay revealed that the enzyme recognizes a ten amino acid stretch of the substrate, from P6 to P4′ (Steinkuhler et al. 1996; Zhang et al. 1997), with the P1 Cys being the major determinant of cleavage site recognition (Bartenschlager 1999). The structures confirmed earlier modeling studies that placed a Phe residue at the bottom of the S1 subsite in position to allow van der

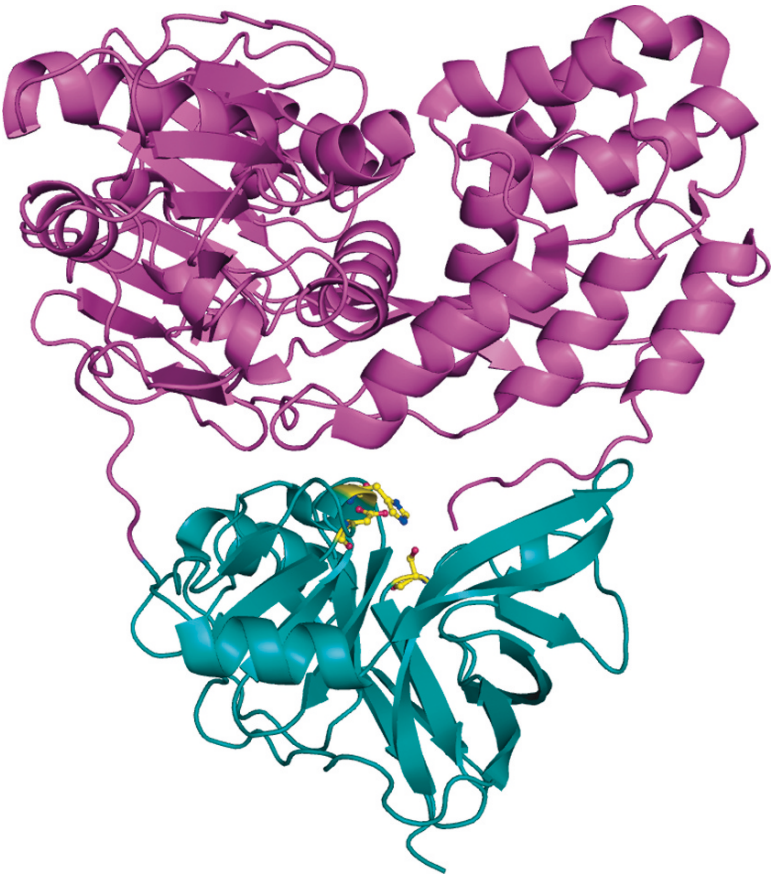

Fig. 3 A ribbon diagram of the HCV NS3/4A protease 1CU1 (Yao et al. 1999). The serine protease domain is shown in *cyan* with the catalytic triad highlighted in *yellow*, and the helicase domain is in *magenta*

Waals interactions with the P1 Cys, and also located basic Arg and Lys residues in position to interact with the P6 acidic residue. More detail of the binding of the N-terminal (i.e., P residues) part of the substrate was subsequently inferred from NMR studies showing a β-sheet interaction between this N-terminal segment of the substrate and enzyme largely confined to within the C-terminal β-barrel (Cicero et al. 1999).

3.2 Development and Clinical Testing of HCV Protease Inhibitors

Characterization of peptide substrates led to the observation that the N-terminal peptide product was able to inhibit the enzyme (Llinas-Brunet et al. 1998; Steinkuhler

et al. 1998). This proved to be a useful starting point for inhibitor design, first by optimizing the sequence of a peptide inhibitor (Ingallinella et al. 1998) and subsequently using nonpeptidic substituents. A detailed review of HCV protease inhibitor development can be found in Lin (2006) and De Francesco and Carfi (2007).

A major limitation in the development of anti-HCV compounds was the lack of a virus replication system. This was finally overcome with the development of a novel replicon system that directed persistent replication in a cell culture format (Lohmann et al. 1999). Using such a system, it was possible to demonstrate antiviral activity of an NS3/4A inhibitor in a cell culture assay, and demonstrate potency on par with treatment with interferon-α (Pause et al. 2003).

The first inhibitor to be developed to the point of testing in humans was BILN 2061 [celuprevir; (Lamarre et al. 2003)]. This inhibitor is a tripeptide mimetic of substrate residues P1–P3, which included rigidifying the structure by linking the P1 and P3 equivalent side chains in a macrocyclic structure. It is a competitive inhibitor with a Ki in the range of 0.5 nM, with the EC_{50} in cell culture about tenfold higher. In a short-term clinical trial, a 2–3 log decrease in viral RNA load was observed with just two days of drug administration, followed by virus rebound after cessation. These results were confirmed in a larger group of subjects infected with HCV genotype1 (Hinrichsen et al. 2004); however, decreases were more variable in subjects infected with genotypes 2 and 3 (Reiser et al. 2005). Unfortunately, development of this compound has been suspended due to toxicity.

The second NS3/4A protease inhibitor developed to the point of clinical trials was VX-950/telaprevir/TVR. This is a mimetic of P1–P4 peptide representing the cleavage site at NS5A/5B. The novel feature of its design is the inclusion of an α-keto-amide group at the C terminus. In this position the keto carbonyl is able to form a reversible covalent bond with the Ser139 nucleophile at the active site (Perni et al. 2006). This inhibitor has a Ki of 7 nM and an EC_{50} about 50-fold higher in cell culture replicon assays. In an initial phase 1 dosing study with administration over a 2 week period, there was a 3 log drop in viral RNA load in the blood in 90% of subjects (Reesink et al. 2006), although the maximum drop was seen between 3 and 7 days followed by rebound of virus in the blood. Coadministration of telaprevir and interferon-α blocked the rebound within this 2 week period (Forestier et al. 2007).

The third NS3/4A inhibitor developed was SCH 503034 (boceprevir). This inhibitor also uses the keto-amide strategy to form a covalent bond with Ser139, and in this case representing a P1–P3 mimetic, although the P3 capping group occupies S4 (Venkatraman et al. 2006). An initial phase 1 dosing study showed potency in humans which was enhanced with coadministration of interferon-α (Sarrazin et al. 2007).

Other NS3/4A inhibitors are in earlier stages of development. TMC435350, a macrocyclic inhibitor, has shown good potency in vitro and high bioavailability (Simmen et al. 2007). ITMN-191 is another macrocyclic inhibitor that is entering early clinical trials. It appears to have a two-step binding mechanism that results in noncovalent binding but slow dissociation (Rajagopalan et al. 2007).

3.3 Combination Therapy

Treatment of HCV has strong parallels with the treatment of HIV-1, specifically the need to suppress viral replication. The major advantage with HCV treatment compared to HIV-1 treatment is that sustained suppression of HCV can lead to the complete clearance of virus, that is, a cure. As with HIV-1 treatment, the use of several agents together gives better sustained viral response compared with the use of single agents. The current therapy of ribavirin and interferon-α has a 70% success rate with 24 weeks of treatment in people with genotypes 2 and 3, while this treatment is successful in only 50% of subjects with genotype 1 and after 48 weeks of treatment (http://digestive.niddk.nih.gov/ddiseases/pubs/chronichepc/). Future studies will initially combine new agents with the approved therapies (ribavirin and interferon-α); however, the use of combinations of new agents with known virus-specific targets is a major goal. Experimental evidence generated using inhibition of a replicon in a cell culture-based assay supports the use of agents with different viral targets (Wyles et al. 2007). Important milestones will be to avoid the use of interferon-α and to reduce the time on therapy to reach sustained viral response.

Interim results from the PROVE1 and PROVE2 phase 2 trials have been reported in subjects infected with genotype 1 HCV. The design of PROVE1 is to dose telaprevir (VX950) with interferon-α and ribavirin for 12 weeks with a variable therapy tail of interferon-α and ribavirin. At the initial 12 week time point, viral RNA was suppressed in 70% of subjects, compared to 39% receiving only interferon-α and ribavirin (Jacobson et al. 2007); in addition, six of nine subjects receiving all three drugs for 12 weeks but without the tail therapy achieved sustained viral response, with the other three showing evidence of selection of telaprevir resistance. In PROVE2, the 12 week responses were 79% viral RNA suppression for treatment with telaprevir plus IFN-α and ribavirin, but only 63% in dual therapy with telaprevir and IFN-α, compared to 43% with IFN-α and ribavirin (Zeuzem et al. 2007). These results are consistent with the idea that adding combinations of agents will result in improved responses, although an effect on sustained viral response rates has not yet been determined.

Interim results from the SPRINT-1 phase 2 trial of boceprevir (SCH 503034) have been released. In subjects who received boceprevir plus interferon-α and ribavirin, viral RNA loads were suppressed at week 12 in between 70 and 79% of subjects infected with genotype 1 HCV, compared with only 34% in the interferon-α/ribavirin standard of care arm (www.sch-plough.com/schering_plough/news/release.jsp?releaseID = 1064540). However, it is not yet known if this enhanced early response will translate into sustained response.

3.4 Resistance

Resistance to NS3/4A inhibitors was primarily tested using the replicon system in tissue culture and closely parallels what was seen with resistance to HIV-1 PI.

Specifically, incomplete suppression of viral replication allows for the selection of variants with mutations in NS3 that have reduced sensitivity to the inhibitor, and this also holds true in clinical testing. An unresolved question is the extent to which different protease inhibitors select for the same or different resistance mutations. This question is complicated by the fact that different levels of a drug can select for different mutations, and resistance can go through different pathways giving a stochastic feature to the appearance of resistance. Cell culture selection schemes generally involve a relatively small sample size, again adding to the stochastic nature of the results, and resistance evolving in vivo may include low-level variants that are not detected but potentially available to seed the rapid outgrowth of resistant variants with a therapy switch. The lesson to date with HIV-1 protease inhibitors is that improved drug potency has been a much more effective strategy in dealing with resistance within the protease inhibitor drug class than attempts to design drugs that elicit nonoverlapping resistance patterns. Chapter 11 provides more detailed information about resistance development in HCV antiviral therapy.

3.5 The Dengue Virus NS2B/NS3 Serine Protease

Dengue Virus is a member of the *Flavivirus* genus of the Flaviviridae family. Because of the number of people at risk for Dengue Virus infection (2.5 billion), with the attendant risk of progressing to dengue hemorrhagic fever, and the lack of an available vaccine, the development of antivirals against the Dengue Virus protease is an important goal. Unlike HCV, the activating subunit of the Dengue Virus protease comes from a proteolytic fragment of the upstream protein, that is, the NS2B fragment (Arias et al. 1993; Chambers et al. 1993). The recent crystal structure of the dengue virus protease has clarified a number of issues, especially when compared to the structure of the West Nile Virus NS2B/NS3 protease with a bound inhibitor (Erbel et al. 2006). The protease has a similar double β-barrel structure, with the NS2B fragment contributing part of a β sheet. In the inhibitor-bound state, the NS2B fragment has extensive contacts around the NS3 protease domain and also contributes to the substrate binding site. This more intimate interaction helps explain the profound requirement of the NS2B fragment for significant activity (Yusof et al. 2000). A distinctive feature of the protease target cleavage site is the presence of Arg residues at both P1 and P2 (reviewed in Melino and Paci 2007). However, the S1 subsite has a strong stacking interaction with the P1 side chain, while the interaction between P2 and S2 is based on electrostatic interactions (Erbel et al. 2006). Given the known substrates and the catalytic mechanism, drug discovery efforts have been undertaken, although none has progressed to clinical trials (Melino and Paci 2007).

4 Other Viral Proteases

Since many different viruses encode essential proteases, it is possible to envision the development of a potent protease inhibitor for any of these viruses, given the knowledge of the processing site sequence and the catalytic mechanism. At this time, interest in developing inhibitors is limited to settings of viruses that cause chronic disease, viruses that have the potential to cause large scale epidemics, or viruses that are sufficiently ubiquitous that treating an acute infection would be beneficial even if the infection is ultimately self-limiting. Later we review one example of each of these cases.

4.1 The Human Cytomegalovirus Protease

Human Cytomegalovirus (HCMV, Human herpesvirus 5/HHV-5) is in the Herpesviridae family, Betaherpesvirinae subfamily, *Cytomegalovirus* genus, and is a large, enveloped, double-stranded DNA virus. Infection rates in the general population are 80–90%, with asymptomatic infections in healthy individuals. However, becoming immunocompromised or immunosuppressed can result in enhanced replication, leading to pneumonia and retinitis; in addition, congenital infections of newborns can lead to neurologic abnormalities. The available therapies are based on nucleoside analogs and have significant toxicity.

The herpesvirus protease was first described for HSV-1 and HCMV (Liu and Roizman 1991; Welch et al. 1991). The protease, called assemblin, is encoded in the HCMV gene UL80 as the amino-terminal part of a precursor protein. The C-terminal domain of the precursor is the viral assembly protein and cleavage events within the precursor are required for the packaging of viral DNA (reviewed in Brignole and Gibson 2007). The crystal structures of the HCMV protease (Chen et al. 1996; Qiu et al. 1996; Shieh et al. 1996; Tong et al. 1996) and bound to inhibitors (Khayat et al. 2003; Tong et al. 1998) have revealed a number of features of this target (reviewed in Tong 2002). The core of the enzyme is a seven stranded β-barrel structure that represents a novel fold for a serine protease. In addition, the catalytic triad is Ser–His–His, rather than the more familiar Ser–His–Asp, and, combined with the novel fold, represent a third independent evolution of a serine protease (in addition to the trypsin and subtilisin families). The protease crystallized as a dimer, which is the active form of the enzyme (Darke et al. 1996; Margosiak et al. 1996). However, the two active sites in the dimer are displaced from each other and dimerization appears to stabilize the structure of the active site at a distance. The structure changes further when binding substrate (by induced fit) with the substrate/inhibitor binding in an extended conformation and forming an anti-parallel β-sheet to align the scissile bond with the active site. P2 and P4 are solvent exposed while the remaining side chains from $P5-P1'$ interact with the protease.

Given a known mechanism of catalysis, that is, a serine nucleophile, and a consensus substrate sequence (V,L,I)XA/S, it has been possible to carry out drug

discovery efforts. A series of inhibitors based on different chemical backbones have been described, all sharing an activated carbonyl to react chemically with the nucleophile (reviewed in Tong 2002). However, to date, none of these compounds has made it into clinical development. Given this outcome, alternative screens have been developed that will allow high throughput screens in cell-based assays as an alternative to screens based on inhibition of enzymatic activity (for example, Cottier et al. 2006). Finally, the potential for similar drug development efforts exist for other herpesviruses such as HSV-1, HSV-2, and KSHV, although little work has been done to date. An alternative approach to develop an inhibitor has been explored with the KSHV protease by targeting the dimer interface to block the essential step of dimerization (Shimba et al. 2004).

4.2 The Rhinovirus 3C Protease

Rhinoviruses cause a significant fraction of the common colds suffered by the human population. However, members of the *Rhinovirus* genus (Picornaviridae family) include 100 different serotypes that infect humans, making a vaccine strategy impractical. Thus, alternative strategies are needed to intervene in these non-life-threatening but inconvenient infections.

Rhinovirus, like poliovirus, synthesizes a large precursor protein from which all of the mature viral proteins are generated. Two viral proteases are involved in these cleavages: 2A protease cleaves the polyprotein precursor at its own N terminus, while the 3C protease is responsible for additional cleavage events to generate the mature viral proteins. Both proteases can release themselves from the polyprotein precursor. Cleavage by 3C occurs between Gln/Gly, but flanking sequences affect efficiency (reviewed in Racaniello 2001).

Structural analysis of the rhinovirus and the hepatitis A virus 3C proteases (Allaire et al. 1994; Matthews et al. 1994) confirmed earlier predictions that the picornavirus 3C proteases are similar to chymotrypsin-like serine proteases in their fold. An important difference is that the serine nucleophile of serine proteases is replaced with a cysteine; however, the 3C protease is structurally distinct from the eukaryotic cysteine protease class of enzymes.

A variety of designs have been explored in developing inhibitors to the rhinovirus 3C protease (reviewed in Tong 2002). Inhibitor design was aided by structural studies of bound inhibitors (Matthews et al. 1999). One successful design of a 3C inhibitor included an unsaturated ethyl ester to allow the formation of a covalent bond with the protease active site Cys, known as a Michael Acceptor. Using this strategy of an irreversible inhibitor, one compound, rupintrivir, was developed and showed efficacy in the setting of experimentally induced human rhinovirus infections (Hayden et al. 2003). The ability to select for resistance to rupintrivir validated that the 3C protease was the drug target, with mutations at position 130 in the S2 subsite being the most common (Binford et al. 2007). However, rupintrivir failed to show efficacy in the setting of natural infection (Patick et al. 2005). A related

compound (compound 1) was identified that had significant bioavailability when taken orally, but this compound has not been taken into efficacy trials (reviewed in Patick 2006). Limitations in the rapid identification of the infecting agent makes the development of agent-specific therapeutics a challenge, given that a number of viruses can give cold-like symptoms, which likely can be ameliorated only with early intervention.

4.3 The SARS Coronavirus Protease

A new coronavirus was quickly identified after the outbreak of an atypical pneumonia in southern China early in 2003. The new virus eventually caused 8,000 infections with approximately 800 deaths in 29 countries. The condition was named Severe Acute Respiratory Syndrome, SARS, and the causative coronavirus named SARS-CoV. The zoonotic nature of the infection came with the identification of a similar virus in bats (Poon et al. 2005), although it is possible that the bat virus passed through other animal hosts and recombined with other SARS-like coronaviruses prior to infecting humans (Hon et al. 2008). SARS-CoV is not currently circulating in the human population; however, the mysterious appearance and rapid spread of this virus emphasized how vulnerable the human population is to such respiratory infections. This has spurred interest in the development of antivirals that could be used either in treatment or as prophylaxis to complement public health measures in curbing future outbreaks.

The SARS-CoV is classified in the Nidovirales Order, Coronaviridae Family, and *Coronavirus* Genus (www.virustaxonomyonline.com). The genome is approximately 30 kb, and gene expression is accomplished by the generation of 3′ co-terminal subgenomic messages. The replicase gene is encoded in two large overlapping reading frames, termed pp1a and pp1b. The encoded polyproteins undergo extensive proteolytic processing by two viral proteases, with a majority of the cleavage events (11) mediated by the viral 3C-like (3CL, also called main) protease (reviewed in Liang 2006). Cleavage sites are most conserved in the P1 position with Gln, then Ser, Ala, or Gly in the P1′ position, Leu, Met, or Phe in the P2 position, and Ala, Val, Pro, or Thr in the P4 position.

The suggested similarities between the coronavirus 3CL and picornavirus 3C protease was confirmed and extended when the structures of the 3CL protease were determined for several coronaviruses, including SARS (Anand et al. 2002, 2003; Lee et al. 2005; Xue et al. 2008; Yang et al. 2003). The 3CL protease contains three domains, with the two N-terminal domains comprised of a two β-barrel chymotrypsin fold, similar to trypsin/chymotrypsin and the picornavirus 3C protease. The C-terminal domain III is globular, composed of a series of α-helices, and has an unknown function. The crystal structure also confirmed earlier biochemical work that the enzyme is dimeric, with the unusual arrangement of the subunits being perpendicular to each other. Like the picornavirus 3C protease, the 3CL protease replaces the chymotrypsin active site serine with cysteine (Cys145 in the SARS 3CL

protease), which along with His41 makes a catalytic dyad rather than the prototypic catalytic triad. The S1 subsite accommodates Gln, with a specific interaction with a histidine placed at the bottom of the subsite. The hydrophobic P2 side chain is accommodated in a largely hydrophobic S2 subsite. The P3 side chain, in keeping with its poor conservation, is oriented toward the solvent, and the P4 side chain must be a small amino acid to fit into the shallow S4 subsite.

The initial search for an inhibitor of the SARS 3CL protease focused on preexisting drugs and compounds, some tested empirically and others selected based on modeling. The HIV-1 PI nelfinavir and lopinavir/ritonavir have been considered, with the latter actually used clinically in SARS CoV-infected subjects. The rhinovirus inhibitor ruprintrivir and related compounds have also been tested (reviewed in Fear et al. 2007). However, in the absence of any elements of specificity, these preexisting compounds would be expected to have low potency.

More focused efforts on drug development have used either lead compounds identified in the initial screens or started with substrate mimetics. Many of the approaches have used inhibitors that bind irreversibly to the active site Cys and occupy the enzyme subsites, with the most complete studies analyzing enzyme inhibition, inhibition of viral replication, and structural studies of inhibitor bound to enzyme. These studies have included a substrate-based inhibitor with a chloromethyl ketone group (Anand et al. 2003; Yang et al. 2003), substrate-based inhibitors with a Michael Acceptor (Xue et al. 2008; Yang et al. 2005), a peptidomimetic with an aldehyde acceptor (Yang et al. 2006), peptidomimetic inhibitors with a reactive phthalhydrazide group (Yin et al. 2007), and a substrate-based inhibitor with an epoxyketone reactive group (Goetz et al. 2007). This tally represents only a sampling of the drug discovery efforts directed at the SARS CoV 3CL protease.

The appearance of the SARS CoV revealed a new threat to human health. It is now clear that similar viruses, or recombinants of viruses, could emerge from a wide range of animals, analogous to the recurring introductions of influenza virus from migratory bird populations. Thus there may be some advantage to designing less potent inhibitors that have a broad spectrum against coronaviruses, since drug development is a very slow process compared with a spreading epidemic of a respiratory virus. It is likely that being able to blunt the initial acute viremia may be sufficient to provide clinical benefit. Toxicity and potency may be less critical in a short course of therapy compared to treating chronic infections like HIV-1 or HCV. The tools appear to be in place to develop 3CL protease inhibitors that will allow for more targeted clinical intervention in future outbreaks. However, challenge experiments to demonstrate efficacy are not possible, and so potency in model systems and dosing studies in healthy volunteers will likely replace the more lengthy clinical efficacy trials required of typical drugs. In this scenario, SARS CoV inhibitors would be made available during the next epidemic on a compassionate use basis.

Acknowledgments We thank NIH for funding (grants: R01 GM65347 and PO1 GM66524) of the Swanstrom and Schiffer laboratories in the area of viral protease research. Keith Romano and Madhavi Nalam provided assistance with the features. We also thank Leslie Arney and Catherine W. Deschapelles for critical review of the manuscript.

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Anti-Influenza Drugs: The Development of Sialidase Inhibitors

Mark von Itzstein and Robin Thomson

Contents

Abstract Viruses, particularly those that are harmful to humans, are the 'silent terrorists' of the twenty-first century. Well over four million humans die per annum as a result of viral infections alone. The scourge of influenza virus has plagued mankind throughout the ages. The fact that new viral strains emerge on a regular basis, particularly out of Asia, establishes a continual socio-economic threat to mankind. The arrival of the highly pathogenic avian influenza H5N1 heightened the threat of a potential human pandemic to the point where many countries have put in place 'preparedness plans' to defend against such an outcome. The discovery of the first designer influenza virus sialidase inhibitor and anti-influenza drug Relenza™, and subsequently Tamiflu™, has now inspired a number of continuing efforts towards the discovery of next generation anti-influenza drugs. Such drugs may act

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H.-G. Kräusslich, R. Bartenschlager (eds.), *Antiviral Strategies,* Handbook of Experimental Pharmacology 189,

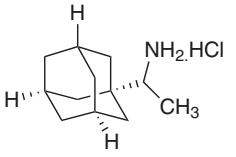
as 'first-line-of-defence' against the spread of influenza infection and buy time for necessary vaccine development particularly in a human pandemic setting. Furthermore, the fact that influenza virus can develop resistance to therapeutics makes these continuing efforts extremely important.

An overview of the role of the virus-associated glycoprotein sialidase (neuraminidase) and some of the most recent developments towards the discovery of anti-influenza drugs based on the inhibition of influenza virus sialidase is provided in this chapter.

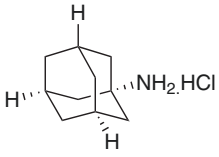
1 Influenza: The Virus and Disease

1.1 General

Influenza over the centuries has had a significant impact on human health and as a result the World Health Organisation (WHO) monitors the appearance of the virus (Kuszewski and Brydak 2000; Taubenberger et al. 2000; Laver and Garman 2002). Until recently, the drugs of choice for the treatment of influenza were the adamantane-based M2 ion channel protein inhibitors, Rimantadine (**1**) and Amantadine (**2**) (Douglas 1990; Wintermeyer and Nahata 1995). These compounds have been useful in the treatment of only influenza A infection, because only the A strains of the virus have an M2 ion channel protein, and they are therefore not effective against influenza virus B strains (Hay 1992; Pinto et al. 1992; Hay et al. 1993). Although these drugs have been found to be effective against influenza virus A infection, both have been reported not only to have significant side-effects (Pinto et al. 1992; Wintermeyer and Nahata 1995), but also lead to the rapid emergence of drug-resistant influenza viral strains (Hay 1992; Hay et al. 1993). As a consequence, these adamantane-based drugs are invariably thought to be of little value as clinically useful therapeutics.



1 Rimantadine

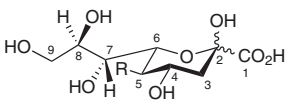


2 Amantidine

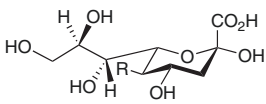
As a result of the emergence of the extremely aggressive avian H5N1 influenza virus, the likelihood of a human influenza pandemic and the possible socio-economic impact is now of major concern. In the absence of strain-independent anti-influenza drugs, there has been significant effort worldwide over the years in the quest for the discovery of novel therapeutic agents against all types of influenza.

1.2 The Virus

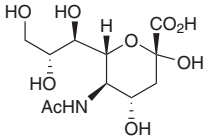
Influenza virus belongs to the orthomyxoviridae family and has a single-stranded, segmented RNA genome. The virus is divided into three serologically distinct types, A, B and C (Webster et al. 1992; Wagner et al. 2002), with types A and B causing disease in human populations (Webster et al. 1997). Influenza A viruses are further sub-divided based on the antigenic properties of the envelope-associated surface glycoproteins, the lectin haemagglutinin (HA) and the glycohydrolase sialidase (neuraminidase, NA) (Webster et al. 1992; Röhm et al. 1996). Both influenza virus surface glycoproteins recognise the sialic acid, *N*-acetylneuraminic acid (Neu5Ac, **3**), found as the terminal carbohydrate unit of upper respiratory tract and lung-associated glycoconjugates (Herrler et al. 1995; Wagner et al. 2002).



3 R = NHAc (Neu5Ac)



3a R = NHAc (α-Neu5Ac)



3a α-Neu5Ac

Haemagglutinin (HA) (Fig. 1) is a trimeric glycoprotein consisting of three identical subunits that are anchored to the virus' lipid membrane. Haemagglutinin is a lectin (Skehel and Wiley 2000; Wagner et al. 2002), and initially acts as the first point of contact for the virus by targeting host cell-surface glycoconjugates containing α-ketosidically-linked terminal *N*-acetylneuraminic acid residues (Couceiro et al. 1993; Skehel and Wiley 2000; Suzuki et al. 2000). Furthermore,

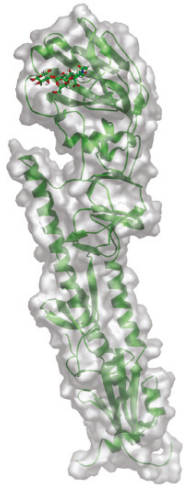


Fig. 1 A view of the influenza virus haemagglutinin (HA) monomer with the receptor fragment α-2,3-sialyllactose [α-Neu5Ac-(2,3)-β-Gal-(1,4)-β-Glc] bound

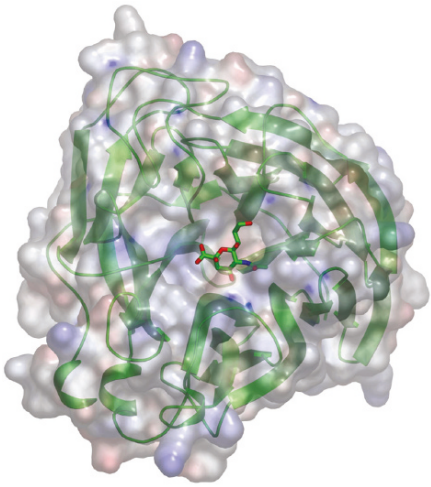


Fig. 2 A view of the influenza virus sialidase (NA) monomer with α-Neu5Ac **3a** bound in the active site

this glycoprotein contributes to the internalisation of the virus through fusion of the viral envelope with the host cell (Skehel and Wiley 2000; Matrosovich and Klenk 2003).

Influenza virus sialidase (Fig. 2) is a tetrameric glycoprotein consisting of four identical subunits (Colman and Ward 1985), and acts as a glycohydrolase that removes α-ketosidically linked terminal *N*-acetylneuraminic acid residues from glycoconjugates.

Both of these surface glycoproteins appear to be essential in the lifecycle of the virus and as such are both considered as drug discovery targets for the development of anti-influenza agents (Meanwell and Krystal 1996a, 1996b; Matrosovich and Klenk 2003). The most significant advances in structure-based anti-influenza drug discovery have arisen from targeting the sialidase function. This chapter reviews the discovery of potent sialidase inhibitors and anti-influenza drugs zanamivir (von Itzstein et al. 1993) and oseltamivir (Kim et al. 1997). Furthermore, an overview of a number of more recent research directions for next generation sialidase inhibitor development is provided.

2 Influenza Virus Sialidase

2.1 Background

Influenza virus sialidase (EC 3.2.1.18) catalyzes the cleavage of terminal α-(2,3 or 2,6)-ketosidically linked *N*-acetylneuraminic acid from a wide range of glycoconjugates, including glycolipids and glycoproteins (Gottschalk 1958; Corfield and Schauer 1982; Corfield et al. 1983). Given the importance of influenza virus

sialidase in the lifecycle of the virus, significant studies on the enzyme have been published over the years (Air and Laver 1989; Colman 1994; Saito and Yu 1995; Matrosovich et al. 2004). The sialidase acts as a pair of biological scissors clipping *N*-acetylneuraminic acid residues both from upper respiratory tract mucins, facilitating movement of the virus (Corfield and Schauer 1982; Herrler et al. 1995; Matrosovich et al. 2004), and from the surface glycoproteins of the newly synthesised virion progeny and the infected host cell surface. The latter action facilitates the release of progeny viruses that would otherwise clump at the infected cell surface through sialic acid–HA interactions and be cleared by the host immune system (Palese et al. 1974b; Palese and Compans 1976).

2.2 Structure and Mechanism

The first X-ray crystal structure of influenza virus sialidase was published in the early 1980s (Colman et al. 1983; Varghese et al. 1983). Several structures of the *apo*-enzyme and complexes with *N*-acetylneuraminic acid **3**, or the naturally occurring sialidase inhibitor 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu5Ac2en, **4**), shown in Fig. 3, have now been published (Baker et al. 1987; Varghese and Colman 1991; Burmeister et al. 1992, 1993; Varghese et al. 1992; Bossart-Whitaker et al. 1993). While the sequence homology between sialidases from influenza A and B viruses is around 30% and between A virus subtypes less than 50%, a number of active site residues are highly conserved between A and B virus and subtypes (Smith and Palese 1989).

The sialidase active site contains a relatively high number of charged amino acids and, in total, there are ten polar (Arg/Asp/Glu) and four non-polar residues that

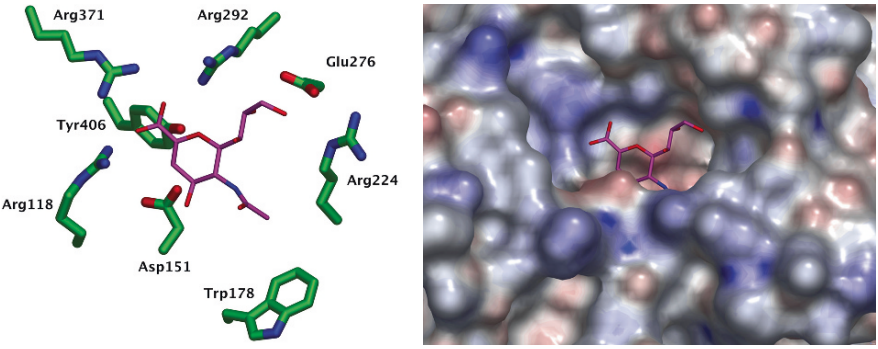
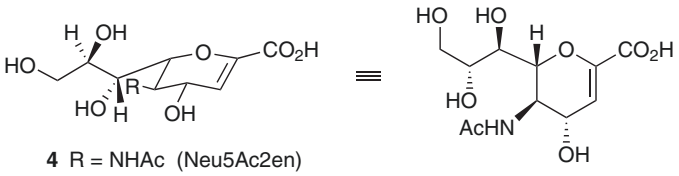


Fig. 3 Sialidase inhibitor Neu5Ac2en **4** bound in the active site of influenza A virus sialidase (from PDB structure 1f8b (Smith et al. 2001)). *Left*: Stick model of **4** surrounded by some important active site residues. *Right*: Electrostatic potential surface rendering of the active site (*blue* – positive, *red* – negative). (Amino acid numbering for influenza A/N2 sialidase is used throughout this review)



appear to be key in the binding of sialic acids and derivatives (Fig. 4) (Colman et al. 1983; Varghese et al. 1992). Both α-Neu5Ac **3a** and Neu5Ac2en **4** are oriented within the active site primarily as a result of charge–charge interactions between the C-1 carboxylate group and a positively charged triarginyl cluster. Additionally, a range of hydrogen bonding, electrostatic and non-polar interactions involving the C-4 hydroxyl group, C-5 acetamido group and glycerol side-chain of sialic acid-based ligands, make important contributions to the overall binding energy. Little, if any, direct role is played by the ring oxygen in positioning or maintaining the substrate within the enzyme's active site (Taylor and von Itzstein 1994).

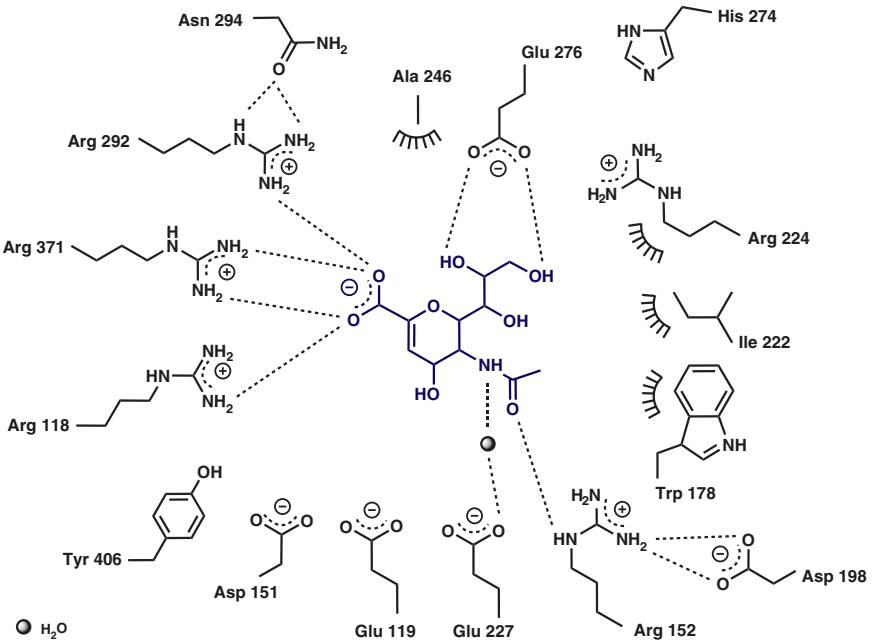
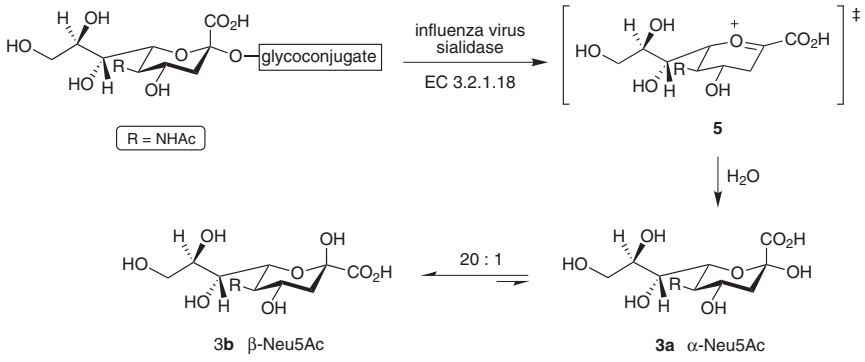


Fig. 4 The influenza A virus sialidase active site showing some key interactions of Neu5Ac2en **4** with conserved residues. Conserved interacting active site residues include Arg118, Arg371, Arg292, Glu276, Arg152 and Glu227. Hydrophobic interactions are made between the C-5 acetamido methyl group and Ile222 and Trp198. Tyr406 and Asp151 are involved in enzyme catalysis

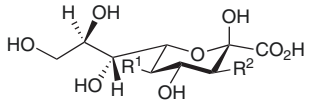
Neu5Ac2en **4**, a putative transition state analogue, inhibits viral and non-viral sialidases alike (Meindl et al. 1974; Holzer et al. 1993), strongly suggesting that sialidases may have a high degree of active site structural homology. Mechanistically, how sialidases act on their substrates has been an area of active research for several decades (Miller et al. 1978; Chong et al. 1992; Thomas et al. 1999; Watson et al. 2003, 2005; Watts et al. 2006). Protein X-ray crystallographic studies of sialidase–α-Neu5Ac **3a** complexes clearly demonstrated that the substrate's conformation distorts from the energetically favoured chair conformation to a twist boat-like conformation upon binding (Varghese et al. 1992). This conformation distortion is facilitated in part by the formation of a salt bridge between the carboxylate and triarginyl cluster (Arg118, Arg371, Arg292) (Varghese et al. 1992; Burmeister et al. 1993). Initially, both kinetic isotope effect measurements and molecular modelling studies suggested that the catalytic reaction proceeds predominantly through an enzyme-stabilised sialosyl cation intermediate **5**, which subsequently reacts stereoselectively with water to afford initially α-Neu5Ac, which then mutarotates to the more thermodynamically stable anomer β-Neu5Ac (Scheme 1) (Chong et al. 1992; Taylor and von Itzstein 1994). In more recent times, a number of studies have shown that while a cation may be formed during the course of a glycohydrolase reaction, the formation of glycosyl–enzyme covalent intermediate appears to be a common feature of large number retaining glycosidases (Ly and Withers 1999; Rye and Withers 2000; Zechel and Withers 2001). In the case of sialidases, irrespective of the source, there is now substantial evidence that a covalent bond is formed between Neu5Ac **3** and a highly conserved active site tyrosine residue positioned directly below the anomeric carbon (C-2) of the *N*-acetylneuraminic acid moiety (Watson et al. 2003; Amaya et al. 2004; Watts and Withers 2004; Watts et al. 2006). However, the fact that Neu5Ac2en (**4**) and structurally related molecules are potent inhibitors of a wide range of sialidases, including influenza virus sialidase, strongly supports the existence of a planar sialosyl cation intermediate **5** at some point along the reaction coordinate.



Scheme 1 The reaction catalyzed by influenza virus sialidase. The initially formed α-anomer **3a** undergoes rapid equilibration with the β-anomer **3b**

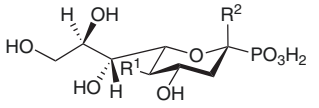
3 Sialidase as a Drug Discovery Target

The role of influenza virus sialidase in the lifecycle of the virus and the conserved nature of the sialidase active site among viral strains has led the enzyme to be considered as an excellent target for antiviral drug design [reviewed, for example, in (Palese and Schulman 1977; Bamford 1995; Wade 1997; Laver and Garman 2002; Wang 2002; von Itzstein 2007, 2008)]. Several approaches to the discovery of sialidase inhibitors from random screening programs (Edmond et al. 1966; Haskell et al. 1970), through to the development of hydrolysis-resistant sialosides that replace the anomeric aglycon oxygen with either nitrogen or sulfur (Khorlin et al. 1970; Suzuki et al. 1990), have been undertaken and much of these developments have been reviewed (Bamford 1995). *N*-Acetylneuraminic acid (Neu5Ac **3**) is itself an inhibitor of sialidases in the millimolar range [K_i 5×10^{-3} M (A/N2)][1] (Walop et al. 1960; Khorlin et al. 1970). Substrate mimetics, including 3-deoxy-3-fluoro-Neu5Ac **6** (Hagiwara et al. 1994), Neu5Ac phosphonate analogues **7** (Chan et al. 1997) and **8** (A/N2) (White et al. 1995), and sialidase resistant thioglycoside **9** (A/N2) (Suzuki et al. 1990), show inhibition constants down to micromolar levels.



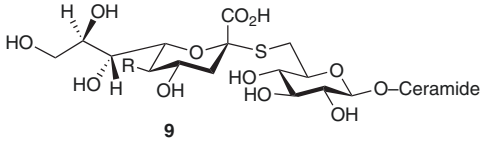
3 R^1 = NHAc, R^2 = H: K_i 5 x 10^{-3} M (A/N2)

6 R^1 = NHAc, R^2 = F: K_i 8 x 10^{-6} M (A/N1)



7 R^1 = NHAc, R^2 = OH: K_i 2 x 10^{-4} M (A/N2)

8 R^1 = NHAc, R^2 = H: IC_{50} ~1 x 10^{-5} M (A/N2)



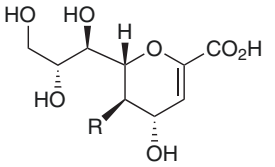
9

K_i 2.8 x 10^{-6} M

Neu5Ac2en **4**, a micromolar inhibitor of influenza virus sialidase [K_i 4×10^{-6} M (A/N2)] (Holzer et al. 1993), was first identified as a very good inhibitor in the late 1960s (Meindl and Tuppy 1969). A series of C-5 modified Neu5Ac2en derivatives provided the first improved in vitro inhibitors compared with the parent compound **4**. The replacement of the C-5 *N*-acetyl moiety with a *N*-trifluoroacetyl group resulted in the most potent inhibitor of this series, 2-deoxy-2,3-didehydro-*N*-trifluoroacetylneuraminic acid **10** [K_i 8×10^{-7} M (A/N1)] (Meindl et al. 1974). While these C-5 modified compounds were also very effective in cell culture assays (Palese et al. 1974a; Palese and Compans 1976), none, including the parent

[1] Inhibition values quoted in this chapter are those from enzyme inhibition assays.

compound **4**, were found to have in vivo activity in an influenza virus infection mouse model. It was reasoned that this may be due to the rapid clearance of these compounds, given their highly polar nature (Palese and Schulman 1977; Nöhle et al. 1982). Furthermore, none of these unsaturated *N*-acetylneuraminic acid derivatives differentiated between sialidases of viral or other origin (Meindl et al. 1974; Holzer et al. 1993).



4 R = $NHC(O)CH_3$ K_i 4 x 10^{-6} M (A/N2)

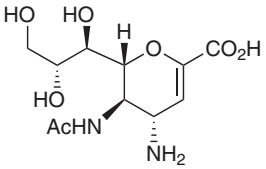
10 R = $NHC(O)CF_3$ K_i 8 x 10^{-7} M (A/N1)

In the following sections, an overview of some of the key developments in the discovery of potent influenza virus sialidase inhibitors is provided. In the first instance, the discovery of the influenza virus sialidase inhibitors that have become the current first-line-of-defence anti-influenza drugs will be described, followed by a description of some of the other important sialidase inhibitor developments to date.

3.1 The Discovery of Zanamivir, the First Potent Designer Anti-Influenza Drug

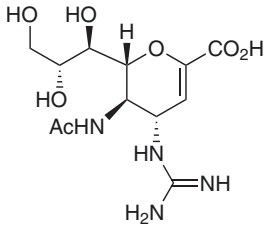
The availability of influenza virus sialidase crystal structures with and without ligands such as α-Neu5Ac **3a** and Neu5Ac2en **4** (Colman et al. 1983; Varghese et al. 1983; Varghese et al. 1992) provided a key turning point in the design of influenza virus sialidase inhibitors. The principles of structure-based drug design enabled the design of the most potent influenza virus sialidase inhibitors ever reported (von Itzstein et al. 1993). Thus, using predictive software programs such as GRID (Goodford 1985), the enzyme's active site was explored for its capacity to favourably accommodate a range of functional groups such as carboxylates, amines, methyl groups and phosphates (von Itzstein et al. 1996). From this study it became clear that replacement of the C-4 hydroxyl group of Neu5Ac2en **4** by a more basic amine group, giving 4-amino-4-deoxy-Neu5Ac2en **11**, should enhance affinity for the active site and improve overall inhibition of the enzyme. Furthermore, it was evident that the C4-hydroxyl binding pocket may also accommodate larger basic groups as an alternative to the amine. Further investigation led to the conclusion that the C-4 guanidino moiety of the Neu5Ac2en derivative **12** could form

substantial interactions with two highly conserved amino acid carboxylate residues (Glu119 and Glu227)[2] via its terminal nitrogens.



11

K_i 5 x 10^{-8} M (A/N2)



12 (zanamivir)

K_i 2 x 10^{-10} M (A/N2)

K_i 7 x 10^{-10} M (B)

Neu5Ac2en derivatives **11** and **12** were readily synthesised and biologically evaluated against sialidases from both influenza virus A and B (Holzer et al. 1993; von Itzstein et al. 1993). Both derivatives were determined to be highly potent, competitive in vitro sialidase inhibitors [**11** K_i 5×10^{-8} M; **12** K_i 2×10^{-10} M (A/N2), 7×10^{-10} M (B)] (von Itzstein et al. 1993). Interestingly, the 4-guanidino derivative **12** exhibited slow binding kinetics that decreased the apparent K_i to 3×10^{-11} M (von Itzstein et al. 1993; Pegg and von Itzstein 1994). Furthermore, both compounds were found to efficiently prevent in vitro and in vivo viral replication (von Itzstein et al. 1993; Woods et al. 1993; Ryan et al. 1994, 1995). Experimentally, both **11** and **12** were found to orient, in general, within the active site in the predicted manner and neither compound caused any rearrangement of amino acids within the catalytic domain. In the case of the guanidino derivative **12**, while the general orientation was in accordance with the predicted binding mode, some specific additional interactions of the guanidinyl moiety were experimentally observed (Fig. 5). Interestingly, the bulkier guanidino substituent was found to displace a water molecule from the C-4 binding domain, accounting for some entropic gain, as well as providing a logical reason for the reported slow binding properties of the compound (von Itzstein et al. 1993). An additional benefit from the installation of the basic functionalities at the C-4 position of the parent compound Neu5Ac2en **4** was that a high selectivity was observed for influenza virus sialidase compared with sialidases from mammalian origin (Holzer et al. 1993; von Itzstein et al. 1993).

GlaxoWellcome (now GlaxoSmithKline) licensed 4-deoxy-4-guanidino-Neu5Ac2en **12** as a lead drug candidate under the generic name zanamivir and gained regulatory approval as a first-in-class sialidase-targeting anti-influenza drug (Relenza™) in 1999. The polar nature of Relenza™ precluded significant oral absorption, and therefore an intraoral inhalation drug delivery method was developed that delivered the drug to the primary site of infection.

[2] Numbering used throughout reflects that reported for influenza virus A N2 sialidase.

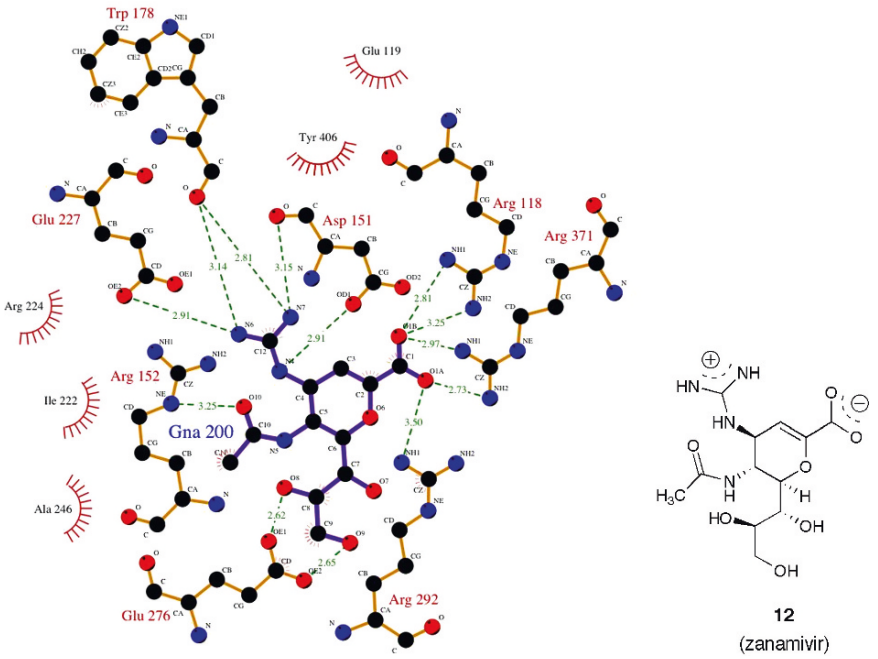


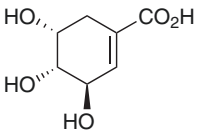
Fig. 5 Key interactions of 4-deoxy-4-guanidino-Neu5Ac2en (zanamivir) **12** with the active site of influenza A virus sialidase [Figure generated from crystal structure data (PDB – 1nnc) using LIGPLOT (Wallace et al. 1995)]. To the right is shown zanamivir **12** in the same orientation

3.2 The Discovery of Oseltamivir

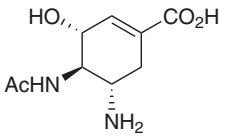
The available structural information for complexes between influenza virus sialidase and zanamivir **12**, Neu5Ac2en **4** and other related molecules indicated that the dihydropyran ring oxygen of these compounds does not appear to play key roles within the active site for substrate binding or catalysis (Taylor and von Itzstein 1994). Thus, it seemed reasonable that replacement of the ring oxygen atom with carbon would not have any impact on the binding affinity. Furthermore, replacement of the ring oxygen may provide opportunities to introduce new functionality that could lead to inhibitor–protein interactions or positively influence the drug-like properties of the resultant compound. Consequently, carbocyclic, cyclohexene-based templates derived from shikimic acid (**13**) were selected as a framework for the development of new sialidase inhibitors (Kim et al. 1997).

A potential major benefit of a cyclohexene-based template was the capacity to mimic more closely the putative transition state sialosyl cation by the placement of a double bond in the correct position. While the ring conformation in Neu5Ac2en **4** is very similar to the sialosyl cation transition state **5**, these two structures are essentially isomers due to the fact that the double bond is either between the ring oxygen and C-2 in **5** or C-2 and C-3 in **4**. The synthesis and biological evaluation of

the precursor alcohols **14** and **15**, having carboxylate, amino, and acetamido groups placed to mimic those of 4-amino-4-deoxy-Neu5Ac2en **11**, provided support for the notion that the position of the double bond in a cyclohexene-based template was important. Compound **14**, with the position of the double bond mimicking that of sialosyl-cation **5**, inhibited influenza A virus sialidase at micromolar levels (IC_{50} 6.3×10^{-6} M), while its positional isomer **15** failed to inhibit the enzyme at concentrations up to 0.2 mM (Kim et al. 1997). This data correlates with the relative inhibitions of the carbocyclic analogues of carbocation **5** (**16**) and of Neu5Ac2en **4** (**17**) against influenza A virus sialidase, where **16** exhibited 40-fold stronger inhibition than the direct Neu5Ac2en analogue **17** (Vorwerk and Vasella 1998).

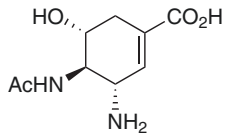


13



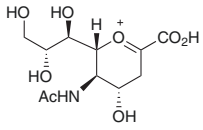
14

IC_{50} 6.3×10^{-6} M (A/N1)

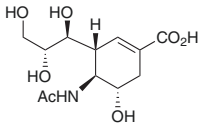


15

IC_{50} $> 2 \times 10^{-4}$ M (A/N1)

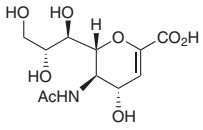


5

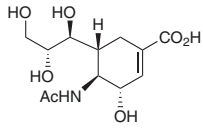


16

IC_{50} 2×10^{-5} M (A)



4

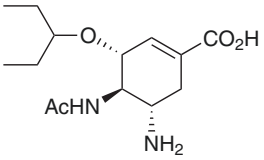


17

IC_{50} 8.5×10^{-4} M (A)

The hydrophobic backbone of the glycerol side-chain in *N*-acetylneuraminic acid-based inhibitors **4** and **12** makes contact with the enzyme's active site. This fact provided hope that wholesale replacement of the very hydrophilic glycerol side-chain with an alkyl substituent may produce a sialidase inhibitor, if the alkyl group was tolerated, with more hydrophobic character that could improve oral bioavailability. To explore this potential, systematic functionalisation of the hydroxyl group of **14**, at the position equivalent to the glycerol side-chain of **4**, with simple hydrophobic ethers was investigated. These studies confirmed that it was possible to replace the glycerol side-chain with a lipophilic moiety and maintain significant influenza virus sialidase inhibition (Kim et al. 1997), with the length, branching and stereochemistry of the alkyl substituent affecting the compound's inhibitory activity. Substantial structure activity relationship (SAR) studies, including variation of the original amino and acetamido substituents of **14** (Kim et al. 1998) (reviewed in Kim et al. 1999; Lew et al. 2000; von Itzstein 2007, 2008), led to the development of optimised structure **18** (GS 4071, oseltamivir carboxylate), with a 3-pentyl ether side-chain and the carboxylate, amino and acetamido groups mimicking 4-amino-4-deoxy-Neu5Ac2en **11**. GS 4071 (**18**) was found to be a potent influenza virus sialidase inhibitor (Kim et al. 1997, 1998), which

displays slow binding kinetics and similar levels of efficacy against influenza A and B sialidases [IC_{50} 1×10^{-9} M (A/N1); 3×10^{-9} M (B)] (Kim et al. 1998). The corresponding guanidino derivative showed only marginally more potent inhibition [IC_{50} 0.5×10^{-9} M (A/N1)] than **18** (Kim et al. 1998).

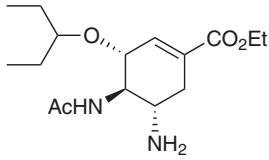


18

IC_{50} 1 x 10^{-9} M (A/N1)

IC_{50} 3 x 10^{-9} M (B)

GS 4071 (oseltamivir carboxylate)



19

GS 4104 (oseltamivir)

The crystal structure of GS 4071 (**18**) in complex with influenza virus sialidase showed that the cyclohexene ring is oriented within the sialidase active site as expected, and key interactions involving the carboxylate, amino and acetamido substituents with the sialidase active site are similar to those observed for Neu5Ac2en and derivatives (Kim et al. 1997, 1998). The 3-pentyl side-chain, however, is accommodated in the glycerol side-chain binding pocket by virtue of a reorganisation of the active site in this region: the side-chain of Glu276 (that interacts with the C-8 and C-9 hydroxyl groups of *N*-acetylneuraminic acid-based inhibitors) is reoriented outwards from the glycerol side-chain binding domain to form a salt-bridge formation with Arg224 (Fig. 6). This rearrangement generates a substantial hydrophobic patch within this region and enables the accommodation of the hydrophobic side-chain.

Despite the presence of the more lipophilic side-chain in **18**, compared to the glycerol side-chain of **12**, a pro-drug strategy was necessary to achieve sufficient oral bioavailability. Thus, the ethyl ester pro-drug form of **18**, oseltamivir (GS 4104, **19**), is now marketed by Roche, under the tradename Tamiflu^{TM}, as an orally administered treatment for influenza virus infection [reviewed in (Doucette and Aoki 2001; Oxford 2005)]. Post absorption, **19** is enzymatically cleaved by esterases in blood and tissue (Eisenberg et al. 1997; Shi et al. 2006) to liberate the active parent compound, **18**.

3.3 Further Developments of Influenza Virus Sialidase Inhibitors

The discovery of the potent in vitro sialidase inhibitory activity and in vivo efficacy of zanamivir **12**, and the increasing availability of 3D structural data for influenza virus sialidases in the 1990s, particularly with Neu5Ac and various inhibitors bound into the active site, provided a platform for further drug discovery efforts targeting

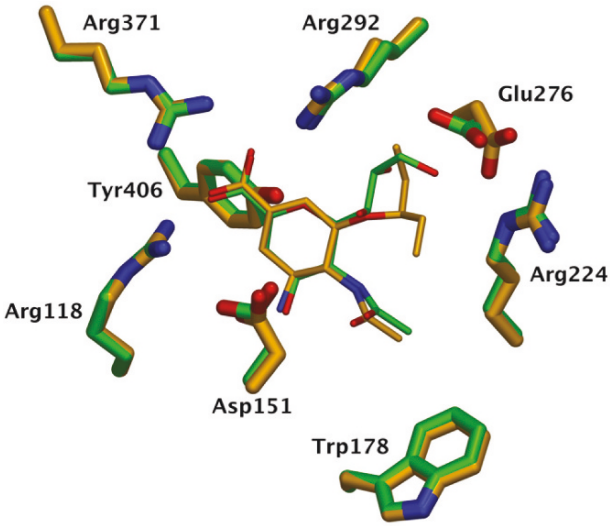


Fig. 6 Superimposition of inhibitors and key active site residues from crystal structures of oseltamivir carboxylate **18** (*brown carbons*, PDB – 2qwk) and Neu5Ac2en **4** (*green carbons*, PDB – 1f8b) in complex with influenza A virus sialidase. Note the alternative conformations of the side-chain of Glu276

the viral sialidase. The subsequent development of oseltamivir carboxylate **18**, with the significant introduction of a hydrophobic side-chain and oral availability of the ethyl ester pro-drug **19**, highlighted further possibilities for inhibitor development. The following section presents a description of the features of the active site that are targeted in designing potent inhibitors, followed by a discussion of some of the more promising recent findings and developments in anti-influenza drug design targeting the viral sialidase that may lead to next generation anti-influenza drugs.

