Topics in Medicinal Chemistry 15

Wibke E. Diederich
Holger Steuber *Editors*

Therapy of Viral Infections

Springer

15
Topics in Medicinal Chemistry

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Editors

Therapy of Viral Infections

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Preface

Even though many viral infections can meanwhile be prevented by prophylactic vaccination, there is still urgent need for the development of new antiviral agents tackling contemporary afflictions of mankind such as HIV, Dengue Fever, West-Nile-Fever, or influenza, to name just a few, as for those infections either no vaccination exits or has to be reformulated annually due to antigenic drift and shift of the virus as in case of influenza.

In the last decades tremendous progress in the development of antiviral agents has been made resulting, e.g., in case of HIV, not only in a significant prolongation but also in an improvement of the quality of the patient's life. Nevertheless, development of resistance towards an approved drug often hampers the clinical use and might in the worst case result in a lack of treatment options for certain patients. Thus, not only the continuous development of new drugs but also the identification and validation of novel drug targets is of utmost importance to ensure that these infections can be adequately treated.

This special issue of *Topics in Medicinal Chemistry* "Therapy of viral infections" focuses on the one hand on recent developments of inhibitors against well-established drug targets and on the other hand new targets on either the virus or its host side are presented.

The issue starts with a review by Demeulemeester, De Maeyer, and Debyser on HIV-1 integrase inhibitors spanning from the first approved inhibitors to recent developments in this intriguing field. In the following, Macchi, Romeo, Chiacchio, Frezza, Giofré Marino-Merlo, and Mastino highlight in their contribution the development of phosphonated nucleoside analogues starting from the well-known nucleoside analogues such as AZT towards the newer derivatives of acyclic nucleoside phosphonates and phosphonated carbocyclic nucleosides.

In the following Steuber, Kanitz, Ehlert, and Diederich provide a comprehensive review of recent advances targeting the Dengue and West-Nile Protease utilizing small molecule inhibitors. Steinmetzer, Hardes, Böttcher-Friebertshäuser, and Garten focus in their contribution on strategies for the development of influenza drugs. Besides approved drugs and their further development, especially alternative strategies such as inhibition of the fusion and the uncoating process, as well as inhibition of HA-activating host proteases and additional innovative strategies that interfere with host mechanisms, are discussed in detail. Finally, Dürr, Keppler,

Christ, Crespan, Garbelli, Maga, and Dietrich provide a comprehensive review on targeting cellular co-factors in HIV therapy, which might offer new opportunities to interfere with virus replication and, moreover, reduce the development of drug resistance.

We are grateful to all authors of this special issue of *Topics in Medicinal Chemistry* "Therapy of viral infections" for the valuable contributions and also would like to thank all reviewers for their very constructive comments.

In addition, we thank the series editors Bernstein, Buschauer, Georg, Lowe, Stilz, Supuran, and Saxena for the invitation and the opportunity to compile this special issue. Finally, we would like to acknowledge the Springer publishing editor Wassermann and the project coordinator Jaeger for the smooth handling of technical aspects related to the special issue.

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HIV-1 Integrase Drug Discovery Comes of Age

Jonas Demeulemeester, Marc De Maeyer, and Zeger Debyser

Abstract Insertion of the viral genome into host cell chromatin is a pivotal step in the replication cycle of the human immunodeficiency virus and other retroviruses. Blocking the viral integrase enzyme that catalyzes this reaction therefore provides an attractive therapeutic strategy. Nevertheless, many years lie between the initial discovery of integrase and the clinical approval of the first integrase strand transfer inhibitor, raltegravir, in 2007. Recently, elvitegravir was second to make it into the clinic, while dolutegravir, a second-generation integrase inhibitor, is close to receiving the green light as well. Viral resistance and cross-resistance among these strand transfer inhibitors however warrant the search for compounds targeting HIV integration through different mechanisms of action. The most advanced class of allosteric integrase inhibitors, coined LEDGINs or non-catalytic integrase inhibitors (NCINIs), has shown remarkable antiviral activity that extends beyond the viral integration step. Time will tell however if they will stand the test of clinical development. Notably, the development of LEDGINs and other integrase inhibitors is aided by recent structural and mechanistic insights into the retroviral integration apparatus. Here we provide an overview of the development of integrase strand transfer and allosteric inhibitors while exploring their mechanisms of action and patterns of viral resistance.

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Keywords Allosteric, HIV, LEDGF/p75–IN interaction, Non-catalytic site integrase inhibitor, Strand transfer

Contents

Abbreviations

1-LTR	1-Long terminal repeat
2-LTR	2-Long terminal repeats
3P	3′-Processing
ADME	Absorption distribution, metabolism, and excretion
AIDS	Acquired immunodeficiency syndrome
ALLINI	Allosteric integrase inhibitor
ART	Antiretroviral therapy
ARV	Antiretroviral
CC_{50}	50% cytotoxic concentration
CCD	Catalytic core domain
CCR5	C–C chemokine receptor 5
CR	Charged region
CTD	C-terminal domain
DKA	Diketo acid
DNA	Deoxyribonucleic acid
EC_{50}	50% Effective concentration
FDA	Food and Drug Administration
HAART	Highly active antiretroviral therapy
HCV	Hepatitis C virus

HDGF	Hepatoma-derived growth factor
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HRP-2	Hepatoma-derived growth factor-related protein 2
HSAB	Hard and soft Lewis acids and bases
IBD	Integrase-binding domain
IC_{50}	50% inhibitory concentration
IN	Integrase
INI	Integrase inhibitor
IRBM	Instituto di Ricerche di Biologia Moleculare
LEDGF/p75	Lens epithelium-derived growth factor/p75
LTR	Long terminal repeat
MACCS	Molecular access system
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCINI	Non-catalytic site integrase inhibitor
NLS	Nuclear localization signal
NMR	Nuclear magnetic resonance
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NTD	N-terminal domain
$PAEC_{50}$	Protein-adjusted 50% effective concentration
$PAIC_{50}$	Protein-adjusted 50% inhibitory concentration
PFV	Prototype foamy virus
PHAT	Pseudo-HEAT analogous topology
PIC	Pre-integration complex
PK	Pharmacokinetic
PPI	Protein–protein interaction
PR	Protease
PrEP	Preexposure prophylaxis
PWWP	Pro–Trp–Trp–Pro domain
RNA	Ribonucleic acid
RT	Reverse transcriptase
SAR	Structure–activity relationship
SIV	Simian immunodeficiency virus
SPR	Surface plasmon resonance
ST	Strand transfer
STD-NMR	Saturation transfer difference nuclear magnetic resonance
tDNA	Target deoxyribonucleic acid
TRN-SR2	Transportin-SR2
vDNA	Viral deoxyribonucleic acid
WT	Wild type

1 Introduction

Human immunodeficiency virus type 1 (HIV-1) is a single-stranded, positive RNA virus of the genus *Lentivirinae*. Its roots can be traced back to Africa to at least four individual zoonotic infections with simian immunodeficiency viruses (SIV) from gorilla and chimpanzee [1]. A disease was however not recognized until the first reports of clustered cases of *Pneumocystis carinii* pneumonia [2] and Kaposi's sarcoma [3] appeared in 1981. It took two more years before the research groups of Montagnier and Gallo isolated a new virus, dubbed lymphadenopathy-associated virus by Montagnier [4] and human T-lymphotropic virus III by Gallo [5], which they suggested to be the etiological agent of a disease known by that time as acquired immunodeficiency syndrome (AIDS). The virus obtained in the two studies was shown to be the same and was renamed human immunodeficiency virus (HIV) in 1986. Today, over 34 million people from all over the world are living with the virus and over 30 million have already passed away. Even though the number of new infections globally is on the decline, it was still estimated at 2.5 million in 2011 [6].

The success of HIV-1 stands in shrill contrast to its apparent simplicity. Its small 9.7 kb genome encodes a mere 15 mature proteins which allow it to elegantly manipulate the infected cell and subvert the innate and adaptive immune responses of the host, resulting in a persistent infection in humans. Viral replication proceeds through a number of characteristic steps, depicted in Fig. 1 (reviewed in [7]). Briefly, after attachment and membrane fusion, the viral core enters the cell and uncoats while the reverse transcriptase (RT) creates a double-stranded DNA copy of the viral genome and a pre-integration nucleoprotein complex (PIC) is formed. The PIC is imported into the nucleus where integrase (IN) catalyzes insertion of the viral DNA (vDNA) into the host chromatin, irreversibly establishing a provirus in the host cell. Transcription and translation take place, giving rise to viral proteins that assemble into budding virions at the cell membrane. Released particles undergo proteolytic maturation by the viral protease (PR), producing novel infectious virions. Each of these steps represents a potential target for antiretroviral therapy (ART). Notably, RT and PR have thus far been targeted most extensively. Clinical use of their inhibitors has markedly improved patient survival and delayed progression to AIDS, resulting in the present-day decrease in incidence as well as in HIV-/AIDS-related mortality.

Since the identification of the first antiviral for clinical use against AIDS, zidovudine (3′-azido-2′,3′-dideoxythymidine, AZT) in 1985, ART has evolved significantly (reviewed in [8]). Treatment developed from AZT monotherapy in the late 1980s over dual therapy of AZT plus zalcitabine (2′,3′-dideoxycytidine, ddC) to the current standard of care, triple therapy. This combination therapy includes at least three antiviral compounds and aims for high synergy, low toxicity, and reduced resistance development. As such, it has come to be known as highly active antiretroviral therapy (HAART) [8]. Thirty years of research and clinical translation have endowed us with a repertoire of 25 US Food and Drug Administration (FDA)-approved compounds,

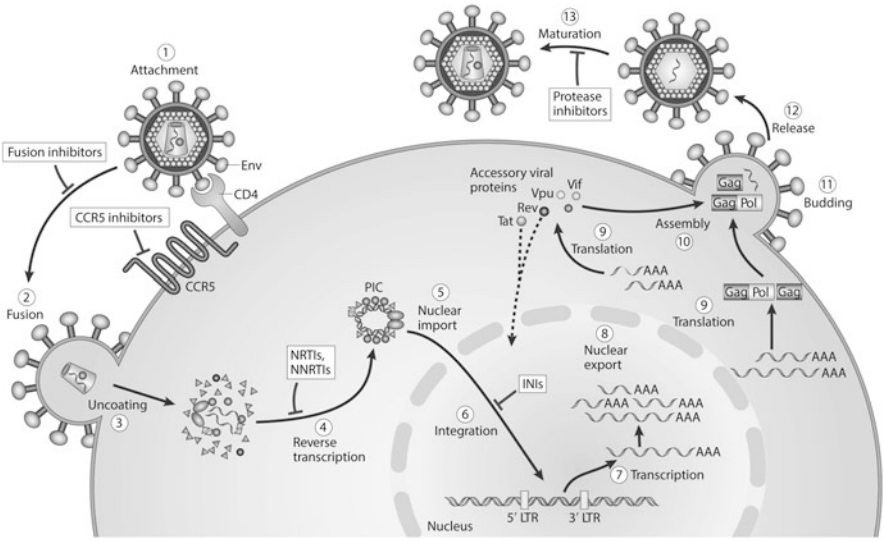


Fig. 1 Replication cycle of HIV-1. After Env-mediated attachment (*1*) of the virus to the cell via the CD4 receptor and binding to the CXCR4 or CCR5 coreceptor, fusion (*2*) of the viral membrane and the cell membrane is initiated. This releases the conical capsid into the cytoplasm where uncoating (*3*) takes place. In the meantime reverse transcriptase creates a double-stranded DNA copy of the viral genome (*4*), which associates with various viral and cellular proteins to form the pre-integration complex (PIC). This nucleoprotein complex is actively imported (*5*) into the nucleus where integrase catalyzes insertion (*6*) of the viral DNA into the host chromatin. Proviral transcription (*7*) and export (*8*) of viral mRNA allow for translation (*9*) of new viral proteins that in turn assemble (*10*) into budding particles (*11*) at the cell membrane. These particles are released (*12*) from the cell and mature (*13*) through polyprotein processing by the viral protease, completing the viral replication cycle. Steps that are targeted in antiretroviral therapy are indicated. CCR5, C-C chemokine receptor 5; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI; non-nucleoside reverse transcriptase inhibitors; INIs, integrase inhibitors; LTR, long terminal repeat. Adapted by permission from Macmillan Publishers Ltd: Nat Rev Microbiol [7], copyright 2012

falling into six distinct classes based on the viral target, which represent the building blocks for HAART (Table 1).

Irrespective of this seemingly large armamentarium of drugs, HIV's vast evolutionary potential allows for rapid emergence of resistant variants. As no cure is available and lifelong treatment is required, the door is always open to resistance development. Additionally, an effective vaccine remains beyond our grasp and hence research efforts focused on exploring novel drug targets in retroviral biology are critical to control the HIV/AIDS pandemic. The main retroviral target that was clinically unexplored until 2007 is the third viral enzyme, IN. Indeed, it is evident from Table 1 that inhibitors of the viral RT or PR far outnumber and predate those targeting IN. There are two main reasons for this. First, for many years a relevant high-throughput screen for IN inhibitors was lacking, and few of the compounds that showed inhibition in vitro retained

Table 1 Antiretrovirals currently approved by the FDA

Approval date	Generic name	Brand name
Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs)		
1986	Zidovudine (ZDV or AZT)	Retrovir
1991	Didanosine (ddI)	Videx
1992	Zalcitabine (ddC)[a]	Hivid
1994	Stavudine (d4T)	Zerit
1995	Lamivudine (3TC)	Epivir
1998	Abacavir (ABC)	Ziagen
2001	Tenofovir disoproxil fumarate (TDF)	Viread
2003	Emtricitabine (FTC)	Emtriva
Protease inhibitors (PIs)		
1995	Saquinavir (SQV)	Invirase
1996	Ritonavir (RTV)	Norvir
1996	Indinavir (IDV)	Crixivan
1997	Nelfinavir (NFV)	Viracept
2003	Atazanavir (ATV)	Reyataz
2003	Fosamprenavir (FPV)	Lexiva
2005	Tipranavir (TPV)	Aptivus
2006	Darunavir (DRV)	Prezista
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)		
1996	Nevirapine (NVP)	Viramune
1997	Delavirdine (DLV)	Rescriptor
1998	Efavirenz (EFV)	Sustiva
2008	Etravirine (ETR)	Intelence
2011	Rilpivirine (RPV)	Edurant
Fusion inhibitors (FIs)		
2003	Enfuvirtide (T-20)	Fuzeon
CCR5 antagonists		
2007	Maraviroc (MVC)	Selzentry
Integrase inhibitors (INIs)		
2007	Raltegravir (RAL)	Isentress
2012	Elvitegravir (ELV)[b]	

[a]Zalcitabine is no longer marketed
[b]At present elvitegravir is only approved as part of the fixed-dose combination Stribild

their effect when assayed on viral replication. Second, although many nuclear magnetic resonance (NMR) and crystal structures (X-ray) for all three domains of HIV-1 IN were solved [9], structural information on the full-length protein and its catalytic assemblies was lacking. Together, these elements rendered IN drug discovery effectively blind for many years. This chapter looks at how integrase drug discovery has recently come of age with the development of two potent inhibitor classes and crucial new insights into the functioning of the enzyme itself. The results do not only epitomize scientific breakthroughs, but represent vital milestones to maintain the success of HAART in the future.

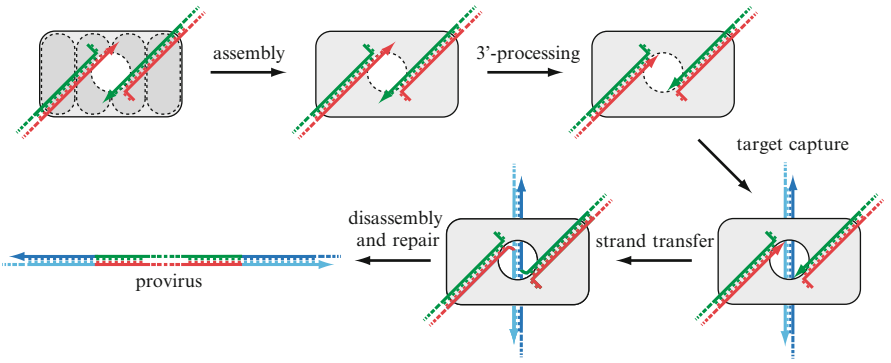


Fig. 2 Schematic overview of the integration process. IN assembles on the vDNA ends and catalyzes the 3′-processing reaction. The tetrameric IN–vDNA assembly known as the intasome can then capture a tDNA strand and carry out strand transfer. Disassembly of the complex and repair of the gapped intermediate by cellular enzymes results in a stably integrated provirus

2 Integration

After nuclear import of the PIC, the viral genome is inserted into host chromatin. Viral IN is the central player here, catalyzing the essential DNA cutting and joining reactions (represented schematically in Fig. 2 and reviewed in [10]). Briefly, following its assembly with the vDNA, IN removes a dinucleotide from both 3′ ends, a step known as 3′-processing (3P). After capture of the host target DNA (tDNA) a tetrameric IN complex with vDNA, the intasome, catalyzes the strand transfer (ST) reaction with the recessed ends. The result is a gapped intermediate where both vDNA strands are joined by their 3′ ends to opposing tDNA strands. The insertion sites are separated from each other by five base pairs in the case of HIV. The gaps are repaired by host cell repair machinery and a provirus is established.

2.1 Integrase: Structure and Function

All retroviral INs contain three structurally conserved domains, an N-terminal, a catalytic core, and a C-terminal domain (NTD, CCD, and CTD, respectively, Fig. 3; see [9–11] for recent structural reviews). The three canonical domains are connected through flexible linkers and are all essential for the biological activity of the enzyme. The NTD is a three-helix bundle stabilized by coordination of a Zn^{2+} ion through its His_2-Cys_2 (HH-CC) zinc-binding motif (Fig. 3a, b) [12, 13]. The CCD embodies the catalytic heart of the enzyme and bears a DD-X_{35}-E motif characteristic of the DDE(D) nucleotidyltransferase family, which includes various transposases as well as RNase H [14]. The signature DDE(D) residues are brought together in the tertiary structure at the active site where they form a triad

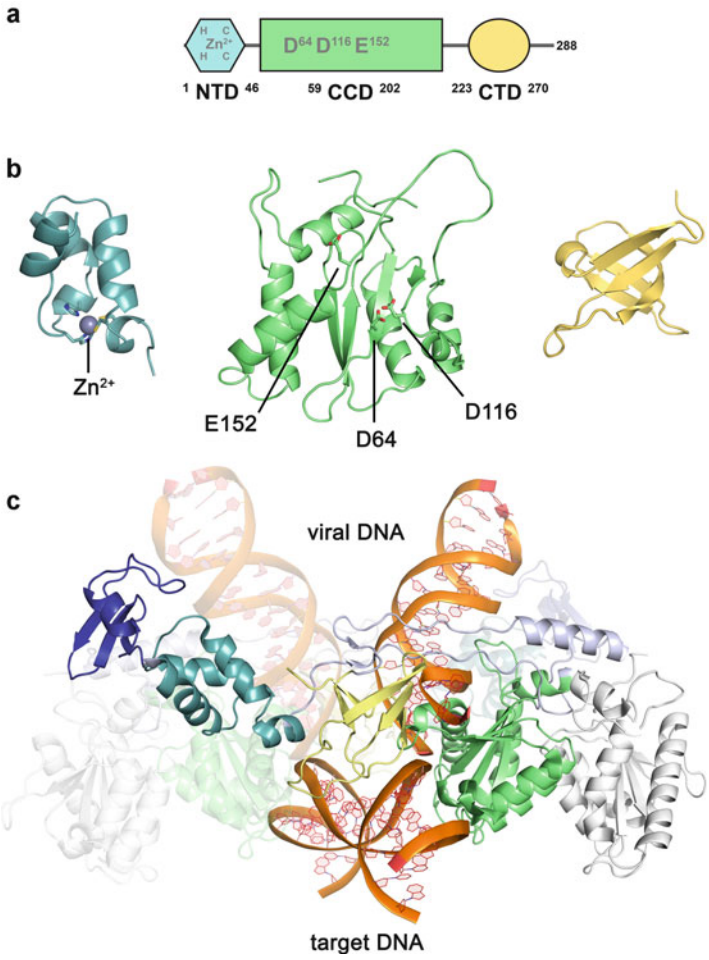


Fig. 3 Structure of retroviral integrase. (**a**) Domain structure and boundaries of HIV-1 integrase. (**b**) From left to right, tertiary structures of the N-terminal [12, 13], catalytic core [15], and C-terminal [16] domains (NTD, CCD, and CTD, respectively). The HH-CC Zn^{2+} binding site and the DDE catalytic triad residues are indicated. (**c**) Structure of the prototype foamy virus (PFV) intasome [18]. One of the two symmetry-related integrase dimers in the assembly is rendered transparent for clarity. The inner monomers containing the two active sites of the complex are color-coded as in (**a**) and (**b**), while the outer supporting monomers are *grey*. The two oligos representing viral DNA ends can be seen to enter the complex from the top while the target DNA lies perpendicular to the paper. PFV integrase contains an additional N-terminal extension domain (NED, colored *deep blue*) on its N-terminus, which is not present in HIV-1 [17]

of carboxylates that coordinate two metal ions (Mg^{2+} or Mn^{2+}) essential for the enzyme's catalytic function (Fig. 3a, b) [15]. The CTD folds into a five-strand β-barrel which is reminiscent of a Src homology 3 domain (SH3, Fig. 3a, b) [16]. All domains are involved in DNA binding and multimerization but the CCD harbors the entire active site.

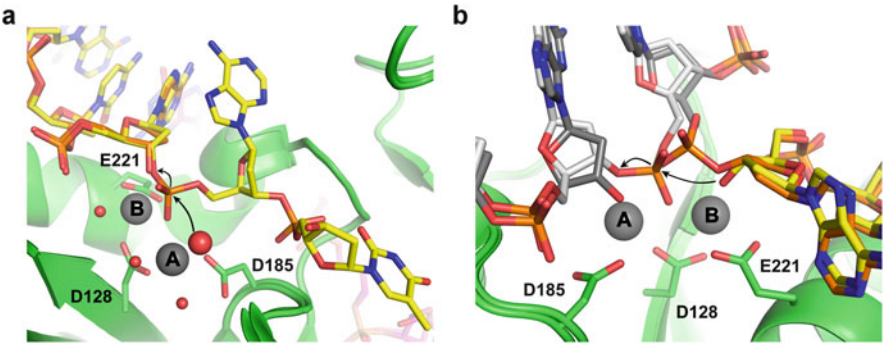


Fig. 4 Integrase catalysis: superposition of the intasome active site before and after (**a**) 3′-processing and before and after (**b**) strand transfer [17, 19]. PFV IN catalytic triad residues D128, D185, and E221 as well as Mg^{2+} ions A and B are indicated. The viral DNA before and after each reaction is shown in *yellow* and *orange*, respectively. Target DNA in (**b**) is shown in *light* and *dark grey*, respectively, before and after

As stated above, full-length IN has proven refractory to structural studies. Fortunately though, the *Retroviridae* family provides ample flavors of IN. Although overall sequence identity is low, structural homology is very strong among these orthologous proteins, allowing their use as a proxy for HIV IN [17]. One of these proteins, the prototype foamy virus (PFV, genus *Spumavirinae*) IN, was ultimately crystallized as a functional intasome assembly with vDNA ends [17]. These complexes were further characterized by inclusion of tDNA and/or inhibitors [18]. The structures unveiled the intimate workings of the integration machinery and represent landmark advances in the field of retrovirology [10, 19]. The intasome consists of a dimer of IN dimers, in which the two inner subunits bridge both dimers and at the same time establish extensive contacts with the vDNA (Fig. 3c) [17]. The unprocessed vDNA ends are partially unwound inside the complex and the scissile phosphodiester bond linking the invariant 3′ CA dinucleotide to the terminal GT is coordinated by Mg^{2+} ion B (Fig. 4a). Activation and alignment of an H_2O nucleophile through coordination by the neighboring Mg^{2+} ion A results in S_N2 nucleophilic substitution on the phosphodiester with the vDNA 3′ hydroxyl as a leaving group [19]. This 3′-processed intasome can accommodate tDNA (forming a target-capture complex) in a groove between the inner monomers, where it is heavily bent (Fig. 3c) [18, 19]. The major groove is pried open, positioning the tDNA scissile phosphodiester bonds in the coordination sphere of Mg^{2+} A (Fig. 4b) [18, 19]. As the vDNA 3′ hydroxyl is still coordinated to Mg^{2+} ion B, this results in catalysis of a second S_N2 reaction where the 3′ hydroxyl attacks the tDNA phosphodiester and the tDNA 5′ of the scissile bond is released [19]. The newly formed phosphodiester is displaced by 2.3 Å from the active site after transesterification, rendering insertion of the vDNA strands into the host genomic DNA scaffold essentially irreversible (Fig. 4b) [18, 19].

The structure of the target-capture complex indicates that most IN-tDNA interactions involve the phosphodiester backbone, imposing little sequence preference on the local integration site [18]. Weak local sequence signatures can be discerned though for different integrases [20, 21]. Much more pronounced are the genus-specific biases among *Retroviridae* for integration into specific genomic contexts. HIV-1 for example will preferentially integrate into the body of actively transcribed genes, whereas murine leukemia viruses (MLV) integrate near the transcription start site [22]. In the case of HIV, this specific bias is brought about by the host protein LEDGF/p75 [23].

2.2 LEDGF/p75: A Crucial Cellular Cofactor of Integration

LEDGF/p75 is a 530 amino acid nuclear protein that is, together with its smaller 333 amino acid splice variant LEDGF/p52, encoded by the *PSIP1* gene located on chromosome 9p22.2 [24]. LEDGF is a member of the hepatoma-derived growth factor (HDGF) family, which is characterized by a conserved N-terminal PWWP domain (Fig. 5a, b). This domain, with its signature Pro–Trp–Trp–Pro motif is a chromatin reader belonging to the Tudor domain "royal family" and is able to bind simultaneously and synergistically to DNA and methyl lysines (i.c. likely H3K36me3) present on histone tails ([25] and reviewed in [26]). The region following the PWWP is presumed to be disordered in LEDGF but contains a classical nuclear localization signal (NLS), two AT-hooks (a minor-groove DNA-binding motif consisting of a Pro–Arg–Gly–Arg–Pro core that preferentially binds AT-rich sequences), and four charged regions (CR1-4, Fig. 5a) [27, 28]. CR2-4 were recently found to constitute a supercoiled-DNA recognition domain (SRD) [29]. Together, these regions constitute the chromatin-binding region of LEDGF, which targets the protein into the body of active transcription units [30]. In the p75 variant but not p52, the SRD is followed by an integrase-binding domain (IBD), a compact right-handed bundle of five α-helices reminiscent of a pair of HEAT repeats (pseudo-HEAT analogous topology, PHAT, Fig. 5) [31]. The C-terminal part of LEDGF/p75 is again disordered. Among HDGF family members, the IBD is only conserved in HDGF-related protein-2 (HRP-2) [32–34].

In 2003, LEDGF/p75 was identified as a binding partner of ectopically expressed HIV-1 IN through co-immunoprecipitation (Fig. 5c) [35]. After its initial implication as binding partner of IN, a crucial role was evidenced for LEDGF/p75 in HIV (and other *Lentivirinae*) replication through mutagenesis, RNA interference, transdominant overexpression of the IBD of LEDGF/p75, and knockout studies [23, 33, 35–40]. Next-generation sequencing and analysis of integration sites in various cell lines demonstrated a tethering and targeting role of LEDGF/p75 [23]. During HIV replication, LEDGF/p75 tethers the PIC via IN to the chromatin [23, 39]. Through the specific chromatin context recognition of LEDGF/p75,

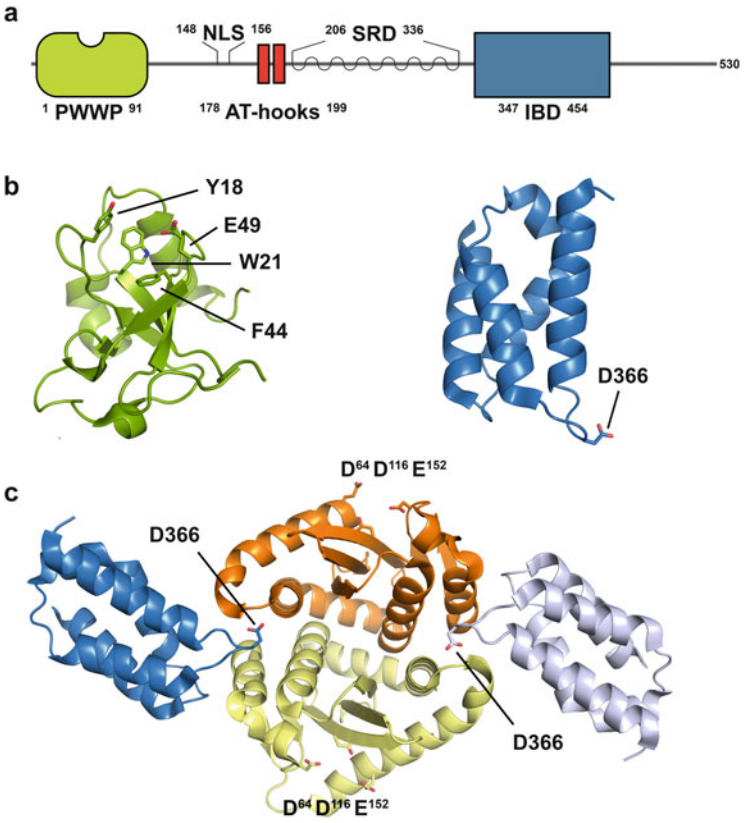


Fig. 5 Structure of LEDGF/p75. (**a**) Domain structure and boundaries of LEDGF/p75. (**b**) Structures of the PWWP [25] and integrase-binding domain (IBD) [31]. Amino acids lining the PWWP aromatic cage binding pocket for methyl lysine are labeled. LEDGF/75 residue D366 in the IBD (shown in *sticks*) is key to the interaction with HIV IN. (**c**) The integrase catalytic core dimer (CCD, *orange* and *pale orange*) bound to two IBDs (*blue* and *pale blue*) [174]. The catalytic triad and LEDGF/p75 D366 are shown in sticks

the intasome and integration are specifically targeted into the bodies of active genes [30]. Additionally, LEDGF/p75 was shown to protect IN from proteolytic degradation, modulate the IN oligomerization state, and stimulate the catalytic activity of IN both in vitro and in vivo [35, 41–43].

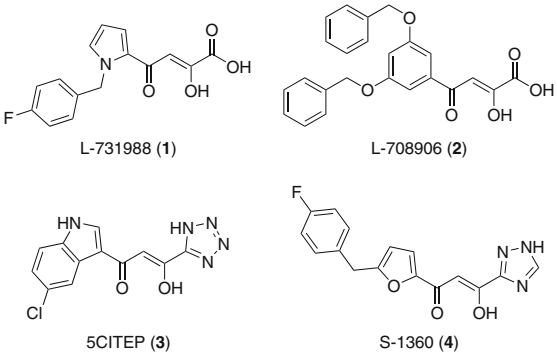
LEDGF/p75 is the best-studied IN cofactor to date, although many other candidates have been described such as transportin-SR2 (TRN-SR2, TNPO3) and integrase interactor 1 (INI1, SMARCB1; see [44, 45] for recent reviews). So far, however, LEDGF/p75 is the only one that has successfully been exploited for IN drug discovery.

3 Integrase Strand Transfer Inhibitors

Initial drug discovery efforts against IN were focused on the assembly with and the processing of viral DNA. This resulted in several inhibitors in vitro, which however did not demonstrate significant antiviral activity. The ball started rolling with the observation that the two enzymatic activities of IN, 3′-processing and strand transfer were separable and that stable complexes poised for ST could be formed in vitro. This led to the development of a high-throughput ST-specific assay at Merck West Point [46]. Screening over 250,000 compounds yielded several inhibitors, the most potent and specific of which all contained a distinct β-diketo acid (DKA) moiety.

3.1 β-Diketo Acids

Two of these potent hits were L-731988 (**1**) and L-708906 (**2**), with 50% inhibitory concentrations (IC_{50}) on ST of 133 and 300 nM and 50% effective concentrations (EC_{50}) against HIV replication in cell culture of 1 and 2 μM, respectively, without apparent toxicity (50% cytotoxic concentration, $CC_{50} > 50$ μM) [47]. These compounds were shown to be bona fide and specific integrase strand transfer inhibitors (INSTIs) and showed no effect on viral entry, reverse transcription, PIC assembly, or 3P. They did however result in an increase of 2-long terminal repeat (2-LTR) circles, a form of viral DNA that is a by-product of abortive integration in the cell. Additionally, resistance selection resulted in T66I and S153Y substitutions in IN close to the active site, corroborating the viral target [47]. DKAs were found to compete with tDNA for IN binding and required the presence of processed vDNA in the complex [48].

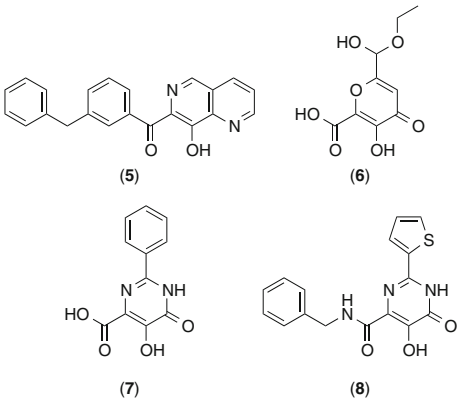


At the same time, scientists at Shionogi & Co., Ltd., developed 5CITEP (**3**), a diaryl diketone in which the DKA carboxylic acid had been subject to bioisosteric replacement by tetrazole [49, 50]. 5CITEP displayed in vitro IC_{50} values of 35 and 0.65 μM against 3P and ST, respectively, but only weak antiviral activity in MT-4 cells ($EC_{50} > 50$ μM) [51]. Crystals of the HIV-1 IN CCD without vDNA were soaked in the presence of this compound and two copies appeared to be bound near

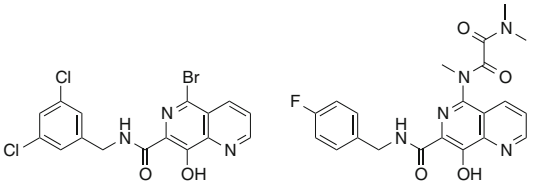
catalytic triad residues D64, D116, and E152 [52]. The resulting binding mode is influenced by crystal packing and the absence of vDNA but clearly shows the coplanar arrangement of the DKA analogous system. Further medicinal chemistry efforts replaced the 5CITEP indole with a variety of substituted heterocycles [49], culminating in the development by Shionogi & Co., Ltd., and GlaxoSmithKline plc of S-1360 (**4**), the first INI to enter clinical trials. S-1360 exhibited potent inhibition of ST in vitro (IC_{50} of 20 nM) and blocked viral replication, as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)/MT-4 assay, with an EC_{50} of 200 nM and a CC_{50} of 12 μM [53]. Note that the in vitro activities in these early days were mostly determined with Mn^{2+} as a cofactor instead of Mg^{2+}, the relevant ion employed by the enzyme during viral replication. The acid replacements in 5CITEP and S-1360 showed a distinct preference for Mn^{2+} as a cofactor, hence (at least partially) explaining the discrepancies between in vitro and antiviral activities [54]. The reduced activity together with an observed rapid clearance through reduction and glucuronidation resulting in low plasma levels of S-1360 likely contributed to the discontinuation of its clinical development [55].

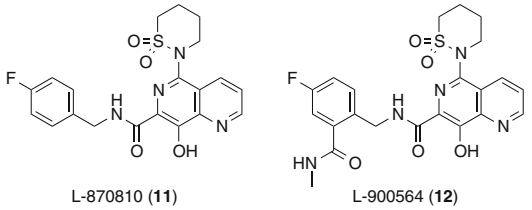
3.2 Raltegravir (MK-0518)

In the meantime at Merck West Point, the nonoptimal diaryl diketone scaffold was replaced by 1,6-naphthyridine (e.g., **5**). This template provided similar strand transfer inhibition and antiviral activity [56]. Simultaneously, at Merck Rome (Instituto di Ricerche di Biologia Moleculare, IRBM), researchers working on the seemingly unrelated hepatitis C virus (HCV) NS5b RNA-dependent RNA polymerase had also identified diketo acids and related meconic acid derivatives (**6**) as lead structures [57, 58]. Further design led them, among others, to a 4,5-dihydroxypyrimidine carboxylic acid template (**7**) [58]. Striking similarities between the compounds from both projects and the Mg^{2+}-dependent catalysis of the two enzymes spurred cross-fertilization between the programs. While simple acid derivatives of **7** showed weak or no activity against HIV integrase, amide **8** proved to be a potent inhibitor of the strand transfer reaction (IC_{50} = 18 nM and EC_{95} = 10,000 nM) [59, 60].



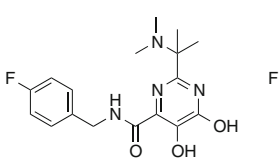
Comparison between the naphthyridine and dihydroxypyrimidine structures suggested replacement of the phenyl ketone by a simpler amide, giving rise to 1,6-naphthyridines with a carboxamide linker at position 7 (e.g., **9**; $IC_{50} = 90$ nM and $EC_{95} = 1{,}250$ nM) [61]. Further merging of the naphthyridine and dihydroxypyrimidine structure–activity relationship (SAR) showed a 4-fluorobenzylamide to provide the highest potency and suggested modification on the naphthyridine 5-position. Reduction of protein binding and in vivo clearance resulted in the oxalyl amide derivative L-870812 (**10**), which inhibited strand transfer with an IC_{50} of 40 nM and viral replication with an EC_{95} of 103 nM. The activity of L-870812 against simian immunodeficiency virus (SIV, $EC_{95} = 350$ nM) allowed evaluation of its oral efficacy in rhesus macaques [62]. This study demonstrated for the first time integrase inhibitor efficacy in vivo. A more potent compound, L-870810 (**11**; $IC_{50} = 10$ nM and $EC_{95} = 15$ nM), proceeded into phase Ib clinical trials [63]. Short-term monotherapy of L-870810 in HIV-1-infected patients proved efficacious in reducing the viral load (as measured by RNA copies/mL) over 1.7 log, and the compound was generally well tolerated. Further optimization of physical properties in the naphthyridine series gave rise to L-900564 (**12**), which showed excellent potency ($IC_{50} = 10$ nM and $EC_{95} = 7$ nM), also against previously identified resistant strains, and a good pharmacokinetic (PK) profile [64]. In the meantime, however, chronic dosing of L-870810 in dogs was found to induce liver and kidney toxicity, and development of the naphthyridine series was put on hold [63].





Fortunately, exploration of the 4,5-dihydroxypyrimidine series had continued in parallel at IRBM [59]. Here as well, the 4-fluorobenzylamide was found to improve potency over the simple benzylamide of **8** [59]. However, the early dihydroxypyrimidines suffered from low cell permeability and high plasma protein binding and efforts were focused on reducing the lipophilicity [60, 65]. This led to substitution on the dihydroxypyrimidine 2-position, which, in analogy to the naphthyridine series 5-position, appeared to support a variety of substituents that

may modulate the physicochemical properties of the series. Compound **13** was the result and showed good activity (IC_{50} = 50 nM, EC_{95} = 60 nM, and protein-adjusted EC_{95}, $PAEC_{95}$ = 78 nM), selectivity (no activity against human DNA polymerases α, β, or γ), and oral bioavailability [60]. This compound however proved toxic to rodents in high doses and an alternative was needed [60, 65].



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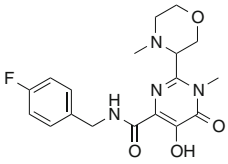

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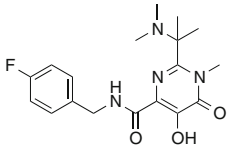

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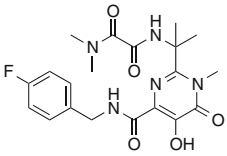

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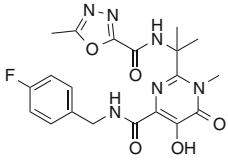
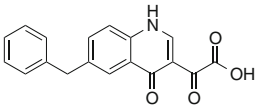


Raltegravir, MK-0518 (**17**)

In parallel, exploration of the related *N*-3-methylpyrimidone series had given rise to **14** (IC_{50} = 62 nM, EC_{95} = 60 nM, and $PAEC_{95}$ = 100 nM) which also showed promise as a clinical candidate until it was found positive in an Ames test for mutagenicity [60, 65, 66]. With the search for a clinical candidate in the two series experiencing difficulties, an attempt was made to combine the optimal properties of both. Unfortunately, the resulting compound (**15**) was less potent in both the strand transfer and viral replication assays. However, the primary amine was available as an intermediate and allowed for rapid preparation of a large set of analogs, among which the *N*,*N*-dimethyl oxalamide (**16**) in analogy to L-870812 (**10**). Despite high potency (IC_{50} = 10 nM, EC_{95} = 74 nM, and $PAEC_{95}$ = 82 nM), good oral bioavailability, and an acceptable toxicity profile in preclinical animal studies, compound development was put on hold due to a suboptimal resistance profile. Fortunately, a search for heterocycles that display a similar pattern of heteroatoms as the oxalamide rapidly led to the discovery the acyloxadiazole and MK-0518 (**17**), which has come to be known as raltegravir or Isentress. MK-0518 showed high potency (IC_{50} = 16 nM, EC_{95} = 17 nM, and $PAEC_{95}$ = 29 nM) also against variants resistant to 1,3-diketo acids, good PK in preclinical animal studies, and high selectivity with respect to nucleotidyltransferases [66, 67]. These qualities allowed the compound to progress through the clinical trials (notably the BENCHMRK [68], STRKMRK [69], and SWITCHMRK [70] studies) and gain FDA approval on 12 October 2007. Twice-daily 400 mg raltegravir induces a fast decay in the viral load and has become a valuable addition to HAART in both treatment-naïve and treatment-experienced patients.

3.3 Elvitegravir (JTK-303/GS-9137)

Meanwhile, in analogy to DKAs, their diaryl diketo and naphthyridine analogs, investigators at Japan Tobacco had conceived a 4-quinolone-3-glyoxylic acid scaffold (**18**) [71]. Surprisingly though, its precursor, the 4-quinolone-3-carboxylic acid **19**, proved to be the more potent strand transfer inhibitor (IC_{50} = 1,600 nM and EC_{50} > 30,000 nM). This indicated that the coplanar arrangement of this β-ketone and carboxylic acid might provide a substitute for the diketo acid motif. Indeed, introduction of halogens on the benzyl group provided a first increase in potency. Further optimization through substitution on the 1- and 7-positions of the quinolone system and combination of the optimal moieties led to the discovery of JTK-303 (**20**; IC_{50} = 7.2 nM and EC_{50} = 0.9 nM) [71–73]. In 2005, Japan Tobacco and Gilead Sciences entered into a license agreement to clinically codevelop the compound, which has come to be known as elvitegravir (JTK-303/GS-9137).



