Antiviral Strategies

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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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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

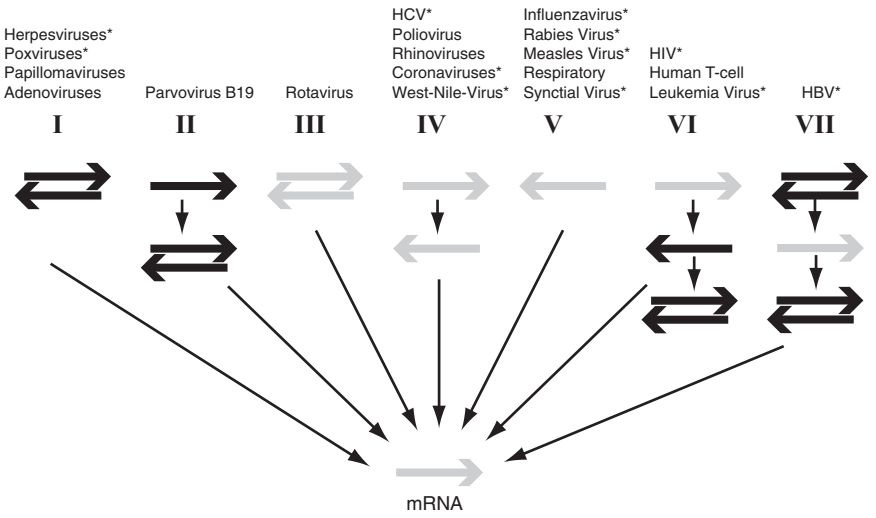

Fig. 1 Classification of viruses by their genome replication strategy according to Baltimore (Baltimore 1971). Examples for important human pathogens falling into the respective class are listed above. *Black*: DNA, *gray*: RNA; *arrows to the right*: (+) strand polarity (i.e., corresponding to mRNA);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

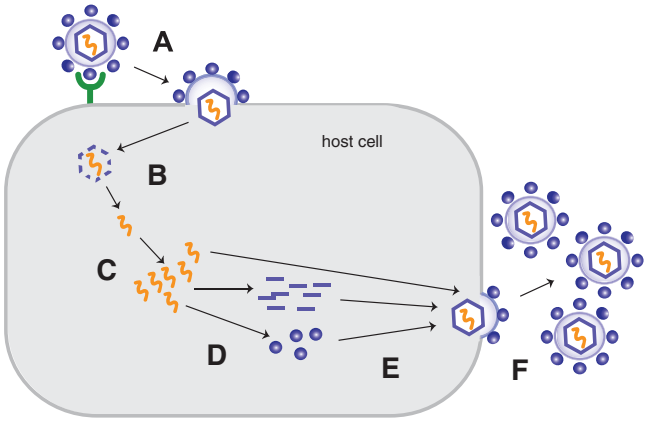

Fig. 2 Basic steps of viral replication: (**a**) binding, (**b**) entry, (**c**) genome replication, (**d**) gene expression, (**e**) assembly, and (**f**) release. Reprinted with permission from Müller and Kräusslich (2008)

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

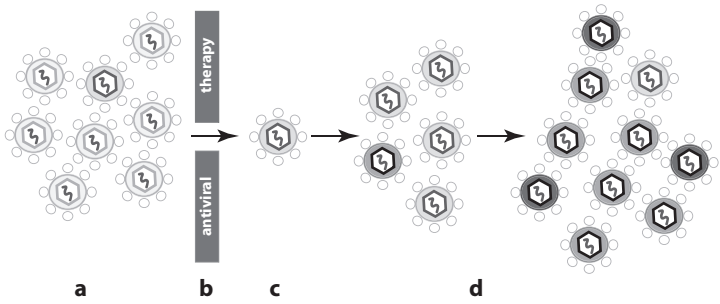

Fig. 3 Stepwise development of antiviral resistance. Because of the rapid mutation rate of viruses, the virus population before treatment (**a**) contains variants, which display by chance a low level of resistance to the drug (indicated by the darker hue). Treatment with suboptimal levels of an antiviral 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.

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