3.3.1 Analysis of Potential Binding Interactions within the Influenza Virus Sialidase Active Site

The active site of influenza virus sialidase is composed of a number of discrete adjoining binding pockets. To facilitate a description of the binding modes of the substrate and known competitive inhibitors of influenza virus sialidase, and as a blueprint for further inhibitor design, the active site can be divided into five regions or subsites (S1–S5) (Stoll et al. 2003) (Fig. 7). Subsites S1, S2, S3 and S5 are occupied by the natural Neu5Ac substrate, while subsite S4 is primarily a hydrophobic region (formed by the side-chains of Ile222, Ala246 and the hydrophobic face of Arg224) and is not occupied by any portion of the sialic acid-based inhibitors. Importantly, the reorientation of Glu276 in the presence of hydrophobic side-chains, as seen with oseltamivir carboxylate **18**, causes the formation of a hydrophobic pocket (bordered by the methylenes of Glu276 and Ala246) within subsite S5. Potential

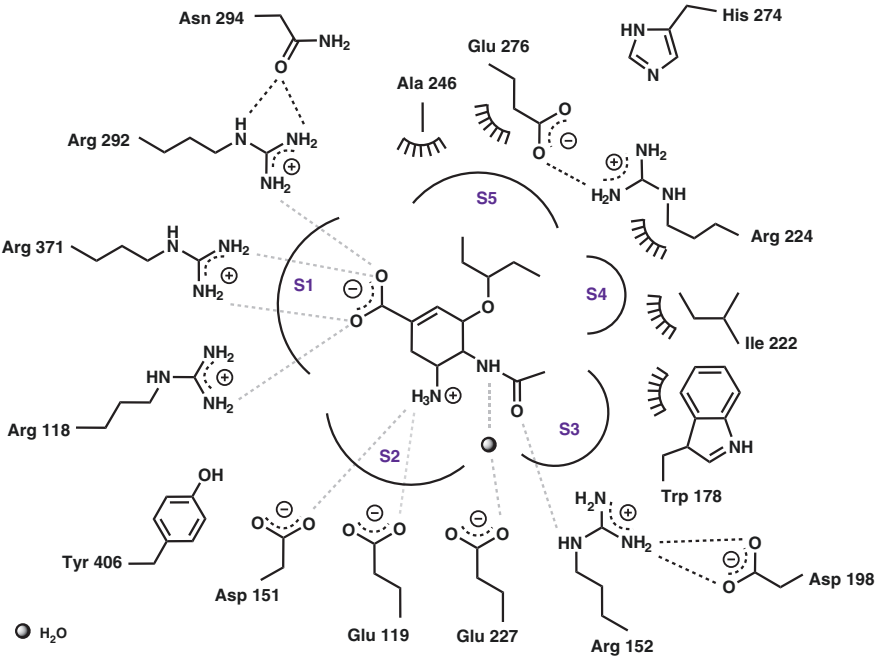
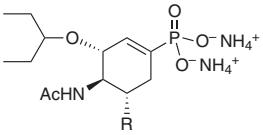


Fig. 7 The influenza virus A sialidase active site showing the five potential inhibitor binding subsites (with S5 containing the hydrophobic pocket formed by reorientation of the Glu276 sidechain), with oseltamivir carboxylate **18** placed in the active site

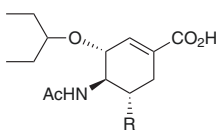
interactions with the five subsites have been taken into account in the design and development of the more recent examples of potent influenza virus sialidase inhibitors described further. A three-dimensional pharmacophore model for predicting sialidase inhibitory activity, developed using 22 known influenza sialidase inhibitors, has also been recently reported (Zhang et al. 2006). Such a model could prove helpful in identifying novel, structurally diverse lead compounds through 3D database searches, and be useful in designing novel sialidase inhibitors.

The principal interactions that anchor the substrate Neu5Ac **3** into the sialidase active site are with subsites S1 and S3. Interaction of the carboxylate group of Neu5Ac **3** to the triarginyl cluster in subsite S1 has been calculated to contribute significantly $(\sim 50\%)$ to the overall binding energy (Taylor and von Itzstein 1994). On the "opposite" side of the active site, the C-5 acetamido group of Neu5Ac orients effectively into the defined pocket of subsite S3, with hydrogen bonding interactions from the acetamido NH and carbonyl groups, and hydrophobic interactions between the acetamido methyl group and the nonpolar region formed by Ile222 and Trp178 (Varghese et al. 1992). It is not surprising therefore that, despite extensive SAR studies, the features of a carboxylic acid and "opposing" acetamido group have been maintained in virtually all potent sialidase inhibitors developed to date.

The possibility of using a phosphonate in place of the carboxylate group was recently explored for oseltamivir carboxylate **18**, with the phosphonate analogue **20** showing improved inhibitory activity relative to **18** against wild type N1 sialidases (Shie et al. 2007). Interestingly, the corresponding phosphonate guanidino analogue **21** showed significantly greater inhibition than the carboxylate form **22**, against the His274Tyr variant A/N1 sialidase that appears under drug pressure from oseltamivir in the clinic.



20 R = NH_2 IC_{50} 0.3 x 10^{-9} M (A, N1)
IC_{50} 526 x 10^{-9} M (A, Mut N1)



18 R = NH_2 IC_{50} 5.9 x 10^{-9} M (A, N1)
IC_{50} 295 x 10^{-9} M (A, Mut N1)

21 R = NHC(NH)NH_2 IC_{50} 0.12 x 10^{-9} M (A, N1)
IC_{50} 7.4 x 10^{-9} M (A, Mut N1)

22 R = NHC(NH)NH_2 IC_{50} 4.1 x 10^{-9} M (A, N1)
IC_{50} 252 x 10^{-9} M (A, Mut N1)

The significant advances made in the design and development of potent and selective influenza virus sialidase inhibitors have arisen through targeting and optimising interactions with the active site in the opposing S2 and S4/S5 subsites. Subsite S2, containing the two negatively charged glutamate residues, Glu227 and Glu119, is occupied by the C-4 hydroxyl group of substrate Neu5Ac **3**. This subsite has been exploited through the incorporation of basic amino or guanidino groups in most of the designed inhibitors. Interestingly, however, in inhibitors that incorporate hydrophobic side-chains that bind efficiently in subsites S4 and S5, the substituent binding in subsite S2 (e.g. hydroxyl vs. amino vs. guanidino) does not have as pronounced an effect on the strength of inhibition as it does in inhibitors with a glycerol side-chain (Lew et al. 2000; Wyatt et al. 2001). This may suggest a balance in the contribution to binding between interactions in the opposing S2 and S4/S5 subsites.

The glycerol side-chain of Neu5Ac **3** interacts through the C-8 and C-9 hydroxyl groups with Glu276 in subsite S5. Replacing the very hydrophilic side-chain, with the objective of creating a drug with improved pharmacokinetic properties, is an area of great interest. The observed reorientation of residue Glu276 in the presence of hydrophobic side-chains to create, with Ala246, a hydrophobic pocket within subsite S5, seen during the development of oseltamivir carboxylate **17**, created a new area to target in anti-influenza drug development. Attempts to incorporate either modifications on the glycerol side-chain, or its replacement with more hydrophobic groups, which still capture significant interactions with the NA active site residues, have been a part of all inhibitor development programmes reported (for some examples, see the following sections).

An interesting feature of the influenza virus sialidase active site that offers the potential for developing inhibitors specific for N1 sialidases, including avian influenza A/H5N1 virus sialidase, has recently been revealed by X-ray crystallography. The

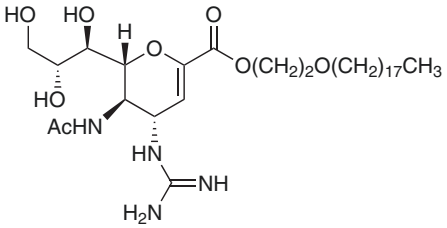
N2 and N1 sialidases of the influenza A viruses currently circulating in humans belong to two phylogenetically distinct groups (Russell et al. 2006): group-1 (N1, 4, 5 and 8) and group-2 (N2, 3, 6, 7 and 9). Until recently, structure-based design of sialidase inhibitors has been carried-out using the three-dimensional structures of sialidases from group 2 and influenza B sialidase. With the recent solution of the crystal structures of a number of group 1 sialidases, including the N1 sialidase from H5N1 avian influenza virus (Russell et al. 2006), it became apparent that the two groups were structurally distinct. Structures of the group-1 sialidases show a flexible protein loop (Amaro et al. 2007) (the so-called 150 loop) and a cavity (the "150 cavity") adjacent to the active site (Russell et al. 2006): this area of the protein has always been seen in the "closed" form in group-2 sialidase structures. The 150 loop is in a more open orientation in the *apo* structure, and so presents a larger active site cavity, but has been shown to close to tightly coordinate bound oseltamivir carboxylate **18**. Other distinctions include a difference in the position of the conserved Asp151, and an altered conformation for conserved Glu119, both in subsite S2. The more open enzyme architecture in the *apo* and initially inhibitor-complexed structures provides new opportunities for inhibitor design. As can be seen from the inhibition data shown throughout the chapter, sialidase inhibitors developed to date have routinely been assayed against influenza A/N1 sialidases, and shown comparable inhibition to A/N2 sialidases; however, none have so far tapped into the potential of the "150 cavity."

3.3.2 Further Developments on the Neu5Ac2en Template: Second-Generation Zanamivir

In the search for potent and selective sialidase inhibitors, significant research has been dedicated to the manipulation of every position on the Neu5Ac2en template. In particular, extensive SAR studies were carried-out on this template during, and after, the development of zanamivir **11**. These studies have been thoroughly reviewed elsewhere (Bamford 1995; Rich et al. 2007). Further developments on the 4-guanidino-4-deoxy-Neu5Ac2en (zanamivir) template, driven by the drug's polar nature that limits its oral bioavailability (Ryan et al. 1994), have been principally directed towards producing derivatives with improved pharmacokinetic properties.

A pro-drug approach for improving the pharmacokinetics of zanamivir has recently shown some promise. The alkoxyalkyl ester **23** of zanamivir, with long alkyl chains chosen to counteract the high hydrophilicity of the molecule, was reported to show significant protective effects against influenza (H1N1) infection in mice upon oral or intraperitoneal administration (Liu et al. 2007).

The majority of studies aimed at developing "second generation" zanamivir with increased lipophilicity, however, have involved replacement or modification of the glycerol side-chain. Significant changes to this side-chain have involved its replacement with a carboxamide function substituted with hydrophobic groups (**24**) (Smith et al. 1998), with cyclic ethers (**25**) (Masuda et al. 2003a), and functionalisation through the C-7 hydroxyl group leading, for example, to multivalent species (**26**) (Watson et al. 2004).



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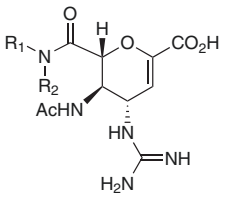
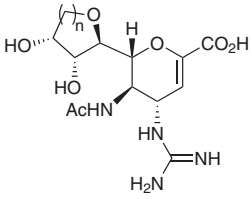
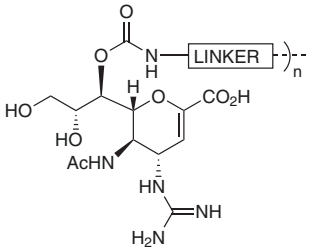


24 R_1, R_2 = H, alkyl, aryl



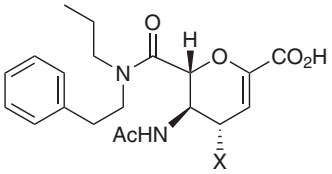
25 n = 1, 2, 3



26 n = 1, 2, 3

The C-6 carboxamide analogues of zanamivir, represented by the general structure **24**, provided an avenue to introduce more hydrophobic side-chains onto the dihydropyran scaffold to interact with the hydrophobic regions of subsites S4 and S5 (reviewed in Islam and von Itzstein 2007). The most active tertiary amides (**24** : R^1, R^2 = alkyl) showed comparable inhibitory activity to their glycerol side-chain counterparts, against influenza A virus sialidase, and good efficacy in a mouse model of influenza A virus infection when delivered intranasally (Smith et al. 1998). As seen with oseltamivir carboxylate **17**, the lipophilic amides induced a conformational reorganisation of the glycerol side-chain binding pocket of both influenza A and B virus sialidases (Smith et al. 1996). Optimal inhibitory activity obtained with one large and one small (ethyl or propyl) alkyl substituent on the tertiary amide derivatives (e.g. **27**) could be explained by the binding of the smaller chain into the small lipophilic pocket (subsite S5) created by reorientation of Glu276, and the larger chain along an extended lipophilic cleft formed between Ile222 and Ala246 at the enzyme surface (Smith et al. 1996). Interestingly, for the most active carboxamides examined, there was a reduced effect on inhibitory potency of the substituent at C-4 in compounds with effective S4/S5 subsite binding (see, e.g. **27**) (Wyatt et al. 2001).

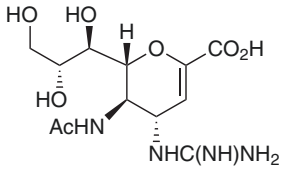
Importantly, there was a general marked selectivity for inhibition of influenza A over influenza B viral sialidases in the carboxamide series (e.g. as seen with **27**) (Smith et al. 1996, 1998), determined from crystallographic and molecular modelling studies (Smith et al. 1996; Taylor et al. 1998) to be due to the relative abilities of each of the sialidases to absorb the structural changes required to accommodate the hydrophobic alkyl chains in the glycerol side-chain binding pocket. In influenza



27 X = H, OH, NH_2, $NHC(NH)NH_2$

IC_{50} 3 to 7 x 10^{-9} M (A/N2)

IC_{50} 1 to 51 x 10^{-6} M (B)



12 (zanamivir)

IC_{50} 2 x 10^{-9} M (A/N2)

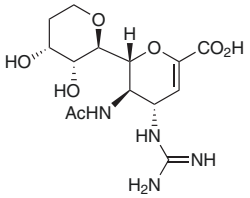
IC_{50} 4 x 10^{-9} M (B)

A sialidase, the rearrangement of the Glu276 side-chain requires little or no distortion of the protein backbone (Smith et al. 1996; Taylor et al. 1998). However, in influenza B sialidase, the required changes are energetically less favourable. While not being pursued further as drug candidates, the carboxamide series of inhibitors highlighted a number of features of the active site for consideration in further drug design efforts, in particular, with inhibitors bearing hydrophobic side-chains.

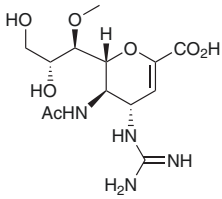
Functionalisation Through the C-7 Position of Zanamivir: The Development of Long Acting Sialidase Inhibitors

In zanamivir, as described earlier, the C-8 and C-9 hydroxyl groups form important interactions with Glu276; however, the C-7 hydroxyl group of the glycerol side-chain makes no direct interactions with the protein and is exposed to bulk solvent (Varghese et al. 1992; Taylor and von Itzstein 1994). This fact led to interest in the development of C-7 functionalised zanamivir derivatives with the aim of increasing compound lipophilicity, while retaining the important contributions of the C-8 and C-9 hydroxyl groups to the overall binding to influenza virus sialidase. This research has generated compounds that have improved pharmacokinetic properties over zanamivir and that show promise as next generation influenza virus sialidase inhibitors.

The glycerol side chain of zanamivir has been replaced by hydroxy-substituted cyclic ethers (e.g. see **25**), in which the C-7 oxygen atom forms part of the ring (Masuda et al. 2003a). When the positional and stereochemical arrangement of ether linkage and the hydroxyl groups on the ring mimicked that of the glycerol side-chain, the compounds were found to be effective inhibitors of sialidase activity and viral growth. The most potent derivative, **28**, showed comparable activity to zanamivir **12** against influenza A virus in both enzyme and plaque reduction assays. Significantly, although this compound may be expected to have only slightly altered hydrophilicity compared to zanamivir, it was reported to have in vivo efficacy by oral administration in a mouse model of influenza A (N1) virus infection, which was similar to that of oseltamivir **19** (based on median survival time) (Masuda et al. 2003a).



28

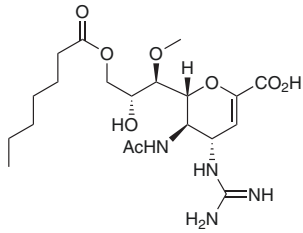


29 (R-125489)

IC_{50} 2.5 x 10^{-9} M (A/N1)

IC_{50} 10 x 10^{-9} M (A/N2)

IC_{50} 16 x 10^{-9} M (B)



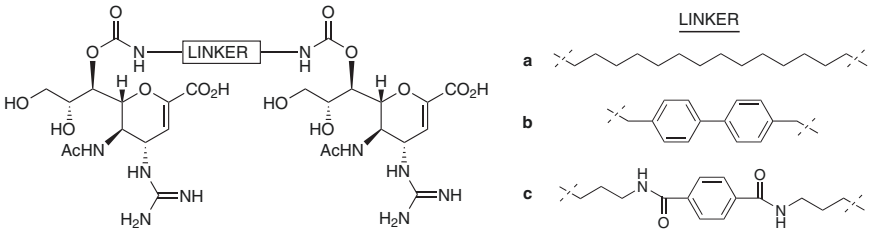
30 (R-118958, CS8958)

A number of studies have investigated the alkylation or acylation of the C-7 hydroxyl group of zanamivir **12**, or an amine in this position (Honda et al. 2002b), with a view to increase lipophilicity. Low nanomolar inhibition, comparable to zanamivir, of influenza A virus sialidase in enzyme assay and viral replication in cell culture was maintained in derivatives *O*-alkylated with alkyl chains, including functionalised alkyl chains, up to 12 carbon atoms in length (Honda et al. 2002a). Similar efficacy was found for a number of *N*-monoalkylated C-7 carbamate derivatives (e.g. of seven atoms in length) (Andrews et al. 1999). These studies demonstrated that the 7-position in the glycerol side-chain could be functionalised with groups of differing size and functionality while retaining potent antiviral activity.

The simple 7-*O*-methyl ether of zanamivir **29** (R-125489) showed comparable sialidase inhibitory potency and slightly improved activity in cell culture to zanamivir (Honda et al. 2002a; Yamashita et al. 2003; Yamashita 2004). A more lipophilic pro-drug form **30** (R-118958, CS8958) of R-125489 **29**, formed by acylation of the C-9 hydroxyl group of **29**, showed a strong protective effect in mice when administered intranasally 4 days prior to infection (with some efficacy also shown 7 days prior to infection), significantly more effective than zanamivir in the same situation (Yamashita 2004). Possibly contributing to this prolonged efficacy in mice was an observed increase in residence time in the lungs after intranasal administration. Prolonged retention of drug in humans was also observed, with the active compound detected in urine of healthy human subjects for more than six days after inhalation of **30** (Yamashita 2004) (reviewed in Klumpp 2004). R-118958 **30** has potential as a long-acting, inhaled, influenza virus sialidase inhibitor, for a once-only and once-weekly prophylactic protection from influenza. At the time of writing, R-118958 is being forwarded in a joint venture between Sankyo (Japan) and Biota (Australia) and successfully completed a Phase I clinical trial in Japan in 2007.

The retention of potent enzyme inhibition and in vitro antiviral activity in the presence of functionalisation with relatively long hydrophobic chains at C-7 of zanamivir has been exploited in the development of di- and poly-valent structures carrying zanamivir (reviewed in Sun 2007). Multivalent presentation of drug may

provide increased binding affinity to the tetrameric sialidase or cross-linking of sialidase proteins. Polymeric display of zanamivir connected to a poly-L-glutamine backbone (Honda et al. 2002c; Masuda et al. 2003b), or more distinct di- (**31**) (Macdonald et al. 2004, 2005), tri- and tetra-meric (Watson et al. 2004) displays on various scaffolds have been investigated. The activity of the multimeric compounds was affected by the length and composition of the linker between zanamivir and the polymer support (Masuda et al. 2003b), or the adjacent zanamivir unit (Macdonald et al. 2004, 2005). The multimeric compounds were generally found to have significantly greater relative inhibitory potency, after correction for valency, than monomeric zanamivir in cell-based viral replication assays (Masuda et al. 2003b; Watson et al. 2004; Macdonald et al. 2005), and consistently showed long-lasting protective effects in mice after intranasal administration.



31

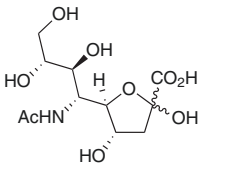
Among the dimeric constructs, a linker length of 16–18 atoms (~20–23 Å) (e.g. **31a**) was found to be optimal, producing inhibitors of influenza virus replication in vitro and in vivo approximately 100-fold more potent than zanamivir, after valency correction (Macdonald et al. 2004). These dimers were able to cause aggregation of isolated sialidase tetramers and whole virus (Macdonald et al. 2004). In general, the dimeric compounds have short to moderate plasma half lives, low clearance and low volumes of distribution. They have no measurable bioavailability after oral dosing, but have high bioavailability after intratracheal dosing in rats (Macdonald et al. 2005). Importantly, the zanamivir dimers provided outstanding protective effects in a mouse model of influenza infection, using a single intranasal dose at significantly lower drug level than that of zanamivir (Macdonald et al. 2004). They also showed extremely long residence times in the lungs of rats, with 100-fold greater concentration than zanamivir at 168 h. These characteristics suggest the possibility for the development of an inhibitor requiring only a single low dose, once a week, for the prevention or treatment of influenza virus infection (Macdonald et al. 2004). In comparison, zanamivir is given twice daily for 5 days. The zanamivir dimers are currently being further developed as long-acting, inhaled sialidase inhibitors (under the class name Flunet®) in a joint venture between Biota (Australia) and Sankyo (Japan).

3.3.3 Inhibitor Design Using Five-Membered Ring Templates

The influenza virus sialidase inhibitors zanamivir and oseltamivir were developed on the 5,6-dihydro-4H-pyran and cyclohexene scaffolds, respectively, with a view to have an inhibitor in which the central ring mimicked the shape of the putative sialosyl cation **5** transition state of the enzyme reaction. Subsequently, there have been a number of research efforts directed at designing potent inhibitors, where alternate cyclic structures, for example an aromatic ring (Chand et al. 2005b), provide a scaffold to appropriately position substituents to interact with the active site (reviewed in Wang 2002; Islam and von Itzstein 2007). Two drug-discovery programmes that have produced potent and selective sialidase inhibitors based on the five-membered ring cyclopentane and pyrrolidine scaffolds, which have less resemblance to the transition state structure **5**, but rather sought to optimise binding with the known subsites of the active site are described later.

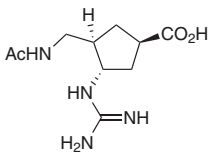
Cyclopentane-Based Inhibitors: The Development of Peramivir

Work in the early 1990s (Yamamoto et al. 1992) showed that the furanose isomer **32** of Neu5Ac gave inhibition of influenza A viral sialidase that approached that of Neu5Ac2en **4** (Babu et al. 2000). Superimposition of crystal structures of **32** and Neu5Ac2en **4** in complex with influenza A virus N9 sialidase (Babu et al. 2000) subsequently revealed that, despite the different positioning of the central ring of each inhibitor in the active site, the four functional groups (carboxylic acid, acetamido group, "C-4" hydroxyl group and the glycerol side-chain) in both the complexes had the similar relative orientations in the active site and similar interactions with the enzyme. This observation led to the conclusion that potent inhibition of influenza virus sialidases depends not on the absolute position of the central ring, but rather on the relative position of the functional groups within the active site (Babu et al. 2000).



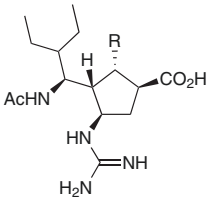
32

IC_{50} 40 x 10^{-6} M (A/N9)



33

IC_{50} 115 x 10^{-6} M (A/N9)



34 R = OH (peramivir)

IC_{50} < 1 x 10^{-9} M (A/N2); 5 x 10^{-9} M (B)

35 R = H

The inhibitory activity of furanosyl nonulosonic acid derivative **32**, and its structure with N9 sialidase, served as a starting point for the structure-based design of

a new class of cyclopentane-based sialidase inhibitors at BioCryst Pharmaceuticals (Babu et al. 2000; Chand et al. 2001). An iterative approach, involving synthesis of target structures as racemic mixtures, followed by crystallisation with N9 sialidase to identify the active isomer, was used to drive the development of potent and selective inhibitors. The relatively simple trisubstituted cyclopentane **33** incorporating three of the key substituents of zanamivir, the carboxylate, the C-5 acetamido group and the C-4 guanidino group to exploit charged interactions in the C-4 pocket, which all occupied the expected positions in the N9 sialidase active site, served as a starting template (Babu et al. 2000). Two iterations of inhibitor design and synthesis focussed on the incorporation, and optimisation, of a side-chain to take advantage of hydrophobic interactions with the S4 and S5 subsites of the active site, as exploited in the development of the cyclohexene-based oseltamivir **19** and the pyranose-based carboxamides **24**. Ultimately, a branched 2′-ethylbutyl side-chain (with analogy to the 3-pentyl side-chain of oseltamivir **19**) was incorporated to take advantage of both the hydrophobic pocket created by the reorientation of Glu276 (S5) and the hydrophobic surface of S4. Compounds **34** and **35** both showed potent inhibition of influenza A and B virus sialidases at the nanomolar level (Chand et al. 2001). As a result of the synthetic approach taken to the enantiomerically pure analogues, compound **34** was more readily synthesised and was therefore chosen for further biological evaluation (Chand et al. 2001).

Further SAR studies on the cyclopentane scaffold have included variation of the hydrophobic side-chain to incorporate a carboxamide substituent (Chand et al. 2004), equivalent to the C6-carboxamide derivatives of zanamivir, and extension of the length of the hydrophobic side-chains (Chand et al. 2005a). Analogues that incorporate a longer 4-heptyl side-chain showed comparable efficacy to **34** upon oral and intranasal administration in mice, and comparable or better efficacy than oseltamivir and zanamivir (Chand et al. 2005a).

Importantly, the crystal structure of **34** complexed with N9 sialidase (Fig. 8) indicated differences in the orientation of the guanidino group in subsite S2, and in its interaction with the active site residues, compared to that of zanamivir (Babu et al. 2000). These differences have implications for "cross-reactivity" of **34** with zanamivir-resistant influenza viruses that have Glu119 mutations in the sialidase S2 subsite (see Sect. 5.1).

Compound **34** (BCZ-1812, RWJ-270201, peramivir) showed selective inhibition of influenza virus sialidases over bacterial and mammalian sialidases (Babu et al. 2000; Bantia et al. 2001; Sidwell and Smee 2002). Successful inhibition of influenza virus infectivity in vitro (Smee et al. 2001) and upon oral administration in vivo [mice (Bantia et al. 2001) and ferrets, reviewed in Sidwell and Smee 2002] led to human clinical trials of orally administered peramivir (Barroso et al. 2005). While orally administrated peramivir successfully completed animal studies and Phase I and Phase II clinical trials, in which the compound was showing neither major side effects nor toxicity (Sidwell and Smee 2002), preliminary results of the Phase III trials (June 2002) "demonstrated no statistically significant difference in the primary efficacy endpoint," possibly due to low bioavailability (Barroso et al. 2005).

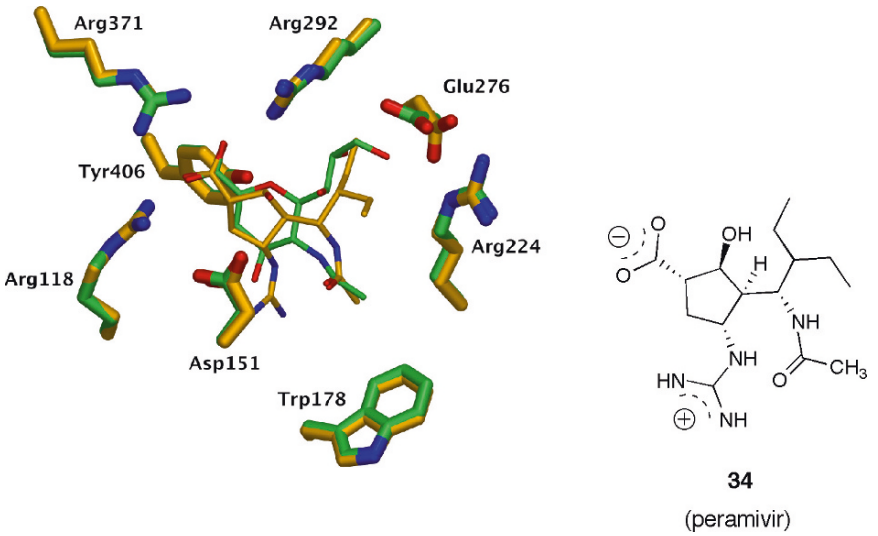


Fig. 8 Superimposition of inhibitors and key active site residues in influenza A virus sialidase: cyclopentane-based inhibitor peramivir **34** (*brown carbons*, PDB – 1l7f), Neu5Ac2en **4** (*green carbons*, PDB – 1f8b). Note the overlap of the carboxyl and acetamido-methyl groups of the inhibitors, and the alternative conformations of the side-chain of Glu276. To the right is shown peramivir **34** oriented as in the crystal structure

Subsequently, the administration of peramivir using both intravenous (BioCryst) and intramuscular (Bantia et al. 2006) formulations has been successfully evaluated in pre-clinical animal models and in Phase I clinical trials (BioCryst), and is currently being evaluated in phase II clinical trials in infected humans. Preliminary studies in mice showed that a single intramuscular injection of peramivir significantly reduced weight loss and mortality in mice infected with influenza A/H5N1, where oseltamivir did not show efficacy using the same intramuscular treatment regime (Bantia et al. 2006). Furthermore, it was demonstrated that the efficacy of a single intramuscular injection of peramivir was comparable to a 5-day course of orally administered oseltamivir (Bantia et al. 2006). In early 2006, the US Food and Drug Administration granted a fast-track designation of peramivir in an injectable formulation.

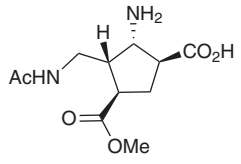
Pyrrolidine-Based Inhibitors: The Development of A-315675

One of the largest classes of inhibitors for glycosyl hydrolases are the imino sugars (El Ashry et al. 2000; Lillelund et al. 2002), in which a nitrogen atom is placed so as to be able to mimic the partial positive charge that is developed on the carbohydrate ring oxygen or at C-1 during glycoside hydrolysis. The prospect that a positively charged amino group could mimic the partial positive charge developed on the glycosidic oxygen in the transition state of sialidase action, led scientists

at Abbott Laboratories to screen 300 α- and β-amino acids for activity against influenza virus A/N2 sialidase (Kati et al. 2001). A 1,3,4-trisusbtituted pyrrolidine identified in this process as having "modest" inhibitory activity specifically against influenza A/N2 sialidase (K_i 59×10^{-6} M) was subsequently used as a starting point for structure-based design (Wang et al. 2001; Stoll et al. 2003) [reviewed in (Wang 2002)]. Key to the iterative structure-based design programme was high-throughput X-ray crystallographic analysis (using A/N9 crystals) for the identification of the active isomer from racemic mixtures and of binding orientations in the active site that could be assessed in the light of biological activity data.

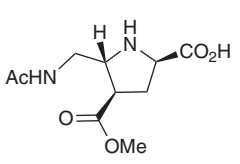
The novel and significant aspect of this inhibitor design process was the identification of an unpredicted hydrophobic interaction within subsite S2, normally the site of charge–charge interactions (e.g. from the guanidino group of zanamivir) (Stoll et al. 2003). This was observed when the tetra-substituted cyclopentane **36** (active isomer of the racemic mixture is shown), a micromolar inhibitor, did not orient as expected with the amino group in subsite S2, but rather with the methyl ester occupying the site. Interaction of the methyl ester is through strong van der Waals interactions with the side-chain methylene groups of Asp151 and Leu135 (Wang 2002) as well as through π-π stacking of the carboxylates of the methyl ester and Glu119 (Stoll et al. 2003). In addition, displacement of two water molecules from subsite S2 on binding of the methyl ester was anticipated to contribute to the inhibitory potency through entropic gain (Stoll et al. 2003).

Inhibitor development integrating the novel hydrophobic interaction with the S2 subsite into the design strategy stemmed from the trisubstituted pyrrolidine **37**, which was equipotent with cyclopentane derivative **36** (Stoll et al. 2003). Hydrophobic side-chains to interact with subsites S4 and S5 were introduced onto the acetamidomethyl side-chain (Stoll et al. 2003), mirroring placement of the glycerol side-chain in the furnose-based Neu5Ac analogue **32** and of the hydrophobic side-chain in peramivir **34**. Within the methyl ester series, nM inhibitory potency against influenza A virus sialidase was achieved with the 3-pentyl-methylene side-chain (**38**).



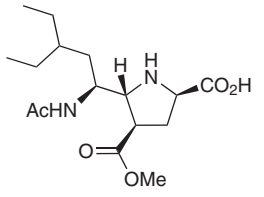
36

K_i 0.7 x 10^{-6} M (A/N2) (rac)



37

K_i 0.9 x 10^{-6} M (A/N2) (rac)

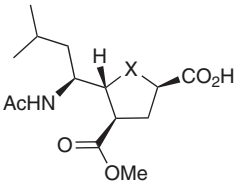


38

K_i 0.8 x 10^{-9} M (A/N2) (rac)

Two concerns about the pharmacokinetic properties of the inhibitor series developed to this point were the zwitterionic character of the pyrrolidine-based compounds and the metabolic liability of the methyl ester (Maring et al. 2005). Attempts to remedy the potential drawback of zwitterionic character through replacement of

the pyrrolidine core with a furan (e.g. **39** → **40**) (Wang et al. 2005) or a cyclopentane ring (Wang 2002), however, resulted in diminished inhibitory potency.

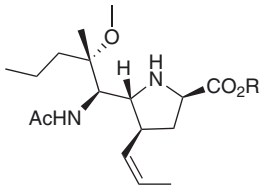


39 X = NH

IC_{50} 41 x 10^{-9} M (A/N2); 56 x 10^{-9} M (B)

40 X = O

IC_{50} 410 x 10^{-9} M (A/N2); 960 x 10^{-9} M (B)



41 R = H (A-315675)

K_i 0.2 x 10^{-9} M (A/N2); 0.14 x 10^{-9} M (B)

42 R = $CH(CH_3)_2$ (A-322278)

That the hydrophobic substituent binding subsite S2 contributed significantly to the inhibitory potency of the developed pyrrolidine series was highlighted by the reduced potency of the corresponding amino derivative against A/N2 sialidase (Stoll et al. 2003; Maring et al. 2005). Efforts to replace the metabolically labile methyl ester led to the incorporation of a *cis*-propenyl group, which showed similar significant van der Waals and π-stacking interactions with subsite S2 residues (Maring et al. 2005). Refinement of the side-chain interacting with S4/S5 resulted in the development of the 2,4,5-trisubstituted pyrrolidine derivative **41** (A-315675, ABT-675), which exhibits nanomolar inhibition both of influenza virus A and B sialidases and influenza virus A and B replication in cell culture (Kati et al. 2002). The *iso*-propyl ester pro-drug of the **42** (A-322278) shows efficacy comparable to oseltamivir upon oral administration in a mouse model of influenza (Ison et al. 2006b) and in ferrets (Mishin et al. 2005). Despite this promising activity, and an encouraging activity profile (complimentary to the other potent sialidase inhibitors) towards sialidase variants (see below) (Mishin et al. 2005), this series of compounds has not been further progressed at this point in time (McCullers 2006).

The optimised S4/S5 binding side-chain of A-315675 **41** interacts through the *n*-propyl group with subsite S4, while the methyl and methoxy groups interact with S5 (Wang 2002). Despite the predominantly hydrophobic substituents accessing subsite S5, apparently no reorientation of the Glu276 side-chain, in either influenza A or B sialidases, is required for high affinity binding of A-315675 (Molla et al. 2002). This is supported by the efficacy of A-315675 (Mishin et al. 2005; Abed et al. 2008) against an oseltamivir-resistant sialidase A strain with a mutation (His274Tyr) that prevents the reorientation of the Glu276 side-chain.

4 Clinical Application of Influenza Virus Sialidase Inhibitors

Zanamivir **12**, oseltamivir carboxylate **18**, peramivir **34** and A-315675 **41** each exhibit time-dependant, or slow-binding, inhibition of influenza virus sialidase (Kati et al. 2002). They showed comparable activity against a number of influenza virus sialidases from A (N1, N2, and N9) and B strains in a fluorescence assay, with K_i values in the low to sub-nanomolar range, with only slight variations (8- to 16-fold) in effectiveness against different medically relevant influenza virus sialidase serotypes, A/N1 and A/N2 and B (Kati et al. 2002). Zanamivir shows slightly greater efficacy of the four inhibitors against influenza B virus sialidase (Kati et al. 2002). Studies of zanamivir, oseltamivir and peramivir in cell culture also showed slight strain-dependant variation in inhibitory potency, but with generally comparable inhibition or slightly better inhibition by peramivir (Bantia et al. 2001; Govorkova et al. 2001; Smee et al. 2001). As anticipated, the sialidase inhibitors efficiently inhibit the release of virion progeny from infected cells, with cell-associated virus particles found in the presence of drug, and extracellular virus levels decreasing in a dose-dependant manner, as shown for peramivir by Smee et al. (2001).

Owing to their different structures, zanamivir **12**, oseltamivir carboxylate **18** and peramivir **34** have different physicochemical properties that determine the most appropriate method of drug delivery in vivo. Zanamivir is only poorly orally bioavailable (Ryan et al. 1994) and is delivered by inhalation or intranasal administration, but has also shown efficacy on intravenous delivery (in experimental influenza A infection) (Calfee et al. 1999). Oseltamivir carboxylate (Mendel et al. 1998), and A-315675 **41** (Ison et al. 2006b), can be administered orally as their ester pro-drug forms, **19** and **42**, respectively. Oseltamivir **19** is also bioavailable through intranasal administration (study in mice) (Bantia et al. 2001). Peramivir **34** can be administered orally, without formation of an ester pro-drug, but appears to have insufficient bioavailability (humans) (Sidwell and Smee 2002; Barroso et al. 2005), and is now being developed for intravenous or intramuscular (Bantia et al. 2006) administration.

The inhaled administration of zanamivir **12** delivers the drug to the epithelium of the lower and upper respiratory tract, the site of influenza infection (Calfee and Hayden 1998; Oxford 2000; Fleming 2003). Intravenous delivery of zanamivir also showed distribution of drug in the respiratory mucosa and protective effect against infection and illness (Calfee et al. 1999). Oseltamivir carboxylate **18**, formed through metabolism of the ester pro-drug **19** by hepatic esterase (Shi et al. 2006), is widely distributed throughout the body, including the upper and lower respiratory tract (He et al. 1999; Doucette and Aoki 2001). Plasma concentrations of oseltamivir carboxylate **18** after administration of the recommended dose exceed the levels known to inhibit viral replication (Oxford 2005). Intramuscular delivery of peramivir **34** is reported to provide high bioavailability in mice (Bantia et al. 2006). The availability of an intramuscular or intravenous formulation of a sialidase inhibitor may be important in treating patients hospitalised with severe and potentially life-threatening influenza, where oral or inhaled dosing is not applicable. It may also be more efficacious in cases of H5N1 infection, where gastrointestinal absorption

may be compromised (Beigel et al. 2005). The more systemic forms of dosing may also provide greater protection against potential systemic infection by H5N1 viruses (Beigel et al. 2005; de Jong et al. 2006).

In the clinical setting, zanamivir **12** and oseltamivir **19** are effective in both the prevention and treatment of influenza A and B infection. Benefit in treatment is restricted to patients treated within 48 h of symptom onset (Fleming 2003). Importantly, the effects of drug treatment are a reduction in the severity of illness, and in the incidence of secondary complications. The term of illness is generally reduced between 1 and 2.5 days. The evaluation of zanamivir (Calfee and Hayden 1998; Oxford 2000; Fleming 2003), oseltamivir (Doucette and Aoki 2001; Oxford 2005) and peramivir (Sidwell and Smee 2002) for the treatment, and prophylaxis, of influenza virus infection has been reviewed. The reader is directed to these reviews for further details of drug pharmacodynamics and clinical trial data.

Zanamivir was generally well tolerated in clinical trials (Fleming 2003). During treatment with oseltamivir, nausea and vomiting have been reported as side effects (Oxford 2005). A small number of severe adverse reactions in children, including neuropsychiatric events and skin hypersensitivity, have, however, been reported, primarily in Japan which has the highest use of oseltamivir (Li et al. 2007).

Of natural concern is the possibility that the influenza virus sialidase inhibitors may inhibit the endogenous human sialidases. There are four known human sialidases (HsNeu 1–4) (Monti et al. 2004) that have significant biological roles (Achyuthan and Achyuthan 2001), and the loss of activity of which has serious biological consequences (Achyuthan and Achyuthan 2001). Zanamivir **12** (Holzer et al. 1993), oseltamivir carboxylate **18** (Mendel et al. 1998; Li et al. 2007) and peramivir **34** (Bantia et al. 2001) all exhibit selective inhibition of influenza virus sialidase over mammalian (human, sheep and rat) sialidases, with inhibition of the mammalian sialidases being four- to fivefold (i.e. up to 100,000 times) less potent than the viral sialidases. The recently published X-ray crystal structure of human cytosolic sialidase (HsNeu2) shows very similar active site architecture to the viral sialidases, but also significant differences in the amino acid residues that interact with the glycerol side-chain (subsite S5) and acetamido group (subsite S3) of the sialic acid-based inhibitor Neu5Ac2en **4** (Chavas et al. 2005). These differences may help to explain, the poor inhibition of mammalian sialidases by the influenza virus sialidase inhibitors, which have been tailored for specific interactions with subsite S4/S5 residues.

Oseltamivir carboxylate **18**, while giving only mM inhibition of HsNeu2, was found to have a slightly increased inhibitory effect on a HsNeu2 mutant (Arg41Gln), which is present in an estimated 9% of the Asian population (but not in European or Afro American populations) (Li et al. 2007). It has been suggested (Li et al. 2007) that inhibition of HsNeu2-R41Q by oseltamivir carboxylate may lead to a reduction in sialidase activity sufficient to cause the neuropsychiatric events and skin hypersensitivity side-effects that have similarities to human sialidase-related disorders.

5 Resistance Development to Sialidase Inhibitors

The influenza virus surface glycoproteins HA and sialidase continually undergo natural and antibody-induced antigenic drift (Webster et al. 1992). However, as the active site residues of sialidase that are involved in interactions with the designed sialidase inhibitors are highly conserved, it was hoped the likelihood of variants with uncompromised infectivity and transmissibility would be reduced. It has been postulated that the viability of "escape" mutants arising under pressure of sialidase inhibitors would be related to how different the inhibitor was to the natural substrate sialic acid (Varghese et al. 1998). This has been borne out to some extent in experimental evidence (Varghese et al. 1998). It is interesting to note that it appears to be significantly more difficult to generate viral resistance to the sialidase inhibitors than to the adamantane-based M2 ion channel blockers (McCullers 2005; Ilyushina et al. 2006).

Virus growth in the presence of the sialidase inhibitors generally gives rise, in the first instance, to variants with changes in the haemagglutinin (HA) glycoprotein and later to changes in the sialidase (Tisdale 2000; Zambon and Hayden 2001). Viruses expressing a haemagglutinin with reduced affinity for the cell surface receptors, may also have reduced reliance on the activity of the sialidase for release of virion progeny from cells (McKimm-Breschkin 2000). Such viruses can show altered sensitivity to the sialidase inhibitors. In addition, because alteration to the strength and/or specificity of HA receptor binding can affect both infectivity and transmission of the virus, mutations in HA can complicate the assessment in cell culture and animal studies of the biological effects of variations in the NA active site (Zambon and Hayden 2001; Gubareva 2004).

Viruses that contain amino acid substitutions in the sialidase that impart resistance to the developed inhibitors have been isolated from serial passage of virus in the presence of drug in cell culture and from the clinical setting (reviewed in McKimm-Breschkin 2000; Zambon and Hayden 2001; Cinatl et al. 2007a; Reece 2007). In addition, influenza B virus variants with reduced drug sensitivity have been isolated from previously untreated patients (Hurt et al. 2006; Hatakeyama et al. 2007). The types of mutations that are observed are sub-type specific. The mutations present in variants isolated from clinical samples are shown in Table 1, and their locations within the sialidase active site are shown diagrammatically in Fig. 9.

A number of sialidase variants (predominantly with changes at Glu119) arise during passage in the presence of zanamivir **12** in vitro (Zürcher et al. 2006) (reviewed in McKimm-Breschkin 2000). However, no viruses with a change in the sialidase causing resistance to zanamivir have yet been isolated from normal patients treated with the drug (Reece 2007), though this may simply be the result of the limited use of this drug in patients. One variant influenza B virus (with Arg152Lys), however, was isolated from an immunocompromised patient (Gubareva et al. 1998). Several sialidase variants have been isolated from patients treated with oseltamivir **19** (Table 1), the much more widely used anti-influenza drug. As can be seen from

Table 1 Influenza virus sialidase variants isolated in the clinical setting

Strain	Mutation[a]	Drug	Level of sialidase activity and/or viral fitness
A/N1	His274Tyr	Oseltamivir[b,c,d,e]	Pathogenicity significantly compromised (ferret)[f] Viral titres reduced *cf* WT[e]; reduced catalytic activity[c]
	Asn294Ser	Oseltamivir[g]	Not stated
A/N2	Glu119Val	Oseltamivir[c,d,h,i]	Replicates and transmissible (ferret)[j] Viral titers ≈ WT[k]; reduced catalytic activity[c]
	Arg292Lys	Oseltamivir[c,h]	v-Poor infectivity (mouse)[l]; not transmissible (ferret)[j] Reduced catalytic activity (< 20% of WT)[c]
	Asn294Ser	Oseltamivir[h]	Not stated
B	Arg152Lys	Zanamivir[c,d,m]	Reduced catalytic activity (3–5% of WT)[c]
	Asp198Asn	Oseltamivir[d,i]	No apparent fitness impairment (ferret)[n]
	Gly402Ser	Oseltamivir[o]	Not stated

[a]Numbering used is that of A/N2
[b]Gubareva et al. (2001)
[c]Zambon and Hayden (2001)
[d]Gubareva (2004)
[e]Le et al. (2005)
[f]Ives et al. (2002)
[g]WHO (2007) (Reviewed in (Reece 2007))
[h]Kiso et al. (2004)
[i]Ison et al. (2006a)
[j]Yen et al. (2005)
[k]Zürcher et al. (2006)
[l]Gubareva et al. (1997)
[m]Gubareva et al. (1998)
[n]Mishin et al. (2005)
[o]Hatakeyama et al. (2007)

the data in Table 1, the isolated variants have different levels of "fitness" in terms of replicative ability and transmissibility. In addition, mutations at the conserved residues can affect the enzyme activity and also the stability of the protein.

5.1 Structural Basis of Resistance, and Cross-Resistance, to Sialidase Inhibitors

The mutations observed in the sialidase under drug pressure, in vitro or in vivo, to date, affect four of the five binding subsites of the sialidase (Fig. 9) – Glu119 affects S2; Arg152, and Asp198 (and Ile222 (Hatakeyama et al. 2007)) affect S3; His274 and Asn294 affect S5; and Arg292 affects both S1 and S5. The amino acid variations arising under drug pressure can be related to the interactions of the inhibitors with active site residues. Conversely, the changes in efficacy of the inhibitors against the mutant sialidases reflect the importance of their binding interactions with the affected subsites. The sensitivity of the clinically significant variants to inhibition by influenza virus sialidase inhibitors (in sialidase assays) is given in Table 2. Only one

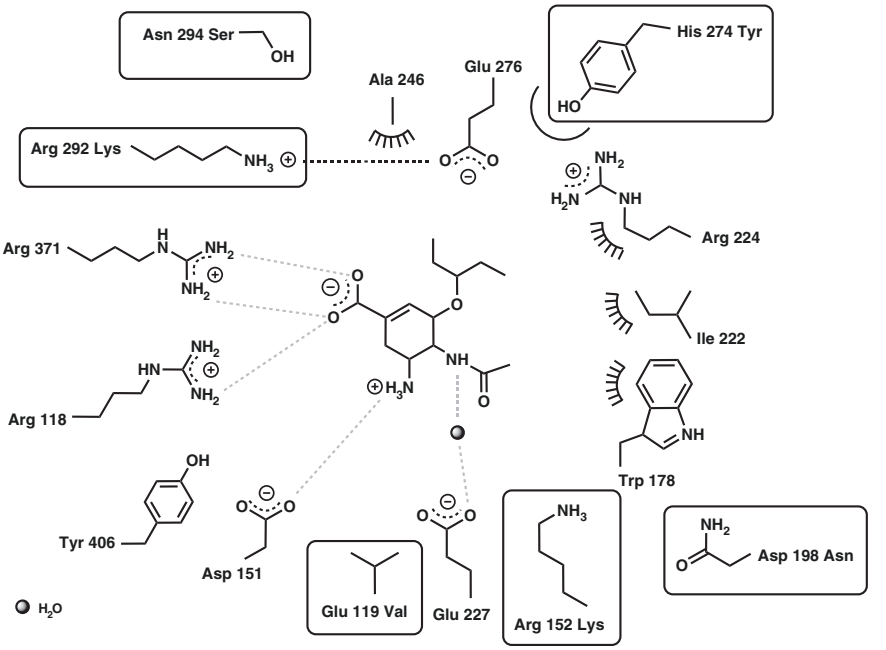
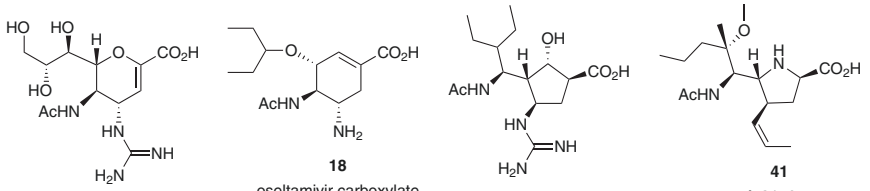


Fig. 9 Diagrammatic representation of influenza A virus active site indicating the amino acid residues that have shown mutation under drug pressure. Oseltamivir carboxylate **18** is shown in the active site

of the clinical variants (B Arg152Lys) shows significant resistance to both zanamivir **12** and oseltamivir carboxylate **18**; however, the sialidase has reduced catalytic activity and so may result in a virus with compromised virulence.



The Glu119 variants appearing under pressure from oseltamivir in the clinic (Kiso et al. 2004; Ison et al. 2006a) and from zanamivir in cell culture (Blick et al. 1995; McKimm-Breschkin 2000) significantly alter the nature of the S2 subsite in which the amino and guanidino groups, respectively, of the two drugs bind. Variations at Glu119 (A/N2, A/N9, B) that appear in the presence of zanamivir (Glu119 Gly, Ala, Asp; reviewed in McKimm-Breschkin 2000) have an adverse affect on

Table 2 Sensitivity of influenza virus sialidase variants isolated in the clinical setting to sialidase inhibitors in sialidase assays[a,b]

Strain	Mutation[d]	Zanamivir		Oseltamivir[c]		Peramivir		A-135675	
		ΔWT	S/R	ΔWT	S/R	ΔWT	S/R	ΔWT	S/R
A/N1	His274Tyr	1	S	> 700	R	100	R	3	S
	Asn294Ser	–	–	113[e]	–	–	–	2[e]	–
A/N2	Glu119Val	1–3	S	> 130	R	1	S	1	S
	Arg292Lys	5	I[f]	> 1600	R	80	R[f]	8	I[f]
	Asn294Ser	–	–	300[g]	–	–	–	–	–
B	Arg152Lys	70	R	100	R	400	R	150	R
	Asp198Asn	9	R[f]; S[h]	9	R	5	S	2	S
	Gly402Ser	7[i]	–	4[i]	–	–	–	–	–

[a]Relative activity of drug against the variant strain (ΔWT) is shown as a fold difference in IC_{50} compared to wild type. Data shown is from Mishin et al. (2005) unless otherwise noted

[b]Sensitivity or resistance of the variant virus to drug (S/R) is given as S, sensitive; R, resistant; I, intermediate. Categorisation is taken from Mishin et al. (2005) and Gubavera (2004)

[c]Oseltamivir carboxylate **18** is used in sialidase inhibition assays

[d]Numbering used is that of A/N2

[e]Abed et al. (2008)

[f]Mishin et al. (2005)

[g]Kiso et al. (2004)

[h]Gubareva (2004)

[i]Hatakeyama et al. (2007)

the stability and activity of the enzyme (McKimm-Breschkin 2000; Zürcher et al. 2006) and on virus replication in vitro (Zürcher et al. 2006). Importantly, however, the oseltamivir-selected Glu119Val variant showed less significant loss of sialidase activity and the virus grew to similar titres as the wild type in cell culture (Zürcher et al. 2006) and was transmissable in ferrets (Yen et al. 2005).

There is significant inhibitor cross-sensitivity observed with the A/N2 Glu119 variants: the Gly and Ala variants are resistant only to zanamivir **12**; the Val variant resistant only to oseltamivir carboxylate **18**, but the Asp variant is resistant to all four developed inhibitors to varying degrees (Mishin et al. 2005). Peramivir **34**, which has a guanidino group that is oriented in S2 differently to that of zanamivir (Babu et al. 2000), retained efficacy against all except the Glu119Asp variant, possibly by virtue of the strong interactions of the hydrophobic side-chain in S4/S5. A-135675 **41**, which has hydrophobic interactions with subsite S2, also retained efficacy against all variants except Glu119Asp. Importantly, the clinically relevant oseltamivir-selected Glu119Val variant is sensitive to zanamivir, as well as peramivir and A-135675 (Mishin et al. 2005).

In influenza A virus N2 subtype, pressure from zanamivir in vitro (Gubareva et al. 1997) and from oseltamivir (Kiso et al. 2004) gives rise to variation in the highly conserved functional residue Arg292, which normally forms a hydrogen bond with the carboxyl group of the substrate Neu5Ac **3**, resulting not unexpectedly in enzymes with lower specific activity (Zambon and Hayden 2001), and a virus of low viability (Gubareva et al. 1997; Yen et al. 2005). The Arg292Lys variant exhibits

minimal resistance to zanamivir **12**, but greater resistance to the inhibitors with large hydrophobic side-chains, in particular, oseltamivir carboxylate **18** (Gubareva 2004; Mishin et al. 2005). In this variant, Lys292 forms a salt bridge with Glu276 that must be broken to enable the reorientation of the Glu276 necessary to accommodate the hydrophobic side-chains of oseltamivir carboxylate **18** and peramivir **34** (Smith et al. 2002). A complex of peramivir bound to the A/N2 Lys292 variant, however, showed the inhibitor bound in the same manner as in the wild type NA, with the reorientation of Glu276 (Smith et al. 2002). A-135675 **41**, where the hydrophobic side-chain binding subsite S5 was designed to avoid a reorientation of Glu276 (Molla et al. 2002), still shows almost full inhibitory potency against this variant (Mishin et al. 2005).

The Asn294 and His274 variants in influenza A sialidase, arising under pressure from oseltamivir in the clinic (Le et al. 2005), are expected to affect the ease with which Glu276 can reorientate to allow binding of hydrophobic side-chains in subsite S5 (Moscona 2005). Interestingly, although His274 does not interact directly with the substrate Neu5Ac **3**, the sialidase with this mutation has reduced catalytic activity (Zambon and Hayden 2001). The effect of replacement of His at position 274 with amino acids of increasing size indicated that the change to Tyr at this position would create a steric barrier to the reorientation of Glu276 (Wang et al. 2002), and so prevent effective binding of oseltamivir carboxylate **18**. This is supported by the significant loss of potency ($>$700-fold) of **18** against the A/N1 His274Tyr sialidase, and also the reduced potency of peramivir **34** (Mishin et al. 2005). Highlighting the subtype specificity of the sialidase mutations is the fact that oseltamivir carboxylate retains essentially full efficacy against the same mutation in an A/N2 sialidase (Wang et al. 2002). The highly oseltamivir-resistant A/N1 His274Tyr variant was sensitive to zanamivir **12** (Le et al. 2005).

Variations have been identified in influenza B viruses in amino acids that surround the binding pocket (S3) of the acetamido group of the substrate Neu5Ac **3** and of each of the inhibitors (Gubareva et al. 1998; Gubareva 2004; Hurt et al. 2006; Hatakeyama et al. 2007). Arg152 normally forms a hydrogen bond to the carbonyl group of the acetamide. It is not surprising, therefore, that the Arg152Lys variant shows a degree of resistance (70- to 400-fold drop in potency) to all the inhibitors (Mishin et al. 2005). This variant, however, also has significantly reduced catalytic activity (Zambon and Hayden 2001).

Are drug resistant viruses already in circulation?

Since the introduction of sialidase inhibitors as antiviral agents in 1999, changes in sialidase sensitivity to the inhibitors in circulating influenza viruses has been monitored (Zambon and Hayden 2001; Monto et al. 2006), with particular attention at the current time given to avian influenza A viruses of the H5N1 subtype (Rameix-Welti et al. 2006; Hurt et al. 2007; Wang et al. 2007). Circulating, non-variant human influenza viruses show a natural variation in sensitivity (up to 10-fold difference) to zanamivir and oseltamivir (Ferraris et al. 2005; Rameix-Welti et al. 2006). Influenza

B virus sialidase variants with reduced drug sensitivity ($\sim$ 5–10% reduced sensitivity *cf* WT) have also been isolated from patients with no prior history of sialidase inhibitor treatment (Hurt et al. 2006; Hatakeyama et al. 2007), suggesting that these variants may have arisen through natural antigenic drift and are circulating in the community (Hatakeyama et al. 2007). The possibility that avian H5N1 viruses may be in circulation that already contain variations in active site amino acids that confer resistance to inhibitors was assessed in two studies in 2006. Analysis of the sialidases from 109 strains of chicken H5N1 virus (isolated before the advent of the sialidase inhibitors) found that the functional residues of the H5N1 sialidases were highly conserved, with only one of the 109 NAs examined having the Ile222Thr replacement (Wang et al. 2007). None of the other mutations (at positions 119, 152, 274 or 292) that appear in variants during cell passage or human treatment with oseltamivir were found (Wang et al. 2007). Similarly, in an assessment of 50 avian influenza A(H5N1) viruses from South East Asia, isolated between 2004 and 2006, showed none of the drug selected variants, but did identify two sialidases with variation at positions 116 and 117, which had reduced sensitivity (in the range of 4- to 63-fold) to zanamivir, oseltamivir and peramivir (Hurt et al. 2007). Most recently and of great concern is the emergence of the human influenza virus H1N1 that has reduced sensitivity to oseltamivir (Rameix-Welti et al. 2008; Sheu et al. 2008).