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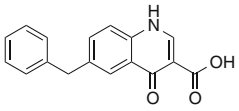

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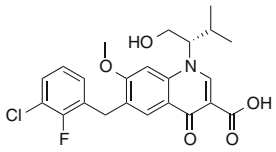


(**20**)
Elvitegravir, JTK-303/GS-9137

In contrast to raltegravir, which is administered twice-daily, elvitegravir is suited for once-daily dosing when used with a pharmacoenhancer such as ritonavir or cobicistat to inhibit cytochrome P450 3A metabolism and efflux transporters in the intestine [74]. Two clinical trials (GS-236-0102 [75] and 0103 [76]) evaluated a coformulation of elvitegravir, cobicistat, tenofovir disoproxil fumarate, and emtricitabine known as Quad or Stribild for use in treatment-naïve HIV-infected patients while the third study (GS-US-183-0145 [77]) directly compared once-daily elvitegravir to twice-daily raltegravir, both with optimized ritonavir-boosted background therapy in treatment-experienced patients. Whereas the fixed-dose combination Stribild was FDA-approved in August 2012, approval for the use of elvitegravir itself in treatment-experienced HIV-infected patients is expected in 2013.

3.4 Mechanism of Action

Owing to the high degree of conservation at the active site of retroviral integrases, INSTIs boast broad-range antiretroviral activity. Notably, also PFV integrase is susceptible to INSTI inhibition [78], which has allowed soaking of PFV intasome crystals with various INSTIs, revealing their long-sought-after mechanism of action [17, 79]. Whereas the first part of the INSTI pharmacophore, the coplanar

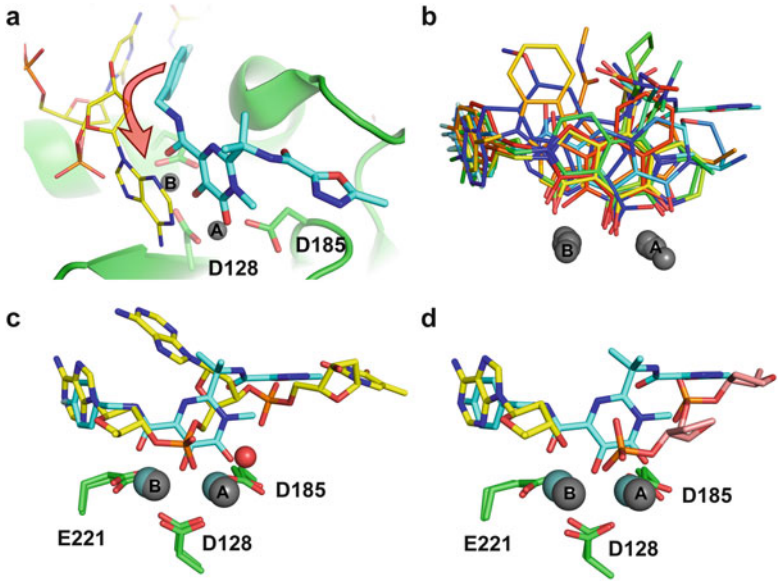


Fig. 6 Mechanism of action of INSTIs. (**a**) INSTI binding to the PFV intasome displaces the 3′ adenosine nucleotide from the active site and sterically precludes tDNA binding [17]. (**b**) Superposition of co-crystallized INSTIs elvitegravir (**20**), dolutegravir (**31**), raltegravir (**20**), MK-0536 (**46**), L-870810 (**11**), GS-9160 (**41**), a β-carboline hydroxamic acid derivative (**35**), MK-2048 (**50**), MB-76 (**62**), and XZ-259 (**63**) at the active site [79, 85]. The superposition illustrates the INSTI pharmacophore of three coplanar metal-coordinating atoms and a distal halobenzyl group. (**c**, **d**) Substrate mimicry of the adenosine, nucleophilic water, or ribose 3′ hydroxyl and phosphate oxygens by INSTIs (i.c. raltegravir) when compared to 3′-processing (**c**) or strand transfer (**d**) [19]. The PFV integrase catalytic triad residues are indicated as well as the two coordinated Mg^{2+} ions

arrangement of three Lewis bases, was widely accepted to coordinate the two active site Mg^{2+} ions, the precise role of the halobenzyl feature remained speculative. Crystal structures show that INSTIs indeed engage the active site metal ions while the halobenzyl inserts into a conserved pocket between a 3_{10}-helix in the active site loop (P145-Q148 in HIV-1 IN) and the 3′-penultimate cytosine base of the vDNA (Fig. 6a, b) [17, 79]. This binding mode displaces the vDNA 3′-dA from the active site, precluding coordination/activation of its 3′-hydroxyl for S_N2 attack on a tDNA phosphodiester bond and effectively disarming the intasome. It also provides mechanistic explanations for the two-step INSTI binding model and the effects of LTR substitutions [80, 81]. Additionally, INSTIs sterically hinder productive tDNA binding, accounting for the observed competition with respect to tDNA [17, 48]. The binding mode of INSTIs can be regarded as substrate mimicry when intasome structures containing either an INSTI, unprocessed vDNA, or processed vDNA and tDNA are compared (Fig. 6c, d) [19].

Like each antiretroviral (ARV), INSTIs have a specific time frame during the HIV replication cycle in which they exert their effect, depending of course on the presence of their target. In the case of INSTIs, this time frame extends from 4 to

12 h postinfection as shown by time-of-addition and washout studies [82]. This is the integration window and it is characterized by the existence of an intasome complex. When the residence times (and dissociation rates, k_{off}) of various INSTIs on wild-type (WT) intasome were determined, these appeared to exceed the half-life of the pre-integration complex (8.8, 2.7, and 71 h, respectively, for raltegravir, elvitegravir, and second-generation inhibitor dolutegravir; see Sect. 3.6) [82–84]. As such, the blocked integration complexes can only be degraded or undergo recombination and repair to form 1-LTR and 2-LTR circles, supporting a notion of functionally irreversible inhibition by INSTIs [82–84]. Next to insights into INSTI binding, the PFV intasome structures also provide valuable clues to the mechanism of resistance [79, 85].

3.5 Resistance Development

Consistent with other antiretroviral drug classes, virological failure associated with the emergence of INSTI resistance is observed in a subset of patients. Resistance to first-generation INSTIs is associated with at least two distinct, but not exclusive, genetic pathways featuring one of the following primary resistance mutations: Q148H/K/R or N155H, along with one or more secondary mutations [86–93]. The N155H pathway confers resistance to both raltegravir and elvitegravir and includes secondary mutations L74M, E92Q, and T97A [75, 76]. Mutations along the Q148H/K/R pathway also induce significant cross-resistance among first-generation INSTIs [75, 76]. Secondary mutations here mainly involve E138A/K or G140A/S. The third Y143C/H/R resistance pathway is unique to raltegravir, as its oxadiazole group establishes direct π-stacking interactions with the tyrosine side chain [79, 94]. It is highly synergistic with a T97A secondary mutation [95]. Similarly, T66A/I/K and S147G are resistance mutations observed in patients failing on elvitegravir treatment, but T66K also decreases susceptibility to raltegravir [96]. Primary INSTI resistance mutations are associated with a significantly decreased viral replication capacity [90]. An even greater reduction in fitness is observed when they are combined, which may explain why they do not readily coexist on the same genome but rather establish independent pathways [96]. Both evolution and stochastic mutation appear to play a role in the appearance of INSTI resistance mutations, while fitness and resistance shape the later selection of resistant viral quasispecies. As such, the Q148 pathway can emerge as the dominant species under prolonged selective pressure due to its superior fitness and replace initial mutations at the other primary positions [73, 97–100]. Importantly, when mutations accumulate along the Q148 pathway, the viruses likewise gain resistance against second-generation INSTIs (such as dolutegravir; see Sect. 3.6), albeit at much lower levels than is seen with first-generation INSTIs [101–103]. Sequential use of raltegravir and elvitegravir is not recommended in either order, due to the significant overlap in their resistance profiles [96, 104]. Similarly to other ARVs, prolonged virological failure under continuous INSTI selective pressure should be avoided in order to minimize (cross-)resistance within the INSTI class [96].

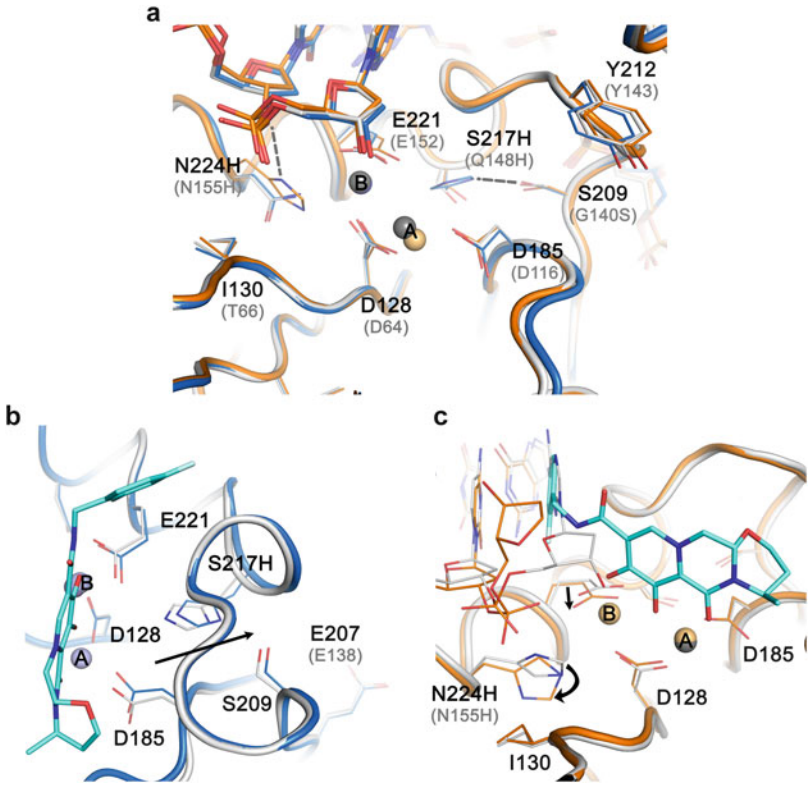


Fig. 7 INSTI resistance mechanisms. (**a**) View of the superposed active sites of the WT (*grey*), S217H (Q148H, *blue*), and N224H (N155H, *orange*) PFV intasomes [79]. *Grey* numbers in between parentheses indicate the homologous positions in HIV-1 IN. (**b**) INSTI (i.e. dolutegravir; see Sect. 3.6) binding to the S217H intasome requires a deformation of the active site 3_{10}-helix [85]. (**c**) Binding of dolutegravir to the N224H intasome entails breaking of the novel N224H–phosphodiester salt bridge and reorganization of E221 and D128 [85]

Most INSTI resistance mutations cluster around the IN active site, but the precise molecular mechanism behind their effect on INSTI binding was unknown. This has changed in recent years, owing again largely to crystallographic studies on the PFV intasome (Fig. 7) [17, 79]. Conservation of the active site allows direct extrapolation to HIV IN for most primary mutations [17, 79]. Nonetheless, most of the secondary resistance mutations reside outside of these regions. Here amino acid identity is low and interpretation becomes more difficult. In this case, homology modeling of the HIV intasome in its INSTI-bound and INSTI-unbound states may provide further insights [96, 105]. The reduced replication capacity of the virus carrying primary INSTI resistance mutation is reflected by the changes on a molecular level: through N155H and Q148H/R/K substitutions, integrase appears to slightly detune its active site while still retaining significant catalytic activity.

Q148H substitution (and K/R by proxy) introduces a slightly larger steric bulk behind the catalytic triad (Fig. 7a, b). In order to accommodate this change D116

shifts a bit, which may affect Mg^{2+} chelation in site A. Whereas INSTI binding to the WT intasome requires almost no conformational changes in IN itself, significant rearrangement is observed upon binding to the Q148H variant (Fig. 7b) [17, 79]. As D116 is forced back into its WT position, steric constraints demand that also H148 moves back, pushing on the active site 3_{10}-helix and weakening its hydrogen bond network [79]. This likely comes at a significant energetic cost, which effectively lowers INSTI potency [79, 83]. G140 is in direct contact with Q148, and G140A/S substitutions further reduce the available space and flexibility to accommodate the conformational changes induced upon INSTI binding (Fig. 7a, b) [79]. E138 establishes indirect contacts with Q148 through hydrogen bonding and van der Waals interactions with H114 and may hence also contribute to the resistant phenotype (Fig. 7b) [96, 105].

In the WT intasome, the N155 side chain amide tethers the catalytic D64 and E152 through hydrogen bonding and pre-organizes the active site to support metal ion coordination at position B. N155H substitution abolishes these hydrogen bonds and instead the imidazole side chain engages the 3′ CpA phosphodiester in a salt bridge (Fig. 7a, c). Consequently, INSTI binding to an N155H intasome requires reorganization/metal binding at site B and disruption of this salt bridge as the adenosine nucleotide is forced to give way to the inhibitor. The entropic and enthalpic penalties associated with these reorganizations are likely to account for the increased INSTI resistance observed in the N155 pathway [79]. The L74M, E92Q, and T97A secondary mutations appear to cluster around the β3–α1 loop. These amino acids support the β4–α2 catalytic loop and their substitutions may hence perturb positioning of D116 [79, 96, 105].

Lastly, Y143C/H/R substitutions result in loss of a direct favorable interaction with raltegravir, explaining the specificity of this pathway (Fig. 7a) [79]. The case is less clear for elvitegravir-specific substitutions at position T66. This amino acid is located adjacent to N155 and T66A/I/K substitutions may similarly affect vDNA 3′ end or Mg^{2+} B binding (Fig. 7a) [96].

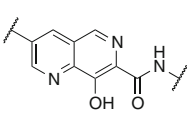
Of note, INSTI resistance seems in part due to the ability of mutations to increase the INSTI dissociation rates (k_{off}) and hence reduce their residence time [82–84]. Second-generation inhibitors such as dolutegravir (see Sect. 3.6) exhibit significantly longer residence times both on WT- and INSTI-resistant complexes, which may underlie in part their higher genetic barrier to resistance [83]. Overall, there appears to be a qualitative relationship between the residence time and in vitro antiviral potency of raltegravir, elvitegravir, and dolutegravir [83].

3.6 Dolutegravir (S/GSK1349572)

Since its approval, raltegravir has become one of the preferred agents in triple therapy as it induces a rapid decline in the viral load, is well tolerated, and has a favorable drug interaction profile. However, twice-daily (2 × 400 mg) dosing is nonoptimal [106] and resistance readily develops [107]. The issue of dosing was partly addressed with elvitegravir which, when taken with a pharmacoenhancer, is amenable

to 150 mg once-daily dosing [75–77]. Unfortunately, elvitegravir has a similar low barrier to resistance development and shows significant cross-resistance to raltegravir-resistant strains, precluding its use in patients failing on raltegravir [98, 108].

In search of an INSTI to surmount the issues above, researchers at Shionogi & Co., Ltd., and GlaxoSmithKline plc had been investigating various scaffolds such as hydroxyquinoline (**21**), hydroxynaphthyridine (**22**), and hydroxynaphthyridinone (**23**), yet none of them were found to be adequate and a novel scaffold was devised [109].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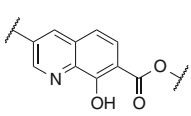
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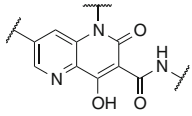
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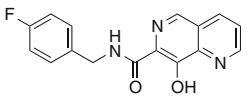
First, by comparing various molecules after superposition of their two-metal chelation motif, the optimal position of the distal aryl was investigated. Whereas the naphthyridine or naphthyridinone scaffolds in, e.g., L-870812 (**10**) and L-870810 (**11**) or compounds **24** and **25**, place the phenyl ring relatively close to the metal chelation motif, scaffold rotation around a pseudo-C2 axis affords a slightly more distal position and concomitant higher activity such as in **26** and **27**. Compounds **24** and **25** display IC_{50} values of 112 and 1,100 nM, while the rotated compounds **26** and **27** inhibit ST with IC_{50} values of 31 and 5 nM, respectively.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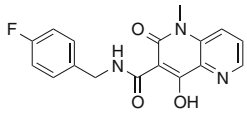
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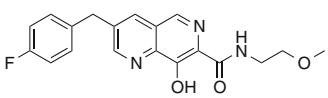
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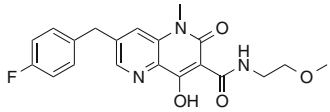
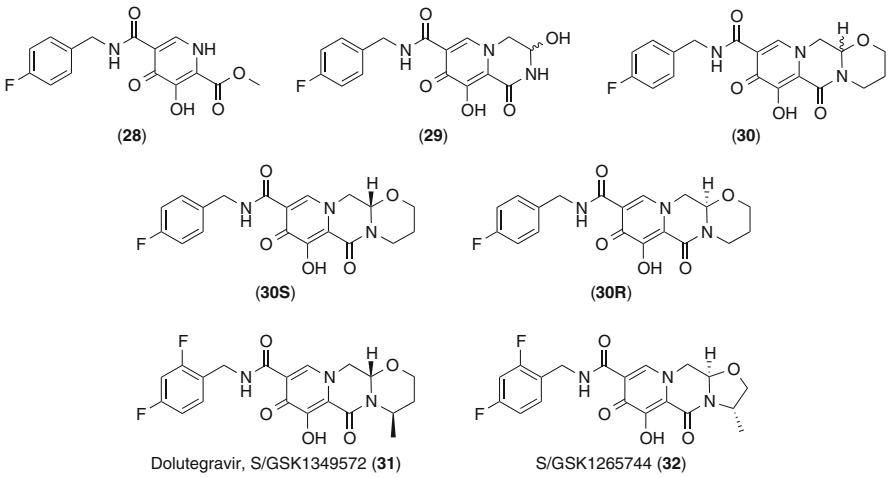

(**27**)

Second, the metal chelation motif itself was investigated. The hard and soft Lewis acids and bases (HSAB) theory predicts that a "hard" metal ion such as Mg^{2+} will prefer coordination by a "hard" ligand such as oxygen instead of the "softer" nitrogen. With this reasoning in mind, the coordinating nitrogen in the naphthyridine scaffold was replaced by oxygen and the ring was opened accordingly due to valence restrictions. Instead, a carboxamide was incorporated on the novel pyridone 5-position, forming a pseudo-ring by intramolecular hydrogen bonding and as such retaining the position of the distal phenyl to mimic the original scaffold. The resulting carbamoyl pyridone scaffold showed excellent potency against wild-type HIV-1 when substituted with an ester or carboxylic acid as the third metal-chelating ion (**28**; $IC_{50} = 18$ nM, $EC_{50} = 10$ nM). However, when an amide was introduced for reasons of stability and cell permeability, significant

losses in potency were observed [109]. This is due most likely to disruption of the coplanar metal-chelating pharmacophore either by unfavorable hydrogen bonding between the amide and the C-3 hydroxyl or by steric hindrance with the pyridone NH [109]. The logical solution was to fix the amide through cyclization and hence the bicyclic carbamoyl pyridones were obtained. When an additional hydroxyl was included (**29**) the compound appeared to retain remarkable activity against Q148K raltegravir-resistant virus ($EC_{50} = 3.8$ nM against WT HIV-1 and a minor 2.1 ± 0.50-fold increase in EC_{50} for the mutant) [110]. This modification however introduced a hemiaminal stereocenter that interconverts relatively easily. In order to lock the center, the hydroxyl was incorporated as an ether in the third ring, giving rise to the tricyclic carbamoyl pyridones (e.g., **30**; $EC_{50} = 3.4$ nM and $PAEC_{50} = 26.9$ nM) [110]. These compounds retained activity against the Q148K and N155H variants and could readily be separated into stable S- and R-stereoisomers (**30S** and **30R**). The two enantiomers showed similar activity against the WT virus but different responses to Q148K and differential serum protein binding [110, 111]. Implementation of an ingenious substrate-controlled diastereoselective synthetic strategy, employing the chiral information from small readily available amino alcohols to control the relative and absolute stereochemistry of the final inhibitors, was key for chemical tractability and further optimization [111]. Exploration of various amino alcohols (derived) from the chiral pool allowed development of the SAR for this series and identification of S/GSK1349572 (better known as dolutegravir, **31**; $IC_{50} = 2.7$ nM, $EC_{50} = 0.51$ nM, $PAIC_{50} = 38$ nM) and its backup compound S/GSK1265744 (**32**; $IC_{50} = 3.0$ nM, $EC_{50} = 0.22$ nM, $PAIC_{50} = 102$ nM) [110–112].



Both molecules are currently codeveloped by Shionogi & Co., Ltd., and ViiV Healthcare (a joint venture between GlaxoSmithKline plc and Pfizer, Inc.) [101]. Notably, S/GSK1265744 is being evaluated as both an oral formulation and a long-acting parenteral injection supporting monthly or even quarterly administration. This infrequent dosing may improve adherence and efficacy both for HIV

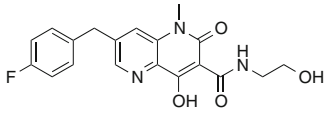
treatment and prevention, the so-called preexposure prophylaxis or PrEP [112–114]. Dolutegravir is progressing steadily through clinical trials. The SPRING-1 and SPRING-2 studies favorably compared once-daily dolutegravir in treatment-naïve HIV-1-infected patients to efavirenz or raltegravir, respectively, against a background of tenofovir/emtricitabine or abacavir/lamivudine [115, 116]. After 96 weeks into these studies, no genotypic or phenotypic INSTI resistance was found, suggesting a higher barrier to resistance development for dolutegravir [117]. The VIKING-1, VIKING-2, and VIKING-3 studies specifically explore the use of dolutegravir in highly treatment-experienced patients who had exhibited virologic failure on a regimen containing raltegravir/elvitegravir and had both genotypic and phenotypic resistances to the drugs [103, 118]. Preliminary results indicate rapid antiviral responses, which were better when dolutegravir was dosed at 50 mg twice – instead of once-daily. Patients whose viruses carried resistance mutations at positions T66, Y143, or N155 responded better than those harboring Q148 pathway substitutions. This response further decreased with increasing number of secondary mutations, notably at positions L74, E138, and G140 [103]. So although a second-generation INSTI like dolutegravir will be useful for management of viruses resistant to first-generation drugs, mutations at position Q148 will have to be monitored closely and therapy adjusted accordingly. In this regard, allosteric targeting of HIV integrase may be of particular interest (see Sect. 4).

3.7 Other (Pre)Clinical INSTIs

A relatively large set of other INSTIs has been explored over the last few years; here we describe those that have received most attention and/or have been in clinical trials.

3.7.1 S/GSK364735

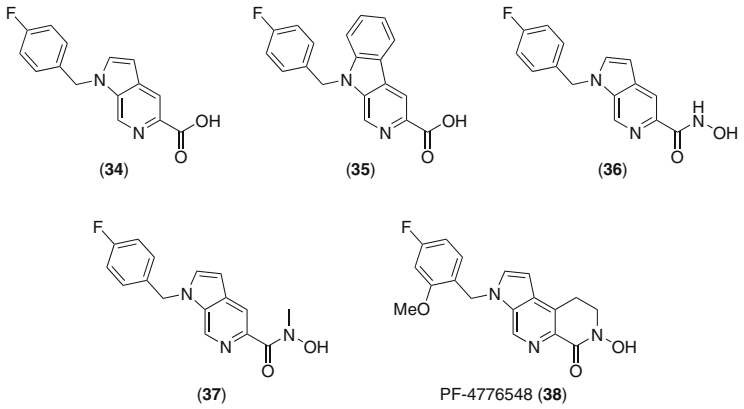
While exploring the hydroxynaphthyridinone scaffold (see Sect. 3.6), researchers at Shionogi & Co., Ltd., and GlaxoSmithKline plc identified S/GSK364735 (**33**) [119–121]. The compound displayed high potency (IC_{50} = 7.8 nM, EC_{50} = 5 nM, and $PAEC_{50}$ = 40 nM) and also had acceptable PK and safety profiles [119, 120, 122]. However, resistance profiling showed considerable overlap with raltegravir/elvitegravir and significant loss of activity was observed for mutations at positions T66, Q148, and N155 [119]. While phase IIa data for S/GSK364735 showed a 2.2 log reduction in the viral load, clinical development was stopped due to hepatotoxicity observed during long-term dosing studies in cynomolgus monkeys [119].



(**33**)

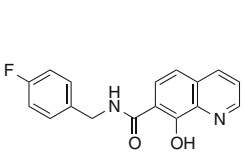
3.7.2 PF-4776548

Screening with a strand transfer assay at Pfizer, Inc., resulted in the identification of azaindole carboxylic acids (e.g., **34**) and β-carboline carboxylic acids (e.g., **35**) as modest hits (IC_{50} values between 840 and 7,350 nM) [123]. When the picolinic acid was replaced by picoline hydroxamic acid in order to complete the 2-metal binding pharmacophore, a 40-fold increase in potency was observed for the azaindole hydroxamic acid (**36**). Hydroxamic acids can however undergo Lossen rearrangement to form potentially mutagenic isocyanates after metabolic activation [124]. To circumvent the rearrangement, the NH was methylated (**37**; IC_{50} = 250 nM, EC_{50} = 32 nM), which slightly affected antiviral potency but rendered the compound inactive in Ames assays [123]. The resulting azaindole *N*-methyl hydroxamic acids are however rapidly cleared through glucuronidation [125]. Medicinal chemistry strategies employed to counter this were both steric crowding of the hydroxyl group and electronic deactivation or complete removal of this grouping [125, 126]. For instance, introduction of polar substituents on the 3-position of the azaindole ring effectively altered the physical properties and attenuated clearance, without impact on the antiviral potency [125]. Similar to the amide-substituted carbamoyl pyridones described above in the development of dolutegravir (see Sect. 3.6) [109], coplanar arrangement of the two-metal binding pharmacophore is energetically disfavored due to steric hindrance between the hydroxamate *N*-methyl and the 4-H of the azaindole system. Again, the solution was to lock the orientation of the hydroxamic acid through cyclization to give rise to the tricyclic *N*-hydroxy-dihydronaphthyridinones and clinical candidate compound PF-4776548 (**38**; EC_{50} = 1.54 nM, CC_{50} = 7.36 μM) [126, 127]. This lead showed high potency and selectivity, as well as good resistance and PK profiles [126]. A microdosing study in male volunteers nonetheless confirmed that PF-4776548 had a high clearance and short half-life, which led to its discontinuation [126].

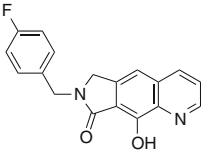


3.7.3 GS-9160

A similar reasoning to fix the coplanarity of the two-metal binding pharmacophore in quinoline carboxamides (**39**; IC_{50} = 2,000 nM, EC_{50} > 10,000 nM) led researchers working at Gilead Sciences to develop tricyclic pyrroloquinoline inhibitors (**40**; IC_{50} = 70 nM, EC_{50} = 100 nM) [128]. Studies of substitution of the pyridine ring [129], introduction of various substituents on the C-5 and C-6 positions [130, 131], and optimization of the benzyl moiety [132] ultimately resulted in the discovery of the C-5 aza pyrroloquinoline analog GS-9160 (**41**), which showed excellent antiviral activity (IC_{50} = 28 nM, EC_{50} = 1.7 nM, $PAEC_{50}$ = 11.4 nM) and good oral bioavailability [131]. GS-9160 was selected for clinical development and showed synergistic effects when combined with approved antivirals [133]. Despite selecting a novel set of resistance mutations in vitro, significant cross-resistance was observed with raltegravir/elvitegravir resistant strains, notably carrying substitutions at positions E92, Q148, and N155 [133]. Additionally, GS-9160 did not support once-daily dosing in healthy human volunteers and clinical development of this compound was discontinued [133].



(**39**)



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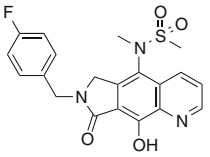
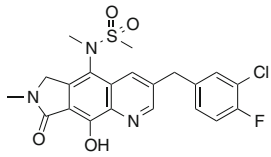


GS-9160 (**41**)



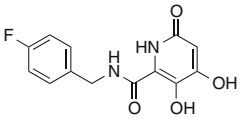
(**42**)

On a side note, and similar to the development of dolutegravir (see Sect. 3.6), a series of "benzyl flipped" pyrroloquinolines was developed [134]. This series essentially features the same pyrroloquinoline core rotated around a pseudo-C2 axis to have the benzyl appended to the pyridine C-3 position [134]. The lead compound (**42**) showed good oral bioavailability, low clearance, and high antiviral activity (IC_{50} = 265 nM, EC_{50} = 1.1 nM, $PAEC_{50}$ = 35 nM) [134].

3.7.4 MK-0536

Slight modification of the 4,5-dihydroxypyrimidine carboxamide scaffold which is the basis of raltegravir (see Sect. 3.2) brought researchers at Merck West Point to 4,5-dihydroxypyridone carboxamides (e.g., **42**) [60, 135]. Here as well, researchers

opted to lock the carboxamide through cyclization such that the two-metal binding pharmacophore is pre-organized in the resulting dihydroxypyridopyrazine-1, 6-diones [135]. These compounds showed potent antiviral activity (**43**; IC_{50} = 10 nM, EC_{95} = 310 nM) and good safety and PK profiles [135]. The tetrahydro-naphthyridine template was put forward as a good target when conceiving analogs for this bicyclic system. Additionally and similar to the 1,6-naphthyridine series (see Sect. 3.2), an *N*-substituted carboxamide was placed in para to the phenolic hydroxyl in order to improve metabolic stability of the series (**44**) [61, 136]. Peralkylation of the amide and pyrimidone nitrogens in combination with a 4-F,3-Cl-benzyl group proved to provide the most optimal antiviral activity and reduced protein binding (**45**; IC_{50} = 19 nM, EC_{95} = 73 nM, $PAEC_{95}$ = 91 nM). Next, a panel of INSTI-resistant viruses (T66I/S153Y, N155S, Q148K, E138A/G140A/Q148K, and F121Y) was used to evaluate the compounds as second-generation inhibitors. Activity against these mutant strains improved significantly with greater steric bulk on the pyrimidine nitrogen and as such an *N*-isopropyl group was included to give rise to MK-0536 (**46**; IC_{50} = 3 nM, EC_{95} = 16 nM, $PAEC_{95}$ = 35 nM) [137, 138]. The resulting compound showed an encouraging PK profile in rats and dogs, but its current level of clinical development is unclear.



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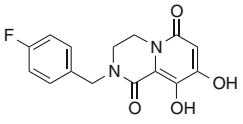

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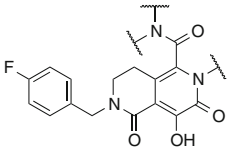

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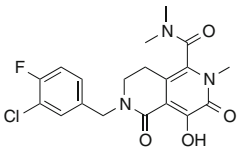

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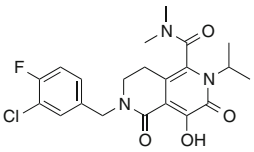
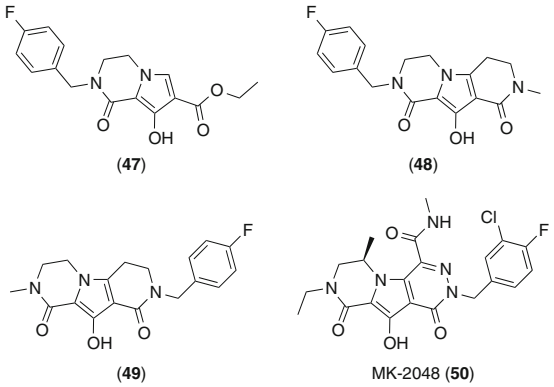


MK-0536 (**46**)

3.7.5 MK-2048

Shrinkage of the pyridone ring in dihydroxypyridopyrazine-1,6-diones (see Sect. 3.7.4 e.g., **43**) to a five-membered pyrrole and replacement of the C-4 hydroxyl by an exocyclic carbonyl group gives rise to a novel 8-hydroxy-3,4-dihydropyrrolo[1,2-*a*] pyrazine-1(2H)-one scaffold that fulfills the two-metal binding pharmacophore (e.g., **47**; IC_{50} = 40 nM, EC_{95} = 3,230 nM) [139]. However, the exocyclic carbonyl, whether being part of a carboxylic acid, ester, or amide, reintroduces flexibility and unwanted hydrogen bonding. In order to circumvent this, another constraint was introduced between the pyrrole and the carboxamide. Two options were explored for this. First, replacement of the pyrrole by pyrazole, which abolishes eclipsing of the

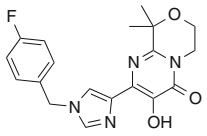
amide and pyrrole protons and replaces it by a potential hydrogen bonding interaction between the amide proton and pyrazole nitrogen [140]. Second, actual cyclization to a lactam gives rise to pseudosymmetrical, tricyclic pyrrolopyrazines (e.g., **48** and **49**; $IC_{50} < 10$ nM, $EC_{95} = 390$ nM, and $IC_{50} < 10$ nM, $EC_{95} = 160$ nM, respectively) [139]. Alkali metal salts, desired for dosing of the latter compounds, were however susceptible to air oxidation. To attenuate this oxidation and stabilize the electron-rich hydroxypyrrole, the dihydropyridone ring was replaced by an electron-withdrawing pyridazinone, yielding stable pyrazinopyrrolopyridazines [141]. Antiviral potency of this novel scaffold against wild type and a panel of strains resistant to diketo acids and naphthyridines (F121Y, T66I/S153Y, N155S) was further optimized by introducing small aliphatic groups in certain positions ultimately giving rise to MK-2048 (**50**; $IC_{50} < 10$ nM, $EC_{95} = 41$ nM) [141, 142].



MK-2048 displayed good PK in preclinical animal species and was taken into clinical development [142]. Its current status however is unclear. Despite the compound retaining significant activity against first-generation INSTI resistance mutations at positions T66, Y143, or N155, it quickly loses potency against mutations arising from the Q148 pathway [97, 143, 144]

3.7.6 BMS-707035

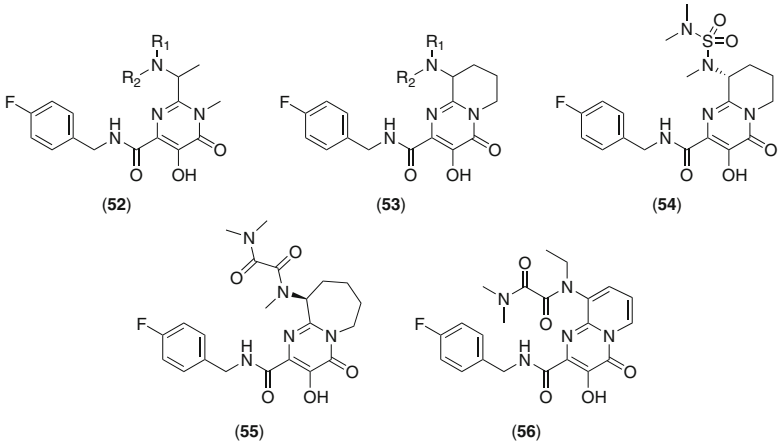
BMS-707035 was an investigational INSTI developed by researchers at Bristol-Myers Squibb Co. The compound was terminated in phase II clinical trials but the reason was not disclosed. Its structure also has not been disclosed, but a recent patent from the company describes a series of potent 3-hydroxy-6,7-dihydropyrimido[2,1-c][1, 4]oxazin-4(9H)-ones [145, 146]. For example, compound **51** displays an $IC_{50} = 3.4$ nM and $EC_{50} = 5$ nM [146]. In this compound series, the metal coordinating amide has successfully been replaced by various azole bioisosteres.



(**51**)

3.7.7 Bicyclic Pyrimidones

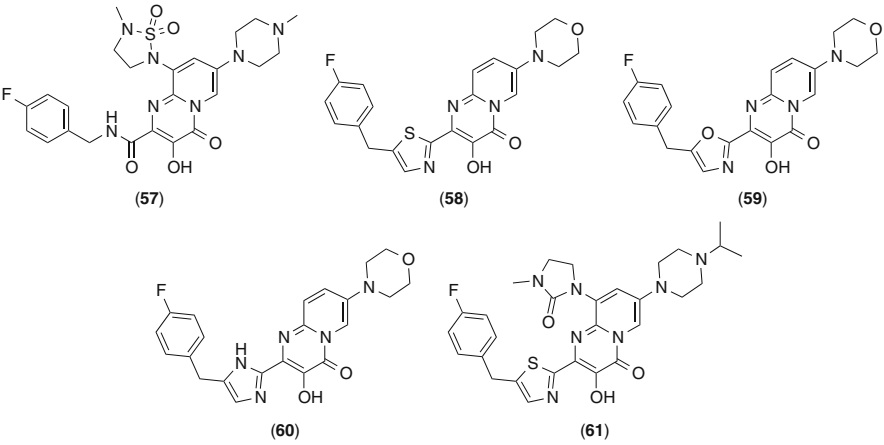
During the development of raltegravir at IRBM (see Sect. 3.2), both *N*-methylation of the dihydroxypyrimidine core [147] and introduction of a β-amino substituent in the 2-position of the ring had proven beneficial [65]. In the next step, researchers attempted to constrain both groups (**52**) through cyclization, giving rise to bicyclic pyrimidones (**53**) [148]. An extensive SAR study was performed to identify a suitable amino substituent on the saturated ring that endowed the molecules with the desired physicochemical properties and conferred nanomolar activity against HIV-1 replication in cell culture [148]. This search resulted in the identification of sulfamide **54** and ketoamide **55**, which inhibited strand transfer with IC_{50} values of 7 and 12 nM and viral replication with $PAEC_{95}$ values of 44 and 13 nM respectively. Compound **55** also exhibited a good PK profile when dosed orally to preclinical species [148]. Further studies sought to remove the benzylic stereocenter and gave rise to fused pyridopyrimidones such as **56** which showed an $IC_{50} = 36$ nM and $PAEC_{95} = 250$ nM [149].



(**52**) (**53**) (**54**) (**55**) (**56**)

Researchers at Avexa Ltd. have also investigated expansion of the hydroxypyrimidone scaffold into a series of tri- and bicyclic systems. They sought to modulate the electronics of the coordinating atoms and introduce further functionality [150]. Selection of the fused pyridopyrimidone scaffold and further

optimization of substituents on the pyridine ring resulted in combination of *N*-methyl piperazine and *N*-methyl thiadiazolidine moieties on compound **57** which displayed a $PAEC_{50} = 4$ nM [150, 151]. Similarly as with BMS-707035 (see Sect. 3.7.6), efforts were put into exchanging the coordinating exo amide group to improve activity in cell culture and PK properties [152]. Bioisosteric replacement by various azoles was investigated, while the exact nature of the azole would allow fine-tuning of the electronic properties of the coordinating atom. In this regard, it was shown that thiazole, oxazole, and imidazole replacement (**58, 59, 60**) allowed the highest antiviral potencies. Compounds **58** and **59** demonstrated IC_{50} values of 20 and 59 nM and EC_{50} values of 16.5 and 6 nM, respectively. The imidazole analog **60** inhibited ST with an $IC_{50} = 45$ nM [152]. Combination of the thiazole replacement together with optimization of the pyridine substituents for activity against INSTI-resistant viruses resulted in a series of potent inhibitors, including **61**, which had EC_{50} values of 6.8, 21, and 8.9 nM against WT, G140S/Q148H, or E92Q/N155H viruses, respectively [153].

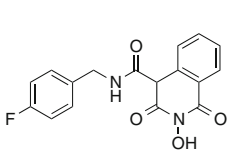


3.8 *Novel Scaffolds Developed at Academia*

Next to the large outflow of INSTIs from pharmaceutical companies, also academic groups have contributed an extensive set of scaffolds and compounds. We will highlight only two series here for which structural and functional data are most complete: 2-hydroxyisoquinoline-1,3(2*H*,4*H*)-diones and dihydroxy-1*H*-isoindole-1-ones.

2-hydroxyisoquinoline-1,3(2*H*,4*H*)-diones were initially conceived as inhibitors of two-metal ion catalytic sites to act as dual inhibitors of the HIV RT RNase H and IN [154]. Further substitution on this scaffold led to the development of potent and selective integrase inhibitors [155, 156]. A lead compound from this

series (MB-76, **62**) remarkably showed similar inhibition against both 3P and ST (IC_{50}^{3P} = 66 nM, IC_{50}^{ST} = 99 nM, and EC_{50} = 2,340 nM), perhaps owing to strong electrostatic interactions between, upon chelation dianionic, MB-76 and the active site Mg^{2+} ions [157]. The minimalistic scaffold limits resistance-susceptible contacts with integrase while efficiently pre-organizing the two-metal binding pharmacophore in a binding conformation [157]. A longer exocyclic linker between the metal-chelating scaffold and the *p*-fluorophenyl may provide additional flexibility similar to dolutegravir in response to a slightly altered INSTI-resistant active site. Resistance selection against MB-76 proved difficult and no significant changes in activity were observed when the compound was assayed against E92Q, Q148H, N155H, or G140S/Q148H resistance mutations [157].



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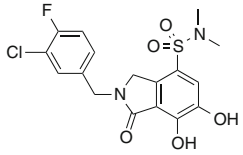


(**63**)

Dihydroxy-1*H*-isoindole-1-ones represent another conformationally constrained scaffold fulfilling the two-metal binding pharmacophore [158, 159]. With an IC_{50} = 77 nM and an EC_{50} = 11 nM, XZ-259 (**63**) is the most potent congener reported for this series [160]. When tested against a panel of Y143R, N155H, or G140S/Q148H raltegravir resistance mutations, XZ-259, despite being less sensitive than raltegravir to the other substitutions, only overcomes the effect of Y143R. Further modifications on this scaffold may address the currently outstanding issues with cytotoxicity and the resistance profile [160].