6 Future Prospects

The design of anti-influenza therapies has long been considered a challenge; the variable antigenic nature of the surface glycoproteins brought about by genetic drift and re-assortment of the segmented viral genome present not only challenges for vaccine development (Cinatl et al. 2007b), but for many years appeared to preclude effective targeting of these proteins for drug development (Meanwell and Krystal 1996a, 1996b). The crystallisation of the sialidase and the identification of the highly conserved nature of the critical catalytic site residues by Colman and co-workers in the early 1980s (Colman et al. 1983), however, provided the basis for rational, structure-based drug design targeting the sialidase. With the benefit of structure-based design, potent and selective inhibitors of influenza virus sialidase have been developed, with two (zanamivir and oseltamivir) now in clinical use, and another (peramivir) undergoing clinical trials. The recently solved structure of avian influenza A virus H5N1 sialidase (Russell et al. 2006), with distinct differences in the *apo* and initially inhibitor-complexed structures, will provide further opportunities for inhibitor design.

In the clinical setting, the need for alternative methods of administration for treatment where oral or inhaled dosing is not appropriate is being addressed with the development of intravenous and intramuscular injection formulations of sialidase inhibitors (Calfee et al. 1999; Bantia et al. 2006). Also of significant interest are "second-generation" zanamivir-based inhibitors that are undergoing clinical trials as long-acting sialidase inhibitors, where once-a-week dosing could be a possibility (Macdonald et al. 2004; Yamashita 2004).

It may be possible to increase the utility of our resources to treat influenza virus infection through combinations of antiviral agents with different modes of action (discussed in Cinatl et al. 2007a; De Clercq and Neyts 2007). The sialidase inhibitors, for example, may be able to be used in conjunction with the adamantane-based M2 ion channel inhibitors (Govorkova et al. 2004; Ilyushina et al. 2006), with Ribavirin (Smee et al. 2002) or with non-influenza virus specific therapeutics such as anti-inflammatory drugs (Carter 2007). Combination therapy may also reduce the potential of resistance development (Ilyushina et al. 2006).

Resistance development to the sialidase inhibitors remains a very important issue. While the dire forecasts of rapid evolution of resistance have so far been proven false, resistant strains have been selected through use of oseltamivir in the clinical setting. The cost of resistance to date, however, seems to be a reduction in enzyme activity and viral fitness, as predicted for alteration of the highly conserved active site, though this is not true for all the variants selected. At present there is also significant cross-sensitivity to zanamivir and oseltamivir seen for the variant sialidases. It is essential that the generation of resistance to inhibitors continues to be carefully monitored. In tandem with this is a real need for the continued development of next generation sialidase inhibitors and inhibitors of other viral targets.

The development of sialidase-based inhibitors as anti-influenza drugs has provided a first line-of-defence to safeguard humanity against a potential pandemic and most importantly to buy time for vaccine and further anti-influenza drug development. Most exciting is that new opportunities exist for next generation sialidase inhibitor development.

Acknowledgements The financial support of the Australian Research Council (ARC) and the National Health and Medical Research Council (NHMRC), and the ARC for the award of a Federation Fellowship (MvI), is gratefully acknowledged. Jeff Dyason is thanked for his assistance in the preparation of this manuscript.

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Other Inhibitors of Viral Enzymes and Functions

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

Contents

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

Abbreviations

DNA	Deoxyribonucleic acid
HIV	Human immunodeficiency virus
SARS	Severe acute respiratory syndrome

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H.-G. Kräusslich, R. Bartenschlager (eds.), *Antiviral Strategies,* Handbook of Experimental Pharmacology 189,

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

1 Introduction

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

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

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

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

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

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

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

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

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

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

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

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

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

3 Integrase Inhibitors

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

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

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

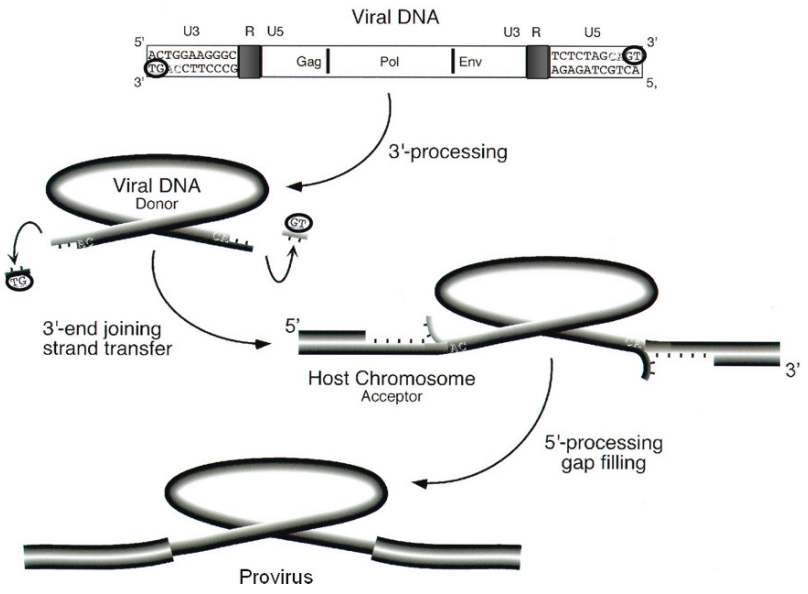

Fig. 1 Integration of HIV DNA into the host genome

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4 Helicase Inhibitors

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

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

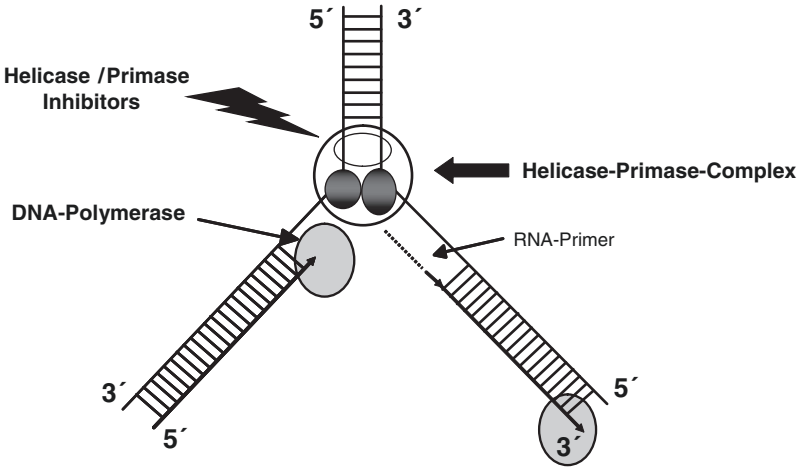

Fig. 2 Herpesvirus DNA-replication fork showing the site of action of helicase/primase inhibitors

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

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

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

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

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

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

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

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

5 Terminase Inhibitors

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

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

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

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

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

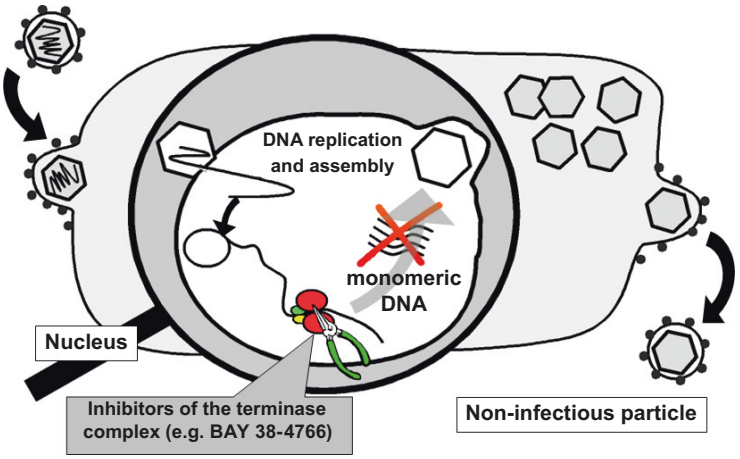

Fig. 3 Representation of the replication cycle of cytomegalovirus showing the site of action of terminase inhibitors

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

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

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

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

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

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

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

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

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

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

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

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

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

6 Maturation/Assembly Inhibitors

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

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

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

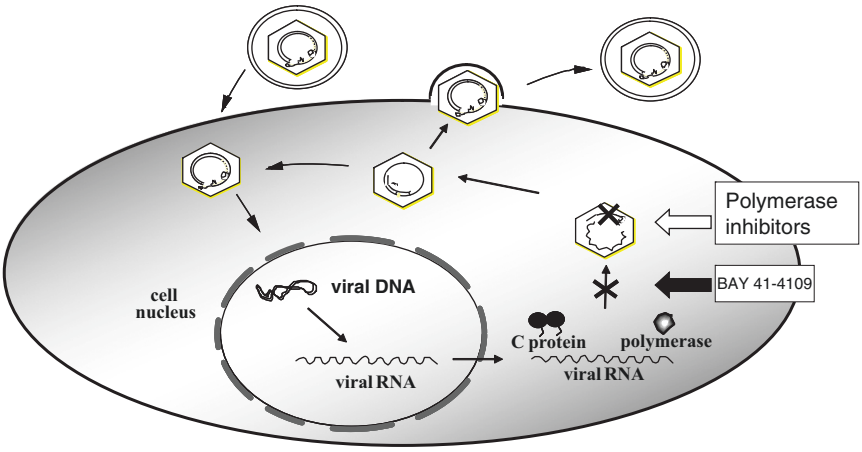

Fig. 4 Replication cycle of HBV illustrating the site of action of polymerase inhibitors and heteroaryl dihydropyrimidine (HAP) assembly inhibitors such as BAY 41–4109

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

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

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

7 Conclusions

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

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Inhibitors of Viral Entry

Tom Melby and Mike Westby

Contents

Abstract The entry of viruses into target cells involves a complex series of sequential steps, with opportunities for inhibition at every stage. Entry inhibitors exert their biological properties by inhibiting protein–protein interactions either within the viral envelope (Env) glycoproteins or between viral Env and host-cell receptors. The nature of resistance to entry inhibitors also differs from compounds inhibiting enzymatic targets due to their different modes of action and the relative variability in

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H.-G. Kräusslich, R. Bartenschlager (eds.), *Antiviral Strategies,* Handbook of Experimental Pharmacology 189,

Env sequences both temporally and between patients. Two drugs that target HIV-1 entry, enfuvirtide and maraviroc, are now licensed for treatment of HIV-1 infection. The efficacy of these drugs validates entry as a point of intervention in viral life cycles and, in the context of HIV treatment, contributes to the growing armamentarium of antivirals which, in multidrug combinations, can effectively inhibit viral replication and prevent disease progression.

Abbreviations

Env	Viral envelope protein
FP	Fusion peptide (amino terminal domain of gp41)
Gp41	Glycoprotein 41 kDa (component of envelope protein complex)
Gp120	Glycoprotein 120 kDa (component of envelope protein complex)
GPCR	G-protein coupled receptor
HAART	Highly active antiretroviral therapy
HIV-1	Human immunodeficiency virus type 1
HR-1	Heptad repeat-1 (domain in gp41)
HR-2	Heptad repeat-2 (domain in gp41)
OB	Optimised background (a drug regimen selected following resistance testing, the latter being used to determine virus susceptibility to available drugs)
MPER	Membrane-proximal external region
TM	Trans-membrane
CT	Cytoplasmic tail
TORO	T-20 vs. optimized regimen only

1 Introduction

Viral entry into target cells is the first step in the replication cycle and has been studied extensively for several viruses. Targeting viral entry as a strategy for therapeutic intervention is potentially attractive in that the site of inhibitory action is likely to be extracellular and therefore relatively accessible to biological interventions (peptides and antibodies) as well as small molecule inhibitors. Also, entry inhibitors and drugs that act on intracellular enzymatic targets select nonoverlapping patterns of viral resistance, potentially making them attractive partners in multidrug regimens for both treatment-naïve and treatment-experienced patients.

Many enveloped viruses share a common mechanism of fusion, mediated by a virus-encoded glycoprotein that contains heptad repeats in its extracellular domain. During the fusion process, these domains rearrange to form highly structured and thermodynamically stable *coiled-coils*. Viruses encoding fusion proteins that have these domains include members of the paramyxovirus family (e.g., respiratory syncytial virus, metapneumovirus, and measles virus), ebola virus, influenza, and members of the retroviridae (e.g., human T cell leukemia virus type-1 and human immunodeficiency virus type-1, HIV-1). Peptide inhibitors of fusion that disrupt the

coiled-coil rearrangement have been described for some viruses (Miller et al. 2007; Mirsaliotis et al. 2008). However, by far, the best characterized case is HIV-1, where a peptide inhibitor of fusion is now an approved drug.

Targeting virus entry is not without its challenges. The envelope and fusion glycoproteins are usually the most variable of all virus-encoded proteins. Indeed, the amino acid sequences can vary substantially both within and between individuals, making the *spectrum* of antiviral activity for any entry inhibitor an important consideration. In addition, successful drug candidates must efficiently inhibit large protein–protein interactions. However, the regulatory approval of two drugs that target HIV-1 entry (enfuvirtide and maraviroc) indicates that these challenges are not insurmountable.

This chapter is intended to provide an overview of HIV-1 entry inhibitors, focusing on the mechanism of action, development, and knowledge of viral resistance to the currently available entry inhibitors, enfuvirtide and maraviroc. Although reversed relative to their order in the entry process, enfuvirtide is discussed first due to the greater clinical experience with this drug.

2 Overview of the HIV-1 Entry Process

HIV-1 entry is mediated by the virus-encoded envelope (Env) proteins. Env comprises two noncovalently linked glycoproteins, gp120 and gp41, that form trimeric spikes outside of virions and infected cells. In broad terms, gp120's functions are threefold: to select which host cells the virus can infect (this defines the viral *tropism*); to stabilize gp41 in an energetically unfavorable state pending the appropriate engagement of a target cell; and to occlude gp41 from immune surveillance and neutralization. The role of the gp41 transmembrane protein meanwhile is to anchor the Env trimers to the virion surface and to bring about the fusion of the viral and host membranes during the entry process.

The entry process can be divided into three stages: viral attachment, coreceptor binding, and fusion (Fig. 1). The first mandatory step in the process of HIV-1

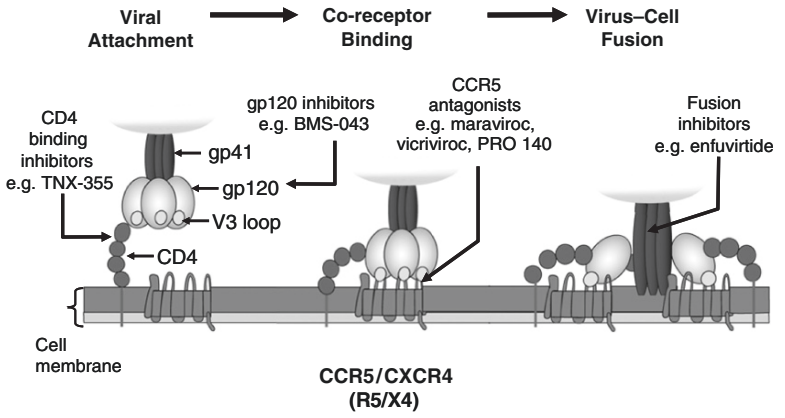

Fig. 1 The HIV-1 entry process

entry is the specific binding of gp120 to CD4, the primary receptor for HIV-1 (Dalgleish et al. 1984; Klatzmann et al. 1984; Maddon et al. 1986). The CD4 receptor is expressed mainly on T-lymphocytes and macrophages and is a member of the immunoglobulin (Ig)-like protein superfamily. The binding of gp120 to CD4 causes a reconfiguration of the variable loops V1/V2 and V3 of gp120 to expose the bridging sheet and form a coreceptor binding site (Kwong et al. 1998; Rizzuto et al. 1998; Wyatt et al. 1998). Coreceptor binding triggers conformational changes in gp120 and gp41, which result in the exposure of the fusion peptide (FP) and its insertion into the target cell membrane. Further rearrangements within gp41 bring the target and viral cell membranes into close proximity, ultimately creating a fusion pore through which the viral core passes into the cell (reviewed by Chan and Kim 1998).

3 Inhibitors of HIV-1 Fusion

3.1 The Fusion Phase of HIV-1 Entry

The fusion phase of HIV entry begins with insertion of the gp41 N-terminal FP into the target CD4+ cell membrane and ends with the enlargement of a fusion pore to allow delivery of the viral core into the target cell. The secondary structure of gp41 is illustrated schematically in Fig. 2. Each domain within gp41 plays an important role in the fusion process; however, the role of the N- and C-terminal heptad repeat (HR) regions will receive particular attention as they represent enfuvirtide's site of action.

In the prefusion envelope complex, gp41 is largely occluded by gp120 and is maintained in a hemi-stable state, ready to be triggered when appropriate target-cell receptors are recognized by gp120 (reviewed in Wyatt and Sodroski 1998). During the attachment and coreceptor binding phases, rearrangements within gp120 expose the gp41 FP, which inserts itself into the target cell (Brasseur et al. 1988; Quintana et al. 2005). The attachment of gp120 to CD4 alone appears sufficient to partially expose the HR-1 and HR-2 domains (He et al. 2003). Additional conformational changes occur following coreceptor binding, which ultimately bring the HR1 and HR2 domains together to form a thermostable six-helix coiled-coil bundle (Chan et al. 1997; Tan et al. 1997; Weissenhorn et al. 1997). Formation of the

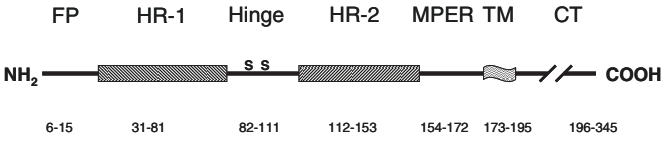

Fig. 2 Secondary structure of gp41

coiled-coil bundle brings the host and viral membranes into close proximity, since regions flanking the distal ends of the HR1 and HR2 domains are buried, respectively, in the cellular and viral membranes. Whether this occurs *prior to* or *drives* the creation of the fusion pore is still in question (Munoz-Barroso et al. 1999; Melikyan et al. 2000); however, formation of the bundle is clearly necessary for viral infection (Furuta et al. 1998).

3.2 Fusion Inhibitor Discovery

Efforts to develop antiretrovirals targeting viral fusion began with the observation that sequences from the regions of gp41 now known as HR1 and HR2 contained a predicted leucine-zipper motif (Gallaher 1987; Wild et al. 1992), suggesting that they might multimerize as part of the fusion process. Subsequently Carl Wild and colleagues in the Matthews laboratory at Duke University explored synthetic peptides derived from the gp41 ectodomain as inhibitors of HIV replication. This work lead to the discovery of a potent peptide fusion inhibitor, dp107, and then the highly potent dp178 (T-20, enfuvirtide, Fuzeon^{TM}) peptide (Wild et al. 1992, 1993). These and other inhibitory peptides (Jiang et al. 1993) were derived from the peptide sequence of the HIV-1 HR1 (dp107, N36) or HR2 (dp178, C34) domains.

Early biochemical studies supported the hypothesis that the HR1 and the HR2 peptides would interact to form a helical structure (Chen et al. 1995; Lu et al. 1995). This hypothesis was strengthened when X-ray structures were resolved for co-crystals of HR1 and HR2 peptides (Chan et al. 1997; Tan et al. 1997; Weissenhorn et al. 1997). The results showed that in the six-helix bundle, three HR1 domains were packed tightly together in the center of the bundle, with the HR2 domains bound in an antiparallel manner in grooves formed along the HR1 core.

3.3 Mechanism of Action of Enfuvirtide

Information obtained from crystallographic structures (Chan et al. 1998; Malashkevich et al. 1998) and binding studies (Chen et al. 1995) strongly suggest that enfuvirtide and other HR2-peptides are dominant-negative inhibitors of viral fusion and act through binding to the HR1 core at an intermediate stage of fusion. This is supported by observations that mutations conferring resistance to enfuvirtide are consistently observed in HR1 (Menzo et al. 2004; Sista et al. 2004; Melby et al. 2006b) and that loss of susceptibility to enfuvirtide is directly proportional to the loss of in vitro binding affinity of mutant HR1 domains for enfuvirtide (Mink et al. 2005). Direct binding to gp120 (Yuan et al. 2004; Liu et al. 2005) has also been proposed to contribute to enfuvirtide's overall antiviral effect and specific contributions to enfuvirtide's activity may also come from adsorption to lipid membranes (Kliger et al. 2001; Saez-Cirion et al. 2002; Veiga et al. 2004) and inhibition of

the formation of the fusion pore (Munoz-Barroso et al. 1998). The precise extent to which any of these properties attributed to enfuvirtide contribute to its clinical efficacy is unknown.

3.4 Clinical Development of Enfuvirtide

The first study to demonstrate the activity of enfuvirtide in HIV-infected patients (Kilby et al. 1998) showed that patients receiving the maximum 100 mg intravenous dose had maximum median declines in HIV-1 RNA of -1.96 $\log_{10}$ copies/mL through 14 days. Several additional studies (Kilby et al. 2002; Lalezari et al. 2003a, b) further demonstrated the safety and efficacy of enfuvirtide and led to the selection of twice-daily subcutaneous injections of a 90 mg nominal dose for testing in the TORO (T-20 vs. optimized regimen only) pivotal clinical trials.

TORO-1 and TORO-2 were randomized, open-label, phase 3 trials comparing enfuvirtide plus an optimized background regimen (OB) to OB alone in patients with previous triple-class treatment experience (Lalezari et al. 2003c; Lazzarin et al. 2003). TORO-1 was conducted in North America, Mexico, and Brazil; TORO-2 was conducted in Western Europe and Australia. Approximately 500 patients were recruited and randomized for each study. At 24 weeks, median change from baseline in HIV-1 RNA for the enfuvirtide + OB vs. OB groups was -1.7 vs. -0.8 in TORO-1 and -1.4 vs. -0.6 $\log_{10}$ copies/mL in TORO-2; similarly, CD4 cell count increases were 76 vs. 32 cells/mm^3 in TORO-1 and 66 vs. 38 cells/mm^3 in TORO-2. Differences between the enfuvirtide and control groups were highly significant with $P < 0.001$ for these and other efficacy comparisons (VL < 400; VL < 50; > 1 log decrease in VL) presented for TORO-1 and TORO-2 (excepting, in TORO-2, change in CD4+ cell count ($P = 0.02$) and VL < 50 copies/mL ($P = 0.01$)). Notably, the proportion of patients who had previously received lopinavir was higher in the TORO-2 study compared with the TORO-1 study (61% for enfuvirtide + OB and 52% for OB only, in TORO-2 vs. 39% and 28%, respectively, in TORO-1); this may account for a somewhat lower overall proportion of responders observed in TORO-2 compared with TORO-1.

Data were pooled from the TORO-1 and -2 studies for 48 week efficacy analyses (Nelson et al. 2005). These generally confirmed the 24 week findings and also demonstrated the durability of virological response – in both the enfuvirtide + OB and OB only groups only about 7% of patients met virological failure criteria between 24 and 48 weeks.

It is worth noting that the development of manufacturing methods and capacity prior to enfuvirtide commercialization was fully as challenging as the demonstration of clinical safety and efficacy. Such large scale peptide synthesis was novel and required an increase in global peptide manufacturing capacity of several orders of magnitude as well as the development of appropriate large-scale synthesis techniques. In 2001, Brian Bray, the Senior Director of Process Research and Development at Trimeris, was awarded an Industrial Innovation Award from the American

Chemical Society's Southeastern Region for work demonstrating the commercial feasibility of the manufacture of enfuvirtide.

3.5 Clinical Virology of Enfuvirtide

3.5.1 Baseline Susceptibility to Enfuvirtide

A number of studies on primary isolates and pseudotyped viruses have demonstrated substantial variability in baseline susceptibility to enfuvirtide (Derdeyn et al. 2000, 2001; Sista et al. 2004; Melby et al. 2006b). Indeed, in virology analyses for the combined TORO studies, baseline susceptibilities for 627 virus envelopes covered a 1,000-fold range, much larger than that seen in naïve patients for drugs targeting the reverse transcriptase or protease enzymes (Harrigan et al. 2001). One of the several factors that may impact in vitro susceptibility is coreceptor usage, which Reeves and colleagues found to impact fusion kinetics and susceptibility to enfuvirtide (Reeves et al. 2002, 2004). Intriguingly, in the heavily treatment experienced TORO study populations, the IC_{50}s of strains with dual/mixed tropism were significantly lower than those of CCR5 using strains or of the rare X4-only strains (Melby et al. 2006a). Reeves and others (Platt et al. 2005) have proposed that susceptibility to enfuvirtide is modulated at least in part by the length of time during which fusion intermediates are accessible to enfuvirtide for binding, a period that is inversely proportional to coreceptor binding affinity and coreceptor density; this is referred to as the "kinetic model" of susceptibility.

Despite the very broad range in in vitro susceptibilities of viruses from enfuvirtide-treatment naïve patients in the pooled TORO studies, there were no convincing correlations between baseline enfuvirtide susceptibility and virological response (Melby et al. 2006b). This could reflect a combination of viral and host factors: for example, minority viral quasispecies with little impact on susceptibility measurements prior to enfuvirtide treatment could rapidly be selected during treatment; also, the greater susceptibility to enfuvirtide of dual/mixed tropic strains relative to R5 strains might tend to equalize virological response if dual/mixed tropism was associated with a greater degree of disease progression and thus potentially with poorer overall response than R5 tropism. Additionally, Heredia and coworkers recently demonstrated an approximately 100-fold range in susceptibility to enfuvirtide for the same HIV-1 strains tested on primary cells from different donors and this variability was strongly and inversely correlated ($P < 0.0001$) with cellular CCR5 expression (Heredia et al. 2007). Because of this lack of correlation with clinical response, baseline phenotypic resistance testing is unlikely to be clinically relevant and is not widely used.

In contrast to the wide range of phenotypic susceptibility of viruses to enfuvirtide, analyses of gp41 sequences at entry into clinical studies show relatively little variation within the gp41 aa 36–45 region representing the binding site of enfuvirtide (described later). The most common polymorphism, N42S ($\sim$16% prevalence

in the TORO studies), was associated in linear regression analyses with enhanced susceptibility to enfuvirtide (Melby et al. 2006b). This polymorphism is relatively rare in subtype B strains but is common in several other subtypes (including C; Cilliers et al. 2004; Aghokeng et al. 2005; Carmona et al. 2005), which may contribute to the higher apparent susceptibility to enfuvirtide of most non-B strains, despite polymorphisms in gp41 relative to subtype B strains (Melby et al. 2006b; Holguin et al. 2007).

3.5.2 Treatment-Emergent Resistance to Enfuvirtide

Rimsky and colleagues first described resistance to enfuvirtide based on serial-passage experiments and reported mutations from wild-type GIV to SIM at gp41 residues 36–38, a very highly conserved region in enfuvirtide naïve isolates (Rimsky et al. 1998; Sista et al. 2004; Melby et al. 2006b). The presence of various mutations in plasma-derived clones was associated with 3.2- to 45-fold reductions in susceptibility to enfuvirtide; binding of enfuvirtide with HR1 cognate peptides was also demonstrably impaired upon introduction of the SIM sequence, supporting the importance of this region for enfuvirtide activity. Mutations in an expanded gp41 aa 36–45 region were confirmed in phase I and II clinical studies (Kilby et al. 1998, 2002; Wei et al. 2002), where substitutions were found to be common at several positions in gp41, including 36, 38, 40, 42, and 43. Similar findings have been reported elsewhere (Marcelin et al. 2004).

The largest body of data on treatment-emergent resistance comes from the TORO studies (Melby et al. 2005, 2006a, b). Genotypic resistance to enfuvirtide, as defined by one or more mutations in gp41 residues 36–45, was observed for viruses from 92.7% of patients failing treatment and for 98.8% (246/249) of viruses with at least a fourfold reduction from baseline in susceptibility to enfuvirtide (Melby et al. 2006b). The most common mutation, either alone or in combination, was V38A, observed in 48% of resistant strains. When present as the only mutation (11.9%), V38A was associated with a 54-fold loss of susceptibility with a range of 10- to 215-fold. Similarly, broad ranges in loss of susceptibility were also observed for other mutations, consistent with an important role for viral genetic context in determining the level of resistance conferred by a given mutation (Greenberg and Cammack 2004; Sista et al. 2004; Labrosse et al. 2006).

Outside the gp41 36–45 region, the most common substitution observed in the primary TORO analyses was N126K, which removes a potential N-linked glycosylation site. Another important secondary mutation, S138A, was reported by Xu et al., particularly in association with the N43D mutation (Xu et al. 2005), a finding subsequently confirmed for longitudinal samples from the TORO studies (Melby et al. 2005). This raises an important point in interpreting the clinical virology studies from TORO, namely that envelope resistance testing was generally performed on samples obtained shortly after virological failure which appears to have limited of those studies ability to detect some secondary mutations.

3.5.3 Enfuvirtide Resistance and Viral Fitness

Early growth competition studies performed by Lu and colleagues (Lu et al. 2004) demonstrated reduced fitness in viruses carrying resistance mutations introduced by site-directed mutagenesis, which was strongly correlated with the degree of enfuvirtide resistance ($R^2 = 0.86,\ P < 0.001$). These findings are consistent with prospective studies of enfuvirtide interruption. One of these showed rapid disappearance of resistant quasispecies from plasma populations associated with an immediate but limited increase in plasma viremia (Deeks et al. 2007), suggesting that enfuvirtide retained modest antiviral activity. Several other studies have also documented a rapid disappearance of enfuvirtide-resistance mutations after discontinuation (Menzo et al. 2004; Sista et al. 2004; Poveda et al. 2005; Kitchen et al. 2006). However, Env is the target of significant immune pressure, which could also contribute to selection of different envelope quasi-species after enfuvirtide discontinuation (Kitchen et al. 2006) and indeed, the extent of reduced fitness associated with enfuvirtide resistance in vivo remains in question (Chibo et al. 2007).

3.5.4 Enfuvirtide Resistance and Pathogenesis

In 2006, Stephano Aquaro and colleagues reported an association between specific enfuvirtide-associated mutations and subsequent CD4+ cell count response independent of viral load in 54 patients continuing failing enfuvirtide treatment (Aquaro et al. 2006). Specifically, Aquaro et al. found that the most common enfuvirtide-resistance-associated mutation, V38A, was associated with continued elevation of CD4+ cell counts, while another mutation, Q40H, was associated with decreasing CD4+ cell counts. These findings were confirmed in a retrospective analysis of data from the TORO studies, which further demonstrated intermediate levels of CD4+ cell response for patients with N43D or with other mutations (Melby et al. 2007b). A theoretical basis for an impact of enfuvirtide-resistance mutations on viral pathogenesis is provided by data indicating that enfuvirtide-resistance mutations impact the coreceptor binding efficiency and fusogenicity of viral envelope (Reeves et al. 2005) and that these characteristics are also associated with the rate of CD4+ cell decline in SIV models (Karlsson et al. 1998; Etemad-Moghadam et al. 2001).

3.5.5 T-1249 and Other Fusion-Inhibitor Peptides

A number of approaches have been followed in attempts to create even more potent and durable inhibitory peptides including various rational approaches to increase peptide binding affinities, stability, and half-life (Dwyer et al. 2007), and the use of peptides such as 5-helix bearing multiple HR domains (Dimitrov et al. 2005).

One second-generation peptide, T-1249, was developed as a follow-on to enfuvirtide and progressed into phase II clinical studies (Lalezari et al. 2004, 2005b). The sequence of T-1249 was derived from HIV-1, HIV-2, and SIV HR-2 domains.

T-1249 demonstrated substantial activity against enfuvirtide-resistant viruses in clinical studies (Melby et al. 2007a); however, development was discontinued due to formulation issues. Additional peptides with more potent activity were subsequently designed, which also showed much improved pharmacokinetic properties (Dwyer et al. 2007); however, the availability of oral agents in other new classes makes the likelihood of the development of these agents uncertain.

4 Chemokine Antagonists

4.1 Identification of HIV-1 Coreceptors

The identification of the chemokine receptors CCR5 and CXCR4 as HIV-1 entry coreceptors occurred in the mid 1990s (Cocchi et al. 1995; Berson et al. 1996; Deng et al. 1996; Dragic et al. 1996; Feng et al. 1996; Liu et al. 1996; Samson et al. 1996). CCR5 and CXCR4 are members of a large superfamily of proteins collectively termed 7-trans-membrane, g-protein coupled receptors (GPCR). Both receptors bind chemotactic cytokines or "chemokines"; SDF-1α (CXCL12) in the case of CXCR4 and MIP-1α (CCL3), MIP-1β (CCL4), and RANTES (CCL5) in the case of CCR5 (Mueller and Strange 2004). CXCR4 is constitutively expressed on a wide variety of cell types, whilst the expression of CCR5 is largely restricted to memory T cells, monocyte-derived macrophages and other antigen presenting cells. CXCR4 is important during fetal development and then later for T cell hemostasis, whilst CCR5 is involved in the recruitment of activated memory T cells and macrophages to sites of inflammation.

4.2 Viral Tropism

The identification of CCR5 and CXCR4 as HIV-1 coreceptors largely explains the cellular tropism of different HIV-1 strains. Some virus strains, typically from end-stage patients, replicate to a high titre in immortalized T-cells and used to be classified as *T-tropic* strains. In contrast, most patient isolates replicate only poorly in culture, their growth being restricted to activated primary human lymphocytes and macrophages (*M-tropic* strains). It is now generally accepted that T-tropic strains preferentially use CXCR4 as their entry coreceptor, whilst most M-tropic strains preferentially use CCR5.

The discovery that HIV-1 coreceptors are key determinants of cellular tropism has led to virus strains being reclassified based on their coreceptor usage (Berger et al. 1998). Thus CCR5-tropic (R5) viruses infect cells via CCR5, CXCR4-tropic (X4) viruses use CXCR4, and dual-tropic (R5X4) viruses can use either receptor for entry. This nomenclature works well for lab strains and cloned virus isolates.

The situation is somewhat more complex for virus sample amplified or isolated directly from patient plasma, as these samples comprise a heterologous mix of related yet genetically distinct "quasi-species." Often these samples contain mixtures of CCR5-tropic (R5), CXCR4-tropic (X4), and/or dual-tropic viruses; these samples are classified in phenotypic tropism assays as dual/mixed tropic (D/M) (Whitcomb et al. 2007).

Data from a number of clinical cohort studies and clinical trials illustrates that HIV-1 is predominantly classified as R5 in treatment-naive patients (Brumme et al. 2005; Moyle et al. 2005). In treatment-experienced patients (with low nadir CD4+ count), there is an increase in CXCR4-using virus, which is almost entirely due to an increase in D/M virus (Moyle et al. 2005; Melby et al. 2006a). Pure X4 virus remains rare and indeed, even in treatment-experienced patients, 47–62% of patients continue to have only R5 virus.

4.3 Discovery of Maraviroc

A high throughput screen was developed at Pfizer Sandwich Laboratories (Kent, UK), which probed the ability of compounds to inhibit the binding of a radiolabeled chemokine MIP-1β to membranes expressing human CCR5. Similar approaches using radiolabeled natural ligands of CCR5, were pursued by scientists at Takeda, Schering, and Merck (Baba et al. 1999; Dorn et al. 2001; Strizki et al. 2001). Active compounds or "hits" from the Pfizer screen were selected as starting points for a comprehensive medicinal chemistry program (Wood and Armour 2005). A parallel screening strategy was employed to select compounds based on improved antiviral activity, pharmacokinetic profile and safety. Two years and approximately one thousand analogues later, UK-427,857 (maraviroc) was nominated as a suitable clinical candidate for the treatment of HIV-1 infection.

Consistent with its mechanism of action, maraviroc selectively inhibits replication of CCR5-tropic viruses, including HIV-1 primary isolates of different clade and wide geographic origin (Dorr et al. 2005). Maraviroc is also not antagonistic when tested in vitro in combination with reverse transcriptase inhibitors, protease inhibitors, or enfuvirtide, supporting its use in multidrug regimens in patients.

4.4 Maraviroc Mechanism of Action

The small molecule CCR5 antagonists that have been described to date are all non-competitive allosteric inhibitors of HIV-1 entry (Watson et al. 2005). They bind to a transmembrane pocket that is near to, but distinct from, the extracellular domains recognized by the viral envelope gp120 (Dragic et al. 2000; Castonguay et al. 2003; Maeda et al. 2004b; Nishikawa et al. 2005; Seibert et al. 2006) (Fig. 3). It is proposed that in binding to CCR5, these compounds stabilize a conformation that is no longer recognized by the viral envelope.

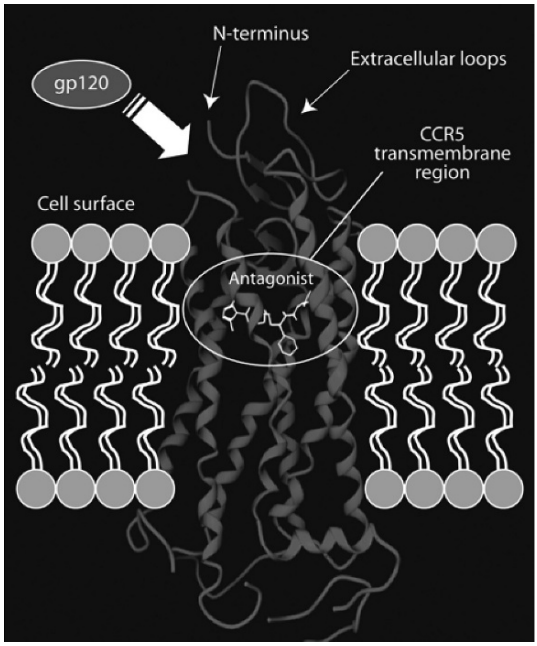

Fig. 3 Model for binding site of maraviroc on CCR5

The binding site of maraviroc on CCR5 was investigated using site-directed mutants of the human recombinant receptor. These mutants were expressed transiently in immortalized cell lines and were tested for their ability to inhibit maraviroc's antagonistic function. Maraviroc was thus shown to have important binding interactions with a tyrosine residue in the third transmembrane region (Y108) and a glutamate residue in the seventh trans-membrane region (E283). Other CCR5 antagonists are thought to bind in a similar region of CCR5. However, modeling suggests that the compounds do not appear to occupy exactly the same space, which may explain the lack of in vitro cross resistance obtained for maraviroc-resistant virus (Westby et al. 2007; discussed later).

4.5 Clinical Development of Maraviroc

Pharmacokinetic studies demonstrated good oral bioavailability of maraviroc and a terminal half-life of 16–23 h following multiple dosing (Abel et al. 2003; Walker et al. 2005). Single doses of up to 900 mg and multiple doses of up to 300 mg BID for 28 days were well tolerated (Abel et al. 2003; Russell et al. 2003; Walker et al. 2005). In Phase 2a studies, treatment-naive HIV-1 patients with R5 virus who received maraviroc monotherapy at doses ranging from 25 mg QD to 300 mg BID for 10 days experienced a median viral load reduction of 1.64 $\log_{10}$ copies/mL and

a maximum VL reduction of 1.84 $\log_{10}$ copies/mL at nadir (Fatkenheuer et al. 2005). The pivotal Ph2b/3 studies were two randomized, placebo-controlled trials (MOTIVATE-1 and −2) in triple drug class experienced patients with R5-virus. Patients were randomized to receive maraviroc (300 mg twice daily or once daily) or placebo, each in combination with an OB regimen. Maraviroc provided significantly superior virologic control and increases in CD4 cell count compared with placebo + OB. At both 24 weeks and 48 weeks, the maraviroc groups had greater decreases in viral load and higher rates of HIV RNA suppression to <400 and <50 copies/mL; these differences were statistically highly significant (Nelson et al. 2007; Lalezari et al. 2007). Trials to examine the safety of maraviroc in patients harboring non-R5 HIV-1 showed maraviroc was safe and well tolerated in this advanced population with documented D/M HIV-1 infection. While superiority was not demonstrated for either the once daily or twice daily maraviroc dose added onto OB vs. OB alone, there was no evidence of virological or immunological decline. In fact, a greater CD4 increase occurred in both maraviroc groups vs. the placebo group, which requires further investigation (Mayer et al. 2006).

4.6 Clinical Virology of Maraviroc

4.6.1 Selection for Virus that Uses CXCR4 for Entry

Serial viral passage data suggest that true coreceptor "switch" of a CCR5-tropic virus to one that can use CXCR4 for entry may not be common (Trkola et al. 2002; Maeda et al. 2004a; Baba et al. 2006). This may be because many CCR5-tropic viruses require the sequential accumulation of multiple mutations to change their tropism (Pastore et al. 2004, 2006). The serial passage of six CCR5-tropic primary isolates and one lab adapted strain (Ba-L) through PBMC in the presence of increasing concentrations of maraviroc did not select for virus that used CXCR4 for entry (Westby et al. 2007). For one isolate, SF162, a change in coreceptor usage was observed during the experiment, but this occurred simultaneously in the maraviroc and drug-free passage control arms, indicating that the selective pressure responsible for the change was exerted by the culture conditions rather than maraviroc per se. SF162 has been shown by others to switch tropism upon serial passage (Harrowe and Cheng-Mayer 1995; Dejucq et al. 2000) and it may therefore represent an atypical rather than a representative CCR5-tropic isolate.

In support of this, the apparent emergence of CXCR4-using virus in some individuals during short-term monotherapy with a CCR5 antagonist appears to result from outgrowth or "unmasking" of preexisting minority CXCR4-using populations (Kitrinos et al. 2005; Westby et al. 2006). More recently, virus clones have been studied in pretreatment and on-treatment samples from 20 anti-retroviral treatment-experienced patients, in whom there was an apparent change in viral coreceptor usage (Lewis et al. 2007). These patients were enrolled in MOTIVATE-1 and −2. An extensive clonal analysis (comprising tropism and sequence determination of 250

envelope clones per patient) identified two patterns of response. For some patients, CXCR4-using clones were identified at a low frequency in the baseline sample and these were genetically very similar to the CXCR4-using virus detected on-treatment. In other cases, the on-treatment CXCR4-using virus was genetically so distinct from the CCR5-tropic virus at baseline (e.g., there were 7–17 amino acid differences in their V3 loops alone) that emergence of a pretreatment CXCR4-using virus (present at baseline but not detected) was by far the most likely explanation.

4.6.2 Selection for Virus that Uses CCR5 but is Resistant to Maraviroc

Some degree of resistance with continued CCR5 use is possible if viruses develop an increased affinity for unbound CCR5 molecules, characterized phenotypically by a classical shift in IC_{50} (Maeda et al. 2000). However, growing in vitro evidence identifies an alternative pathway to resistance for CCR5 antagonists that is characterized by dose–response curves with plateaus in maximal inhibition (Fig. 4). This phenotype is consistent with the ability of the resistant viruses to use inhibitor–receptor complexes for viral entry (Pugach et al. 2007; Westby et al. 2007).

Maraviroc-resistant virus was not selected during prolonged serial passage of the lab-adapted strain Ba-L or 3 clade B primary isolates of Brazilian origin, suggesting that there was a high genetic barrier to maraviroc-resistance under the conditions used (Westby et al. 2007). A similar finding was reported with the discontinued CCR5 antagonist aplaviroc (GW873140), where no detectable resistance was observed after 45 passages of Ba-L through PM1 cells (Maeda et al. 2004a). Resistant viruses were specifically selected from two primary isolates (strains CC1/85 and RU570) following their serial passage through PBL in the presence of increasing maraviroc concentrations. The primary isolate CC1/85 has also been used in serial passage experiments to select for resistance to Schering compounds (Trkola

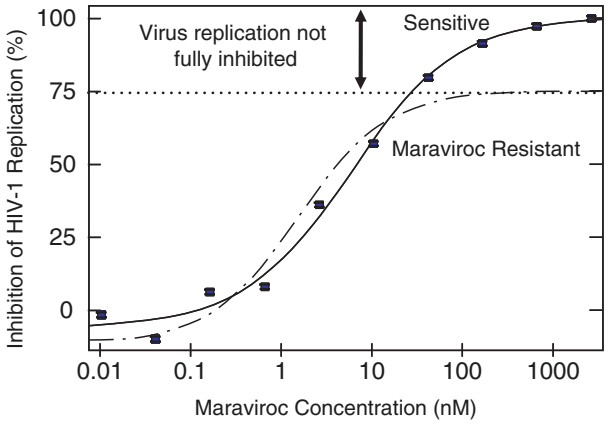

Fig. 4 Plateaus in dose response inhibition curves are a phenotypic marker of resistance to maraviroc

et al. 2002; Marozsan et al. 2005). Site directed mutagenesis of the gp120 envelope from maraviroc-resistant virus confirmed that amino acid mutations in the third variable loop (V3) conferred the resistant phenotype. However, the gp120 mutations selected in the maraviroc-resistant variants of strains CC1/85 and RU570 were different, which suggests that there are multiple genetic pathways to resistance.

Data is now emerging for patients who fail antiretroviral regimens containing maraviroc without emergence of CXCR4-tropic strains. Plateaus in dose response curves (rather than shifts in IC_{50}) appear to be the phenotypic marker of maraviroc resistance in vivo, consistent with the findings from the serial passage experiments (Mori et al. 2007). Furthermore, the gp120 mutations selected in vivo appear to be clustered in the V3 loop but differ between patients. Other groups have described similar results for patients failing vicriviroc (Tsibris et al. 2007).

4.6.3 Cross-Resistance of Maraviroc-Resistant Virus

Cross resistance between the Schering compounds, AD101, Schering-C, and vicriviroc (Schering-D), has been described following serial passage (Trkola et al. 2002; Marozsan et al. 2005). In contrast, virus resistant to the Takeda lead compound, TAK-652, appeared to retain sensitivity to another Takeda CCR5 antagonist, TAK220 (Baba et al. 2006). Similarly, virus selected by serial passage that was highly resistant to maraviroc remained sensitive to other CCR5 antagonists (Westby et al. 2007). This is consistent with the receptor binding site model of CCR5 antagonists described earlier, which suggests that the lead compounds occupy the transmembrane pocket in a different way, selecting distinct conformations of the bound receptor. It is likely therefore that although the resistant virus is able to recognize the CCR5 conformation stabilized by maraviroc, it remains unable to recognize the receptor when another CCR5 antagonist is bound; whether this holds true for clinically derived maraviroc-resistant isolates remains to be confirmed.

4.7 Other Chemokine Antagonists

A large number of CCR5-specific small molecule inhibitors of HIV-1 infection have been described. The development of some early compounds was discontinued, including TAK779 (Baba et al. 1999), Schering-C (Strizki et al. 2001), and aplaviroc (Maeda et al. 2001). Aplaviroc showed promise both in vitro (Maeda et al. 2004a; Watson et al. 2005) and in vivo (Lalezari et al. 2005a), but it was discontinued in September 2005 during Phase 2b clinical trials when hepatotoxicity was reported in some patients.

Schering recently recommenced Phase 3 trials of vicriviroc (Schering-D). This compound has a favorable QTc profile compared to Schering-C (Strizki et al. 2005) and has shown activity in clinical trials (Este 2002). Incyte has been developing INCB9471, which was efficacious and well-tolerated when given once daily in a

phase 2a monotherapy trial (Cohen et al. 2007; Erickson-Viitanen et al. 2008). Pfizer has recently published on a second CCR5 antagonist, PF-232,798, which is currently in phase 2 clinical evaluation for the treatment of HIV-1 infection (Dorr et al. 2008). PF-232798 is an imidazopiperidine small molecule antagonist with considerable structural differentiation from maraviroc and the potential for once daily dosing. Takeda continue to nominate candidate molecules, the latest of which is TAK652 (Baba et al. 2006). There are also CCR5-specific antibody approaches being pursued by Progenics Pharmaceuticals (Trkola et al. 2001; Murga et al. 2006) and Roche (Ji et al. 2007). Since these are parenterally administered but are projected to have a long half life, their place in the HIV treatment armentarium will be distinct from the oral CCR5 antagonists.

Two CXCR4 antagonists from Anormed have progressed to clinical development. AMD3100 and AMD070 are potent antivirals selective for X4 strains in vitro but both development programs have since been terminated for HIV. A pivotal Phase 2a clinical study by Hendrix and colleagues revealed that CXCR4 is likely a flawed drug target for HIV-1. Viral responses in most enrolled patients were poor due to the fact that the patients had significant levels of circulating R5 virus which was not inhibited by AMD3100 (Hendrix 2004). This might argue for coadministration of a CCR5 antagonist and a CXCR4-antagonist (thereby inhibiting R5, X4, and dual tropic viruses simultaneously). However, there are safety concerns with chronic inhibition of the CXCR4 receptor. AMD3100 administration leads to temporal increases in white blood cell count, which is a result of rapid mobilization of hemopoeitc stem and progenitor cells. This side effect has led to AMD3100 being redeveloped for transplantation.

5 Attachment Inhibitors

Early attempts to inhibit gp120 binding to CD4 with soluble, recombinant CD4 were abandoned because of poor clinical response, attributable to insufficient activity of the CD4 against primary HIV-1 isolates (Schooley et al. 1990; Collier et al. 1995). Progenics Pharmaceuticals designed PRO-542, a tetravalent CD4-IgG2 fusion protein incorporating four copies of the Env-binding domains of CD4. This did show some limited efficacy in two Phase 1 studies in HIV-1 infected adults and children (Jacobson et al. 2000; Shearer et al. 2000), but further clinical development has since been terminated. Ibalizumab (TNX-355) is a humanized IgG4 monoclonal antibody targeted towards CD4 rather than gp120. This compound demonstrated antiviral efficacy in HIV-1 infected patients (Kuritzkes et al. 2004) and in vitro experiments suggested that combinations of Ibalizumab with other entry inhibitors may be synergistic (Zhang et al. 2006).

Two orally bioavailable compounds that target HIV-1 attachment have been described. BMS-806 and BMS-043 bind to gp120 (reviewed by Kadow et al. 2006) and demonstrate variable activity when tested in vitro against panels of virus isolates (Lin et al. 2003). BMS-043 has shown efficacy in short-term monotherapy studies

in HIV-1 infected individuals (Hanna et al. 2004). These compounds appear to exert their antiviral activity by stabilizing a protein confirmation in their target (gp120) that prevents engagement of CD4 and hence inhibits downstream entry events (Ho et al. 2006). However, their variable spectrum of activity against diverse clinical isolates will prevent these compounds from progressing and compounds targeting gp120 with a broad spectrum of activity have yet to be disclosed.

6 Concluding Remarks

The successful development and regulatory approval of enfuvirtide and maraviroc have shown that viral entry is a valid target for therapeutic intervention. In pivotal clinical trials, each compound has demonstrated equivalent/superior potency and safety to drug classes targeting the viral enzymes. Coupled with the recent approvals of other antiretrovirals, these compounds have helped revolutionize the treatment of HIV-1 patients harboring drug-resistant strains such that fully suppressive regimens are once again the goal of treatment across the treatment-experience continuum. Furthermore, their discovery and successful development proves that targeting virus entry is a viable therapeutic strategy for treating diseases resulting from virus infections.

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Interferons and Their Use in Persistent Viral Infections

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Contents

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H.-G. Kräusslich, R. Bartenschlager (eds.), *Antiviral Strategies,* Handbook of Experimental Pharmacology 189,

Abstract In 2007, the world celebrated the 50th anniversary of the discovery of interferon (IFN) by Isaacs and Lindenmann. Subsequently, the IFN-α gene was cloned, fully sequenced and IFN-α was produced in recombinant form. Recombinant IFN-α is now used as the basis for treatment of chronic hepatitis C virus infection and can also be used to treat certain forms of chronic hepatitis B virus infections. IFNs have also been used in other viral infections, although with less success. The antiviral mechanisms of IFNs are reviewed in this chapter as well as the utility of IFNs in the treatment of persistent viral infections.

Abbreviations

CARD	Caspase recruitment domains
ds	Double-stranded
ERK 1/2	Extracellular signal-regulated kinase 1/2
GAF	γ-IFN activation factor
GAS	γ-IFN activation site
HAART	Highly active antiretroviral therapy
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDV	Hepatitis D/delta virus
IFN	Interferon
ISRE	IFN-stimulated response element
LRR	Leucine-rich repeats
MDA-5	Melanoma differentiation-associated gene 5
MEK 1/2	Mitogen-activated/extracellular signal-regulated kinase 1/2
NK	Natural killer cells
PKR	RNA activated protein kinase
PRR	Pattern-recognition receptors
RIG-1	Retinoic acid-inducible gene 1
RLR	Retinoic acid-inducible gene 1 (RIG-1)-like helicase receptors
TIR	Toll/IL-1R/resistance
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing IFN-β
TRIM	Tripartite motif protein

In 2007, the world celebrated the 50th anniversary of the discovery of interferon (IFN) by Isaacs and Lindenmann (Isaacs and Lindenmann 1957). Human IFN-α was the first cytokine to be purified to homogeneity. Subsequently, the IFN-α gene

was cloned, fully sequenced and IFN-α was produced in recombinant form in *E. coli* (Nagata et al. 1980). Recombinant IFN-α is now used as the basis for treatment of chronic hepatitis C virus (HCV) infection and can also be used to treat certain forms of chronic hepatitis B virus (HBV) infections. IFNs have also been used in other viral infections, although with less success.

1 Interferons

IFNs are natural glycoproteins produced by the cells of most vertebrates in response to the challenge by foreign agents, such as infectious organisms (viruses, bacteria, fungi, and parasites), and by tumor cells. IFNs can be produced by cells of the innate and adaptive immune systems and by non-immune cells such as fibroblasts and epithelial cells.

1.1 Type I IFNs

Type I IFNs form a superfamily of innate cytokines that comprise IFN-α (alpha), with 13 human subtypes, IFN-β (beta), IFN-ω (omega), IFN-τ (tau), IFN-κ (kappa), IFN-ε (epsilon), IFN-λ (lambda), and IFN-ζ (zeta) (Pestka et al. 2004). Only IFN-α, IFN-β, IFN-ω, IFN-κ, and IFN-ε are expressed in humans. All IFN-α subtypes are secreted by leukocytes, whereas IFN-β is also produced by fibroblasts. IFN-τ is exclusively expressed in ruminants at a specific stage of pregnancy (Martal et al. 1998), whereas IFN-κ is expressed by human keratinocytes (LaFleur et al. 2001).

There are 17 human type I IFN genes, all clustering on chromosome 9. They are intronless and encode secretory signal peptide sequences that are proteolytically cleaved prior to secretion from the cell. Type I IFNs are genetically and structurally closely related. They range in length from 161 to 208 amino acids and have apparent molecular weights of 15–24 kDa (Table 1) (Chen et al. 2004). The different subtypes of human IFN-α have approximately 50% amino acid sequence identity, whereas IFN-α shares approximately 22% amino acid identity with human IFN-β and 37% with human IFN-ω (Chen et al. 2004).

1.2 Type II IFNs

There is only one known type II IFN, IFN-γ, discovered in 1965 (Wheelock and Sibley 1965). IFN-γ is exclusively produced by immune cells, such as activated thymus-derived T cells and natural killer (NK) cells, after stimulation by foreign antigens or mitogens in the early stages of the innate immune response (Boehm et al. 1997). The human IFN-γ gene maps to chromosome 12. IFN-γ is a noncovalent

Table 1 Characteristics of the principal human IFN genes

	Type I IFNs			Type II IFN	Type III IFNs	
	IFN-α	IFN-β	IFN-ω	IFN-γ	IFN-λ1 (IL-29)	IFN-λ2/3 (IL-28A/B)
Gene	*IFNA*	*IFNB*	*IFNW*	*IFNG*	*IFNL*	*IFNL*
Chromosomal localization	Chromosome 9	Chromosome 9	Chromosome 9	Chromosome 12	Chromosome 19	Chromosome 19
Number of amino acids	165 or 166	166	172	143	181	175
Number of structural genes	13	1	1	1	5	6
Number of introns	None	None	None	3	5	6
Receptor	IFNAR-1/2	IFNAR-1/2	IFNAR-1/2	IFNGR-1/2	IFNLR-1/IL10R2	IFNLR-1/IL10R2
Producing cells	Hematopoietic cells, mainly leukocytes	Fibroblasts and some epithelial cell types	Hematopoietic cells, mainly leukocytes	Immune cells, mainly T cells and NK cells	Hematopoietic cells, mainly monocytes	Hematopoietic cells, mainly monocytes

homodimer composed of two identical 17-kDa polypeptide chains 166 residues long (Ealick et al. 1991). The 23-residue hydrophobic signal sequence is removed by proteolytic cleavage prior to secretion from the cell (Table 1) (Boehm et al. 1997).

1.3 Type III IFNs

Recently, a novel class of type I-like human IFNs, named IFN-λ1 or IL-29, IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B), was identified (Dumoutier et al. 2003; Sheppard et al. 2003). The three IFN-λ genes cluster on human chromosome 19 and comprise 5 exons for IFN-λ1 and 6 for IFN-λ2 and IFN-λ3, and several introns (Table 1). They encode 20- to 22-kDa secreted monomeric proteins of 196 to 200 amino acids. Type III IFNs have also been identified in other species such as mice, birds, and fish.

2 IFN Production during Viral Infections

Production of types I and III IFNs is an essential component of the innate immune response, the first line of defense against an invading pathogen (Gale and Foy 2005). IFNs exert auto- and paracrine actions within a few hours in response to a viral infection. Their protective function is dual: they induce an antiviral cellular state and facilitate the clearance of infected cells by activating apoptosis, in synergy with other proapoptotic agents such as tumor necrosis factor (TNF) family members, depending on the intracellular level of double-stranded (ds) RNAs (Clemens 2003).

2.1 Viral Triggering of IFN Production

Most cell types recognize incoming RNA or DNA viruses, owing to the presence of intracellular RNAs with a 5′ triphosphate end (i.e. lacking a 5′ cap structure) or dsRNA structures generated during viral RNA replication. Indeed, dsRNA replication intermediates are essential for the synthesis of new RNA genomes, whereas DNA viruses often have overlapping open reading frames lying in opposite orientations, and these generate mRNA transcripts that can base-pair to form dsRNA stretches. The conserved molecular patterns of dsRNAs are recognized by pattern-recognition receptors (PRR), of which the best known are Toll-like receptors (TLR) and retinoic acid-inducible gene 1 (RIG-1)-like helicase receptors (RLR) (Yoneyama et al. 2004). TLRs are present at the cell surface and in endosomes and recognize various microbial structures, whereas RLRs are expressed ubiquitously in the cytoplasm and recognize only viral structures. Their activation leads to the production of both type I and III IFNs, and this production appears to be regulated by

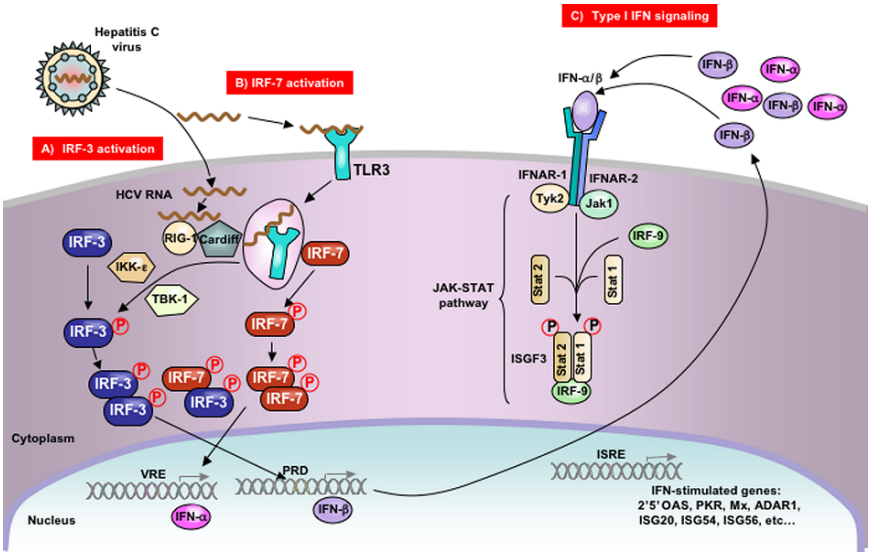

Fig. 1 Type I IFN production and signaling pathways. (**a**) Interferon regulatory factor 3 (IRF-3) activation. HCV RNA binding to retinoic acid-inducible gene 1 (RIG-1) or Toll-like receptor 3 (TLR-3) results in the phosphorylation and activation of IRF-3 by the tumor necrosis factor receptor-associated factor-associated nuclear factor κB (NF-κB) activator binding kinase 1 (TBK-1) or inhibitory κB kinase ε (IKK-ε) protein kinases. The dimer of phosphorylated IRF-3 translocates to the cell nucleus, interacts with its transcription partners, and binds to the cognate-DNA positive regulatory domain (PRD) in the promoter region of IRF-3 target genes, including IFN-β, resulting in IFN-β production and secretion. (**b**) IRF-7 activation. IRF-7 is a transcription factor and an IFN-stimulated gene (ISG). It is activated after expression by viral components through signaling pathways that overlap with the pathways of IRF-3 activation. IRF-7 phosphorylation, dimerization, and heterodimerization with IRF-3 allow it to bind its cognate virus-responsive element (VRE) in the promoter region of IFN-α genes, resulting in the production of various IFN-α subtypes that further signal ISG expression. (**c**) Type I IFN signaling. Type I IFN binding to the receptor signals the activation of the associated Tyk2 and Jak1 protein kinases to direct the phosphorylation and assembly of a Stat1–Stat2 heterodimer and trimeric IFN-stimulated gene factor 3 (ISGF3) complex containing IRF-9. The ISGF3 complex locates to the cell nucleus, where it binds to the IFN-stimulated response element (ISRE) on target genes to direct ISG expression. Adapted from Gale and Foy (Gale and Foy 2005) and Stephen J. Polyak, presentation at the Post-Graduate Course of the 41st Annual Meeting of the European Association for the Study of the Liver, Vienna (Austria), April 26–27, 2006, with permission

a common mechanism (Ank et al. 2006; Onoguchi et al. 2007; Uze and Monneron 2007). Type I IFN production is described in Fig. 1.

2.1.1 TLR Pathways

At least 12 different TLRs have so far been identified in mammals (Uematsu and Akria 2007). TLRs are type I transmembrane PRRs that possess an extracellular domain containing leucine-rich repeats (LRR), a transmembrane domain, and an

intracellular signaling domain known as the Toll/IL-1R/resistance (TIR) domain. The difference in the amino acid number and the molecular weight of different TLRs is principally related to differences in the number of LRRs in their extracellular domain, which allow them to recognize different ligands. The TIR domain can interact with various adaptor molecules, such as myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adaptor inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM), thereby activating intracellular signaling pathways.

2.1.2 RLR Pathways

In cell types other than dendritic cells, RLRs such as RIG-1 and melanoma differentiation-associated gene 5 (MDA-5) appear to play a key role in the response to viral infections (Kato et al. 2005). RIG-1 contains two tandem caspase recruitment domains (CARD), which are needed to activate NF-κB and IRF-3. Double-stranded RNA binding to the RNA helicase domain of RIG-1 induces a conformational change that exposes the CARD domains to downstream signaling proteins. MDA-5 also contains two CARD domains that can activate the IFN-β promoter (Creagh and O'Neill 2006; Seth et al. 2006). A recent study has shown that the tripartite motif protein 25 (TRIM25), a ubiquitin ligase, catalyzes RIG-1 ubiquitination, a crucial step in the cytosolic pathway by which RIG-1 elicits host antiviral responses (Gack et al. 2007).

2.2 IFN Gene Induction

The induction of type I and III IFN gene transcription is principally controlled by transcription factors NF-κB, IRF-3, and IRF-7 (Fig. 1). In its inactive state, NF-κB is held in the cytosol by members of the inhibitory κB (IκB) family. In the presence of incoming viruses, the IκB kinase (IKK) is activated and phosphorylates IκB, which is subsequently ubiquitinated and degraded by the proteasome. Free NF-κB translocates into the nucleus, where it increases the transcription of target genes. Like NF-κB, the inactive form of IRF-3 is cytosolic. In response to a viral infection, IRF-3 is phosphorylated by two IKK-like kinases: TNF receptor-associated factor (TRAF)-associated NF-κB activator (TANK) binding kinase 1 (TBK-1) and IKKε. IRF-3 phosphorylation leads to its dimerization and translocation into the nucleus (Hayden et al. 2006; Seth et al. 2006). IRF-7 is expressed at low levels in most cells. It is also activated through phosphorylation by TBK-1 and IKKε (Honda et al. 2005). NF-κB, IRF-3, IRF-7, and nuclear architecture proteins assemble into a complex in the nucleus that remodels the chromatin in IFN gene promoters, resulting in an increase in transcriptional initiation.

3 Responses to IFN

3.1 IFN-Induced Signaling Pathways

On binding to its receptor, the IFN molecule is sandwiched between the two chains of the receptor, thus forming a ternary complex that activates the canonical Jak/Stat pathway through activation of the transduction elements located in the intracytoplasmic tail of each receptor subunit (Fig. 1).

3.1.1 Type I and III IFNs

The similarity of the transcriptional responses induced by type I and type III IFNs has been confirmed by microarray analysis of Jak/Stat pathway gene expression in various cell lines (Doyle et al. 2006; Dumoutier et al. 2004). Figure 1 shows type I IFN signaling through the Jak-Stat pathway. Type I and III IFN binding to their specific receptor triggers the activation of Jak1 and Tyk2 through tyrosine transphosphorylation. The activated kinases induce the formation of the IFN-stimulated gene factor-3 (ISGF3), itself composed of two elements, ISGF3α and ISGF3γ. ISGF3α is formed of two cytoplasmic peptides, Stat1 (p91) and Stat2 (p113), which share 42% nucleotide identity. ISFG3γ is a member of the IRF family, which has been renamed IRF-9. The IRF-9 protein is a single polypeptide with an apparent molecular weight of 48 kDa. It comprises two domains: a conserved amino-terminal DNA-binding domain and a carboxy-terminal Stat-binding domain. IRF-9 is mostly cytoplasmic and inactive in untreated cells. It migrates to the nucleus after IFN treatment.

The phosphotyrosyl residues of activated Jak proteins serve as docking sites for Stat1 and Stat2, both of which are phosphorylated on a single tyrosine residue by activated Jak proteins. Stat1 and Stat2 phosphorylation induces their release from the IFNARs and their dimerization through their SH2 domain. Stat1–Stat2 heterodimers then move to the nucleus to form the ISGF3 complex, together with IRF-9 (Fig. 1). ISGF3 then interacts directly with the IFN-stimulated response element (ISRE), a DNA sequence that characterizes ISGs. These may contain one or several ISRE sequences in their promoter sequence. Other Stat proteins have been shown to be activated by type I and III IFNs, but their involvement in IFN responses may be restricted to certain cell types (Takaoka and Yanai 2006).

3.1.2 Type II IFN

IFN-γ receptor subunits IFNGR1 and IFNGR2 trigger the activation of Jak1 and Jak2 through tyrosine phosphorylation. Jak1 and Jak2 activation leads to the formation of a multimeric complex, γ-IFN activation factor (GAF), that transduces the signal to the target DNA sequence in ISGs, known as the γ-IFN activation site (GAS). GAF is a homodimer of tyrosine-phosphorylated Stat1 proteins. GAF exists in latent form in the cell cytoplasm. It can be rapidly activated following IFN-γ

binding to its receptor by phosphorylation at Stat1 tyrosine residue 701. Once activated, GAF translocates into the nucleus and binds GAS sequences to induce ISG transcription. It was recently shown that Stat1–Stat2 heterodimers can also activate GAS elements (Takaoka and Yanai 2006). In addition, a novel *c-Jun*-dependent signal transduction pathway induced by type II IFN has been identified. This pathway involves protein kinases other than Jaks, including extracellular signal-regulated kinase 1/2 (ERK1/2) and mitogen-activated/extracellular signal-regulated kinase 1/2 (MEK1/2). This pathway appears to operate in parallel with the canonical Jak/Stat signaling pathway (Gough et al. 2007).

3.2 IFN Antiviral Effectors

The antiviral state induced by different types of IFNs is mediated by various IFN-induced proteins. The best-known antiviral effectors produced as a result of IFN cascade induction are shown in Table 2. They include 2′–5′ oligoadenylate synthetase (2′–5′OAS), double-stranded RNA activated protein kinase (PKR), and myxovirus (Mx) proteins. Additional effectors include RNA-specific adenosine deaminase 1 (ADAR1), the 20-kDa ISG product (ISG20), ISG54 and ISG56, and IFN-stimulated micro RNAs (Pedersen et al. 2007).

3.3 Immunomodulatory Properties of IFNs

In addition to their direct antiviral properties described earlier, IFNs exhibit potent immunomodulatory properties that contribute to their antiviral effects by activating

Table 2 Interferon-induced antiviral effectors

Short name	Name	Function(s)
2′-5′ OAS	2′-5′ oligoadenylate synthetase	Activate a latent endoribonuclease, RNAse L, which degrades viral and cellular mRNAs and rRNAs
PKR	Double-stranded RNA-activated protein kinase	A serine threonine kinase that phosphorylates eIF2α on serine residue 51 when activated
MxA and MxB	Mixovirus proteins A and B	IFN-induced GTPases that block intracellular transport of viral components
ADAR1	Adenosine deaminase acting on RNA 1	Involved in RNA editing
ISG20	IFN-stimulated gene product of 20 kDA	A member of the DEDD exonuclease superfamily with RNAse and DNAse activity
ISG54 and ISG56	IFN-stimulated gene products of 54 and 56 kDA	Inhibit translation
miRNA	IFN-induced micro RNAs	Specifically target viral transcripts

cells other than those that are infected (Pestka et al. 1987). IFNs can stimulate the effector function of NK cells, cytotoxic T lymphocytes, and macrophages, upregulate the expression of major histocompatibility complex (MHC) class I and class II molecules, induce immunoglobulin synthesis by B cells, and stimulate the proliferation of memory T cells. This offers various modes to control viral replication, by modulating the innate and adaptive immune responses (Guidotti and Chisari 2001). Type I IFNs act through activation and maturation of dendritic cells, leading to MHC upregulation. They can also upregulate various chemokines, chemokine receptors, and costimulatory molecules, which, in turn, stimulate CD4-positive and CD8-positive T cell responses and modulate T lymphocyte responses through the promotion of Th1 differentiation (Solis et al. 2006).

IFN-γ is a major immunoregulatory cytokine involved in the regulation of nearly all phases of immune and inflammatory responses, including activation and differentiation of T cells, B cells, NK cells, and macrophages. The expression of class II MHC molecules on immune cells is principally regulated by IFN-γ, especially on monocytes and macrophages. IFN-γ also increases the transcription of the β_2-microglobulin light chain of MHC class I molecules. In addition, IFN-γ regulates T cell functions and modulates neutrophil functions by modulating the surface expression of various molecules, including integrins and chemokine receptors (Gattoni et al. 2006a, b).

4 Therapeutic Forms of IFN

4.1 Standard IFN-α

Different forms of IFN-α have been available for the treatment of chronic hepatitis B and C, including IFN-α2a and IFN-α2b. The administered dose was 3–5 megaunits three times a week subcutaneously.

4.2 Pegylated IFN-α

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

Two forms of pegylated IFN-α have been approved by the Food and Drug Administration (FDA) and by the European Medicines Agency (EMEA) for the

Table 3 Pharmacological parameters of pegylated IFN-α molecules approved for the treatment of chronic hepatitis C

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

[a]After subcutaneous injection

treatment of chronic hepatitis C in adults, namely pegylated IFN-α2a and pegylated IFN-α2b, in which the IFN-α molecules are linked to PEG molecules of different sizes (Table 3).

4.3 Other IFNs

Other IFNs that can be used in the treatment of viral infections include IFN-β, IFN-ω, a molecule that is more potent than its nonglycosylated form, which itself has activity comparable to that of IFN-α (Buckwold et al. 2007), and that will be delivered continuously by an implantable device, IFN-γ and IFN-λ1, a pegylated form of which will soon be available.

New IFN-α molecules are also being developed. They include albumin-IFN-α2b (Alb-IFN-α2b, Human Genome Sciences, Rockville, Maryland, and Novartis, Basel, Switzerland), a novel 87.5-kDa recombinant protein consisting of an IFN-α2b molecule attached to a human albumin moiety. Alb-IFN-α2b has a half-life of up to 159 h, allowing dosing at intervals of 2–4 weeks (Balan et al. 2006). Pharmacokinetic studies in monkeys showed approximately 140-fold slower clearance and an 18-fold longer half-life than IFN-α after a single subcutaneous injection (Osborn et al. 2002).

Novel IFN-α variants have been created experimentally by means of DNA shuffling ("gene-shuffled" IFN-α). In this technique, natural IFN-α genes are fragmented and then reassembled through recombination, a process that mimics evolution. Gene-shuffled IFN variants may have increased antiviral and antiproliferative activity (Chang et al. 1999). A library of clones has been generated (Maxygen,

Redwood City, California, and Roche) and two clones have been selected for their improved in vitro activity relative to human IFN-α. One of them has been pegylated but its development has been halted because of strong immune reaction.

IFN alphacon-1 (Infergen, Amgen, Thousand Oaks, California) is a synthetic recombinant "consensus" IFN-α molecule created by scanning the sequences of several natural IFNs and assigning to the recombinant molecule the most frequently observed amino acid at each position (Keeffe and Hollinger 1997). The intrinsic antiviral activity of this IFN appears to be 10- to 100-fold higher than that of standard IFNs (Blatt et al. 1996; Ozes et al. 1992). The future of consensus IFN-α will depend on the development of a pegylated or otherwise pharmacologically modified form. Multiferon (Viragen, Plantation, Florida) is a highly purified, multi-subtype natural human IFN-α derived from human leukocytes. Medusa® (Flamel Technologies, Lyon, France) is a self-assembled poly-aminoacid nanoparticle system that can be used as a protein carrier for novel long-acting native protein drugs. Medusa IFN-α2a and Medusa IFN-β are currently in early clinical development. Various types of orally administered IFN-α are also being developed.

5 IFN-Based Treatment of Hepatitis C Virus Infection

5.1 Treatment of Chronic HCV Infection with IFN-α

Chronic HCV infection is curable, and cure is the goal of antiviral therapy. Successful treatment is characterized by a sustained virological response (SVR), defined by undetectable HCV RNA in a sensitive assay (detection limit $\leq$ 50 international units (IU)/ml) 6 months after the end of therapy. Recent large-scale follow-up studies have shown no relapse or recurrence after 4–6 years in more than 99% of patients who have an SVR (McHutchison et al. 2006; Swain et al. 2007).

The choice of IFN-α as a potential treatment for chronic hepatitis C in 1986 was empirical (Hoofnagle et al. 1986). At this time, the causative agent of chronic "non-A, non-B" hepatitis had not yet been identified, and there was no way of evaluating HCV replication or, thus, the antiviral activity of a drug. In the first cohort of 10 patients with chronic non-A, non-B hepatitis treated with IFN-α, a significant decline in alanine aminotransferase (ALT) levels was observed in 8 patients, and liver histology had improved at the end of therapy in the three patients who were biopsied (Hoofnagle et al. 1986). Ten years later, 5 of the 10 patients were free of infection (Lau et al. 1998).

5.1.1 Standard IFN-α and Pegylated IFN-α Monotherapy

The first National Institutes of Health (NIH) Consensus Development Conference on Management of Hepatitis C, held in 1997 (1997), recommended that standard

IFN-α be used at the same dose for 48 weeks for the treatment of chronic hepatitis C. However, the SVR rates were still only 12–20% with this treatment schedule (Di Bisceglie and Hoofnagle 2002; Lindsay et al. 2001). The development of pegylated IFN-α was found to ensure sustained drug exposure. Pegylated IFN-α2a is administered at a fixed dose of 180 μg/week, whereas pegylated IFN-α2b is administered at a weight-adjusted dose of 1.5 μg/kg/week. Both pegylated IFNs have been reported to yield a twofold higher SVR rate than the corresponding standard IFN-α when administered alone for 48 weeks

5.1.2 IFN-α-Ribavirin Combination Therapy

Ribavirin is a guanosine analogue with a broad spectrum of activity against DNA and RNA viruses (Sidwell et al. 1972). Ribavirin modestly and transiently inhibits HCV replication in vivo (Pawlotsky et al. 2004), but it efficiently prevents relapses during IFN-ribavirin combination therapy (Bronowicki et al. 2006). The underlying mechanisms are unknown. In the first trial of standard IFN-α and ribavirin combination therapy, HCV was eradicated in 40% of patients who received the combination, but in none of those on IFN-α monotherapy (Brillanti et al. 1994). Further randomized controlled trials (McHutchison et al. 1998; Poynard et al. 1998) led to the approval of the standard IFN-α-ribavirin combination as the standard treatment for chronic hepatitis C, before the development of pegylated IFNs (1999).

Ribavirin is administered at a dose of 0.8–1.2 g/day, depending on body weight and the HCV genotype (2002). Higher doses may be necessary for heavy patients. The addition of ribavirin increased the SVR rate to 41% and 43%, respectively, compared to 16% and 19% with standard IFN-α2a and IFN-α2b monotherapy (McHutchison et al. 1998; Poynard et al. 1998). In the three main registration trials (randomized controlled studies involving patients without cirrhosis), pegylated IFN-α plus ribavirin gave global SVR rates of 54–56%, compared to 18–39% with pegylated IFN-α monotherapy (Fried et al. 2002; Hadziyannis et al. 2004; Manns et al. 2001). The SVR rates ranged from 76% to 84% in patients with HCV genotype 2 or 3 infection and from 42% to 52% in patients with HCV genotype 1 infection. Little information is available on patients with other genotypes, but the SVR rates in patients with HCV genotype 4 infection appear to be close to those in patients with genotype 1 infection. Pretreatment variables that correlated with sustained viral eradication included HCV genotypes 2 and 3, lower baseline viral load, lower body weight, younger age, and milder hepatic fibrosis (Fried et al. 2002; Hadziyannis et al. 2004; Manns et al. 2001). In pivotal trials approximately 10% of patients discontinued therapy because of adverse events, and dose reductions were required in about 30% of cases (Fried et al. 2002; Hadziyannis et al. 2004; Manns et al. 2001). Neutropenia and thrombocytopenia were frequently associated with IFN-α administration, and hemolytic anemia was the most frequent adverse effect of ribavirin administration (Fried et al. 2002; Hadziyannis et al. 2004; Manns et al. 2001). Ribavirin-associated anemia can be severe and demand discontinuation or dose reduction.

5.1.3 Optimized Pegylated IFN-α–Ribavirin Combination Therapy

Pegylated IFN-α-ribavirin combination therapy can be improved by increasing the dose of pegylated IFN-α and/or ribavirin in selected patients and by tailoring the length of treatment to the virological response.

Elevated Doses

The optimal dose of ribavirin is still uncertain. The approved dose is 0.8 g/day for patients infected with HCV genotypes 2 and 3, and 1.0–1.2 g/day in patients (weighting less and more than 75 kg, respectively) infected with HCV genotypes 1, 4, 5, and 6. However, several studies have suggested that higher serum ribavirin concentrations are associated with higher SVR rates (Jen et al. 2000, 2002; Lindahl et al. 2005). In patients infected with HCV genotype 1, the probability of an SVR increases with the AUC of ribavirin concentrations (Snoeck et al. 2006). In addition, the proportion of patients achieving an SVR is higher with a standard weight-based dose of ribavirin than with a low dose (Jensen et al. 2006). Although adverse effects are more frequent and more serious, the use of high ribavirin doses (average 2.5 g/day) to treat patients with high-viral-load HCV genotype 1 infection is feasible and leads to an SVR in 9 out of 10 patients (Lindahl et al. 2005). The use of higher ribavirin doses is offset by the potential increase in drug-related toxicity (principally hemolytic anemia), especially at doses above 15 mg/kg. Epoetin (or darbepoetin, an epoetin prodrug) can be used to reduce the incidence and severity of anemia. However, neither the FDA nor the EMEA has approved the use of these drugs in the treatment of chronic hepatitis C, and the cost-effectiveness of this approach has been questioned (McHutchison et al. 2007b). In patients with HCV genotype 2 or 3 infection, the SVR rate is not strongly influenced by the dose of ribavirin, and 0.8 g/day is probably sufficient to maximize the chances of achieving an SVR (Fried et al. 2002; Hadziyannis et al. 2004; Manns et al. 2001). Whether lower doses of ribavirin could be sufficient to achieve the same SVR rates is currently under investigation.

In a so-called difficult-to-treat patient population (HCV RNA $>$ 800,000 IU/ml and body weight $>$ 85 kg), raising the doses of both pegylated IFN-α and ribavirin significantly increased the SVR rate (Fried et al. 2006). In another study, weight-based ribavirin administration up to 1.4 g/day was more effective than a fixed dose of 0.8 g/day in patients infected with HCV genotype 1 (Jacobson et al. 2005). In a small number of cirrhotic patients infected with HCV genotype 1 who had not responded to a previous course of pegylated IFN-α and ribavirin, 180 μg of pegylated IFN-α2a every 5 days, combined with ribavirin, induced an SVR in several cases (Hézode et al. 2006). Ongoing trials are assessing more frequent administration and higher weekly doses of pegylated IFN-α, and higher doses of ribavirin, in nonresponder and difficult-to-treat patients. Patients receiving such reinforced therapy must be carefully monitored for toxicity, and the merits and drawbacks of growth factor administration should be considered.

Tailoring the Treatment Duration to the Virological Response

Current guidelines state that the length of treatment should be tailored to the HCV genotype (Pawlotsky 2006). Patients infected with HCV genotypes 1, 4, 5, and 6 should be treated for 48 weeks (with 1.0–1.2 g/day ribavirin), whereas patients infected with HCV genotypes 2 and 3 should be treated for 24 weeks only (with 0.8 g/day ribavirin) (2002; Hadziyannis et al. 2004). Treatment must be stopped at week 12 in genotype 1-infected patients who do not have a 2-log drop in their HCV RNA level. Rapid virologic responses (RVR) are defined by an HCV RNA level below 50 IU/ml at week 4 of therapy. Several recent reports suggest that patients with an RVR could qualify for shorter treatment, which would improve adherence and reduce the cost of therapy. Conversely, extending treatment duration beyond 48 weeks may achieve an SVR in patients infected with HCV genotype 1 who have a slow virologic response, defined as an HCV RNA decline of more than 2 log IU/ml but a value above 50 IU/ml at week 12. For more detailed information, see the chapter by Zeuzem, this volume.

5.2 Treatment of Acute HCV Infection with IFN-α

HCV infection is rarely diagnosed in the acute phase, as most acutely infected individuals are asymptomatic. Between 50% and 90% of patients develop chronic infection, however, and this warrants early therapy. After occupational exposure with a known date, treatment should not be started before the acute episode characterized by alanine aminotransferase elevation, but it should always be started within 24 weeks after the onset of symptoms. The optimal treatment schedule for acute hepatitis C is controversial. Pegylated IFN-α monotherapy at the standard dose for 24 weeks yielded SVR rates close to 100% in symptomatic patients referred to tertiary care centers (De Rosa et al. 2006; Jaeckel et al. 2001; Santantonio et al. 2005; Wiegand et al. 2006). Shorter therapy may be envisaged (Calleri et al. 2007). Combination with ribavirin is recommended if a first course of pegylated IFN-α monotherapy fails to eradicate the infection. Viral elimination appears to be independent of the HCV genotype and the HCV RNA level (Calleri et al. 2007; De Rosa et al. 2006; Jaeckel et al. 2001).

5.3 Future Perspectives of IFN-Based HCV Therapy

5.3.1 Other IFNs

Several studies have tested IFN-β for chronic hepatitis C, achieving response rates similar to those obtained with IFN-α and with similar or fewer adverse effects (Barbaro et al. 1999; Castro et al. 1997; Habersetzer et al. 2000; Montalto et al.

1998; Villa et al. 1996). Recent reports from Japan suggest that daily IFN-β administration is highly effective in patients with low or moderate HCV RNA levels (Horiike and Onji 2003; Shiratori et al. 2000). Twice-daily administration of IFN-β as induction therapy has also been reported to be effective (Kim et al. 2005; Nakajima et al. 2003). It is unlikely, however, that IFN-β will be used in routine clinical practice unless it is pegylated or otherwise modified, and until specific clinical trials are done.

IFN-ω (Intarcia Therapeutics, Emeryville, California) has been reported to be well tolerated and safe, in patients infected with various HCV genotypes, at doses of 15–120 μg three times weekly for 12 weeks, with dose-dependent virological and biochemical responses (Plauth et al. 2002). At a dose of 25 μg daily, IFN-ω induced a 2-log HCV RNA decline at week 12 in two-thirds of 74 patients infected with HCV genotype 1 (Gorbakov et al. 2005). In a recent trial, SVR was achieved in 6% and 36% of patients receiving the same dose of IFN-ω without and with ribavirin, respectively (Novozhenov et al. 2007). A new trial of IFN-ω, delivered continuously by an implantable device, will start soon.

IFN-γ has potent activity against HCV in the subgenomic replicon system (Dash et al. 2005; Frese et al. 2002; Lanford et al. 2003). Synergistic immunomodulatory effects of IFN-γ1b and IFN-α have been reported (Wang et al. 2006). However, a pilot study of IFN-γ at a dose of 100–400 μg three times per week showed no antiviral efficacy in patients infected with HCV genotype 1 who had not responded to standard therapy or who had relapsed (Soza et al. 2005).

IFN-λ1 exhibits dose- and time-dependent inhibition of HCV replication in various models, independently of type I and II IFN receptors and induced pathways (Marcello et al. 2006). A pegylated form of IFN-λ will soon enter clinical evaluation.

5.3.2 New IFN-α Molecules

Alb-IFN-α2b has been reported to induce a dose-dependent antiviral response in previously untreated patients and in nonresponders to the pegylated IFN-α and ribavirin combination (Bain et al. 2006; Balan et al. 2006). The results of a phase II trial in untreated genotype 1-infected patients were recently reported: the SVR rates were not significantly different among four groups of patients receiving either the standard pegylated IFN-α2a and ribavirin combination or alb-IFN-α2b at doses of 900 μg every two weeks, 1,200 μg every 2 weeks and 1,200 μg every 4 weeks (Zeuzem et al. 2007). End-of-treatment responses to alb-IFN-α2b administered every 2 or 4 weeks in combination with ribavirin are similarly frequent in patients infected with HCV genotypes 2 and 3 (Bain et al. 2007). Higher doses of alb-IFN-α2b given every 4 weeks are currently under investigation, and the product will soon enter phase III clinical evaluation.

Consensus IFN-α has been used in various populations of HCV-infected patients but the published results are variable. Consensus IFN-α gave a higher response rate than the combination of standard IFN-α and ribavirin in patients who relapsed

after standard IFN-α monotherapy (Miglioresi et al. 2003). In patients in whom the standard IFN-α-ribavirin combination failed, an SVR rate of approximately 30% was obtained with consensus IFN-α (Bocher et al. 2006). In contrast, an SVR was achieved in only 8% of patients who did not respond to standard IFN-α2b plus ribavirin (Moskovitz et al. 2003). Recently, a direct comparison of consensus IFN-α-ribavirin and pegylated IFN-α-ribavirin showed similar SVR rates of 37% and 41%, respectively, in previously untreated patients infected with HCV genotype 1 (Sjogren et al. 2007). Nevertheless, there is no clear evidence that consensus IFN-α is superior to other IFNs when given at equivalent doses. The future of consensus IFN-α will depend on the development of a pegylated or otherwise pharmacologically modified form.

5.3.3 Enhanced IFN-α-Based Therapy

The results of IFN-α-based therapy can theoretically be improved by using better-tolerated drugs that mimic the action of ribavirin and/or by using HCV inhibitors that, when combined, substantially reduce HCV replication. Since the mechanism of action of ribavirin is still unknown, no credible alternative approach is currently available. In contrast, many specific inhibitors of the HCV replication cycle are in preclinical development and several have reached clinical development (Pawlotsky et al. 2007). A number of them are being tested in combination with pegylated IFN-α, with or without ribavirin.

The NS3/4A serine proteinase inhibitors telaprevir (VX-950, Vertex Pharmaceuticals, Cambridge, Massachusetts) and boceprevir (SCH 503034, Schering-Plough Corporation, Kenilworth, New Jersey) have now advanced to phase II clinical trials. In a recent trial, HCV RNA became undetectable (below 10 IU/ml) in all 12 patients receiving the triple combination of pegylated IFN-α2a, ribavirin, and telaprevir for 28 days (Rodriguez-Torres et al. 2006). Preliminary results of the PROVE 1 phase II trial showed that, after 12 weeks of treatment with telaprevir plus both pegylated IFN-α and ribavirin, HCV RNA was undetectable ($<10\,\mathrm{IU/ml}$) in significantly more genotype 1-infected treatment-naive patients than in the arm receiving only the dual combination of pegylated IFN-α and ribavirin, without telaprevir (70% and 39%, respectively). Telaprevir administration was associated with more frequent dermatological (especially rash and pruritus) and gastrointestinal side effects (McHutchison et al. 2007a). Telaprevir was withdrawn after 12 weeks in all the groups, and the patients will continue on pegylated IFN-α and ribavirin for various times. Other trials of the triple combination given for 12 or 24 weeks and a dual combination of pegylated IFN-α and telaprevir (without ribavirin) are undergoing in treatment-naive and nonresponder patients (PROVE 2 and PROVE 3).

In nonresponders to IFN-α-ribavirin, the antiviral effect of boceprevir appeared to be strictly additive to that of pegylated IFN-α2b (Sarrazin et al. 2007). In an ongoing phase II clinical trial, higher doses of boceprevir are being administered to treatment-naive patients, in combination with pegylated IFN-α and ribavirin.

Resistance is a problem when these drugs are administered alone and will need to be carefully monitored when they are used in combination with IFN-α and ribavirin.

Inhibitors of the RNA-dependent RNA polymerase belong to two categories: nucleoside/nucleotide inhibitors target the catalytic site of the enzyme, and non-nucleoside inhibitors target allosteric sites of the RdRp (Pawlotsky et al. 2007). Three RdRp inhibitors have been tested in clinical trials, including two nucleoside inhibitors, valopicitabine (NM283, Idenix Pharmaceuticals, Cambridge, Massachusetts, and Novartis) and R1626 (Roche), and a non-nucleoside inhibitor, HCV-796 (ViroPharma, Exton, Pennsylvania, and Wyeth Pharmaceuticals, Madison, New Jersey) (Pawlotsky et al. 2007).

In a phase IIb trial involving 190 HCV genotype-1-infected treatment-refractory patients, valopicitabine had a dose-dependent additive effect to that of pegylated IFN-α2a (Afdhal et al. 2006). Frequent gastrointestinal side-effects have been reported and the development of valopicitabine has been halted. In a phase II trial in which HCV genotype 1- and non-1-infected patients received the combination of pegylated IFN-α2b and HCV-796 for 14 days, the HCV RNA level fell more than with pegylated IFN alone (3.3–3.5 log IU/ml in the combination groups and 1.6 log IU/ml in the pegylated IFN-α group), and no viral breakthroughs due to resistance selection occurred during the 14 days of administration (Villano et al. 2007). R1626, combined with pegylated IFN alpha and ribavirin, has recently progressed to phase II clinical development. Further studies are needed to establish the potential benefits and drawbacks of adding HCV replication cycle inhibitors to the pegylated IFN-α-ribavirin combination, the current standard of care.

6 IFN-Based Treatment of Hepatitis B Virus Infection

HBV-infected patients can be subdivided into two groups according to the presence or absence of circulating hepatitis B e (HBe) antigen (Ag). HBeAg-negative patients do not produce HBeAg because the infecting virus harbors precore and/or core promoter nucleotide substitutions (Carman et al. 1989). They generally have lower, fluctuating HBV DNA levels and a more severe course of disease. Chronic HBV infection currently is not curable, because covalently closed circular DNA (cccDNA) persists in the hepatocyte nucleus. Antiviral treatment of chronic hepatitis B has a triple aim: (1) to slow the progression of fibrosis to cirrhosis; (2) to prevent hepatic failure; and (3) to prevent hepatocellular carcinoma. Profound and sustained inhibition of HBV replication is necessary if these goals are to be achieved. Treatment can consist of short-term therapy, generally with IFN-α, or long-term (possibly life-long) therapy with specific nucleoside/nucleotide analogue inhibitors of HBV replication.

The virological response, which is assessed by measuring HBV DNA levels in serum during and after therapy, is the best predictor of the outcome of antiviral treatment. It has been suggested that HBV viral load should be reduced to less than 2,000 IU/mL (de Franchis et al. 2003; Keeffe et al. 2006; Liaw et al. 2005;

Lok and McMahon 2007), but the ideal outcome is undetectable HBV DNA ($<$ 10–30 IU/ml) in highly sensitive real-time PCR-based assays. In HBeAg-positive patients, loss of HBeAg followed by the emergence of anti-HBe antibodies ("e" seroconversion) indicates a sustained response to therapy when it persists after treatment cessation. HBs seroconversion (loss of HBsAg and emergence of anti-HBs antibodies) is the most desirable endpoint, as it indicates a complete response with sustained remission from HBV disease. It is rarely achieved with current therapies.

6.1 Treatment of Chronic HBV Infection with IFN-α

IFN-α was first used empirically in chronic hepatitis B in 1986 (Peters et al. 1986). The effect of human recombinant IFN-α on lymphocyte proliferation and differentiation was studied in 18 patients with chronic hepatitis B. Inhibition of immunoglobulin synthesis was observed, and the authors postulated that the immunomodulatory effect of IFN-α could be important in the therapeutic response of chronic hepatitis B (Peters et al. 1986). The first study to evaluate the antiviral efficacy of IFN-α involved nine patients, who received different doses administered three times a week for two weeks. Two of them entered sustained remission, with undetectable HBV DNA, loss of HBeAg, and ALT normalization (Dooley et al. 1986). Two forms of IFN-α have been used in the treatment of chronic hepatitis B, namely standard and pegylated IFN-α.

6.1.1 Standard IFN-α Monotherapy

Standard IFN-α has been used in chronic HBV infection at a dose of 5 million units (MU) daily or 9–10 MU three times a week (de Franchis et al. 2003). A 4- to 6-month course was initially recommended, but it soon appeared that longer treatment (12 months or more) could result in more frequent and more durable responses (Hui et al. 2006; Lampertico et al. 1997; Lampertico et al. 2003).

HBeAg-Positive Patients

A meta-analysis of 15 randomized controlled trials involving HBeAg-positive patients showed that, after 12–24 weeks of treatment, HBeAg was lost in 33% of cases, with HBe seroconversion in 18% of them, compared to 12% in the control arm (Wong et al. 1993). HBeAg relapsed in 10–30% of patients who received the shortest treatments (Lau et al. 1997; Lok et al. 1993; Niederau et al. 1996; van Zonneveld et al. 2004). Clearance of HBsAg and seroconversion to anti-HBs antibodies were rare and occurred late (5–10% of patients 1 year after treatment in European studies). HBsAg was lost in 11–25% of sustained HBe seroconverters after 5 years (Bortolotti et al. 2000; Fattovich et al. 1998; Niederau et al. 1996).

Standard IFN-α treatment has been shown to reduce the risk of cirrhosis and hepatocellular carcinoma (Lin et al. 2007).

HBeAg-Negative Patients

The use of IFN-α-based therapy is more controversial in HBeAg-negative patients, in whom sustained responses such as HBe seroconversion cannot be expected. Four randomized controlled trials showed a reduction in the HBV DNA level at the end of treatment in 60–70% of patients, compared to 10–20% of untreated controls (Fattovich et al. 2000; Lampertico et al. 1997; Manesis and Hadziyannis 2001; Pastore et al. 1992). However, about half the virological responders relapsed after treatment discontinuation, and relapses continued to occur for up to 5 years post-therapy (Papatheodoridis et al. 2001). Despite the high relapse rates, a sustained virological response (sustained inhibition of viral replication) was achieved in about one-third of patients 5 years after treatment (Kaymakoglu et al. 2007a). Longer IFN treatment periods (12 months or more) are associated with higher rates of sustained virological response (Lampertico et al. 1997, 2003).

Retreatment of nonresponders with standard IFN-α

Two small studies of repeat therapy with standard IFN-α involved both HBeAg-positive and HBeAg-negative patients (Carreno et al. 1999; Manesis and Hadziyannis 2001). Retreatment of nonresponders and responder-relapsers resulted in sustained virological responses in 33% and 35% of cases, respectively. However, the results were difficult to interpret, as the first IFN-α dose regimen and the interval before retreatment were both variable.

6.1.2 Pegylated IFN-α Monotherapy

Pegylated IFN-α2a, but not pegylated IFN-α2b, has been approved by the American and European authorities for the treatment of chronic hepatitis B, based on two large pivotal trials involving HBeAg-postive and HBeAg-negative patients (Lau et al. 2005; Marcellin et al. 2004). Pegylated IFN-α2b has also been tested in HBeAg-positive and HBeAg-negative patients (Flink et al. 2006b; Janssen et al. 2005; Kaymakoglu et al. 2007b; Zhao et al. 2007).

HBeAg-Positive Patients

In a clinical phase II trial, the efficacy of various doses of pegylated IFN-α2a (90–270 μg weekly) administered for 24 weeks was studied in treatment-naïve patients, by comparison with standard IFN-α2a at a dose of 4.5 MU three times a week

(Cooksley et al. 2003). At the end of follow-up, 24 weeks after the end of therapy, HBeAg had been lost by 35% of patients receiving pegylated IFN-α2a 180 μg weekly and by 25% of patients receiving standard IFN-α. In addition, an endpoint combining HBeAg loss, HBV DNA $< 5.0 \times 10^6$ (6.7 $\log_{10}$ copies/mL), and ALT normalization was reached by 24% of pegylated IFN-α-treated patients, whatever the dose regimen, compared to 12% of patients receiving standard IFN-α (Cooksley et al. 2003). A larger clinical trial tested pegylated IFN-α2a monotherapy at a weekly dose of 180 μg (Lau et al. 2005). At the end of follow-up, HBeAg seroconversion had occurred in 32% of the patients and ALT had normalized in 41% of patients. The approved pegylated IFN-α2a treatment schedule for HBeAg-positive patients is currently 180 μg weekly for 48 weeks. It is possible, however, that a lower dose and/or shorter treatment may be sufficient (Zhao et al. 2007), and further studies are therefore needed (Hui et al. 2006). With pegylated IFN-α2b, 52 weeks of therapy yielded a 29% seroconversion rate 24 weeks after the end of treatment (Janssen et al. 2005).

Several recent studies have suggested that the HBV genotype influences treatment outcome, HBV genotype A or B infection tending to respond better to pegylated IFN-α than HBV genotype C or D infection (Flink et al. 2006b; Janssen et al. 2005; Zhao et al. 2007). However, more data are needed before using the HBV genotype to tailor treatment.

HBeAg-Negative Patients

One large trial evaluated 48 weeks of treatment with pegylated IFN-α2a in HBeAg-negative patients (Marcellin et al. 2004). After 24 weeks of follow-up, 36% of the patients had normal ALT levels and HBV DNA levels below 20,000 copies/mL. HBsAg was lost in 4% of cases. In a trial of pegylated IFN-α2b given for 48 weeks, 26% of patients achieved an HBV DNA level below 400 copies/mL (Kaymakoglu et al. 2007b).

Nonresponders to Standard IFN-α and Lamivudine

In two published cohort studies, pegylated IFN-α was effective in approximately one-third of HBeAg-positive patients in whom standard IFN-α or lamivudine had failed (Flink et al. 2006a; Leemans et al. 2006).

6.1.3 Combination Therapy with IFN-α Alpha and Nucleoside/Nucleotide Analogues

A few studies have evaluated standard IFN-α2a and -α2b combined with lamivudine, whereas more recent trials tested pegylated IFN-α combined with nucleoside/nucleotide analogues. The aim was to determine whether combination therapy

could increase antiviral efficacy, reduce adverse effects, and hinder the emergence of analogue-resistant viral variants. Simultaneous and sequential protocols were both tested.

Simultaneous Combination Therapy

Several studies have tested lamivudine and standard IFN-α given simultaneously for 16–24 or 48–52 weeks. In one trial, involving HBeAg-positive patients, sustained HBe seroconversion and undetectable HBV DNA (<1.6 pg/mL) were significantly more frequent at the end of follow-up in patients receiving the combination than in patients treated with lamivudine alone (33% and 15%, respectively) (Barbaro et al. 2001). In contrast, lamivudine plus standard IFN-α was not more effective than lamivudine alone in HBeAg-negative patients (Akarca et al. 2004; Yurdaydin et al. 2005). The incidence of lamivudine resistance was nonetheless lower when IFN-α was coadministered (Jang et al. 2004; Santantonio et al. 2002).

Conflicting results have been reported with pegylated IFN-α and lamivudine combination therapy. In a randomized controlled trial involving HBeAg-positive patients, a sustained virological response (HBV DNA $<$ 500,000 copies/mL) and HBe seroconversion were more frequent in patients receiving pegylated IFN-α2b 1.5 μg/kg/week and lamivudine 100 mg daily than in patients receiving lamivudine monotherapy (Chan et al. 2005a, b). In contrast, there was no difference in the rate of ALT normalization or histological improvement. Lamivudine resistance was more frequent with lamivudine monotherapy than with combination therapy (40% and 21% of patients, respectively). Another trial involving HBeAg-positive patients favored pegylated IFN-α2a or -α2b plus lamivudine over lamivudine monotherapy, but not over pegylated IFN-α monotherapy, despite an additive antiviral effect (Lau et al. 2005). In another study involving HBeAg-positive patients, 52 weeks of treatment with pegylated IFN-α2b yielded an HBe seroconversion rate of 29%, whether IFN-α2b was given alone or combined with lamivudine (Janssen et al. 2005). The combination did not improve liver histological status (van Zonneveld et al. 2006), but it induced less resistance to lamivudine. In HBeAg-negative patients, lamivudine combination with pegylated IFN-α2a or -α2b did not improve the rate of ALT normalization or HBV DNA suppression at the end of follow-up compared to pegylated IFN-α alone. Nevertheless, the decline in viral load was more pronounced and lamivudine resistance was less frequent with the combination (Kaymakoglu et al. 2007b; Marcellin et al. 2004).

One trial assessed the combination of pegylated IFN-α2b and adefovir dipivoxil given for 48 weeks to both HBeAg-positive and HBeAg-negative patients. There was no control arm. Both liver cccDNA and serum HBsAg levels fell strongly, and 4 (17%) of the 26 patients seroconverted to anti-HBs at the end of follow-up (Wursthorn et al. 2006).

Sequential Combination Therapy

The use of sequential combination therapy is based on the notion that lowering the HBV DNA level with nucleoside/nucleotide analogues, thereby permitting partial immune recovery, might improve the subsequent efficacy of pegylated IFN-α. One trial compared 8 weeks of lamivudine monotherapy followed by 16 weeks of lamivudine/standard IFN-α combination therapy, with 52 weeks of lamivudine monotherapy in HBeAg-positive patients (Sarin et al. 2005). The rate of sustained virological responses, defined by HBe seroconversion and undetectable HBV DNA ($<1.4 \times 10^5$ copies/mL) 24 weeks after the end of treatment, was significantly higher with the combination than with lamivudine monotherapy. A recent study of HBeAg-positive patients conducted by the same group compared 4 weeks of lamivudine monotherapy followed by 24 weeks of pegylated IFN-α therapy with 24 weeks of pegylated IFN-α monotherapy (Sarin et al. 2007). Six months after the end of treatment, HBV DNA was undetectable (below 4,700 copies/mL) in 50% of the patients who received sequential therapy and 15% of patients on pegylated IFN-α monotherapy. HBeAg loss was also more frequent in the sequential therapy group (39% vs. 15%, respectively) (Sarin et al. 2007).

In HBeAg-negative patients, 12 weeks of lamivudine monotherapy followed by 36 months of pegylated IFN-α2b therapy (including 3 months of concomitant administration) has been compared with 48 weeks of lamivudine monotherapy (Vassiliadis et al. 2007). At the end of follow-up, the rate of ALT normalization was significantly higher with the sequential therapy than with lamivudine alone. No difference in the proportion of patients with undetectable HBV DNA was observed. Similar results were obtained in a Chinese cohort study (Shi et al. 2006).

6.2 Treatment of Chronic HBV Infection with Other Type I IFNs

6.2.1 Standard IFN-β Monotherapy

The antiviral efficacy of IFN-β administered for 24 weeks at a dose of 3 million units daily has been studied in a small series of HBeAg-positive patients. HBe seroconversion was observed in half the patients and ALT normalization in four patients out of five (Kagawa et al. 1993). Sequential therapy with lamivudine and IFN-β has been tested in HBeAg-positive patients (Enomoto et al. 2007). A sustained virological response was achieved in only 7 (29%) of the 24 patients, 24 weeks after the end of therapy. In a pilot study of IFN-β therapy in 29 patients in whom IFN-α therapy had failed, HBV DNA became undetectable in 6 patients (21%) (Munoz et al. 2002).

6.2.2 Standard IFN-λ

The antiviral efficacy of IFN-λ has been evaluated in vitro in human hepatocyte-derived cells. IFN-λ reduced HBV replication but the results suggested that antiviral efficacy in vivo would be limited (Hong et al. 2007).

6.3 Treatment of Chronic HBV Infection with Type II IFN

IFN-γ exhibits antiviral activity against HBV in vitro (Parvez et al. 2006). In addition, IFN-γ was found to reduce hepatic fibrosis by 63% after 9 months, compared to 24% in untreated controls (Weng et al. 2005).

7 IFN-Based Treatment of Hepatitis D/Delta Virus Infection

Worldwide, 15 million HBsAg carriers are also infected with hepatitis D/delta virus (HDV) (Gaeta et al. 2000). This situation represents a major therapeutic challenge, as most of these patients have advanced liver disease, including cirrhosis in 60–70% of cases, and hepatocellular carcinoma (Fattovich et al. 2000; Saracco et al. 1987). No specific HDV inhibitors have been developed, and IFN-α-based treatment is more difficult in HBV–HDV infection than in HBV monoinfection. HDV RNA levels in serum can be used to monitor treatment efficacy. The endpoint of therapy is HDV RNA clearance and ALT normalization, and this is sometimes achieved after the end of treatment. A sustained response can lead to HBsAg clearance from serum.

7.1 Standard IFN-α Monotherapy

To date, standard IFN-α, but not pegylated IFN-α, has been approved by the American and European authorities for the treatment of chronic hepatitis D. Clinical pilot trials conducted in the early 1990s suggested that IFN-α could inhibit HDV replication. The sustainability of this inhibition depended on the dose and duration of IFN-α therapy, and relapse was frequent (Farci et al. 1994; Gaudin et al. 1995; Madejon et al. 1994; Rosina et al. 1991). A complete response, characterized by normalization of ALT levels and undetectable HDV RNA, was observed six months after the end of treatment in 50% of patients receiving a high dose of standard IFN-α, compared to 21% and 0% of patients receiving a low dose of IFN-α and no treatment, respectively (Farci et al. 2004). IFN-α treatment has been shown to provide a clinical benefit, with regression of advanced hepatic fibrosis. No pretreatment characteristics have been shown to predict the response to IFN-α, and the possible influence of the HDV genotype on the response is not known (Niro et al. 2005b).

Several strategies have been developed to improve the efficacy of standard IFN-α treatment; in particular, treatment has been prolonged, for up to 12 years, but tolerability was poor (Lau et al. 1999b).

7.2 Pegylated IFN-α Monotherapy

Three recent studies evaluated the efficacy and safety of pegylated IFN-α monotherapy in patients with HBV–HDV infection (Castelnau et al. 2006; Erhardt et al. 2006; Niro et al. 2006). The first tested was pegylated IFN-α2b, given for 48 weeks to 14 patients. A virological response, defined by undetectable HDV RNA, was achieved in 43% of patients. ALT normalization was more frequent at the end of follow-up than at the end of therapy (Castelnau et al. 2006). The second study, a randomized controlled trial, evaluated the efficacy of 72 weeks of pegylated IFN-α2b therapy. A virological response was achieved in 25% of patients (Niro et al. 2006). The last study assessed the efficacy and safety of 48 weeks of pegylated IFN-α2b administration in 12 patients. A sustained virological response, defined by undetectable HDV RNA and ALT normalization, occurred in only 2 patients (17%) (Erhardt et al. 2006). The different rates of sustained virological response might be explained by different frequencies of cirrhosis in the three study populations.

7.3 IFN-α Combination with Nucleoside/Nucleotide Analogues

Drugs such as ribavirin that may directly reduce HDV replication and specific inhibitors of HBV replication such as lamivudine and adefovir dipivoxil have been tested in combination with IFN-α in patients with chronic hepatitis D.

7.3.1 Standard IFN-α Plus Ribavirin

Ribavirin inhibits HDV replication in hepatocyte cultures (Di Bisceglie 1997). However, ribavirin monotherapy is not effective in patients with chronic hepatitis D. A pilot study evaluated the efficacy of ribavirin, 15 mg/kg, administered for 16 weeks to 9 patients. At the end of follow-up, HDV RNA levels were reduced in only one patient and ALT levels never normalized (Garripoli et al. 1994). The efficacy of standard IFN-α, 9–10 MU three times a week, combined with ribavirin, 1,000–1,200 mg daily, has been evaluated in two studies each lasting 24 months (Gunsar et al. 2005; Kaymakoglu et al. 2005). Ribavirin did not increase the efficacy of IFN-α. Another trial tested the efficacy of a 72-week course of pegylated IFN-α2b alone vs. 48 weeks of pegylated IFN-α2b plus ribavirin followed by 24 weeks of pegylated IFN-α2b alone. A virological response was achieved in 25% and 18% of patients, respectively, while the rates of biochemical response were similar (Niro et al. 2006).

Thus, ribavirin is ineffective in chronic hepatitis D, whether given alone or in combination with IFN-α.

7.3.2 Standard IFN-α Plus Lamivudine

Lamivudine monotherapy has no effect on HDV (Lau et al. 1999a; Niro et al. 2005a), and lamivudine adjunction only slightly improves the efficacy of standard IFN-α (Canbakan et al. 2006; Wolters et al. 2000).

7.3.3 Pegylated IFN-α Plus Adefovir Dipivoxil

A recent randomized multicenter trial compared the efficacy of pegylated IFN-α2a monotherapy, adefovir monotherapy, and the pegylated IFN-α2a/adefovir combination administered for 48 weeks to patients with chronic hepatitis D. Adefovir did not inhibit HDV replication, and the combination had no additional benefit compared with pegylated IFN-α monotherapy in terms of the HDV RNA level (Yurdaydin et al. 2006).

8 Treatment of HIV Infection with IFN-α

IFN-α was one of the first drugs to be tested against human immunodeficiency virus (HIV) in the 1980s. Recombinant human IFN-α inhibits HIV replication in normal peripheral blood mononuclear cells (Ho et al. 1985). However, the ability of IFN-α to inhibit HIV replication in vitro depends largely on the viral isolate, the target cells, the IFN concentration, and the inoculum. IFN-α appears to interfere with the assembly and/or release of newly formed virions (Fernie et al. 1991; Hansen et al. 1992; Poli et al. 1989). In addition, IFN-α may inhibit viral protein synthesis by blocking viral RNA translation (Coccia et al. 1994), and may also prevent cell infection (Gendelman et al. 1990). In vivo, IFN-α has been suggested to block de novo infection rather than virion production, a finding supported by in vitro studies showing that IFN-α is more effective when used before rather than after viral challenge (Neumann et al. 2007). IFN therapy of HIV infection was abandoned because of its adverse effects, its modest inherent antiviral efficacy, and the development of highly active antiretroviral therapy (HAART) (Lane 1994).

8.1 Standard IFN-α

Several trials have evaluated the antiviral efficacy of standard IFN-α in HIV-infected patients also receiving zidovudine, in comparison with zidovudine alone.

The combination was not more effective than zidovudine monotherapy, either in adults (Fernandez-Cruz et al. 1995; Fischl et al. 1997; Krown et al. 1999) or in infants (Giovannini et al. 1992).

8.2 Pegylated IFN-α

In a prospective pilot study involving nine patients coinfected by HIV and HCV genotype 1, HIV replication showed a slow continuous decline during the first week of pegylated IFN-α administration, and there was no rebound when pegylated IFN-α was withdrawn (Neumann et al. 2007). Pegylated IFN-α was also reported to display antiretroviral activity in two patients with refractory HIV-related Kaposi's sarcoma (van der Ende et al. 2007) and in HIV-infected patients with condylomata acuminata (Brockmeyer et al. 2006). Finally, pegylated IFN-α has been shown to participate in early control of viral replication during primary HIV-1 infection when combined with antiretroviral drugs (Emilie et al. 2001). However, there was no control group in this study, and further investigations are therefore needed.

9 Treatment of Other Viral Infections with Type I IFN in Humans

Table 4 is illustrating the role of type I IFN in other human viral infections (Hodson et al. 2007; Isomura et al. 1982; Kalil et al. 2005; Lewis and Amsden 2007; Phillpotts et al. 1984; Rahal et al. 2004; Sainz et al. 2004; Scagnolari et al. 2004; Solomon et al. 2003).

Table 4 Treatment of viral infections other than chronic viral hepatitis and HIV with type I IFNs

Virus	Protection	References
Avian influenza A virus	No significant protection	Isomura et al. 1982; Phillpotts et al. 1984
Coronavirus	IFN-β more protective than IFN-α Synergistic effect of IFN-α and IFN-β	Sainz et al. 2004; Scagnolari et al. 2004
Cytomegalovirus (CMV)	No significant reduction of the risk of CMV disease Reduced CMV viremia on IFN-α treatment	Hodson et al. 2007
Arthropod-borne virus infections	Under investigation	Kalil et al. 2005; Lewis and Amsden 2007; Rahal et al. 2004; Solomon et al. 2003

10 Conclusion

In conclusion, IFNs have proven to be invaluable tools in the fight against chronic viral hepatitis. In these indications, their antiviral properties play a major role and it remains unclear whether their immunomodulatory properties are also important. Disappointing results obtained with purely immunomodulatory molecules, such as interleukins or Toll-like receptor agonists suggest that, if immunomodulation plays any role, potent inhibition of viral replication is also needed. The role of IFNs in the treatment of viral infections other than hepatitis B and C remains elusive.

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Nucleic Acids-Based Therapeutics in the Battle Against Pathogenic Viruses

Joost Haasnoot and Ben Berkhout

Contents

Abstract For almost three decades, researchers have studied the possibility to use nucleic acids as antiviral therapeutics. In theory, compounds such as antisense oligonucleotides, ribozymes, DNAzymes, and aptamers can be designed to trigger the sequence-specific inhibition of particular mRNA transcripts, including viral genomes. However, difficulties with their efficiency, off-target effects, toxicity, delivery, and stability halted the development of nucleic acid-based therapeutics that can be used in the clinic. So far, only a single antisense drug, Vitravene for the treatment of CMV-induced retinitis in AIDS patients, has made it to the clinic. Since the discovery of RNA interference (RNAi), there is a renewed interest in the development of nucleic acid-based therapeutics. Antiviral RNAi approaches are highly effective in vitro and in animal models and are currently being tested in clinical trials. Here we give an overview of antiviral nucleic acid-based therapeutics. We focus on antisense and RNAi-based compounds that have been shown to be effective in animal model systems.