Recently a systematic dissection and evaluation of the metal binding pharmacophore was reported [161]. Retaining the *p*-fluorobenzyl moiety and varying the metal binding group allowed direct identification of a number of important features: (a) hard Lewis bases better match the hard character of the Mg^{2+} ions; (b) 5- and 6-membered chelate rings appear to be the most common and allow for optimal metal–ligand bond lengths and angles; and (c) the hardest, highly electron-dense and preferentially anionic donor should occupy the μ-bridging position between the two Mg^{2+} ions [161]. Several molecules in this study confirm the detrimental effect of steric hindrance in the coplanar metal binding conformation on activity and hence indirectly support "locking" of the scaffold as observed in various second-generation inhibitors. Of note, the authors described two, upon chelation dianionic, *N*-oxide-containing scaffolds similar to MB-76. These inhibitors may also display stronger electrostatic interactions with the active site Mg^{2+} ions, which could underlie their relatively strong effect on 3′-processing [157, 161].

4 Allosteric or Non-catalytic Site IN Inhibitors

Although the development of INSTIs represents a significant milestone in the field of HIV-1 drug discovery, the steady emergence of (cross-)resistance against this inhibitor class warrants research into novel, allosteric, or non-catalytic site IN inhibitors (ALLINIs or NCINIs) with unique mechanisms of action. Allosteric literally translates from Greek as "other place," describing inhibitors that bind a region distinct from the active site. In the case of IN, we can envision allosteric inhibitors that induce conformational changes at the active site, interfering with substrate binding or catalysis. Allosteric inhibitors may also modulate IN oligomerization and hence disrupt the precise and dynamic equilibrium required for IN activities. Last, they may directly inhibit protein–protein interactions (PPI) between IN and an important cellular cofactor.

A large number of approaches and compounds have been described, with varying degrees of success (reviewed in [162–164]). Notably, the groups of Wielens and Rhodes have reported on a fragment-based drug discovery campaign against the HIV-1 IN CCD [165–168]. Two parallel approaches were employed to identify hits from two closely related 500-compound fragment libraries. One strategy used surface plasmon resonance (SPR) for hit identification while the other employed saturation transfer difference nuclear magnetic resonance (STD-NMR) and both were followed by X-ray crystallography to guide structure-based elaboration of the hits [165–168]. These studies have led to the identification and characterization of four potentially druggable pockets on the HIV-1 IN CCD [165–172]. However, so far only one single binding site has allowed development of nanomolar activity allosteric inhibitors and has led compounds in (pre)clinical development. This is the case of small molecules binding to the **LEDG**F/p75–**IN** interaction site on HIV-1 IN, hence referred to as LEDGINs [173].

4.1 LEDGINs

In 2005 Cherepanov et al. reported the crystal structure of the HIV-1 IN CCD dimer in complex with the LEDGF/p75 IBD (Fig. 5c) [174]. Not much later initial data appeared indicating that the LEDGF/p75–IN interaction may provide a valuable and tractable target for the development of novel ARVs. Overexpression of an IBD-only fragment showed a dominant negative effect and potently inhibited HIV-1 replication [175, 176]. Additionally, peptides derived from the LEDGF/p75 IBD interface were shown to modulate IN oligomerization and to inhibit IN catalytic activity [177]. Hence, research efforts focused on developing a small-molecule inhibitor of the interaction intensified [168, 178–182].

4.1.1 Discovery of 2-(Quinolin-3-yl)Acetic Acids

The main breakthrough however came in 2010, when Christ et al. reported the structure-based design of small-molecule PPI inhibitors targeting the LEDGF/p75 binding pocket on HIV-1 integrase, which potently inhibited HIV replication [173]. The authors started off with a diverse 200,000-compound commercial library, which was filtered with a set of 2D descriptors defining small-molecule PPI inhibitor chemical space. After analysis of the different (co-)crystal structures of HIV-1 IN (notably the complexes with the LEDGF/p75 IBD [174], with tetraphenyl arsonium [183] and CCD structures displaying crystal packing in the region [184]), a consensus pharmacophore, describing the molecular recognition patterns in the LEDGF/p75 binding site of HIV-1 IN, was constructed [173]. Next, molecules emerging from the pharmacophore query were docked into the binding pocket and scored through a consensus scoring algorithm. The best scoring molecules were retained and visually inspected for fulfillment of the pharmacophore hypothesis. Finally, 25 compounds were selected, purchased, and tested for inhibition in an in vitro bead-based IN–LEDGF/p75 interaction assay (AlphaScreen, PerkinElmer). One compound (LEDGIN 1, **64**) inhibited the AlphaScreen signal by 36% at 100 μM. Commercial analogs were sought using MACCS (molecular access system) structural fingerprints, which led to the identification of LEDGIN 2 (**65**), showing an $IC_{50}^{IN–LEDGF/p75}$ of 27.27 μM in the interaction assay. Medicinal chemistry efforts resulted in bioisosteric replacement of the tetrazole by a carboxylic acid and removal of the unstable secondary ketimine, giving rise to 2-(quinolin-3-yl)acetic acid LEDGIN 3 **(66).** LEDGIN 3 displayed an $IC_{50}^{IN–LEDGF/p75}$ of 12.2 μM and inhibited viral replication with an EC_{50} of 41.9 μM without apparent cellular toxicity ($CC_{50} > 150$ μM). Soaking of this compound into crystals of the HIV-1 IN CCD resulted in additional electron density in the LEDGF/p75 binding pocket. Comparison to the structure of the IBD–CDD complex revealed mimicry of LEDGF/p75 residues I365, D366, and L368 by the compound phenyl, acid, and chlorine functions, respectively, validating the pharmacophore model (Fig. 8).

Fueled by the structural insights, introduction of hydrophobic bulk on the acetic acid 2-position was proposed to fill up a hydrophobic region of the binding site [173, 185]. Indeed, compound potency further progressed into the single-digit micromolar range (CX05168, **67**; $IC_{50}^{IN–LEDGF/p75} = 1.37$ μM, $EC_{90} = 5.36$ μM, and $CC_{50} = 59.8$ μM). Notably, along with the compounds' increased potency against the LEDGF/p75–IN interaction, allosteric inhibition of IN catalytic activity in the absence of LEDGF/p75 became apparent as CX05168 showed an $IC_{50}^{ST} = 54.88$ μM against ST [173, 185]. Further medicinal chemistry work included replacement of the quinoline benzene by thiophene, various substitutions on the 4-phenyl group, and optimization of the hydrophobic bulk in the acetic acid 2-position [173, 185, 186]. This resulted in CX05045 (**68**; $IC_{50}^{IN–LEDGF/p75} = 0.58$ μM, $EC_{90} = 1.86$ μM, and $CC_{50} = 72.2$ μM) and CX14442 (**69**; $IC_{50}^{IN–LEDGF/p75} = 0.046$ μM, $EC_{90} = 0.114$ μM, and $CC_{50} = 96$ μM). Importantly, CX14442 also inhibited IN catalytic activities, both ST and 3P, with IC_{50} values of 146 and 727 nM, respectively, when the compound was preincubated with HIV-1 IN before addition of LTR or tDNA substrate [185]. These compounds were

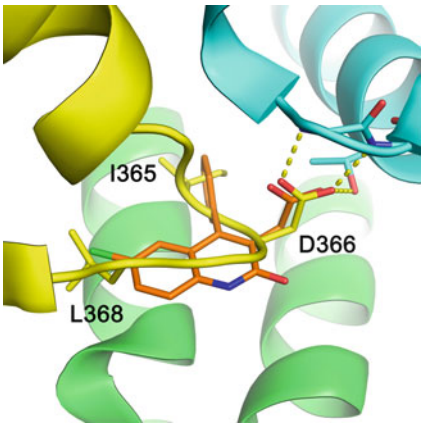
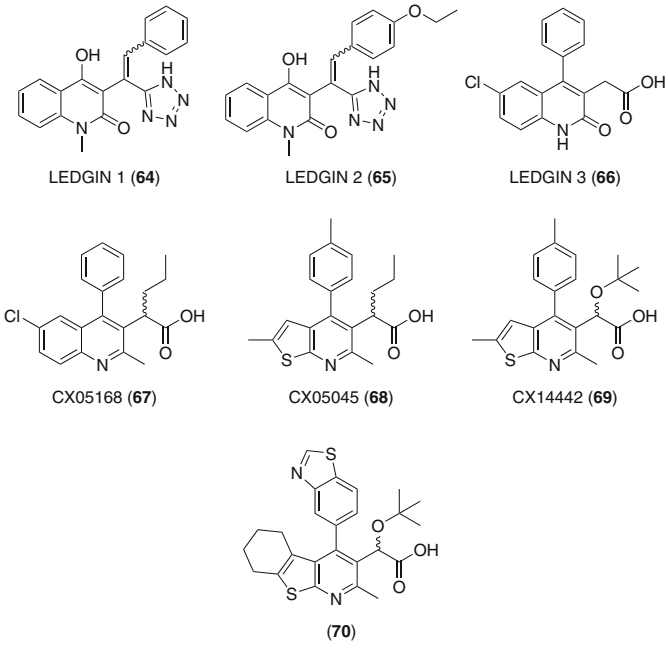


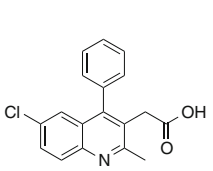
Fig. 8 Mimicry of LEDGF/p75 residues I365, D366, and L368 by the LEDGIN 3 phenyl, acetic acid and chloro groups [173]

licensed to Pfizer, Inc./ViiV Healthcare for preclinical development [186]. Both the University of Leuven and Pfizer have recently disclosed novel structures in follow-up patents in which the original quinoline is replaced by other heterocyclic systems (e.g., **70**; EC_{50} = 282 nM) [187–190].

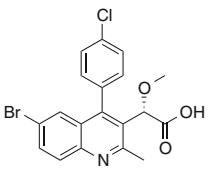


Independently, researchers at Boehringer Ingelheim GmbH performed a high-throughput IN 3P screen to discover novel IN inhibitor classes and identified the 2-(quinolin-3-yl)acetic BI-A (**71**) as a hit (IC_{50}^{3P} = 9 μM and EC_{50} > 40 μM) [191]. Structure-guided medicinal chemistry optimization resulted in the development of BI-B (**72**; IC_{50}^{3P} = 28 nM, $IC_{50}^{IN–LEDGF/p75}$ = 1.5 μM, EC_{50} = 0.45 μM,

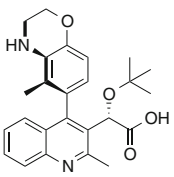
and $CC_{50} > 50$ μM) and BI-C (**73**; $IC_{50}^{3P} = 3$ nM, $EC_{95} = 10$ nM, and $CC_{50} > 80$ μM). On a side note, discrepancies in the potency of these compounds on the IN–LEDGF/p75 interaction or on IN catalytic activities between these compounds and those discussed above are likely attributable to differences in order of addition, preincubation, or general assay format or conditions [185, 192]. BI-C displayed excellent ADME (absorption, distribution, metabolism, and excretion) characteristics: favorable metabolic stability, low cytochrome P450 inhibition, high permeability, and excellent physicochemical properties [191]. As such, a compound from this class [193–196] that also showed excellent PK in preclinical animal species was advanced into phase I clinical trials [191, 197–201]. BI 224436 ($PAEC_{95} = 22$ nM and $CC_{50} > 90$ μM) was given to 48 healthy subjects in escalating doses and showed good tolerability and no serious adverse events [201]. However, clinical studies on BI 224436 were put on halt and the program was licensed to Gilead Sciences in October 2011 for further development [202]. Since then, Gilead has disclosed three other structures from this series: GS-A, GS-B, and GS-C (**75**, **76**, and **77**, respectively) and has also published a patent describing compounds where the quinoline core is rotated (e.g., **78** and **79**; $EC_{50} = 11$ and 543 nM, respectively) or rotated and further expanded (e.g., **80**; $EC_{50} = 72$ nM) [202, 203]. Further patents from Gilead claim series of naphthalene [204] or benzothiazole acetic acid derivatives [205]. The company's ambition to bring a compound from this class into the clinic is further evidenced by two more patent applications: the first on a process for the large-scale synthesis of several 2-(quinolin-3-yl)acetic acids [206] and the second on solid-state forms for formulation of one of these compounds [207].



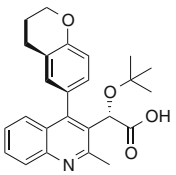
BI-A (**71**)



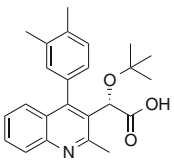
BI-B/BI-1001 (**72**)



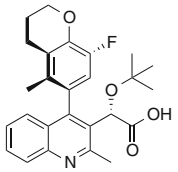
BI-C (**73**)



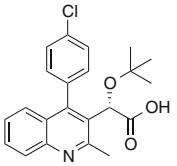
BI-D (**74**)



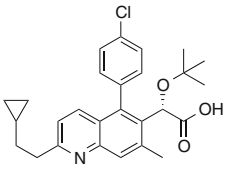
GS-A (**75**)



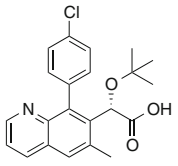
GS-B (**76**)



GS-C (**77**)



(**78**)



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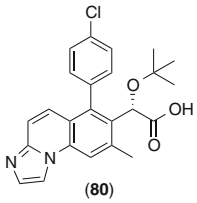


(**80**)

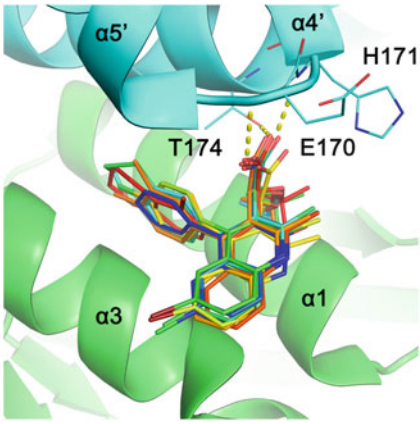


Fig. 9 Binding mode of 2-(quinolin-3-yl)acetic acids in the LEDGF/p75 binding pocket on the IN CCD dimer interface [173, 192, 202, 208]. Alpha helices from both monomers are indicated, as well as residues E170, H171, and T174, which engage the compounds' carboxylic acid group

4.1.2 Mechanism of Action

Since their initial discovery, several of these inhibitors have been soaked into HIV-1 IN CCD crystals [173, 185, 192, 202, 208, 209], in each case showing a similar binding mode at the LEDGF/p75 interaction site on the HIV-1 IN dimer interface (Fig. 9). The binding pocket is formed between the α4-loop-α5 region of the first CCD monomer and the α1 and α3 helices of the second, resulting in two symmetry-related binding sites on the IN dimer. The compounds' carboxylic acid group is strongly engaged in a tripartite hydrogen bonding interaction with the E170 and H171 backbone amide and the T174 side chain hydroxyl and represents a key pharmacophore feature. Simultaneously, the quinoline largely lies on top of the N-terminal part of the α3-helix. The quinoline 4-phenyl and the acetic acid 2-alkyl(oxy) group occupy the cleft between the α1- and α3-helices on one monomer and the α5-helix on the other, effectively bridging the dimer interface (Fig. 9) [173, 191].

The dynamics and temporally ordered assembly of HIV-1 IN with viral DNA and LEDGF/p75 are crucial for successful completion of the viral replication cycle [41, 210, 211]. LEDGIN binding deeply perturbs this IN assembly through two interwoven mechanisms: (a) direct competition with the LEDGF/p75 cofactor, blocking chromatin tethering of the PIC [34, 173, 192, 202, 212], and (b) stabilization of IN dimers, stimulating untimely and unproductive multimerization which prevents productive assembly with DNA [185, 192, 202, 210, 213, 214]. Although the precise extent of their relative contributions remains a matter of discussion, it is likely that both effects add to inhibition of HIV-1 integration [185, 192, 202]. Indeed, like INSTIs, LEDGINs increase the number of 2-LTR circles, but the junctions of these circles carry significantly fewer deletions, consistent with a block in productive IN–DNA assembly [173, 202]. However, not only during integration does IN play a pivotal part but also during reverse transcription and late stages such as virus assembly, as indicated by pleiotropic effects on viral replication observed for various IN mutants [215]. Consequently, since LEDGINs also bind to IN in the absence of DNA, it may come as no surprise that these compounds were recently shown to

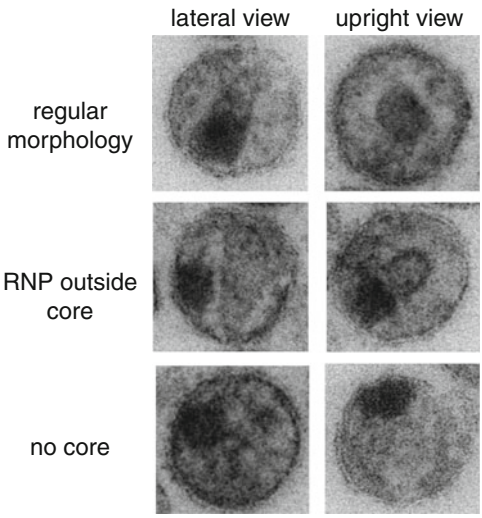


Fig. 10 Transmission electron microscopy images reveal morphological aberrations in viruses produced in the presence of LEDGINs. Reproduced with permission from [216]

exert a late effect as well [185]. When LEDGINs are present during the late stages of viral replication, the virions that are produced are significantly less infectious [185, 208, 209, 216]. Transmission electron microscopy analysis revealed that 60–90% of viral particles produced in the presence of LEDGINs display aberrant morphology (Fig. 10); either no conical core is formed or the electron-dense ribonucleoprotein complex is mislocalized outside an empty core [208, 216].

When these particles are allowed to infect cells, defects are detected in the early replication steps (reverse transcription, nuclear import, and integration) [208, 216]. LEDGINs appear to be able to engage IN as a part of the Pol polyprotein precursor and induce its oligomerization as well [216]. Inside the viral particles produced in the presence of the compounds, IN itself also shows a higher degree of multimerization [208, 216]. Perturbed assembly hence appears to lie at the basis of the observed defects during maturation and subsequent early steps. However, a role for LEDGF/p75 during the late stage of viral replication has not been excluded, and data have been presented suggesting decreased infectivity in the absence of LEDGF/p75 [217, 218].

It is clear that LEDGINs have a multimodal mechanism of action, which endows them with very steep dose–response curves [185, 191, 192, 202]. In contrast to INSTIs, which target a single intasome complex (slope ~1), LEDGINs are believed to act on a larger subset of IN molecules which are required during the relevant steps of the replication cycle [192, 208, 216]. The resulting intermolecular cooperativity is also observed with the PI and NNRTI classes and allows more potent antiviral activity [219]. Combination experiments have also suggested that combination of LEDGINs and INSTIs leads to additive or even synergistic effects [185].

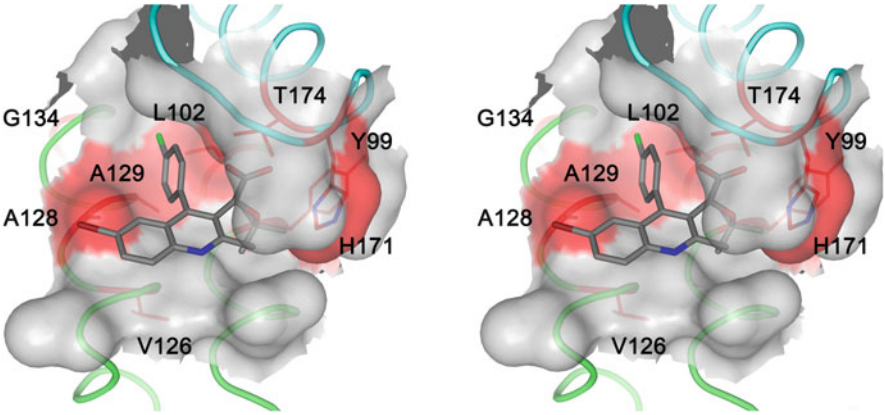


Fig. 11 Wall-eyed stereo rendering of the LEDGIN binding site. Positions of resistance mutations observed in vitro under selective pressure from various 2-(quinolin-3-yl)acetic acid-type LEDGINs are indicated in *red* [173, 185, 202, 220]

4.1.3 Resistance

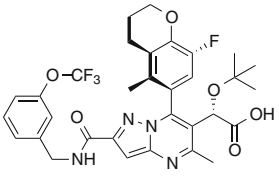
LEDGINs inhibit a wide range of HIV-1 subtypes, but HIV-2 is insensitive due to an A128M substitution perturbing the binding pocket [173, 185]. Importantly, these allosteric inhibitors retain activity against all INSTI-resistant strains [173]. Various groups have reported resistance selection experiments with LEDGINs in cell culture [173, 185, 202, 220]. Most of the amino acids found to be substituted under LEDGIN selective pressure are in direct contact with the compounds or are part of the helices that line the pocket; these include Y99H, L102F, V126I, A128T, A129T, G134E, H171Q/T, and T174I (Fig. 11) [173, 185, 202, 220].

Two more substitutions are observed where the resistance mechanism is less clear: V201I and N222K [202, 220], which are located at either end of the CCD–CTD linker. Inversely, raltegravir was found to retain activity against all resistant strains selected. Of note, several of these mutations have been shown to result in a pronounced reduction in viral replication capacity [220], and the resistance profile of more potent congeners appears different from that of earlier compounds [185, 202, 220].

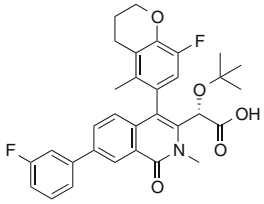
4.1.4 Related 2-Aryl-2-alkoxy-acetic Acid Derivatives

Patent literature indicates that major pharmaceutical companies Bristol-Myers Squibb Co., GlaxoSmithKline plc, and Shionogi & Co., Ltd., are also actively engaged in the development of LEDGIN-class compounds. Researchers at Bristol-Myers Squibb Co. have replaced the initial quinoline by a pyrazole[1,5-a]pyrimidine core which is further substituted on the 2-position (e.g., **81**; $EC_{50} = 9$ nM)

[221, 222]. Whereas GlaxoSmithKline plc has explored series of isoquinolines, 7-azaindoles and pyrrolopyridones substituted on the pyrrole nitrogen (e.g., **82**-**84**; EC_{50} = 10, 10, and 50 nM, respectively) [223–225]. Shionogi has shrunk down the quinoline system to a sole substituted aromatic ring (e.g., **85**-**87**; EC_{50} = 90 nM, 40 nM, and EC_{50} = 1.2 nM, respectively) or differently oriented bicyclic systems (**88**; EC_{50} = 4.4 nM) [226, 227]. Also the French *Laboratoire* BIODIM and the Korea Research Institute for Chemical Technology have recently published patents that describe 2-alkoxy-acetic acid analogs (e.g., **89** and **90** from BIODIM with EC_{50} = 1.6 and 2.2 μM) [228–230].



(**81**)



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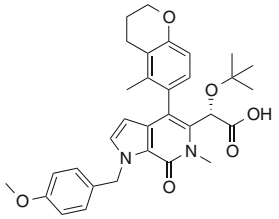
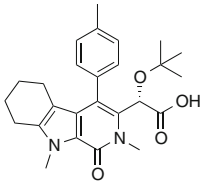


(**83**)



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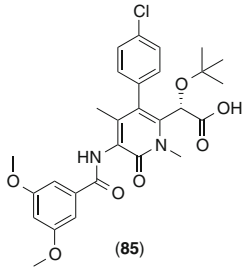

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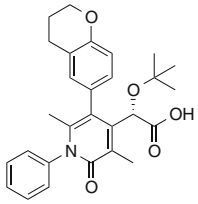

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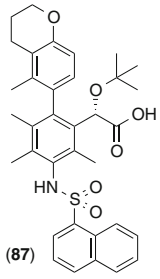
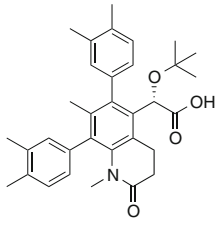
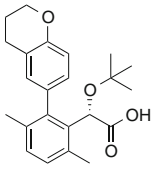


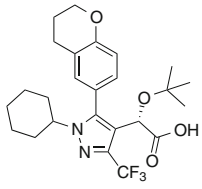
(**87**)



(**88**)



(**89**)



(**90**)

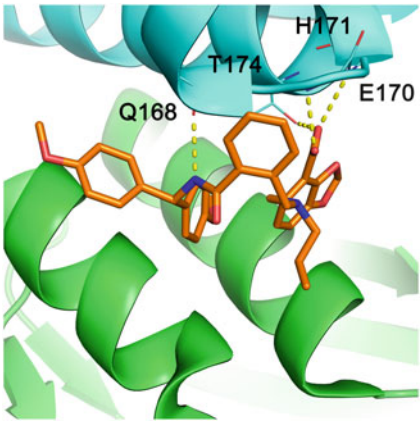
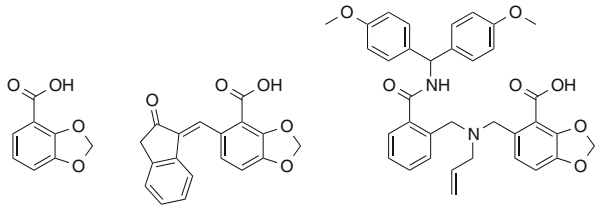


Fig. 12 Interactions of 1,3-benzodioxole-4-carboxylic acid (84) in the LEDGF/p75 binding pocket [168]. Besides hydrogen bonding of the carboxylic acid to E170, H171, and T174, the compound engages the backbone carbonyl of Q168 with its amine. One of the *p*-methoxyphenyl groups also penetrates deeper into the pocket than currently published 2-(quinolin-3-yl) acetic acids [168]

4.1.5 1,3-Benzodioxole-4-carboxylic Acids

Aside from 2-(quinolin-3-yl)acetic acids, one other class of LEDGINs has been disclosed for which there is evidence in the form of crystal structures. In their fragment-based drug discovery efforts (see Sect. 4), the groups of Wielens and Rhodes have also identified several fragments that, upon soaking into HIV-1 IN CCD crystals, displayed (partial) electron density in the LEDGF/p75 binding pocket [166–168]. One of the SPR hit compounds was 1,3-benzodioxole-4-carboxylic acid (**91**). Similarity and substructure searches identified analog **92** and further structure-based design as well as a series of small serendipities led to **93** which inhibited the IN–LEDGF/75 interaction with an $IC_{50} = 8.1$ μM and viral replication with an $EC_{50} = 29$ μM without apparent toxicity ($CC_{50} > 100$ μM).



The compounds also inhibited G140S/Q148H and E92Q/N155H INSTI-resistant viruses with a similar EC_{50}, supporting the allosteric mode of action of the compounds. Interestingly, compound **85** established novel contacts with the LEDGF/p75 binding pocket that were not previously observed with the 2-(quinolin-3-yl)acetic acids (Fig. 12). First, it engages the backbone carbonyl of Q168 in a hydrogen bonding interaction that was also detected with a series of cyclic peptides based on the LEDGF/p75 interacting loop [231]. Second, it appears that one of its

p-methoxyphenyl groups inserts deeper into the binding pocket than 2-(quinolin-3-yl) acetic acids, indicating these and other classes may be expanded in this direction as well.

Future optimization, resistance selection, and evaluation of cross-resistance with 2-(quinolin-3-yl)acetic acids will demonstrate whether these novel contacts into the dimer interface will raise the barrier toward resistance selection for this class of LEDGINs.

5 Conclusions

Control of human immunodeficiency virus type 1 (HIV-1) infection still poses major problems. Although the current standard of care, highly active antiretroviral therapy (HAART), has markedly improved survival and delayed progression to AIDS, it is not without drawbacks. A cure is lacking and lifelong treatment is required, opening the door to side effects and resistance development. While an effective vaccine remains beyond our reach, research efforts focused on exploring novel drug targets in retroviral biology remain essential to control the AIDS pandemic. Although INSTIs were last to be added to the HAART armamentarium, they have already claimed a first-line position in antiretroviral therapy. Nonetheless, resistance mutations have been found to arise under first-generation INSTI selective pressure, and significant cross-resistance is observed. Second-generation INSTIs such as dolutegravir seem to have a higher genetic barrier toward this resistance development. When dosed twice-daily, dolutegravir can be a treatment option for patients failing on raltegravir or elvitegravir. Close monitoring of patients will be required though as further accumulation of secondary mutations along the Q148 INSTI resistance pathway may lead to a reduced virological response to dolutegravir as well [103, 118].

Allosteric targeting of IN could provide future treatment options for these patients. Most promising in this respect are compounds of the LEDGIN class, which engage the LEDGF/p75 binding pocket on HIV-1 IN and do not show any cross-resistance with current ARVs. Despite impressive antiviral data showing potent inhibition of multiple steps during viral replication and steep dose–response curves, these compounds are still in pre- or early clinical development. Initial results show good tolerability and PK profile indicating the feasibility of once-daily dosing [199, 201]. Trials with BI 224436 were halted however and the current developmental status of this specific series is unclear. Nonetheless, various major pharmaceutical companies appear to be actively exploring LEDGIN-class compounds and we may soon see several of these inhibitors in clinical trials.

These last years have witnessed the coming of age of the field of IN drug discovery, from clinical approval of the first INSTI and development of second-generation INSTIs to discovery of potent multimodal allosteric inhibitors. These milestones, together with unprecedented insights into the structure and molecular mechanism of the retroviral integration machinery, hold great promise for years to come.

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Phosphonated Nucleoside Analogues as Antiviral Agents

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Abstract The present review is focused on the description of synthesis and antiviral activities of both acyclic and carbocyclic nucleoside phosphonates, endowed with an antiviral potential. Despite the outstanding results in antiviral therapy of acyclovir and azidothymidine, a major drawback concerning the use of nucleoside analogues (NA) is the retention of their stability following triphosphorylation within the host cell. The instability of the phosphate forms of NA has been, at least partially, overcome by the introduction of phosphate groups in the molecular structure. This approach gives rise to two main classes of compounds endowed with ascertained or potential antiviral activity, such as acyclic nucleoside phosphonates (ANP) and phosphonated carbocyclic nucleosides (PCN). Regarding ANP, a higher affinity for HIV reverse transcriptase (RT), with respect to NA, and the potent inhibition of HIV and hepatitis B virus (HBV) have been reported

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for some of them. Regarding PCN, some phosphonated cyclopropyl and cyclopentyl carbanucleosides, characterized by the presence of one or more phosphonic groups and by replacement of the endocyclic oxygen atom with a methylene group, showed to be good inhibitors of HBV and HIV infection. Another class of PCN is represented by phosphonated N,O-nucleosides (PCOAN). PCOAN encompass homo phosphonated-, phosphonated- and truncated phosphonated-N,O-nucleosides. Some PCOAN have been shown to directly inhibit RT activity of both murine and human retroviruses and to block HTLV-1 infection in vitro. The flexibility of the phosphonated NA structure suggests the possibility to develop new analogues endowed with antiviral activity towards a broad range of DNA or RNA viruses.

Keywords Acyclic nucleoside phosphonates (ANP), Antivirals, HBV, HCV, HIV, HTLV-1, HSV, Phosphonated carbocyclic nucleoside (PCN), Phosphonated N,O-nucleosides (PCOAN)

Contents

Abbreviations

3TC, lamivudine	(−)-L-3′-thia-2′,3′-dideoxycytidine
ABC, abacavir	Cyclopentenyl-N^6-cyclopropylaminopurine
ACV	Acyclovir
adefovir	PMEA
AdF	(5′S)-5-fluoro-1-isoxazolidin-5-yl-1H-pyrimidine-2,4-dione

ADV	Adefovir dipivoxil
ANP	Acyclic nucleoside phosphonates
APC	Apricitabine
ATL	Adult T-cell leukemia
AZT, zidovudine	3′-Azido-3′-deoxythymidine
BMS986001	Festinavir
CMV	Cytomegalovirus
d4T, stavudine	2′,3′-Didehydro-2′,3′-dideoxythymidine
ddC, zalcitabine	2′3′-Dideoxycytidine
ddI, didanosine	2′3′-Dideoxynosine
ddNMP	Dideoxynucleotide monophosphate
EBV	Epstein–Barr virus
ETC, entecavir	BMS-200475
Fd4AP	5-[(6-Amino-purin-9-yl)-4-fluoro-2,5-dihydro-furan-2-yloxymethyl] phosphonic acid disodium salt (50)
FPMPA	2,6-Diaminopurine
[(−) FTC, emtricitabine]	(−)-L5-fluoro-3′-thia-2′,3′-dideoxycytidine
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDP	Hexadecyloxypropyl
HAM/TSP	HTLV-1 associated-myelopathy/tropical spastic paraparesis
HMCV	Human cytomegalovirus
hOAT1 and hOAT3	Human renal organic anion transporters types 1 and 3
HPMPC, cidofovir	(S)-1-(3-Hydroxy-2-phosphonylmethoxypropyl)cytosine
HSV	Herpes simplex virus
HTLV-1	Human T-cell leukemia/lymphotropic virus type 1
MRP4	Multidrug resistance protein 4
NA	Nucleoside analogues
NNRTI	Non-nucleoside reverse-transcriptase inhibitors
NRTI	Nucleoside reverse-transcriptase inhibitors
ODE	Octadecyloxyethyl
PBMC	Peripheral blood mononuclear cells
PCN	Phosphonated carbocyclic nucleosides
PCOAN	Phosphonated N,O-nucleosides
PMCG	9-[1-(Phosphonomethoxycyclopropyl) methyl] guanine
PMCG	9-[1-(Phosphonomethoxycyclopropyl)methyl]guanine 55
PMEO-DAPy	2,4-Diamino 6-[2-(phosphonomethoxy)ethoxy]pyridine
PMPA	9-(2R,S)-2-phosphonylmethoxypropyl derivatives of adenine
R7128	Mericitabine
TDF	Tenofovir disoproxil fumarate
TPCOAN	Truncated phosphonated N,O-nucleosides 119
VZV	Varicella-zoster virus

1 Introduction

The continuous effort in the development of new antiviral agents is the consequence of the urgent demand for new therapeutic agents in which an improved biological activity against viruses is assisted by a low toxicity towards the host cell. The aim of this review is to summarize the most recent knowledge in literature regarding the synthesis and the biological activity of nucleoside analogues characterized by the presence of one or more phosphonate groups and by a well-proved or a potential antiviral activity. However, suitable reference to the past studies on the structure, the activity and the therapeutic efforts, regarding the wider and prototype class of pharmacologically active antiviral agents represented by the nucleoside analogues (NA), will be also reported.

2 Nucleoside Analogues as Antiviral Agents: An Historical Overview

The term NA refers to compounds in which structural modifications of the heterocyclic bases and/or the sugar moiety of natural nucleosides have been performed (Fig. 1). The development of NA as antivirals achieved a fundamental importance when acyclovir (ACV), an acyclic nucleoside analogue where the sugar unit is lacking, was found to be the first drug able to efficiently counteract herpes simplex virus (HSV) type 1 and 2 infection, in the 1970s. ACV is a guanosine analogue which is activated following phosphorylation promoted firstly by virus thymidine kinase, encoded by UL23 gene of HSV, and successively by cellular kinases (Fig. 2) [1].

In addition, other nucleoside analogues such as the prodrugs valaciclovir, valganciclovir, famciclovir and foscarnet have been clinically used for infections sustained by other components of the herpes virus family, encompassing also varicella-zoster virus (VZV) and cytomegalovirus (CMV) (for a complete review see [1]).

A remarkable input to the research on NA is derived, then, from the urgent need to find a therapeutic approach to withstand human immunodeficiency virus (HIV) infection. In the 1980s, the research on antiretrovirals was highly fruitful and in 1986 the in vitro anti-HIV activity of the prototype antiretroviral drug, 3′-azido-3′-deoxythymidine (AZT, zidovudine), was demonstrated by Mitsuya and Broder [2, 3]. The drug was originally intended to treat cancer, but it was associated with a high side effect profile. In 1987 AZT became the first drug to be approved by the Food and Drug Administration (FDA) for the treatment of AIDS and HIV infection. The designation “nucleoside analogue” was successively adopted for all structurally similar antiviral compounds, derived from the structural similarity of AZT to the building blocks of nucleic acids constituting DNA and RNA. AZT differs from the thymidine by the replacement of the hydroxyl groups in the 3′ position with a $–N_3$

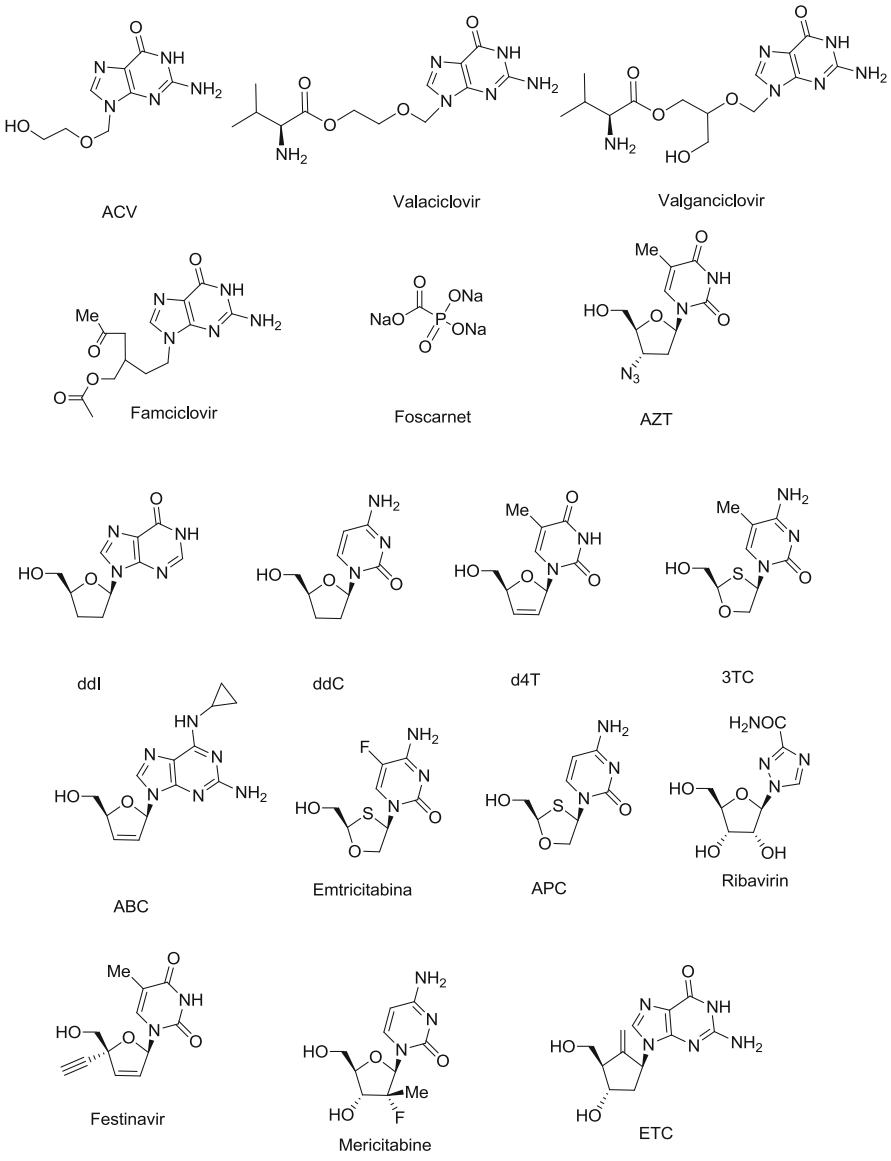


Fig. 1 Molecular structures of representative nucleoside analogues

group, which is unable to form the 5′-3′ phosphodiester linkage, essential for DNA elongation by DNA polymerases, including HIV reverse transcriptase (RT). This effect justifies the definition of AZT and other drugs as belonging to the same class of nucleoside reverse-transcriptase inhibitors (NRTI). The putative target of NRTI is the HIV-1 RT, an error-prone DNA polymerase, biochemically distinct from cell DNA polymerases [4]. In order to be properly incorporated by HIV-RT, NRTI must be

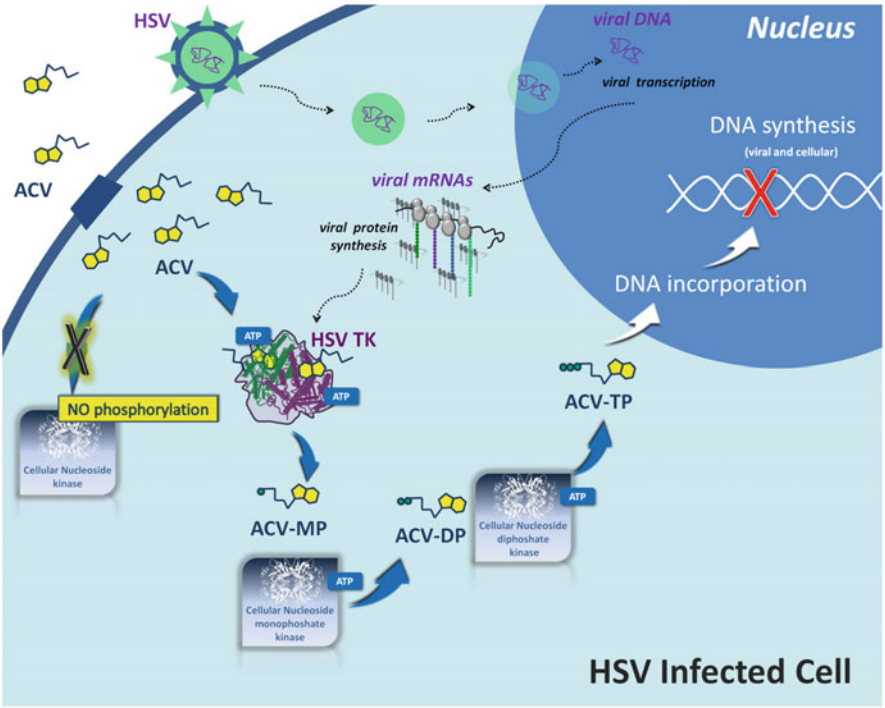


Fig. 2 Phosphorylation steps of acyclovir. The acyclic guanosine analogue acyclovir (ACV) is activated into the triphosphate compound (ACV-TP), in herpes simplex virus (HSV) infected cells, by both viral and cellular kinases. The first phosphorylation step is performed exclusively by HSV thymidine kinase (HSV TK). Successively, acyclovir monophosphate (ACV-MP) is transformed into acyclovir diphosphate (ACV-DP) and ACV-TP by host cell kinases

phosphorylated to their active 5′ triphosphate forms by cellular kinases, and this process might be influenced by the cell cycle phase of the host cell [5]. The 5′-triphosphate forms of NRTI are incorporated into DNA chain as dideoxynucleotide monophosphate (ddNMP) after the removal of the β- and the γ-phosphate groups.