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H.-G. Kräusslich, R. Bartenschlager (eds.), *Antiviral Strategies,* Handbook of Experimental Pharmacology 189,

Abbreviations

AIDS	Acquired immune deficiency syndrome
AAV	Adeno-associated virus
CMV	Cytomegalovirus
HIV-1	Human immunodeficiency virus type 1
HBV	Hepatitis B virus
HCV	Hepatitis C virus
RSV	Respiratory syncytial virus
SARS	Severe acute respiratory syndrome
RNAi	RNA interference
siRNA	Small interfering RNA
shRNA	Short hairpin RNA
RISC	RNA-induced silencing complex
PS	Phosphorothioate
OMe	2′-*O*-Methyl-RNA
MOE	2′-*O*-Methoxy-ethyl-RNA
PNA	Peptide nucleic acid
LNA	Locked nucleic acid
MF	Morpholino phosphoroamidates

1 Introduction

The most efficacious method to avoid infection with pathogenic viruses is immunization of individuals with prophylactic vaccines. However, for most viruses such vaccines are not available. Antiviral drugs that block replicating virus in infected individuals are therefore of great importance in the battle against virus infections. Ideally, these drugs should induce strong and specific inhibition of virus replication without affecting cellular processes. This type of drugs remained fiction until Zamecnik and Stephenson reported the sequence-specific inhibition of Rous sarcoma virus replication by antisense DNA oligonucleotides in 1978 (Zamecnik and Stephenson 1978). This paper and subsequent reports on the sequence-specific knock-down of genes by antisense technology made a big impression on the scientific community that is comparable to the current RNAi boom. However, the development of antisense-based therapeutics stuttered and almost came to a stop because of problems with toxicity of the oligonucleotides, their instability in serum, and the problem of delivery to the right target cells. A new generation of nucleic acid-based antiviral compounds were subsequently developed, such as ribozymes, DNAzymes, and decoy RNAs, but they faced similar problems. Some of the problems could be solved using chemically modified nucleic acids instead of normal DNA/RNA oligonucleotides. For example, modifications such as phosphorothioate (PS) DNA or 2′-*O*-methyl-RNA (OMe) increased the affinity of the oligonucleotides for their target sequence and increased serum stability (see Fig. 1).

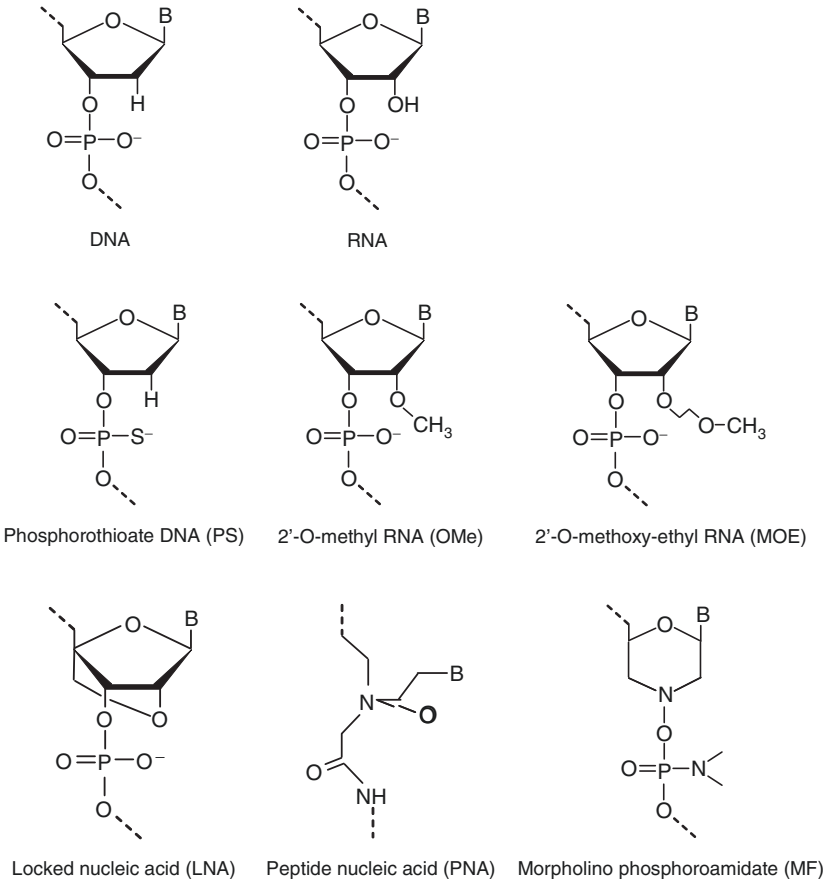

Fig. 1 Chemical structures of backbone modifications used in therapeutic nucleic acid analogs. Shown are the unmodified DNA/RNA chemical structures in addition to a selection of first (PS), second (OMe, MOE), and third generation (PNA, LNA, MF) nucleic acid modifications

New hope for nucleic acid-based therapeutics came in 2001 when Elbashir and coworkers showed that synthetic small interfering RNAs (siRNAs) induced sequence-specific knock-down of genes in mammalian cells without triggering the interferon response (Elbashir et al. 2001). RNAi was immediately recognized as a potentially powerful new tool to combat a variety of viruses. Indeed, profound inhibition has been reported for many important human pathogenic viruses, and several potential antiviral siRNA compounds are currently being tested in clinical trials (Haasnoot et al. 2007). Despite these promising data, it is becoming increasingly clear that some of the problems that were earlier encountered by antisense drugs also apply to RNAi-based therapeutics. Although RNAi-mediated silencing is highly sequence-specific, therapeutic siRNAs can cause unwanted silencing of cellular mRNAs (Birmingham et al. 2006; Fedorov et al. 2006; Jackson et al. 2003;

Kleinman et al. 2008). In addition, synthetic siRNAs are also subject to degradation in vivo by nuclease activity. Besides side effects and instability, the efficient and specific delivery of the RNAi inducers to the target cell still requires optimization. Here we summarize the current status of nucleic acid-based antiviral therapeutics. The focus will be on antiviral strategies using antisense and RNAi technology. Additionally, antiviral ribozymes and aptamers will be discussed briefly, with a focus on recent studies. Gene therapy approaches and delivery systems are the subject of Chapter 11 of this book.

2 Antisense-Based Antiviral Therapeutics

Antisense technology is based on the sequence-specific binding of antisense DNA oligonucleotides to complementary sequences in the target mRNA (Van Aerschot 2006). After binding to the target RNA, the oligonucleotide can block translation via steric hindrance of the elongating ribosome or induce RNA cleavage by activation of RNase H, the enzyme that specifically recognizes DNA/RNA duplexes. Although antisense technology can induce profound sequence-specific inhibition of gene expression in some settings, major problems include instability, delivery, and unwanted side effects of the oligonucleotides. Chemical modification of the oligonucleotides was found to improve the efficacy (Fig. 1).

The first generation of chemical modification was designed to enhance nuclease resistance of the oligonucleotide in serum (Stein et al. 1997). The PS modification, in which the oxygen atom in the DNA backbone is replaced by a sulfur atom, has been widely used. The advantage of the PS-modified oligonucleotides is that it allows RNase H cleavage of the target sequence. A disadvantage is that the PS modification introduces a negative charge that causes nonspecific interactions with other molecules, for example, cellular proteins. As a result, PS-modified oligonucleotides are notorious for inducing severe side effects that are toxic for cells. A well-known example of an antiviral PS-modified oligonucleotide is Vitravene for treatment of cytomegalovirus (CMV)-induced retinitis in AIDS patients. This is a 21-nucleotide that inhibits the expression of viral proteins from the major immediate-early transcriptional unit (Vitravene study group, 2002). Until now, Vitravene is the only antisense-based drug that has received FDA approval and is used in the clinic. Although Vitravene provides a marked reduction of disease severity, the compound is currently not widely used mainly because AIDS patients on antiretroviral therapy rarely develop CMV-induced retinitis.

Second generation oligonucleotides are based on modifications of the sugar ring. For example, OMe and 2′-*O*-methoxy-ethyl (MOE) modifications (Fig. 1) increase the stability of the oligonucleotide and the affinity for the target mRNA when compared to PS-modified oligonucleotides. In contrast to the PS modification, the OMe and MOE-modified oligonucleotides do not allow cleavage by RNase H, but they rather block translation via steric hindrance of the ribosome during translation. For optimal activity, the oligonucleotide has to be targeted to sequences that are at or

near the translation initiation site. The OMe modification has also been used in combination with PS modifications in the so-called gap-mers, in which the central nucleotides carry the PS modification and the flanking nucleotides the OMe modification (Monia et al. 1993). This approach combines the benefits of both the first and second generation modifications. An example of a second generation-modified antiviral oligonucleotide is GEM-92, which was developed by Hybridon (currently discontinued). GEM-92 is a gap-mer, with OMe modified flanks and PS modified central sequences (Turner et al. 2006).

Third generation oligonucleotides are DNA and RNA analogues with extensive modifications of the phosphate backbone and the sugar ring. Examples are peptide nucleic acids (PNA), in which the backbone has been replaced by a peptide linker, morpholino phosphoroamidates (MF), and locked nucleic acids (LNA) (Fig. 1) (Iversen 2001; Larsen et al. 1999; Petersen and Wengel 2003). These modifications provide further improved nuclease resistance and target affinity. In addition, the PNA and MF modifications allow enhanced cellular uptake. To combine all benefits, third generation modifications can be used in combination with unmodified residues in chimaeric gap-mers. LNA modifications are widely used in various applications. It introduces a 2′-O, 4′C-methylene bridge that locks the ribose in a C3′-endo conformation. This "locked" conformation results in a strong increase in the affinity for the target mRNA (Petersen and Wengel 2003). LNA modifications were reported to cause less toxic side effects when compared to the first and second generation modifications (Wahlestedt et al. 2000). However, LNA-modified oligonucleotides were recently shown to induce profound toxicity in hepatocytes in mice (Swayze et al. 2007).

Despite these intense efforts to test different chemical modifications, there is so far little success in developing potent and safe antivirals. For hepatitis C virus (HCV), McHutchison et al. reported in vivo side effects of a 20-nucleotide PS-modified oligonucleotide (ISIS-14803) (McHutchison et al. 2006). In a test group of 28 patients, only 3 patients responded to the treatment by a reduction in the HCV viral load. The researchers concluded that further studies are needed to evaluate this novel agent and its side effects. Previously, ISIS Pharmaceuticals reported a 3.8 log reduction in plasma virus in patients with chronic HCV infection, using ISIS-14803 (www.isispharm.com).

Warfield et al. reported profound inhibition of Ebola virus in mice and rhesus macaques using antisense MF oligonucleotides (Warfield et al. 2006). Combination of several oligonucleotides targeting mRNAs for VP24, VP35, and the RNA polymerase protected the animals, both therapeutically and prophylactically. The modified oligonucleotides protected 75% of the macaques from lethal Ebola virus infection. Similarly, MF oligonucleotides were recently found to profoundly inhibit the murine hepatitis coronavirus. Treatment protected mice against a normally lethal viral challenge, but there were some indications of toxic effects (Burrer et al. 2007).

A different antiviral approach uses intracellular expression of unmodified antisense molecules corresponding to viral sequences. Such an intracellular immunization approach may require a gene therapy protocol to deliver the antiviral transgene. In a phase I clinical trial, Levine et al. used intracellularly expressed

antisense transcripts to inhibit HIV-1 replication. The advantage of using extended antisense sequences is that multiple viral sites are targeted simultaneously (Levine et al. 2006). A conditionally replicating lentiviral vector was used to transduce CD4 T cells ex vivo for intracellular expression of antisense sequences against the HIV-1 envelope gene. The modified CD4 T cells were infused back into the same patient and were apparently well tolerated in all patients. One of the five patients showed a sustained decrease in viral load (Levine et al. 2006). Alternatively, short antisense molecules against sequences in the HIV-1 gag gene were shown to inhibit virus replication in transduced CEM cells (Gu et al. 2006).

3 Ribozymes as Antiviral Therapeutics

Ribozymes represent a class of natural RNAs that catalyse RNA cleavage and ligation reactions as well as peptide bond formation. In most cases, these reactions are self-processing events that take place in a sequence-specific manner. It was found, however, that ribozymes can be designed to specifically cleave other RNA molecules in *trans* by modification of the substrate recognition domains. Because of their sequence specificity and multiple turnover or catalytic properties, such ribozymes have been extensively studied as potential therapeutics (Peracchi 2004). Of the nine classes of ribozymes, the hammerhead and hairpin ribozymes have received a great deal of attention. These ribozymes are relatively small (50–100 nucleotides) and can direct cleavage of single-stranded target RNA. Many ribozymes that are used in therapeutic applications have been improved by in vitro selection procedures, in which the most active molecules were selected from partially randomized sequences. In vitro selection has also generated DNAzymes that structurally and functionally resemble ribozymes, but that have a DNA instead of RNA backbone (Santoro and Joyce 1997). These DNAzymes can have a catalytic activity similar to that of ribozymes, but they are more stable in vivo.

Therapeutic ribozymes have been used both as synthetic RNA that is transfected into cells and as intracellularly expressed RNA. Because unmodified ribozymes are rapidly degraded in serum, synthetic ribozymes require chemical modification to extend the half life and to improve the therapeutic potential of ribozymes (Gonzalez-Carmona et al. 2006; Jakobsen et al. 2007). Antiviral ribozymes have been extensively tested in gene therapy settings (Haasnoot et al. 2007). In these studies, the ribozyme is placed under control of a suitable promoter and inserted in a vector to transduce the target cells. Anti-HIV-1 ribozymes have been studied in several clinical trials. Macpherson et al. have tested an anti-tat ribozyme in a phase I clinical trial (Macpherson et al. 2005). In preclinical studies, this ribozyme was shown to decrease HIV-1 replication and virus-induced pathogenicity in T cell lines and peripheral blood T-lymphocytes. In the clinical trial, $CD4^+$ T-lymphocytes were transduced ex vivo using a retroviral vector for expression of the ribozyme. The transduced cells were given back to the HIV-positive patients. This gene therapy was found to be safe and was shown to inhibit HIV-1 replication. Li and coworkers have

used a ribozyme in combination with an siRNA and a decoy RNA to inhibit HIV-1 replication (Li et al. 2003, 2005b). The ribozyme is targeted towards the mRNA for the cellular CCR5 protein, which functions as essential coreceptor for HIV-1 entry. The decoy is the viral TAR RNA hairpin structure, which is supposed to bind the viral Tat protein and cellular cofactors that are needed for viral gene expression. $CD34^+$ hematopoietic progenitor cells were also transduced with a retroviral vector for the expression of the ribozyme. These cells differentiated normally into mature macrophages and showed significant resistance to viral infection. When the ribozyme was used in combination with the Tat/Rev siRNA and the TAR decoy, the transduced cells showed long-term viral resistance in vitro and the transduced hematopoietic stem cells developed normally into T cells in SCID-hu mice. These cells were able to resist HIV-1 infection ex vivo (Anderson et al. 2007).

4 Aptamers and Decoy RNAs as Antiviral Therapeutics

In contrast to other nucleic acid-based antiviral strategies, decoy RNAs or small RNA/DNA aptamers act via specific binding to a viral target protein rather than to viral RNAs. The interaction of the decoy with the target protein prevents it from being active and hence blocks virus replication (James 2007). The HIV-1 TAR decoy described earlier is a well-known example. Another decoy RNA to inhibit HIV-1 corresponds to the Rev responsive element (RRE). Interaction of the RRE with the viral Rev protein is required for the transport of single and unspliced viral RNAs from the nucleus to the cytoplasm. Overexpression of an RRE decoy sequesters the Rev protein, thereby blocking nuclear export of viral RNAs and thus virus replication (Bahner et al. 1996). This RRE decoy has earlier been tested in a phase I clinical trial in which $CD34^+$ stem cells were transduced ex vivo with a retroviral vector for the expression of the RNA inhibitor (Kohn et al. 1999). There was no evidence that expression of the decoy affected cell function, but there were also no detectable changes in the subject's plasma HIV-1 level.

Besides overexpression of a decoy RNA that resembles a natural viral RNA motif, there is also an interest in the use of artificial small nucleic acid aptamers that have a high affinity for a certain target protein. These aptamers were identified in large randomized pools of sequences by repetitive rounds of selection for binding with the highest affinity to the target protein (James 2007). The early targets for aptamer development were viral polymerases. For example, potent aptamers were selected that inhibit HIV-1 reverse transcriptase (RT) and HCV RNA polymerase (Bellecave et al. 2003; Burke et al. 1996; Tuerk et al. 1992). These aptamers are not similar to any viral sequences, indicating that the high affinity binding aptamers do not necessarily have to be analogues of natural substrates. It has been shown that HIV-1-specific RT aptamers can potently inhibit RT enzymes from multiple HIV-1 subtypes, suggesting that these aptamers can provide broad protection (Held et al. 2007). However, naturally resistant virus strains exist against some aptamers, which means that these antiviral should be used in combination with other HIV-1 inhibitors.

5 Antiviral RNAi-Based Strategies

RNAi has only recently been added to the list of nucleic acid-based therapeutics. In eukaryotic cells, RNAi plays an important role in the regulation of gene expression via microRNAs (miRNA). These are small noncoding RNAs that are expressed as primary miRNAs (pri-miRNAs) and processed by the cellular proteins Drosha and Dicer into mature miRNA of 21–25 nucleotides (Fig. 2). In mammals, miRNAs typically direct translational inhibition of cellular mRNAs bearing complementary sequences in the 3′ UTR (Grimson et al. 2007). RNAi has also been proposed to play a role in the cellular defense against viruses and silencing of transposable elements by generation of virus-specific siRNAs (Bennasser et al. 2005; Soifer et al. 2005; Triboulet et al. 2007; Yang and Kazazian 2006). Recently, it was shown that transposable elements in mouse oocytes are potently silenced by RNAi-related

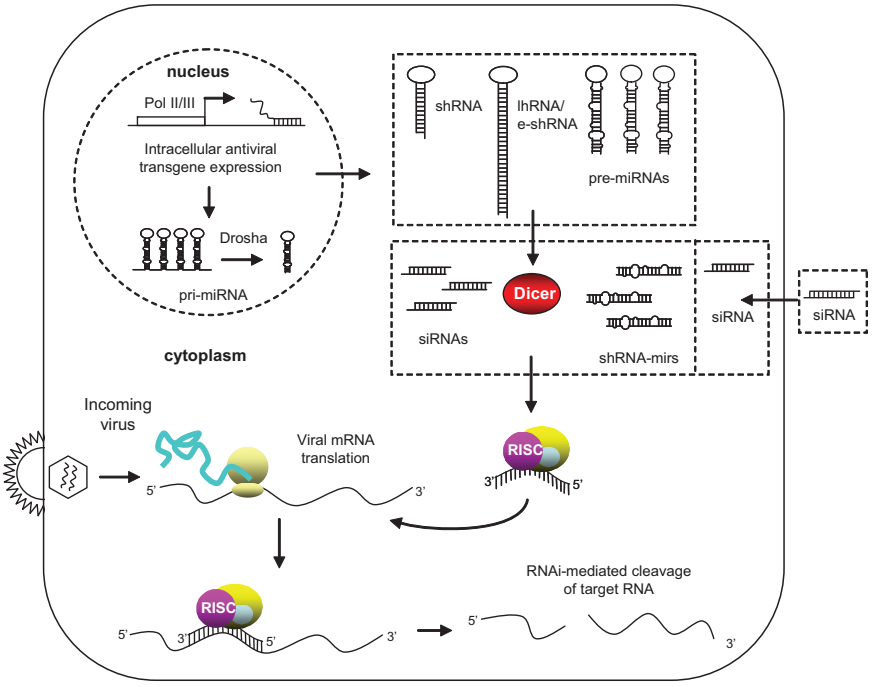

Fig. 2 RNAi inducers used in antiviral strategies. In general, RNAi is induced either by transfection of synthetic siRNAs into cells, or by stable or transient intracellular expression of double-stranded siRNA precursors (shRNA, e-shRNA, lhRNA, or pri-miRNAs). After transcription in the nucleus shRNAs, lhRNAs and e-shRNAs are exported to the cytoplasm and subsequently diced into mature siRNAs. Pri-miRNAs modified to encode antiviral siRNAs first undergo cleavage by Drosha before they are exported to the cytoplasm. Here the antiviral pre-miRNAs (also called shRNA-miRs) are processed by Dicer into the mature miRNAs. After loading of the antisense strand of the siRNAs/miRNAs into RISC, the complex will target and cleave viral transcripts bearing the complementary sequences

mechanisms involving small RNAs (Tam et al. 2008; Watanabe et al. 2008). Both miRNAs and siRNAs are loaded into the RNA-induced silencing complex (RISC) and target RNAs for, respectively, translational repression or destruction (Fig. 2). Although the antiviral function of RNAi is well established in plants, insects, and nematodes, it is still debated whether RNAi has a similar role in mammals, but the circumstantial evidence is growing in support of this idea (Andersson et al. 2005; Bennasser et al. 2005; Cullen 2006; Grimson et al. 2007; Soifer et al. 2005; Tam et al. 2008; Voinnet 2005; Watanabe et al. 2006b, 2008; Yang and Kazazian 2006).

The discovery that exogenously delivered synthetic siRNA and intracellularly expressed short hairpin RNA (shRNA) trigger gene silencing in mammalian cells has made RNAi a powerful technique for generating genetic knock-downs (Brummelkamp et al. 2002; Elbashir et al. 2001). It also allowed the development of RNAi-based therapeutics for prevention of virus transmission or treatment of virus replication. An overview of therapeutic strategies used to induce antiviral RNAi responses is shown in Fig. 2. Synthetic siRNA duplexes used to induce RNAi are generally 21 nucleotides long with 2-nucleotide 3′ overhangs and are modeled after the natural Dicer cleavage products. Once the antisense strand (guide) of the antiviral siRNA is loaded into RISC, the complex will target the viral RNAs in a sequence-specific manner. Alternatively, transient transfection of plasmids that express antiviral shRNAs is also used to induce RNAi. These shRNAs are typically 19–29 base pairs long, with a small apical loop and a 3′-terminal UU overhang. The shRNAs are usually expressed in the nucleus under control of a polymerase III promoter (U6 or H1) and are translocated to the cytoplasm by Exportin-5. The shRNAs are processed in the cytoplasm by Dicer into functional siRNAs.

The technology of shRNA-induced RNAi has been significantly improved by using shRNA variants that more closely resemble natural miRNAs. This second generation shRNAs (shRNA-mirs) can be designed using the increased knowledge on the biochemistry of the RNAi mechanism and miRNA biogenesis. These shRNA-mirs are expressed as larger transcripts under control of polymerase II promoters, and contain bulged nucleotides and internal loops as present in pri- and pre-miRNAs (Boden et al. 2004; Du et al. 2006; Liu et al. 2008; Silva et al. 2005). Compared to shRNAs, the shRNA-mirs show significantly increased activity.

Another possibility to induce RNAi is intracellular expression of long hairpin RNAs (lhRNAs) (Akashi et al. 2005; Konstantinova et al. 2006; Liu et al. 2007; Nishitsuji et al. 2006; Watanabe et al. 2006a; Weinberg et al. 2007). In contrast to transfection of dsRNA larger than 30 bp, intracellular expression does not seem to induce the interferon response. Efficient inhibition by lhRNAs has been reported for HIV-1, HCV, and HBV. Besides conventional lhRNAs, extended-shRNAs (e-shRNAs) have also been successfully used to trigger a potent RNAi response. These e-shRNAs encode individually effective siRNAs that are stacked to form a lhRNA structure (Liu et al. 2007). The potential advantage of a lhRNA or e-shRNA is the generation of multiple siRNAs from a single precursor molecule, which may prevent viral escape.

5.1 Chemically Modified siRNAs

Similar to antisense oligonucleotides, synthetic siRNAs are relatively instable in vivo due to degradation by nuclease activity. The siRNA half life must be increased to be effective as antiviral therapeutic. This would allow treatment with infrequent administration of small amounts of siRNAs, which also increases the prophylactic potential of siRNAs. In case of influenza virus, individuals at risk could be treated with such stabilized antiviral siRNAs during the seasonal epidemic and thus avoid infection. Chemical modifications that were earlier used for antisense compounds have been successfully used to increase the half life of siRNAs in vivo while retaining their activity (Morrissey et al. 2005a, b; Rana 2007). For example, introducing a PS-linkage at the 3′ end renders siRNA resistant to 3′ exonuclease activity, whereas OMe modification gives resistance to endonucleases. However, some of the modifications that increase the stability have a negative effect on the siRNA activity (Czauderna et al. 2003). Modification of the 5′ end resulted in loss of siRNA activity, emphasizing its importance. Modifications in the antisense (passenger) strand can stabilize the siRNA, without a negative effect on the activity of the sense (guide) strand. LNA modification dramatically increases the potency of siRNA in vitro and in vivo without toxicity (Elmen et al. 2005).

5.2 RNAi-Mediated Inhibition of HIV-1 and Virus Escape

Numerous research groups have studied RNAi as an alternative method to block HIV-1, using both transient transfection of synthetic siRNAs and stably expressed shRNAs (Arrighi et al. 2004b; Das et al. 2004; Jacque et al. 2002; Lee et al. 2002; Martinez et al. 2002; Novina et al. 2002; Qin et al. 2003; Surabhi and Gaynor 2002; Ter Brake et al. 2006; Westerhout et al. 2006). Long-term virus suppression by an RNAi gene therapy approach is perhaps best suited to target HIV-1 and other viruses that cause a chronic infection. Lenti-, retro-, and adeno-associated virus (AAV) vectors can be used to stably transduce cells with constructs that express an antiviral shRNA, resulting in cellular resistance to the virus. Transduction of $CD34^+$ hematopoietic stem cells with anti-HIV-1 RNAi constructs ex vivo and grafting these cells back into the patient will result in a resistant cell population that might reconstitute the patients' immune system (Berkhout 2005; see also Chapter 11).

Prolonged in vitro culturing of resistant cells with HIV-1 resulted in the emergence of viral escape variants that are no longer targeted by the siRNAs. These RNAi escape variants contain point mutations or deletions within the siRNA target sequences (Das et al. 2004). In addition, an escape virus was described with a mutation outside the target sequence. This mutation triggered escape by inducing a conformational change in the local RNA secondary structure that rendered the target sequence inaccessible for RISC (Westerhout et al. 2005). Escape from RNAi has also been reported for poliovirus, hepatitis A virus, HBV, and HCV (Boden et al. 2003; Das et al. 2004; Gitlin et al. 2005; Kusov et al. 2006; Ter Brake and

Berkhout 2005; von Eije et al. 2008; Westerhout et al. 2005; Wilson and Richardson 2005; Wu et al. 2005b). These combined results show that a single siRNA therapy is not sufficient to obtain long-term inhibition of virus replication. Escape from RNAi is reminiscent of the evolution of drug resistant HIV-1 variants in patients on antiretroviral therapy. Only the combined use of multiple HIV-1 drugs (highly active antiretroviral therapy or HAART) can permanently block virus replication without the emergence of resistant variants. Similarly, the combined expression of multiple siRNAs would be required to persistently block virus replication. This combinatorial approach should ideally target viral sequences that are essential and well conserved among different virus strains. The emergence of HIV-1 escape variants was delayed in cells expressing two siRNAs when compared to cells expressing a single siRNA (Ter Brake et al. 2006). In cells expressing four potent siRNAs, no viral escape was observed (Ter Brake et al. 2008).

lhRNAs have also been used to induce an anti-HIV-1 RNAi response (Barichievy et al. 2007; Konstantinova et al. 2006, 2007; Liu et al. 2007). These lhRNAs can be processed into multiple effective siRNAs, thus preventing the chance of viral escape. Although lhRNA have been shown to effectively inhibit HIV-1 replication, there is currently no data on their ability to prevent viral escape. The use of multiple siRNAs or lhRNAs should take into account the increased danger of side effects due to interference with cellular miRNA processing and function.

To prevent viral escape from RNAi, an alternative strategy is to target host factors that are essential for viral replication. However, there are few host factors that can be targeted without affecting host cell viability (Arrighi et al. 2004a; Liu et al. 2004; Ping et al. 2004; Qin et al. 2003; Zhou et al. 2004). For HIV-1, a well known example is the CCR5 coreceptor for HIV-1. This coreceptor is important for virus entry, yet mutation of the CCR5 gene does not affect the fitness of the host (Liu et al. 1996; Samson et al. 1996). Recently, Brass et al. (2008) published a large-scale siRNA screen to identify host factors required for HIV-1 replication (Brass et al. 2008). More than 250 HIV-dependency factors were identified, some of which may represent potential targets for therapy.

5.3 RNAi-Based Strategies Against HBV

Despite the availability of an effective HBV vaccine, the virus is still a major health problem with approximately 350 million persons infected worldwide. Hepatitis an infection of the liver that is caused by a variety of RNA viruses (hepatitis A virus, hepatitis B virus, hepatitis C virus). RNAi has been used to inhibit HBV replication both in vitro and in vivo (Carmona et al. 2006; Ely et al. 2008; Hamasaki et al. 2003; Klein et al. 2003; Konishi et al. 2003; Weinberg et al. 2007; Ying et al. 2003). HBV is a member of the Hepadnaviridae and its genome is a 3.2-kb double-stranded circular DNA. Synthetic siRNAs and shRNA expression constructs showed potent inhibition of HBV replication in mice (Chen et al. 2005, 2007; Giladi et al. 2003; McCaffrey et al. 2003; Morrissey et al. 2005b; Shin et al. 2006; Wu et al. 2005b;

Xuan et al. 2006; Ying et al. 2007). The RNAi-based therapy needs to be provided for a long period because HBV causes a chronic infection of the liver. Transduction of the hepatocytes in vivo with a vector expressing anti-HBV shRNAs could provide such long-term protection. Chen et al. have indeed demonstrated long-term inhibition of HBV in mice transduced with an AAV vector to deliver the shRNA inhibitor. HBV DNA, mRNA, and protein levels were reduced in the liver of the transgenic mice, whereas the amount of circulating virus dropped by 2–3 logs. Virus inhibition was still apparent 120 days after vector administration (Chen et al. 2007). In addition, lhRNAs and shRNA-mirs could efficiently inhibit HBV replication in vitro and in mice (Ely et al. 2008; Weinberg et al. 2007).

5.4 RNAi-Based Strategies Against Respiratory Viruses

Respiratory viruses are perhaps best suited to target with RNAi-based therapeutics, because the upper airways and lungs are relatively easy to reach as target tissues. Already several studies reported therapeutic effects in animal models and in clinical trials. For example, siRNAs against influenza virus were found to reduce virus titers in the lungs of infected mice when siRNAs were administered through hydrodynamic intravenous injection (Ge et al. 2004). Similarly, virus titers were reduced when mice were given DNA vectors, either intravenously or intranasally, that express antiviral shRNAs (Ge et al. 2004). Moreover, reduced virus-induced mortality was scored in treated mice, even when the siRNA was delivered after virus infection, suggesting that siRNAs can act both in a prophylactic manner and in the treatment of established infections. In another study, synthetic siRNAs also protected mice against a lethal influenza virus challenge (Tompkins et al. 2004). This siRNA treatment induced a broad protection against the pathogenic avian influenza A viruses of serotypes H5 and H7.

Another respiratory virus for which RNAi therapeutics are being developed is respiratory syncytial virus (RSV). In fact, RSV was the first virus for which RNAi-mediated inhibition was demonstrated (Bitko and Barik 2001) and synthetic siRNAs are currently entering phase II clinical trials. RSV belongs to the paramyxoviridae and is an enveloped, nonsegmented, negative-strand RNA virus. RSV is a major cause of respiratory illness in both the upper and lower respiratory tract, typically leading to common cold-like symptoms that in some cases – especially in young children – lead to more serious illness such as bronchiolitis and pneumonia. In mice, replication of RSV and also human parainfluenza virus could be blocked by synthetic siRNAs (Bitko et al. 2005). Synthetic siRNAs and intranasal administration of plasmids expressing shRNAs resulted in a significant decrease of the RSV load (Zhang et al. 2005). Similar to siRNA-induced inhibition of influenza virus, the siRNAs against RSV were effective both before and after infection with RSV. Alnylam has presented the results of a phase I clinical trial with its leading siRNA drug candidate ALN-RSV01 (http://www.alnylam.com/). The drug was found to be safe and well tolerated when administered intranasally in two phase I clinical studies

(DeVincenzo et al. 2008). Phase II clinical trials in naturally infected RSV patients were initiated in April 2008. ALN-RSV01 is expected to advance into the pediatric patient population by the second half of 2008.

RNAi against the severe acute respiratory syndrome coronavirus (SARS-coronavirus) and other coronaviruses has also been studied extensively. Both siRNAs and plasmid-derived shRNAs were effective in blocking virus replication in cell culture infections (He et al. 2003; Lu et al. 2004; Ni et al. 2005; Pyrc et al. 2006; Wang et al. 2004; Wu et al. 2005a; Zhang et al. 2004). Potent siRNA inhibitors against the SARS-coronavirus spike and pol mRNAs were also tested for efficacy and safety in a rhesus macaque SARS model. The siRNAs were administered intranasally as prophylactic, concurrent with viral challenge, or early post-exposure (Li et al. 2005a). Similar to the mice studies with anti-influenza siRNAs and anti-RSV siRNAs, both prophylactic and therapeutic effects were observed. siRNA treatment caused a significant reduction in viral RNA levels, SARS-like symptoms, and lung histopathology. These combined findings suggest that low dosages of inhaled or intravenously administered siRNAs or shRNA plasmids may provide an easy and efficient basis for prophylaxis against respiratory viruses in certain situations – for example, a pandemic influenza threat – or antiviral therapy of already infected individuals.

5.5 RNAi Side Effects and Off-Target Effects

RNAi is a highly specific tool for inducing gene knock-down. However, studies show that high expression of siRNA/shRNAs can induce interferon responses and other unwanted side effects (Bridge et al. 2003; Jackson and Linsley 2004). Cellular transcripts containing partial complementarity with an siRNA can also be targeted for RNAi-mediated knock-down (Birmingham et al. 2006; Fedorov et al. 2006; Jackson et al. 2003). These off-target effects are of great concern in the development of safe RNAi-based antivirals, because they will have an impact on cell viability (Fedorov et al. 2006; Jackson and Linsley 2004). High concentrations of siRNA/shRNAs may also interfere with cellular miRNA processing and function. This problem is caused by the fact that shRNA processing uses the cellular pathway for miRNA processing. In particular, Exportin-5, which is required for nuclear export and stability of miRNAs, was found to be a limiting factor in the RNAi pathway that could be saturated by exogenous shRNAs. In extreme cases, such side effects can have severe consequences, as illustrated by the mice that died after treatment with an AAV vector for overexpression of shRNAs in the liver (Grimm et al. 2006). This study also showed that there is an shRNA-threshold for toxicity. Because of our limited knowledge on cellular miRNA function, we should not underestimate the impact of shRNAs on these processes. Moderate expression of highly active shRNAs is essential for the development of safe RNAi therapeutics, and candidate shRNA/siRNA inhibitors should be screened for off-target effects. Inducible promoters are useful to regulate shRNA expression to avoid off-target effects. To avoid

toxic off-target effects of antiviral RNAi therapeutics, viruses should ideally be targeted transiently, locally, and with a low dosage. This could be realized for acute respiratory viral infections, but treatment of chronic viral infections may require a durable gene therapy approach. An elegant strategy to reduce potential off-target effects of the siRNA passenger strand is the use of so-called small internally segmented interfering RNAs (sisiRNA) (Bramsen et al. 2007). These sisiRNAs are composed of an intact antisense strand complemented with two shorter 10–12 nt sense strands. Because of its segmented nature the passenger strand is inactive, whereas the antisense strand can still efficiently induce a potent RNAi response.

Delivery of the siRNAs to the right target cells will also limit the impact of potential off-target effects. Progress has been made by using nanoparticles loaded with siRNAs. These particles, ranging between 25–40 nm in diameter, can be used to load high concentrations of siRNAs into a protected environment. The nanoparticles can be targeted to specific cells or tissues by decorating their surface with ligands that target specific cell surface receptors. Ligands such as transferrin, Her2 antibodies, folate, and integrin-targeting peptide have already been successfully used to target siRNA particles to cells bearing the corresponding receptors (Guo et al. 2006; Hu-Lieskovan et al. 2005; Schiffelers et al. 2004; Tan et al. 2007). These results are promising and will contribute to the further development of safe and efficient siRNA delivery systems.

6 Conclusions

The development of nucleic acid-based therapeutics is not as straightforward as researchers had initially anticipated. Stability, toxicity, specificity, and delivery of the compounds continue to be challenging issues that need further optimization. In recent years, researchers have come up with intricate solutions that have greatly improved the efficacy of potential antisense, ribozyme, as well as RNAi-based therapeutics. Clinical trials for all these types of nucleic acid-based therapeutics are underway. So far, data from several trials and studies in animal models look promising, in particular, the therapies that trigger the RNAi pathway. However, history has shown that compounds that do well in phase I or phase II clinical trials may still fail in phase III. A striking example is the nonspecific suppression of angiogenesis by siRNA via toll-like receptor 3 (Kleinman et al. 2008). It will become clear in the near future which compounds will make it as a new class of antiviral therapeutics.

Acknowledgements We thank Truus Abbink for critical reading of the manuscript. RNAi research in the Berkhout lab is sponsored by ZonMw (Vici grant and Translational Gene Therapy program), NWO-CW (Top grant), the European Union (LSHP-CT-2006–037301), and the Technology Foundation STW (grant AGT.7708).

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Antiviral Gene Therapy

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H.-G. Kräusslich, R. Bartenschlager (eds.), *Antiviral Strategies,* Handbook of Experimental Pharmacology 189,

Abstract This chapter describes the major gene therapeutic approaches for viral infections. The vast majority of published approaches target severe chronic viral infections such as hepatitis B or C and HIV infection. Two basic gene therapy strategies are introduced here. The first involves the expression of a protein or an RNA that inhibits viral replication by targeting crucial steps of the viral life cycle or by interfering with a cellular factor required for virus replication. The major limitation of this approach is that primary levels of gene modification have generally not been sufficient to reduce the availability of target cells permissive for virus replication to a level that significantly decreases overall viral load. Thus, investigators have banked on the expectation that gene-protected cells have a sufficient selective advantage to accumulate and gain prevalence over time, a prediction that so far could not be confirmed in clinical trials. In vivo levels of gene modification can be improved, however, by introducing an additional selectable marker. In addition, a secreted antiviral gene product that exerts a bystander effect could significantly reduce overall virus replication despite relatively low levels of gene modification. In addition to these direct antiviral approaches, several strategies have been developed that employ or aim to enhance host immune responses. The innate immune response has been enhanced, for example, by the in vivo expression of interferons. Alternatively, T cells can be grafted with recombinant receptors to boost adaptive virus-specific immunity. These approaches are especially promising for chronic virus infection, where natural immune responses are evidently not sufficient to effectively control virus replication.

Abbreviations

AAV	Adeno-associated virus
ADA	Adenosine deaminase
Ad	Adenovirus
AIDS	Acquired immunodeficiency syndrome
CGD	Chronic granulomatous disease
cTCR/rTCR	Chimeric/recombinant T cell receptor
CTL	Cytotoxic T lymphocyte
CTVI	Capsid targeted viral inactivation
EBV	Epstein Barr-virus
EC	European Commission
HAART	Highly active antiretroviral therapy
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPC	Hematopoietic progenitor cells
IN	Integrase
maC46	Membrane-anchored C46
MGMT	O6-methylguanine-DNA-methyltransferase

rSV40	Recombinant simian virus-40
RT	Reverse transcriptase
SCID	Severe combined immunodeficiency
Td	Transdominant
Wt	Wild-type

1 Introduction

Initially, the definition of the term "gene therapy" was restricted to "the insertion of normal DNA directly into cells to correct a genetic defect" (Webster's New World: Medical Dictionary). Today, gene therapeutic strategies target a much broader range of different diseases and the definition has been widened. This is reflected by the definitions used in drug legislation as found, for example, in Part IV of the EC Directive 2003/63/EC: "… gene therapy medicinal product shall mean a product obtained through a set of manufacturing processes aimed at the transfer, to be performed either in vivo or ex vivo, of a prophylactic, diagnostic or therapeutic gene (i.e., a piece of nucleic acid), to human/animal cells and its subsequent expression in vivo. The gene transfer involves an expression system contained in a delivery system known as a vector, which can be of viral, as well as nonviral origin." Of note, this definition requires that the gene product is expressed in vivo, so that therapeutic nucleic acids (ribozymes, aptamers, and siRNAs) per se are not automatically gene therapeutic drugs. Accordingly, therapeutic RNA or protein will only be discussed in this chapter, if expressed in vivo. A detailed review of the application of therapeutic RNA is given in Chap. 9 of this book.

Gene transfer technology advanced remarkably during the last two decades, leading to considerable progress in clinical application. Treatment of patients with hereditary monogenetic diseases, such as primary immunodeficiencies, has been especially successful, and curative effects were obtained for gene therapy in patients with X-linked severe combined immunodeficiency (X-SCID) (Cavazzana-Calvo et al. 2000). In addition, long-term immune reconstitution has also been attained with stem-cell gene transfer for adenosine deaminase (ADA) deficiency and chronic granulomatous disease (CGD) (Aiuti et al. 2002a; Ott et al. 2006). However, with the first clear clinical benefits of gene therapy, severe safety issues with regard to the use of integrating gene transfer vectors also became evident as discussed further in Sect. 4.2 (Hacein-Bey-Abina et al. 2003a).

2 Basic Gene Therapeutic Approaches for Viral Diseases

There are three principal gene therapeutic approaches for viral diseases. First, several genes that inhibit virus replication have been developed. This approach has been termed "intracellular immunization" (Baltimore 1988). Second, genetic approaches

have been developed that aim to specifically increase antiviral immunity by arming autologous T cells with recombinant receptors targeting viral antigens (Engels and Uckert 2007). Finally, all therapeutic or even prophylactic vaccination strategies that aim to enhance antiviral immunity and use nonviral or viral vectors to express the viral antigens could formally be classified as gene therapy. Therapeutic vaccinations, however, have been reviewed recently in detail by others and are not covered here (Puls and Emery 2006). In this chapter, we focus on the first two approaches.

3 Chronic Viral Infections as Targets for Antiviral Gene Therapy

In general, severe diseases that lack effective therapeutic options are preferred targets for experimental therapies such as gene therapy. In severe chronic diseases, sustained expression of a therapeutic gene product may be necessary to achieve a therapeutic benefit. In contrast, direct administration of the therapeutic gene product (antibody, siRNA, cytokine, etc) is expected to be sufficient for most acute diseases. Consequently, gene therapeutic strategies mostly target severe chronic diseases, such as cancer and chronic viral infections. Among the chronic viral infections, most studies have focused on gene therapeutic strategies for human immunodeficiency (HIV-1), hepatitis B (HBV), and hepatitis C virus (HCV) infections.

4 Gene Transfer Vectors

4.1 Integrating Vectors Derived from Retroviruses

The major target cells for HIV gene therapy are hematopoietic progenitor/stem cells (HPC) and T cells. In addition, immunogene therapy approaches for a broad range of different viruses as discussed later involve gene transfer of recombinant antigen receptors to T lymphocytes. For these therapeutic strategies, the gene transfer vector must support long-term expression in proliferating cells. This requirement is fulfilled by several of the currently available vector systems, of which only the two candidates that have been used more extensively for antiviral gene therapeutic strategies are discussed here. These are gammaretroviral and lentiviral vectors. Both vector types have been reported to mediate stable random integration of the transgene expression cassette into the host genome. The potential risks associated with vector integration are discussed later.

Of note, therapeutic gene transfer to T cells and HPC is generally performed ex vivo after removal of the target cells from the patient. The gene-modified cells are then reinfused. This technically demanding procedure is not an option for the majority of patients with HIV infection, and future research must give the development of technologies that allow efficient in vivo gene transfer top priority.

Vectors have been derived from several retroviral genera such as the gammaretroviruses, lentiviruses, and spumaviruses (foamy virus vectors) (Park et al. 2005). Only the former two vector types have been tested in clinical trials. Two issues related to retroviral vector technology are highly relevant for gene therapy of HIV infection. First, unlike gammaretrovirus vectors, lentiviral vectors derived from HIV may compete with wild-type (wt) HIV genomic RNA for encapsidation and virus production, although the efficacy of this process is discussed controversially (Richardson et al. 1993). In addition, lentiviral vectors are trafficked by wt HIV, leading to spread of the vector genome in HIV-infected patients, thereby increasing the level of in vivo gene modification (Bukovsky et al. 1999; Dropulic et al. 1996).

A second crucial relevant difference between the two vector systems is that gammaretroviral vectors can only transduce dividing cells, while lentiviral vectors mediate gene transfer also into resting cells. Nevertheless, both retroviral vector types require ex vivo cytokine stimulation of T cells and HPC for efficient gene transfer. This necessity may be especially problematic for gene therapy of AIDS, as ex vivo stimulation and expansion can impair the functionality of T cells and HPC significantly and these cells are already injured considerably in HIV-infected patients. Stem cells are known to lose capacity to repopulate and regenerate when stimulated with cytokines, and T cells have been reported to loose their capacity to respond to recall antigens (Ferrand et al. 2000; Sauce et al. 2002). Prestimulation periods required for gammaretroviral vector gene transfer are longer than for lentiviral vectors, which is a clear advantage of lentiviral gene transfer. This advantage may be crucial when developing effective in vivo gene delivery regimen.

4.2 Genotoxicity of Integrating Vectors

All vectors more or less have the potential to integrate into chromosomal DNA and thus could occasionally activate cellular genes with a transforming potential, unless integration can be exclusively targeted to a “safe harbor”. Despite some advances in basic research, at present, no vector with a sufficiently high degree of site-specific integration is available for clinical use. The genetic and epigenetic consequences of unspecific integration depend on the nature of the affected cellular gene(s) and the degree of interference induced by transgene insertion, which may lead to activation, fusion, truncation, or extinction of the cellular message. Functional consequences of random vector insertion have been recognized in the form of enhanced clonal fitness, without major alterations of differentiation (“clonal dominance”) (Kustikova et al. 2005; Ott et al. 2006), and in the manifestation of overt malignant disease such as leukemia (Hacein-Bey-Abina et al. 2003a; Li et al. 2002) or sarcoma (Seggewiss et al. 2006). To date, five cases of leukemia associated with retroviral insertional mutagenesis have been described, all after initially successful stem cell gene transfer for X-SCID. Peer-reviewed studies of the underlying mechanisms are available for two of these patients (Hacein-Bey-Abina et al. 2003b;

Howe et al. 2008). Of note, more than 30 other severely ill patients with two types of SCID have received therapeutically effective gene therapy without clinical or molecular evidence of major side effects in prolonged follow-up.

The risk of transformation induced by vector genotoxicity is expected to depend upon the type of cell. Of special interest for gene therapy of viral infections is that clinical observations and additional preclinical studies support the hypothesis that mature T cells are less prone to transformation than HPC (Newrzela et al. 2008). Additional relevant parameters for vector safety are the average gene copy number per cell (Kustikova et al. 2005), the type and architecture of the vector used (Modlich et al. 2006; Montini et al. 2006), and the engraftment rate of the mutated cells. The latter is strongly influenced by environmental, potentially disease-specific conditions (Baum 2007). Measures that may strongly reduce the risk of genotoxic side effects in future gene therapy studies include rational redesign of vector type and architecture, improving cell culture conditions (used in case of ex vivo gene transfer), and avoiding clinical conditions that result in a strong replicative stress for transplanted cells.

4.3 Nonintegrating Vectors Derived from Adenovirus, Adeno-Associated Virus, and HBV

For intracellular immunization strategies of hepatitis B and C, lentiviral vectors have been tested (Matskevich et al. 2003; Parouchev et al. 2006). However, as the turnover of hepatocytes is thought to be much slower than that of HPC or T cells, nonintegrating vectors that carry a reduced risk of insertional mutagenesis are favored (Nowak et al. 1996).

Adenoviral (Ad) vectors of the first generation have been studied extensively in the past and result in highly efficient gene transfer to the liver in various animal models. Ad vectors can be produced at high titers and show broad cell tropism and great stability. However, a problem is their tendency to elicit toxic effects in vivo, partly owing to the intracellular de novo production of immunogenic viral proteins (Raper et al. 2003). This may ultimately lead to the elimination of transduced cells by an innate or adaptive immune response. In the newer generation of highly attenuated gutted Ad vectors, all adenoviral coding sequences have been deleted. These vectors allow the transfer of up to 35 kb of foreign DNA and were found to induce less CTL activity, thus supporting long-term gene expression in small and large animal models after liver-directed gene transfer for several months (Ehrhardt and Kay 2002; Ehrhardt et al. 2003). However, the viral proteins contained in the gutted vector particles may still elicit host responses (Volpers and Kochanek 2004).

The small adeno-associated virus (AAV) vectors can be easily produced, have not been found to cause major toxicity, and persist in the transduced hepatocyte typically as an episomal transcriptionally active DNA molecule. Because of the limited packaging capacity for foreign DNA of 4.6 kb, these vectors have been preferentially used to express small transgenes and shRNAs directed against HBV or HCV

RNA in liver cells (Grimm and Kay 2006). A recently published clinical trial in patients with severe hemophilia B involving AAV-2-mediated factor IX expression in the liver suggests that cell-mediated immunity targeting AAV capsid antigens can destroy liver cells and thereby limit transgene expression. The authors suggest that immunomodulation may be necessary to achieve long-term expression from AAV vectors in the liver in humans (Manno et al. 2006). In addition, novel AAV vectors derived from other viral serotypes, such as AAV-8, may allow more efficient gene transfer in vivo as these are not neutralized by patient antibodies and target hepatocytes more efficiently. Moreover, vectors with self-complimentary AAV genomes, although they have a reduced packaging capacity, express transgenes in hepatocytes 10- to 100-fold more efficiently than conventional single-stranded AAVs (Gao et al. 2006). These improved AAV vectors are an interesting tool especially for the delivery of shRNAs.

HBV-based vectors efficiently target quiescent hepatocytes and HBV-specific promoter and enhancer elements allow hepatocyte specific gene expression (Protzer et al. 1999). In addition, a very favorable ratio of infectious to defective particles renders HBV-based vectors good candidates for liver-directed gene transfer. Improved HBV vectors, in which HBV gene expression was abolished (Untergasser and Protzer 2004), were used in chimpanzees to treat chronic HCV infection and did not show any toxicity (Shin et al. 2005).

In conclusion, several vector systems are available in the molecular therapy toolbox, each suited for specific applications.

5 Genes that Inhibit Viral Replication

5.1 Major Antiviral Gene Products

The first basic gene therapeutic strategy to be discussed here involves the expression of antiviral genes and has been termed “intracellular immunization” (Baltimore 1988). The gene product that mediates the antiviral effect is either a protein or an RNA. Both have advantages and disadvantages. Proteins can be immunogenic, while RNA is not. On the contrary, some antiviral proteins can be secreted and thus act on nontransduced cells to exert a bystander effect. Examples are secreted CD4 (sCD4), neutralizing antibodies, and cytokines (Gay et al. 2004; Morgan et al. 1994; Sanhadji et al. 2000). As discussed later, this may be a possibility to effectively suppress overall virus replication despite low gene marking levels. Antiviral gene products can either target the virus genome or viral gene products directly or act by inhibiting cellular cofactors that are essential for virus replication. The latter strategy is clearly less prone to induce escape mutations but has a greater risk of toxicity.

5.2 Targets in the Viral Life Cycle

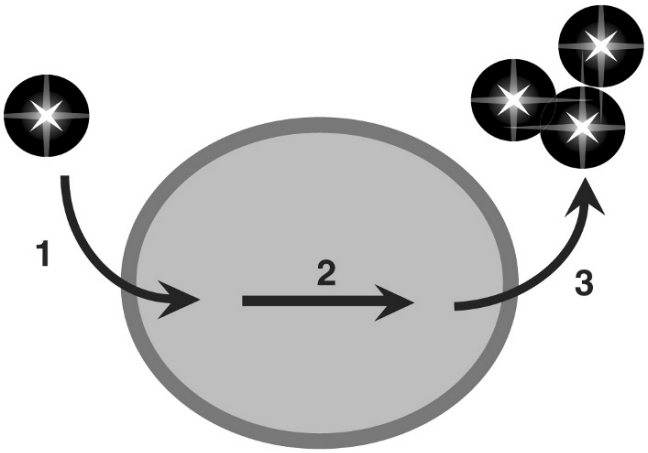
Intracellular antiviral strategies have two major potential modes of action. First, if in the patient a sufficient fraction of cells can be genetically modified to obviate virus replication, the virus load is expected to decline due to a lack of permissive target cells. For HIV-infection, an additional antiviral effect can be achieved if sufficient T helper cell clones specific for HIV antigens are protected against viral replication. These gene-protected helper cells could support the immunologic control of viral replication, without the risk of infection or virus-induced cytotoxity, which both are enhanced by HIV antigen activation.

The basic problem of intracellular antiviral strategies for HIV is that the total number of target cells for a virus in the patient is generally large, more than 10^{11}, so that direct genetic modification of the entire target cell population will not be feasible in the foreseeable future. Application of an antiviral gene is therefore not expected to lead to a substantial level of gene protection, with a significant reduction of susceptible target cells, unless the genetically protected cells have a selective advantage over the nonmodified cells and accumulate with time (Lund et al. 1997). Indeed, several antiviral genes have been developed that confer such a selective advantage in infected cell cultures.

In vivo selection of gene-protected cells is expected to be much slower for hepatitis than for HIV-infection, as the turnover of virus-infected hepatocytes (half-life 10–100 days for HBV) is much slower than that for HIV-infected T cells (half-life 1 day) (Nowak et al. 1996). Noninfected hepatocytes also have a longer life span of approx. 5 months relative to the average T cell with a half-life of about 80 days. However, vector can be applied locally with greater efficacy to the liver than to hematopoetic cells in vivo, and vector systems (Ad and AAV) preferentially used for in vivo gene transfer to the liver are highly efficient. Thus, higher primary levels of gene transfer could be achieved for the liver than for the hematopoietic system, although additional in vivo selection of gene-protected hepatocytes may be slow.

Since HCV persistence requires continuous replication, effective long-term suppression of HCV replication in an infected cell will lead to viral clearance from the infected cell. HBV deposits several copies of a covalently closed circular (ccc) DNA genome as a persistence form in the nucleus of infected cells and thus is difficult to eliminate. HIV-1, once integrated as a provirus, will persist throughout the lifespan of the infected cell. Thus, especially for HBV and HIV, the ability of gene-protected cells to become dominant cell populations, and the overall effect on viral load, is expected to depend crucially on which steps of the viral life cycle are inhibited by the antiviral gene product. This hypothesis has been described in detail and thoroughly evaluated by mathematical modeling previously and will only be outlined here briefly (von Laer et al. 2006b).

Based on the predicted effects on HIV-1 and T cell dynamics, antiviral genes have been grouped into three classes (Fig. 1) (von Laer et al. 2006b). Early inhibitors are classified as class I, inhibitors of intracellular reproduction of the viral genome and production of viral gene product are class II, and late inhibitors are considered class III. A comprehensive list of types of antiviral genes reported to date for each class is found in Table 1 (von Laer et al. 2006a).



Infection prevented Protection from CPE Protection from CTL Selective advantage	Infection is established Viral replication blocked Protection from CPE Partial protection from CTL Selective advantage	Infection is established Viral genes expressed Viral genome replicated Production of infectious virions blocked No protection from CPE No protection from CTL No selective advantage

Fig. 1 Antiviral genes inhibit virus replication at different stages of the viral life cycle. Early inhibitors prevent the establishment of the viral genome in the target cell (class I, e.g., entry inhibitors, RT inhibitors for HIV). Intermediate inhibitors prevent viral gene expression or amplification of the viral genome (class II, e.g., siRNAs, antisense RNAs). Late inhibitors prevent virion assembly or release, or inactivate the mature virions (class III, e.g., transdominant core proteins, capsid-targeted virion inactivation, CTVI). A list of antiviral genes in each class is found in Table 1

Genes in the first group are clearly expected to confer a selective advantage to the cell and lead to the accumulation of gene-modified, noninfected cells. Genes of the second group also confer a selective advantage. However, gene-modified cells that harbor a suppressed proviral genome are expected to accumulate in the patient. Because of accumulation of these "pseudolatently" infected cells, class II gene modification will not necessarily produce a substantial reduction of the overall viral load unless the antiviral activity is strong and sustained (von Laer et al. 2006b). Another concern with class II genes is that they may ultimately convert HIV into an insertional mutagen, as a cell expressing effective class II genes may accumulate several integrated HIV proviruses without undergoing cell lysis. Late antiviral gene products (assembly/release inhibitors, capsid-targeted viral inactivation), class III, do not confer a selective advantage at all. These considerations are clearly in favor of the inclusion of early inhibitory antiviral genes in intracellular antiviral strategies.

Table 1 Exemplary antiviral genes/sequences with proposed mode of action

Target	Examples	Virus targeted	References
Class I			
Virus receptor binding	Coxsackievirus receptor trap: hCAR-hDAF:Fc	Coxsackievirus	Lim et al. 2006
CD4 receptor binding	sCD4: Truncated secreted CD4	HIV-1	Morgan et al. 1994, 1994; Sanhadji et al. 2000
Co-receptor binding	CCR5-RNAi: RNAi targeting CCR5	HIV-1	Bai et al. 2000, 2001; BouHamdan et al. 2001; Feng et al. 2000; Lee et al. 2004; Steinberger et al. 2000
	Anti-CCR5 ribozyme		
	Intracellular scFV against CXCR4, CCR5		
Fusion	maC46: Membrane-anchored antiviral peptide	HIV-1	Egelhofer et al. 2004; Hildinger et al. 2001; Lee et al. 2004; Sanhadji et al. 2000
	Secreted neutralizing human mab 2F5		
	Membrane-anchored anti-gp41 scFv		
Reverse transcription	ScFv-RT Intracellular scFv to RT	HIV-1	Shaheen et al. 1996; Yamamoto et al. 1999
	Intracellular scFV against HBV core	HBV	
Integration	ScFv to IN: Intracellular scFv to IN	HIV-1	Goldstein et al. 2002; Kitamura et al. 1999; Levy-Mintz et al. 1996

(Continued)

Table 1 *(Continued)*

Class II			
Genome replication	Dominant-negative EBNA 1	EBV	Nasimuzzaman et al. 2005
	RNA decoys from HCV stem-loop structures in NS5B coding region sequester replication complexes	HCV	(Zhang et al. 2005)
Tat-transactivation	Anti-hCyclinT1 intrabodies tdTat: Transdominant HIV Tat protein scFV to Tat; Tar decoy	HIV-1	Bahner et al. 1996; Bevec et al. 1996; Duan et al. 1994; Malim et al. 1989; Malim et al. 1988; Reddy et al. 1999
Nuclear export of unspliced RNA	RevM10: Transdominant HIV Rev protein	HIV-1	Habu et al. 2005; Hotchkiss et al. 2004; Li et al. 1998a, b; Yamada et al. 1994
	Dominant negative Sam68, a Rev homolog scFv to Rev; RRE decoy		
Degradation of viral RNA	Small-guide RNAs directing specific cleavage of HIV RNA by tRNase ZL	HIV-1	Kim et al. 1999; Nash et al. 2005; Pan et al. 2004; Weinberg et al. 2000; Welch et al. 1997
	Ribozymes	HIV-1, HBV, HCV, HPV	Korf et al. 2005; Rossi 2006; Uprichard et al. 2005; Wilson and Richardson 2005
	shRNA	HIV-1, HBV, HCV	Joshi et al. 1991; Mautino and Morgan 2000; Nash et al. 2005; Tung and Bowen 1998; zu Putlitz et al. 1998

(Continued)

Table 1 *(Continued)*

Target	Examples	Virus targeted	References
Translation of viral RNA	Antisense RNA	HIV-1, HBV, HCV, HPV	Bai et al. 2003; Fraisier et al. 1998; Kolb et al. 2006; Lee et al. 2003; Matsugami et al. 2003; Pearson et al. 1990; Poznansky et al. 1999; Sullenger et al. 1990, 1991
Class III			
Packaging of viral genome	Antisense to packaging signal	HIV-1	Joshi et al. 1991; Trono et al. 1989
	Transdominant HIV gag protein		
	Dominant negative mutant HBV core	HBV	(Scaglioni et al. 1996)
Infectivity of particles	ER retained CD4 chimera	HIV-1	Chaloin et al. 2002; Joshi and Prasad 2002; Kobinger et al. 1998; Okui et al. 1998; San Jose et al. 1998; Vallanti et al. 2005
	F12-vif: Transdominant Vif		
	Fusion protein of HIV-1 VPR fusion proteins		
	Aptamers to HIV-1 RT		
	Capsid-nuclease-fusion protein	Dengue 2 virus	Qin and Qin 2006
	Core-nuclease fusion proteins	HBV	Beterams and Nassal 2001; Gong et al. 2005

5.3 Antiviral Proteins

Several types of antiviral proteins have been described. Dominant-negative mutants of viral proteins inhibit the function of the wt proteins. Examples are transdominant (td) Rev and Tat mutants, which competitively inhibit binding of their wt counterparts to the viral RRE and TAR RNA elements, respectively. TdTat thereby inhibits transcription from the HIV provirus and tdRev blocks nuclear export of unspliced viral genomic RNA (Malim et al. 1989; Pearson et al. 1990). Expression of transdominant EBV EBNA-1 was found to reduce viral genome loads and impair growth of EBV-transformed B cells in vitro (Nasimuzzaman et al. 2005). Dominant negative mutants of HBV core protein were reported to potently inhibit HBV production in hepatoma-derived cell lines (Scaglioni et al. 1994, 1996). Correspondingly, expression of mutated cellular proteins can interfere with essential functions of their wt cellular counterparts within the viral life cycle. Examples are inhibition of virus entry by expression of soluble viral receptors such as sCD4 for HIV-1 and hCAR:Fc and hDAF:Fc for coxsackievirus (Lim et al. 2006). Expression of mutated eIF-5A was found to inhibit the expression of unspliced HIV-1 genomic RNA (Bevec et al. 1996; Morgan et al. 1994).

Antibodies that bind and inactivate viral proteins can be expressed within the cell as single-chain fragments (anti-HIV-1 Rev, anti-HIV-1 IN, anti-HIV-1 RT, and anti-HBV core) or secreted as a neutralizing antibody into the supernatant (Levy-Mintz et al. 1996; Sanhadji et al. 2000; Shaheen et al. 1996; Yamamoto et al. 1999). In a strategy termed capsid-targeted viral inactivation (CTVI), viral-capsid protein or other virion-associated proteins have been used as a carrier to guide a degrading enzyme into virus particles, thus specifically inhibiting virus replication by inactivating the virion. Reports on CTVI have been published for HIV-1, HBV, and dengue virus (Beterams et al. 2000; Beterams and Nassal 2001). Finally, ectopic expression of certain cytokines has been shown to inhibit viral replication. Examples are the interferons and interleukin-16 discussed in more detail in Sect. 8.3 (Cremer et al. 2000; Dumortier et al. 2005; Lauret et al. 1994; Sanhadji et al. 1997; Zhou et al. 1997).

A membrane-anchored anti-HIV peptide C46 (maC46) expressed from a retroviral vector (M87o) has been developed by the authors (Egelhofer et al. 2004; Hildinger et al. 2001; Lohrengel et al. 2005). C46 is comprised of 46 amino acids, is derived from the second heptad repeat of the HIV-1 envelope glycoprotein gp41, and effectively inhibits fusion of the viral and cellular membranes during virus entry (Melikyan et al. 2006). The 36 C-terminal amino acids of C46 correspond to the fusion inhibitory peptide C36 (T-20/Enfuvirtide), the first HIV fusion inhibitor approved for clinical use. MaC46 is expressed as a fusion protein with an N-terminal signal peptide, which targets the peptide through the ER to the cell surface and a C-terminal linker followed by a membrane-spanning domain. Expression of maC46 was shown to effectively inhibit a broad range of HIV isolates, including viruses resistant to C36/T-20. In HIV-1-infected cultures, T cells that express maC46 have a strong selective advantage, rapidly accumulate and gain prevalence.

The accumulated cells do not carry an HIV provirus (Egelhofer et al. 2004). This peptide has been clinically evaluated as described later.

5.4 Antiviral RNAs

Antisense RNAs, RNA decoys, ribozymes, small interfering RNAs, and RNA aptamers are potential tools in antiviral gene therapy. The application of the antiviral RNAs is described in detail in Chap. 9 of this volume by J. Haasnoot and B. Berkhout.

6 Clinical Trials Involving Intracellular Antiviral Strategies

To our knowledge, all antiviral gene-therapeutic strategies tested in clinical trials to date target HIV-infection.

6.1 Stem Cell- vs. T Cell-Based Gene Therapy Strategies for HIV-Infection

HIV replicates primarily in T helper cells and in monocytes (von Laer et al. 1990). As monocytes cannot proliferate and expand in vivo, direct introduction of antiviral genes into this cell population is not expected to lead to a substantial reduction of overall virus replication. However, T cells as well as HPC both have a high regenerative capacity and are attractive targets for intracellular antiviral strategies. Clinical gene therapy trials have targeted T-helper cells and HPC. Arguments in favor and against both cell types have been put forward.

For effective treatment of HIV infection, regeneration of the T cell repertoire could be crucial. Gene modification of HPC has the potential to restore a normal T cell repertoire and, above all, allow the regeneration of HIV-specific T helper cells, if thymic functions are present or reactivated in the treated patient. In contrast to HAART, regeneration of the T cell repertoire after stem cell gene therapy could be allowed to occur in the presence of replicating HIV, for example, during structured treatment (HAART) interruptions. Thereby, the developing genetically protected immune cells could mount an efficient de novo response to HIV without risking viral infection facilitated by viral antigen activation. In contrast, T cell clones lost from the repertoire can obviously not be regained by autologous T cell transfers.

A further advantage of the HPC modification is that monocytes/macrophages derived from gene-modified HPC are also protected. For both HPC and T cells, protocols involving mild conditioning with myelotoxic or lymphotoxic drugs,

respectively, allow 10% and higher long-term gene marking, even in the absence of a selective advantage (Aiuti et al. 2002b; Ott et al. 2006; Dudley et al. 2002b; Morgan et al. 2006). The main limitation of stem cell gene transfer is the risk of insertional mutagenesis, which can lead to the preferential expansion of individual hematopoietic cell clones or even overt leukemia/lymphoma as discussed earlier (reviewed in Baum 2007). This risk is predicted to be lower in T cells (Newrzela et al. 2008). In conclusion, current T cell gene therapies have been associated with less toxicity, but also have a lower therapeutic potential than stem cell gene transfer in the treatment of HIV infection.

6.2 Clinical Trials Involving T Cell Gene Transfer of Antiviral Genes

Several clinical studies indicate that antiviral genes can protect against HIV-associated cell death in vivo and thereby may prolong the survival of T cells after adoptive transfer (as summarized in Table 2. The mechanism of action of the different antiviral genes has been introduced in Sects. 5.3 and 5.4). A selective advantage for autologous T lymphocytes transduced with *tdRev* was found in all three trials performed with this transgene (Ranga et al. 1998; Travel et al. 2001; Woffendin et al. 1996). However, in none of these trials, a sustained absolute accumulation of gene-modified cells, and accordingly no effect on viral load, was observed.

Two clinical studies with CD4+ lymphocytes expressing an antiviral were performed. Marking in both studies was low but there was some indication for a possible selective advantage conferred by the ribozyme (Buchschacher and Wong-Staal 2001; Macpherson et al. 2005).

The first clinical evaluation of a lentiviral vector has recently been reported (Levine et al. 2006). A conditionally replicating HIV-1-derived vector expressing an antisense gene against HIV-1 envelope was tested. Gene-modified autologous CD4+ T lymphocytes were infused in five patients with chronic HIV infection. Self-limiting vector mobilization was observed but did not lead to a substantial increase in the levels of gene modification. Interestingly, one of the four patients showed a sustained decrease in viral load. The mechanism is currently unclear as the levels of gene modification were far too low to substantially reduce target cell availability (Levine et al. 2006).

Recently, the first clinical trial involving class I antiviral gene modification was performed testing safety and potential antiviral activity of autologous T cells expressing the membrane-anchored antiviral peptide maC46 in 10 AIDS patients with severe immunodeficiency and HAART failure (van Lunzen et al. 2007). While a significant rise in T helper cell counts was seen, viral load was not affected. Gene marking could be detected throughout the 1-year follow-up, but the levels were too low to account for the marked rise in CD4 counts.

Table 2 Gene therapy trials for HIV infection

Gene	Target cell	Mode of action	References
CD4zeta	T cells	Target T cells to HIV Env protein expressing cells	Deeks et al. 2002; Mitsuyasu et al. 2000; Walker et al. 2000
tdRev	T cells	Inhibits HIV-1 Rev-mediated transactivation of HIV-1 gene expression	Morgan et al. 2005; Ranga et al. 1998; Woffendin et al. 1996
Ribozyme	T cells	Specifically cleaves viral RNA	Buchschacher et al. 2001; Macpherson et al. 2005
maC46(M87o)	T cells	Inhibits fusion of viral and cellular membranes during entry	Lunzen et al. 2007
Anti-HIV-1env Anti-sense	T cells	Binds transcript thereby mediating degradation and hindering translation	Levine et al. 2006
RRE decoy	CD34+ bone marrow cells	Traps Rev and thus inhibits transactivation of HIV-1 gene expression	Kohn et al. 1999
RevM10	CD34+ bone marrow cells	Inhibits HIV-1 Rev-mediated transactivation of HIV-1 gene expression	Podsakoff et al. 2005
RevM10	CD34+ bone marrow cells after conditioning	Inhibits HIV-1 Rev-mediated transactivation of HIV-1 gene expression	Kang et al. 2002a, b
Ribozyme	CD34+ bone marrow cells	Specifically cleaves viral RNA	Amado et al. 2004

In vitro studies have clearly shown that the *maC46* transgene product effectively protects cells from HIV infection and thus indeed confers a strong selective advantage to the gene-modified cells in HIV-infected cell cultures (Egelhofer et al. 2004). However, a clear accumulation of gene-modified cells over time was not seen in the treated patients. There are several possible explanations for this discrepancy. First, the gene-modified cells may have downregulated expression of the transgene in vivo. Antiviral efficacy of maC46 correlates strongly with the level of maC46 expressed on the cell surface (Egelhofer et al. 2004). In addition, the

gene-modified cells may have lost their repopulation potential during ex vivo expansion. Transferred autologous T cells only carry T cell receptors with specificities already present within the reduced T cell repertoire in the patient, with which they must compete to survive and proliferate. In addition, the percentage of HIV-infected T cells ($<1\%$) (von Laer et al. 1990) and thus the total turnover of T helper cells are much lower in patients than in HIV-infected T cell cultures. Therefore, the selective pressure is relatively low in the HIV-infected patient, most likely much lower than that for the gene-corrected cells used successfully to genetically correct T cell precursors and mature T cells in children with different forms of SCID (Aiuti et al. 2002b; Bordignon et al. 1995; Hacein-Bey-Abina et al. 2002; Kawamura et al. 1999). Finally, the architecture of the lymph nodes, which provide a major support for T cell regeneration, is destroyed in these advanced patients with persistently high levels of virus replication. All these parameters must be considered when optimizing future gene therapeutic regimens. For example, HIV-infected patients with a short history of high-level virus replication when lymph node function is not yet severely impaired are expected to profit most from gene therapeutic strategies.

6.3 Clinical Trials Involving Stem Cell Gene Transfer of Antiviral Genes

Several clinical trials involving genetic modification of HPC have been published (listed in Table 2). In a study performed by Kohn and coworkers, mixtures of autologous bone marrow HPC transduced with a retroviral vector expressing an antiviral *RRE* decoy or with a control vector were transplanted into 4 HIV-infected children without prior conditioning. Gene marking was only detected sporadically during the 1-year follow-up (Kohn et al. 1999). A similar study was recently reported by the same group for two children who were cotransplanted with autologous bone marrow CD34+ cells modified to express *transdominant RevM10* or a control gene. Although here again gene marking was low, a reappearance of blood mononuclear cells containing the *RevM10* gene, but not the control gene, occurred concomitant, with a rise in the HIV-1 viral load during a period of nonadherence to the antiretroviral regimen (Podsakoff et al. 2005). This finding indicates that *RevM10* expressing cells may have had a selective advantage during active virus replication.

In a further study, 10 HIV-infected adults were cotransplanted with mobilized peripheral blood progenitors cells modified to express an antiviral ribozyme or a control vector. Transgene expression was detected in myeloid and in T cells for up to 2 years after infusion. There was no indication for a survival advantage of gene-protected cells relative to cells modified with the control, and the levels of gene modification were extremely low ($<0.01\%$). Thus, not unexpectedly, no substantial impact on viral load was observed (Amado et al. 2004). No adverse events related to the gene modification were reported in any of these clinical trials. However, for stem cell gene therapy of AIDS to be effective, levels of gene marking would have to be increased substantially.

Higher levels of gene modification of 10% and more have been achieved by combining transplantation of gene-modified HPC with a mild myelosuppressive conditioning in SCID patients (Aiuti et al. 2002b; Ott et al. 2006). Although not broadly applicable in HIV-infected individuals, a myelosuppressive regimen may be justified for patients with AIDS-related malignancies. Recent studies indicate that patients with AIDS-related lymphoma may profit from high dose therapy followed by autologous or even allogeneic stem cell transplantation (Kang et al. 2002b; Krishnan et al. 2005; Re et al. 2003). In addition, these patients may benefit from genetic modification of the transplant with an antiviral gene. A first step in this direction was reported by Kang and coworkers, who treated two AIDS patients with non-myeloablative conditioning followed by transplantation of gene-modified allogeneic HPC for refractory hematologic malignancies (Kang et al. 2002a, b). The antiviral gene-product expressed in this study was tdRe*v*, and the control gene was *gp91phox*. The marking data did not suggest a survival advantage of cells carrying the therapeutic gene. In addition, levels of gene marking for both genes were much lower than what has been reported for patients treated for inherited immunodeficiencies, possibly due to competition by the cotransplanted unmanipulated graft. In conclusion, as for T cell-based gene therapies, the major challenge in stem cell gene therapies for AIDS will be to develop technologies that achieve therapeutic gene-modification and expression levels.

7 Strategies to Potentially Overcome Insufficient Gene Marking

7.1 In Vivo Enrichment of Gene-Protected Cells by Coexpression of a Selectable Marker

The frequency of transduced cells typically remains below the therapeutic threshold as the percentage of cells that can be genetically modified in vivo is limited by several factors as discussed earlier. In addition, the relative selective advantage of cells protected by an antiviral gene may not be sufficient to allow accumulation of gene-modified cells to therapeutic levels. In this case, levels of gene modification could be increased by coexpressing the anti-HIV gene(s) with a gene conferring a selective advantage at the level of long-lived hematopoietic stem cells (e.g., selection marker).

Generally, two options to achieve a selective advantage in hematopoietic cells are available: selective expansion of transduced cells (for example by *Hox* genes) or transfer of a drug resistance gene that allows selective elimination of nontransduced cells. Regarding the latter strategy, MGMT (O6-methylguanine-DNA-methyltransferase) is a DNA repair protein with promising chemoprotective/selective potential. MGMT specifically counteracts the genotoxic effects of alkylating agents by repairing alkylations at the O6-position in guanines. The selective advantage mediated by *MGMT* overexpression is especially effective in hematopoietic stem cells, because alkylating agents are highly toxic for this

population (Milsom and Fairbairn 2004). Furthermore, inhibitors of endogenous MGMT (e.g., O6-benzylguanine) used in combination with potent MGMT variants (e.g., P140K, G156A) that are resistant to this inhibitor have been developed (Davis et al. 1999). Using retroviral gene transfer of *MGMT P140K*, this combination allows efficient selection of gene-modified hematopoietic stem cells in large animal models (Neff et al. 2006). *MGMT* has been successfully coexpressed with therapeutic transgenes. An example is the coexpression with the antiviral membrane-anchored peptide maC46 in murine and human hematopoietic stem cells in vitro and in vivo (Schambach et al. 2006). MaC46-MGMT expressing murine bone marrow cells were diluted with freshly isolated competitor cells and transplanted into lethally irradiated recipients. Starting with a low level of chimerism, transduced cells were clearly enriched after one round of chemotherapy with BCNU (carmustin) and O6-benzylguanine. These preclinical data suggest that *MGMT* is currently one of the most powerful tools for drug-mediated selection of gene-modified cells also for antiviral gene therapy. Consequently, clinical phase I trials with *MGMT* are ongoing or in preparation (Cornetta et al. 2006).

To translate this approach into clinical scenarios, the risk-benefit assessment of chemotherapy administration in already immunocompromised patients would favor situations in which cytotoxic drugs are indicated anyhow, such as in AIDS-related lymphomas, where alkylating agents are part of the standard regimens.

7.2 Antiviral Gene Products with a Bystander Effect

Secreted antiviral gene products are expected to produce a bystander effect on neighboring nonmodified cells. However, the number of reports on this approach is still very limited. Examples are the secretion of neutralizing antibodies, soluble virus receptors, and interferons from gene-modified cells (Gay et al. 2004; Morgan et al. 1994; Sanhadji et al. 2000; Shin et al. 2005). In principle, these gene products hold considerable promise, as an overall antiviral effect could be achieved, even at relatively low levels of gene marking. For secreted antiviral gene products against HIV-1, the modification of both T cells as well as HPC appears to have great therapeutic potential, as gene-modified cells would be expected to home to lymphatic tissues, which are also the major sites of viral replication, leading to high local concentrations of the secreted antiviral gene product.

8 Gene Therapeutic Strategies that Act via Host Immune Effector Mechanisms

8.1 Enhancing Host Innate Immune Response

For hepatitis B and C, local production of antiviral cytokines at the site of infection, the liver, is expected to be better tolerated than systemic application. Studies

in HBV-transgenic mice showed that IFNγ either secreted in the liver by adoptively transferred T cell (Guidotti et al. 1996) or expressed in liver cells following adenoviral gene transfer (Dumortier et al. 2005) efficiently controlled HBV replication. However, studies in the woodchuck models of HBV infection only showed limited effects (Fiedler et al. 2004; Jacquard et al. 2004; Zhu et al. 2004). In a pilot study, liver-directed *IFN*γ gene delivery was investigated in chronically HCV-infected chimpanzees employing HBV-based as well as adenoviral vectors. In both animals investigated, HCV viremia did not drop although a transient increase of intrahepatic IFNγ mRNA was associated with homing of HCV-specific and nonspecific T cells into the liver (Shin et al. 2005).

In addition, studies evaluating interferon alpha/beta and interleukin-16 expression have shown substantial antiviral effects against HIV-1 in vitro and in a humanized mouse model (Cremer et al. 2000; Lauret et al. 1994; Sanhadji et al. 1997; Zhou et al. 1997).

8.2 Adoptive T Cell Transfer

Therapeutic efficacy of adoptively transferred cytotoxic T lymphocytes (CTL) has been demonstrated in clinical trials for cytomegalovirus (CMV)-associated disease (Walter et al. 1995), for EBV-associated lymphoma (Rooney et al. 1995) and nasopharyngeal carcinoma (Comoli et al. 2005) as well as for chronically active EBV infection (Savoldo et al. 2002).

A broad and vigorous T cell response generally accompanies elimination of HBV as well as HCV infection. By contrast, patients with chronic hepatitis B or C tend to have late, transient, or narrow T cell responses. In a long-term follow-up of HBV-infected patients receiving HPC transplants from HBV-immune individuals, 20 of 31 recipients cleared their HBV infection (Hui et al. 2005). In principle, these results encourage the development of adoptive T cell transfer strategies for the treatment of chronic viral hepatitis. However, it is still controversial whether induction of an efficient T cell response is the cause or the consequence of viral clearance. Furthermore, T cell responses do not only contribute to virus control but also to disease pathology (Rehermann and Nascimbeni 2005).

Although there is substantial evidence demonstrating that CTL are crucial for the control of HIV replication, the adoptive transfer of HIV-specific CTL has not resulted in clear clinical benefits so far (Hess et al. 2004; Schmitz et al. 1999; Walker and Levy 1989). Virus load was generally unaffected (Lieberman et al. 1997; Tan et al. 1999). The poor therapeutic activity of autologous CTL transfer may be explained by findings showing that HIV-specific CTL derived from infected patients have functional defects. (Appay et al. 2000). On the contrary, increased functionality of HIV-specific CTL is coupled to the ability to control HIV replication in long-term non-progressors, in patients with structured treatment interruptions after treatment of acute HIV infection, and in chronically infected patients who spontaneously control virus infection (Hess et al. 2004; Migueles et al. 2002).

Taken together, these results have encouraged further studies on adoptive T cell transfer for the treatment of chronic viral infections using either in vitro expanded, unmodified, or receptor-modified, retargeted T cells as discussed in the following (Engels and Uckert 2007). The next generation of adoptive T cell-based immunotherapies will probably rely on the ability to endow "fit" cells with elevated cell-surface expression of high-affinity, specific receptors by gene-transfer technology (Gattinoni et al. 2006).

8.3 Grafting T Cells with Recombinant Receptors to Enhance Virus-Specific Immunity

Transfer of a T cell receptor-encoding gene can redirect antigen specificity and thereby increase the number and potentially the activity of CTL for a specific target antigen (reviewed in Engels and Uckert 2007). Two types of recombinant antigen receptors for T cells have been described. In the first approach, expression of the alpha and beta chain genes of a high avidity T cell receptor (rTCR) cloned from a natural CTL clone selected in vitro is used to redirect antigen specificity of gene-modified CTL. This strategy was shown to be effective against tumor cells in vitro and in murine models (Chamoto et al. 2004). CTL expressing recombinant Gag- or Pol-specific rTCR were found to specifically lyse HIV-infected target cells in vitro (Cooper et al. 2000; Ueno et al. 2004).

The second approach utilizes an artificial chimeric T cell receptor (cTCR) composed of an antigen binding domain, which is frequently a single-chain antibody, and a signal transducing unit, often derived from the CD3 zeta chain optionally fused to the costimulatory CD28 signaling domain (Abken et al. 2003). Again this approach has been primarily used for redirecting T cells for tumor recognition, but was adapted for the treatment of viral infections. CTL have been gene-modified to target HIV-infected cells by ectopic expression of cTCRs with different antigen binding domains, such as the extracellular domain of CD4 (which binds gp120) or single-chain antibodies specific for HIV envelope glycoproteins gp41 or gp120 (Masiero et al. 2005; Roberts et al. 1994; Walker et al. 2000; Yang et al. 1997).

Both rTCR and cTCR have advantages and disadvantages. The major disadvantage of the rTCR is their MHC restriction, which limits application to MHC-matched individuals. In addition, presentation of viral antigens on MHC molecules may be limited by downregulation of MHC molecules or by reduced endolysosomal antigen processing in chronic virus infection (Basta et al. 2005). Furthermore, the expression of rTCR chains in T cells in addition to the endogenously expressed molecules could impair T cell function or cause unwanted effects by mixed TCR heterodimers and requires careful rTCR design (Sommermeyer et al. 2006).

A drawback of the more artificial cTCR is that important components of the TCR signaling complex may not be recruited upon receptor binding, leading to incomplete activation of gene-modified T cells and insufficient survival signals (Germain and Stefanova 1999). Since single chain antibody fragments almost

exclusively recognize native proteins (and not MHC/peptide complexes), cTCR can only be used to redirect T cells against proteins on the surface of target cells. This is especially problematic when targeting tumor cells. For virus-infected cells, it is less of an issue, but clearly restricts the application of cTCR-grafted T cells to infections with enveloped viruses, since predominantly viral envelope proteins are displayed on intact surfaces of infected cells.

In HIV infection, the gp120/gp41 complex is only expressed during late stages of the replication cycle in cells that start to actively produce virus and that only have an extremely limited lifespan anyhow (Pomerantz et al. 1990). However, especially for HIV infection, redirected CTL should act as early as possible after infection to reduce the lifespan of virus-producing cells to a level that leads to a significant reduction of the overall viral load and thus cTCR may not be the best choice. In contrast, for viruses such as HBV and HCV, which do not follow an early/late shift in their replication cycle and thus express envelope proteins early after infection, targeting of infected hepatocytes by cTCR-grafted T cells is more promising. Especially in HBV infection, in which persistently infected, cccDNA positive cells continuously produce the hepatitis B surface antigen (HBsAg), redirecting T cells towards HBsAg may allow elimination of residual infected cells after antiviral treatment.

HBsAg-specific cTCR were designed by fusing a single chain antibody fragment to the transmembrane and cytoplasmic regions of the costimulatory CD28 molecule followed by the CD3 ξ-signaling domain. These chimeric receptors, when retrovirally delivered, enabled primary human T cells to recognize HBsAg-positive hepatocytes, to release IFNγ and IL-2 and, most importantly, kill cells productively infected with HBV. When coincubated with HBV-infected primary human hepatocytes, these grafted T cells selectively eliminated HBV-infected, cccDNA-positive target cells (Bohne et al. 2008; Bohne and Protzer 2007).

8.4 Clinical Trials Employing TCR-Grafted T Cells

A chimeric receptor composed of CD4 and the signal-transducing domain of the zeta chain (CD4zeta) has been tested in three clinical trials in HIV-infected individuals. A trial involving the transfer of either CD4zeta-modified syngeneic CD8+ T cells alone or together with gene-modified CD4+ T cells suggests that CD4zeta-expressing CD4+ T cells provide help and thereby support survival of the cotransferred CD8+ cells (Walker et al. 2000). In a second trial, trafficking of transferred autologous CD4zeta-modified T cells to rectal tissue was associated with a decrease in rectal tissue-associated HIV RNA (HIV-1 is known to replicate in mucosa-associated lymphoid tissue) (Mitsuyasu et al. 2000). In a third phase II trial in subjects on effective HAART treatment, the transfer of unmodified T cells was compared to CD4zeta-modified T cells. A substantial decrease of HIV RNA in rectal mucosa as well as in virus that could be recovered by coculture form blood lymphocytes was observed after infusion of gene-modified but not of unmodified T cells (Deeks et al. 2002). However, substantive changes in plasma viral load were seen in none of the three trials.

Currently, these local effects do not justify broad application of gene-modified HIV-specific T cells, and for treatment of HBV or HCV infection no in vivo data are available yet. However, high levels of antigen-specific T cells were found to be associated with therapeutic efficacy after adoptive T cell transfer for cancer and were achieved by mild conditioning (Dudley et al. 2002a, b; Morgan et al. 2006). Furthermore, adoptive transfer of rTCR-grafted T cells improved the clinical course of CMV infection during severe immunosuppression. These findings clearly encourage the further development of adoptive T cell transfer protocols for chronic viral infections.

9 Outlook

Several antiviral genes that inhibit replication of HIV-1, HBV, HCV, and other viruses effectively in tissue culture or even in small animal models have been developed. However, despite major improvements in gene transfer technology, levels of gene modification and expression in vivo have not been sufficient to clearly achieve a therapeutic effect in human viral diseases. For viruses with a prominent tissue tropism, such as hepatitis B and C virus, AAV or gutted Ad vectors allow direct local in vivo transfer of high vector doses, leading to relatively good levels of gene marking in the target cell population. For viruses that show a more disseminated replication pattern, such as HIV-1, primary levels of gene marking will most likely not be sufficient to achieve a therapeutic benefit. Here, gene therapy can only be effective, if the antiviral genes or coexpressed selectable markers confer a selective advantage sufficient to support accumulation of gene-modified cells to high levels. Alternatively, secreted transgene products that exert a bystander effect on nonmodified viral target cells may be therapeutically effective, despite low levels of gene modification. Alternative promising approaches may arise from gene therapy strategies that employ and enhance host immune responses.

The central long-term goal of gene-therapy will be to develop technologies for efficient direct in vivo gene transfer. This will be the only chance for gene therapeutic regimen to successfully compete with classical antiviral drugs in the future.

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Antiviral Resistance and Impact on Viral Replication Capacity: Evolution of Viruses Under Antiviral Pressure Occurs in Three Phases

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Contents

Abstract Resistance development is a major obstacle to antiviral therapy, and all active antiviral agents have shown to select for resistance mutations. Aspects of antiviral resistance development are discussed for specific compounds or drug classes in the previous chapters, while this chapter provides an overview regarding the evolution of different viruses (HIV, HBV, HCV, and Influenza) under pressure of antiviral therapy. Virus replication is an error prone process resulting in a large number of variants (quasispecies) in patients. Resistance evolution under suboptimal therapy can be schematically distinguished into three phases. (1) preexisting variants less sensitive to the respective drug are *selected* from the quasispecies population, (2) outgrowing variants *acquire* additional mutations increasing their resistance, and

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H.-G. Kräusslich, R. Bartenschlager (eds.), *Antiviral Strategies,* Handbook of Experimental Pharmacology 189,

(3) compensatory mutations *accumulate* to overcome the generally reduced replicative capacity of resistant variants. Successful therapy should be aimed at suppression of all existing viral variants, thus preventing selection of minority species and their subsequent evolution. This implies that the amount of mutations required for first escape to the viral regimen (genetic barrier) should be larger than the expected number of mutations present in viruses in the quasispecies. Accordingly, combination therapy can achieve complete inhibition of replication for most HIV, HBV, and Influenza infected patients without resistance development. However, resistant viruses can become selected under circumstances of suboptimal antiviral therapy and these resistant viruses can be transmitted. Proper use of drugs and worldwide monitoring for the presence and spread of drug resistant viruses are therefore of utmost importance.

Abbreviations

HIV	Human immunodeficiency virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
OC	Oseltamivir carboxylate
RC	Replicative capacity

1 Introduction

In this chapter we describe the current insights into the evolution of viruses under pressure of antiviral therapy and the potential impact on viral fitness. As most recent work in this field has been done in the field of human immunodeficiency virus (HIV), we use the evolution of this virus as the basis for the chapter. Subsequently, we describe resistance evolution for Hepatitis B virus (HBV), where large progress has been made in recent years. Furthermore, we describe the resistance development for Hepatitis C virus (HCV), for which a very active drug development program is undertaken by several pharmaceutical companies. Finally, we discuss resistance evolution for Influenza.

Our understanding of evolution and the role of viral diversity, resistance, and fitness has expanded greatly over the last decade, but is still incomplete. Undoubtedly, our insights will improve in the years to come.

2 Human Immunodeficiency Virus (HIV)

2.1 HIV Diversity

Worldwide, the HIV-1 epidemic is mainly fuelled by heterosexual intercourse. HIV is also spread by homosexual contact, by transfusion of contaminated blood

products, by intravenous drug use, and by mother-to-child transmission. The risk and efficiency of transmission depends on several viral and host factors, like HIV-1 concentration, viral phenotype, route of transmission, and susceptibility of the host. As a result, only a relatively small amount of infectious virus from the source population encounters susceptible cells and is able to establish a new productive infection. Recently infected individuals therefore harbor a relatively homogenous viral population, especially as compared to chronically infected individuals (Delwart et al. 1994; Wolfs et al. 1992). The HIV-1 population within a chronically infected individual consists of a complex and dynamic swarm of viral variants referred to as a quasispecies (Domingo et al. 1997). The continuous high replication leading to the production of 10^{10} viral particles a day, lack of proofreading by the viral reverse transcriptase, and recombination lead to the generation of massive numbers of genetically distinct viral variants in an infected individual. The complete viral quasispecies is subject to selection and evolution and its genetic variability will allow the viral population to respond to different selective pressures. Within the quasispecies, wild type is defined as the most fit and most common individual sequence. However, it is important to realize that the total number of mutants taken together is far greater than the number of wild type viruses in a patient population. It is therefore predicted that any genome selected at random from the population is likely to harbor a mutation as compared with wild type and is less fit (under those circumstances). However, such a mutant virus may display an increased fitness under different circumstances in the patient, such as antiretroviral therapy or changed immune pressure.

In HIV literature, viral fitness and replicative capacity (RC) are often interchanged, but viral fitness comprises not only the intrinsic viral replication capacity but also other viral and host/environmental factors. Therefore, an in vivo analysis of the kinetics of a viral variant over time is required to provide the best estimate of true viral fitness. In this chapter, we focus on RC, ignoring other viral and environmental components. The impact of a certain drug resistance associated mutation on the intrinsic viral RC can be best estimated in a more defined in vitro system (Quinones-Mateu and Arts 2001). In vitro, the impact of drug resistance-associated mutations on viral RC can be estimated using either HIV-1 primary isolates or recombinant viruses, in which part of the drug exposed virus is cloned in a neutral HIV-1 backbone. The viruses are tested in parallel mono-infections (replication curves) or mixture-infections (competition experiments). In general, viral replication curves can only discern large differences in viral RC, whereas the replication competition experiments can be used to detect even minute differences.

Initially, it was assumed that the HIV-1 population is infinite, evolution is deterministic, and antiretroviral resistance development is definite (Coffin 1995). However, our research amongst others has demonstrated that the effective population size, defined as the average number of HIV variants that produces infectious progeny is relatively small (Leigh Brown 1997; Leigh Brown and Richman 1997; Nijhuis et al. 1998). This can be explained because the majority of virus particles that are produced harbor deleterious mutations resulting in noninfectious virus. Also limited target cell availability and inactivation of potentially infectious viruses by the host

immune system is reducing the effective population size. These estimations of the relatively small effective population size are supported by the observation that current HIV-1 therapy is capable of inhibiting viral replication when given under ideal circumstances.

2.2 Mechanisms of HIV Resistance

More than 20 antiretroviral compounds have been approved for treatment of HIV-1 infection. These drugs belong to different classes targeting different steps in the viral life-cycle as described in the chapters on individual drug classes: viral binding and entry into the host cell, reverse transcription of the viral RNA into DNA, integration of the DNA into the host cell genome, and maturation by the viral protease. Current HIV-1 therapy is successful in most patients being treated. However, if the patient cannot comply with the regimen or if the patient became infected with a drug-resistant virus from the beginning, replication may occur and can give rise to resistance development. Considerable progress has been made to identify the molecular basis of drug resistance and to understand the mechanisms causing resistance.

A general mechanism of resistance is reducing the affinity of the antiretroviral compound for its mutant target protein. Resistance mutations associated with reduced affinity are observed during treatment failure with a fusion inhibitor, non-nucleoside reverse transcriptase inhibitors (NNRTI), integrase inhibitor, and protease inhibitors as reviewed in Chaps. 3, 4, 6, and 7 (Hazuda et al. 2007; Hsiou et al. 2001; King et al. 2002; Mink et al. 2005).

Nucleoside and nucleotide reverse transcriptase analogues (NRTI) lack a 3′ hydroxyl group and as a result no additional nucleotides can be incorporated into the growing DNA chain. Two NRTI resistance mechanisms are identified: impairment of the incorporation of the antiretroviral drug (discrimination) and removal of the analogue from the terminated DNA chain (excision) as reviewed in Chap. 3 (Arion et al. 1998; Meyer et al. 1999; Sarafianos et al. 1999).

In addition to these target gene-based resistance mechanisms, also nontarget gene-based resistance has been proposed, particularly affecting the substrates of HIV protease (Doyon et al. 1996, 1998; Maguire et al. 2002; Prado et al. 2002). Recently, we described a novel substrate-based HIV-1 protease inhibitor drug resistance mechanism (Nijhuis et al. 2007). We demonstrated the in vitro selection of viruses resistant to all protease inhibitors in the absence of a single substitution in the viral protease. Full genomic sequencing revealed the presence of protease cleavage site substitutions in the Gag polyprotein in all resistant viruses. These changes when introduced in a reference strain conferred protease inhibitor resistance by enhancing the processing efficiency of the altered substrate by wild-type protease. Selection of such substitutions allows the virus to overcome a protease inhibitor-induced reduction in protease activity and represents a general mechanism of resistance to all known protease inhibitors.

Resistance mutations have been described for all clinically used anti-HIV drugs based on both in vitro selection and sequence analysis of viruses from patients failing therapy. This led to the description of specific resistance patterns for individual compounds. Resistance profiles for individual drugs or drug classes are described in the chapters dealing with the respective class. Knowing drug- or class-specific resistance patterns also allowed the establishment of bioinformatics tools to predict the expected resistance against specific compounds based on sequence analysis of viral RNA amplified by RT-PCR from patient blood. These tools are continuously updated and provide an important diagnostic aid to guide HIV combination therapy (http://www.hiv-grade.de; http://hivdb.stanford.edu/; http://www.hivfrenchresistance.org/; http://www.rega.kuleuven.be/cev/). Besides genotypic analysis, resistance testing may also involve phenotypic analysis where the patient-derived sequences are cloned into a standard proviral plasmid and viruses obtained after transfection of this pool are tested for RC and resistance in tissue culture (Hertogs et al. 1998; Petropoulos et al. 2000). This latter method is primarily useful for new drugs with less validated resistance patterns, while genotypic testing is generally sufficient for drugs with an established resistance pattern.

2.3 Evolution of HIV Resistance During Antiretroviral Therapy

Resistance development is not an all-or-nothing phenomenon and in general gradually increases over time. Overall, three phases in the evolution of antiretroviral drug resistance can be discerned (Fig. 1).

First phase: In the first phase, preexisting virus variants with a reduced susceptibility to the antiretroviral compound are selected from the viral population in the patient. Since most of these drug-resistant variants differ by only one or two nucleotides from the wild-type sequence, they preexist in the viral quasispecies. The natural occurrence of drug resistance-associated mutations in the viral population before onset of treatment was already demonstrated more than 10 years ago, before the large scale introduction of antiretroviral therapy (de Jong et al. 1996; Najera et al. 1994). Although the impact of a single or double mutation on viral replication capacity may differ, in general they all replicate less well than the original wild type virus (Quinones-Mateu and Arts 2001). Furthermore, selection of particular drug resistant variants from the viral population is associated with a strong reduction in the viral population size (Delwart et al. 1998; Nijhuis et al. 1998).

Second phase: In the second phase, the initially selected drug resistant mutant viruses are allowed to replicate in the presence of a suboptimal antiretroviral regime. This continuous replication results in the generation and selection of novel amino acid changes and a further increase in drug resistance. In this second phase, viruses are being generated that have not been observed in the patient before and in general have a reduced replication capacity as compared to the original wild-type virus.

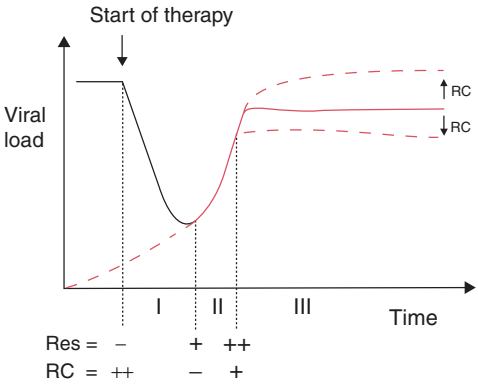


Fig. 1 Three phases in viral evolution during suboptimal therapy. The black line represents wild type virus, whereas the red line represents mutant virus (Res stands for the level of resistance and RC for replication capacity).

Third phase: The third phase is characterized by the generation of novel compensatory mutations, increasing the reduced replication capacity of the first and second phase drug-resistant viruses. Selection of compensatory amino acid changes was first described for protease resistant variants and involves the selection of amino acid changes in the viral protease itself and the substrate of the viral protease, the Gag protein (Doyon et al. 1996; Mammano et al. 1998; Nijhuis et al. 1999; Quinones-Mateu and Arts 2001; Zhang et al. 1997). We have demonstrated that continuous replication during nonsuppressive therapy may result in the selection of a novel protease resistant variant that can replicate even better than the original wild-type protease variant (Nijhuis et al. 1999). Compensation of the initial reduction in viral replication capacity has also been observed for reverse transcriptase, integrase, and fusion inhibitor resistant virus variants (Hazuda et al. 2007; Huigen et al. 2008; Kleim et al. 1996; Labrosse et al. 2006). Investigation of the viral population size indicates that the originally observed bottlenecking may be transient and the variation in the viral population as observed before therapy reappears (Delwart et al. 1998).

2.4 Genetic Barrier

The challenge in the treatment of HIV infection is to overcome the plasticity of the HIV population. We have learned that a HIV-1 regimen fails when just one or two mutations, which are preexisting in the viral population, are sufficient to confer clinically relevant levels of resistance, whereas any HIV regimen requiring more than three mutations will be successful in patients capable of taking this regimen as prescribed. Based on these observations the term genetic barrier has been introduced. Genetic barrier is defined as the number of mutations required to escape the drug-selective pressure in the patient. It is important to realize that for a virus to be able to escape the pressure of a drug (combination), two conditions have to be met. First of all the virus has to be resistant to the drug (combination). However, resistance per se is not sufficient; the virus has also to be able to replicate at

sufficient amounts. Several approaches have been developed to increase the genetic barrier of drug regimens. First of all, novel inhibitors with an intrinsic high genetic barrier have been designed. The protease inhibitor darunavir (see chapter by Anderson et al., this volume) is an example of a compound with such an high intrinsic genetic barrier (De Meyer et al. 2005; Dierynck et al. 2007). Second, for some drugs, increasing the drug concentration of the inhibitor, for instance through ritonavir-boosting (see chapter by Anderson et al., this volume), can increase the genetic barrier. Finally, a very successful approach to increase the genetic barrier is to combine two or more inhibitors, which do not share resistance patterns (discussed in more detail in chapter by Hofmann and Zeuzem, this volume). Not all combination therapy regimens were shown to be sufficiently effective. For instance, it was demonstrated that a low genetic barrier could explain early virological failure to a specific combination of three reverse transcriptase inhibitors (didanosine, tenofovir, and efavirenz) as was observed in several patients (Podzamczer et al. 2005). This low genetic barrier was explained by the fact that one mutation could confer resistance to two compounds. The L74V change caused resistance to both didanosine and tenofovir. But, in addition, it helped to restore the reduced replication capacity caused by the efavirenz-resistance mutation G190E. Consequently, a virus can escape this triple drug combination by selection of just two mutations, resulting in a resistant and replication competent virus. Also treatment with other triple reverse transcriptase inhibitors combinations consisting of lamivudine and tenofovir combined with either abacavir or didanosine showed a high rate of virological failure. Although negative drug–drug interactions and potential pharmacokinetic limitations may have an effect, the low genetic barrier of these regimens (e.g., two mutations M184V and/or K65R) seems to play a role in the early virological failure (Gallant et al. 2005; Landman et al. 2005; Lanier et al. 2005; Luber 2005; Ruane and DeJesus 2004).

The variation of the viral population in a patient, which is a reflection of the actual population size, is an important factor influencing the genetic barrier. But also the quantity of the minority species may contribute to therapy outcome. For instance, a single dose nevirapine (NNRTI) monotherapy is often used to prevent mother-to-child transmission. Under these conditions, the preexisting single NNRTI-resistant mutants have a clear growth advantage over wild–type, resulting in an increase of resistant variants. Over time after the intervention, the amount of resistant mutants may decrease again. Nevertheless, several studies have reported a higher virological failure on a subsequent NNRTI-containing HAART regimen in these patients even when the NNRTI-resistant mutant was present as a minority species (Jourdain et al. 2004; Lecossier et al. 2005; Lockman et al. 2007). The genetic barrier may also be affected by natural variation in the viral target gene. HIV-1 subtypes and recombinant forms are very unevenly distributed throughout the world, with the most widespread being subtypes B and C (Thomson and Najera 2005). Historically, subtype B has been the most common subtype in the US, Japan, Australia, and Europe. Although this is still the case in Europe, other subtypes are becoming more frequent and now account for at least 25% of infections. Subtype C and A, as well as the CRF02_AG and CRF01_AE recombinant forms are of special concern, since they

represent the predominant subtypes in Africa and Asia where HIV is spreading most rapidly. Until recently, antiretroviral compounds were being designed for use against subtype B virus. Analysis of other HIV-1 subtypes has revealed many polymorphisms within the target genes of the inhibitors that are implicated in facilitating the emergence of resistance (Kantor 2006).

Also transmission of drug resistant variants may compromise the genetic barrier of a drug regimen. Several studies have shown that in areas with ongoing access to antiretroviral therapy, about 10% of the newly diagnosed HIV-1 patients are already infected with a drug-resistant variant (SPREAD Programme 2008; Weinstock et al. 2004; Wensing and Boucher 2003). A large prospective study involving 33 countries in Europe (European HIV Resistance Network) has demonstrated that the prevalence of viruses with drug-resistant mutations is 9%. The majority of strains harbor single drug resistance associated mutations. Although most of these single drug resistance-associated mutations do not confer resistance on their own, it was shown that their presence results in faster acquisition of drug resistance and compromises the genetic barrier (Garcia-Lerma et al. 2004). Finally, in a situation in which we have performed in vitro selection experiments using an intrinsic high genetic barrier protease inhibitor, we have demonstrated that HIV-1 can use a short-cut to protease resistance development (Nijhuis et al. 2007). In this situation, an alternative protease resistance mechanism based on two mutations in the gag cleavage site sequence was selected. The mutations confer broad class protease inhibitor cross-resistance but are not generally observed, which is most likely related to their poor replication capacity.

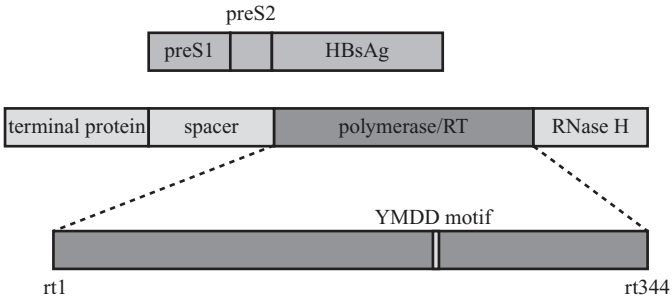
Taken together, the genetic barrier cannot be simply defined by the sum of resistance mutations, but also the genetic background of the virus, the variation in the viral population, interaction of mutations, and their impact on viral replication play a role.

3 Hepatitis B

The hepatitis B virus (HBV) genome is one of the smallest viral genomes (approximately 3,200 base pairs) and encodes only one viral enzyme, namely the HBV reverse transcriptase (RT). Like the HIV RT, the HBV RT is an error-prone enzyme lacking proofreading activity. In combination with a high virus production, this results in an HBV quasispecies.

3.1 Evolution of HBV Resistance During Lamivudine Therapy

HBV RT has been the target for the development of several anti-HBV agents during the last decade. The first HBV RT-inhibitor approved for treatment of chronic infection was the nucleoside analogue lamivudine, which had already been used



Lamivudine	L80I	V173L L180M	M204I/V/S
Adefovir		A181V	N236T
Entecavir*		I169T T184G	S202I/G M250V

*in the background of lamivudine resistance mutations

Fig. 2 The HBV polymerase and envelope proteins, whose reading frames overlap each other, and position of the main HBV drug-resistant mutations

clinically as inhibitor of HIV RT. Lamivudine exerts its antiviral activity by competing with dCTP and once incorporated into DNA causes immediate chain termination. Although lamivudine therapy results in a clear reduction in HBV viremia, breakthrough due to the emergence of resistant variants constitutes a significant problem. Lamivudine-resistant variants appear in 14–32% of patients after 1 year of therapy, increasing to 53–76% after 3 years (Lai et al. 2007). Lamivudine resistance is mainly due to selection of variants with a single amino acid change within the YMDD catalytic domain of HBV RT, with the major resistance mutations being rtM204V and rtM204I (Fig. 2) (Bartholomeusz and Locarnini 2006; Durantel et al. 2005). These changes affect the same positions as lamivudine-resistance mutations in HIV RT and cause structural alterations resulting in steric hindrance of lamivudine binding to HBV RT and high-level resistance (>100-fold) (Das et al. 2001; Locarnini and Mason 2006). The high-level of lamivudine resistance caused by the rtM204V change can be further increased by the selection of an additional change at codon 180 (rtL180M) (Ono et al. 2001). Whether or not other mutations infrequently observed during lamivudine treatment (rtI169T, rtT184S, and rtQ215S) can also further increase the level of resistance remains to be investigated.

Changes at rtM204 also result in a marked decrease in viral RC (Sheldon et al. 2006). This decrease in viral replication can be partially restored by mutations elsewhere in the HBV polymerase. For instance, the rtL180M change not only increases the level of lamivudine resistance, but also partially restores the decreased RC of the rtM204V variant. Other mutations that have been described to act as compensatory

mutations include rtL80I and rtV173L (Delaney et al. 2003; Warner et al. 2007). It thus appears that the evolution of lamivudine resistance in Hepatitis B also occurs in several phases.

Another nucleoside analogue belonging to the same class as lamivudine is telbivudine. Clinical resistance to telbivudine has to be studied in more detail, but the first in vivo data and several in vitro results suggest that tebivudine's resistance profile is quite similar to that of lamivudine (Lok et al. 2007).

3.2 Evolution of HBV Resistance During Adefovir Therapy

Adefovir dipivoxil is another antiviral agent approved for chronic HBV treatment. It is an analogue of the nucleotide adenosine and results in DNA chain termination once incorporated. Although the selection of resistant variants is also a problem with adefovir dipivoxil, resistance to adefovir is less frequent than to lamivudine, with adefovir resistant variants appearing in approximately 2% of patients after 96 weeks of therapy (Hadziyannis et al. 2006). The major resistance mutations for adefovir are rtN236T and rtA181V, which result in only a small increase in resistance (approximately one- to tenfold) (Borroto-Esoda et al. 2007; Locarnini and Mason 2006). It is currently not known what effect these changes exert on RC. Several other changes have been implicated in adefovir failure (rtL80V/I, rtV84M, rtV214A, rtS85A, rtQ215S, rtP273H, and rtN238T/D), but their exact role remains to be determined (Shaw et al. 2006).

3.3 Evolution of HBV Resistance During Entecavir Therapy

Entecavir is a deoxyguanosine analogue, which also results in chain termination once incorporated. It is currently not fully understood how HBV develops resistance to entecavir. In patients with prior failure to lamivudine, entecavir resistance developed by selection of additional mutations (rtI169T, rtT184G, rtS202I/G, or rtM250V) on top of two previously selected lamivudine-resistance mutations (rtL80M + rtM204V) (Shaw et al. 2006). This suggests that HBV may have a high genetic barrier for developing entecavir resistance, since at least three mutations were required for resistance. In naïve patients treated with entecavir monotherapy for 96 weeks, viral rebound was seen in approximately 3% of patients (Colonno et al. 2006). However, population phenotypic testing of the overall susceptibility of the quasispecies showed that entecavir susceptibility was virtually unchanged from baseline. In addition, changes in RT that emerged during entecavir treatment were analyzed in phenotypic assays using recombinant viruses and also here entecavir susceptibility remained virtually unchanged from baseline (Colonno et al. 2006). Further studies are warranted to investigate this in more detail.

In summary, all antiviral agents licensed for treatment of chronic HBV infection can lead to resistance. Currently, the effect of most resistance-associated mutations on RC is unknown. It is important to keep in mind that the HBV polymerase gene completely overlaps with the envelope gene. As a consequence, resistance mutations can cause collateral amino acid changes or even stop codons in the HBV surface antigen, making their selection unlikely or impossible. The effect of resistance mutations also altering the viral surface protein on diagnostic detection, vaccine escape and pathogenicity warrants further investigation.

4 Hepatitis C

Currently, there is no approved antiviral therapy specifically targeting hepatitis C virus (HCV). The development of an HCV replicon system and our improved understanding of the structure and function of HCV proteins have led to the development of several classes of specific HCV inhibitors. NS3-4A protease inhibitors and NS5B polymerase inhibitors are furthest in development as discussed in Chaps. 2–4 (De and Migliaccio 2005; Manns et al. 2007; Pawlotsky et al. 2007).

The HCV genome encodes a polyprotein, which is cleaved by viral and cellular proteases, including the HCV nonstructural protein NS3-4A (Lindenbach and Rice 2005). Several inhibitors of NS3-4A have been designed and are currently in clinical trials (e.g., telaprevir (VX-950), boceprevir (SCH503034), SCH6, BI12202, MK-7009, TMC435350, and ITMN-191) (De and Migliaccio 2005; Manns et al. 2007).

One of the proteins cleaved from the HCV polyprotein is the nonstructural protein NS5B, an RNA-dependent RNA polymerase. NS5B is required for the replication of the genomic RNA (Lindenbach and Rice 2005). Several NS5B inhibitors have been developed and are currently or have been in clinical trials. Based on their mechanism of action they can be divided into two classes: the nucleoside analogues that result in chain termination once incorporated (e.g., valopicitabine (NM-283) MK-0608, R1479, PSI-6130, and R7128) and the non-nucleoside inhibitors, which block the enzyme by preventing a conformational transition needed for the initiation of RNA synthesis (e.g., JTK003, BILB1941, VCH-759, PF-866554, HCV-371, A-878837, A-782759, GSK625433, HCV-796, and GS-9190) (De and Migliaccio 2005; Manns et al. 2007).

4.1 Evolution of HCV Resistance in the Presence of Antiviral Agents

Like HIV and other RNA viruses, HCV replicates as a quasispecies due to a high level of viral replication in combination with an error-prone replication strategy. Therefore, the issue of drug-resistance is expected to be a major challenge in this case as well. Indeed, resistance to HCV antiviral agents has already been

demonstrated both in vitro and in vivo. For the HCV protease inhibitors, it has been observed that resistance can occur by selection of variants with only one or two amino acid changes within the protease gene, giving rise to either low- or high-level resistance (Table 1) (De and Migliaccio 2005; Manns et al. 2007; Sarrazin et al. 2007; Tong et al. 2006; Yi et al. 2006). It is currently not known whether resistance to HCV protease inhibitors or viral RC can also be influenced by mutations outside the protease gene, for instance, in the protease cleavage sites. For the NS5B polymerase inhibitors, no in vivo resistance data are available yet. In vitro experiments have shown that single or double amino acid changes in the polymerase gene can confer low to moderate levels of resistance against the nucleoside inhibitors (Le et al. 2006a; Migliaccio et al. 2003). Also for non-nucleoside inhibitors, several resistance mutations have been described (Table 2). Again, single or double amino acid changes in NS5B affecting the inhibitor binding site have been shown to confer moderate to high-level resistance (Howe et al. 2006; Kukolj et al. 2005;

Table 1 Mutations associated with resistance to HCV NS3-4a protease inhibitors in vitro and in vivo (Lin et al. 2007; Manns et al. 2007)

	V 36	F 43	T 54	R 109	R 155	A 156	V 170
Telaprevir (VX-950)	A/M	–	A	–	K/T	S/V/T	–
Boceprevir (SCH503034)	A/M/L	S/C	A	–	K/T/Q/M	S	A/T
SCH6	–	–	–	K	–	V/T	–

Table 2 Mutations associated with resistance to HCV NS5B polymerase inhibitors in vitro (Lin et al. 2007; Manns et al. 2007)

	Examples of drugs	Mutations
Nucleoside inhibitors	Valopicitabine R7128 MK-608	S282T
	R1479	S96T, N142T
Non-nucleoside inhibitors	JTK003 BILB1941	P495A/L/S/T, P496A/S, V499A
	VCH-759 PF-866554 HCV-371	T19P, M71V, A338V, L419M, M423I/T/V, A442T, I482L
	A-878837 A-782759 GSK-625433	G46A, H95Q/R, C316Y, S368A/T, I392F, N411S, M414L/T, E446D, Y448H, Q514R, C451R, G554D, Y555C, G558R, D559G, V581A, Y586C
	HCV-796	V201A, C316F/S/Y, I363V, S365A/L/T, M414I/V

Lu et al. 2007; Tomei et al. 2003, 2004). It remains to be investigated whether the resistance level of these variants with single or double amino acid changes can be further increased by selecting additional mutations.

As for HIV, the selection of HCV drug resistant variants can be accompanied by a decrease in RC. For HCV protease inhibitor resistant variants, the level of resistance seems to be inversely related to viral fitness (Sarrazin et al. 2007; Tong et al. 2006; Yi et al. 2006). There is some evidence that viral RC can be restored by the selection of compensatory mutations within the protease gene (Sarrazin et al. 2007; Tong et al. 2006; Yi et al. 2006). However, further research is warranted to investigate to what extent viral fitness can be restored and by which mutations. Also for the nucleoside and non-nucleoside inhibitors, the selection of resistance results in a fitness defect. It remains to be investigated whether or not compensatory mutations can be selected.

Overall, the genetic barrier to resistance against HCV inhibitors currently in development appears to be fairly low, with only one or two amino acid changes necessary for escape. Keeping in mind the lessons learned from HIV, this implicates that future HCV antiviral therapy will have to be based on a combination of (antiviral) agents with different modes of action and resistance profiles to be successful. Some in vitro studies have already shown that resistance development is more difficult when two different HCV antiviral agents are combined (Le et al. 2006b; Mo et al. 2005). The genetic barrier for HCV resistance development in vivo remains to be determined however.