From the discovery of AZT, a number of NRTI have been designed, sharing structural similarities with each other and mimicking endogenous nucleosides, such as 2′3′-dideoxynosine (ddI, didanosine), 2′3′-dideoxycytidine (ddC, zalcitabine), 2′, 3′-didehydro-2′,3′-dideoxythymidine (d4T, stavudine), and (−)-L-3′-thia-2′, 3′-dideoxycytidine (3TC, lamivudine), cyclopentenyl-N^6-cyclopropylaminopurine (ABC, abacavir), (−)-L-5-fuoro-3′-thia-2′,3′-dideoxycitidine [(−) FTC, emtricitabine] [6]. NRTI are used also in combination among them, trizivir (ABC, AZT, 3TC) or combivir (AZT, 3TC), or in combination with non-nucleoside reverse-transcriptase inhibitors (NNRTI). More recently, a novel NA, apricitabine (APC), a deoxycytidine analogue, was approved for HIV −1 infection treatment. Its structure is similar to that of

lamivudine, but APC was found to be active versus HIV strain bearing mutation M184 that confers resistance to lamivudine and emtricitabine [7, 8]. Even more recently, festinavir (BMS986001), a thymidine analogue similar to stavudine, was found to be less toxic [9].

A special mention in the chronology of NA deserves also ribavirin, one of the most used ribonucleoside analogue used in therapy. The mechanism of action of ribavirin is not unique and is not entirely clear. However, it is generally assumed that the antiviral activity of ribavirin is due to its competition with the guanosine triphosphate intracellular pool. Ribavirin is efficacious towards both DNA viruses (herpesviruses, adenoviruses, and poxviruses) and RNA viruses (HIV, influenza A virus, hepatitis C virus, respiratory syncytial virus) [10]. The combination of ribavirin with pegylated-interferon α is the backbone treatment in patients with hepatitis C virus (HCV) infection, reaching a sustained virological response in 75% of the treated individuals with genotypes 2 or 3. Other NA compounds, the synthesis of which and first evidence for antiviral activity date back at least 15 years ago, only recently have been demonstrated to be endowed with an actual therapeutic efficacy. The BMS-200475 (ETC, entecavir), a ribonucleoside analogue, was used for hepatitis B virus (HBV) infection. It is a cyclopentyl guanine, with an exo carbon-carbon double bond, that has been shown to be an efficacious and selective inhibitor of HBV polymerase in cultured liver cells [11]. Inhibition occurred following triphosphorylation of the compound in mammalian cells by cellular enzymes [12]. Thus, ETC acts as a structural terminator, presumably by introducing what is called "structural distortion" to preclude the enzyme from optimal interaction with the 3′ end of the growing DNA chain. More recent studies have shown that ETC is effective in both NA-naive and, at least partially, NA-experienced chronic hepatitis B patients [13].

The development of new therapies for the treatment of HCV infection is another intensive area of research for NA. In particular, high effort has been pursued in the research on inhibitors of one of the most important target in HCV, i.e., NS5B RNA-dependent RNA polymerase. A number of NA are in the pipeline such as mericitabine (R7128), which is the prodrug of PSI-6130, under clinical development for HCV chronic infection [14]. It is a ribonucleoside, (2′*R*)-2′-deoxy-2′-fluoro-2′-methylcytidine, which upon phosphorylation into 5′ triphosphate form inhibits HCV NS5B, acting as non-obligate chain terminator. Moreover, compounds in which the oxygen atom of the ribose unit is replaced by alternative heteroatoms, such as azaribonucleosides, have been reported to be endowed with antiviral activity in the cell-based HCV replicon assay [15].

In addition to HIV, HBV and HCV infections, NA were found to have some activity also towards infections sustained by other viruses. For example, a prodrug of the ribonucleoside analogue, 2′-fluoro-2′-deoxy-uridine and their corresponding phosphoramidates, was recently found to be efficacious in influenza virus infection [16].

Although treatments with NA have remarkably changed the course of virus infections, and particularly of HIV infection, the side effects of NA unavoidably influence the response to therapy. The toxicity of NA varies for each analogue and

relies on different markers of damage and tissue specificity [17]. Experimental models revealed that mitochondria were a major target of NA toxicity, showing disruption of a broad range of mitochondrial functions. Moreover, not less important toxic effects were also reported [18–22]. A complex double-edged, both inducing and inhibitory, regulatory action on apoptosis controlling genes has been recently reported for AZT [23].

Besides the toxicity, another major drawback of antiviral therapy with NA is the development of resistance. In the case of HIV infection, HIV-RT is highly prone to accumulate errors due to its lack of proofreading properties, thus generating mutated viruses [24]. Accumulation of one or more mutations in HIV-RT could lead to resistance of the virus to drug treatment. Two are the main mechanisms underlying the resistance to anti-HIV NA. One is the decreased incorporation of the triphosphorylated forms of NA into the growing DNA chain in comparison with normal dNTP or the decreased binding of the same activated forms to RT in competition with natural dNTP. Both effects are defined as discrimination effect.

The second mechanism of resistance is associated with the increased excision of NA from the DNA chain due to a process opposite to polymerization, defined as pyrophosphorolysis.

The outcome of resistance mutations was greatly favored by monotherapy and antagonized by combination therapy. However, despite the important results achieved by the latter, it still contributes to a number of mutations that accelerate the outcome of multi-drug-resistant HIV strains. In addition, also suboptimal therapy might select resistant virus strains.

In the following sections of this review we will focus the attention on the synthesis and the biological activities of acyclic nucleoside phosphonates (ANP), and cyclopropyl, cyclopentyl, and cyclohexyl carbanucleoside phosphonates, endowed with an antiviral potential, paying particular attention to compounds that appeared in literature in the last decade.

3 Acyclic Nucleoside Phosphonates

One of the metabolic drawbacks of NA is the retention of their stability following the triphosphorylation inside the host cell. To overcome the instability of triphosphate NA, several strategies have been proposed to increase their resistance towards the phosphohydrolase or to ensure a more efficient phosphorylation within the target cells. These approaches have led to the design of new classes of nucleotide prodrugs. One of the first described family of nucleotide analogues is represented by the ANP, which contain a phosphonate group linked to the acyclic chain. The development of ANP represents one of the remarkable progresses in the research on antiviral agents. In the phosphonated form these compounds are able to bypass the initial enzymatic phosphorylation step, undergoing only two phosphorylation steps in the host cell and showing resistance to pyrophosphorolysis [25]. In addition, ANP were found to have a higher affinity for RT with respect to NA, acting as potent inhibitors of infections sustained by both hepatitis and immunodeficiency

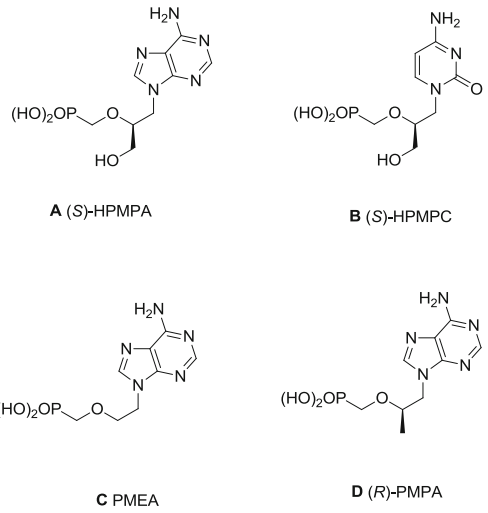


Fig. 3 Acyclic nucleoside phosphonates

viruses. ANP are converted by kinases into the corresponding diphosphorylated phosphonates and interfere with nucleic acid biosynthesis acting as DNA chain terminators.

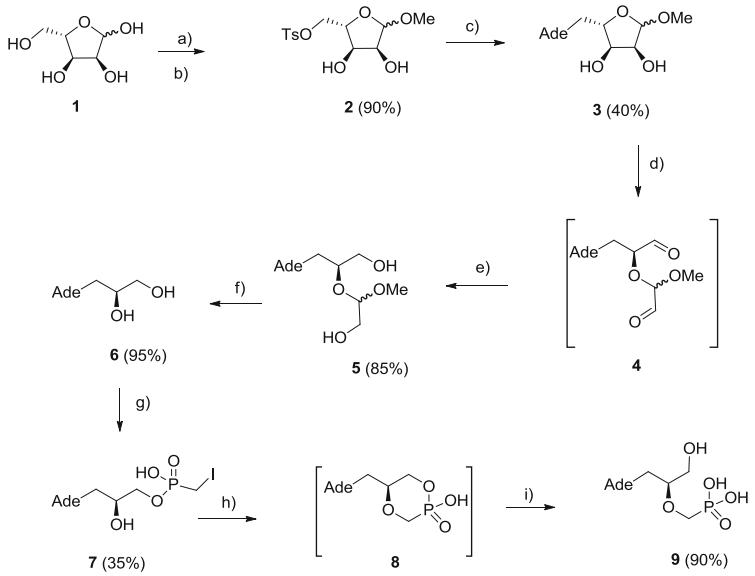
The structure of four ANP prototypes, designed with letters from **A** to **D**, is reported in Fig. 3.

3.1 Compounds of Structure A

The (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine, (*S*)-HPMPA, opened the era of ANP [26–28]. HPMPA is endowed with a strong activity against a variety of DNA viruses, including HSV-1, HSV-2, VZV, CMV, poxviruses, adenoviruses, and HBV. A recent synthetic approach towards (*S*)-HPMPA and its enantiomer starts from L(+)- or D(−)-ribose, respectively [29]. L(+)-ribose **1** was converted into its 5-tosyl derivative **2** and coupled with adenine, to afford the L-9-(*α*,*β*-1-methyl-5-ribofuranosyl)adenine **3**. This product was oxidized to dialdehyde **4** with sodium periodate, reduced with $NaBH_4$ to give **5**, which by treatment with formic acid afforded the diol **6**. The subsequent reaction with iodomethylphosphonic acid, with the formation of **7**, is followed by an intramolecular cyclization to **8**, from which **9** has been isolated, after treatment with water (Scheme 1).

The corresponding enantiomer (*R*)-HPMPA was similarly obtained from D(−)-ribose.

The activity against *CMV* and adenovirus is markedly increased when **9** is transformed into the hexadecyloxypropyl (HDP) **15** or octadecyloxyethyl (ODE) **16** esters. Noteworthy, while (*S*)-HPMPA is virtually inactive against HIV-1, HDP, and ODE, esters are active in nanomolar range and show also activity against HCV replication with EC_{50} of about 1 μM.



Scheme 1 Reagents and conditions: (*a*) AcCl/MeOH, RT, 16 h; (*b*) TsCl/Py, 4°C, 16 h; (*c*) adenine, NaH, DMF, 80°C, 5 days; (*d*) $NaIO_4$, 4°C, 1 h; (*e*) $NaBH_4$, 4°C, 3 h; (*f*) H^+, 6 h; (*g*) $ICH_2PO(OH)_2$/DCC/Py, 16 h; (*h*) NaH/DMF, 6 h, H_2O, 3 h; *i*) glacial AcOH, 3 h

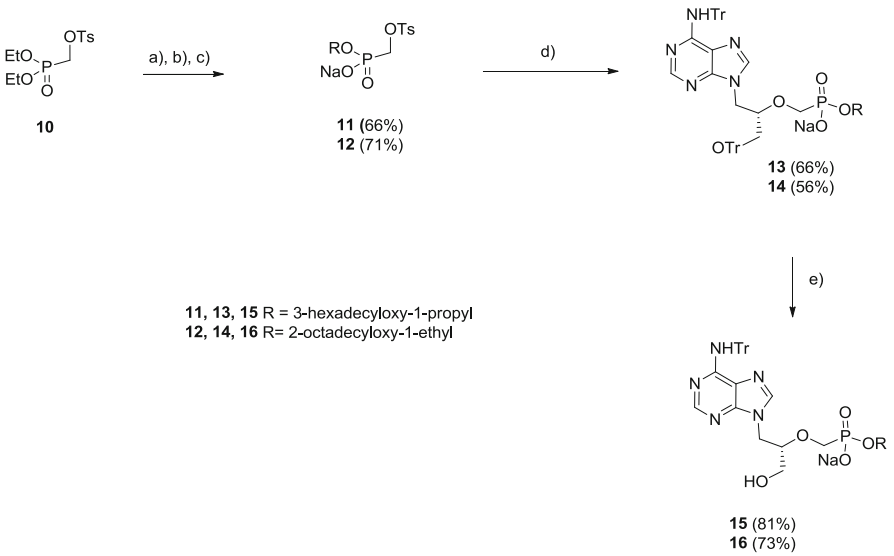
The synthesis of ODE-(*S*)-HPMPA and HDP-(*S*)-HPMPA has been described by Hostetler and Beadle et al. [30] and it is reported in Scheme 2.

3.2 *Compounds of Structure B*

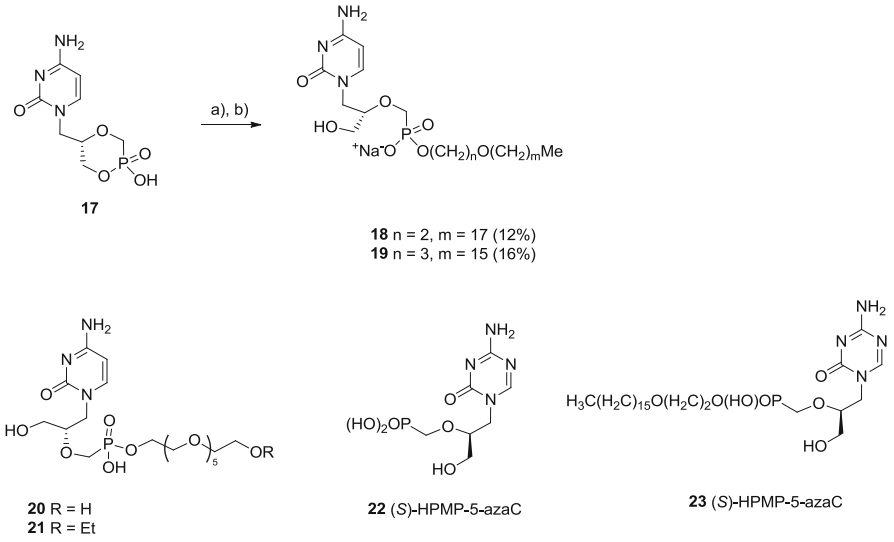
(*S*)-1-(3-Hydroxy-2-phosphonylmethoxypropyl)cytosine B (HPMPC, cidofovir) was firstly reported in 1987 [31]. The antiviral activity spectrum of cidofovir is similar to that of HPMPA: compound is active against virtually all DNA viruses, including polyoma-, papilloma-, adeno-, herpes- and poxviruses [32].

Similarly to (*S*)-HPMPA, alkoxyalkyl esters of cidofovir have been designed as prodrugs, in order to increase the oral bioavailability and reduce the toxicity of cidofovir. The ODE and HDP derivatives **18** and **19** are the most interesting compounds: in particular, pharmacokinetic and safety studies are currently being developed in humans for the use of novel lipid conjugate of **18** in the prevention of dsDNA viral infections [33].

Two new prodrugs of (*S*)-HPMPC, **20** and **21**, have been recently synthesized [34]. These compounds inhibit the in vitro replication of different herpesviruses and poxvirus vaccinia virus with an activity equivalent to that of cidofovir. The

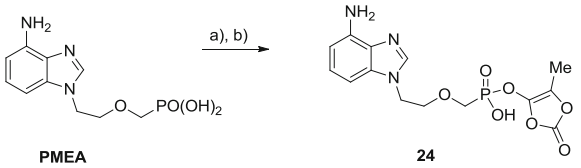


Scheme 2 Reagents and conditions: (*a*) TMSBr, CH_2Cl_2; (*b*) oxalyl chloride, N,N-DMF (0.5 mL), 0°C, 5.5 h; (*c*) 3-hexadecyloxy-1-propanol or 2-octadecyloxy-1-ethanol, Et_2O, Py, 3 h, aq $NaHCO_3$, 1 h; (*d*) (*S*)-9-[3-trityloxy-2-hydroxypropyl]-N^6-trityladenine, NaH, Et_3N (solvent), 50°C, 12 h; (*e*) 80% aq AcOH, 60°C, 1 h



Scheme 3 Reagents and conditions: (*a*) ODE-Br, ODP-Br, or HDP-Br, N,N-DMF, 80°C, 6 h; (*b*) 0.5 M NaOH, 1.5 h, AcOH

synthetic scheme towards these prodrugs starts from the cyclic form of cidofovir **17**, which undergoes a nucleophilic substitution by the corresponding haloderivative (Scheme 3) [35].



Scheme 4 Reagents and conditions: (*a*) Bu_4NOH, MEOH; (*b*) (5-methyl-2-oxo-1,3-dioxolen-4-yl)methylbromide, DMF

Acyclic nucleoside **22**, the (*S*)-HPMP-5-azaC, is characterized by the replacement of pyrimidine ring by a triazine system. This compound shows, against DNA viruses, an activity comparable to that of the parent compound [32]. The insertion of an alkoxyalkyl group, as in its hexadecyloxyethyl (HDE) ester **23**, leads to the enhancement of the antiviral activity against infections sensitive to cidofovir, in particular CMV, HSV, HPV, adeno- and poxivirus infections [36].

3.3 Compounds of Structure C

Amphiphilic prodrugs of PMEA (adefovir) have been prepared by its conversion into alkoxyalkyl esters (Scheme 4): the (5-methyl-2-oxo-1,3-dioxolen-4-yl) methyl ester of PMEA **24** showed significant activities against HIV and herpesviruses [34].

3.4 Compounds of Structure D

Holy and De Clercq have described in 1991 the 9-(2*R*,*S*)-2-phosphonylmethoxypropyl derivatives of adenine (PMPA) and 2,6-diaminopurine (FPMPA) as potent and selective antiretroviral agents [37]. Later, they showed that the anti-HIV activity of this class of compounds resided in (*R*)-enantiomers (Fig. 4) [38].

(*R*)-PMPA (the well-known tenofovir) has been selected as a drug and actually plays a key role in the control of HIV infections [39].

A reported kilogram-scale synthesis of tenofovir (*R*)-PMPA develops in a three-step sequence [40]: (1) condensation of adenine with (*R*)-propylene carbonate **25**; (2) alkylation of the corresponding (*R*)-9-(2-hydroxypropyl)adenine **26** with diethyl *p*-toluenesulfonyloxymethanephosphonate using lithium *t*-butoxide and (3) hydrolysis of ester **27** with bromotrimethylsilane (Scheme 5).

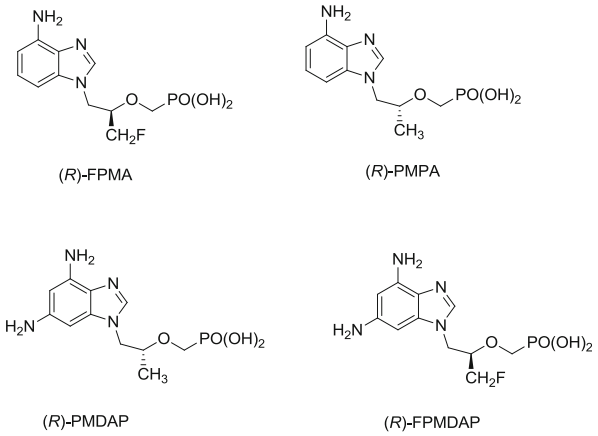
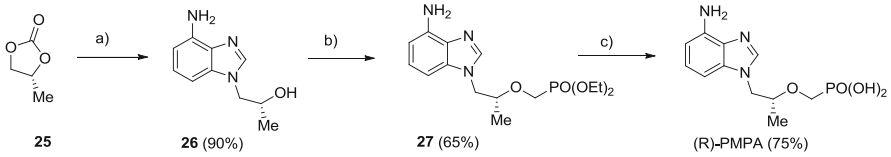


Fig. 4 Structures of (*R*)-FPMA, (*R*)-PMPA, (*R*)-PMPDAP, and (*R*)-FPMDAP



Scheme 5 Reagents and conditions: (*a*) adenine, NaOH, DMF, 140°C, 20 h; *t*-BuOLi, diethyl *p*-toluenesulfonyloxymethanephosphonate, 35°C, 1 h, aq AcOH; (*c*) TMSBr, MeCN

3.5 2,4-Diaminopyrimidine Nucleosides

DAPys, the so-called 2,4-diaminopyrimidine derivatives, constitute a novel class of ANP where the disubstituted pyrimidine ring is linked, through an oxygen bridge, to either the 2-phosphonylmethoxyethyl or 2-phosphonomethoxypropyl moiety (Fig. 5).

The synthesis of 2,4-diamino 6-[2-(phosphonomethoxy)ethoxy]pyridine (PMEO-DAPy) **28** is reported in Scheme 6 [41]. The reaction of diisopropyl 2-chloroethoxymethyphosphonate **33** with 2,4-diamino-6-hydroxypyrimidine leads to a mixture of diesters **34** and **35** in low yields. Compound **34** was isolated and transformed into the free phosphonic acid **28** (78.3%).

The synthesis of 5-Br-PMEO-DAPy **29** and 5-Me-PMEO-DAPy **30** is described in Scheme 7 [42]. Compound **29** has been prepared starting from derivative **34**, intermediate in the synthesis of PMEO-DAPy, by reaction with bromine followed by reaction with TMSBr, and hydrolysis. 5-Me-PMEO-DAPy **30** has been synthesized by coupling reaction of **36** performed with Me_3Al and $Pd(PPh_3)_4$ and subsequent hydrolysis of **37** and **38**.

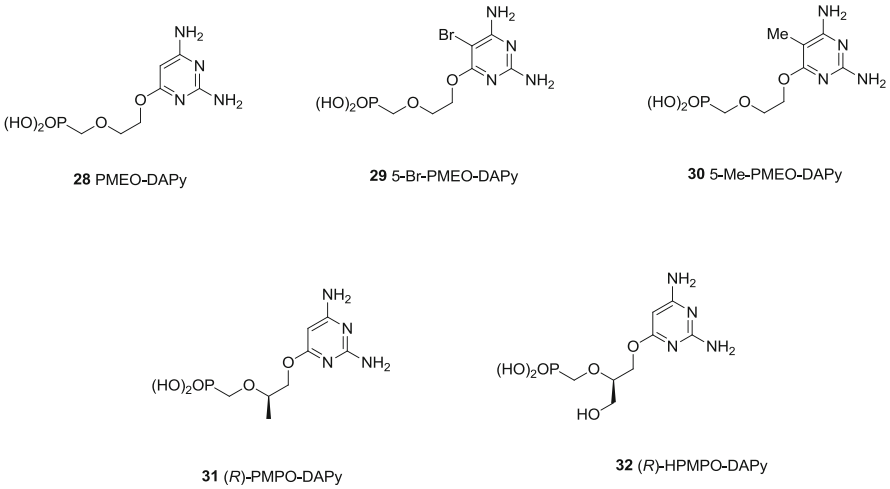
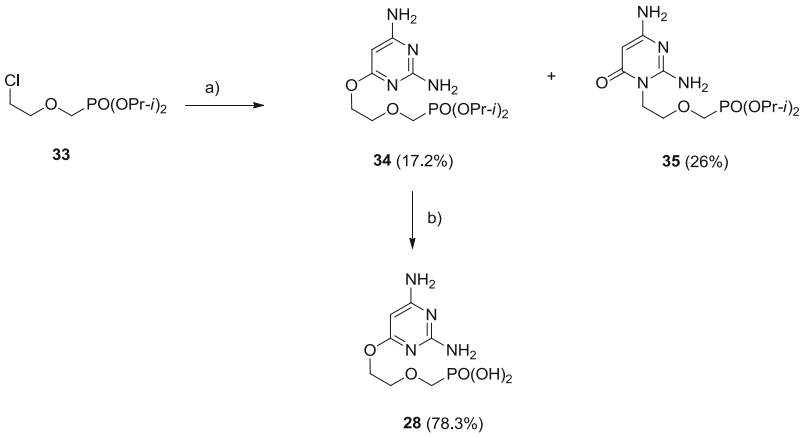


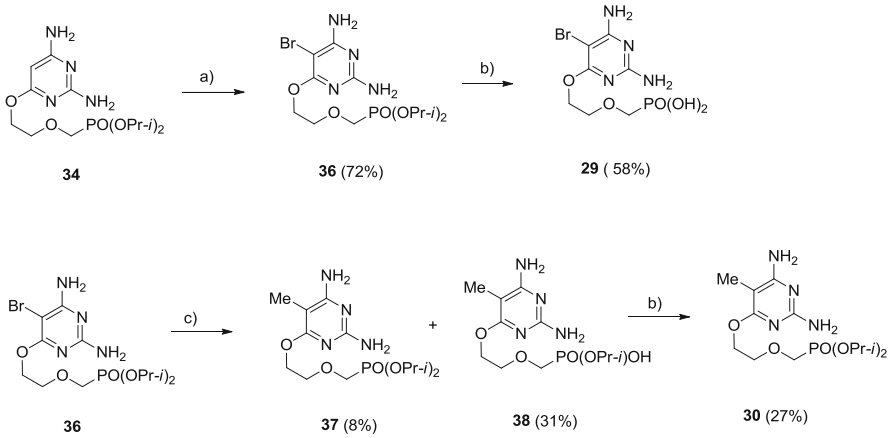
Fig. 5 2,4 Diaminopyridine nucleosides



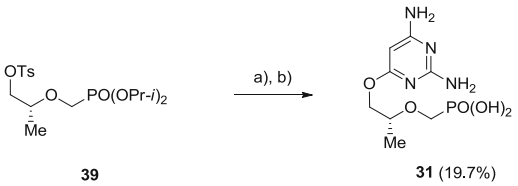
Scheme 6 Reagents and conditions: (*a*) 2,4-diammino-6-hydroxypyrimidine, Cs_2CO_3, DMF, 80°C, 30 min then 100°C 16 h; (*b*) TMSBr, MeCN 14 h, RT, aqueous NH_3

(*R*)-PMPO-DAPy **31** has been synthesized starting from the (*R*)-2-(diisopropyl-phosphoryl)-methoxypropyl tosylate **39** (Scheme 8). (*R*)-PMPO-DAPy has showed pronounced antiherpes and anti-retroviral activity comparable to that of **28**, whereas the (*S*) enantiomer was virtually devoid of antiviral activity.

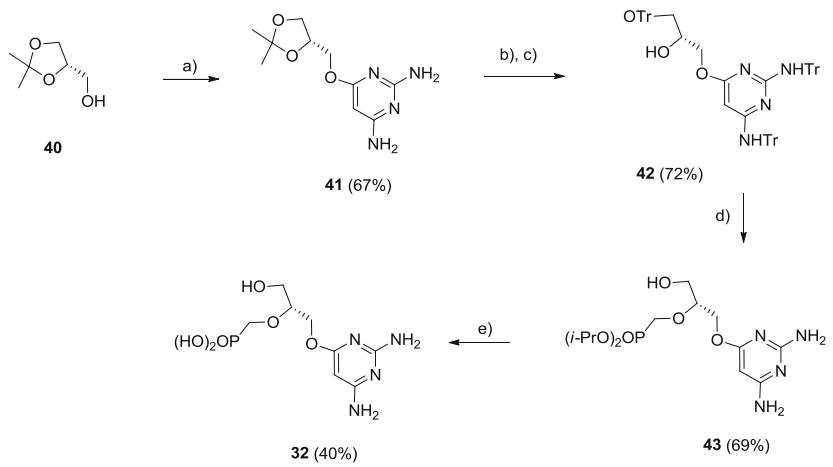
Finally, compound **32** has been prepared according to Scheme 9 in five steps, involving a sequence of reactions that starts from compound **40** [43].



Scheme 7 Reagents and conditions: (*a*) Br_2, DMF/CCl_4, Cs_2CO_3, DMF, 80°C, 30 min then 100°C 16 h; (*b*) TMSBr, MeCN 14 h, RT, aqueous NH_3; (*c*) $AlMe_3$, $Pd(Ph_3P)_4$, THF



Scheme 8 Reagents and conditions: (*a*) 2,4-diammino-6-hydroxypyrimidine, DBU, DMF, 90–100°C, 24 h; (*b*) TMSBr, MeCN 14 h, RT, aqueous NH_3



Scheme 9 Reagents and conditions: (*a*) NaH, THF, 2,4-diamino-6-chloropyrimidine, 50°C, 12 h; (*b*) H_2SO_4, RT 12 h; (*c*) trityl chloride, DMAP, Py, 50°C, 15 h; (*d*) diisopropyl *p*-toluenesulfonyloxymethylphosphonate, NaH, 3d, RT; (*e*) TMSBr, MeCN, 12 h

Table 1 Antiviral activity of acyclic nucleoside phosphonates

Compound	Structural analogy	Molecular targets	Virus	References
Class				
representative compounds				
HPMPA (*S*)-*HPMPA*	Adenine analogues	Viral DNA polymerase, HBV-RT	Herpesviruses, poxviruses, adenovirus, HBV	[26, 30]
HPMPC *Cidofovir*	Cytosine analogues	Viral DNA polymerase	Herpesviruses, poxviruses, adenovirus, polyomavirus, papillomavirus	[31, 32, 34–36, 44]
PMEA *Adefovir*	Adenine analogues	Viral DNA polymerase, HIV-RT	Herpesviruses, HIV	[34, 45–47]
PMPA *Tenofovir*	Adenine analogues	HIV-RT, HBV-RT, HTLV-RT	HIV, HBV, HTLV-1	[37–40, 48–52]
DAPy *PMEO-DAPy* (*R*) - *HPMPOyDAPy*	Pyrimidine analogues	HIV-RT, HBV-RT, viral DNA polymerase	HIV-1, HIV-2, HBV, herpesviruses, poxviruses, adenovirus, papillomavirus	[41–43, 59–62]

3.6 *Highlights on Antiviral Activity and Toxicity of ANP*

Currently, three ANP are in clinical use (Table 1). Cidofovir was the first ANP to be approved in 1996 by FDA for CMV infection in HIV patients and is also in use for HPV infection [44]. Adefovir dipivoxil (ADV) is an orally bioavailable prodrug, chemically defined as a nucleotide analogue of adenosine monophosphate. It was previously used in HIV infection, but it was discontinued because of toxic effects in 1999. In 2002 FDA approved its use for HBV infection in hepatitis B e antigen (HBeAg)-positive as well as hepatitis B e antigen-negative patients [45]. The results of a phase III study, encompassing 500 individuals with chronic HBeAg-positive infection, treated with ADV versus placebo, showed that the ADV groups had greater histologic improvement, decreased viral load and higher rates of HBeAg seroconversion [46]. A study published in 2004 demonstrated that adding ADV to lamivudine treatment in individual resistant to lamivudine gave better results in the clearance of HBeAg and in the decreasing of viral load, in comparison with switching to monotherapy with ADV in lamivudine-resistant individuals [47]. ADV was well tolerated and the most serious side effect was ascribed mainly to nephrotoxicity, taking place more often in individual suffering of kidney dysfunction. Tenofovir is a 9-[2-(*R*)-(phosphonomethoxy) propyl] adenine which requires, similarly to other ANP, two phosphorylation steps by cellular kinases to be activated. Its spectrum of antiviral activity includes HIV and hepadnaviruses [39, 48]. In addition, it has been also shown that tenofovir was able to protect peripheral blood mononuclear cells from healthy donors against human T-cell leukemia/lymphotropic virus type 1 (HTLV-1) infection in vitro [49]. However,

the tenofovir prodrug, tenofovir isoproxil fumarate (TDF), when used as a monotherapy, was unable to inhibit viral replication in HTLV-1-infected patients affected by tropical spastic paraparesis [50]. Conversely, when adopted in HIV infection in combination therapy, TDF caused a significant decline in plasma HIV-1 RNA levels in randomized, double-blind placebo-controlled clinical trials in HIV-1 infected individuals, including those with nucleoside resistance mutations [51, 52]. TDF use was then approved by FDA in a single-tablet formulation with emtricitabine (Truvada) in 2004 or with efavirenz and emtricitabine (Atripla) in 2006. Tenofovir, like other ANP, provides a long-term antiviral response, leads poorly to the emergence of drug resistance and is well tolerated in vivo, in HIV-infected patients [51, 52], although alteration of renal proximal tubules has been reported [53].

ANP are endowed with a relative limited toxicity. At the cellular level, the limited toxicity of tenofovir seems due to a low capability to produce mitochondrial dysfunction. This seems to be related to the poor capacity of tenofovir to act as a substrate for human cellular and mitochondrial α, β, γ and ε DNA polymerases in vitro [54]. It has been shown that tenofovir has limited effects in vitro on the proliferation of renal proximal tubule epithelial cellsand erythroid and myeloid progenitors [55]. Conversely, in vivo studies demonstrated that acyclic nucleotides are prone to accumulate in kidney, since they are secreted through glomerular filtration and tubular secretion [56]. Accumulation of ANP in renal proximal tubules has been shown to be regulated by human renal organic anion transporters types 1 and 3 (hOAT1 and hOAT3) [57]. On the other hand, multidrug resistance protein 4 (MRP4) counteracts the action of the transporter by reducing accumulation of ANP in tubular cells [58]. Thus modulation of ANP transporter plays a critical role for accumulation of the drugs within basal membrane of proximal tubule epithelial cells and related toxicity.

Regarding the antiviral activity of 2,4-diaminopyrimidine nucleosides, it has been pointed out that 6-[2-(phosphonomethoxy)ethoxy]pyrimidines bearing amino group concomitantly on both C-2 and C-4, or an amino on C-2 and an OH group on C-4, exhibited antiviral activity [59]. Some of these compounds have shown promising antiviral activity. The prototypes PMEO-DAPy 28, 5-Br-PMEO-DAPy 29, 5-Me-PMEO-DAPy 30, and (*R*)-PMPO-DAPy **31** have shown activity against different HIV-1 isolates and HIV-1 mutant strains, HIV-2 and also HBV, while (*R*)-HPMPO-DAPy **32** has shown activity against herpes-, adeno, pox- and papillomaviruses (Fig. 5) [42, 43, 59–62]. In particular Balzarini et al. in 2002 reported that the prototypes 6-PMEO 2,4-diaminopyrimidine and 6-PMPO 2,4-diaminopyrimidine derivatives showed a potent activity against HIV replication, in CEM and MT-4 cells as well as in primary culture of peripheral blood mononuclear cells and that they were endowed with a limited cytotoxicity. Further studies have shown how 5-substituted PMEO-pyrimidine derivatives, methyl, bromo, formyl, cyano, chloro, were endowed with an anti-HIV-1 and anti-HIV-2 antiretroviral activity in CEM cells, comparable with that of adefovir and tenofovir. In particular PMEO-5-Me-DAPy exhibited an EC_{50} of 0.06 μg/mL, 25 times lower than that showed by ADV (EC_{50} 1.5 μg/mL). In addition, it has to be underlined that all the 5-substituted PMEO-pyrimidine

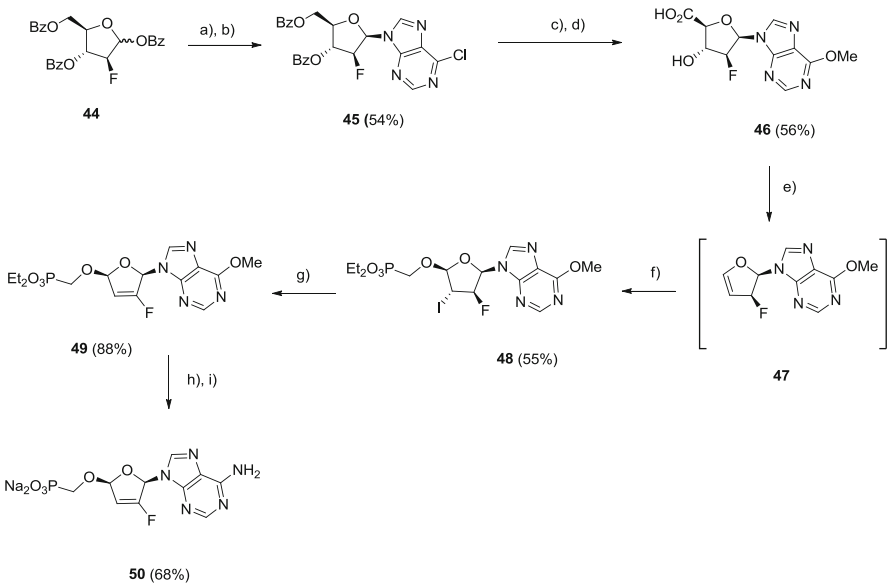
derivatives were not toxic in cell culture except for PMEO-5-Me-DAPy with a CC_{50} of 3.4 μg/mL, i.e., a value that was in any case 5 and 37 times lower than that of adefovir and tenofovir, respectively [61].

3.7 Resistance to ANP

ANP are associated with few resistance mutations in viral polymerases, differently from NA. Some of them deserve to be described. The primary mutation in HIV-RT conferring resistance to tenofovir is the K65R, which is sheared with some NA such as ddI, abacavir and emtricitabine. This mutation decreased the sensitivity of HIV viral isolates to tenofovir in vitro [63]. Moreover, also HIV-RT from patients not responding to therapy with tenofovir exhibited the K65R mutation [64]. Studies about the processivity of tenofovir on wild type and mutant HIV strains, respectively, revealed that K65R mutation containing viruses were unable to bind or incorporate tenofovir [65]. Another mutation which equally seems to confer resistance in HIV-RT to ANP is the K70E. This mutation was first identified following treatment with adefovir [66]. After stopping the therapy with ADV in HIV infection, the resistance mutation disappeared. The re-emergence of the K70E resistance mutation was observed in patients receiving treatment with ABC, 3TC and TDF [67]. Molecular studies revealed that K70E mutation confers resistance to tenofovir and NRTI by preventing the positioning of the NRTI-TP in the active center of HIV-RT, which results in increased discrimination process, due to a failure of the catalytic efficiency of polymerase [68]. A few studies have investigated the resistance to pyrimidine analogue phosphonates. Recently, it was reported that acyclic pyrimidine nucleoside phosphonate analogues, presenting an amino group at C-2 of the pyrimidine ring together with an amino group at C-4, maintained activity against HIV laboratory strains bearing L100I, K103N, Y181C, and Y188H mutations in the RT [43].This activity was maintained also versus three clinical isolates either untreated, HIV-1/L1S, or bearing NRTI-specific mutations, HIV-1/L6S, or bearing mutation to PMEA, HIV-1/L6S/PMEA [43].

3.8 GS-9148: New Promising Phosphonated Nucleoside

Recently, the synthesis of GS-9148 (phosphonomethoxy-2-fluoro-2,3-dideoxydide-hydroadenosine **50**; Fd4AP) [69], a novel nucleotide HIV reverse-transcriptase inhibitor, has been reported. GS-9148 is an adenosine derivative equipped with a 2′,3′-dihydrofuran ring containing a 2′-fluoro group. This compound was prepared starting from commercially available 2-fluoro-1,3,5-tri-*O*-benzoyl-D-arabinofuranose **44**, in nine steps, according to Scheme 10. Thus, fluorosugar **44** was transformed into **45**, by reaction with HBr and addition of 6-chloropurine sodium salt. The chloropurine derivative **45** was converted into 6-methoxy acid **46** by hydrolysis



Scheme 10 Reagents and conditions: (*a*) HBr/Ac, CH_2Cl_2; (*b*) NaH, 6-chloropurine, MeCN; (*c*) K_2CO_3, MeOH; (*d*) Jones' reagent, acetone; (*e*) DIAD, Ph_3P, CH_2Cl_2; (*f*) $HOCH_2PO_3Et_2$, IBr, CH_2Cl_2; (*g*) AcOH, MeOH, NaClO; (*h*) NH_4OH aq, Δ; *i*) 2,6-Lutidine, TMSBr, MeCN

and selective Jones oxidation. This acid led to compound **48** via the glycal intermediate **47**, by Mitsunobu activation followed by treatment with IBr and diethyl hydroxymethylphosphonate. **48** was transformed into unsaturated derivative **49** using buffered sodium hypochlorite oxidation of the iodide, followed by elimination. Finally, the target **50** was obtained from **49** by reaction with NH_4OH followed by hydrolysis with trimethylsilylbromide and $NaHCO_3$ treatment.

Molecular studies revealed that GS-9148 exhibited 5–10 times less affinity than cidofovir, adefovir and tenofovir for hOAT1 and 60–100 times lower transport efficiency by the transporter [70]. The poor affinity for the transporter, demonstrated in vitro, very likely is the cause of a lower accumulation of GS-9148 in kidney of dog observed in vivo in comparison with the other ANP [70]. Although GS-9148 was found less toxic than tenofovir, its oral bioavailability needed to be improved. The phosphoamidate prodrug of GS-9148, GS-9131, was optimized to increase the delivery of GS-9148-DP into lymphoid cells by 200-folds relative to that of GS-9148 [71]. Further studies have demonstrated that GS-9131 inhibited HIV replication in CD4+ primary cells with EC_{50} (3.7 nM) lower than that of tenofovir diphosphate (EC_{50} 1.5 μM) [72]. Therefore, given the pharmacokinetic properties of GS-9131 and its ability to load lymphoid cells with long intracellular half-life and to be efficiently phosphorylated, it can be considered as a potential candidate for HIV antiretroviral therapy. In addition, it has been reported that GS-9148 selected a unique HIV resistance mutation. Biochemical studies have shown that the HIV-1 mutant RT

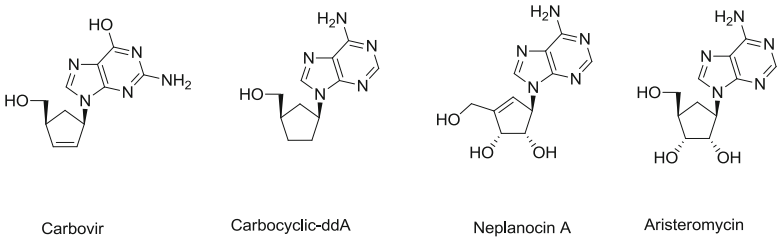


Fig. 6 Relevant carbocyclic nucleosides

Q151L exhibited resistance to GS-9148 by blocking its incorporation in the DNA growing chain. This was found to be peculiar for GS-9148 since the Q151L resistance mutation did not affect the incorporation of other adenosine analogues such as tenofovir [41].

4 Phosphonated Carbocyclic Nucleosides (PCN)

Carbocyclic nucleosides, the class of compounds by which phosphonated carbocyclic nucleosides derive, are structural analogues of natural and synthetic nucleosides, characterized by the replacement of the endocyclic oxygen atom with a methylene group. Due to the absence of the labile glycosidic bond, these analogues are chemically and enzymatically more stable than the natural nucleosides. Figure 6 reports some members of this family: carbovir and carbocyclic-ddA, naturally occurring carbo-nucleosides, show a good activity against HIV. Natural neplanocin A and aristeromycin, and other synthetic derivatives, exhibit powerful antitumor and antiviral activities [73].

4.1 Phosphonated Cyclopropyl Nucleosides

Phosphonated cyclopropyl nucleosides, reported here, can be classified in different groups depending on the relative position of nucleobase and phosphonic group (Fig. 7).

4.1.1 Compounds of Structure I

The 9-[1-(phosphonomethoxycyclopropyl)methyl]guanine **55** (PMCG), containing a cyclopropyl moiety at the 2′-position, is endowed with a selective anti-HBV activity ($EC_{50} = 0.5$ μM). The synthetic route involves, as the key step, a titanium-mediated Kulinkovich cyclopropanation and develops in seven steps starting from

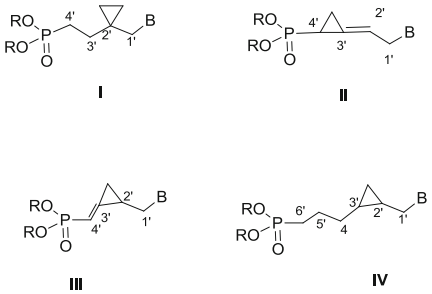
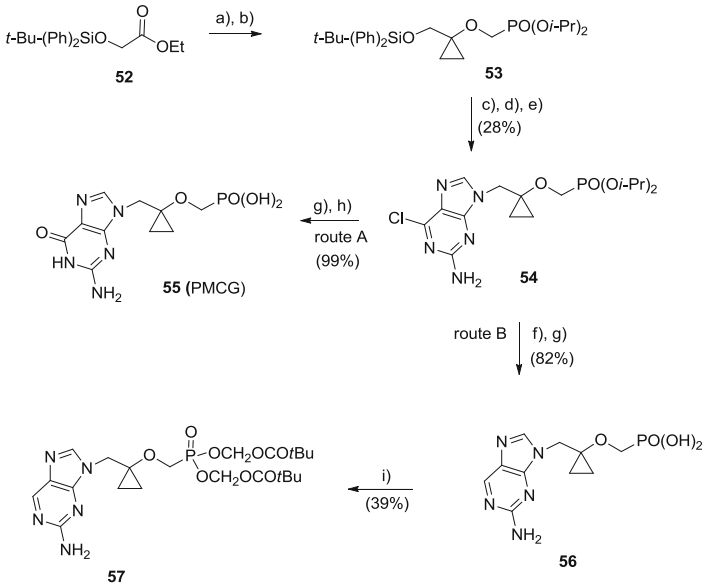


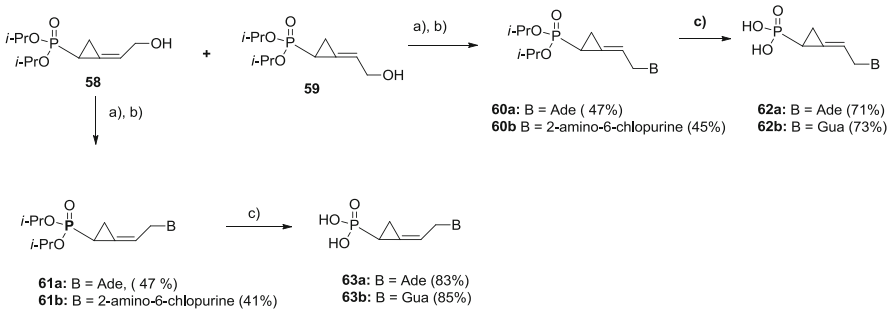
Fig. 7 Phosphonated cyclopropyl nucleosides



Scheme 11 Reagents and conditions: (*a*) CH_3CH_2MgBr, $Ti(O\textit{i}\text{-}Pr)_4$ (0.25 equiv), 10 h, THF 0−25°C; (*b*) $BrCH_2P(O)(O\textit{i}\text{-}Pr)_2$, LiI (cat.), *t*-BuOLi, DMF, THF, 60°C, 4 h; (*c*) NH_4F, MeOH, reflux, 10 h; (*d*) MsCl, TEA, MDC, 0−25°C; (*e*) 6-chloroguanine, NaH, DMF, 80°C, 4 h; (*f*) H_2, 5% Pd/C, THF, 1 atm, 18 h; (*g*) TMSBr, MDC, reflux, 18 h; (*h*) 2 N HCl, reflux, 6 h; *i*) chloromethyl pivalate, TEA, 1-methyl-2-pyrrolidinone, 25°C, 48 h

ethyl 2-acetate **51**. Its conversion into the cyclopropanol derivative **52** (80% yield), by reaction with ethylmagnesium bromide and titanium (IV) isopropoxide, is followed by the reaction with diisopropylbromomethylphosphonate to give **53**, from which the target phosphonated **55** was obtained (Scheme 11) [74].

Hydrogenation of **54**, followed by hydrolysis and etherification with chloromethyl pivalate (39% yield), afforded the orally available Dipivoxil **57** (Scheme 11 route b).



Scheme 12 Reagents and conditions: (*a*) Ph_3P, CBr_4, CH_2Cl_2; (*b*) adenine or 2-amino-6-chloropurine; (*c*) HCl

4.1.2 Compounds of Structure II

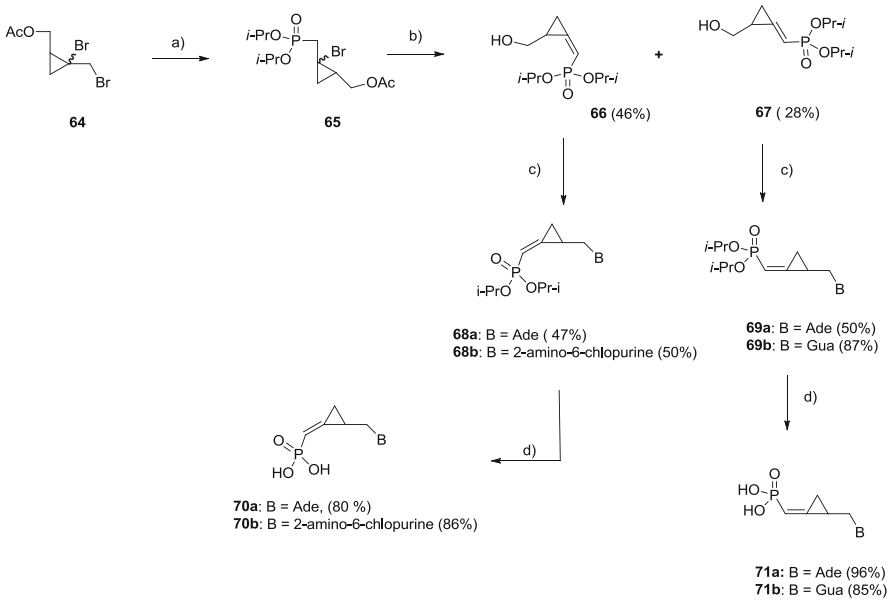
An easy entry towards this class of compounds is reported in Scheme 12. In particular, diisopropyl (*Z*)- and (*E*)-(2-hydroxyethylidene)-1-cyclopropylphosphonate **58** and **59** were converted into the corresponding nucleotides **60** and **61** utilizing an Appel reaction, followed by nucleophilic amination with adenine or 2-amino-6-chloropurine. The subsequent acidic hydrolysis led to adenine and guanine analogues **62** and **63** [75] (Scheme 12). Phosphonates **61a** and **61b** are potent inhibitors of Epstein–Barr virus (EBV) replication.

4.1.3 Compounds of Structure III

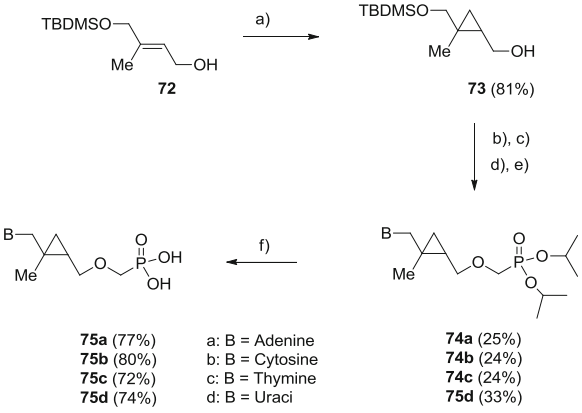
Reversed methylenecyclopropane phosphonated nucleosides **70** and **71** have been synthesized starting from 1-bromomethylcyclopropane 64, which was converted into the bromocyclopropyl phosphonate **65** by Michaelis–Arbuzov reaction. The subsequent β-elimination, followed by nucleobase introduction and hydrolysis, furnishes the target phosphonates **70** and **71** (Scheme 13) [76].

4.1.4 Compounds of Structure IV

Compounds bearing a methyl substitution at the cyclopropyl ring have been synthesized by Kim and coworkers [77], employing the Simmons-Smith reaction as key step. Thus, the allylic alcohol **72** was reacted with $ZnEt_2$ and CH_2I_2 to form the cyclopropyl alcohol **73** that was converted, according to standard procedures, into the phosphonated nucleosides **74a–d** (Scheme 14). The phosphonic acid nucleosides **75a–d**, obtained by hydrolysis of **74a–d**, have been evaluated for their antiviral activities. Unfortunately, all compounds proved to be inactive against HIV-1, HSV-1, HCMV, and CoxB3, except **74b** that shows a low activity against CoxB3 and HIV-1 (EC_{50} 43.5 and 55.7 mg/mL, respectively).

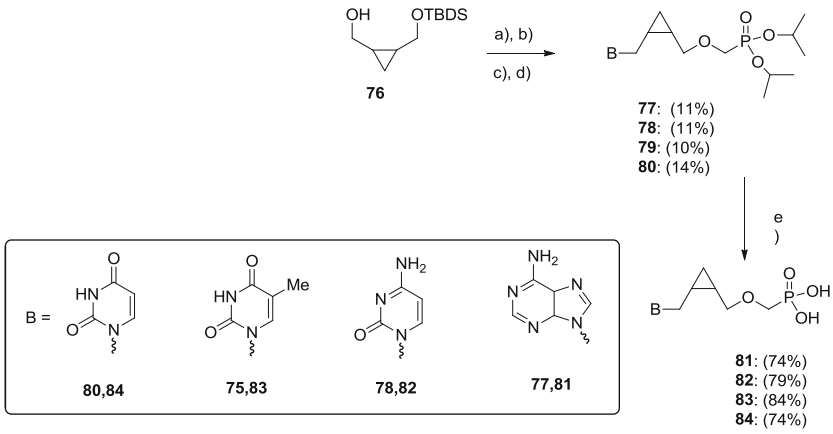


Scheme 13 Reagents and conditions: (*a*) triisopropyl phosphite, 120°C, 48 h; (*b*) NaOH, 40 min., RT; (*c*) Ph_3P, adenine or 2-amino-6-chloropurine, DEAD 0°C, 12 h RT, (*d*) HCl



Scheme 14 Reagents and conditions: (*a*) CH_2I_2, Et_2Zn, CH_2Cl_2; (*b*) diisopropyl bromomethylphosphonate, LiI, *t*-BuOLi,DMF; (*c*) TBAF, THF; (*d*) MsCl, TEA, CH_2Cl_2; (*e*) adenine, cytosine, thymine or uracil, K_2CO_3, 18-C-6, DMF; (*f*) Me_3SiBr, CH_2Cl_2

Hong et al. [78], applying the same methodology above reported [77], have prepared various unsubstituted cyclopropyl phosphonates of structure IV, starting from the *cis*-[2-({[*t*-butyl(dimethyl)silyl]oxy}methyl)cyclopropyl]methanol **76** (Scheme 15). Only the adenine derivative **81** exhibited moderate activity against human cytomegalovirus (HMCV) (EC_{50} = 22.8 μg/mL), with cytotoxic activity to the host cell at a concentration higher than 100 μg/mL.



Scheme 15 Reagents and conditions: (*a*) diisopropyl bromomethylphosphonate, LiI, *t*-BuOLi, DMF; (*b*) TBAF; (*c*) MsCl, TEA, CH_2Cl_2; (*d*) uracil, thymine, cytosine, K_2CO_3, adenine, 18-C-6, DMF; (*e*) Me_3SiBr, CH_2Cl_2

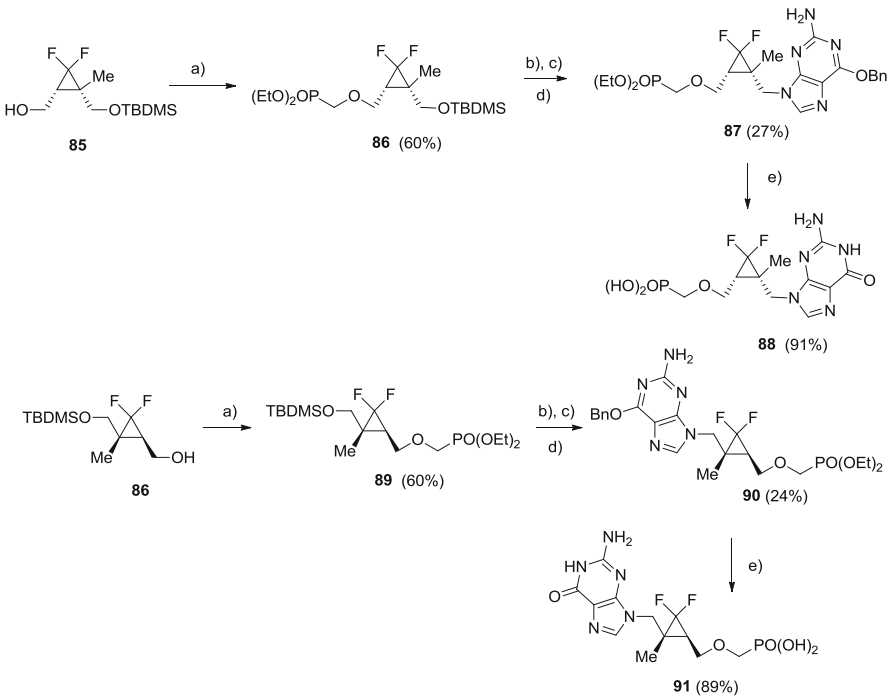
In the same context difluoro cyclopropyl analogues **88** and **91** have been prepared involving, as key intermediates, the difluoro-cyclopropyl alcohols **85** and **86** (Scheme 16) [79]. Phosphonated nucleoside **88** exhibits in vitro anti-HIV-activity similar to that of PMEA ($EC_{50} = 2.4$ μM).

4.2 Phosphonated Cyclopentyl Nucleosides

Starting from commercially available desymmetrized cyclopentanediol **92**, 4′-C-branched carbocyclic nucleoside phosphonates **95** and **96** have been recently synthesized (Scheme 17) [80]. Thus, **92**, by reaction with 6-chloropurine, was converted in **93**, which was deacetylated and oxidized with Dess–Martin reagent to intermediate **94**. **94** was treated with organocerium reagents and transformed into compounds **95** and **96** by reaction with diisopropyl bromomethylphosphonate and ammonia. Furthermore, compound **96** was converted in its diphosphonate **97** and finally in **98**, through Lindlar hydrogenation. The biological assay performed on these compounds have shown that compound **96** is a potent inhibitor of HIV-RT.

In the same context, the synthesis of 4′-ethyl-5′-norcarbocyclic adenosine phosphonic acid analogues **101** and **104** has been reported [81]. The synthetic route starts from the racemic 4-ethyl-4-(4-methoxybenzyloxy)-cyclopent-2-enol **99**, which can be easily converted into the target compounds by methods above reported (Scheme 18).

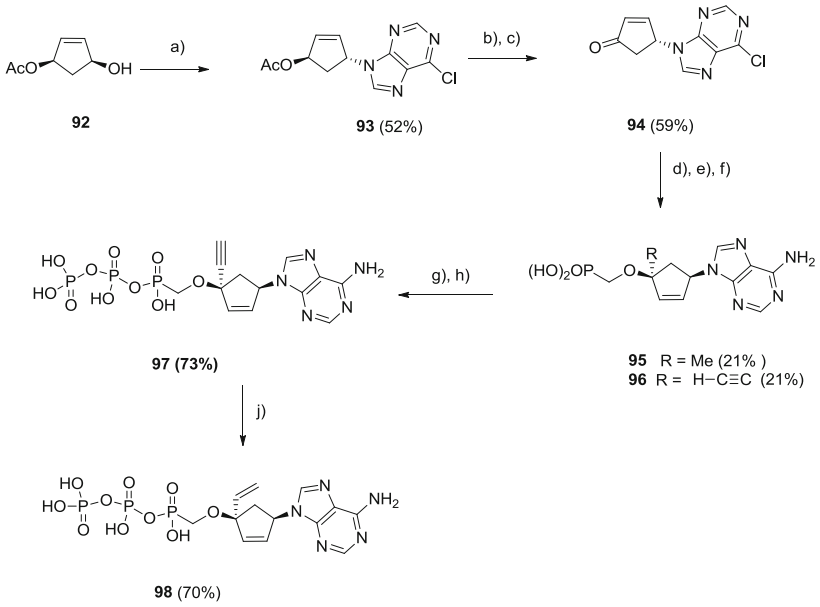
Only derivatives **100** and **101** have shown a moderate antiviral activity against HIV-1 ($EC_{50} = 55$ and 21 μM, respectively), while compounds **102** and **104** did not show any interesting activity.



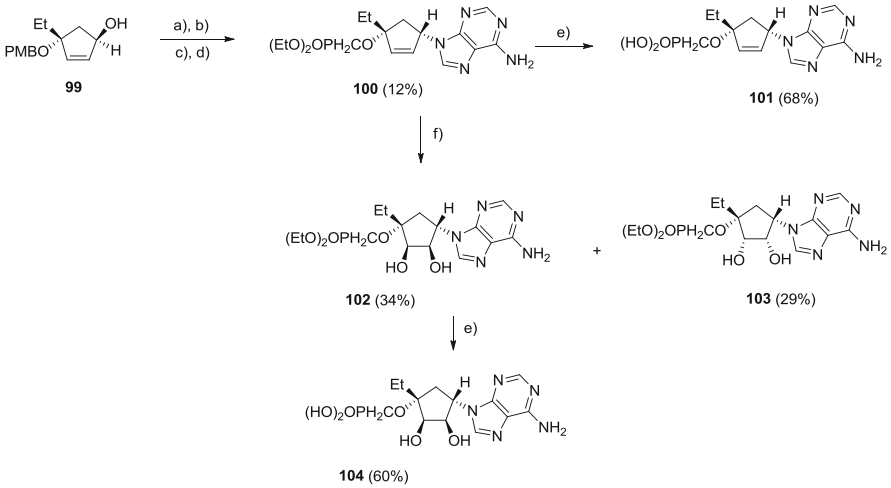
Scheme 16 Reagents and conditions: (*a*) $(EtO)_2POCH_2OTf$, LiO-*t*-Bu, THF; (*b*) TBAF; (*c*) NBS, Ph_3P, CH_2Cl_2; (*d*) 2-amino-6-benzoyloxypurine, K_2CO_3, DMF; (*e*) Me_3SiBr, DMF, RT, aq. HCl, RT, NaOH, H_2O

6′-Fluoro-6′methyl-5′-noradenosine nucleoside phosphonic acid **109** and its SATE (S-acyl-2-thioethyl) prodrug **110** have been prepared from the key fluorinated alcohol **106**, obtained through a selective ring-opening of epoxide **105**. Coupling of **106** with N^6-bis-Boc-adenine under Mitsunobu conditions, followed by phosphonation and deprotection, gave the nucleoside phosphonic acid **109**, from which the final *t*-Bu-SATE prodrug **110** was obtained by reaction with AS-2-hydroxyethyl-2,2-dimethyl-propanethioate, in the presence of 1-(2-mesitylenesulfonyl)-3-nitro-1*H*,1,2,4-triazole (Scheme 19) [82].

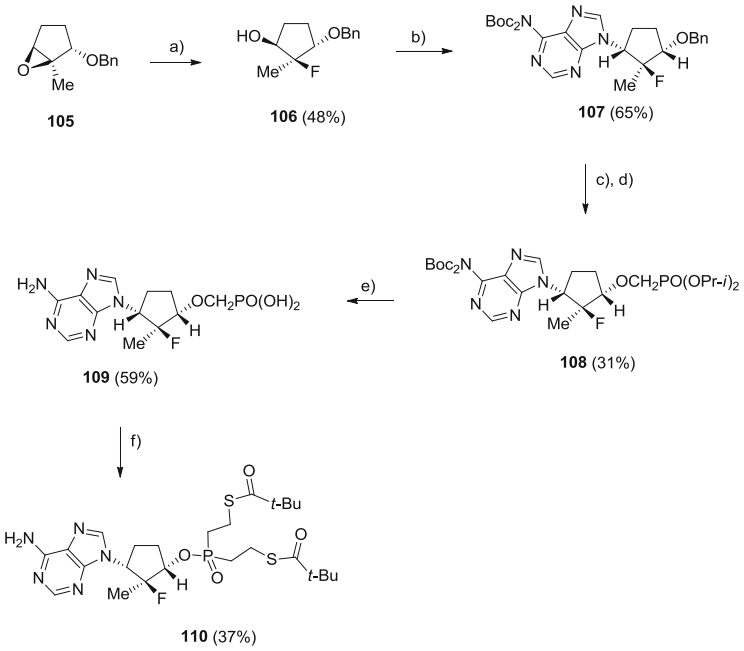
The phosphonic nucleoside analogues **109** and its prodrug **110** showed an antiviral activity against HIV-1 with $EC_{50} = 62$ and 16.7 μM, respectively. The increased anti-HIV activity for the neutral phosphodiester **110** is the result of increased cellular uptake, followed by intracellular release of the parent phosphonic acid.



Scheme 17 Reagents and conditions: (*a*) 6-chloropurine, Ph_3P, DIAD, dioxane; (*b*) $MeOH/NH_3/H_{20}$ 8:1:1; (*c*) Dess-Martin reagent; (*d*) $CeCl_3$, MeMgBr or TMS—≡, THF, −78°C; (*e*) diisopropyl bromomethylphosphonate, *t*-BuOLi, THF, 50°C; (*f*) NH_3, MeOH, 50°C; (*g*) TMSBr, 2,6-lutidine, DMF, 50°C, then MeOH, NH_4OH; (*h*) Et_3NHCO_3, $(lm)_2CO$, tributyl ammonium pyrophosphate; (*j*) Lindlar catalyst, quinoline, H_2, H_2O



Scheme 18 Reagents and conditions: (*a*) DIAD, 6-chloropurine, THF; (*b*) DDQ, CH_2Cl_2/H_2O, RT; (*c*) $(EtO)_2POCH_2OTf$, LiOBu-*t*; (*d*) NH_3/MeOH, 70°C; (*e*) 2,6-lutidine, TMSBr, MeCN; (*f*) OsO_4, NMO,acetone/*t*-$BuOH/H_2O$



Scheme 19 Reagents and conditions: (*a*) $(NH_4)_2SiF_6$, 47% HF, CsF; (*b*) DIAD, Ph_3P, N^6bis-BOC-adenine; (*c*) $Pd(OH)_2$, cyclohexene, MeOH, Δ; (*d*) $(i\text{-}PrO)_2POCH_2Br$, LiI, LiOBu-*t*, DMF, 60°C; (*e*) TMSBr, MeCN, 60°C, 12 h; (*f*) Bu_3N, MeOH, S-2-hydroxyethyl 2,2-dimethylpropanethioate, 1-(2-mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole, RT, 6 h

4.3 Highlights on Antiviral and Biological Activities of Phosphonated Cyclopropyl and Cyclopentyl Nucleosides

Carbovir is the prototype of non-phosphonated carbocyclic nucleosides endowed with antiviral activity (Table 2). The recent importance of carbovir in antiviral therapy is owed to the fact that it is the in vivo relevant anabolite of abacavir. Actually, abacavir is the cyclopropylaminopurine analogue of carbovir. Early studies showed a moderate activity versus HIV infection (EC_{50} 2.4 μM) in peripheral blood mononuclear cells (PBMC), with a cytotoxicity towards PBMC and Vero and CEM cell lines at concentrations higher than 100 μM [72]. The 9-[1-(phosphonomethoxycyclopropyl) methyl] guanine (PMCG) is frequently regarded as an "adefovir-like" nucleotide, able to block HBV infection in HepG22.2.15 cells with a EC_{50} of 0.5 μM and a cytotoxicity 2,000 times higher than that able to inhibit HBV infection. Conversely no activity versus HIV was ever shown [73]. Clinical studies have shown a remarkable HBV DNA suppression in patients treated with 60 mg or higher dose of PMCG over 28 days of therapy [74, 83]. However large clinical studies are needed to confirm these results. Among the phosphonated cyclopentyl nucleosides, the novel carbocyclic nucleoside phosphonate compound **96**, bearing a 4-ethynyl

Table 2 Antiviral activity of phosphonated carboyclic nucleosides

Compound	Structural analogy	Molecular targets	Virus	References
Class				
representative compounds				
Phosphonated cycloproyil nucleosides	Purine and Pyrimidine monophosphate analogues	Viral DNA-polymerase, HIV-RT	HBV, EBV, CoxB3, HIV-1, HMCV	[74–79]
PMCG				
Phosphonated cyclopentil nucleosides	Purine and Pyrimidine mono- di-phosphate analogues	HIV-RT	HIV-1	[80–82]
SATE				
Phosphonated N, O-nucleoside (PCOAN)	Purine and Pyrimidine monophosphate analogues	Retroviral RT	AMV, MLV, HTLV-1, HIV-1	[85–91, 94–98]
Truncated Phosphonated N, O-nucleosides (TPCOAN)				
Phosphonated N, O-Psiconucleosides				

group, exhibited inhibition of HIV infection in MT-2 cells with EC_{50} of 0.13 μM, 2–5 times weaker than that of abacavir. Interestingly, compound **96** inhibited HIV-1 RT activity with an EC_{50} comparable to that of abacavir triphosphate (EC_{50}, 0.13 μM) [79]. The evaluation of the resistance profile indicated a 2.2-fold lower susceptibility to compound **96** of HIV mutant strain bearing K65 mutation. However, this resistance is negatively counterbalanced by the 30 times less susceptibility of strain with M184V mutation, which is the common mutation in treated HIV-positive patients [79]. The phosphonic nucleoside analogue prodrug **110** is potentially an important improvement from the biological point of view, since it counteracts the ionic character of the phosphonic acid which represents a drawback for cell permeability. Esterification of the phosphonic acid with two SATE groups helps the delivery of phosphonate drug into the cells. The antiviral effect of the newly phosphonate **109** versus the prodrug **110** activity was assayed in HIV infection of MT-4 cells. The results revealed that the SATE derivative was four times more active than the parent nucleotide in inhibiting HIV infection, although it showed higher cytotoxic effect versus uninfected cells [81].

4.4 Phosphonated N,O-Nucleosides (PCOAN)

With the aim to bypass the first limiting step of phosphorylation [84], phosphonated N,O-nucleosides, as mimetic of monophosphate nucleosides [85], have been synthesized (Fig. 8). In this context, the groups of Romeo and Chiacchio have synthesized homo phosphonated-, phosphonated- and truncated phosphonated-N,

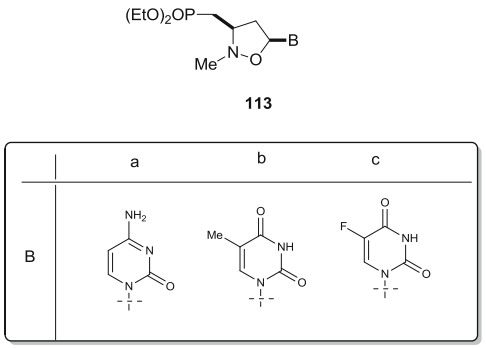
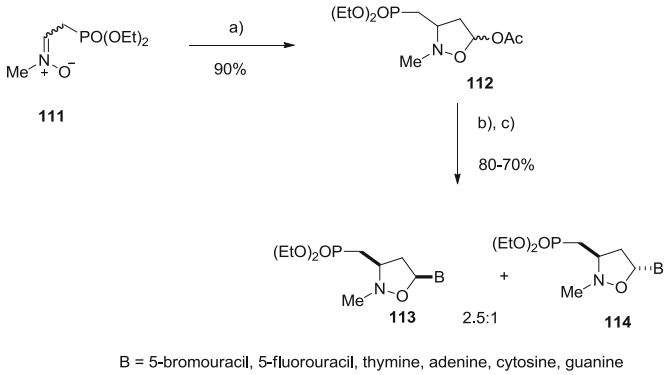


Fig. 8 PCOAN (compounds 113): (**a**) AdC-P; (**b**) AdT-P; (**c**)AdF-P



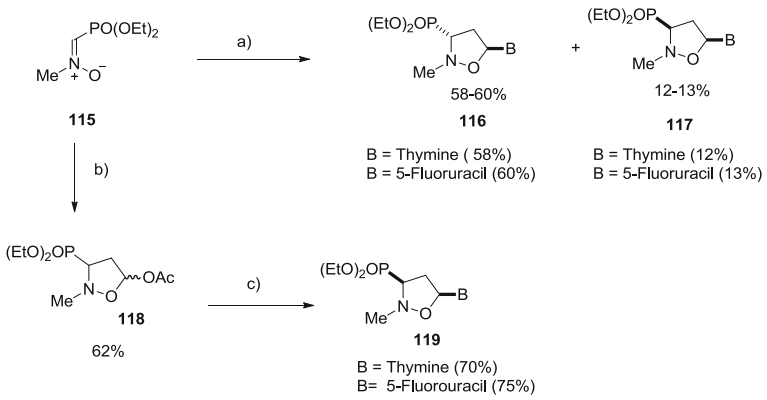
B = 5-bromouracil, 5-fluorouracil, thymine, adenine, cytosine, guanine

Scheme 20 Reagents and conditions: (*a*) vinyl acetate (30 mL), 60°C, 24 h; (*b*) 5-Bromuracil, 5-fluorouracil, thymine, adenine, cytosine or guanine (0.62 mmol), MeCN, bis(trimethylsilyl)-acetamide (BSA) (2.54 mmol), refluxed for 15 min; (*c*)TMSOTf, 55°C, 6 h

O-nucleosides, by exploitation of the 1,3-dipolar cycloaddition methodology [86–88], starting from nitrones containing a phosphonic group [89–91].

The route towards phosphonated carbocyclic 2′-oxa-3′-aza- nucleosides (PCOAN) **113** is described in Scheme 20 [89, 90]. The cycloaddition of phosphonated nitrone **111** with vinyl acetate affords a mixture of *cis*/*trans* isoxazolidines **112**, which were converted, according to the Vorbrüggen nucleosidation, into phosphonated N, O-nucleosides **113** and **114**, containing thymine, 5-fluorouracil, 5-bromouracil, cytosine, adenine and guanine, in 2.5:1 ratio, respectively (Scheme 20).

Cis stereoisomers **113** show low levels of cytotoxicity, assessed by conventional methods to detect viability. Noteworthy, the pyrimidinyl derivatives were endowed with an interesting biological activity [89, 90].



Scheme 21 Reagents and conditions: (*a*) vinyl thymine or vinyl 5-fluoruracil, MeCN, 100 W, 90°C; (*b*) $PhCH_3$, vinyl acetate, 60°C, 40 h; (*c*) thymine or 5-fluorouracil, BSA, TMSOTf, 70°C

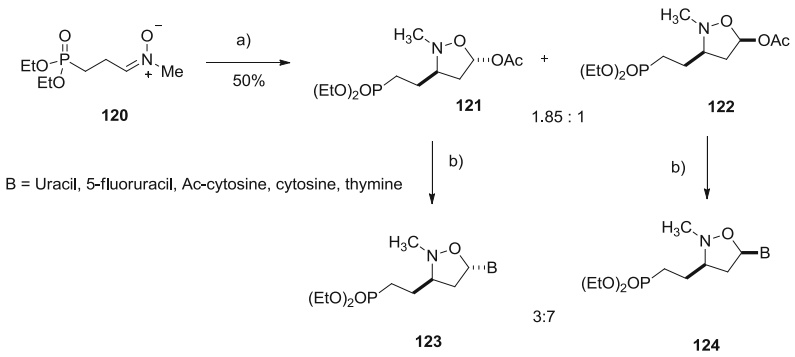
4.5 Truncated Phosphonated N,O-Nucleosides (TPCOAN)

Truncated phosphonated N,O-nucleosides **119** (TPCOAN) [91], containing a diethylphosphonate group directly linked at C3 of the isoxazolidine ring, have been obtained by using the phosphonated nitrone **115** as dipole. The reaction of **115** with vinyl nucleobases was performed under microwave irradiation, with formation of the *trans* nucleosides **116** as main adducts. The corresponding *cis* derivatives **117** were obtained in high yield by a two-step procedure, involving the vinyl acetate cycloaddition, followed by nucleosidation (Scheme 21).

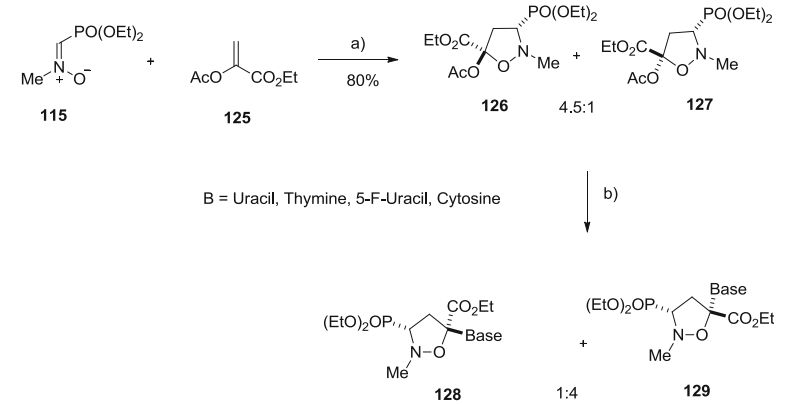
Cis-TPCOAN **119** are able to completely inhibit the RT of Avian Moloney Virus (AMV) and HIV, at concentrations 1 ± 0.1 nM, a level comparable with that of tenofovir (1 nM) and tenfold lower than that of AZT (10 nM). MTS assays indicate a very low toxicity ($CC_{50} > 500$ μM) in comparison with AZT (CC_{50} 12.14 μM) [91].

Homologous derivatives **123** and **124** have been prepared starting from nitrone **120**. Its reaction with vinyl acetate afforded a mixture of *trans*/*cis* adducts (1.85:1 ratio) **121** and **122**, which were then converted into α- and β-anomers **123** and **124**, in a ratio varying from 1:9 (N-acetylcytosine) to 3:7 (thymine and 5-fluorouracil) (Scheme 22). All the products have been evaluated for their ability to inhibit the RT of avian myeloblastosis retrovirus, and no significant activity was observed [92].

A rationalization for the lack of antiviral activity of full-length phosphonated N, O-nucleosides can be supported by the data reported by Sigel [93]. Viral polymerases recognize and use triphosphates nucleosides, complexed with metal ions such as an Mg^{++} and Mn^{++}; the type of complexation determines the reaction pattern of nucleotides. Thus, in the case of phosphonated N,O-nucleosides, the proximity of the N- to P-atom could be discriminant with regard to the biological activity. In full-length nucleotides **124**, the N-atom cannot assure a positive contribute because a 7-membered ring should be formed by chelation, while short length and truncated PCOAN could form 6- six or 5-membered chelates, thus facilitating the bond break between P-α and P-β and allowing the transfer of the nucleotide group with release of pyrophosphate.



Scheme 22 Reagents and conditions: (*a*) vinyl acetate, 100 W, 60°C, 20 min; (*b*) uracil, 5-fluorouracil, Ac-cytosine, cytosine, thymine, BSA, TMSOTf, 70°C, 6 h



Scheme 23 Reagents and conditions: (*a*) THF, reflux, 24 h; (*b*) isoxazolidines **126** and **127**, MeCN, TMSOTf, silylated Thy (overall yield 72%), 5-Fu (overall yield 80%), U (overall yield 71%), Ac-Cy (overall yield 61%), Cy(42% from Ac-Cy)

4.6 Phosphonated N,O-Psiconucleosides

Natural psicofuranosyl nucleosides, characterized by the presence of a hydroxymethyl group at the anomeric carbon atom, are endowed with interesting biological activities. Chiacchio and Romeo [94–96] have designed an easy entry towards the new class of truncated phosphonated N,O-psiconucleosides, starting from the reaction of the phosphonated nitrone **115** with ethyl 2-acetyloxyacrylate **125** [97]. The cycloaddition leads to a mixture of *trans*/*cis* isoxazolidines **126** and **127** (4.5:1 ratio; 80% yield). The subsequent coupling with silylated nucleobases, performed at 70°C, produces, as expected, the *cis*-anomers **129** as almost exclusive compounds (Scheme 23).

Biological tests show that the *cis*-anomers **129** inhibit the RT of AMV, HTLV-1 and HIV. The 5-fluorouracil derivative is the more promising compound, acting on AMLV and on HIV at a concentration of 1 and 10 nM, respectively. The inhibitory activity towards HTLV-1 and HIV was tenfold higher than that of tenofovir and was similar to that of AZT. Moreover, this compound does not show any cytotoxicity according to MTS assay [97].

4.7 Highlights on Antiretroviral and Biological Activities of Phosphonated N,O-Nucleosides

Series of studies were performed by the group directed by Macchi and Mastino to assess the antiviral and biological activities of PCOAN. Actually PCOAN were evaluated for their activity towards both the HTLV-1 RT activity and the HTLV-1 infection in vitro. The ability of the newly synthesized compounds to inhibit RT activity was firstly determined by means of a novel, cell-free assay [98]. This assay measured the effect of PCOAN on the RT activity of commercial avian myeloblastosis virus RT (AMV-RT) and Moloney murine leukemia virus RT (MLV-RT), using, as a template, RNA isolated from stable transfectants expressing constitutively the glycoprotein D of HSV-1. All phosphonates, AdC-P (nucleoside 113 containing cytosine), AdT-P(nucleoside 113 containing thymine) and AdF-P (nucleoside 113 containing 5-fluorouracil) completely inhibited the formation of amplified products at 10 nM concentration, while the (5′S)-5-fluoro-1-isoxazolidin-5-yl-1H-pyrimidine-2,4-dione (AdF), a nucleoside compound showing biological activity, but lacking RT inhibitory activity due to the absence of a hydroxymethyl group in the furanose ring, was actually unable to inhibit RT activity in this assay. PCOAN were then tested to assess their possible cytotoxic effect on lymphoid and monocytoid cells. In particular, the ability of the new compounds to specifically induce apoptosis and to inhibit the cell metabolic activity was assayed. The results indicated that AdC-P and AdT-P did not induce apoptosis or other toxic effects neither in lymphoid cells nor in monocytoid cells. Conversely, AdF-P was shown to cause detectable levels of toxicity [89]. Successively, the activity of PCOAN was tested versus both HTLV-1 RT activity and HTLV-1 infection in vitro [89]. HTLV-1 is an oncogenic human retrovirus endemic in certain areas of the world including Japan and North and South America, where about 5% of the estimated 20 million HTLV-1-infected people develop HTLV-1-associated diseases [99], such as adult T-cell leukemia (ATL), HTLV-1 associated myelopathy/tropic spastic paraparesis (HAM/TSP), or other minor inflammatory diseases. HTLV-1 preferentially infects T lymphocytes with a CD4+ phenotype. In HTLV-1 infection viremia is essentially

a "cytoviremia," since the virus is cell associated. In fact, cell-free virions are rarely infectious, and spreading of the virus in vivo does not require the extracellular release of viral particles. The spread of the virus within an individual host is most commonly recognized as being through cell divisions, and the clonal expansion of infected cells ensures a constant level of viral load. However, recently, it has been highlighted that the HTLV-1 viral load in vivo is also sustained by cell-to-cell contact, involving an horizontal spread of viral particles. Unfortunately, although a number of strategies for therapeutic intervention have been pursued, limited advances have been achieved.

NA have been used for therapy in ATL and in TSP patients. They were used in combination with IFN in ATL or in a different cocktail including AZT, 3TC and TDF in TSP patients. ATL patients exhibited remission for several years during treatment, while TSP patients gave a poor response to NA. Apparently NA utilized in HIV infection provided limited results in HTLV-1 infection. Thus it was worthwhile to investigate whether newly synthesized nucleoside/nucleotide analogues could be useful in HTLV-1 infection. Parallel experiments carried on with AZT, as positive control, revealed that PCOAN inhibited RT activity at a concentration of 10 nM, comparable to that of AZT. The activity of PCOAN was investigated also on HTLV-1 infection in vitro. PCOAN were able to completely inhibit HTLV-1 cell-to-cell transmission in vitro when added in co-treatment, at a concentration of 1 μM, similar to what has been observed for tenofovir and AZT. The inhibition of HTLV-1 infection was ascribed to the block of HTLV-1 RT, as demonstrated in the cell-free HTLV-1 RT inhibition assay.

5 Conclusions

In this review we have tried to summarize the present knowledge concerning the antiviral potential of both old and newly synthesized phosphonated nucleoside analogues (Fig. 9). A high input to continue research in this field is driven from the clinical results in antiviral therapy obtained with the known prototype compounds and from encouraging antiviral effects demonstrated in vitro by newly synthesized compounds. Further substitutions of a number of moieties have shown the flexibility of phosphonated nucleoside analogues structure. This is an additional impulse to design and develop new phosphonated nucleoside-based analogues, endowed with an antiviral activity towards a broader range of DNA or RNA viruses.