5 Influenza

Two classes of inhibitors for influenza virus are currently available (Hayden 2006). The M2 proton channel inhibitors amantadine and rimantadine and the neuraminidase (NA) inhibitors oseltamivir carboxylate and zanamivir. Chapter 5 provides more details about the class of NA inhibitors.

5.1 M2 Proton Channel Inhibitors

Amantadine and rimantadine inhibit influenza A virus replication by interfering with uncoating of the virus inside the cell. Resistance to these compounds occurs rapidly in vitro and was found to be caused by selection of single preexisting nucleotide changes, leading to amino acid changes in the M2 ion channel protein. These changes confer high levels of cross resistance to both inhibitors without large changes in viral fitness in animal models (Abed et al. 2005). Resistant viruses also appeared rapidly in clinical application during rimantadine treatment. These variants were capable of causing typical influenza illness and transmission of resistant viruses was observed among close contacts (Hayden et al. 1989).

Since 2004, several publications have reported the presence of primary resistance in an increasing population of viral isolates obtained from humans and birds (Bright et al. 2005). Recently, a specific change at position 31 from serine to asparagine conferring resistance was found in over 70% of Asian human isolates and over 90% of North American isolates from untreated individuals (Bright et al. 2006). It can be assumed that this change causes at least some decrease in viral RC because it is otherwise difficult to explain why this single mutation was not observed previously in circulating influenza strains. Accordingly, it may be predicted that currently circulating viruses containing the Ser31Asn mutation have acquired compensatory changes that prevent reversion to serine at position 31. Further research in this direction is needed to confirm this hypothesis. The high prevalence of resistance mutations in an untreated population of influenza A-infected persons indicates, however, that amantadine and rimantadine are of limited clinical use.

5.2 Neuraminidase Inhibitors

Two neuraminidase inhibitors (oseltamivir carboxylate and zanamivir) are approved for prevention and treatment of infections with both influenza A and B viruses as discussed in chapter by Itzstein and Thomson, this volume. Oseltamivir carboxylate (OC) has gained most use because it can be taken orally, whereas the current formulation of zanamivir has to be inhaled. In addition, the WHO recommends oseltamivir for treatment of clinically confirmed cases of H5N1 and for post-exposure prophylaxis to control recent H5N1 avian influenza outbreaks.

Our understanding of resistance evolution against NA inhibitors has developed during the last decade. Viruses with reduced susceptibility to OC have been selected both in vitro and in vivo. Two mechanisms can account for the reduced virus susceptibility to NA inhibitors. A neuraminidase independent mechanism involves mutations in or close to the HA receptor binding site. The affinity of HA for its receptor has been shown to be an essential feature for the susceptibility to NA inhibitors (Gubareva et al. 2001). Changes in HA decrease viral dependency on the activity of NA (Staschke et al. 1995). In one set of experiments, changes in HA were selected initially (A28T in HA1 and R124M in HA2) and upon further selection a change in NA (R292K) was added (Tai et al. 1998).

Most frequently, NA inhibitor resistance is caused by mutations within NA however. Natural variation in sensitivity due to variation in the NA sequence has been observed (Rameix-Welti et al. 2006). In addition, the specific mutation pattern observed upon exposure to NA inhibitors depends on the NA type (Abed et al. 2006; Ferraris and Lina 2008). For N2, the most frequent change observed upon OC application in vitro or in vivo (from treated adults and children) is R292K, whereas E119V/A/D and N294S are found less frequently. The most frequent change for N1 is H274Y, with N294S being less common. For influenza B, a change at position R512K has been observed. The H274Y and E119V mutations confer resistance to oseltamivir and not zanamivir, while E119G confers resistance only to

zanamivir, and all other changes confer resistance to both drugs (N294S not tested for zanamivir).

The biological properties of changes in NA have been extensively studied using enzymatic studies and by looking at replication and pathogenicity in animal models. Invariably, changes in NA lowered the activity of the enzyme (Aoki et al. 2007). These animal experiments generated interesting results, but it is important to keep in mind that the animal model systems have not been validated against clinical or epidemiological data yet. As a consequence of the lack of validation, the field suffers from a lack of consensus definitions/terminology and consensus experimental conditions to determine RC and pathogenicity as well as virulence and transmissibility.

Nevertheless, viruses harboring the changes mentioned earlier can replicate under cell culture conditions. The R292K and N294S mutations confer a reduction in replicative capacity, virulence, and transmissibility in animal models (ferrets and mice) (Abed et al. 2006). An exception is a H3N2 isolate with a E119V change, which had similar infectivity and transmissibility as the pre-therapy isolate from the same patient in the ferret model (Herlocher et al. 2004). A detailed study evaluated the effect of the H274Y change in N1 using a virus selected in vitro (Ives et al. 2002). Compared to the respective wild-type, this virus replicated less well in cell culture and to lower levels in the lungs of infected mice. Infection of ferrets showed a reduced infectivity of H274Y compared to wild type.

The appearance of viruses with reduced susceptibility to OC has been reported in 50 treated children in Japan infected in 2002/2003 with H3N2 (Kiso et al. 2004). In this study, using a low dose (2 mg/kg body weight twice daily) of OC, three different resistance mutations were identified: the most frequent mutation occurred at position 292 (lysine to arginine), twice a mutation at position 119 (glutamine to valine), and once at position 294 (asparagine to serine). The reduction in IC_{50} to OC was impressive ranging from 280- to 110,000-fold, with the K292R mutation giving rise to the highest level of resistance (28,000–110,000 increase in IC_{50}). The amount of resistant viruses detected in nasal swabs/aspirates or throat swabs determined by quantitative PCR was not significantly lower in a small number of children studied (two children at 4 days and three out of six at 5/6 days) compared to the nontreatment situation. These data are in contrast to a study performed in the USA, where age and weight tailored doses were used for the treatment of 147 children (Whitley et al. 2001). None of these children shed resistant viruses.

Recently, two fatal cases of avian influenza H5N1 with resistant virus were reported in older children who were treated with OC (de Jong et al. 2005). For both patients the appearance of H274Y mutant sequences was observed in longitudinally collected throat swabs, and viral isolates with the same change were isolated. Both children were dosed at 75 mg/kg twice daily, but OC was interrupted after 5 days. Remarkably, the response in throat swabs was predictive in this relative small series of eight reported cases of clinically recognized H5N1 infections: a 5 log reduction in viral titer was observed in the four cases that survived, whereas the RNA response after initiation of therapy was limited to 1–2 log in the four patients who died. In

the two cases with resistant virus, the viral RNA during treatment remained at levels comparable to pre therapy.

Clearly one cannot draw firm conclusions from two cases, but this remarkable finding may argue that there is no severe reduction in RC for resistant H5N1 viruses in natural infection. Unfortunately, the RC of the isolated viruses containing the H274Y was not reported. The high titers maintained in the two patients suggest, however, that either the H274A mutation in H5N1 has no strong effect on RC or secondary changes in the virus compensate for an initial reduction in RC conferred by H274Y. Further studies are urgently needed to relate the presence of resistant virus to pathogenicity/virulence in failing patients. These studies should systematically address the amount of in vivo replication of NA inhibitor resistant viruses and relate it to replicative properties determined in vitro.

Similar to the situation observed for the M2 channel inhibitors where transmission of drug resistance has become a major concern, a similar problem starts to arise for the NA inhibitors. The 2007/2008 epidemic of H1N1 across the world was shown to be partly due to the spread of an OC resistant strain carrying the H274Y mutation. This result was not anticipated, given the reduced replication of H274Y mutants in H1N1 in vitro and in animal models, but may be viewed differently in light of the results with H5N1 variants. More research need to be conducted to investigate whether these and other resistant viruses have acquired compensatory mutations.

6 Conclusions

The evolution of antiviral resistance for viruses discussed in this chapter (HIV, HBV, HCV, and Influenza virus) shares some common features. Replication in vivo results in the generation of viral variation and selection of preexisting viruses from the population occurs under particular conditions. This will only happen when the escaping viruses have a sufficient level of both resistance and RC. In most cases, the resistance level subsequently increases further by the gradual acquisition of further mutations. Additional compensatory mutations then accumulate that help to restore full RC in the third stage.

Successful therapy should be aimed at suppression of all existing viral variants, thus preventing selection of minority species and their subsequent evolution. This implies that the number of mutations required for first escape from the drug regimen (genetic barrier) should be larger than that present in the population of viruses in the quasispecies. This goal can be achieved by the right combination of drugs for most HIV- and HBV-infected patients and for some HCV subtypes and most Influenza-infected patients. However, given that for all of these viruses, drug-resistant viruses can become selected under circumstances of suboptimal antiviral therapy and that these resistant viruses can be transmitted, it is mandatory to maintain proper use of drug combinations and to monitor worldwide for the presence and spread of drug resistant viruses.

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Antiviral Combination Therapy for Treatment of Chronic Hepatitis B, Hepatitis C, and Human Immunodeficiency Virus Infection

Wolf Peter Hofmann, Vincent Soriano, and Stefan Zeuzem

Contents

Abstract This chapter reviews the main chemotherapeutic strategies used against human infections caused by agents responsible for the most important chronic viral illnesses, namely hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV). There is no doubt that most current knowledge about combination antiviral therapy has been developed in the battle against HIV. The availability of more than 20 antiretroviral drugs has permitted to explore their efficacy when given in combination, an opportunity that unfortunately has only been possible since recent years for chronic hepatitis C and still is in the early stages

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H.-G. Kräusslich, R. Bartenschlager (eds.), *Antiviral Strategies,* Handbook of Experimental Pharmacology 189,

for chronic hepatitis B. However, new antiviral compounds targeting each of these viruses are developed rapidly and will provide further opportunities to explore the efficacy of combination antiviral therapy. While sufficient suppression of HIV RNA and HBV DNA can only be achieved by long-term administration of potent antiviral drugs, HCV RNA may be completely eradicated from the infected individual after a limited duration of treatment.

Abbreviations

ALT	Alanine aminotransferase
HAART	Highly active anti-retroviral therapy
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitor
RT	Reverse transcriptase
PI	Protease inhibitor
INI	Integrase strand transfer inhibitor

1 Combination Therapy in Chronic Hepatitis B

1.1 Introduction

Despite the availability of an effective and safe vaccine against hepatitis B for more than two decades, HBV infection is still a major health problem worldwide. More than two billion individuals have been infected with HBV, and chronic infection affects approximately 350 million patients (Lavanchy 2004). While HBV infection in adults is mostly asymptomatic and self-limiting in >80% of the cases, transmission at a younger age is associated with a high risk of chronic infection. Vertical transmission of HBV leads to chronic infection of the newborns in up to 90% of the cases.

Individuals with chronic hepatitis B have a high risk to develop cirrhosis and, once cirrhosis is present, the 5-year cumulative risk to develop hepatocellular carcinoma is about 10–15% (Fattovich et al. 2004).

1.2 Approved Drugs for the Treatment of Chronic Hepatitis B

Over the last 5–10 years, the clinical management of chronic hepatits B has experienced significant advances mainly due to the introduction, evaluation, and approval

of a number of nucleoside and nucleotide (nucleos(t)ide) analogs with specific antiviral activity against HBV (Lok and McMahon 2007). Furthermore, the evaluation of pegylated forms of interferon-α as an immunomodulatory treatment in chronic hepatitis B has contributed to improve antiviral strategies (see chapter by Chevaliez and Pawlotsky, this volume, for further details). Treatment may be initiated with any of the currently approved drugs (standard interferon-α, peginterferon-α2a, lamivudine, adefovir, entecavir, telbivudine, and tenofovir).

1.3 Treatment Objectives in Chronic Hepatitis B

The main treatment objective is the sustained suppression of HBV DNA to reduce hepatic necroinflammation and progression of liver fibrosis. Furthermore, seroconversion to anti-HBe should be pursued in HBeAg positive individuals, as it has been shown that seroconversion in these individuals is associated with improved outcome. Seroconversion to anti-HBs is rarely observed during antiviral therapy, indicating that eradication of HBV is a rather unlikely event with the currently available antiviral treatment.

In general, patients with high HBV DNA ($>20,000\,\mathrm{IU/mL}$), increased ALT levels ($\geq$twofold above the upper limit of normal) over a 3–6 months period, and/or histological evidence of chronic hepatitis are eligible for antiviral treatment. However, in HBeAg negative patients, who have lower HBV DNA concentrations ($>2,000\,\mathrm{IU/mL}$), antiviral therapy should equally be considered when liver histology shows significant necroinflammation and/or fibrosis (Lok and McMahon 2007). HBV DNA concentrations of about 2,000 IU/mL have been shown to distinguish inactive HBsAg carriers from individuals with HBeAg negative chronic hepatitis B (Manesis et al. 2003). It has to be noted that independently of the presence or absence of HBeAg, individuals with HBV DNA concentrations $>2,000\,\mathrm{IU/mL}$ may already have an overall increased risk for the development of cirrhosis and hepatocellular carcinoma (Chen et al. 2006; Liaw et al. 2004). In a recent study, it was demonstrated that continuous treatment with lamivudine delays clinical progression by significantly reducing the incidence of hepatic decompensation and the risk of hepatocellular carcinoma (Liaw et al. 2004).

1.3.1 Interferon-α

Today, 180 μg of peginterferon-α2a once weekly for 48 weeks is recommended in patients with compensated liver disease. However, treatment schedules that were chosen in clinical trials vary between 24 weeks to 2 years and optimal treatment durations and dose remain to be defined.

The HBV genotype has been shown to predict treatment response in patients receiving peginterferon-α (Fung and Lok 2004). Generally, patients infected with HBV genotypes A and B show higher rates of HBeAg loss and HBe seroconversion as compared to those infected with HBV genotypes C and D.

1.3.2 Nucleos(t)ide Analogs

The introduction of oral nucleos(t)ide analogs has substantially enhanced treatment options for patients with chronic hepatitis B (for more information, refer to chapter by De Clercq and Neyts, this volume). Currently, three nucleoside analogs (lamivudine 100 mg/day, entecavir 0.5–1 mg/day, telbivudine 600 mg/day) and two nucleotide analogs (adefovir dipivoxil 10 mg/day and tenofovir 245 mg/day) are licensed as antiviral therapy for chronic hepatitis B in many countries. In general, nucleos(t)ide analogs are marked by their excellent side effect profile. However, drawbacks of nucleos(t)ide therapy include the infinite duration of treatment in patients with HBe-Ag negative disease, different antiviral potencies and genetic barriers implicating the potential occurrence of drug resistance.

In HBeAg positive patients, nucleos(t)ide therapy may be discontinued 6–12 months after HBeAg seroconversion is achieved. Rates of HBeAg seroconversion have been shown to increase with longer duration of nucleos(t)ide therapy (e.g., ~20% and 40% after 1 and 3 years of lamivudine monotherapy, respectively) (Leung et al. 2001). However, HBeAg seroconversion rates during nucleos(t)ide therapy seems to be less durable as compared to those seen with interferon-α. HBeAg negative patients generally require an indefinite treatment duration, as HBV DNA levels and ALT levels rebound after therapy is discontinued, which is associated with progression of liver disease.

The efficiencies of the different nucleos(t)ide analogs to induce HBe seroconversion, suppression of HBV DNA, and normalization of ALT levels in patients with chronic hepatitis B after 48–52 weeks of treatment are shown in Table 1 (Chang et al. 2006; Dienstag et al. 1999; Hadziyannis et al. 2000, 2003; Lai et al. 1998, 2006a, b; Lau et al. 2000; Liaw et al. 2000; Marcellin et al. 2003; Tassopoulos et al. 1999).

Table 1 Efficiency of approved nucleos(t)ides as monotherapy in treatment naive patients with chronic hepatitis B according to HBeAg status

Substance	Class	Oral dosage per day	Efficiency at Week 48/52 HBeAg *positive* patients			Efficiency at Week 48/52 HBeAg *negative* patients	
			Loss of serum HBV DNA[a]	HbeAg sero-conversion	ALT nor-malization	Loss of serum HBV DNA[1]	ALT nor-malization
Lamivudine	Nucleoside	100 mg	40–44%	16–21%	41–75%	60–73%	60–79%
Adefovir	Nucleotide	10 mg	21%	12%	48%	51%	72%
Entecavir	Nucleoside	0.5–1.0 mg	67%	21%	77%	90%	78%
Telbivudine	Nucleoside	600 mg	60%	22%	68%	88%	74%
Tenofovir	Nucleotide	245 mg	76%	20%	69%	93%	77%

[a]HBV DNA was measured by hybridization or branched chain DNA assays (lower detection limit 20,000–200,000 IU/mL) and PCR assays (lower detection limit approximately 50 IU/mL). For references see text

1.3.3 Resistance to Nucleos(t)ide Analogs

The potential emergence of drug-resistant mutations represents a major drawback during treatment with nucleos(t)ide analogs (Zoulim 2006). In clinical practice, resistance is most easily detectable by a virologic breakthrough, which precedes an increase of ALT levels in the majority of cases. Virologic breakthrough is defined by an increase in serum HBV DNA by >1 log 10 above the nadir after achieving a virologic response. Biochemical breakthrough is defined by an increase in ALT above the upper limit of normal after achieving normalization during continued treatment. Genotypic resistance, which should be confirmed in clinically suspected cases, is defined by the detection of a mutation that has been shown to confer resistance to the drug by in vitro studies.

Drug resistance emerges most frequently during lamivudine therapy and to a lesser extent during treatment with adefovir, telbivudine, and rarely during entecavir treatment. For lamivudine, drug resistance rates of 14–32% after 1 year of treatment and 60–70% after 5 years of treatment have been reported. Adefovir drug resistance rates at 1, 2, 3, 4, and 5 years in HBeAg negative patients were 0.3%, 11%, 18%, and 29%, respectively (Lok and McMahon 2007). During telbivudine treatment, resistance defined as virologic breakthrough (1 log above nadir) with resistance mutations after 2 years of treatment in the GLOBE study was found in 21.6% of HBeAg-positive patients and in 8.6% of HBeAg-negative patients (Lai et al. 2006a). Resistance rates during entecavir treatment are generally low. Virologic breakthrough was observed in 3% of treatment-naive patients by week 96, only two individuals had new virologic breakthrough detectable by year 3, and none by year 4. However, in patients with preexisting lamivudine resistance, virologic breakthrough during entecavir treatment was detectable in 7% by week 48, in 16% by week 96, and in 15% by year 4 (Colonno et al. 2006, 2007). So far, no genotypic resistance has been observed during tenofovir treatment.

1.4 Combination Therapy in Chronic Hepatitis B

In the treatment of HIV and HCV infection, combination therapies have been proven to be more effective than monotherapies. In case of HBV infection, various combination therapies have been evaluated but none of them has shown to induce higher rates of sustained response as compared to monotherapy (Yuen and Lai 2007). Although several combination therapies were found to lower the rates of lamivudine resistance, data are lacking for nucleos(t)ide analogs that have an a priori low risk of drug resistance. Combination of a nucleos(t)ide analog with (peg)interferon-α seems to be a logical approach, as these two classes of substances have different modes of action. In the last years, interferons and nucleos(t)ide analogs were either administered to the patients simultaneously, staggered, and/or sequentially, all for a finite treatment duration.

1.4.1 (Peg)Interferon-α in Combination with Lamivudine

Several trials have been conducted to compare the combination of (peg)interferon-α and lamivudine with lamivudine and/or (peg)interferon-α alone (Chan et al. 2005; Janssen et al. 2005; Lau et al. 2005; Marcellin et al. 2004). In all studies, a greater suppression of on-treatment HBV DNA and higher rates of sustained virologic response after discontinuation of treatment were observed in the combination therapies as compared to lamivudine alone, but not to (peg)interferon-α alone. Overall, lower rates of lamivudine resistance were found in the combination therapies. However, no evidence for HBV drug resistance to (peg)interferon-α is currently known. One major limitation of the design of these studies is the definite treatment duration for lamivudine and (peg)interferon-α combination therapy, as it is well established today that nucleos(t)ide treatment should be considered as a long-term treatment. New approaches in combination therapy for chronic hepatitis B include the sequential administration of lamivudine and peginterferon-α. It is known that lower concentrations of pretreatment HBV DNA are associated with greater sustained response rates during peginterferon-α monotherapy. In two recent studies, the sustained virologic response rates have been shown to be increased when lamivudine was administered 4–20 weeks prior to initiation with peginterferon-α (Sarin et al. 2007; Serfaty et al. 2001). In another study, a staggered treatment regimen in HBeAg positive patients was applied. After peginterferon-α was given for 8 weeks, a combination of peginterferon-α and lamivudine was administered for 24 weeks, followed by lamivudine monotherapy for further 28 weeks (Chan et al. 2005). In the staggered regimen, higher rates of HBeAg seroconversion were observed as compared to lamivudine monotherapy. However, before recommendations for combination therapy can be made, larger studies are needed to evaluate the impact of different regimens of peginterferon-α in combination with nucleos(t)ide analogs on HBeAg seroconversion rates, sustained viral suppression, and overall clinical outcome.

1.4.2 Antiviral Combination Therapy with Lamivudine and Other Nucleos(t)ide Analogs

So far, a limited number of trials have been conducted comparing treatment response of lamivudine monotherapy with regimens consisting of lamivudine and a second nucleos(t)ide analog. For the combination of lamivudine and adefovir, no beneficial effect on sustained virologic response rates has been found in comparison to lamivudine monotherapy (Sung et al. 2003). In another trial, the combination of lamivudine and telbivudine has been compared with both lamivudine and telbivudine monotherapy (Lai et al. 2005). Combination of the two nucleosides was inferior in all parameters as compared to telbivudine monotherapy. Taken together, currently available data suggest that the combination of two nucleos(t)ide analogs cannot be recommended as initial treatment for chronic hepatitis B. However, in the case of lamivudine resistance, the addition of adefovir to the ongoing therapy,

Table 2 Clinical management of antiviral resistance in hepatitis B (modified according to Lok and McMahon (2007))

Lamivudine resistance	Add adefovir or tenofovir
Adefovir resistance	Add lamivudine or telbivudine
Entecavir resistance	Switch to or add adefovir or tenofovir
Telbivudine resistance	Add adefovir or tenofovir

rather than a switch, has become a recommended treatment strategy mainly to prevent hepatitis flares that have been observed during the transition period from one drug to another and to reduce the risk of drug resistance to adefovir. Furthermore, adefovir should be added as early as genotypic resistance becomes evident (Fung et al. 2006; Lampertico et al. 2005). An exclusion for the add-on strategy represents treatment of lamivudine resistant hepatitis B with entecavir, as it has been shown that the presence of lamivudine-resistant variants is associated with increased rates of viral breakthrough during entecavir treatment (Colonno et al. 2006). Current recommendations for resistant hepatitis B treatment are shown in Table 2.

2 Combination Therapy in Chronic Hepatitis C

2.1 Combination Therapy with Ribavirin and Development of Pegylated Interferons

Ribavirin is a guanosine analog synthesized more than 35 years ago, which possesses broad-spectrum antiviral activity against several RNA and DNA viruses in vitro (Sidwell et al. 1972). When administered as monotherapy in patients with chronic hepatitis C, ribavirin induces a decline of serum alanine aminotransferase (ALT) levels while no effect on sustained virologic response is detectable (Di Bisceglie et al. 1992).

Although the mechanisms of action are not entirely understood, the addition of ribavirin to interferon-α in the late 1990s has been found to substantially improve treatment outcomes, with mean sustained virologic response rates of 41% (Brillanti et al. 1994; McHutchison et al. 1998; Poynard et al. 1998). However, differences between genotype 1 and genotype 2, 3-infected patients remained evident (Fig. 1). Sustained virologic response rates for patients with genotype 1 were 28–36% compared to 61–79% for patients with genotype 2 and 3 infection (Fried et al. 2002; Manns et al. 2001; McHutchison et al. 1998; Poynard et al. 1998). The reasons for the markedly different virologic response rates between genotype 1 on the one hand and genotype 2,3-infected patients on the other hand are not known. Several HCV proteins (core, envelope (E) 2, non structural (NS) 3, NS5A) have been associated with interferon-α resistance mechanisms in vitro, and extensive sequencing studies of the respective HCV genes isolated from patients showed a potential

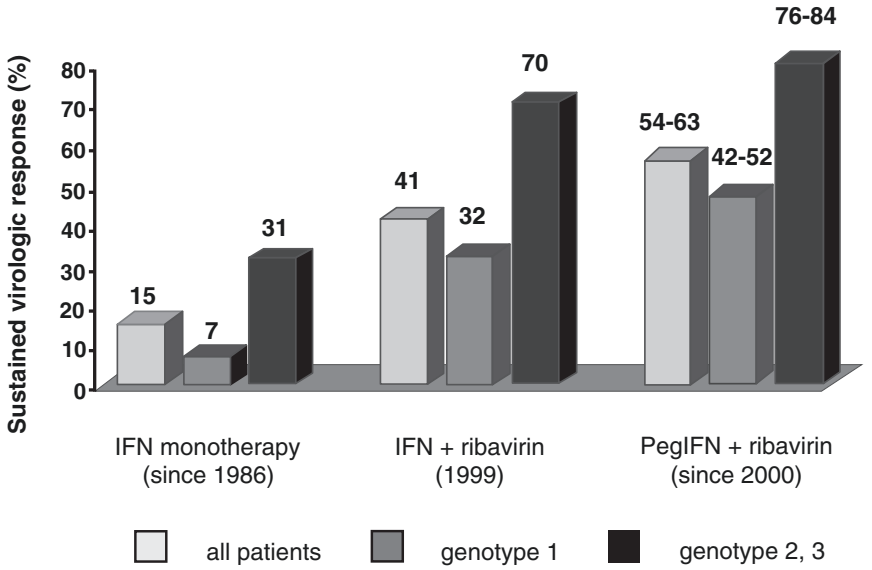

Fig. 1 Sustained virologic response rates from pivotal trials in patients with chronic hepatitis C according to treatment regimen and HCV genotype. For references see text

importance of amino acid variations within the NS5A protein of patients with HCV genotype 1 infection, which correlated with sensitivity to interferon-α-based therapy (Wohnsland et al. 2007).

The development of pegylated (peg) interferons that possess a sustained absorption, a slower rate of clearance, and a longer half life than unmodified interferons led to further improvement of sustained virologic response rates especially for patients infected with genotype 1 (Fig. 1) (Fried et al. 2002; Manns et al. 2001; Zeuzem et al. 2000).

At present, two types of peginterferon-α are approved for the treatment of chronic hepatitis C: peginterferon-α2a and peginterferon-α2b, which differ in size and form of the linked polyethylene glycol molecule (40 vs. 12 kDa). Both peginterferons are injected subcutaneously once a week (see chapter by Chevaliez and Pawlotsky, this volume, for further details).

2.2 Current Combination Therapy in HCV Genotype 1 and 4–6

Patients infected with HCV genotype 1, 4, 5 or 6 are treated with peginterferon-α in combination with ribavirin for 48 weeks (Table 3). In the pivotal trials, sustained virologic response rates achieved with patients infected with these genotypes were 46–51% with peginterferon-α2a and ribavirin and 42–48% with peginterferon-α2b and ribavirin. Different baseline parameters (female gender, age <40 years, low body

Table 3 Standard combination therapy for chronic hepatitis C

Genotype[a]	Dose of peginterferon	Dose of ribavirin	Duration (weeks)	Early discontinuation or prolongation of therapy
1	Peginterferon-α2a 180 μg weekly, or peginterferon-α2b, 1.5 μg/kg weekly	1,000 mg or 1,200 mg daily, according to body weight ($\leq$ 75 kg or $>$75 kg)	48	*Rapid response*: discontinue at week 24 if baseline HCV RNA level $<$600.000 IU/mL and HCV RNA undetectable by week 4 *Slow response*: consider prolongation to 72 weeks of treatment if HCV RNA is positive by week 12 but negative by week 24 *Nonresponse*: discontinue at week 12 if $<$2 log 10 IU/mL decline in HCV RNA levels or at week 24 if HCV RNA is still detectable
2 or 3	Peginterferon–α2a, or peginterferon-α2b, doses as above	800 mg daily as fixed dose	24	*Rapid response*: discontinue at week 12–16 if HCV RNA is $<$400,000 IU/ml at baseline and undetectable by week 4 *Slow response*: consider treatment prolongation to 48 weeks *Nonresponse*: unusual

[a]HCV genotypes 4, 5, and 6 infection should be treated according to the regimen recommended for HCV genotype 1 infected patients. For references see text

weight, low degree of fibrosis, HCV RNA serum concentration $<6–8 \times 10^5$ IU/ml) significantly correlated with the probability of sustained virologic response. However, even in individuals with favorable baseline characteristics, reduction of therapy duration from 48 to 24 weeks was associated with significantly lower sustained virologic response rates. Only a subset of individuals who have low baseline HCV RNA levels ($<$600,000 IU/mL) and in whom HCV RNA is undetectable early during therapy (week 4) can be discontinued from treatment by week 24 without a significant loss of virologic response (Zeuzem et al. 2006). Treatment outcome of patients with high baseline viral load is still poor. In one trial with a limited number of patients, high doses of ribavirin (mean dose 2,540 mg/day) combined with standard doses of peginterferon alfa-2a were shown to result in sustained virologic response in 9 out of 10 patients (Lindahl et al. 2005). The mechanisms by which ribavirin contributes to improved sustained virologic response rates have not been clarified. Accumulating evidence exists that ribavirin may act as an RNA virus mutagen, thus

driving HCV into error catastrophe (Asahina et al. 2005; Hofmann et al. 2007). However, if that is the case, it remains unclear why ribavirin monotherapy does not affect viremia.

Only few smaller studies have investigated the treatment of patients infected with HCV genotypes 4, 5, and 6 (Diago et al. 2004; Yuen and Lai 2006). The data suggest that these patients should be treated like patients infected with HCV genotype 1 (Hoofnagle and Seeff 2006). Thus, treatment duration and the administered dose of ribavirin have great influence on treatment outcome. A shortening of the treatment duration to 24 weeks or a reduction of the ribavirin dose was reported to lower the sustained virologic response rates.

For early prediction of the outcome of virologic response in HCV genotype 1 patients, the initial decline of serum HCV RNA concentration should be considered. Thus, identification of patients with non-sustained virologic response is possible at an early stage of therapy (Berg et al. 2003; Davis et al. 2003). Treatment should be discontinued in patients who do not achieve a 2 log10 HCV RNA decay, or in whom residual serum HCV RNA $>30,000\,\mathrm{IU/mL}$ by week 12 is still detectable (negative prediction value 98–100%). For the remaining patients, HCV RNA should be assessed after 24 weeks of therapy on the basis of a highly sensitive (qualitative) HCV RNA assay (detection limit 10–50 IU/ml). If HCV RNA is detectable by week 24, treatment should be discontinued, as there is virtually no chance to achieve a sustained virologic response (negative prediction value 98–100%).

2.3 Current Combination Therapy in HCV Genotype 2 and 3

In the first pivotal trials using peginterferon-α in combination with ribavirin, treatment was given for 48 weeks for all HCV genotypes. Patients with genotype 2 and 3 infection treated with peginterferon-α2b or -α2a plus ribavirin achieved sustained virologic response rates of 82% and 76%, respectively, confirming the favorable results achieved by standard interferon and ribavirin combination therapy. Subsequent studies therefore investigated the possibility of shortening therapy to 24 weeks for patients with genotype 2 and 3 infection without compromising the antiviral efficacy. It was shown that similar sustained virologic response rates (78–81%) were achieved in patients treated for 24 weeks compared to those treated for 48 weeks (Hadziyannis et al. 2004; Zeuzem et al. 2004). As expected, treatment-associated adverse events leading to treatment discontinuation or dose reduction occurred at substantially lower rates during the shortened treatment course. Consequently, 24 weeks of treatment has been established as the standard care for genotypes 2 and 3 infection (Table 3). In patients infected with genotype 2 or 3 treated for 24 weeks, assessment of HCV RNA by week 12 is not necessary, since the overall virologic response rates are very high.

Further reduction of treatment duration to gain even better tolerability and lower side effects without compromising the sustained virologic response rates was

recently investigated in four clinical trials (Dalgard et al. 2004; Mangia et al. 2005; von Wagner et al. 2005; Yu et al. 2007). In these studies, reduction of treatment duration to 12–16 weeks in patients with an rapid virologic response defined as undetectable HCV RNA ($<$50–600 IU/ml) by week 4 was associated with similar sustained virologic response rates (82–100%) as compared to the standard treatment duration of 24 weeks (80–98%). However, in the largest prospective study performed in this population, including 1,469 treatment-naive patients with HCV genotypes 2 and 3 (ACCELERATE study), the overall sustained virologic response rates were significantly greater after 24 weeks rather than after 16 weeks of treatment (76% vs. 65%). This difference could be attributed to a higher relapse rate with 16 weeks of treatment (Shiffman et al. 2007). However, subgroup analyses suggest that shortening of treatment courses may be possible in those patients who achieve a rapid virologic response by week 4.

2.4 Emerging Strategies in Antiviral Combination Therapy

Detailed sub-analyses of a variety of clinical trials have provided information about host and viral factors influencing the virologic response in the treatment of chronic hepatitis C. The most important factors include the HCV genotype, HCV RNA concentration at baseline, age, weight, gender, ethnicity, liver enzymes, and stage of fibrosis (Mihm et al. 2006; Pawlotsky 2005).

To further improve sustained virologic response rates, different treatment approaches are currently under investigation. For example, individualized therapy durations on the basis of the HCV RNA concentration at baseline and early during therapy are the subject of clinical studies (Berg et al. 2006; Zeuzem et al. 2005a). In addition, triple therapy with other antiviral compounds, such as amantadine, has been evaluated in multiple studies, leading to contradictory results (Mangia et al. 2004).

Recently, multiple new compounds with different modes of action are being studied in ongoing preclinical and clinical trials (e.g., long acting interferons, Toll-like receptor agonists, therapeutic vaccination strategies, selective antivirals targeting viral proteins NS3/4A, NS5A, or NS5B, cyclophilin inhibitors, IRES inhibitors, replicase inhibitors, antisense compounds, etc.).

Most promising, results of phase I/phase II clinical trials with several selective antiviral drugs have recently been published or have been presented at international meetings. These antiviral agents are directed against the HCV NS3/4A serine protease and the HCV NS5B RNA dependent RNA polymerase. So far, early clinical phase I/II study results for protease inhibitors (e.g. BILN2061, telaprevir (formerly VX-950), and boceprevir (SCH503034)) as well as polymerase inhibitors (e.g. valopicitabine (formerly NM283), R1626, and HCV-796) are available (Afdhal et al. 2004; Hinrichsen et al. 2004; Reesink et al. 2005; Reiser et al. 2005; Zeuzem et al. 2005b). Table 4 gives an overview of selective antiviral compounds that are

Table 4 Specifically targeted antiviral therapy for hepatitis C (Stat-C) currently in clinical development

Compound	Company	Study phase
I. NS3/4 serine protease inhibitors		
Ciluprevir (BILN 2061)	Boehringer Ingelheim	Stopped
Telaprevir (VX950)	Vertex/Tibotec	Phase 2
Boceprevir (SCH 503034)	Schering Plough	Phase 2
GS9132/ACH 806	Gilead Sciences/Achillion	Stopped
ITMN191	InterMune/Roche	Phase 1
II. NS5B RNA-dependent RNA polymerase inhibitors		
Nucleoside inhibitors		
Valopicitabine (NM283)	Idenix/Novartis	Stopped
R1626 (prodrug of R1479)	Roche	Phase 2
Non-nucleoside inhibitor		
HCV 796	ViroPharm/Wyeth	Phase 2
III. NS5A inhibitors		
A831	Arrow Therapeutics/Astra Zeneca	Phase 1
IV. Cyclophilin inhibitors		
DEBIO-25	Debiopharm	Phase 1
NIM 811	Novartis Pharma	Preclinical

currently under investigation as monotherapy or in combination with peginterferon-α and/or ribavirin. So far, none of these compounds has been established as a standard treatment for hepatitis C.

Because of the high error rate of the NS5B RNA polymerase, the selection of isolates that are resistant to compounds targeting the NS3/4A protease or the NS5B polymerase must be considered as a major limitation for these therapies in patients with chronic hepatitis C. Whether the combination of selective antiviral drugs as well as combination therapy with peginterferon-α will prevent or at least delay the development of resistance remains to be clarified in future studies. In general, it seems clear that the treatment of HCV infection with direct antivirals is associated with a rapid development of drug resistance comparable to that seen in the treatment of human immunodeficiency virus (HIV) infection.

2.4.1 HCV NS3/4A Protease Inhibitors in Combination with Interferons

In patients with chronic HCV genotype 1 infection, a short-term monotherapy with the protease inhibitors BILN2061 (2 days), boceprevir, and telaprevir (14 days) led to a decrease in HCV RNA concentrations between 1.5 and 4 log10 HCV RNA IU/mL. BILN2061 and telaprevir showed particularly high antiviral efficacy, resulting in suppression of HCV RNA levels below the detection limit of a highly sensitive assay ($<10\,\mathrm{IU/ml}$) in several patients. However, after the end of dosing, HCV RNA levels increased to concentrations at baseline in all patients. Recently, first

results of clinical trials comparing combination therapies of boceprevir or telaprevir with peginterferon-α were reported (Kieffer et al. 2006; Sarrazin et al. 2007). Overall, combination of NS3 protease inhibitors with peginterferon-α led to a stronger decline of HCV RNA load as compared to the decline seen in protease inhibitor monotherapy.

The development of resistance against HCV NS3/4 protease inhibitors will become a major challenge for the clinical use of these new compounds. Clinical trials of telaprevir (VX-950) have shown that mutations at different positions are rapidly selected (Sarrazin et al. 2005). In vitro studies indicate that cells bearing replicons with those mutations are associated with different levels of resistance to telaprevir (<10-fold change to >40-fold change in sensitivity). However, telaprevir-resistant mutants remain susceptible to interferon-α, at least in the replicon system. Likewise, replicon mutants that are resistant to boceprevir are still sensitive to interferon-α (Tong et al. 2006).

2.4.2 HCV NS5B Polymerase Inhibitors in Combination with Interferons

Results of phase I/phase II studies with the NS5B polymerase inhibitor valopicitabine as a monotherapy showed a decrease of HCV RNA concentrations of approx. 1 log10 HCV RNA IU/ml in treatment-naive patients or interferon-α nonresponders. Interim analysis of ongoing studies showed improved antiviral efficacy for combination therapy with valopicitabine and peginterferon-α. Valopicitabine (NM283) is the oral prodrug of 2′-*C*-methyl-cytidine (NM107) and is cleaved to the free nucleoside that is converted to the active triphosphate by the cellular machinery. No in vivo resistance data for Valopicitabine are available yet, but it was shown that the 2′-*C*-methyl-nucleoside NM107 is susceptible to resistance development in vitro (Bichko et al. 2005). However, due to safety issues, further development of valopicitabine was stooed recently.

R1479 (4′-azidocytidine) is a potent inhibitor of the NS5B polymerase and efficiently blocks HCV replication in cell culture. R1626 is a prodrug of R1479. A multiple dose ascending study was designed to evaluate the antiviral activity of R1626 in previously untreated patients with chronic hepatitis C genotype 1 infection. A dose-dependent median reduction of HCV RNA was observed ranging from 0.8 log10 to 4.1 log10 IU/ml.

HCV-796 is a non-nucleosidic NS5B polymerase inhibitor with potent antiviral activity in vitro. A phase 1b study was performed to determine the antiviral activity, pharmacokinetics, and safety of HCV-796 in patients with chronic HCV infection. Maximum antiviral effects were achieved after 4 days of treatment with a mean reduction of HCV-RNA of 1.4 log10 IU/ml. Combination of HCV-796 with pegylated interferon-α led to a greater reduction of viral RNA load (3.3–3.5 log10 IU/ml) after a 14 days treatment interval.

3 Antiretroviral Therapy

3.1 The Goal of Antiretroviral Therapy

The first drug to be found active against HIV was zidovudine (AZT). However, its efficacy was low and hampered in the short-mid term by the development of drug resistance. It took a decade to discover the "magic" of combination therapy against HIV, mainly thanks to the approval of the first protease inhibitors (PI) in 1996. When any given PI is administered in combination with two nucleoside analogs, most patients reach undetectable viremia and experience dramatic gains of CD4+ counts. As many more antiretroviral drugs have become available since then (there are nearly 25 at the moment), the goal of antiretroviral therapy has clearly been established in the achievement of plasma HIV-RNA load <50 copies/ml (Hammer et al. 2006). In this setting, drug resistance does not develop and almost all patients experience immune recovery and lack of clinical progression. In other words, keeping patients with undetectable viremia under HAART, HIV is no longer a deadly disease and may resemble a chronic condition such as diabetes or hypertension. The mode of transmission (sexual and blood-borne) remains the most important difference to other chronic conditions, since it may represent a threat for others.

HIV can not be eradicated, which means that once HAART has been initiated it will never be stopped. Antiretroviral therapy can control but not eradicate HIV infection, because the virus integrates into the host cell genome (the "provirus") and thus becomes a "cellular gene." Since treatment may cause side effects and reduce the quality of life of patients, it is currently advised to delay the initiation of HAART until the CD4+ count is below 350 cells/mm^3 (Hammer et al. 2006). Using triple drug combination therapy, almost all compliant patients achieve undetectable viremia within 12 weeks and experience a significant gain of CD4+ count. However, viral elimination is not feasible and therefore once treatment is stopped for any reason, viral rebound occurs and loss of CD4+ T lymphocytes is resumed. Planned treatment interruptions have been explored in patients on successful HAART for many years. However, recent study results were discouraging as treatment interruption has been shown to be associated with poorer clinical outcome (El-Sadr et al. 2006).

3.2 Classification of Antiretroviral Drugs

There are currently six major antiretroviral drug families (Table 5). Nucleoside reverse transcriptase inhibitors (NRTI) are nucleoside analogs (discussed in more detail in chapter by De Clercq and Neyts, this volume) and were the first approved antiretroviral agents. They include drugs such as AZT, didanosine (ddI), stavudine (d4T), abacavir (ABC), and lamivudine (3TC), the latest used at doses of 300 mg daily as anti-HIV agent (100 mg/day is the dosing approved for treatment of HBV

Table 5 Classification of antiretroviral drugs for the treatment of HIV infection

RT inhibitors		Protease inhibitors	Integrase inhibitors	Entry inhibitors		Others
NRTI	NNRTI			Fusion inhibitors	Co-receptor antagonists	
Zidovudine	Nevirapine	Saquinavir	Raltegravir	Enfuvirtide	Maraviroc	Bevirimat
Didanosine	Efavirenz	Indinavir	Elvitegravir		Vicriviroc	
Stavudine	TMC125	Lopinavir				
Lamivudine		Fosamprenavir				
Abacavir		Atazanavir				
Tenofovir		Tipranavir				
Emtricitabine		Darunavir				

RT reverse transcriptase, *NRTI* nucleoside reverse transcriptase inhibitors, *NNRTI* Non-nucleoside reverse transcriptase inhibitors

infection). Five years ago, the first nucleotide analog, tenofovir (TDF), was approved as anti-HIV agent, but also shows anti-HBV activity (see above). All these drugs act as chain-terminators, competing with the natural nucleosides and blocking the elongation of the cDNA chain synthesis carried out by the HIV-1 reverse transcriptase (RT), which utilizes the viral RNA as template. All NRTI should undergo phosphorylation within the target cells to be converted into its active form.

The combination of two NRTI has been the most recommended backbone of triple drug regimens so far. However, not all combinations of NRTI are advised. The two thymidine analogs, AZT and d4T, cannot be taken together since they compete intracellularly for enzymes required for phosphorylation, thus leading to partial antagonism. Moreover, ddI and TDF should not be taken together since both are adenosine analogs and share intracellular metabolic pathways, potentially resulting in increased risk of toxicities, including loss of CD4+ count, pancreatitis, hyperglycemia, and lactic acidosis (Barreiro et al. 2005).

Non-nucleoside reverse transcriptase inhibitors (NNRTI) act by blocking the enzymatic activity of the viral RT after inducing a conformational change of the catalytic site upon binding to a nearby position (see chapter by Zimmermann et al., this volume). The two most important agents within this family are nevirapine and efavirenz. They are given as only one (efavirenz) or two (nevirapine) pills daily, and therefore are very easy to take. Unfortunately, resistance to these agents develops rapidly (<4 weeks) when provided without other active drugs or in patients with low adherence to therapy. In all these situations, patients are unlikely to reach complete suppression of virus replication, which is the best way to prevent selection of drug resistance (Havlir and Richman 1996).

Protease inhibitors (PIs) inhibit the maturation of the virus, through blocking the enzymatic activity of the viral protease. Defective viruses are released, which are unable to establish a productive viral infection. Given that almost all PIs are metabolized in the liver by cytochrome p450, the majority (all but nelfinavir) are generally given with low-doses of ritonavir, which blocks their metabolism and therefore acts as a pharmacokinetic enhancer. In general, ritonavir-boosted PIs are amongst the

Table 6 Recommended antiretroviral regimens as initial HIV therapy according to the IAS-USA panel (Hammer et al. 2006)

2 NRTI	3rd drug
Tenofovir + Emtricitabine	Efavirenz or Nevirapine
Zidovudine + Lamivudine	
Abacavir + Lamivudine	Lopinavir/r or Atazanavir/r or Fosamprenavir/r or Saquinavir/r

most potent antiretroviral agents, with a high genetic barrier for drug resistance. They lead to $\sim$2 log 10 reductions in plasma HIV-RNA and generally more than five protease resistance mutations are needed to compromise their antiviral activity (de Mendoza et al. 2006). The latest two approved PIs, named tipranavir (TPV) and darunavir (DRV), are the most potent drugs within this family (Clotet et al. 2007; de Mendoza et al. 2007). Current guidelines recommend to initiate antiretroviral therapy with two nucleoside analogs plus either one NNRTI or a ritonavir-boosted PI (Table 6) (Hammer et al. 2006). However, given the robustness of PIs, most experts prefer to use them over NNRTI in patients with very advanced HIV disease, such as those presenting for the first time with very low CD4+ counts and high viral load (Barreiro et al. 2002). Chapter by Anderson et al, this volume, provides more details about the class of protease inhibitors.

Integrase inhibitors (INI) are a new family of antiretroviral drugs, currently represented by two compounds, raltegravir and elvitegravir (Garcia-Gasco et al. 2005). Raltegravir acts as specific strand-transfer inhibitor and is characterized by an improved pharmacokinetic profile (Embrey et al. 2005). In October 2007, raltegravir was approved by the U.S. Food and Drug Administration (FDA) for the treatment of HIV-1 as part of combination antiretroviral therapy in treatment-experienced patients (see chapter by Anderson et al., this volume, more details).

Two classes of entry inhibitors have been developed. The first entry inhibitor approved as HIV therapy was enfuvirtide, a fusion inhibitor. In contrast with all other antiretrovirals, this drug must be administered subcutaneously and twice a day, which represent important disadvantages to the patient. It is very potent and generally reserved for heavily antiretroviral-experienced patients with virologic failure. Unfortunately enfuvirtide shows a low genetic barrier for resistance (Fig. 2) and should be administered in combination with at least one other active drug.

The second group of antiretroviral drugs belonging to the family of entry inhibitors are the coreceptor antagonists. HIV enters target cells using the CD4 molecule expressed over the membrane surface, which acts as its main receptor. A second binding of the virus is required to enter cells, and is provided by some chemokine receptors, named CCR5 and CXCR4. Given that HIV strains use preferentially one or the other, viruses are split into two categories, R5 and X4. While R5 viruses predominate in all patients during the first years of infection, X4 viruses tend to be selected as immunodeficiency progresses and nearly half of patients with AIDS show X4 strains. Two CCR5 antagonists are in the last steps of clinical

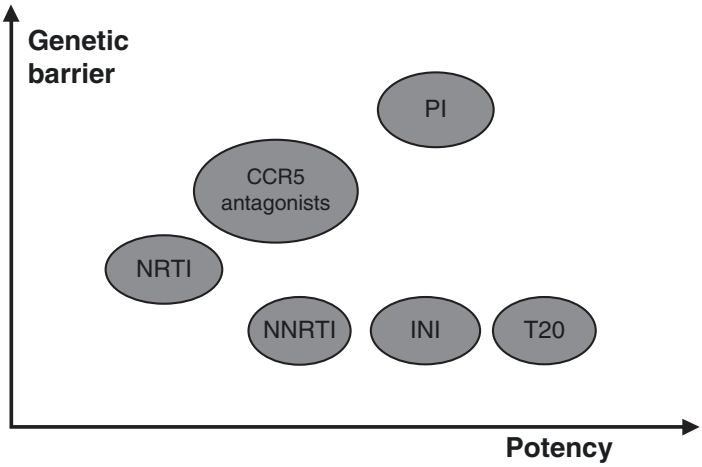

Fig. 2 Potency and genetic barrier for resistance according to different antiretroviral drug families

development, Maraviroc and Vicriviroc. They have been recently approved for use only in antiretroviral-experienced patients, in whom unfortunately X4 viruses are more prevalent and these compounds cannot be used. Of note, testing for HIV tropism must be performed before prescribing these drugs, and patients with X4 viruses should not be treated with CCR5 antagonists (Poveda et al. 2006). The MOTIVATE trials have shown that Maraviroc is a potent and relatively well tolerated drug, which in combination with other antiretroviral agents permits to regain undetectable viremia in a significant proportion of heavily treatment-experienced patients with R5 viruses. For more information about entry inhibitors, refer to chapter by Melby and Westb.

3.3 Antiretroviral Drug Resistance

As is the case with HBV and HCV, antiviral therapy against HIV is characterized by the development of drug resistance due to the large genetic variability of HIV in each infected person (see chapter by Nijhuis et al., this volume, for more information). Resistance mutations preexist for almost all antiviral agents before treatment is initiated. In fact, selection of resistance from preexisting viral variants is more common than new generation of mutant viruses under therapy. However, resistance mutations to multiple compounds are only rarely seen within the same viral genome, at least in drug-naïve individuals. Therefore, prescription of triple drug therapy is almost always capable of completely suppressing HIV replication. In such a scenario, it should be highlighted that virological failure as a consequence of evolving drug resistance can only occur when treatment compliance is poor and the virus is exposed to incomplete drug pressure (Havlir and Richman 1996). However, once

drug resistance has emerged, keeping the same drug regimen may allow the virus to accumulate more drug resistance mutations, which frequently results in broader cross-resistance at least to agents of the same drug family. In addition, impairment of viral fitness frequently imposed by drug resistance mutations is often compensated by second site mutations, thus leading to resistant viruses that are fully replication competent. Therefore, antiretroviral regimens should be modified as soon as possible once virological failure is recognized.

The mechanisms of resistance to antiretroviral drugs vary according to agents (Clavel and Hance 2004) and are described in more detail in Chaps. 4, 6, 7, and 11 in this volume. Resistance to NRTI can occur due to reduced affinity for the drug thus favoring binding of physiological nucleosides or due to mutations that favor the removal (excision) of the last incorporated nucleoside analog from the growing cDNA chain (Johnson et al. 2006). Resistance to NNRTI rapidly develops as a consequence of mutations that lead to conformational changes in the RT pocket where the drugs bind (Johnson et al. 2006). Given that almost all currently available protease inhibitors are boosted with low-dose ritonavir (generally 100 mg/12h), resistance to these agents generally requires the accumulation of multiple mutations in the protease gene (de Mendoza and Soriano 2004). In clinical practice, selection of an individual PI often is based on considerations such as toxicity profile, opportunity for once daily dosing, and expected potency. Resistance to INI develops rapidly as a consequence of drug exposure in the absence of complete virus suppression. In this way, these drugs share a low genetic barrier for resistance with the family of NNRTI. Moreover, broad cross-resistance exists between raltegravir and elvitegravir (Garcia-Gasco et al. 2005). Resistance to enfuvirtide, the only approved fusion inhibitor today, develops as a consequence of mutations in the gp41 envelope gene. Although the drug is very potent, a single change may abolish its inhibitory activity. Interestingly, CD4+ cell gains may persist in some cases even in the absence of apparent antiviral activity (Poveda et al. 2004). Finally, resistance to CCR5 antagonists may result from two mechanisms. On the one hand, a shift to use the alternative CXCR4 coreceptor to entry into cells; on the other hand, viral escape as a result of selection of mutations within the gp120 envelope gene, which allows the virus to resume its interaction with CCR5 (Poveda et al. 2006).

Wide and prolonged use of antiretroviral drugs has resulted in the emergence of drug-resistant viruses that can be transmitted. Transmission of drug-resistant HIV currently occurs in around 15% of new infections in developed countries (de Mendoza et al. 2005a, b). Therefore, drug resistance testing is currently recommended prior to initiation of antiretroviral therapy. As exposure has been limited so far to NRTI, NNRTI, and PI, drug resistance to these agents should be excluded before recommending their use. For the future, as new drug families are introduced and begin to be widely used, resistance testing should expanded to exclude resistance mutations to all these new compounds as well.

3.4 Side Effects of Antiretroviral Therapy

The dramatic decrease in the morbidity and mortality of HIV-infected individuals in the last decade, due to the wide use of HAART, has been somewhat tempered by the emergence of mid-long term toxicities. A characteristic body fat redistribution and metabolic changes, including dyslipidemia and insulin resistance, are amongst the most prevalent and worrisome consequences (Carr et al. 2003). As HIV-infected individuals have increasing life expectancies, the risk for cardiovascular complications has emerged as an important cause of morbidity and mortality and preventive measures should be considered to minimize their impact (Weber et al. 2006).

Long-term antiretroviral therapy is also associated with fatigue and low drug adherence, which in turn may result in treatment failure. For this reason, simplification strategies aimed to reduce pill burden, daily number of pill intakes, and to minimize side effects are increasingly pursued. Moreover, pharmaceutical companies are pushed to produce fixed drug co-formulations. This is the case for Trizivir®, which combines in a pill AZT, lamivudine, and abacavir, which is taken twice a day; or Truvada®, which combines TDF and emtricitabine in a single pill, which is provided once a day. More recently, a further step has been taken merging in a single pill Truvada® with efavirenz (Atripla®), which represents the first HAART regimen given as only one single daily pill.

3.5 Antiretroviral Combination Therapy for Drug-Naive and for Treatment-Experienced Patients

With so many antiretroviral drugs to choose, individualization of therapy is becoming the rule. Several tools are currently used to identify the most convenient combination drug regimen for a given patient (Table 7). Besides CD4+ counts and viral load, other tools such as drug resistance testing, viral tropism, plasma drug levels, and pharmacogenomics are increasingly used to provide further insights about how best to tailor therapy for a given patient (Rodriguez-Novoa et al. 2006).

The priorities in antiretroviral therapy may change according to specific characteristics of the patient's virus population. Primarily, therapy should be potent enough to maximize the chances of achieving undetectable viremia; thereafter, other consid-

Table 7 Diagnostic tools used for monitoring and optimizing antiretroviral therapy

CD4+ cell counts
Plasma HIV RNA
Genotypic and phenotypic drug resistance testing
Plasma drug levels
Pharmacogenomics
Immune activation markers
Viral tropism

erations become relevant, including tolerance and convenience. This must be pursued in both drug-naïve and antiretroviral-experienced patients, although choices are limited in the latter group of patients. To ensure maximal activity of prescribed drugs, drug resistance testing should be performed before initiating antiretroviral therapy. While in drug-naïve individuals resistance testing may identify subjects who have acquired a drug-resistant virus, in antiretroviral-experienced patients, the knowledge of the resistance profile is required to devise rescue intervention. Of course, this is not enough to assure success, which also depends on the convenience of the prescribed drug regimen. In other words, prescription of potent, but poorly tolerated regimens should be avoided. In general, cross-resistance exists and should be presumed for most compounds within the same drug family but not for the rest.

3.6 Antiretroviral Therapy in Special Clinical Settings

Prevention of HIV transmission in specific situations is yet another aim of antiretroviral therapy. This is the case for (1) HIV-infected pregnant women before delivery, (2) HIV-serodiscordant couples who wish to have children by natural sexual intercourse (Barreiro et al. 2006), and (3) post-exposure prophylaxis. As for patients in whom HAART is provided because of low CD4+ counts, vertical, sexual, and parenteral transmission of HIV is very efficiently minimized when viral load becomes undetectable using HAART for short periods of time. In the case of mothers infected with HIV, single dose nevirapine was shown to be effective to diminish to less than 5% transmission of HIV to the offspring. Triple drug therapy is recommended to be provided for 6 weeks following accidental inoculations of contaminated blood or other fluids to personnel working in health care units, or following high-risk sexual behavior with an infected person.

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Socio-Economic Impact of Antiviral Intervention

S. Fleßa and P. Marschall

Contents

Abstract In this paper we present a meta-analysis of the Cost-of-Illness of HIV/AIDS and the socio-economic impact of antiretroviral therapy. We distinguish between provider costs, direct household costs, and indirect costs. There is a growing number of publications on provider costs in different countries, but the methodology and the degree of precision between these papers make it difficult to give a good estimate of the current provider costs of treating HIV/AIDS cases. There seems to be a declining interest in health economic analysis of HIV/AIDS, and usually data is rather obsolete at the date of publication. In addition, we know hardly anything about household costs not covered by health insurances (e.g., transport to the provider, special diet). There are more studies on indirect costs, but even these studies are difficult to compare due to methodological differences.

Even under these conditions we can state that in highly developed countries HAART is cost-effective. Because of an increase of life expectancy, the life-time provider costs increase under this drug regime. But, on the contrary, the indirect

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H.-G. Kräusslich, R. Bartenschlager (eds.), *Antiviral Strategies,* Handbook of Experimental Pharmacology 189,

costs strongly decrease. In particular for employed and young HIV/AIDS cases in the USA and in Europe, HAART is an investment that pays back. In countries and in compartments of the population (e.g., unemployed, pensioners) where the loss of labor can be neglected, the positive effects of HAART on the indirect costs do not necessarily justify its costs.

The cost-effectiveness analysis of antiviral therapy has to be seen under the precondition that no long-term effects, such as drug resistance, occur. Future analysis might show that we strongly underestimated the long-term costs of HIV/AIDS.