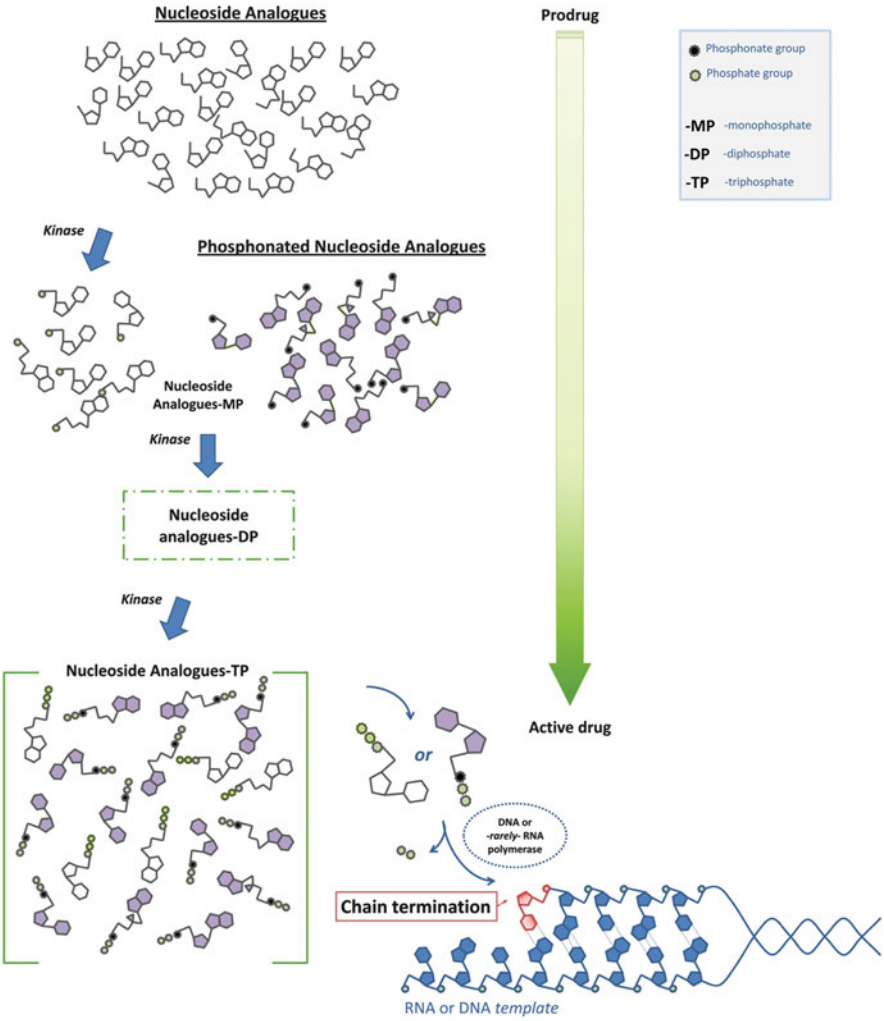


Fig. 9 Transformation from prodrug into active drug of nucleoside analogues and phosphonated nucleoside analogues. The nucleoside analogues are activated into the triphosphate (TP) by three phosphorylation steps performed by cellular kinases. Phosphonated nucleoside analogues can bypass the first phosphorylation steps

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Recent Advances in Targeting Dengue and West Nile Virus Proteases Using Small Molecule Inhibitors

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Abstract Targeting viral proteases represents an attractive concept in the field of anti-infective lead discovery and has been exploited successfully yielding a number of drugs on the market. Next to their essential role in degrading the virus-encoded polyprotein to functionally relevant units, viral proteases can be involved in further processes relevant to viral replication such as interaction to host proteins. Despite the promising nature of this drug discovery concept in general, lead discovery strategies against flavivirus-caused diseases such as Dengue- and West Nile virus infections are still at a comparably early stage. In the present contribution, recent advances targeting the proteases of Dengue and West Nile viruses are reviewed.

Keywords Dengue virus, Drug discovery, Inhibitor design, Viral protease, West Nile virus

Contents

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Abbreviations

Abz	*o*-Aminobenzoic acid
Ac	Acetyl
ACMC	7-Amino-3-carbamoylmethyl-4-methyl-coumarin
Ag	Agmatine, 4-aminobutylguanidine
AMC	4-Methylcoumaryl-7-amide
BHK21	Baby hamster kidney cells
Boc	*t*-Butoxycarbonyl
Bz	Benzoyl
Cit	Citrulline
cmpd(s)	Compound(s)
CNS	Central nervous system
DEV	Dengue virus
DEV^{pro}	Dengue virus protease
DNS	Dansyl
EDDnp	*N*-(2,4-dinitrophenyl)ethylenediamine
FL	Full length
GCMA	*trans*-(4-guanidino)cyclohexylmethylamide
HTS	High-throughput-screening
ITC	Isothermal titration calorimetry
Itz	Iminothiazolidine
2-Naph	2-Naphthoyl
nL	Norleucine
nY-NH_2	3-Nitrotyrosineamide
O	Hydroxyprolin
Orn	Ornithine
PDB	Protein data bank
pG	Pyroglutamyl
Ph	Phenyl
Phac	Phenacetyl
PI	Protease inhibitor
*p*NA	*para* nitroanilide
SAR	Structure–activity relationship
SAXS	Small-angle X-ray scattering
SPR	Surface plasmon resonance
WNV	West Nile virus
WNV^{pro}	WNV protease
Z	Benzyloxycarbonyl

1 Introduction

Due to their robust and severe emergence in recent decades, infections caused by the flaviviruses Dengue virus (DEV) and West Nile virus (WNV) are of strong interest to the pharmaceutical research community. As neither a chemotherapeutic agent nor prevention by immunization is currently available, the therapeutic options are only limited to a symptomatic treatment. An increasing number of cases and deaths indicates that specific treatment options are urgently demanded [1–3].

The four DEV serotypes have considerably expanded their geographic distribution in recent years. With 2.5 billion people at risk, more than 50 million documented cases, and about 12,500–25,000 deaths annually, DEV is robustly emerging in a growing number of more than 100 countries. Central issue to the emergence of Dengue as a public health problem is the distribution of efficient mosquito vectors: the primary vector, *Aedes aegypti* sprang up from Africa during the slave trade in the fifteenth through nineteenth century, moved towards Asia facilitated by the commercial exchange in the eighteenth and nineteenth centuries as well as distribution by the transport of war material and troops, e.g. in World War II, and has up to now spread globally due to the increased travel and trade activities in times of today [1, 4]. A secondary vector, *Aedes albopictus*, has dramatically expanded in recent years, even into regions within Europe, such as Italy and Germany, where this vector made use of vehicles such as caravans for further infiltration of novel yet unthreatened regions. In addition, the latter mosquito vector experienced further distribution by the globalization of trade, particularly of tires from used vehicles, which contributed to the dispersal of eggs of immature forms of these arboviral vectors into new territories that are not limited to economically "poor" regions [2, 5]. In addition to the geographic expansion of the DEV by means of infected travelers [6, 7], changes of the global climate conditions and the subsequent adaptations and response by humans are expected to further contribute to viral expansion [8, 9]. While the climate change will probably influence the emergence of viruses transmitted by, e.g. ticks, birds, or mosquitos, a recent study suggests that the increased installation of domestic water supplies as consequence of the expected reduced rainfall in southeast Australia will lead to an expansion of the Dengue risk [10]. Concerningly, almost all types of water reservoirs such as pots, flower vases, bottles, fountains are expected to be exploited by the arboviral vectors for breeding purposes. Thus, appropriate vector management is one of the key options nominated by the WHO to curtail further distribution of DEV. A recent strategy makes use of the release of bioinsecticides and natural biological enemies such as the soil bacterium *Bacillus thuringiensis israelensis* or the mosquito fish *Gambusia affinis* to affect mosquito larvae populations [11, 12].

However, it is to expect that these methods alone will not be sufficient to reduce the increasing Dengue burden. On the contrary, the robust viral expansion over the recent years has led to the call for a "World Dengue Day", in analogy to the World AIDS day, to increase awareness and commitment at all levels to reduce Dengue incidence and burden [13, 14].

Although most of the Dengue infections are asymptomatic, a wide variety of clinical manifestations may arise, ranging from mild febrile illness to severe and fatal disease. The former WHO classification of 1997 distinguished between undifferentiated fever, Dengue fever, Dengue haemorrhagic fever (DHF) and Dengue shock syndrome (DSS), with the latter present in subclasses III and IV of a four-level sub-classification of Dengue haemorrhagic fever. To reduce complexity and facilitate an appropriate classification, this disease taxonomy was simplified in 2009 towards a two-group grading, comprising uncomplicated Dengue (with or without warning signs), and severe Dengue patients [1, 15–17]. Patients who recover without major complications are considered as Dengue patients, whereas those with any of the following conditions are classified as severe Dengue: plasma leakage resulting in shock (pulse pressure of less than 20 mmHg), accumulation of serosal fluid sufficient to cause respiratory distress or both, severe bleeding or severe organ impairment. Shock can occur during the critical phase (3–7 days since onset of fever) either for increased fluid leakage without an adequate fluid intake or because of internal bleeding, facilitated by thrombocytopenia and deranged clotting. In the former situation, the packed cell volume (PCV) rises while it decreases in the latter case. Monitoring for evidence of fluid leakage to approximate the onset of the critical phase, stringent monitoring during the critical phase to identify bleeding such as mucosal and skin bleeds, monitoring of clinical parameters such as postural drop in blood pressure, postural tachycardia, and urine output to control the fullness of the intravascular compartment will help to avoid death in severe Dengue. However, only a symptomatic management is available to date, mostly comprising fluid management as key component, such as oral fluid administration, blood transfusions, dextran solutions (second line), combined with an antipyretic treatment using paracetamol [18, 19].

Increasing evidence suggests that most cases of DHF are associated with a secondary DEV infection after a symptomless or only less affecting primary infection, e.g. during childhood. The concept of antibody-dependent enhancement [20] rationalizes well that DHF occurs at least 10-fold more frequently in infections with a second serotype of DEV than during primary infection. In addition, infants of mothers who had previously suffered from DEV infection during pregnancy are at high risk of developing DHF when infected with the virus. Primary DEV infections usually result in non-complicated Dengue fever and the acquirement of humoral and cellular immunity that is available long-term and protects the host from re-infection by the same serotype. While this immune response is cross-reactive in nature, it does not confer long-term cross protection against other serotypes. Instead, Dengue pathogenesis relies on immunological processes associated with a secondary infection. In this context, antibodies (Ab) acquired by a primary infection that do not neutralize viral infectivity of a secondary infection are able to form Ab-DEV immune complexes. These immune complexes yield increased entry into mononuclear phagocytes by Fc receptor-mediated endocytosis of viruses and subsequently increased viral replication [21–25].

In contrast to yellow fever, where a vaccination was comparably early available, the presence of four DEV serotypes does not only aggravate the onset of secondary

infections by a different serotype but has also complicated the successful development of appropriate immunologic prevention against Dengue virus infections. While immunization by a monovalent vaccination yields antibodies against only one serotype and thereby may exacerbate the situation after an infection with a different DEV serotype, the development of a tetravalent vaccination was highly challenging so far. Apart from the requirement to combine appropriate antigens of all four DEV serotypes, the development of such a vaccination was hampered by the absence of appropriate in vivo models, even non-human primates inoculated with DEV have been described to develop neither Dengue fever nor DHF that could be used as a correlate for pathogenesis in humans. In addition, the correlation of the presence of neutralizing antibodies towards the extent of protection turned out to be quite complex [25–27].

A remarkable breakthrough towards a vaccinating agent has recently been achieved by the recombinant tetravalent, attenuated Dengue vaccine ChimeriVax™ by Sanofi Pasteur. Even though this vaccination was well tolerated and data from a recent clinical Phase IIb study could prove for the first time that a safe vaccine against Dengue is possible, its efficacy was found to be as low as 30%. In addition, the immunity-generating effect caused by multi-valent vaccinations is more complex than expected: whether or not a successful immunization is achieved may depend on various additional factors such as the kind of infection prior to the immunization procedure and the presence of antibodies generated thereby, the type of chimerization of the immunogenic agents or the type of donation at one single or several different sites [27–29]. Facing such a complex behaviour of the immunogenic response, the importance to have access to further therapy options such as small molecule enzyme inhibitors affecting viral replication is strongly emphasized.

Similarly to Dengue, also infections caused by the West Nile Virus (WNV) have gained severe distribution in recent years. While the virus was first isolated in Uganda in 1937, it is endemic in Africa and southern Europe. However, its appearance in the USA in 1999 led to a massive geographic extension from Canada to Argentina in 2008, causing thousands of deaths and disabled patients [3, 30, 31]. WNV is a zoonotic virus that is transmitted via mosquitos and mainly infects birds; however, viral adaptation towards infection of mammals highlights the potential to produce novel strains of increased pathogenicity. Infections of humans or horses by WNV can lead to fever and severe neurologic diseases such as meningitis, encephalitis or flaccid paralysis. During the decades after its first discovery, WNV infections were described in Africa, Asia and around the Mediterranean Sea. In 1999, WNV reached the focus of public interest after its emergence in the USA, where, starting from New York City, the epidemic spread within only few years over the entire continent to countries in Latin America. In humans, most infections appear asymptomatic; however, 20% of the infected individuals develop flu-like symptoms with high fever within 2–14 days after infection. In about 1% of the infection cases, WNV can lead to severe neuroinvasive disease, among which the mortality rate can reach 20%. At particular high risk are older and immune-suppressed persons. In 2010, major outbreaks occurred in Romania and Greece, cases of limited numbers

were reported from Russia, Italy, Turkey, Israel and Morocco. Considering only the US region, about 30,000 human cases were observed between WNV's first emergence in 1999 and 2010, associated with more than 1,100 deaths. This trend continued in 2012 with the highest number of WNV cases per year since 2003, reported in the USA. The numbers of 5,674 WNV disease cases including 2,873 neuroinvasive cases and 286 deaths only for the USA in 2012 strongly underline the increasing menace of this arboviral infection [31–34].

Interestingly, while WNV had not caused severe mortality in birds until the late 1990s, from 1998 onwards avian mortality was associated with outbreaks in Israel and the US epidemic, where a dramatic decrease concerning the populations of many American bird species was observed. This can be regarded as an alarming signal towards the development of novel WNV strains of higher pathogenicity and virulence. Horses are even more susceptible to develop severe symptoms than humans, with mortality rates as high as 40%. Such mortality of horses or birds can be regarded as important indicator for WNV circulation and prediction of outbreaks within the human community [34].

In contrast to DEV infections, the pathogenicity of WNV is strongly related to its ability to cause neuroinvasive complications and to affect the central nervous system. Subsequent to the injection by the insect vector, WNV is expected to cause an initial infection of the dendritic cells within the skin. The latter transport the virus to draining lymph nodes, resulting in the spread into the blood stream and peripheral organs. After an incubation period of about one week, the virus enters the central nervous system by crossing the blood–brain barrier (BBB), while the serum virus load decreases. Several mechanisms have been suggested for this translocation, among them an increasing permeability of the BBB by local production of TNFα, entry after disruption of the BBB, or direct axonal transport via peripheral neurons [33, 34].

An infection with WNV is in most cases transient and causes lifelong immunity. Next to innate and cellular immune mechanisms, humoral response mechanisms such as generation of neutralizing antibodies contribute to the control of WNV infections. B-cell-deficient mice or IgM-deficient mice die rapidly after WNV infection, but are protected by passive donation of IgG. However, as described above for DEV infections, the presence of non-neutralizing antibodies (e.g. because of targeting an unsuitable virus epitope) may lead to antibody-mediated enhancement of the infection. However, this does not reach a similar extent as a secondary infection with a DEV serotype deviation from that of the primary infection. Thus, for WNV immunization the protecting effect of vaccination could be exploited, however, so far only veterinary WNV vaccines (formalin-inactivated whole WNV particles) are on the market to protect horses and geese [34].

Interestingly, the strong incidence of severe WNV pathogenicity in elderly people can be interpreted in the context of affected T-cell activity. As was shown in a rodent model, WNV-specific T-cells from older mice are affected in their cytokine and lytic granule production. In addition, the traffic of T-cells into the CNS is affected in older mice. These and other observations rationalize the higher incidence of severe WNV cases in the elderly [34].

Caused by the increasing number of outbreaks and the strong emergence of flaviviral infections, research has generated a broad knowledge on the virus biology and the characteristics of virus–host interactions. An ongoing task for presence and future will consist in the translation of this knowledge towards antiviral agents and lead candidates. In the following, current approaches towards this goal, focusing on the inhibition of the flaviviral NS2B-NS3 protease are highlighted.

2 Structural and Functional Features of the Virus-Encoded Proteases and Their Implications Towards Lead Discovery

The flaviviral NS3 protein is an about 70 kDa-sized protein possessing several enzyme activities involved in polyprotein processing and RNA replication [35–38]. The virus-encoded polyprotein precursor consists of three structural (C, prM, E) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). This 375 kDa polyprotein is processed by host proteases and a virus-encoded protease activity localized within the N-terminal domain of NS3 ($NS3^{pro}$). The cleavage events are assumed to occur in virus-induced membranous structures evolved from the rough endoplasmatic reticulum (rER) and are known as convoluted membranes or paracrystalline arrays [35]. Whereas cleavage in-between the junctions C-prM, prM-E, E-NS1, NS4A-NS4B, and likely NS1-NS2A is performed by the host signal-peptidase located within the lumen of the ER, the remaining peptide bonds between NS2A-NS2B, NS2B-NS3, NS3-NS4A and NS4B-NS5 are cleaved by the virus-encoded $NS3^{pro}$ [35]. Cleavage at the NS2B/NS3 site is performed in *cis*, but is not necessary for protease activity [39]. Similar to the viral proteases discussed before, the protease activity of the flaviviral NS3 is essential for viral replication and its inhibition is to be considered a valuable intervention strategy for the treatment of infections.

The activity of $NS3^{pro}$ depends significantly on the association of a 40-amino acid region of NS2B acting as a cofactor to $NS3^{pro}$, resulting in the formation of a heterodimeric complex. The NS2B protein is a small (~14 kDa) polypeptide with a central hydrophilic part (amino acid residues 49–89) involved in binding to NS3. In addition, two terminal hydrophobic regions are responsible for membrane association of the NS2B-NS3 complex and stabilization of NS3 [35–40]. The co-localization of NS2B and NS3 in convoluted membranes suggests the latter ones as the location for polyprotein processing by NS2B-$NS3^{pro}$, whereas Golgi-derived vesicle packets, the compartment presumably involved in RNA replication by NS3 and NS5, lack the presence of NS2B. Even though the minimal requirements for proteolytic activity comprise NS3 residues 1–160 (WNV) or 1–167 (DEV2), the maximum activity concerning WNV $NS3^{pro}$ for an optimized fusion construct containing 44 amino-acid residues from the essential NS2B covalently

connected via a G_4SG_3 linker to the NS3^{pro} domain has been observed for the N-terminal 184 residues [40].

In addition to the essential function of polyprotein processing, NS3 was revealed to participate in further relevant cellular processes. (a) For DEV2, it could be shown that NS3 interacts with the nuclear receptor-binding protein (NRBP), a polypeptide implicated in Golgi-ER trafficking. This interaction affects the intracellular distribution of NRBP and is putatively involved in the generation of virus-induced membrane structures. (b) Furthermore, for DEV2, the proteolytic activity of NS3^{pro} and NS2B-NS3^{pro} could be related to the induction of host–cell apoptosis, whereas mutation of the NS3 proteolytic site to an inactive mutant significantly reduced the ability of NS3 to induce apoptosis [35]. (c) Even though the transition of WNV through the blood–brain barrier seems to require host proteases such as matrix metalloproteinase-9 [41] instead of proteolytic activities adherent to virus-encoded proteases, host proteins, e.g. myelin basic protein, have been demonstrated to be susceptible to NS2B-NS3^{pro} degradation [42]. Likely, NS2B-NS3^{pro} inhibitors will not only prevent polyprotein processing but also interfere with pathogenic events inherent to the *trans*-cleavage activity of the viral protease to host proteins.

2.1 General Structural Properties of Flaviviral NS3^{pro}

First sequence and structural comparison studies carried out in 1989 analysed the flavi- and pestiviral genome and suggested the presence of a serine protease related to the trypsin family, comprising a catalytic triad of His, Asp and Ser [43]. One year later, this prediction was verified for NS3 from the Yellow Fever Virus by mutagenesis and characterization of segments of the NS3 gene [44].

Unfortunately, the first reports on experimental structure determinations [45, 46] making use of X-ray crystallography were based on fabricated data exploiting structural data of HCV protease [47] that became available shortly before. The corresponding publications and deposited structural data were recently retracted and will not be considered further in this contribution [48–50].

The native Flavivirus protease consists of the N-terminal domain of the full-length NS3 protein and its NS2B cofactor. The hydrophilic region of NS2B strongly interacts with NS3^{pro}, whereas both N- and C-terminal moieties form two hydrophobic helices putatively acting as membrane anchors. Most crystal structures available to date have been obtained from NS3 constructs containing the isolated protease domain together with the NS2B component included into a fusion protein linked to the NS3 N-terminus, thus lacking the C-terminal helicase part. As the first protease crystal structures of DEV2 and WNV published by Erbel et al. in 2006 demonstrated, NS2B-NS3^{pro} adopts a chymotrypsin-like fold comprising two β-barrels, each formed by six β-strands, embedding the catalytic triad (His 51, Asp 75, Ser 135) between them [51]. Since then, a number of publications have contributed to our current understanding of the structural features required for protease activity, ligand binding and lead discovery efforts. Table 1 provides an

Table 1 Overview on publicly available crystal structures from Dengue and West Nile virus proteases

Protein	PDB entry	Resolution (Å)	Inhibitor-type ligand/comment	References
DEV2 NS2B-NS3^{pro}	2FOM	1.5	–	[51]
DEV3 NS2B-NS3^{pro}	3U1J	1.8	Aprotinin	[52]
DEV3 NS2B-NS3^{pro}	3U1I	2.3	Bz-nKRR-H	[52]
DEV1 NS2B-NS3^{pro}	3L6P	2.2	–	[53]
DEV1 NS2B-NS3^{pro}	3LKW	2.0	10-Residue internal deletion mutant	[53]
DEV4 NS2B-NS3 (FL)	2VBC	3.15	Protease-helicase	[54]
DEV4 NS2B-NS3 (FL)	2WHX	2.2	Protease-helicase, ADP (helicase)	[55]
DEV2 NS2B-NS3^{pro}	4M9K, 4M9M, 4M9I, 4M9F, 4M9T	2.65-1.46	Wild-type protease and A125C mutant structures at different pH values	[56]
WNV NS2B-NS3^{pro}	2FP7	1.68	Bz-nL-KRR-H	[51]
WNV NS2B-NS3^{pro}	2IJO	2.3	Aprotinin	[57]
WNV NS2B-NS3^{pro}	2GGV	1.8	H51A mutant	[57]
WNV NS2B-NS3^{pro}	2YOL	3.2	3,4-Dichlorophenylacetyl-KK-GCMA	[58]
WNV NS2B-NS3^{pro}	3E90	2.45	Naph-KKR-H	[59]

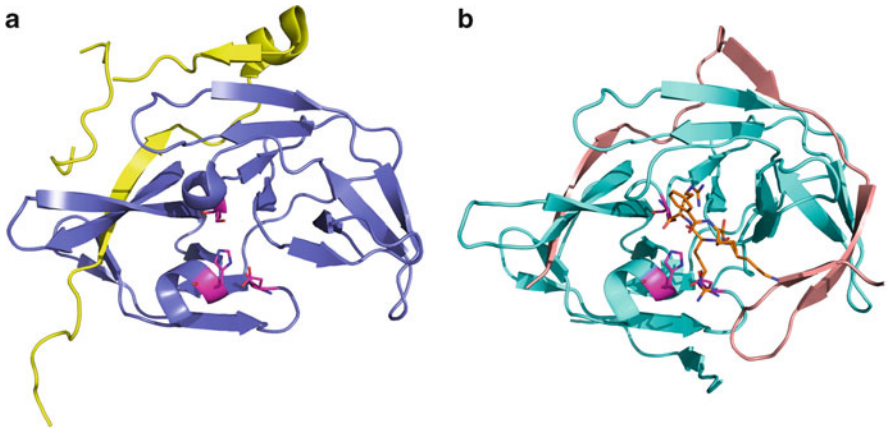


Fig. 1 "Open" (**a**) and "Closed" (**b**) conformation of DEV proteases. The NS3 core of the proteases is shown in *dark* (DEV2, **a**) and *light blue* (DEV3, **b**), the arrangement of the NS2B cofactor is shown in *yellow* (**a**) and *light red* (**b**), respectively. For orientation purposes, the catalytic triad is shown as *sticks coloured in magenta*, a peptidic ligand bound to DEV3 NS2B-NS3 is shown as *orange sticks*. Note that in the open conformation (**a**) the cofactor does not contribute to the substrate binding site, while in the closed conformation (**b**) the β-hairpin moiety does contribute to the substrate binding pockets

overview on the publicly available X-ray structures from proteases of different DEV serotypes and WNV and their references.

In DEV2 NS2B-NS3^{pro}, the hydrophilic domain of NS2B forms a link between the two β-barrels and contributes an anti-parallel β-strand to each of them. The arrangement of the catalytic triad of the NS2B-bound NS3^{pro} suggests an elaborated hydrogen-bond network between the catalytic residues, particularly two hydrogen bonds between His 51 and Asp 75 [51]. Sharing a sequence identity of about 41%, the overall fold observed for the NS2B-NS3^{pro} from WNV and DEV2 is very similar, with only subtle deviations in length and location of secondary structure elements. On the contrary, the NS2B cofactor is more divergent, with a sequence identity of 19% overall and only 10% with respect to the extra-protease residues.

Interestingly, throughout the NS2B-NS3^{pro} crystal structures presently available, a remarkable conformational plasticity of the NS2B peptide is observed: whereas in those protease structures in complex with a small-molecule inhibitor within the active site, NS2B forms a belt around NS3^{pro} by contributing one β-strand to the N-terminal β-barrel and two β-strands as β-hairpin motif to the C-terminal β-barrel; in the unbound state, the latter β-hairpin does not contribute to the C-terminal β-barrel [36, 51, 52]. On the contrary, the latter residues exhibit various conformations, to some extent depending on the crystallographic packing environment. The fold adopted by NS2B appears relevant to structure-based ligand design of inhibitors of WNV NS3^{pro}, as the NS2B β-hair pin tip in the ligand-bound state of NS2B-NS3^{pro} partly contributes to the formation of the S2 as well as the S3 pockets (Fig. 1) and may thereby directly interact with the bound ligand. Recent studies have shown a similar relevance of NS2B towards ligand binding to DEV2

NS2B-NS3^{pro}. Due to the importance of the structural NS2B organization, the nomenclature of the "closed conformation" for the NS2B β-hairpin contributing to the active site (observed with ligands bound) and the open conformation for NS2B β-hairpin pointing away from the active site (with unbound ligand) has recently been introduced [60].

2.2 Site-Specific Features of Flaviviral Proteases

Even though WNV and DEV proteases show a remarkably high sequence conservation (about 50%) and protein fold architecture, they possess different sequence preferences in their cleavage sites and deviating affinities for structurally comparable inhibitors [51, 52, 60–62].

As elucidated by peptide profiling, DEV2 preferred Lys over Arg in P3, Arg over Lys in P2 and Arg over Lys in P1 position. In contrast, in WNV protease P2, Lys is preferred over Arg. This deviating cleavage preference could be attributed to the different amino acid equipment in the S2 pocket of both proteases: While DEV2 possesses the smaller Ser 83 residue (Thr in DEV3), WNV has a more bulky Asn 84 at the corresponding position and is therefore narrower than DEV protease. On the prime side, DEV protease prefers Ser at P1′ and possesses only minor specificity for P2′, while WNV^{pro} prefers Gly at both substrate positions. A comparison of both proteases (DEV3 and WNV) bound to the same peptide suggests that this deviation is caused by the side chain inventory of the two proteases: In the neighbouring positions 131 and 132, DEV^{pro} contains Lys and Pro, respectively, while WNV^{pro} possesses Pro and Thr at the corresponding position. Transplantation of the DEV residues into the WNV proteases yielded a reconstitution of the cleavage preference resembling to DEV^{pro}. This is consistent with the experimentally elucidated shape deviation of the WNV and DEV S1´and S2´pockets adherent to the amino acid exchange [60, 61].

A deviating behaviour between the two proteases has also been reported to the inhibitory properties of small-molecule ligands, as the WNV protease shows higher susceptibility to inhibition by small peptide-shaped inhibitors. A series of di- and tripeptide aldehyde inhibitors addressing the unprimed protease pockets showed higher potency towards WNV^{pro} by two orders of magnitude [63]. This deviation has recently been attributed to the NMR-based observation that in WNV^{pro} the NS2B β-hairpin adopts predominantly the closed state, while similar NMR studies on DEV^{pro} suggested a more open or disordered state for the same protein region, in agreement with the high plasticity of NS2B observed in DEV^{pro} crystal structures [64]. For WNV^{pro} being rather pre-organized in closed state, inhibitor binding would be associated with a less pronounced entropic penalty compared to DEV^{pro}, rationalizing the higher ligand affinity towards WNV protease [60]. However, this observation might have been biased as most affinity determinations have been carried out on artificially designed enzymes with NS2B connected via an non-natural linker, e.g. G4SG4 or similar, to NS3^{pro}. Interestingly, a recent NMR

study on the unlinked NS2B-NS3 protease complex revealed that the conformational disorder observed for the fused NS2B-NS3^{pro} construct might be an artificial property adherent to the non-natural enzyme constitution, as the NS2B-NS3^{pro} construct in absence of the covalent linker exhibits structured behaviour resembling the closed state of the enzyme [65]. In addition, a recent SAXS study on WNV^{pro} with NS2B cofactor and NS3 artificially linked by design provided evidence that the picture of open and closed conformations of NS2B might be a rather crystal structure-biased observation for the unbound (ligand/inhibitor-free) state in solution which appeared fully dynamic and not bound to the structured NS3^{pro} domain [66]. Thus, the artificial character of protein constructs, assay systems, and limits of the applied biophysical methods has to be kept in mind when interpreting their results with respect to structure–activity correlations and selectivity features.

2.3 Substrate Specificity

Substrates for DEV-2 protease were initially derived from the P6-P1 cleavage sites of, e.g. NS2A/NS2B (Table 2, entry 1), NS2B/NS3 (Table 2, entry 2), NS3/NS4A (Table 2, entry 3), and NS4B/NS5 (Table 2, entry 4) or the P6–P6′ sites, respectively, indicating a strong prevalence for basic residues in the P2 (R) and P1 position (R) and for smaller residues such as G, S, A in P1′ (see above). Further variation of substrates differing in the number of non-prime and prime site residues as well as chromophores used are listed in Table 2 (entries 5–9) with Bz-nLKRR↓ACMC exhibiting the highest k_{cat}/K_m = 1,121,000 M^{-1} s^{-1} reported as yet. However, there seems to be no correlation between substrate affinity and cleavage rate [72]. Profiling of the P4–P4′ specificities of the three other DEV subtypes by screening a combinatorial peptide substrate library revealed that the cleaving sites of all four serotypes are comparable [67, 68, 74]. The activity of the NS2A/NS3 protease construct was shown to be pH-dependent with the maximum activity between pH 8.5–9.0 for DEV protease and pH 9.5 for WNV [69, 76]. For the latter, the susceptibility to the ionic strength of the buffer and to detergents utilized has been demonstrated [76–78].

In case of WNV protease, for efficient substrate cleavage K is favoured at P2, R at P1, and G in P1′ and P2′ [57, 61, 79]. First substrates for WNV protease were deduced from the corresponding cleavage sites like NS2A/NS2B (Table 3, entry 1), NS2B/NS3 (Table 3, entry 2), NS3/NS4A (Table 3, entry 3) and NS4B/NS5 (Table 3, entry 4). Moreover, it has been shown that some fluorescent furin substrates are also efficiently cleaved (Table 3, entry 5). Substrates with hydrophobic non-natural amino acids in P4 showed comparable K_m and k_{cat} values as Ac-LKKR↓pNA (Table 3, entry 6) does, with 2-Naph-KKR↓pNA (Table 3, entry 7) being the most efficiently processed substrate within this series. Incorporating unnatural amino acids in P3 resulted in a decrease in k_{cat}/K_m-values, mainly caused by a reduction of the corresponding k_{cat}. The tetrapeptide 2-Naph-KOrnR↓pNA (Table 3, entry 8), modified in P4 and P2, was also effectively processed [82]. Further variation

Table 2 Substrates of DEV-2 protease

Entry	Substrate	k_{cat}/K_m (M^{-1} s^{-1})	References
1	Ac-RTSKKR↓pNA	903 ± 56[a]	[67]
	DNS-RTSKKR↓SWPLNE	6.3[a]	[68][b], [69][a]
	Abz-RTSKKR↓SWPLNEQ-EDDnp	5,700[a]	[70]
2	Ac-EVKKQR↓pNA	220 ± 6[a]	[67]
	DNS-EVKKQR↓AGVLWD	1.6[a]	[68][b], [69][a]
	Abz-EVKKQR↓AGVLWDQ-EDDnp	3,400[a]	[70]
3	Ac-FAAGRK↓pNA	425 ± 30[a]	[67]
	DNS-FAAGRK↓SLTLNL	12.0[a]	[68][b], [69][a]
	Abz-FAAGRK↓SLTLNLQ-EDDnp	1,200[a]	[70]
4	Ac-TTSTRR↓pNA	883 ± 22[a]	[67]
	DNS-TTSTRR↓GTGNIG	10.9[a]	[68][b], [69][a]
	Abz-TTSTRR↓GTGNIGQ- EDDnp	1,500[a]	[70]
5	Abz-RRRR↓SAG-nY-NH_2	11,087[a]	[71]
	Abz-RRRR↓XXXX-nY-NH_2	[a,c]	[72]
	Abz-RRRR↓HHGN-nY-NH_2	$k_{cat:}$ 0.08 min^{-1}; K_m: 4.34 μM[a]	[72]
	Abz-RRRR↓DDGN-nY-NH_2	k_{cat}: 0.07 min^{-1}; K_m: 79.59 μM[a]	[72]
	Abz-SAAQRR↓GRIGRNQ-EDDnp	21,000[a]	[70]
	Abz-ARRR↓SQ-EDDnp	14,800[a]	[70]
	Abz-AKRR↓SQ-EDDnp	31,800[a]	[70]
6	pERTKR↓AMC	97[d]	[73]
7	Bz-nLKRR↓ACMC	112,100 ± 18,500[d]	[74]
	Bz-nLKTR↓ACMC	40,300 ± 10,200[d]	[74]
	Bz-nLTRR↓ACMC	16,700 ± 2,000[d]	[74]
	Bz-TKRR↓ACMC	18,300 ± 2,100[d]	[74]
	Bz-TTRR↓ACMC	2,200 ± 200[d]	[74]
8	Boc-QRR↓AMC	107[d]	[73]
	Boc-GRR↓AMC	172[d]	[73]
		265 ± 18[e]	[75]
9	Z-RR↓AMC	88[d]	[73]

[a]Assay was performed at pH 9.0
[b]Assay was performed at pH 8.0
[c]56 substrates were examined
[d]Assay was performed at pH 8.5
[e]Assay was performed at pH 9.5

of substrates differing in the number of non-prime site residues as well as chromophores used are listed in Table 3 (entries 9–15).

2.4 Inhibitor Design Concepts

Screening of general serine protease inhibitors revealed that only aprotinin (**1**), a 58 amino acid polypeptide, inhibits the DEV-2 protease as well as the WNV protease effectively with an IC_{50} of 65 nM and a K_i of 26 nM, respectively. The

Table 3 Substrates of WNV protease

Entry	Substrate	k_{cat}/K_m (M^{-1} s^{-1})	References
1	Ac-DPNRKR↓pNA	1,756 ± 96[a]	[76]
2	Ac-LQYTKR↓pNA	1,233 ± 86[a]	[76]
3	Ac-FASGKR↓pNA	4,222 ± 313[a]	[76]
	Ac-DFASGKR↓AMC	245±84[b]	[80]
4	Ac-KPGLKR↓pNA	1,827±124[a]	[76]
		5,694 ± 345[a]	[81]
5	Boc-RVRR↓AMC	1,791[c]	[42]
	pG-RTKR↓AMC	23,000[c]	[42]
6	Ac-LKKR↓pNA	30,117 ± 2,248[a]	[82]
7	2-Naph-KKR↓pNA	42,603 ± 5,472[a]	[82]
8	2-Naph-KOrnR↓pNA	42,889 ± 3,152[a]	[82]
9	pGRTKR↓AMC	88,000 ± 1,200[c]	[61]
10	Boc-RVRR↓AMC	5,500 ± 500[c]	[61]
11	pG-RTKR↓AMC	88 ± 12[c]	[79]
12	Boc-GRR↓AMC	601 ± 51[a]	[75]
		222 ± 35[b]	[80]
13	Boc-GKR↓AMC	1,316 ± 66[a]	[75]
14	Boc-LRR↓AMC	405 ± 44[a]	[75]
15	Boc-LKR↓AMC	822 ± 33[a]	[75]

[a]Assay was performed at pH 9.5
[b]Assay was performed at pH 9.0. Substrate inhibition was observed
[c]Assay was performed at pH 8.0

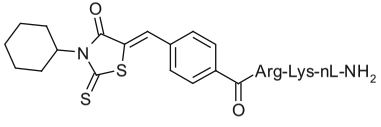
X-ray structures of the aprotinin-DEV-3 protease as well as the WNV aprotinin complex have been determined (see Sect. 2.1 and Fig. 2). Other commonly used inhibitors such as 4-(2-aminoethyl)benzenesulfonylfluoride*HCl (**2**) or *N*-Tosyl-L-phenylalanine chloromethyl ketone exhibited only weak inhibition whereas, e.g. the soybean trypsin inhibitor, 4-amidinophenylmethanesulfonylfluoride or benzamidine were shown to be inactive in case of DEV-2 protease (Table 4, entries 1–3) [67, 76]. Likewise, WNV protease is not susceptible towards benzamidine, leupeptin, phenylmethylsulfonyl fluoride or tosyl-L-lysine chloromethyl ketone, to name just a few inhibitors tested [75, 76].

2.4.1 Substrate-Based Peptide-Type Ligands

DEV Protease

Following a classical approach for the design of serine protease inhibitors, amidation of the C-terminus or introduction of an α-keto amide moiety into short peptide sequences derived from the P6–P1 cleavage sites of DEV-2 protease led to the first peptidic inhibitors, albeit exhibiting only moderate affinity in the mid micromolar range (Table 3, cmpds **4**–**8**). Within a series of substrate-based tetrapeptides bearing different warheads, the boronic acid Bz-nL-KRR-$B(OH)_2$ (**9**) was

Table 4 Substrate-based peptide-type ligands

Compound	Substance	Affinity (DEV-2)[a]	Assay pH	References
1	Aprotinin	IC_{50} = 65 nM[b]	9.0	[67]
		25 pM[c]	8.5	[74]
2	4-(2-aminoethyl)benzenesulfonyl fluoride*HCl	20–30% inhibition at 500 μM	9.0	[67]
3	*N*-Tosyl-L-phenylalanine chloromethyl ketone	20–30% inhibition at 1 mM	9.0	[67]
4	Ac-EVKKQR-$CONH_2$	66.68 μM	9.0	[83]
5	Ac-RTSKKR-$CONH_2$	12.14 μM	9.0	[83]
6	Ac-FAAGRK-$CONH_2$	25.87 μM	9.0	[83]
7	Ac-FAAGRR-α-keto-SL-$CONH_2$	47 ± 3 μM	7.5	[67]
8	Ac-TTSTRR-α-keto-SL-$CONH_2$	220 ± 55 μM	7.5	[67]
9	Bz-nL-KRR-$B(OH)_2$	0.043 μM	8.5	[84]
10	Bz-nL-KRR-CF_3	0.85 μM	8.5	[84]
11	Bz-nL-KRR-thiazole	42.8 μM	8.5	[84]
12	Bz-nL-KRR-benzoxazole	82.9 μM	8.5	[84]
13	Bz-nL-KRR-NH_2	127.5 μM	8.5	[84]
14	Bz-nL-KRR-$NHSO_2CF_3$	>500 μM	8.5	[84]
15	Bz-nL-KRR-H	5.8 μM	8.5	[84]
		9.5 μM	8.5	[63]
16	Bz-KRR-H	1.5 μM	8.5	[85]
17	Bz-RKnL-NH_2	15.6 ± 1.1 μM	9.0	[86]
18		K_i1 = 9.3 ± 3.4 μM K_i2 = 4.4 ± 2.1 μM EC_{50} = 16.7 ± 1.2 μM	9.0	[87]
19	WYCW-NH_2	4.8 μM	9.0	[88]
20	Ac-WYCW-NH_2	18.0 μM	9.0	[88]
21	Itz-CTRRGLPVCGSEE GPTNCGRRS	1.39 ± 0.35 μM	9.0	[89]

(continued)

Table 4 (continued)

Compound	Substance	Affinity (DEV-2)[a]	Assay pH	References
22	Itz-CRRSWPVCTRRS GRRSEESGCVPLG	3.03 ± 0.75 μM	9.0	[89]
23	 NGVCCGYKLCHOC	9.0 ± 0.4 μM	9.0	[90]
24	 CGYKLC	12.5 ± 0.3 μM	9.0	[90]
25	 CGKRKSC	1.4 ± 0.1 μM	9.0	[90]
26	 CAGKRKSG	2.2 ± 0.2 μM	9.0	[90]
27	CAGKRKSG	42.0 ± 4.2 μM	9.0	[90]
28	 RGGRLCYCRRRFCVCVGR	5.85 ± 1.82 μM	8.5	[91]
29	 GICRCICGRGICRCICGR	IC_{50} = 21.4 ± 1.6 μM[d]	8.5	[92]

[a]If not otherwise stated, K_i-values are reported. K_i1 and K_i2 refer to a mixed inhibition mechanism
[b]A non-covalently bound complex (CF40.NS3pro) was used
[c]A covalently bound complex (CF40-Gly-NS3pro185) was used
[d]Determined at 37°C; 28°C: 46.1 ± 1.7 μM; 40°C: 14.1 ± 1.2 μM

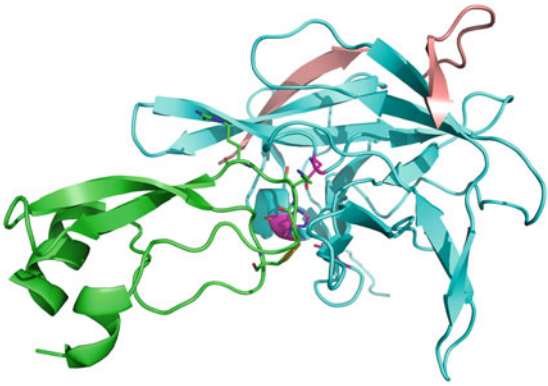


Fig. 2 DEV3 NS2B-NS3 protease with bound aprotinin. The NS3 protease core is shown in *light blue*, with the bound NS2B cofactor represented in *light red* and the catalytic triad as *magenta-coloured sticks*. Aprotinin is shown in *green*. Note that in this crystal structure the NS2B peptide is partly disordered and not included to the structural model

identified, being the most active peptide-derived inhibitor reported so far. Interestingly, replacement of the boronic acid moiety by other physicochemically different warheads (**10**–**13**) resulted in a significant decrease in affinity, with the trifluoromethylsulfonamide analogue (**14**) being even inactive [84]. Noteworthy, the tripeptidic aldehyde Bz-KRR-H (**16**), derived from Bz-nL-KRR-H (**15**), was slightly more potent (Table 4). Besides peptidic inhibitors, also retro-peptides in which the C- and N-termini are interchanged have been investigated [86]. Based on the tetrapeptide Bz-nLKRR-NH_2 (**13**), the tripeptide Bz-RKnL-NH_2 (**17**) lacking the original P1-substituent was designed resulting in an eight-fold increase in potency (Table 4). Incorporation of either a rhodanine- or a thiazolidinone-moiety into the N-capping benzoyl moiety resulted in peptide hybrids. Thorough SAR-studies revealed that the substitution pattern of the heterocyclic nitrogen atom has a tremendous effect on the affinity of the compounds, with hydrophobic, rather flexible substituents being favored. Overall, the thiazolidinones were more potent than the respective rhodanines. However, rhodanine **18** did not only display affinity in the one-digit μM range but was also shown to exert antiviral activity in cell culture (EC_{50} = 16.7 μM) and in a Dengue virus titer reduction assay (65% reduction at 50 μM). [87] Recently, based on the screening results of an octapeptide library utilizing a proteochemometric modeling approach, the first uncharged tetra-peptidic inhibitors exhibiting activity against all four serotypes in the range from 4.2 to 75.1 μM were found (Table 4, cmpds **19** and **20**) [88].