Abbreviations

COI	Cost-of-Illness
FC	Friction cost approach
GNP	Gross national product
HAART	Highly active antiretroviral therapy
HC	Human capital approach
QALY	Quality-adjusted life years
WP	Willingness-to-pay approach

The proceeding chapters give witness of an overwhelming scientific development. Antiviral intervention has become a standard, and the worldwide availability of this innovation is perceived as a humanitarian matter of course with a value of its own. However, such an important therapy progress has to be seen in competition with other allocations of scarce funds. Health care resources are limited – in one country more than in another, but in principle funds invested to antiretroviral intervention will not be available for prevention of HIV/AIDS or for the cure of other diseases. This fundamental scarcity calls for a rational utilization of existing resources and a scientific calculation of the socio-economic impact of antiretroviral intervention.

This chapter adds the socio-economic dimension to the medical or technical perspectives of the proceeding contributions of this book. As its health economic terminology and approach might be unfamiliar to some readers, we start with a section on methodology. In particular, we present an overview of the concept of "Cost-of-Illness" (COI) and of relevant health economic evaluation techniques. In Sect. 2, we present the basic findings of a meta-analysis of the socio-economic costs of HIV/AIDS and of the socio-economic impact of antiviral intervention. The major findings are reflected in Sect. 3. The chapter closes with a speculation on long-term socio-economic costs of antiviral intervention.

1 Methodology

The studies presented in the Sect. 2 of this paper give a comprehensive picture of the Cost-of-Illness (COI) of HIV/AIDS. In a broad sense, these COI are the difference

of the economic potential of a population without and with HIV/AIDS. The knowledge of these costs is in particular important for calculating the efficiency of antiretroviral therapy by comparing the COI with and without these interventions.

1.1 Concept of Cost-of-Illness

The concept of COI was first developed by Rice (Rice 1966, 1967; Rice and Cooper 1967; Rice et al. 1985). It assumes that resources could be used for another purpose if a disease did not exist. Figure 1 demonstrates the basic concept of COI. Most health economic studies assessing the total costs of a disease use this methodology (e.g., Keith and Shackleton 2006; Welte et al. 2000; Leidl et al. 1999; Henke et al. 1997; Xie et al. 1996; Harwood et al. 1983; Hodgson and Meiners 1979).

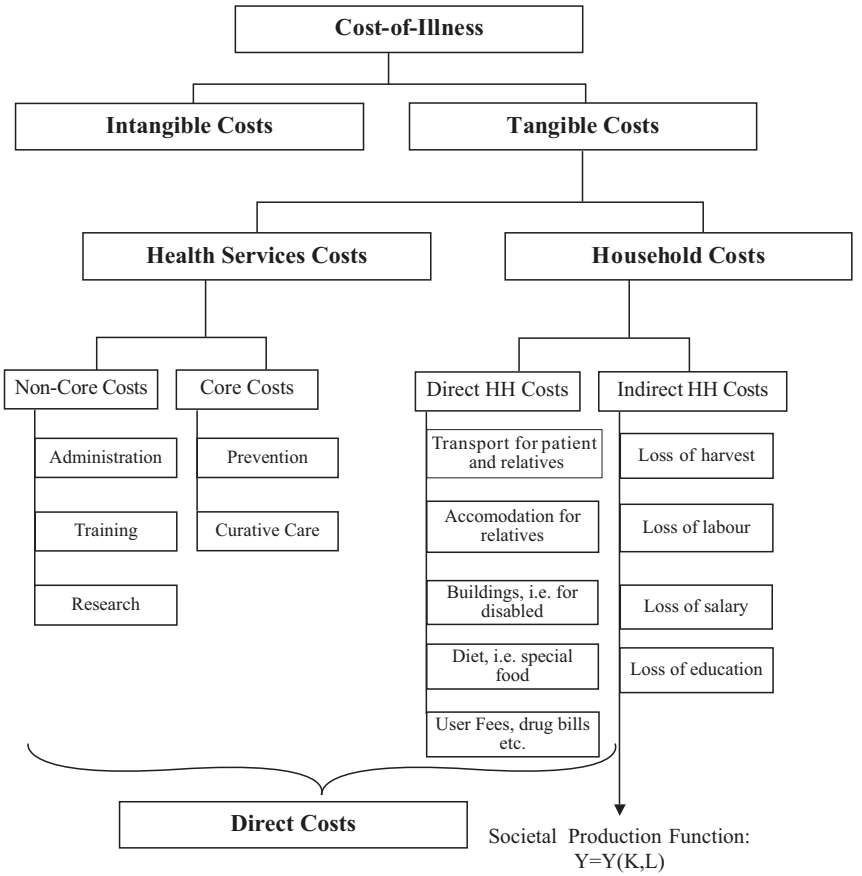

Fig. 1 Concept of Cost-of-Illness

Households have direct and indirect costs. *Direct household costs* imply payments for transport to and from the health services, accommodation for the accompanying relative, special buildings for disabled patients (e.g., changed bathroom), costs for diet, and the re-education, for instance new training after a paralysis. Private households also have to bear direct payments for user fees and drugs, which are an income of the providers. There must be clearing between these components of the COI to avoid double-counting.

Indirect household costs summarize all lost opportunities. During the time of illness, a patient and a caring relative cannot work. Therefore, wage-earners lose salaries as well as the economy production force. Sick parents do not have time to take care of their children so that their education will suffer. Therefore, morbidity leads to indirect costs. The term "socio-economic costs" is used for the total of direct and indirect costs as both have to be shouldered by the society.

Direct and indirect household costs are so-called tangible costs because a monetary value can be attached to them. Pain, psychological pressure, reduced joy of life, and social prestige are reductions in the quality of life, which do not have a natural monetary value. These *intangible costs* are sometimes evaluated as well and a monetary value is attached to them. For instance, an interviewer might seek the "willingness to pay" of a sick person for being healed (Breidert 2006; Gertler and Gaag 1990).

In countries with an existing (social) health insurance system, it is usually rather simple do receive a close-to-reality estimate of the *provider Costs-of-Illness*. The insurance pays the bills of general practitioners, specialists, hospitals, pharmacies, laboratories, etc. so that the total costs per patient can easily be determined. However, in some countries we cannot receive this data, and sometimes confidentiality regulations do not permit the transfer of insurance data, so that, for instance, provider costs of difference phases of HIV/AIDS can be calculated. In this case, a sample of patient files has to be analyzed with permission of the patients so that the provider costs can be recorded.

The calculation of direct household costs of HIV/AIDS is quite difficult. First, resource consumption is hardly documented, so that patients have to be interviewed or be asked to keep household diaries for all expenditure due to their disease. Second, it is frequently not easy to allot a certain expenditure to a specific disease. Co-payments for drugs, practitioner, and hospital services as well as transport to and from the provider are easily allocated to the COI of this disease. But other direct household costs might be even higher, such as the costs of a special diet, but it is very difficult to analyze whether these costs are really incurred due to this illness. Studies demonstrate that direct household costs might be small in developed countries, but they might make up to 50% of the total COI in developing countries (Su et al. 2006).

The calculation of indirect COI can be based on different methodologies, and there is no generally accepted standard for all circumstances. The most common approach to calculate the indirect costs of illness is the *human capital method*. The loss of welfare of a society in the form of nongenerated commodities and services mainly depends on the lost working hours. The method assumes that if a person had

not been sick or had not died from a disease, this person could have worked and contributed to the welfare of the society. Therefore, the disease induced a loss of productivity of the factor labor, which has to be valued with its marginal contribution to the total production. However, as this is very difficult, most studies use the wage rate as a substitute (e.g., Henke et al. 1997; Rice et al. 1990; Rice 1967). The human capital is calculated as the net value of future production of a human being.

The calculation of indirect costs of HIV/AIDS can be based on this methodology if we assume that all AIDS-patients were fully working before they fell sick. However, this is not always the case. Nonpaid activities are difficult to consider in this approach, so that the loss of work of, for instance, a housewife suffering from AIDS cannot be valued easily with the average wage rate. Other concepts of calculating indirect household costs have been developed. For instance, instead of calculating the human capital based on wage rates, one could use the gross national product or the gross domestic product as an indicator of the productivity. However, if patients do not have a wage income (e.g., housewife, pensioner), this approach cannot be applied.

The *friction cost method* (Koopmanschap et al. 1995) assumes that the human capital approach has a tendency to overestimate the indirect household costs. If a worker is seriously sick or dies, his job will be vacant for some time until a new person is hired or trained. However, the loss will not be there for many years. Therefore, the loss of human capital is calculated not until the time of retirement, but until the time of replacement. This approach is correct for unskilled labor in a situation of unemployment. It fits neither to a fully employed economy nor to a subsistence farmer society where there is no substitution of a father who died.

Consequently, there is no golden standard of calculating indirect Costs-of-Illness. The estimates based on different methodologies might differ significantly, and so will the total Costs-of-Illness of HIV/AIDS. It is possible to analyze the quality of studies whether all components (such as indirect costs of caring relatives etc.) have been included. Whether the methodology applied is "best" cannot always be determined.

Figure 1 summarizes the different components of the total COI.

1.2 Economic Evaluation of an Intervention

The costs of an intervention have to be compared with the results of this intervention (Drummond et al. 2004). These results can be outputs, outcomes, and impacts (Fig. 2). An *output* is the direct result of a production process. Agents of production (resources) are transformed to generate a certain commodity or service (output). For instance, equipment, reagents, and the knowledge of a laboratory technician are used to perform a certain resistance test. Other examples of outputs are contacts, admissions, or prescriptions.

The individual client uses these outputs to transform it into an *outcome* for himself. For instance, a hospital cannot produce health, but merely health services. The

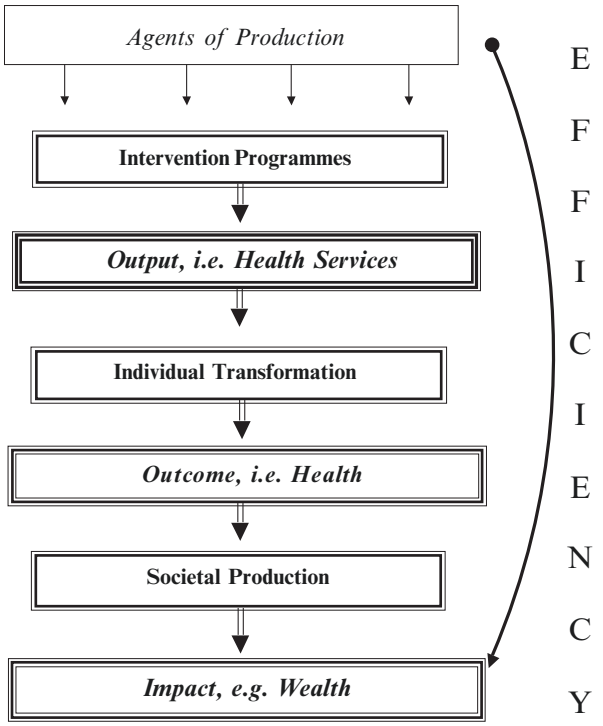

Fig. 2 Basic model of output, outcome and impact

compliance of the patient is significant for the production of the outcome health out of the product health services. Finally, the *impact* of the outcome on a broader system is again a transformation process. For instance, a healthy population will most likely have a higher gross national product, that is, economic strength is a function of agents of production, of outputs of production, and of outcomes.

The health economic evaluation model and the COI model are closely related. The consumption of agents of production causes direct costs. Indirect costs are a monetary expression for the loss of economic wealth, that is, the impact of a health intervention is the reduction of indirect costs. The increase of health is reflected by the reduction of intangible costs.

A health economic evaluation calculates the *efficiency* of the transformation processes. For instance, we can compare the consumption of agents of production with the output, the outcome, or the impact of this production process. Table 1 demonstrates some possible comparisons and indicators.

The comparison between these components can be done using different methods (e.g., Murray and Evans 2003; Edejer et al. 2003; Muenning 2002; Drummond et al. 2004). First, all inputs and results can be expressed in monetary terms, that is, outputs, outcomes, and impacts are transformed to currency units and compared with the costs (difference or quotient). This *cost-benefit analysis* is rarely done in

Table 1 Examples for indicators of health economic evaluation

		Compared with	
	Output: health services	Outcome: health	Impact: wealth, growth, etc.
Input: agents of production	Cost per admission; annual cost per HIV/AIDS-patient; Cost per prescription; profit, return on investment, cost recovery, sustainability, coverage, sales; etc.	Cost of antiviral intervention in comparison to mortality, progression, quality of life, expectancy of life); number of health care workers per life year gained	Cost of antiviral intervention in comparison to increase of gross national product due to increased work force
Output: health services		Admission of HIV/AIDS patients to hospital compared to mortality due to AIDS	Specialist AIDS-consultancy services compared to gross national product
Outcome: health			Comparison of health indicators (incidence, prevalence, life expectancy, life years gained, QALYs etc.) and economic indicators (e.g. wealth, growth etc.)

the field of HIV/AIDS, as the economic value of intangible costs is very difficult to assess. Consequently, a *cost-effectiveness analysis* is used that compares the costs of an intervention with the direct effect, for example, increase of life expectancy. Sometimes several effects are combined to one index number (e.g., quality of life and life expectancy to a Disability Adjusted Life Year, DALY).

Based on this methodology, we can expect the following from a health economic analysis of an intervention:

1. Direct provider COI
2. Direct household COI
3. Indirect COI
4. Intervention cost
5. Health effects of an intervention (incidence, prevalence, life years gained, QALYs, etc.)
6. Comparison of intervention cost and direct provider COI
7. Comparison of intervention cost and total direct COI
8. Comparison of intervention cost and health effects
9. Comparison of intervention cost and indirect costs

The following chapter will analysis a number of published studies on COI on HIV/AIDS and on antiviral intervention. For each study we analyse whether it can

contribute to our understanding of the COI of AIDS and/or of the consequences of a certain intervention. As there is no literature on the socio-economic impact of resistance testing, this aspect had to be neglected. The dearth of data in this field calls for further research.

2 Literature Review

2.1 Direct Cost-of-Illness

As discussed previously, direct COI are the economic representation of resource consumption and have a natural monetary value. They can occur on the provider and the household side. This chapter analyses the direct COI in four steps: Provider cost before HAART (Sect. 2.1.1), provider cost in the HAART era (Sect. 2.1.2), lifetime provider costs (Sect. 2.1.3), and direct household cost (Sect. 2.1.4).

2.1.1 Provider Cost Before HAART

From the early beginning of treating HIV/AIDS, most health economic studies focussed on the calculation of provider costs. During the first years there had been a clear dominance of research on hospital costs for patients with AIDS, in particular, in the *United States of America*.

Scitovsky et al. (1986) calculated the average cost per AIDS-related hospital admission as US$ 9,024 ranging from US$ 7,026 to US$ 23,425. A more comprehensive picture is presented by Scitovsky and Rice (1987), who estimated provider cost of the AIDS epidemic in the United States in 1985, 1986, and 1991, based on prevalence estimates provided by the Center for Disease Control (CDC). They predicted that the core provider costs of AIDS would rise from US$ 630 million in 1985 to US$ 1.1 billion in 1986 and to US$ 8.5 billion in 1991. The authors compared their estimates of the cost of AIDS in the USA with the estimates for end-stage renal disease (US$ 2.2 billion), traffic accidents (US$ 5.6 billion), lung cancer (US$ 2.7 billion), and breast cancer (US$ 2.2 billion). They concluded that the core provider costs of AIDS were relatively low in comparison with the provider costs of all illness as well as the costs of these other diseases. However, they also assessed the non-care costs (e.g., for research) to rise from US$ 319 million in 1985 to US$ 542 million in 1986 and to US$ 2.3 billion in 1991.

Hellinger (1988, 1991, 1992) developed a model that estimated the provider costs of AIDS using incidence-based measures derived from US data. His findings for inpatients were similar to those by Scitovsky and Rice (1987). He added the costs of outpatient antiretroviral medications and estimated an increase of provider costs from US$ 5.8 billion in 1991 to US$ 10.4 billion in 1994 and to US$ 15.2 billion in 1995.

Solomon and Hogan (1992) analyzed the Michigan Medicaid payment records for the period of 1985–1989. Payments data were merged with data from the Michigan Death Registry and the AIDS Surveillance Registry. The payments rose steadily with age through the 36–45-years-old group, declining slightly among older adults. Men received services with average cost of US$ 1,522 per month, with a median monthly cost of US$ 792. Women received services with average cost of US$ 777 per month, with a median monthly cost of US$ 119. On average, the HIV-related health care provided through the Michigan Medicaid Program cost about US$ 1,300 per month per person, an amount substantially lower than other estimates. The authors pointed out that there were several possible reasons for that result, for example, that their data included many persons who were still in the early stages of infection, and high costs at the end stages of the disease had been excluded by the protocol. The bulk of the cost associated with treating persons with HIV infection was for inpatient hospital services. Among men the next highest cost was on average for drugs. Among women, physician fees were the next largest expense. A possible reason for the observed gender discrepancy could have been differences between gay males in intravenous drug users of both sexes.

The study of Anderson and Mitchell (1997) examined whether the AIDS-specific home and community-based waiver program, which was implemented in Florida in 1990 as an alternative to institutional care, was effective in reducing Medicaid expenditures per beneficiary during its first 2 years of operation. Therefore, the authors used Medicaid claims data and country information to estimate the effect of the waiver on expenditures controlling for nonrandom program selection. Their results indicate that persons with AIDS who used waiver services incurred monthly expenditures that were on average 22–27% lower than otherwise similar nonparticipants. The authors concluded that home and community-based care for AIDS patients resulted in lower expenditures per beneficiary.

The economic impacts of HIV/AIDS disease have also been analyzed in Europe. Beck (1995) studied the AIDS-related costs in a national AIDS referral center in *London*. He concluded that share of total drug cost increased between 1985 and 1989 from 5.0% to 30.0%. The median survival time from the date of the diagnosis of AIDS was 14.6 months before the introduction of Zidovudine (1987) and 21.0 months afterwards.

Based on the years 1990/1991, Papaevangelou et al. (1995) calculated the cost of HIV/AIDS inpatients and outpatients in two hospitals in Athens, *Greece*. The average annual cost per person was estimated to be US$ 8,428, consisting of costs for Zidovudine (US$ 1,343), outpatient care (US$ 1,122), and inpatient care (US$ 5,963). A quite similar temporal data background was used by Rovura and Leidl (1995) for collecting data to estimate individual healthcare costs of HIV/AIDS patients in Catalonia, Spain, according to the disease stage. Annual patient cost for early stage, intermediate, and AIDS, respectively, was US$ 648, US$ 9,707, and US$ 24,915, whereby the share of inpatient cost increased from 0.15% in the early share to 84% during AIDS.

Kyriopoulos et al. (1995) compared different estimates of HIV/AIDS healthcare expenditure in Greece based on study years 1987–1993. Average annual cost per

Table 2 Examples of provider cost-of-illness in the pre-HAART era [US$ p.a.]

Study	Country	Study year	Total provider costs per year [*1,000,000]	Annual provider costs per patient
Scitovsky et al. (1986)	USA	1984		9,024
Scitovsky and Rice (1987)	USA	1985	949	
		1986	1,662	
		1991	10,869	
Solomon and Hogan (1992)	USA	1986–1989		15,600
Diomidous and Sissouras (1990)	Greece	1988		6,350
Hatzakis and Trichopoulos (1990)	Greece	1989		38,482
Papaevangelou et al. (1995)	Greece	1990–1991		24,160
Paparizos et al. (1996)	Greece	1994		14,396

patient ranged between US$ 6,350 (Diomidous and Sissouras 1990) and US$ 38,482 (Hatzakis and Trichopoulos 1990), whereby the later included Zidovudine costs.

Table 2 summarizes these basic studies before the era of HAART. It is obvious, that *there is a wide range between the results of these studies and we have to admit that we have little reliable data on the provider costs before 1995. The methodologies used are very different, so that comparison of results becomes difficult. In particular, the extent of considering special cost categories and the quality of data might have influenced the corresponding results*. Borleffs et al. (1990) supposed that precise data on the utilization of health care facilities by HIV patients were often not available.

2.1.2 Provider Cost in the HAART Era

Highly active antiretroviral therapy (HAART) based on protease inhibitors or non-nucleoside reverse transcriptase inhibitors was introduced in the industrialized world during the second half of the 1990s. The majority of studies are based on data from the *United States of America*.

In 1997, Haburchak (1997) conducted a study that showed that monthly costs due to HIV were significantly greater than previously recorded. This could have coincided with the approval of more antiretroviral drugs. In this study, the authors estimated that the monthly costs reflected an additional US$ 1,033 for monthly drug costs with current treatment guides. Another study by Holtgrave and Pinkerton (1997) demonstrated that the introduction of protease inhibitor-based regimens had also increased the monthly cost of caring for AIDS patients. Depending on the level of access to care, the monthly costs ranged from US$ 3,274 to US$ 4,087.

The introduction of protease inhibitor-based regimens seemed to be responsible for a change within the structure of provider costs. There is some evidence that the share of drugs increased, whereas the importance of hospitalization declined. For instance, Hellinger (1993) estimated that drug costs to account for about 10% of the total provider costs, whereas the inpatient hospitals costs were responsible for some

68%. After the introduction of HAART, this structure changed. Moore and Chaisson (1997) calculated that drug costs accounted for 21% and inpatient hospital costs for 56% of the total provider costs. The results of Gebo et al. (1999) are quite comparable to the latter. The authors tried to determine how the costs to Medicaid of patients in Maryland, USA, infected with HIV hanged in the setting of highly active antiretroviral treatment between January 1995 and December 1997. When data were stratified according to the use of a protease inhibitor-containing regimen, it was found that hospital inpatient payments decreased significantly for patients on a protease inhibitor-containing regimen, whereas drug payments increased significantly (Table 3).

Hellinger and Fleishman (2000) derived estimates for costs of treating people with HIV disease in the United States using patient-based, payer-based, and provider-based approaches. Based on insurance data from 1996, they calculated average annual cost of treating a person with HIV disease between US$ 20,000 and US$ 24,700.

The study of Hellinger and Encinosa (2004) was the first claims-based study of the privately insured population of persons with HIV disease. The database comprised of all claims paid on behalf of 24 large US-American employers (governments and large private corporations) for all inpatient and outpatient services, including pharmaceutical charges. Claims consisted of the gross covered payments, deductibles, co-payments (i.e., out-of-pocket expenses), and net payments due from the insurer to the provider. The authors compared the use of antiretroviral therapy and other health care resources in 2000 by women and men with HIV disease. Using a simple comparison, Hellinger and Encinosa found that the average drug cost was US$ 9,037 for a man and US$ 3,893 for a woman. Furthermore, they identified that the out-of-pocket expenses comprised 10% of the total expenses for men (US$ 1,617 out of US$ 16,405) and 4% for women (US$ 405 out of US$ 10,397). The authors concluded that there were significant differences in the utilization of costly, new drug therapies between men and women.

Hutchinson et al. (2006) conducted an incidence-based COI analysis to estimate the lifetime cost of HIV/AIDS resulting from new infections diagnosed in 2002. Having used data from the HIV/AIDS Reporting System of the Centers for Diagnose Control and Prevention, stages of disease at diagnoses and the proportion of cases by race/ethnicity were determined. The authors estimated provider costs and mortality-related productivity losses were estimated using data on cost, life expectancy, and antiretroviral therapy from literature. The authors estimated the costs of new HIV infections in the United States in 2002 at US$ 36.4 billion, composed of US$ 6.7 billion in medical costs and US$ 29.7 in productivity losses, whereby the medical costs per case were highest for whites (US$ 180,900) and lowest for Afro-Americans (US$ 160,400).

The number of studies for other countries is limited. Krentz et al. (2003) analyzed the provider costs of providing medical care to patients with HIV/AIDS in Southern Alberta (*Canada*) between April 1995 and April 2001. The authors collected all patient-specific provider costs including the cost of drugs (HIV and non-HIV drugs), outpatient care (including physician costs and laboratory testing), and

Table 3 Examples of provider cost-of-illness in the HAART era [US$ p.a.]

Study	Country	Study year	Annual provider costs per patient (US$ p.a.)
Holtgrave and Pinkerton (1997)	USA	1996	44,166
Hellinger and Encinosa (2004)	USA	2000	Men: 16,405 Women: 10,397
Hellinger and Fleishman (2000)	USA	1996	22,000
Krentz et al. (2003)	Canada	1995/1996	5,772
		1996/1997	7,524
		1997/1998	8,868
		1998/1999	8,868
		1999/2000	9,036
		2000/2001	8,940
Beck et al. (2004)	Canada	1997–2001	9,445 (non-AIDS patient) 11,754 (AIDS-patient)
Easterbrock et al. (1998)	England	1996	7,324 (asymptomatic) 11,864 (symptomatic) 31,758 (AIDS)
Kyriopoulos et al. (2001)	Greece	1996	14,737
		1997	13,403
Stoll et al. (2002a, b)	Germany	1997	31,812
		2000	24,029
		2001	21,926
Tramarin et al. (2004)	Italy	1994	15,515
		1998	10,312
Bozette et al. (2001)	France	1996	20,300
		1998	18,300
Yazdanpanah et al. (2002)	France	1998	11,748 (high CD4 stratum) 15,132 (low CD4 stratum)
Caekelbergh et al. (2007)	Belgium	2005	49,439.40 (CD4: 0–51) 31,322.85 (CD4: 51–100) 16,627.52 (CD4: 101–200) 8,650.57 (CD4: 201–350) 3,226.24 (CD4:351–500) 2,889.43 (CD4 > 500)
Kimura (2002)	Japan	1999	17,858–21,431; outpatient 37,007–170,155; inpatient

inpatient and nursing care (acute, long-term, and palliative). Laboratory costs included all HIV-related laboratory tests (CD4 cell count, viral load determination, serology, and chemistry). The costs of community-based care included the costs of acute, long-term, and palliative nursing care. According to their calculations,

cost per patient per month increased from US$ 481 in 1995/1996, before the use of HAART, to US$ 739 in 1997/1998, when HAART was widely used. During the following 3 years, mean overall costs per patient per month remained stable. The authors found that antiretroviral drugs accounted for 30% (US$ 145 per patient per month) of the total cost in 1995/1996 and increased (based on Can-$) to 69% (US$ 516 per patient per month) in 2000/2001.

Beck et al. (2004) presented an analysis of the cost-effectiveness of highly active antiretroviral therapy in Canada. They compared the cost-effectiveness from 1991 to 1995 (pre-HAART period) with the period from 1997 to 2001 (HAART period) for non-Aids and Aids groups. For the first group, they calculate total cost of US$ 4265 in the pre-HAART period and US$ 9445 in the HAART-period, whereas 66% and 84% were spent on antiretrovirals. The incremental cost per life year gained was US$ 14,587, that is, the HAART technology is rather cost-effective. For the Aids patients, the total costs were US$ 9,099 in the pre-HAART period and US$ 11,764 in the HAART period, whereas 29% were for antiretrovirals in the pre-HAART era and 72% in the HAART era. The incremental cost per life year gained by introducing HAART was US$ 12,813, so that HAART seems cost-effective in Canada.

The use and cost of HIV service provision in *England* in 1996 was analyzed by Easterbrock et al. (1998). Standardized activity and case-severity data was collected prospectively in ten English HIV clinics. 5,440 patients attended the services during the first six months of 1996 and 5,708 patients during the second term. Cost estimates per patient-year for HIV service provision in 1996 varied from US$ 7,324 for asymptomatic patients to US$ 11,864 for symptomatic non-AIDS patients, and to US$ 31,758 for patients with AIDS. Easterbrock et al. (1998) concluded that different combinations of antiretroviral therapy affected the cost estimates differently.

To compare the epidemiological, clinical, and economic impacts of the HIV epidemic in *Italy* prior to and after the introduction of HAART, Tramarin et al. (2004) conducted a prospective and observational study with a multi-center design. They used data collected on an AIDS cohort from 1994 and updated data from a comparable cohort in 1998. Mortality and medical costs of 251 patients were measured in 1994 and in 1998, respectively. A considerable difference was observed in mortality (33.9% in 1994 vs. 3.9% in 1998). The cost per patient per year was US$ 15,515 in 1994 and US$ 10,312 in 1998. Based on the comparison of the two cohorts between both years, the authors concluded that after the introduction of HAART, hospital-based provision shifted from an inpatient-based to an outpatient-based service, with major focus on pharmaceutical care.

Flori and le Vaillant (2004) studied the temporal relationship between the uptake of the more aggressive antiretroviral therapy and the use and cost of hospital treatment for HIV-infected patients in *France* from 1995 to 2000 from a hospital perspective. The authors found that during this period the proportion of patients on ARV treatment increased from 69.5% to 97%, with a large rise in the use of polytherapy. This increase was most notable for patients with CD4 cell counts above 500. ART expenditures per patient increased between the study years by 220%, reaching US$ 1,886 in 2000. Unlike that, inpatient hospitalization fell by 60% and average length of stay declined. Thus hospital costs (excluding ART) decreased to US$ 2,137 in 2000.

Bozette et al. (2001) examined expenditures for the care of adult HIV-infected patients since the introduction of highly active antiretroviral therapy. They interviewed a representative random sample of 2,864 patients in early 1996 and followed them for up to 36 months. They estimated the average expenditure per patient per month on the basis of self-reported information. According to their calculations, the mean expenditure was US$ 1,792 per patient per month at base line in early 1996, but it declined to US$ 1,359 for survivors in 1997, since the increases in pharmaceutical expenditures were smaller than the reductions in hospital costs. After adjustments for the interview date, clinical status, and deaths, the estimated annual expenditure declined from US$ 20,300 per patient (1996) to US$ 18,300 (1998).

Yazdanpanah et al. (2002) calculated the resource use and cost for different stages of HIV infection in *France* based on a clinical database of HIV-infected patients between 1994 and 1998. The total costs attributable to bed-day and day-care inpatient care included the mean cost of each inpatient day times the length of stay, as well as total number of laboratory tests, dosage and quantity of medications, and total number of procedures. The total cost attributable to outpatient care included the mean physician and nurse fees per visit, as well as total number of laboratory tests and total number of procedures. In the absence of an AIDS-defining event, the average total cost of care ranged from US$ 797 per person-month in the highest CD4 stratum to US$ 1,261 per person-month in the lowest CD4 stratum.

Caekelbergh et al. calculated the direct costs of HIV/AIDS in *Belgium* from the health care pay perspective. On the basis of 150 patients, they determined the costs of antiretrovirals, outpatient and inpatient resource use for the year 2005. They realize that the costs strongly depend on the CD4+ T-cell count, that is, the annual costs per patient are on average about US$ 2,900 for a patient with a CD4+ T-cell count $>$500, US$ 3,200 (CD4: 351–500), US$ 8,650 (CD4: 210–350), US$ 16,600 (CD4: 101–200), US$ 31,300 (CD4: 51–100), and US$ 49,400 (CD4: 0–50), respectively. Consequently, the early detection of an HIV-infection as well as proper management that prohibits disease transition is of high cost-importance.

An analysis to study the impacts of the introduction of new antiretroic therapeutic schemes on the cost for AIDS treatment in *Greece* was conducted by Kyriopoulos et al. (2001). They used data recorded at a university hospital in Athens. 1996 was the last year with the implementation of dual antiretroic therapy, and 1997 was the first year of triple antiretroic treatment application. The data were based on a randomized sample of 60 HIV-infected patients at various stages of the HIV disease. Cost estimations included the number of hospital days, clinic visits, physician salaries, laboratory tests, day care services, drugs used, and any other extra expense required for each patient. Annual cost per patient was US$ 14,737 in 1996 and US$ 13,403 in 1997. Their cost comparison showed that the amount of each cost component decreased, with the exception of the medication cost. Patient costs for antiretroic drug were more than double from 1996 to 1997. It is helpful to compare results from Kyriopoulos et al. (2001) with the results of a study by Paparizos et al. (1996), who estimated the costs in Greece in 1994 based on the same methodology and on a similar sample of AIDS patients. There is no significant difference in the total annual provider cost per patient. Unlike that, the composition

of the patient's cost structure changed: the share of hospitalization decreased (1994: 13.92%; 1996: 6.21%; 1997: 5.47%). On the contrary, the share of medication costs continuously increased (1994: 60%; 1996: 64%; 1997: 71%).

Stoll et al. (2002b) examined provider costs in a *German* monocentric cohort of HIV-infected patients after the introduction of HAART. According to their findings, mean provider costs per capita decreased from US$ 31,812 in 1997 to US$ 21,926 in 2001. The costs of HAART per capita decreased significantly from US$ 15,739 in 1997 to US$ 14,336 in 2001. Also quite impressive was the continuous decrease of expenditures for additional drug therapy (-43.3%) and hospitalization (-52.1%), respectively. However, the costs caused by HAART increased from 49.5% of all provider costs in 1997 to 65.4% in 2001.

Finally, Kimura (2002) estimated the socio-economic impact of HIV/AIDS in Japan and estimated the inpatient and outpatient costs of treating HIV-infected adults. He calculated costs of US$ 17,858 to US$ 21,431 p.a. for outpatients, whereas 83% were due to antiretrovirals. The cost estimates for inpatients with Aids varies strongly. Inpatients with a CD4+ count from 200–499 had costs of US$ 37,007 p.a., inpatients with a CD4+ count between 50 and 199 had costs of US$ 76,197 p.a., and inpatients with a CD4+ count of less then 50 had average cost of US$ 170,155 p.a.

With these findings we can conclude that the drug costs strongly increased since the introduction of HAART. However, other provider costs strongly declined with the introduction of this drug regime, so that the total costs remained stable or declined. HAART is – at least in the short-term analysis given in this literature review – cost-effective.

2.1.3 Lifetime Provider Costs

Estimates of the lifetime COI are needed for temporal and international comparisons and for assessment of the efficiency of prevention strategies. During the first years of HIV/AIDS treatment, direct lifetime costs were only estimated by simple projections based on retrospective data. Later, specific statistical tools were adopted to estimate life expectancy and lifetime costs. The results of lifetime estimates are very sensitive to imputed assumptions. Table 4 demonstrates some studies in this field.

Table 4 Lifetime provider cost-of-illness [US$ p.a.]

Study	Country	Study year	Lifetime provider costs per patient
Scitovsky et al. (1986)	USA	1984	27,571
Hellinger (1993)	USA	1992	69,000–119,000
Hurley et al. (1996)	Australia	1992–1993	93,000–70,000
Gable et al. (1996	USA	1995	94,726
Yazdanpanah et al. (2002)	France	1997–1998	189,797
Schackman et al. (2006)	USA	2004 (projection)	385,200–351,100

Scitovsky et al. (1986) calculated the mean lifetime inpatient charges of patients with AIDS who died and who had received all their inpatient care at *San Francisco* General Hospital. Based on the data from 1984, they estimated costs of US$ 27,571. Life expectancy increased, and so did the lifetime costs. Based on US-data from 1992, Hellinger (1993) estimated lifetime costs of treating a person with HIV from the time of infection until death (over 12.4 years) as approximately US$ 119,000, while the estimated provider cost from AIDS development until death was approximately calculated at US$ 69,000. According to the author, these estimates defined upper bounds, because they assumed that persons would continuously receive treatment from the moment of infection until death. This figure is very similar to the findings of Beck (1995), based on data from a London hospital, who estimated lifetime costs of treating a person with HIV infection at US$ 117,600.

Gable et al. (1996) estimated lifetime costs of treating a person with HIV to be US$ 94,726, using an expert panel and several cost data sources to produce treatment protocols for opportunistic infections and primary antiretroviral therapy.

Holtgrave and Pinkerton (1997) also estimated the lifetime costs after the diffusion of new combination therapies. Having discounted the costs of care, their lifetime estimates ranged from US$ 71,143 to US$ 424,763. The ranges reflect different levels of access to care.

Based on data from 1992 to 1995, Moore and Chaisson (1997) analyzed combined economic and clinical data in patients from the Johns Hopkins HIV Service, the provider of primary and specialty care for a majority of HIV-infected patients in the Baltimore metropolitan region. By using the Kaplan–Meier technique and Markov modeling, they calculated the lifetime costs of Medicaid patients in Maryland, USA. According to their estimates, the total payment to providers was US$ 133,000. The authors included all HIV-related and non-HIV-related inpatient care, outpatient clinic visits, pharmacy costs, and home health care costs. This approach was also used by Holtgrave and Pinkerton (1997) for calculating life expectancy after the diffusion of new combination therapies. Their estimates, based on an international expert panel, ranged from US$ 71,143 to US$ 424,763, whereby the ranges reflected different levels of access to care. As persons live longer, they consume greater health care resources, driving the overall health care costs associated with HIV infection even higher. The decreasing death rate from AIDS, however, has not been accompanied by a decrease in the number of persons newly infected with HIV disease.

Schackman et al. (2006) projected HIV medical care costs in the USA in 2004 US$. From the time of entering HIV care, the discounted lifetime direct provider cost was calculated as US$ 385,200, whereas 73% were due to antiretrovirals. For patients with a CD4+ count of less than 200, the discounted lifetime cost was US$ 354,100.

Based on two clinics specialized in AIDS treatment, Papaevangelou et al. (1995) calculated lifetime costs per patient in *Greece* at US$ 24,160, consisting of drug costs (US$ 9,022), costs for outpatient care (US$ 963), and inpatient care (US$ 14,175).

The goal of the study, which was conducted by Hurley et al. (1996), was to estimate the average present value, at both the time of diagnosis and the time of seroconversion, of the lifetime cost of health care for HIV infection. *Australian* data on the monthly cost of HIV-related health care for homosexual men were linked with published data on disease progression using survival analysis methods. Future costs were discounted at 5% per annum. For a patient diagnosed when his CD4+ count fell below $500 \times 106/\mathrm{L}$, the average present value in 1992–1993 lifetime cost was US$ 93,000 of which 49% was for inpatient bed-days. Unlike that, the average present value of lifetime cost for a man infected in 1992–1993 and diagnosed when his CD4+ count fell below 106/L was US$ 70,000.

The discounted und undiscounted lifetime cost of HIV disease in *France* was estimated by Yazdanpanah et al. (2002) by using a clinical computer-based, probabilistic simulation model of the natural history of HIV infection. The discounted lifetime costs of treating a person in 1997/1998 were estimated at US$ 189,797. Results ranged between US$ 138,356 and US$ 224,386, provided that clinical management of HIV infection began a CD4 count of 378 cells. The undiscounted costs were US$ 346,158 over a projected life expectancy of 16.4 years. They ranged between 188,023 and 335,248, depending upon the stage of illness.

A final assessment is difficult as the number of studies on lifetime costs of HIV/AIDS is very small, and the last few years have seen only few publications in that field. However, with an increasing life expectancy due to HAART, we can expect that the provider lifetime COI will strongly increase.

2.1.4 Direct Household Cost-of-Illness

HIV/AIDS cause expenditure for households, which strongly differ from provider costs. Patients need resources to travel to the provider (bus fare, care mileage). At least in the final stages of the disease, patients are frequently accompanied by a relative or close friend living in a hostel close to the hospital. The expenditure for this hostel is household cost. In some cases, patients need equipment or changes of buildings, which are not paid by the health insurance. They frequently need another diet with higher costs, in particular, to prevent side-effects of drugs. Thus, household COI can be high.

However, *we know hardly anything on these costs*. Providers document all costs and health insurances keep files with all expenditure. Households have no documentation on such costs, and direct household costs are hardly recovered by health insurances. Thus, there is a major research field waiting to be covered. Merely Bozette et al. (2001) based their cost assessment on household documentations, but they do not sufficiently distinguish between household and provider costs.

In a nut-shell: *Out knowledge of the direct COI of HIV/AIDS has strongly increased in the last 10 years. Depending on the level of care in a particular country, provider cost per case might be as high as US$ 25,000 p.a., and the discounted lifetime costs in the HAART era will be more than 100,000 US$ per case. There can be no doubt that AIDS will cause higher costs for the patients and his household, but we know almost nothing about these costs*.

2.2 Indirect Cost-of-Illness

Most of the HIV-related economic studies calculate direct costs. However, the socio-economic costs of HIV/AIDS are far greater. For instance, patients as well as family members and friends who provide care incur costs that are not related to payments but to lost income (indirect cost). This cost category includes the loss of wages for a wage earner, the loss of labor for a non-wage earner (e.g., pensioner, household), the loss of harvest for a farmer, and other losses (e.g., loss of education and chances for children of AIDS patients). Some studies address this issue.

In addition to their calculation of direct costs in the *USA*, Scitovsky and Rice (1987) also determined indirect costs attributable to the loss of productivity, resulting from morbidity and premature mortality in the US. The authors used the human capital approach. Indirect costs were estimated to rise from US$ 3.9 billion in 1985 to US$ 7.0 billion in 1986 and US$ 55.6 billion in 1991 (Table 5).

Hanvelt et al. (1994) estimated the nationwide indirect costs of mortality due to HIV/AIDS in *Canada*. A descriptive, population-based economic evaluation study was conducted. Data from Statistics Canada were used, which contained information about all men aged 25–64 years for whom HIV/AIDS or another selected disease was listed as the underlying cause of death from 1987 to 1991. Based on the human capital approach, the present value of future earnings lost for men was calculated. The estimated total loss from 1987 to 1991 was US$ 2.11 billion, with an average cost of US$ 558,000 per death associated with HIV/AIDS. Future production loss due to HIV/AIDS was more than double during the period 1987 to 1991, from US$ 0.27 to US$ 0.60 billion. A more comprehensive update of this study was presented by Hanvelt et al. (1996). The same database and the same data section but for the calendar years 1987–1993 was used. The indirect cost of future production due to HIV/AIDS in Canada based on the human capital approach for that period was estimated to be US$ 3.28 billion. The authors also calculated the willingness-to-pay to prevent premature death due to HIV/AIDS, which was estimated based on

Table 5 Indirect cost-of-illness [US$ p.a.][a]

Study	Country	Study year	Total annual indirect costs [*1,000.000.000]	Annual indirect costs per case
Scitovsky and Rice (1987)	USA	1985	3.9 (HC)	
		1986	7.0 (HC)	
		1991	55.6 (HC)	
Papaevangelou et al. (1995)	Greece	1990		11,774 (HC)
Lambert (1995)	France	1992	3.1 (HC)	
Stoll et al. (2002a)	Germany	1997		24,639 (HC) 2,421 (FC)
Hanvelt et al. (1994)	Canada	1987–1991	2.1 (HC)	
Hanvelt et al. (1996)	Canada	1987–1993	3.28 (HC) 5.2 (WP)	

[a]See abbreviations

the premiums for insurance to cover lost income. According to their calculations, the society would be willing to commit up to US$ 5.2 billion to prevent these deaths. The annual loss attributed to HIV/AIDS death increased from US$ 0.23 billion to US$ 0.72 billion.

Indirect cost for patients in two AIDS clinics in Athens, *Greece*, based on the human capital approach were calculated by Papaevangelou et al. (1995). The authors estimated average annual indirect cost at US$ 11,774. Lifetime indirect cost per case was calculated at up to US$ 59,047.

Based on prevalence estimates and mortality rates for the *French* AIDS epidemic, Lambert (1995) calculated indirect cost by using the human capital approach in 1992 as US$ 3.054 billion. Future indirect costs up to 2020 were simulated under different scenarios of the HIV prevalence. According to a pessimistic scenario, indirect cost would rise until 2010 (US$ 9.381 billion) and then keep almost stable until 2020 (US$ 9.069 billion). If the infection rate could be reduced, indirect costs would decrease to US$ 1.507 billion in 2020.

Mullins et al. (2000) estimated the indirect costs of the HIV epidemic in *England* in 1997–1998 from both a public-sector and societal perspective, including the costs of statutory, community, and informal services, and disability payments. The economic consequences of reduced productivity were estimated using the wage rate based on the human capital approach. Disability payments were excluded from the societal perspective, whereas the degree of lost economic productivity varied within a sensitivity analyses (full loss vs. partial loss of productivity). Annual indirect costs from the public-sector and societal perspectives, respectively, ranged from US$ 5,252 to US$ 6,515 per person-year for asymptomatic non-AIDS, and US$ 16,499 to US$ 34,825 for patients with AIDS. Estimated population-based indirect costs from the public-sector perspective varied between US$ 181 million and US$ 241 million for 1997–1998, respectively, comprising between 58% and 124% of direct treatment costs for triple drug therapy in England during 1997. From societal perspective, estimated population-based costs varied between US$ 138 million and US$ 198 million in 1997–1998, comprising between 45% and 102% of direct treatment costs and cost of care, respectively, during 1997.

Beside their calculation of direct costs, Stoll et al. (2002a) also examined indirect costs in a *German* sample of HIV-infected patients after the introduction of HAART. To emphasize the implications of different approaches of indirect costs, the authors determined both costs based on the human capital approach and costs calculated on the friction cost approach. They concluded that indirect costs based on the friction approach per patient in 1997 (US$ 2,421) add up to only one-tenth of the amount derived from the human capital approach (US$ 24,639).

HIV/AIDS-induced mortality and morbidity of workers can result in significant economic *loss to business*, including direct cost due to increased insurance premiums paid by employers, costs due to increased benefits paid by employers, indirect costs due to lost time due to illness, lost and reduced productivity, and other costs, like cost to new training and hiring of staff. Farnham and Gorsky (1994) used a Markov model to calculate the expected medical, disability, employee replacement, life insurance, and pension costs to a business firm in the US for an HIV-infected

employee. They calculated baseline costs for 1994 about US$ 17,000. The maximum expected five-year cost to a business firm for an HIV-infected employee was estimated at US$ 32,000, with an average expected cost of US$ 17,000 per year. Employment-based costs were most influenced by the type of benefits provided under employer-based health insurance plans. The authors demonstrated that these expected costs from the employer perspective was much less than the lifetime medical AIDS costs to society for an individual, which was estimated at more than US$ 85,000.

Liu et al. (2002) also calculated the economic cost of an HIV infection for the employer. Based on a simulation model to predict the comprehensive lifetime economic costs of HIV-infected workers to an employer, they predicted total lifetime costs of US$ 90,000 for the employer, whereas 2/3 are direct expenses on health insurance premium, life insurance premium, benefits, etc., and 1/3 loss of productivity.

Indirect costs strongly differ with methodology. The human capital approach leads to much higher indirect costs and the friction cost method. Furthermore, both methodologies lead to a strong difference of indirect costs for countries with different gross national products, that is, an infection with HIV in Greece leads to merely 10% of the indirect costs of an infection in the USA. This might make this calculation questionable. On the contrary, we realize *that the loss for the society due to lost labor might be higher than the treatment costs*. The socio-economic cost of HIV/AIDS is billions, mainly caused by disability to work and early death. Thus, *HAART is strongly reducing indirect costs by keeping patients' ability to work and prolonging life expectancy. At least with the human capital approach (which fits best for a highly qualified, fully employed adult between 25 and 65), the reduction of indirect COI by HAART is higher than the direct costs of treatment per annum.*

2.3 Total Cost-of-Illness

There are only few papers that include both direct and indirect COI. The most frequent cited AIDS cost figure in the United States in the middle of the 1980 was an estimate of the lifetime hospital costs and economic losses from disability and premature death of the first 10,000 patients with AIDS in the United States (Hardy et al. 1986). The authors extrapolated data of surveys done in New York City, Philadelphia, and San Francisco and estimated the lifetime hospital costs of these patients at US$ 1,473 billion or US$ 147,000 per AIDS patient. This estimate was based on the assumption of a lifetime use of 168 hospital days, an average survival time of 392 days, and an average charge per hospital day or US$ 878. The authors noted that the length-of-stay varied between geographic locations and explained this variation by the differing patient management practices in the different geographic regions. The costs associated with the management of AIDS patients in New York were significantly higher than those in San Francisco. Hardy et al. estimated losses incurred for the 8,387 years of work that will be lost from disability and from the premature death of the 10,000 patients would be over US$ 4.7 billion, almost $3\frac{1}{2}$ times of direct hospital costs.

In the early period of the pandemic, before the development of sensitive and reliable instruments to diagnose early infection, only patients with advanced AIDS presented to health care facilities. As a result, the estimates and projects of the costs for HIV/AIDS patients based on observed health care utilization were high. For example, Scitovsky and Rice estimated the annual costs of AIDS care in the United States in 1985, 1986, and 1991 to be US$ 630 million, US$ 1.1 billion, and US$ 8.5 billion, respectively; these costs represented the direct and indirect costs of HIV infections (Scitovsky and Rice 1987; Scitovsky 1988, 1989). More than 80% of these costs stemmed from losses in productivity, a reflection of the fact that AIDS has afflicted primarily working-age adults. The great increase in total costs by 1991 is caused by a projected increase in the prevalence.

Based on their estimates of direct and indirect costs of two clinics in Athens specialized in AIDS treatment, Papaevangelou et al. (1995) estimated total annual costs per case for 1990 as US$ 20,202.

3 Discussion

There is a growing body of literature on the costs of HIV/AIDS (Hornberger et al. 2007), but all studies address only one branch of the COI tree (Fig. 1). Until now there is not a single publication fully covering all aspects of COI of HIV/AIDS, and different methodologies (such as human capital approach vs. friction cost method) make comparison difficult. In addition, the time lag between the year of the study and the publication is between 3 and 10 years. That is one reason why we know so little about the costs of the last 5 years: results are not yet published.

Compared with the standard health economic methodology applied in COI studies for other diseases (e.g. Keith and Shackleton 2006; Welte et al. 2000; Leidl et al. 1999; Henke et al. 1997; Xie et al. 1996), the quality of health economic analysis of HIV/AIDS is not always satisfactory as far as costs are concerned (Levy et al. 2006). Sometimes it is not clear whether researchers included both inpatient and outpatient medications in their analyses. Equally important, many of the early studies used costs and charges interchangeably; data using charges may not accurately reflect true costs. Drummond and Davis (1988) also argued that there have been incorrect estimates of the survival times and costs in all these early studies, since there were no explicit adjustments made for disease severity.

Costs in treating persons with HIV disease vary over time, according to the consequences of utilized drugs and guidelines, and because of the special treatment at different stages of illness. This explains partly the enormous range in lifetime costs. Estimated costs also vary across geographic regions – even between East Coast and West Coast USA. During the 1980s and the early 1990s, the majority of healthcare costs for HIV-infected patients were mainly due to hospitalization. Therefore, total costs were influenced strongly by different lengths of hospital stay (Hellinger 1998). However, cost estimates for treatment varied widely in several US studies.

For example, Scitovsky et al. (1990) estimated the costs of treating individuals with AIDS in the first 12 months following diagnosis to be between US$ 34,450 and US$ 36,900 per person-year. Seage et al. (1990) indicated an estimate of US$ 55,800 per person-year between March 1984 and February 1986, while Andrulis et al. (1992) estimated US$ 30,000 per person-year.

Comparisons between European studies are also difficult. Tolley and Gyldmark (1993) reviewed costs of treatment, care, and support for HIV-positive and AIDS patients in eleven European countries, which were based on data from the second half of the eighties. The authors inflated cost figures to 1990 prices and converted them from local currency to US$ by using national healthcare-specific price indices and health-specific purchasing power parities. The standardized cost estimates ranged between US$ 1,700 (social care per HIV-positive) and US$ 28,200 (hospital care per AIDS person-year), with the exception of a Greek study, which produced an adjusted cost estimate for the hospital treatment and care of AIDS patients of US$ 70,400 per person-year.

The cost structure strongly changed with the introduction of HAART. The use of potent combinations of nucleoside reverses transcriptase inhibitors, protease inhibitors, and non-nucleoside reverse transcriptase inhibitors have resulted in a great decline in the rates of HIV disease progression. The costs associated with hospitalization decreased markedly, but the proportion of patients receiving these therapies, the drug costs, and the costs of associated illnesses increased dramatically with time. Because of country-specific price discrepancies, costs of antiretroviral therapy in country comparisons vary. This is quite obvious for industrialized vs. developing countries (e.g. Bautista et al. 2003; Ministry of Health (Brazil) 2003; Levi and Vitoria 2002). However, beside this general trend figure there are substantial variations between the concrete results of studies.

Is there any conclusion that we can draw? With all carefulness and in due respect to the strong differences between countries, we can state that *the reduced costs of hospitalization justify the high cost of HAART in countries where hospitalization and long-term care are provided to all HIV/AIDS patients. In countries with low hospital services (e.g., developing countries) this might be different. On the contrary, HAART is strongly increasing the number of years for which we have to finance this expenditure as the life expectancy is strongly increasing. The lifetime provider costs have to be compared with the reduced lifetime indirect costs of HIV/AIDS. In countries with a high gross national product, we can conclude that the reduction of indirect costs due to HAART is higher than the increase of lifetime provider costs so that HAART seems a wise investment – not only from a humanitarian, but also from an economic point of view. In countries with a low gross national product and, in particular, for people without a strong contribution to the society (pensioners, unemployed etc.), it is questionable whether the reduced indirect costs really justify HAART. There it remains an issue of ethics and humanity whether the society is able and willing to support this group of the society even if this investment does not pay back.*

4 Strategic Costs

The majority of studies presented earlier have a limited time horizon and merely document the annual cost of HIV/AIDS in a particular year. Cost of treating side-effects of HAART and patient compliance are frequently neglected. Bautista et al. (2003) showed that drug resistance due to poor compliance might lead to higher costs, and Tornero et al. (2005) estimated for Spain that this effect might be about 20% of total provider costs. Other authors (e.g., Becker and Shakur 2001; Moatti et al. 2004; Baggaley et al. 2005; Munakata et al. 2006) also suggest that HIV/AIDS costs for patients might be underestimated if long-term effects (such as resistance) are neglected.

The long-term consequences of antiviral intervention can be excluded in short-term, operational studies, but they must be considered in a long-term analysis of strategic costs. It is obvious that this kind of analysis must remain imprecise, rough, and sometimes even speculative. However, this is not unusual for strategic management. Long-term plans and analyses must include all possible consequences of interventions, even if they might be unlikely in the nearer future. Their direct and indirect costs and risks must be assessed and included into the analysis. Causal loop diagrams, as demonstrated in Fig. 3, are frequently used to indicate the interdependencies of system components. Systems of such complexity can hardly be understood

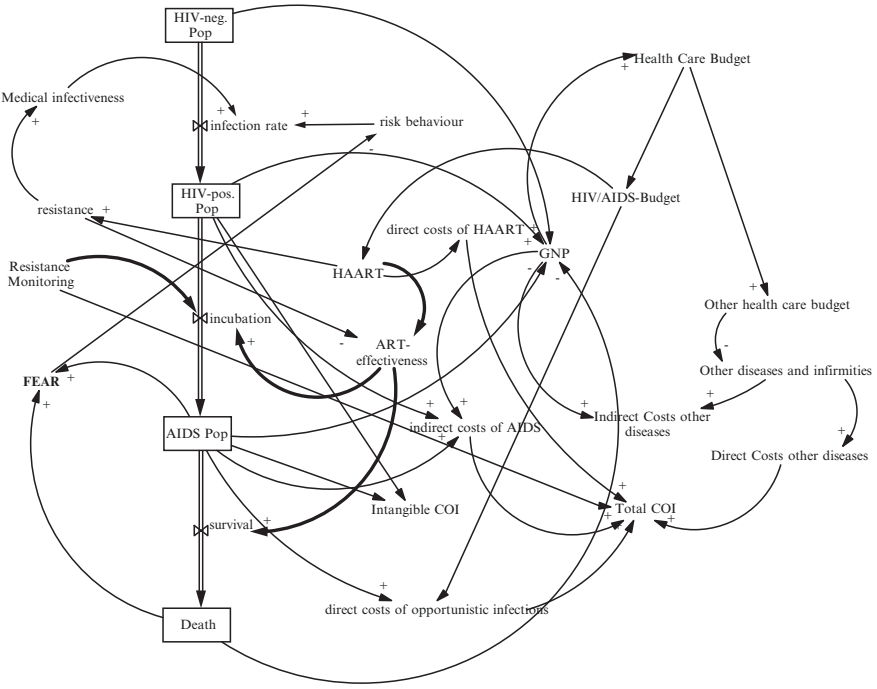

Fig. 3 Causal loop diagram of Costs-of-AIDS

by humans, but they can be handled by System Dynamics Models (Milstein and Homer 2006; Joffe and Mindel 2006, 473–479, Sterman 2000; Goodman 1988; Clark 1988). As Homer and Hirsch (2006, 452–458), Sterman (2006, 505–514), and Jones et al. (2006, 488–494) show, they are in particular helpful in predicting the long-term epidemiological and socio-economic impacts of short-term interventions.

The bold lines demonstrate the short-term system. It is obvious that no feedback loops exist between antiviral intervention and other elements of the system. Therefore, the total COI can be calculated by just adding the independent cost components.

In a long-term perspective, a number of important feedback loops might exist, with tremendous consequences on the COI and the cost-effectiveness of intervention programmes. Some of these loops were discussed in the Special Issue on HIV/AIDS of the Lancet (Vol. 368, August 5–11, 2006), but we would like to stress their importance for the long-term socio-economic impact of antiviral intervention.

First, antiretroviral drugs might cause resistance. Already today we see that "emergence of drug resistance is the most common reason for treatment failure" (Simon et al. 2006), and "drug-resistant HIV-1 is transmissible and can be detected in up to 20% of newly infected individuals in countries with broad access to antiretrovirals. The prevalence of drug resistance in the untreated population remains low in regions with poor access to treatment." Drug resistance will definitely have an impact on costs, as more expensive drugs will have to be developed and applied, and it might also increase the medical infectiveness with a resulting increase of AIDS cases.

Second, sexual behavior might change if the "good message" of a treatment of HIV/AIDS spreads. Already today we have an increase of incidence in some countries, such as Germany. This might be partly due to the long-term consequence of a reduced "horror."

Third, there is a strong interdependency between AIDS and economic strength (express in gross national product, GNP). On the one hand, patients treated with antiviral drugs are longer able to contribute to the economic wealth of a nation, on the other hand, the drug regime requires high resources. Assuming a fixed health care budget, these costs have to be deducted from the health care resources of other diseases. Antiviral intervention might result in higher COI of other diseases, but without appropriate budgets for these neglected diseases.

Strategic management and long-term cost analysis cannot provide exact figures, but they should point at risks and potential shortcomings. *Antiretroviral therapy seems quite economically favorable in the short-run, but we might expect the highest costs still to come*. This is particularly true for resource-poor countries, such as Sub-Saharan Africa, where health care resources invested in therapy of AIDS patients might be missing for prevention of HIV and prevention and cure of other diseases.

Does this call for a cancellation of antiviral interventions? Definitely not! But it calls for a courageous long-term analysis of the COI of AIDS and of the cost-effectiveness of certain interventions, and it calls for the political will to reallocate public budgets towards the health care sector, as the highest socio-economic costs are most likely still to come.

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