Iminothiazolidine derivatives **21** and **22** were identified by altering the molecular framework of cyclic peptides based on the structure of kalata B1 by incorporating substrate recognition sequences [89].

Screening of a natural peptide library consisting of crude venom extractions of different cone snail species resulted in identification of the cyclic peptide MrIA (**23**) from *C. marmoreus* possessing a K_i of 9 μM. A truncated, single loop construct of MrIA (MrIA-SL, **24**) inhibits DEV-2 protease equally well (K_i = 12.5 μM), whereas the linear peptide of **24** was shown to be inactive, indicating that the cyclization is crucial for activity. Based on these results, cyclic peptides containing two to three basic residues were designed, mostly showing affinity in the one-digit

micromolar range (e.g. cmpds **25** and **26**). Again, the linear peptide (**27**) was significantly less active [90].

Rothan et al. discovered the polycyclic peptides protegrin-1 (**28**) and retrocyclin-1 (**29**) inhibiting DEV-2 protease with a K_i of 5.85 μM and an IC_{50} of 21.4 μM (37°C), respectively. Protegrin-1 as well as retrocyclin-1 was also shown to significantly reduce viral replication in a cell-based assay in a dose-dependent manner, thus being the first peptidic inhibitors for which cellular activity was proven [91, 92].

WNV Protease

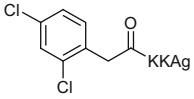
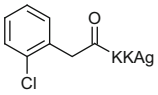
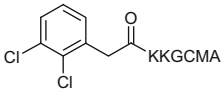
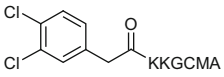
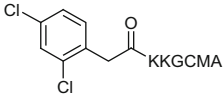
Evaluation of a tetrapeptide aldehyde inhibitor library comprising 27 peptides deduced from ligand **30** resulted in a slight improvement of the affinity for derivatives bearing D-nL, A, or F at P4 or K at P2 (**31**–**33**) [93]. D-Arg-based peptides (**34**–**36**) were shown to be efficient inhibitors of WNV with K_i-values ranging, dependent on the chain length, from 478 to 1 nM. Moreover, the nona-Arg-derivative (**35**) was proven to reduce the virus production in infected primary neurons [42]. Based on their earlier profiling results of WNV substrate specificity, Stoermer et al. systematically varied the N-terminus of the tripeptide aldehyde X-KKR-H, which resulted in identification of 12 reversible competitive inhibitors (**37**–**39**) with K_i-values in the low nanomolar range (6–160 nM). Replacement of one positively charged amino acid either in P2 or in P3 by a citrulline moiety resulted in a reduced affinity (**40**–**41**). The antiviral activity of **37** was proven in a plaque reduction assay [94]. Likewise, replacement of Arg in P1 by agmatine (4-aminobutylguanidine) in the 4-Ph-PhacKKR-tripeptide (**38**) leading to **42** resulted in a significant drop of affinity [95]. Interestingly, N-capped dipeptides such as **43** retain activity in the sub-micromolar range thereby exhibiting high ligand efficiencies [96]. Hammamy et al. optimized the N-capping moiety as well as the P1 position of the X-KKR-tripeptide successively. For the P1 position, incorporation of a trans-(4-guanidino)cyclohexylmethylamide (GCMA) moiety was most beneficial (**44**). Screening of the N-capping moiety led to several one-digit μM inhibitors, with the dichloro-substituted phenylacetyl derivative (**45**) being slightly more potent than the mono-substituted representative (**46**). Combination of both optimized residues resulted in the most potent inhibitors (**47**–**49**) within this series with K_i-values of 120, 130 and 140 nM, respectively [58]. A similar approach was pursued by Lim et al. with **46** being the most potent derivative (Table 5) [97].

2.4.2 Non-peptidic Ligands, Screening Hits and Derivatives Thereof

DEV-Protease

As many of the inhibitors have also been tested against WNV, the corresponding affinity data are provided in parentheses.

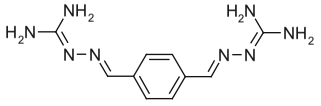
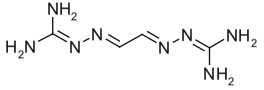
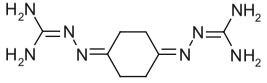
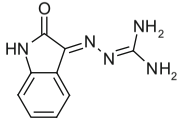
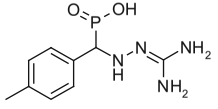
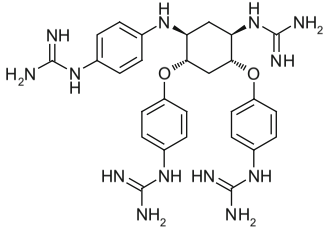
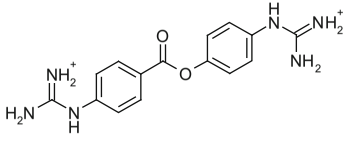
Table 5 Substrate-based peptide-type ligands

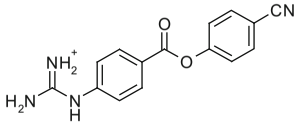
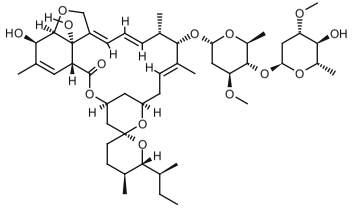
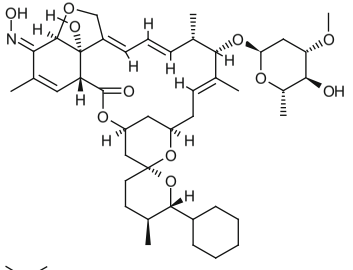
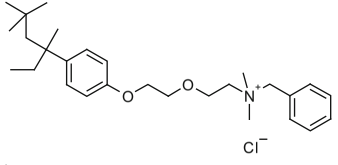
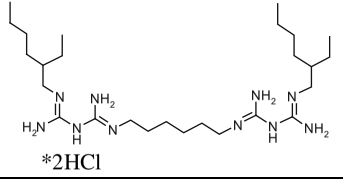
Compound	Substance	Affinity WNV[a]	References
1	Aprotinin	26 ± 4 nM[b]	[42]
30	Bz-nL-KRR-H	IC_{50} = 4.1 mM[c]	[93]
31	Bz-F-KRR-H	IC_{50} = 1.2 mM[c]	[93]
32	Bz-A-KRR-H	IC_{50} = 0.7 mM[c]	[93]
33	Bz-nL-KKR-H	IC_{50} = 1.9 mM[c]	[93]
34	hexa-DArg-NH_2	478 ± 20 nM[b]	[42]
35	nona-DArg-NH_2	6 ± 1 nM[b]	[42]
36	dodeca-DArg-NH_2	1 ± 1 nM[b]	[42]
37	Phac-KKR-H	9 nM IC_{50} = 1.6 μM[d]	[94]
38	4-Ph-Phac-KKR-H	6 nM IC_{50} = 56 ± 4 nM[c]	[94] [63]
39	Abz-KKR-H	160 nM	[94]
40	Phac-CitKR-H	111 nM	[94]
41	Phac-KCitR-H	2.07 μM	[94]
42	4-Ph-Phac-KKAg-H	2.05 ± 0.13 μM[b]	[95]
43	Ac-KR-H	IC_{50} = 0.17 ± 0.04 μM[c]	[96]
44	Phac-KK-GCMA	1.2 μM[c]	[58]
45		0.6 μM[c]	[58]
46		0.7 μM[c] IC_{50} = 2.6 ± 0.3 μM[b]	[58] [97]
47		0.12 μM[c]	[58]
48		0.13 μM[c]	[58]
49		0.14 μM[c]	[58]

[a]If not otherwise stated, K_i-values are reported and the assay was performed at pH 9.5
[b]Assay was performed at pH 8.0
[c]Assay was performed at pH 8.5
[d]Antiviral activity in a plaque reduction assay

By searching the Available Chemical Directory for compounds bearing a guanidine moiety aimed to mimic the P1-substituent, six bis- and 17 mono-aminoguanidino derivatives were retrieved, 20 of which were commercially available. Four substances (**50**, **51**, **53**, **54**) were shown to exhibit K_i-values in the micromolar range against DEV-protease, whereas compound **52** displays only millimolar activity (Table 6). For all other mono-aminoguanidino derivatives, no

Table 6 Non-peptidic ligands of DEV-protease

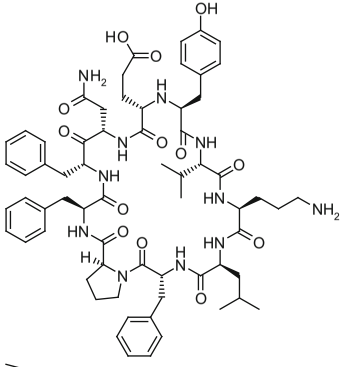
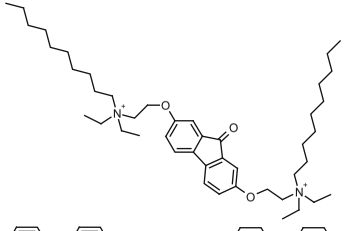
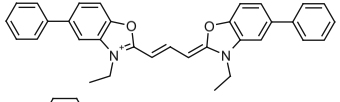
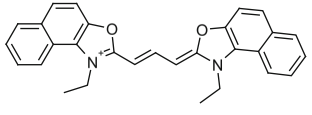
Compound	Substance	Affinity DEV-2 protease[a] (affinity WNV-protease)[a]	Assay pH (WNV)	References
50		44 ± 5 μM (35 ± 5 μM)	8.5 (8.5)	[98]
51		423 ± 50 μM (337 ± 56 μM)	8.5 (8.5)	[98]
52		1,783 ± 113 μM (1,088 ± 162 μM)	8.5 (8.5)	[98]
53		23 ± 2 μM (16 ± 2 μM)	8.5 (8.5)	[98]
54		14 ± 2 μM (13 ± 1 μM)	8.5 (8.5)	[98]
55		7.1 ± 0.5 μM (3.6 ± 0.3 μM)	8.8 (8.8)	[99]
56		2.0 μM (4.6 μM)	8.5 (8.5)	[100]

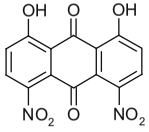
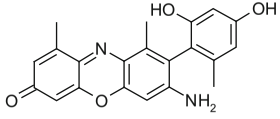
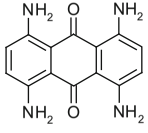
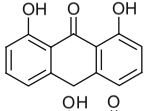
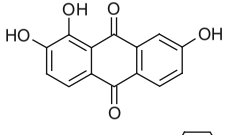
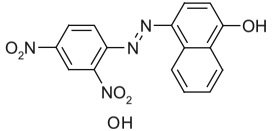
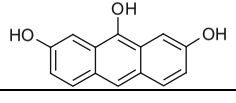
57		31.1 μM (16.0 μM)	8.5 (8.5)	[100]
58		K_i1: 79 ± 21 μM K_i2: 35 ± 4 μM (K_i1: 55 ± 18 μM) (K_i2: 16 ± 4 μM)	9.5 (9.5)	[101]
59		K_i1: 63 ± 18 μM K_i2: 45 ± 8 μM (K_i1: 15 ± 9 μM) (K_i2: 28 ± 6 μM)	9.5 (9.5)	[101]
60		K_i1: 322 ± 81 μM K_i2: 160 ± 39 μM (K_i1: 141 ± 20 μM) (K_i2: 307 ± 80 μM)	9.5 (9.5)	[101]
61		K_i1: 41 ± 3 μM K_i2: 84 ± 16 μM (K_i1: 12 ± 1.2 μM) (K_i2: 28 ± 5 μM)	9.5 (9.5)	[101]

*2HCl

(continued)

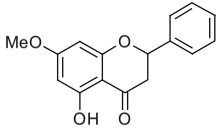
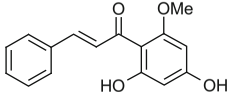
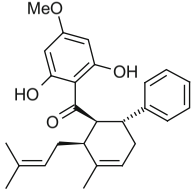
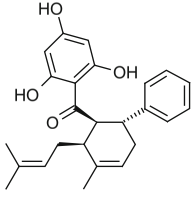
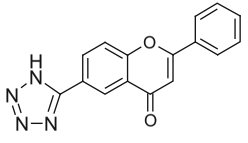
Table 6 (continued)

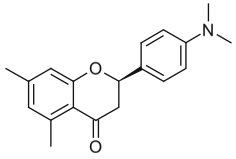
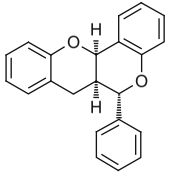
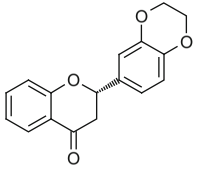
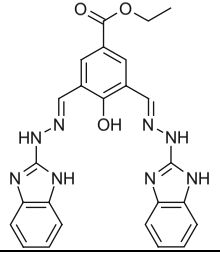
Compound	Substance	Affinity DEV-2 protease[a] (affinity WNV-protease)[a]	Assay pH (WNV)	References
62		12 ± 1.5 μM (2 ± 0.2 μM)	9.5 (9.5)	[101]
63		IC_{50} = 15.43 ± 2.12 μM	9.0	[102]
64		IC_{50} = 20.48 ± 4.12 μM	9.0	[102]
65		IC_{50} = 27.00 ± 1.32 μM	9.0	[102]

66		432 ± 46 μM EC_{50} = 4.2 ± 1.9 μM[b]	9.5 9.0	[103] [104]
67		EC_{50} = 35 ± 8 μM[b]	9.0	[104]
68		K_i1: 158 ± 32 μM K_i2: 43 ± 3 μM	9.5	[103]
69		K_i1: 47 ± 15 μM K_i2: ± 35 μM	9.5	[103]
70		K_i1: 215 ± 119 μM K_i2: 20 ± 2 μM	9.5	[103]
71		K_i1: 15 ± 3 μM K_i2: 10 ± 1 μM	9.5	[103]
72		K_i1: 7 ± 5 μM K_i2: 3 ± 1 μM	9.5	[103]

(continued)

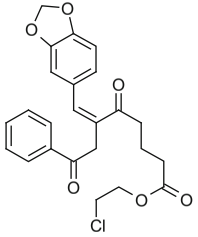
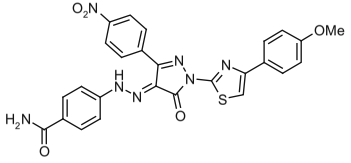
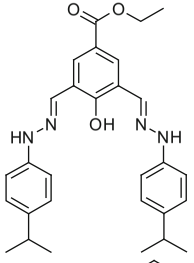
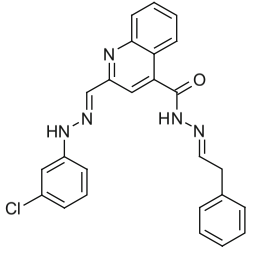
Table 6 (continued)

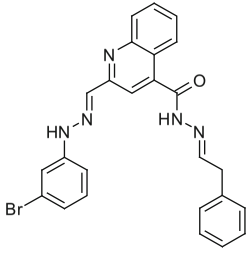
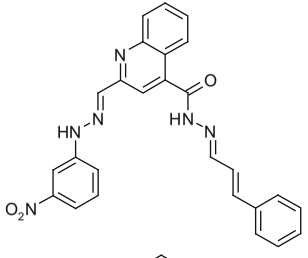
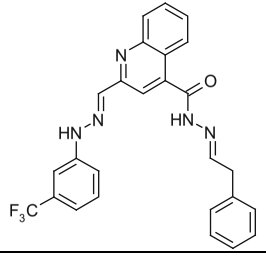
Compound	Substance	Affinity DEV-2 protease[a] (affinity WNV-protease)[a]	Assay pH (WNV)	References
73		345 ± 70 μM	8.5	[105]
74		377 ± 77 μM	8.5	[105]
75		25 ± 8 μM	8.5	[105]
76		21 ± 6 μM	8.5	[105]
77		69 ± 9 μM	8.5	[106]

78		121 ± 14 μM	8.5	[106]
79		510 ± 120 μM	8.5	[106]
80		186 ± 38 μM	8.5	[106]
81		IC_{50} = 13.1 ± 1.03 μM	9.0	[107]

(continued)

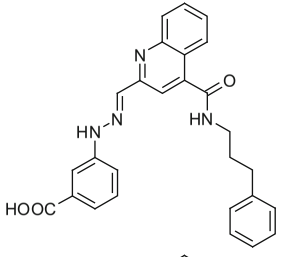
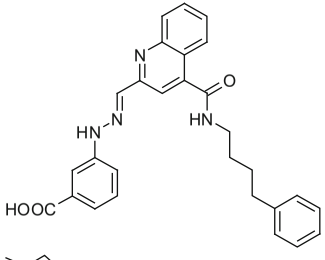
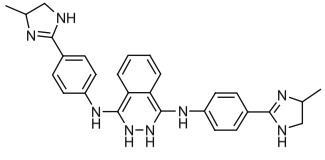
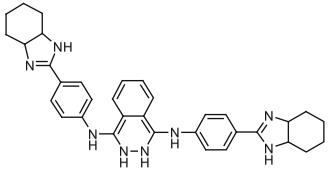
Table 6 (continued)

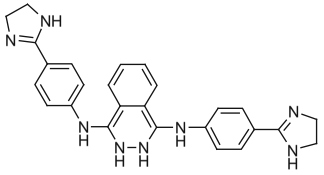
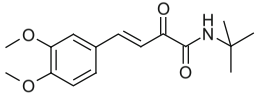
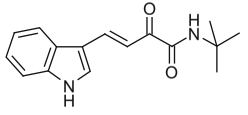
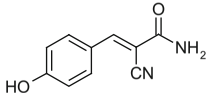
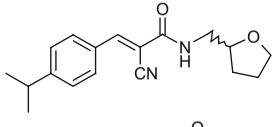
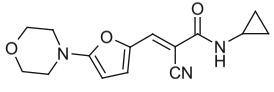
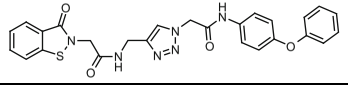
Compound	Substance	Affinity DEV-2 protease[a] (affinity WNV-protease)[a]	Assay pH (WNV)	References
82		$IC_{50} = 242.4\ \mu M$	9.0	[107]
83		$IC_{50} = 219.9\ \mu M$	9.0	[107]
84		$IC_{50} = 39.46 \pm 1.43\ \mu M$ $IC_{50} = 8.0\ \mu M$[d] $CC_{50} = 29.7\ \mu M$[c]	9.0	[107]
85		$IC_{50} = 28.12 \pm 1.96\ \mu M$ $IC_{50} = 61.3\ \mu M$[d] $CC_{50} = 19.7\ \mu M$[c]	9.0	[107]

86		$IC_{50} = 9.45 \pm 0.78$ μM $IC_{50} = 24.7$ μM[d] $CC_{50} = >100$ μM[c]	9.0	[107]
87		$IC_{50} = 21.96 \pm 2.05$ μM $IC_{50} = 74.9$ μM[d] $CC_{50} = 25$ μM[c]	9.0	[107]
88		$IC_{50} = 19.8 \pm 1.15$ μM $IC_{50} = 73.4$ μM[d] $CC_{50} = 16.4$ μM[c]	9.0	[107]

(continued)

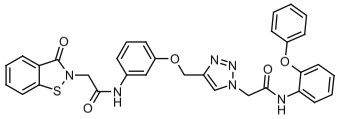
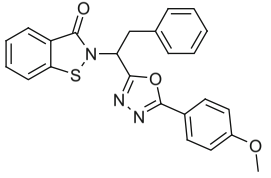
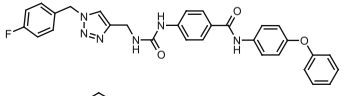
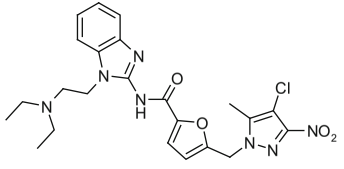
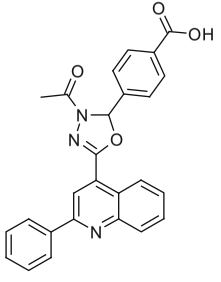
Table 6 (continued)

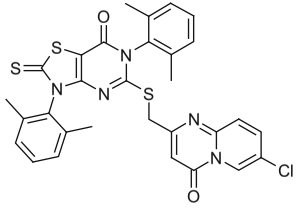
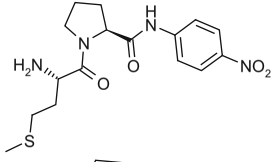
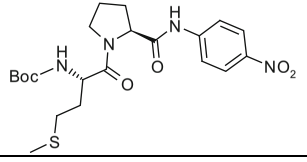
Compound	Substance	Affinity DEV-2 protease[a] (affinity WNV-protease)[a]	Assay pH (WNV)	References
89		$IC_{50} = 41.24 \pm 5.53$ μM $IC_{50} = 30.9$ μM[d] $CC_{50} = 64.3$ μM[c]	9.0	[107]
90		$IC_{50} = 35.28 \pm 4.36$ μM $IC_{50} = 11.0$ μM[d] $CC_{50} = 49.7$ μM[c]	9.0	[107]
91		$IC_{50} = 6 \pm 2.6$ μM $K_D = 7.2 \pm 3.2$ μM[d] $K_D = 7.3$ μM (ITC) $K_D = 3.7$ μM (SPR) ($IC_{50} = 24.5$ μM) ($K_D = 21$ μM)[d]	7.5 (7.5)	[108, 109]
92		$IC_{50} = 1.1 \pm 0.2$ μM $K_D = 7.7 \pm 2.0$ μM[d] $K_D = 13$ μM (ITC) $K_D = 0.3$ μM (SPR) ($IC_{50} = 22$ μM) ($K_D = 90$ μM)[d]	7.5 (7.5)	[108]

93		$IC_{50} = 10.2 \pm 6.0$ μM $K_D = 16.3 \pm 0.3$ μM[d] $K_D = 40$ μM (ITC) $K_D = 4.3$ μM (SPR) ($IC_{50} = 16$ μM)	7.5	[108]
94		36.1 ± 6.8% inhibition at 50 μM (31.0 ± 0.5% inhibition at 50 μM)	9.0 (9.0)	[110]
95		39.1 ± 0.7% inhibition at 50 μM (40.6 ± 0.7% inhibition at 50 μM)	9.0 (9.0)	[110]
96		35.7 μM (44.6 μM)	9.0 (9.0)	[111]
97		98.1 μM (101 μM)	9.0 (9.0)	[111]
98		184 μM (>200 μM)	9.0 (9.0)	[111]
99		$IC_{50} = 4.87 \pm 0.07$ μM	9.5	[112]

(continued)

Table 6 (continued)

Compound	Substance	Affinity DEV-2 protease[a] (affinity WNV-protease)[a]	Assay pH (WNV)	References
100		4.77 ± 0.05 μM	9.5	[112]
101		3.75 ± 0.06 μM (4.22 ± 0.07 μM)	9.5 (9.5)	[113]
102		8.77 μM (5.55 μM)	9.5 (9.5)	[114]
103		4.0 ± 0.4 μM (DEV-4 protease)	7.5	[115]
104		4.9 ± 0.3 μM (DEV-4 protease)	7.5	[115]

105		3.4 ± 0.1 μM (DEV-4 protease)	7.5	[115]
106		4.9 μM	9.0	[116]
107		10.5 μM	9.0	[116]

[a]If not otherwise stated, K_i-values are reported; K_i1 and K_i2 refer to a mixed inhibition mechanism
[b]Concentration at which the ELISA signal had been reduced 50% in the cell-based replication assay
[c]CC_{50} values were calculated with nontransfected cells
[d]Competition assay
[e]Replicon assay

detectable inhibition was observed [98]. **50–54** were shown to be equally potent against WNV.

Guanidinylated 2,5-dideoxystreptamine derivatives were identified via screening of a 12,000 member-comprising in-house library at PanThera Biopharma exhibiting IC_{50}-values in the one-digit micromolar range. However, based on the screening results it was not possible to deduce an SAR. Compound **55** possessing a K_i of 7.1 and 3.6 μM against DEV and WNV-protease, respectively, was shown to be inactive in a cell-based assay [99].

The two phenylguanidine derivatives **56** and **57** were found following a fragment-based drug design approach. Utilizing a drug-like subset of the ZINC-database, subsequent fragment generation with RECAP, docking, and scoring followed by fragment linking, and finally visual inspection of the best scored molecules rendered 23 representatives, which were tested in an *in vitro* assay. Two substances (**56**, **57**) were found to be active against DEV-protease with a K_i of 2.0 and 31.1 μM, respectively [100]. They also exert affinity towards WNV in the μM range.

HTS of the MicroSource Spectrum collection comprising 1,040 compounds that had reached clinical trials before, 800 natural products as well as 160 substances that had previously been proven to exhibit toxicity finally rendered eight compounds that showed an >80% inhibition of DEV-2 protease at a 100 μM concentration. As six of these compounds also strongly inhibited the serine protease trypsin, they were excluded from the hit set. Via retesting of the library with slightly altered criteria (≥50% inhibition of DEV-2 protease and ≤20% inhibition of trypsin at 100 μM), additional six substances were identified summing up to a total of eight compounds in this screening. For three of the substances, neither structural information nor inhibition constants have been published, as the initial results were not reproducible in the following kinetic experiments. For ivermectin (**58**), a macrocyclic lactone, selamectin (**59**), a structurally related lactone, the antiseptic methylbenzenethonium chloride (**60**), as well as the antibiotic alexidine (**61**), a mixed-competitive mode of inhibition could be determined. For tyrothricin (**62**), an antibiotic, a competitive inhibition mode was observed for DEV and WNV-protease, respectively [101].

63, **64** and **65** were identified by HTS of 41,600 compounds, purchased from Chemical Diversity Laboratory, all three exhibiting low two-digit micromolar IC_{50}-values; however, **64** and **65** were excluded from further studies due to toxicity to BHK21 cells. **63** is thought to disturb the protein–protein interaction of NS2B/NS3 as it strongly impedes with RNA replication rather than viral translation. Mutation in NS2B (R55K and E80K) resulted in a pronounced decrease in affinity of **63** in the transient Dengue virus replication assay (WT-EC_{50}: 0.17 ± 0.01 μM: KK-Mutant-EC_{50}: 12.55 ± 4.79 μM) [102].

Compounds **66** and **67** were identified by virtual screening of a subset of an in-house database from Mayo Clinic comprising 2.5 million compounds using a meanwhile withdrawn X-ray structure by Murthy et al. Nevertheless, out of 20 compounds identified, 13 fulfilled the solubility criteria. Five of these reduced DEV-2 protease activity (only rel. fluorescence data provided) and two were also significantly

active in a cell-based replication assay [104]. To further optimize the screening hit **66**, a similarity search using Pubchem and Sigma Aldrich's similarity search was performed and additional filters such as clogP and predicted solubility were applied. Finally, 23 analogues were purchased for further testing, rendering 10 analogues that were soluble at 10 μM. Four of those (three anthracene-based and one azo dye, **68–71**) were proven to exceed the activity of the initial hit **66** and four were equally potent. Further docking studies using an apostructure (2FOM) finally led to the discovery of **72**, the most active compound described within the study [103].

The flavonoid pinostrobin (**73**), the chalcone Cardamonin (**74**), and the dihydrochalcones panduratin (**75**) and 4-hydroxypanduratin (**76**) isolated from the rhizomes of the fingerroot *Boesenbergia rotunda*, were shown to inhibit DEV-2 protease in the two- to three-digit micromolar range, out of which the dihydrochalcones turned out to be significantly more active [105]. Following up, Heh et al. screened a subset of the ZINC database encompassing those compounds possessing ≥50% similarity to the backbone structures of flavanones, flavones and chalcones, in total 13,341 compounds. Four compounds (**77–80**) finally fulfilled the selection criteria for the docking process, e.g. $\Delta G_{\text{dock}} < \Delta G_{\text{dock of}}$ *R*-pinostrobin, and were shown to exhibit activity in the mid-micromolar range with compound **77** being the most active identified within this study [106].

By virtual screening of ~600,000 compounds of the ACD database and experimental evaluation of 27 predicted hits, three compounds (**81–83**) were identified inhibiting DEV-2 protease with IC_{50}-values ranging from 13.1 to 242 μM. Further optimization by functional group modification and scaffold hopping resulted in the design of 35 analogues, out of which 16 were shown to exhibit moderate affinity in the DEV-2 protease activity assay with IC_{50}-values in the range from 9.45 to 41.24 μM. Eight compounds (**81**, **84–90**) were also proven to exert activity in a DEV-2 luciferase reporter replicon-based assay with CC_{50}-values in the low two-digit micromolar range [107].

Screening of an in-house library of ~1,000,000 compounds resulted in the identification of one compound class, which, however, was synthetically challenging to alter. Thus, a structurally related substance (**91**) was used as initial lead for further structural optimization. Three of the compounds (**91–93**) were shown to bind in the catalytic pocket of DEV-2 protease via displacement titrations utilizing bovine pancreatic trypsin inhibitor. Besides inhibition constants, K_D-values were determined via ITC, SPR and a fluorescence competition assay [108].

The design and synthesis of a library of compounds bearing a β,γ-unsaturated α-ketoamide moiety as electrophilic warhead resulted in identification of several compounds that showed moderate inhibition of DEV at 50 μM. Two of the compounds **94** and **95** were shown to inhibit DEV replication dose dependently in a cell-based assay [110]. In continuation of their work, Nitsche et al. finally altered the electrophilic warhead to a nitrile functionality retaining the cinnamyl moiety. Thorough SAR led to the discovery of three low molecular weight compounds possessing K_i-values in the mid-micromolar range (**96–98**) [111].

By screening an in-house library, a benz[d]isothiazol-3(2H)-one derivative was identified. The exploration of this initial hit by synthesizing two focused libraries

finally resulted in **99** and **100** which turned out to be most potent derivatives displaying activity in the one-digit μM range [112]. In a second series, an 1,3,4 oxadiazole was attached to the benzothiazol-3(2H)-one scaffold. These compounds were nearly equally potent against DEV and WNV with **101** possessing a K_i-value of 3.75 and 4.22 μM, respectively [113].

Based on a molecular modeling approach, aminobenzamides were exploited as inhibitors of DEV and WNV. Out of 26 synthesized compounds, four exhibited activity in the μM range, out of which **102** represented the most potent derivative [114].

Via virtual screening of 300,000 compounds, 36 compounds were selected and tested for their inhibitory effect on DEV. Seven diverse compounds exhibited activity with IC_{50}-values ranging from 86.7 to 3.9 μM. For compounds **103**, **104** and **105**, K_i-values were determined for a competitive binding mode with 4.0, 4.9 and 3.4 μM respectively [115].

Compounds **106** and **107** were identified by screening an in-house library of compounds at Nanjing University. Structural modification of the methione-proline-scaffold revealed that almost all changes had a negative impact on the compounds' activity [116].

WNV

HTS of about 32,000 compounds resulted in the identification of 212 compounds that showed an inhibition of WNV activity of at least 50%. By filtering these initial hits applying, e.g. Lipinski's rule of five, 98 compounds were retrieved and further clustered by structural similarity. Finally, seven compounds were evaluated in more detail. Among the selected compounds, 8-hydroxy-quinoline-derivatives were found to be the most active ones with K_i-values up to 3.2 μM. **109** displayed an EC_{50} of 1.4 μM in cultured vero cells [117]. Further modification of these compounds to deduce a first structure–activity relationship revealed that the 8-hydroxy-quinoline-moiety is essential for activity and that small changes in the architecture can lead to a significant decrease in affinity. For **110**, a slight improvement in potency was observed [118, 119].

After virtual screening of the NCI database (~275,000 compounds) for allosteric inhibitors targeting the NS2B cofactor binding site in the NS3 domain and subsequent re-docking of the top 80 initial hits, the top 50 commercially available compounds were selected and tested for their inhibitory effect on WNV and DEV protease. Fifteen structurally diverse compounds exhibited activity with IC_{50}-values ranging from 0.26 to 9.83 μM and 2.04 μM to $>$ 100 μM against WNV and DEV protease, respectively. The most potent screening hit, bis-diazo-compound **111**, showed a 10-fold selectivity towards WNV [120].

Initial screening of an in-house library of 110 compounds identified 8 substances inhibiting WNV. So far three of which have been evaluated in more detail. Thorough SAR studies of one hit, a zwitterionic tetraazaphenanthren-4-one derivative (**112**), finally led to several one-digit μM inhibitors with **113** being an

uncompetitive one with an IC_{50}-value of 5.41 μM [121]. To optimize the second screening hit, 2-thiolo-6-aminobenzothiazole derivative **114**, a focused library of 39 compounds was evaluated. Interestingly, the *p*-des-amino derivative **115** was more potent than the screening hit. Introduction of a *p*-ethyl group at the phenyl substituent gave rise to the most potent derivative **116. 116** is an allosteric inhibitor of WNV and DEV, with IC_{50}-values of 3.3 and 42.9 μM, respectively [122]. Structural optimization of the third screening hit (**117**) bearing a 1*H*-pyrrol-2(*5H*)-one scaffold resulted in the identification of **118**, which was shown to be an uncompetitive inhibitor of WNV with a K_i-value of 1.8 μM. Noteworthy, (–)-**118** is significantly more active than the (+)-enantiomer [123].

By HTS of a 65,000 compounds-comprising library of the NIH Molecular Libraries Small Molecule Repository, 126 compounds were retrieved showing promising activity at 10 μM. Further evaluation of these initial screening hits led to the discovery of 15 compounds. Ten of these possessed a 5-amino-1-phenylsulfonyl-pyrazol-3-yl scaffold. Several of the compounds showed sub-micromolar activity, with the uncompetitive inhibitor **119** being the most active screening hit [124]. Albeit their affinity, they were prone to hydrolysis at pH 8. Replacement of the labile ester bond, e.g. by an alkene, and substitution of the amino functionality (**120**) resulted in significantly more stable but, however, in less potent compounds [125].

Utilizing a fragment-based docking approach employing a diversity subset of the ZINC database, five compounds were finally selected for testing. **121** and **122** exhibited μM activity in an enzymatic assay. Additionally, K_d-values were obtained via a tryptophan fluorescence assay and NMR spectroscopy [126].

In a virtual HTS approach utilizing the 6 million compounds-comprising iResearch library, 11,715 compounds bearing at least one positive charge or 5 or more H-bond donors were selected. Upon docking, minimization, and visual inspection, 22 compounds were finally selected for affinity determination by NMR spectroscopy. Six of the tested compounds showed specific binding with **123** being the most active derivative with a K_d of ~40 μM [127].

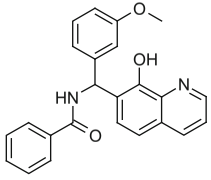
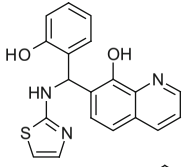
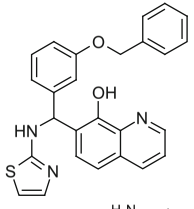
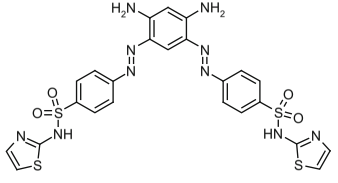
Within a series of 1-oxo-1,2-dihydroisoquinolines, **124** showed the highest affinity with a IC_{50}-value of 30 μM [128].

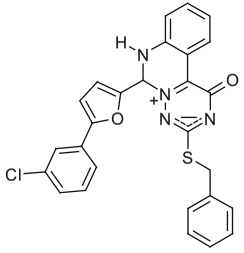
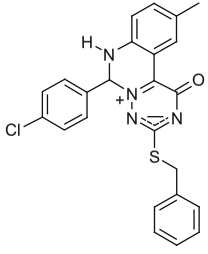
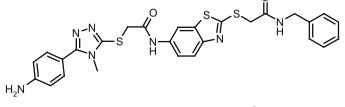
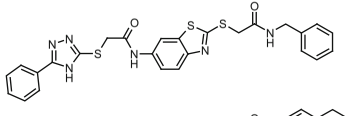
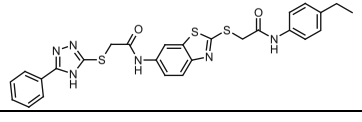
The protoberberine alkaloid Palmatine (**125**), possessing an IC_{50}-value of 96 μM, was shown to exert antiviral activity with an EC_{50}-value of 3.6 μM in vero cells (Table 7) [129].

3 Considerations on Emerging Resistance Against Flavivirus Protease Inhibitors

As observed from most diseases with prevalent genetic instability and variation of the nucleic acid inventory by dysregulated cellular processes or loss of proofreading activities upon amplification of the genetic material, also inhibitors against

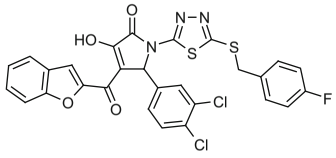
Table 7 Non-peptidic ligands of WNV

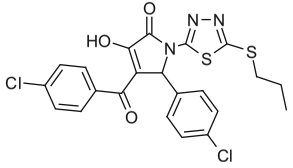
Compound	Substance	Affinity WNV-2[a] (affinity DEV protease)[a]	Assay pH (DEV)	References
108		3.2 ± 0.34 μM (28.6 ± 5.1 μM)	9.5 (9.5)	[117]
109		3.4 ± 0.59 μM (30.2 ± 8.6 μM) EC_{50} = 1.4 ± 0.3 μM	9. 5 (9.5)	[117]
110		IC_{50} = 1.06 ± 0.07 μM IC_{50} = 3.46 ± 0.06 μM (IC_{50} = 2.99 ± 0.06 μM)	9.5 (9.5)	[118, 119]
111		IC_{50} = 0.26 μM (IC_{50} = 2.75 μM)	8.0	[120]

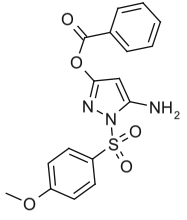
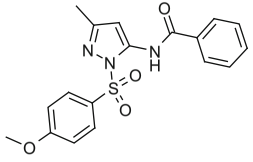
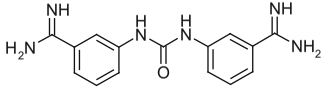
112		$IC_{50} = 96.6 \pm 4.16$ μM	8.0	[121]
113		4.47 ± 0.37 μM	8.0	[121]
114		$IC_{50} = 59.0 \pm 2.9$ μM	8.0	[122]
115		$IC_{50} = 14.3 \pm 2.5$ μM	8.0	[122]
116		$IC_{50} = 3.3 \pm 0.19$ μM	8.0	[122]

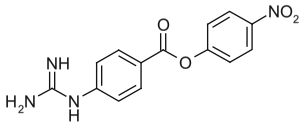
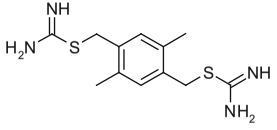
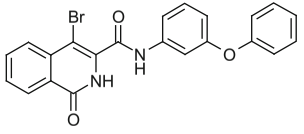
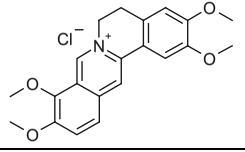
(continued)

Table 7 (continued)

Compound	Substance	Affinity WNV-2[a] (affinity DEV protease)[a]	Assay pH (DEV)	References
117		$IC_{50} = 34 \pm 1.8$ μM	8.0	[123]

118		for (−)-118: 1.8 ± 0.6 μM
		$IC_{50} = 2.2 \pm 0.7$ μM
		for (+)-118: $IC_{50} = 90.4 \pm 8.3$ μM
			8.0	[123]

119		$IC_{50} = 0.105$ μM	8.0	[124]
120		$IC_{50} = 9.2$ μM	8.0	[125]
121		$IC_{50} = 2.8 \pm 0.1$ μM $K_d = 15 \pm 1.6$ μM[b] $K_d = 90 \pm 40$ μM[c]	7.5	[126]

122		$IC_{50} = 34.2 \pm 0.1$ μM $K_d = 16 \pm 1.8$ μM[b]	7.5	[126]
123		$K_d = \sim 40$ μM[c]	8.5	[127]
124		$IC_{50} = 30$ μM	9.5	[128]
125		$IC_{50} = 96$ μM $EC_{50} = 3.6$ μM	8.0	[129]

[a]If not otherwise stated, K_i-values are reported; K_i1 and K_i2 refer to a mixed inhibition mechanism
[b]K_d determined by a tryptophan fluorescence assay
[c]K_d determined by NMR

flaviviral proteases suffer from their progressing inactivation by increasing resistance throughout exposure of the antiviral agent. Particularly caused by the error-prone activity of the virus-encoded RNA-dependent RNA polymerase, the generation of drug-resistant flaviviral variants is strongly enhanced. While inhibitor design against proteases without mutational variation can be assumed to be comparatively well achievable, hitting a target enabled to "move" facilitated by genetic instability has to be considered as significant challenge [3, 130]. Typically, insights into resistance mechanisms are derived from clinical observations after drug exposure and selection of resistant strains throughout antiviral therapy, or detection of mutations within the target protein in replicon-based cell assays. As lead discovery against DEV or WNV infections is still at a very early stage, in absence of clinical trials and resistant isolates from treated patients, only the latter approach might provide insights, but only very few studies describe explicitly resistance mutations to occur within the protease region. However, there are some lessons to be learned from more advanced treatment regimens, e.g. targeting the NS3-4A protease of the hepatitis C virus or the protease of the human immunodeficiency virus (HIV) 1.

The hepaciviral protease NS3-4A is to high extent structurally and functionally related to the NS2B-NS3 proteases of DEV and WNV [131], and might therefore serve as a promising surrogate to study mechanisms of resistance development. Even though NS3-4A protease inhibitors contribute as "direct-acting antiviral agents" (DAAs) and pave the way towards a new era of HCV therapy and interferon-free regimens, the application of the first generation protease inhibitors either approved (telaprevir, boceprevir) or close to approval is not suitable as a single therapy because of the acquirement of resistance within only few days and should only be co-administered with interferone/ribavirine. Interestingly, recent contributions have shown that drug-resistant variants do not arise as new viral mutations, but pre-exist constitutively as minor subtypes. Three major factors have been identified that influence viral resistance within the host: (1) the genetic barrier to resistance, i.e. the number of mutations required to achieve drug resistance, (2) the in vivo fitness of the variant population, i.e. the ability to survive and replicate and (3) the drug exposure in the relevant cellular compartment [132].

An "old" concept to overcome resistance that was already derived from research experiences in the field of anti-HIV lead discovery bases on the idea that resistance-associated mutants still must need to be able to fulfill their proteolytic function at all different substrate sites. This concept emerged under the terminus "substrate envelope hypothesis": an inhibitor is assumed to be less susceptible to be affected by mutations if its spacial requirements closely resemble the consensus volume of the substrates [133, 134]. This consensus volume can be obtained from the superimposition of target X-ray structures in complex with their substrates derived from the different polyprotein cleavage sites. Interestingly, this concept experienced some renaissance, as particularly for HCV NS3-4A it was recently shown that mutations conferring resistance accumulate in regions where the drug (candidate) molecules occupy regions of the enzyme binding pocket apart from the consensus substrate envelope. In addition, ligands that interact to protein residues explicitly involved in the catalytic activity show even lower susceptibility to resistance

development, as mutations at these sites affect the fitness of the viral subpopulation [135, 136]. Even though some inhibitors that fulfill this property exhibit a certain degree of robustness against resistance development, following this strategy will become difficult in case of tandem mutations occurring at protease and cleavage site simultaneously [137]. In some contrast to the substrate-envelope hypothesis, experience from HIV protease inhibitor discovery shows that mutations affecting substrate binding and catalytic efficiency are tolerated as well, as long as the virus maintains sufficient fitness to replicate [138].

Further lessons from HIV-1 protease drug discovery efforts provide evidence that the concept of ligand residence time and drug action has also implications for the acquirement of drug resistance by mutational change of the target properties. According to this concept, ligands that reside longer on their target binding site do not only show a higher efficacy, but are thereby also prevented from binding to other proteins or degradation processes. While saquinavir binds to wild-type HIV-1 protease with a residence time of 11.9 min, for the L90M, G48V and L90M/G48V mutants this parameter amounts only to 52.6, 7.8 and 1.9 s, respectively. This observation provides the basis for the emerging paradigm that tight-binding inhibitors with slow-off kinetics are privileged in discovery efforts against resistance-compromised targets [138–140].

4 Conclusions

Due to the essential function of proteases in viral replication, they represent attractive targets for drug discovery. The design of appropriate inhibitors is facilitated by the experience of medicinal chemists and drug designers that has been collected in more than two decades of industrial and academic research. Thus, targeting viral proteases generally provides an essential contribution towards being prepared for future viral outbreaks. Interestingly, despite extensive efforts described in this contribution that have been invested into the discovery of chemical starting points and hit-to-lead optimization, most synthesized compounds inhibit Dengue or West-Nile proteases with only moderate affinities in the micromolar range. The further optimization of affinity, depeptidization and replacement of reactive warheads to increase the drug-likeliness appear necessary to pave the way towards lead structures for further profiling. The influence of resistance mutations onto ligand affinity might be of structural origin, although changes of the dynamic properties are likewise involved as well. Studies linking the structural properties of resistant mutants to the thermodynamic fingerprint of ligand binding will hopefully improve the current understanding and contribute to novel strategies in order to overcome susceptibility to resistance. Awareness for the massive health menace has been created in the mindset of the society, its scientists, and even football fans are currently advised against the putative risk exposure at the next World Cup in Brazil, where “football fever could be a dose of Dengue” [141]. New public-health information systems such as DengueMap (http://www.healthmap.org/dengue/en/.

Accessed 20 Jan 2014), DengueTrends (http://www.google.org/denguetrends/intl/en_gb/. Accessed 20 Jan 2014) and ArboNET (http://www.cdc.gov/westnile/resourcepages/survResources.html. Accessed 20 Jan 2014) have been created and are publicly available to monitor the risk of putative outbreaks. It remains to hope that this awareness translates as well into preparedness with respect to the development of medication remedies and vaccination.

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Strategies for the Development of Influenza Drugs: Basis for New Efficient Combination Therapies

Torsten Steinmetzer, Kornelia Hardes, Eva Böttcher-Friebertshäuser, and Wolfgang Garten

Abstract Only four drugs are approved for the specific treatment of acute influenza infections worldwide. These drugs address either the viral neuraminidase or the M2-channel but suffer from poor efficacy and the rapid emergence of drug resistances. Some additional drugs are launched in few countries, but their efficacy is relatively low and often limited to certain influenza strains. Ideally, new drugs should possess a broad potency against all influenza viruses and be less susceptible to the development of resistances. The propagation cycle of the influenza virus offers many possibilities for the development of new anti-influenza drugs. Various compounds addressing viral targets reached clinical development, from which the most-advanced candidate is the polymerase inhibitor favipiravir. A promising strategy could be also the inhibition of host targets and signaling pathways, e.g., by already approved kinase inhibitors, anti-inflammatory drugs, or immunomodulatory agents. However, the benefit of these agents has to be demonstrated in controlled clinical trials. A broader arsenal of approved drugs will offer the possibility for more efficient combinatorial therapies in the future. The first proof of concept studies for such multiple drug therapies in infected mice revealed beneficial effects. Moreover, this strategy should reduce the rapid emergence of resistant influenza strains.

Keywords Antiviral drugs, Combination therapy, Drug resistance, Drug targets, Influenza virus, Inhibitors

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Contents

Abbreviations

dsRNA	Double-stranded RNA
HA	Hemagglutinin
HCV	Hepatitis C virus
HPAIV	Highly pathogenic avian influenza virus
HTS	High-throughput screening
IFN	Interferon
NA	Neuraminidase
nAb	Neutralizing antibody
NAI	Neuraminidase inhibitor
NEP	Nuclear export protein
NP	Nucleoprotein
RdRp	RNA-dependent RNA polymerase
RNP	Ribonucleoprotein
SA	Sialic acid

1 Introduction

Seasonal flu epidemics still cause thousands of deaths each year. Most of them are among risk patients, but nevertheless, a certain number of people die every year from influenza without any medical history. For the 2009 pandemic H1N1 outbreak, 18,631 laboratory-confirmed deaths have been reported to the WHO worldwide, although global estimates are more than ten times higher [1]. The related costs for loss of working hours and hospitalization of sick people are a strong socioeconomic burden. Prophylactic vaccination offers the best protection, but is regularly used by too few people. The vaccine has to be reformulated annually because of continuous changes of the virus genome due to point mutations

persistently producing many changes in virus proteins including the surface proteins. Besides this antigenic drift, it can also come to a reassortment of the segmented genome leading to an antigenic shift of virus progeny. Despite high antigenic variability of the viral surface glycoproteins, there is progress in the development of universal influenza vaccines against common antigenic epitopes found in all virus strains. In case of a pandemia, it normally takes several months to develop a new vaccine. Therefore, novel and effective anti-influenza drugs are urgently needed. So far, only few drugs have been launched worldwide, although in some countries, additional approvals have been granted. Many of these drugs suffer from limited efficacy, side effects, and the rapid emergence of resistances. Starting from the approved drugs, we will provide an overview about present developments in the field of anti-influenza agents. These strategies are directed against various steps in the viral propagation cycle, which is shortly summarized below.

2 Structure and Replication of Influenza Viruses

The enveloped influenza viruses belong to the family of *Orthomyxoviridae*. They can be divided into the serologically different types A, B, and C, whereas infections with the type C in men are usually only associated with minor symptoms and thus not further discussed in this article. The genome of the subtypes A and B consists of eight single negative-stranded RNA segments, which encode for about 12 different proteins [2, 3]. The viral envelope is covered on its surface by multiple copies of membrane-integrated glycoprotein spikes consisting of receptor-binding trimeric hemagglutinin (HA), to a lesser extent of the sugar-cleaving homotetrameric neuraminidase (NA) and of a few membrane-spanning proton channel M2 tetramers. The inner side of the envelope is covered by the matrix protein M1. The virus interior contains the ribonucleoprotein (RNP) complex consisting of the viral RNA genome, the nucleoprotein NP, and the virus-encoded heterotrimeric polymerase complex buildup by PB1 (polymerase basic protein 1), PB2 (polymerase basic protein 2), and PA (polymerase acidic protein) [4]. The RNA segment of PB1 encodes for two more proteins, PB1-F2 and PB1-N40. PB1-F2 is a small nonstructural protein with apoptotic functions [2], whereas the role of PB1-N40 is still unknown. Influenza A viruses encode for two further proteins, which are present only in infected cells. The first nonstructural protein NS1 protects against host cell antiviral responses, and the second one is NEP (nuclear export protein), formerly known as NS2, which is responsible for the nuclear export of vRNP.

The replication cycle can be roughly divided into ten steps (Fig. 1); in principle all of them offer possibilities for blocking virus propagation [5]. After attachment of the virus via multiple binding of its HA trimers to terminal *N*-acetyl neuraminic acid (sialic acid) of glycoconjugates (glycoprotein, glycolipids) on host cells (step 1), the virus enters the interior of the cell by endocytosis (step 2). The subsequent fusion of the viral envelope with the endosomal membrane enables the release of the RNP complexes into the cytosol (uncoating, step 3). The RNP complexes are

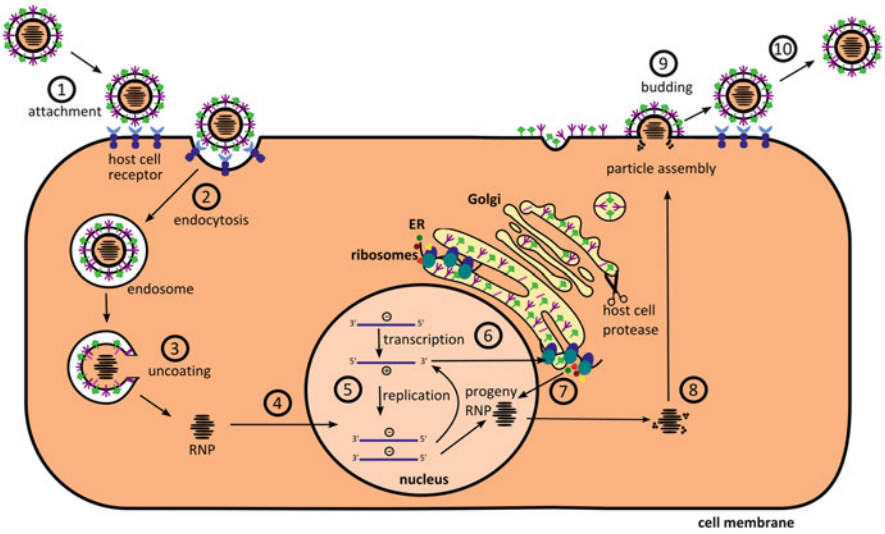


Fig. 1 Multiplication cycle of influenza virus. Initial virus attachment is followed by viral entry via endocytosis and acidification of the endosomal lumen and viral interior. After fusion of the viral and endosomal membranes, the RNPs are released into the cytosol, followed by their transport into the nucleus, where transcription and replication take place. Viral mRNAs are exported into the cytosol, where the viral proteins NP, PA, PB1, and PB2 are synthesized and translocated back into the nucleus for RNP formations. Newly formed RNPs are transported to the cytosol and further to the cell membrane, where they assemble with HA, NA, and M2, which reach the cell surface via the secretory pathway. Finally, virus progeny is released from cells by viral NA

transported into the nucleus (step 4), where the viral RNA-dependent RNA polymerase (RdRp) complex catalyzes the transcription and replication of the viral RNA (step 5). The viral mRNA is transported into the cytosol for translation (step 6). The newly synthesized proteins NP, PA, PB1, and PB2 are imported into the nucleus forming RNP complexes together with the minus-stranded RNA segments for progeny viruses (step 7). These RNP complexes are exported from the nucleus and transported together with the M1 protein to the cell surface (step 8). HA, NA, and M2 are transported along the secretory pathway to the plasma membrane, where the RNPs are wrapped up in HA- and NA-enriched areas, and the new viruses are subsequently released from the cell by budding (step 9). Progeny viruses are finally liberated by the viral NA activity from the cell surface and from each other, followed by dissociation of the weakly bound SA (step 10).

3 Approved Drugs and Their Further Development

3.1 Neuraminidase Inhibitors (NAIs)

The most important drugs targeting influenza A and B viruses are the inhibitors of the viral neuraminidase, a homotetrameric sialidase anchored into the viral envelope, which fulfills several functions. Firstly, it improves the mobility of virus particles in the upper respiratory tract by cleavage of mucins, and secondly, it removes viruses trapped via binding to sialic acids of non-receptor molecules on the host cell surface. Thirdly, NA catalyzes the release of progeny virions after budding by removal of the terminal *N*-acetylneuraminic acid residue (**1**, sialic acid (SA)), from cell surface glycoconjugates, and fourthly, it removes (SA) residues from the viral HA and NA proteins, thereby preventing the aggregation of progeny viruses [6]. The development of effective sialidase inhibitors was strongly accelerated after the crystal structures in complex with sialic acid derivatives became available [7, 8]. Molecular modeling suggested the replacement of the 4-OH group of the ligand (**1**) by a positively charged substituent, which should fill the space around the NA side chains of residues Glu119 and Glu227. This resulted in the discovery of the highly polar guanidine analogue (**2**) (4-guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuraminic acid, zanamivir), meanwhile marketed under the trade name Relenza® as a powder for oral inhalation (Figs. 2 and 3).

Several modifications to reduce its strong polarity, including a movement of the double bond and the replacement of the glycerol and guanidine groups by an isopentyl ether and amino group, respectively, provided the orally available ethyl ester prodrug oseltamivir (**3**) containing a cyclohexene core structure. Oseltamivir, marketed as Tamiflu®, is rapidly converted in man to the active NA inhibitor oseltamivir carboxylate. During the 2009 H1N1 pandemia, a third NA inhibitor, the cyclopentyl-derivative peramivir (**4**) [10], was temporarily authorized in the

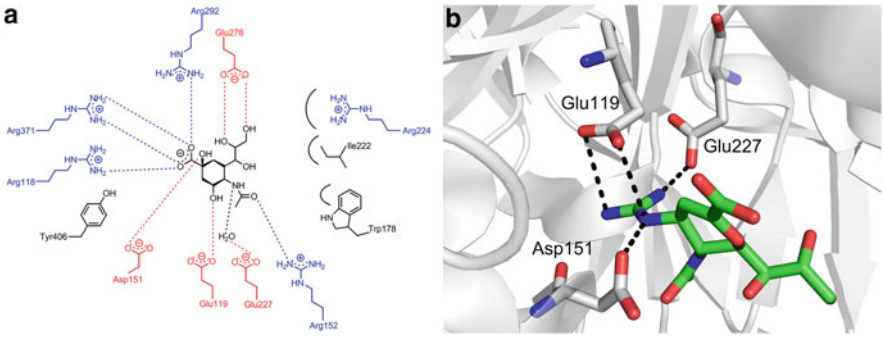


Fig. 2 (**a**) Schematic interactions found in the complex between influenza neuraminidase and *N*-acetylneuraminic acid (**1**), bound in a boat-like conformation. This figure was adapted from [6]. (**b**) Structure of zanamivir in complex with an N8 neuraminidase (2htq.pdb) [9]. A key step in the development of zanamivir was the replacement of the 4-OH substituent in (**1**) by a guanidine group

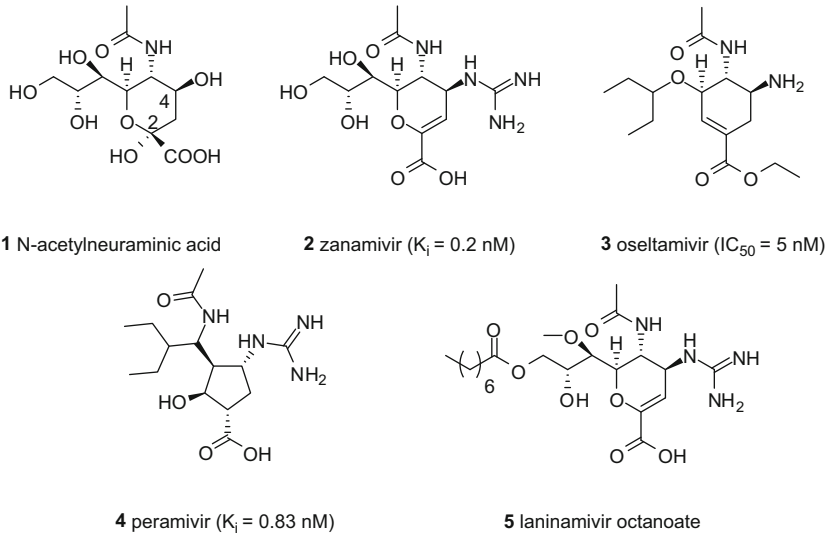


Fig. 3 Structures of *N*-acetylneuraminic acid and approved neuraminidase inhibitors

USA for parenteral treatment in emergency situation. Meanwhile, peramivir is approved in Japan and South Korea. A prolonged and improved antiviral activity provides Inavir®, the octanoyl prodrug (**5**) of laninamivir. Otherwise, the structure of laninamivir is nearly identical to zanamivir (**2**), although it contains an additional methylation on the inner glycerol hydroxy group. Meanwhile, Inavir® (**5**) is approved as an inhalable dry powder formulation in Japan.

The efficacy of oseltamivir is often hampered by rapid resistance developments caused by point mutations of various active site residues. Especially, the His274Tyr mutant of NA, which was also found among the 2009 pandemic flu and highly pathogenic avian influenza H5N1 virus strains, is much less susceptible to oseltamivir. The sterically more demanding Tyr side chain induces a shift of the residue Glu276, which weakens the affinity to the pentanyl group of oseltamivir (**3**) and peramivir (**4**), whereas the analogous glycerol moiety of zanamivir still enables sufficient binding to the displaced Glu276. A few additional active site mutations reduce the efficacy of the NA inhibitors (NAIs); therefore, the development of new and more effective derivatives is ongoing. Various strategies for the design of improved NAIs were recently reviewed [11]. All NAIs consist of a cyclic core scaffold, which is decorated with the functional groups targeting the various NA subsites. Based on the used core, the NAIs can be roughly divided into compounds with a flexible scaffold and inhibitors with a central aromatic and therefore more rigid ring system. The flexible core structures comprise three major types, the pyranose (Fig. 5), cyclohexene, and cyclopentane derivatives (Fig. 6).

The NAs can be divided into the two phylogenetic groups 1 or 2. All NAs contain a 150-loop adjacent to their active site. This loop can exist in a relatively closed conformation in case of the group 2 members (e.g., N2, N3, N6, N7, or N9)

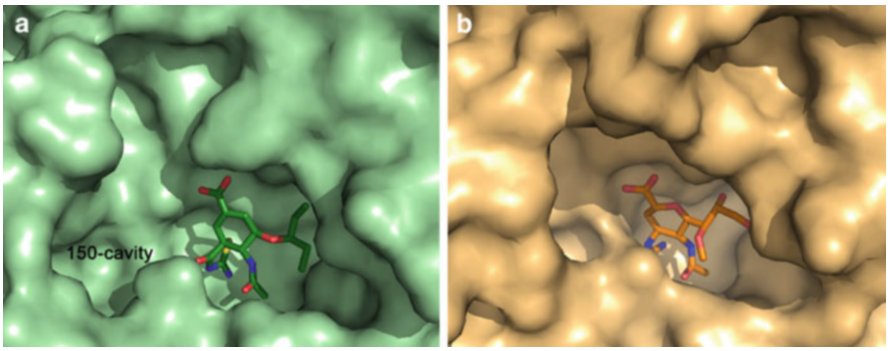


Fig. 4 Crystal structures of the active sites of N8 neuraminidase (4mju.pdb) [12] in complex with inhibitor (**17**) (**a**) and N6 neuraminidase (4qn6.pdb) [13] inhibited by laninamivir, the active form of the prodrug (**5**) (**b**). N8 belongs to the group 1 NAs often possessing an open 150-cavity, whereas N6 is a typical group 2 NA missing this subpocket of the active site

or in a more open form found for the group 1 NAs (e.g., N1, N4, N5, and N8). The already approved NAIs were designed based on the group 2 structures, which are missing an additional binding pocket, named 150-cavity [9]. This pocket only exists in the group 1 NAs, due to their more open active site (Fig. 4). It was proposed that this structural difference should enable a further optimization of the inhibitors against the group 1 NAs.

For targeting the 150-cavity in the group 1 NAs, several C-3-substituted pyranose analogues have been prepared such as (**6**) [14], or the pyranose core was modified with more bulky C-4 substituents (**7**) [15] (Fig. 5). However, most of these analogues suffered from reduced potency in the micromolar range, and compound (**6**) was still more potent against N2 ($IC_{50} = 0.1$ μM) compared to N1 ($IC_{50} = 3.5$ μM). Furthermore, it was recently reported that the NA from the pandemic 2009 H1N1 virus is an atypical group-1 NA, which is lacking the 150-cavity [16]. Therefore, targeting the 150-cavity could be less efficient against such NAs. An improved subnanomolar inhibitory potency against various NAs, including the His274Tyr mutant, was found for the phosphonate analogue of zanamivir (**8**) [17]. Additional inhibitors were designed by elongation of the C-1 carboxamide structure, which targets the so-called 430-cavity. The fluorophenyl derivative (**9**) inhibits the NAs of H3N2, H5N1, and H1N1 viruses with IC_{50} values <15 nM and possesses an improved half-life compared to zanamivir [18].

The C-1 phosphonate modification was also exploited for the design of NAIs based on the cyclohexene scaffold, used in oseltamivir. The phosphonate derivatives (**10**–**13**) are potent NAIs in the nanomolar range. Especially, the guanidine analogues (**12**) and (**13**) are also effective against the His274Tyr mutant, whereas it was weakly inhibited by the amines (**10**) and (**11**) (Fig. 6). However, a relatively low bioavailability of less than 12% was observed for compound (**13**), which was even more reduced with analogues (**10**–**12**). An improved subnanomolar N1 inhibition was found for the ring isomer (**14**), whereby the authors argue that the

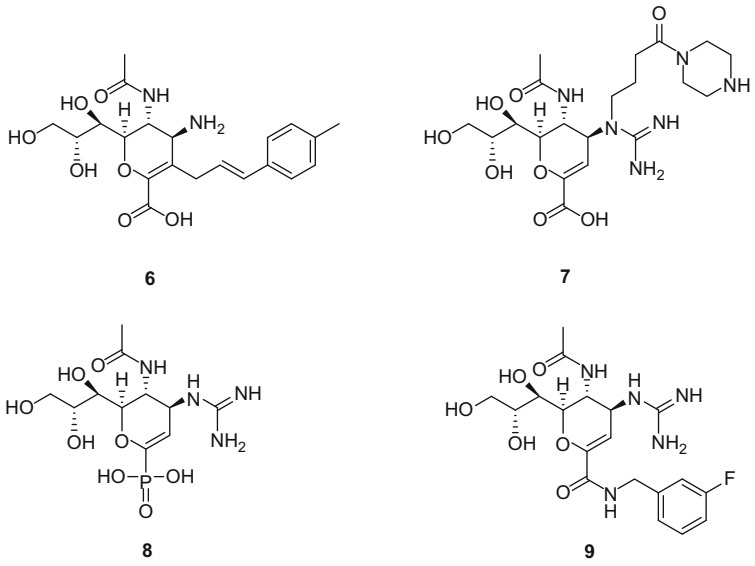


Fig. 5 Experimental neuraminidase inhibitors based on a pyranose core

isomeric double bond influences the ring conformation providing a better orientation of the C-3 guanidine toward the 150-loop [19].

Several oseltamivir derivatives bearing substituted guanidines to address the 150-cavity have been recently described. However, only small groups such as methyl or hydroxyl were tolerated (**15**, **16**), whereas all compounds with larger substituents displayed dramatically reduced potency [20]. Therefore, it seems to be impossible to address the 150-cavity by attaching larger groups to the terminal guanidine nitrogen. A strong activity against a H3N2 strain in cell culture was recently reported for the novel spirolactam (**17**), which was also crystallized in complex with an N8 NA (Fig. 4a) [12]. Otherwise, compound (**17**) was a relatively poor inhibitor of the tested H1N1 strain. Moreover, novel orally available oseltamivir analogues have been developed by incorporation of an acetamidine moiety at C-3 position. Compound (**18**) exhibits subnanomolar affinity for various N1 and N2 NAs and retains acceptable affinity against the H274Y N1 variant ($IC_{50} = 14.5$ nM). For its *N*-hydroxy prodrug (**19**), an excellent oral bioavailability in rats (31%) was determined [21]. A few NAIs have been prepared based on the cyclopentane core, whereas most analogues suffered from relatively poor activity. An exception is the deguanidinylated peramivir analogue (**20**), which retains relatively strong inhibitory potency ($K_i = 7.5$ nM) (Fig. 6) [22].

Besides the already described scaffolds, intense work was performed to develop NAIs containing an aromatic core segment, which would alleviate the synthesis. So far, all of these analogues possess significantly reduced affinity; selected examples

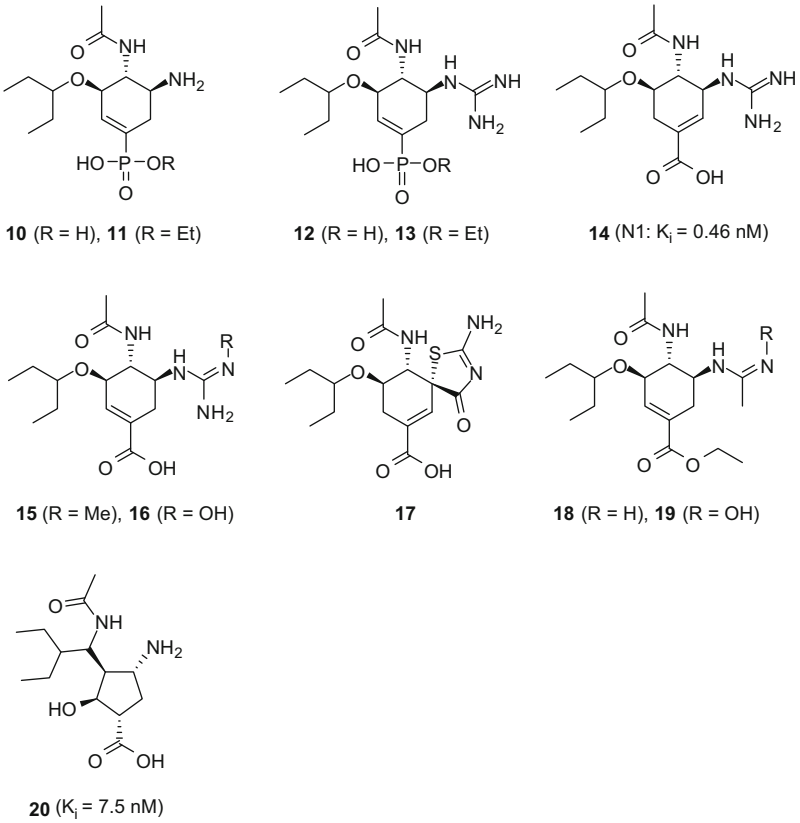


Fig. 6 Experimental neuraminidase inhibitors based on a cyclohexene (**10**–**19**) or cyclopentane (**20**) core

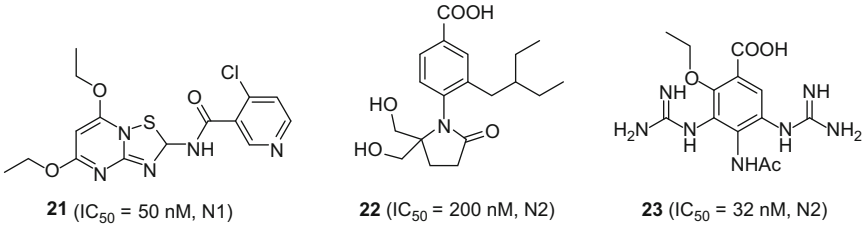


Fig. 7 Experimental neuraminidase inhibitors containing an aromatic core scaffold

(**21**–**23**) are shown above (Fig. 7) [23–25]. Moreover, a few natural products have been identified as moderately potent NAIs, which also possess aromatic core systems. Among them are several chalcones, flavonoids, or other polyphenols; some examples have been summarized in a recent review and therefore will not further be discussed [11].

Among the newly developed NAIs are a few leads with a promising overall profile. However, it is currently unknown whether some of these candidates will enter a clinical development as a next-generation neuraminidase inhibitor.

3.2 M2 Inhibitors

The homotetrameric matrix protein M2 (Fig. 8) is a pH-gated proton channel spanning the viral lipid envelop. After endocytotic virus uptake, it enables the conductance of protons into the viral lumen, which facilitates the dissociation of the RNPs from the matrix protein M1 during uncoating. Moreover, when it resides in the trans-Golgi network (TGN), it contributes to the pH equilibrium between the TGN and the host cell cytoplasm, thereby preventing the premature conformational rearrangement of the newly synthesized HA in the secretory pathway.

The M2 channel inhibitors amantadine (**24**) and rimantadine (**25**) (Fig. 9) have been launched as the first drugs against influenza A strains in 1966 and 1994, respectively; however, they are inactive against the influenza B virus. Moreover, due to various mutations in the transmembrane domain of M2, whereby S31N, L26F, and V27A are the most prominent ones, both inhibitors are nearly ineffective against the presently circulating strains. Therefore, it was recommended to stop their continued usage [27]. The structurally very different spiranamine (**27**) [28, 29], which was derived from the long known HTS hit BL-1743 (**26**) [30], is active

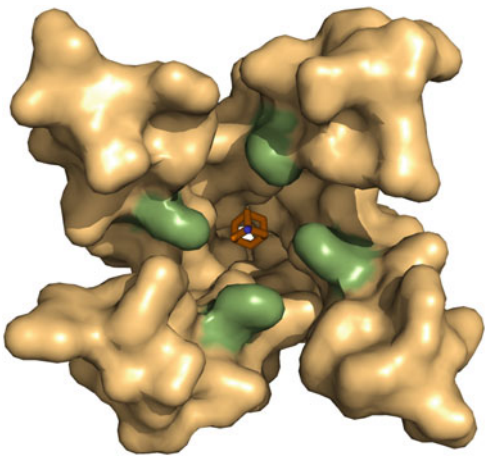


Fig. 8 Complex of amantadine (**24**, shown as *sticks* with *orange carbon atoms*, nitrogen in *blue*) within the pore of the homotetrameric M2 channel provided with a beige surface. The complex was determined by X-ray crystallography (3c9j.pdb) [26]. After acidification of the endosomal lumen, the four histidine residues of the channel, shown with a *green surface*, become protonated. This disrupts the H-bond network between the imidazole side chains found in the closed form at higher pH and opens the channel, which enables the entry of protons from the endosomal lumen through the viral envelope into the lumen of the virus

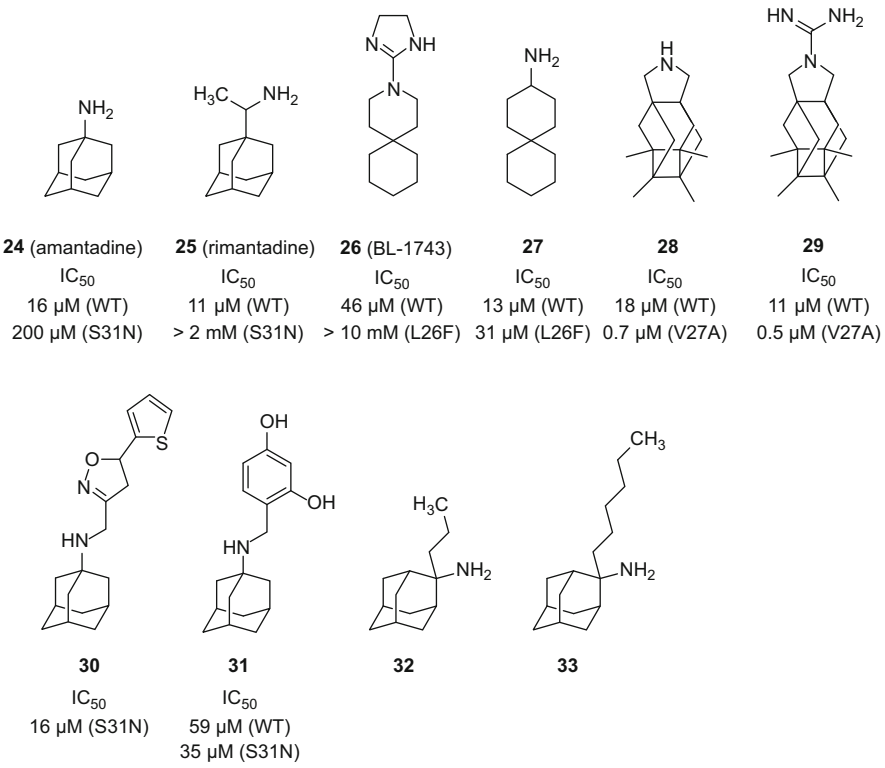


Fig. 9 Approved (**24**, **25**) and experimental M2-channel inhibitors

against the L26F and V27A variants. However, it lacks inhibition of the S31N mutant, which is now with more than 95% the predominant form in the transmissible amantadine-resistant H1N1, H3N2, and H5N1 strains. A very similar selectivity profile was found for a series of polycyclic pyrrolidines, such as compounds (**28**) and (**29**) [31].

The structure determination of the M2 channel by X-ray analysis [26] and NMR [32] (the available pdb codes have been listed in a recent review [27]) has supported the development of new analogues to overcome the resistance problem. The S31N mutation affects the size and polarity of the binding site for the adamantane group in the pore of the M2 homotetramer, which finally leads to a ~12-fold drop in potency of amantadine (**24**) using an electrophysiological assay, whereas the drop in affinity for rimantadine (**25**) is even more pronounced [33]. Very recently, two series of elongated amantadine derivatives including compounds (**30**) [34] and (**31**) [33] have been disclosed, which exhibit high inhibitory potency against the S31N mutant in an electrophysiological assay in oocytes. The NMR structure of inhibitor (**30**) (2ly0.pdb) revealed that the adamantane moiety binds very similar as known from the amantadine (**24**) and rimantadine (**25**) complexes making van der Waals and hydrophobic contacts to the wall of the pore. The ammonium group is located

in the center of the channel and is stabilized by water-mediated hydrogen bonds to carbonyl groups of the channel. Moreover, experimental structures and MD simulations suggest that the ammonium group can adopt multiple binding modes [34]. The new S31N M2 inhibitors showed potent inhibitory effects in plaque formation assays when MDCK cells were infected with amantadine-/rimantadine-resistant influenza strains ($EC_{50} = 153$ nM for (**30**) versus 328 nM for amantadine (**24**) against a WT strain). These are the first examples that in principle also the S31N M2 mutant is a druggable target. However, so far, it is not known if one of these compounds has been further evaluated in animal models.

Various 2-amino-2-alkyl-adamantanes including the propyl and hexyl derivatives (**32**) and (**33**) have been recently described as inhibitors for virus propagation of some strains containing the S31N mutation in M2, but not for all of them [35]. Surprisingly, the authors could not find a significant blockade of the M2 channel when performing electrophysiological measurements in HEK cells expressing the full-length S31N M2 protein; therefore, a different target seems to be involved. A few mechanisms have been proposed including pH buffering of the endosomal or viral lumen, stabilization of HA against acid-induced conformational change and stabilization of the lipid–water interfaces, and subsequent protection of the envelope–endosomal membrane fusion. Moreover, the comparison of HA sequences of sensitive and insensitive strains suggested that these C-2 substituted adamantanes might block the HA-mediated virus binding to the host cell receptor.

A completely different approach to target M2 is the inhibition of its translation by the small-interfering RNA construct AVI-7100 (Serepta, Cambridge, MA). AVI-7100 inhibits also the translation of the matrix protein and is a phosphorodiamidate morpholino oligomer with enhanced resistance to enzymatic degradation [36]. Based on results from phase 1 studies reported in September 2014, AVI-7100 is well tolerated in human (see NCT01747148).

4 Alternative Strategies

4.1 Anti-adhesion Drugs

The initial step in virus replication is the HA-mediated binding of the virus to sialic acid (SA) containing glycoprotein/glycolipid receptors on the surface of host cells within the respiratory tract of infected persons. Blocking this step by anti-adhesion drugs should provide an antiviral effect. However, simple monomeric SA derivatives suffer from poor stability and weak HA-binding affinity in the millimolar range. Adapted from nature, where the virus enhances its affinity by parallel multiple binding of HA trimers to host cell receptors, various bivalent, tetravalent, und polyvalent SA conjugates have been developed. Among them, the dimeric

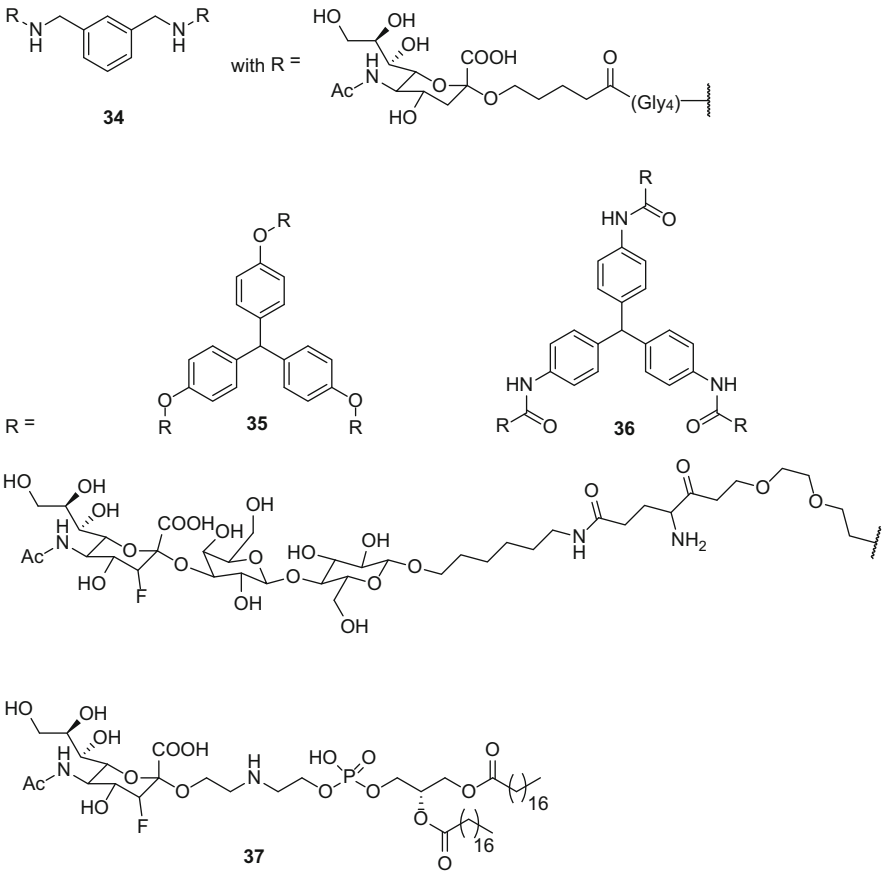


Fig. 10 Anti-adhesive SA analogues

compound (**34**) (Fig. 10) binds approximately 100-fold tighter to a whole virus compared to a monovalent ligand [37]. However, it was necessary to incorporate a spacer with a sufficient length of approximately 55 Å between both SA units to improve potency. In addition, SA was conjugated to various polyacrylamides [38, 39]. Such polymers inhibit the influenza-induced agglutination of red blood cells and have been used as aerosols, where they protect mice from H1N1 influenza strains [40]. However, the toxicity of polyacrylamide and acrylamide limits the application of such conjugates. Moreover, two trivalent SA conjugates based on a trityl core have been described, whereby the SA-containing arms have been coupled via a phenol ether (**35**) or amide bond (**36**) (Fig. 10). Interestingly, only the trisphenol derivative (**35**) showed an inhibition of H1N1 virus replication in MDCK cells ($IC_{50} = 400$ μM).

A different strategy for multivalent SA presentation could be the preparation of liposomes. For this purpose, the SA-distearoyl phosphatidylethanolamine

derivative (**37**) was developed, in which the C-3 position is modified with an axial fluorine atom. The liposomes were produced from distearoylphosphatidylcholine and cholesterol together with 10 mol% of the sialylphospholipid (**37**). A dual inhibitory mechanism was found for the obtained liposomes; besides blocking the HA and therefore the initial virus attachment, they also disturb the release of the progeny virus by sialidase inhibition [41]. In virus-infected MDCK cells, an IC_{50} value of 5.6 μM was determined based on the cytopathic effect. Additional examples of such SA-derived anti-adhesion drugs have been reviewed [3]. However, the synthesis of larger SA conjugates is a relatively laborious task, which might be a clear drawback compared to the straightforward preparation of small-molecule drugs. Furthermore, very often the activity of these SA conjugates strongly depends on the used influenza strains.

Besides such SA derivatives, various peptides have been identified as inhibitors for virus attachment. A screening of a set of cell-penetrating peptides provided a 20-mer entry blocker derived from the signal sequence of fibroblast growth factor 4 (RRKKAAVALLPAVLLALLAP), which inhibits virus replication of a H5N1 strain in cell culture with an IC_{50} value of 4.5 μM [42]. For several other strains, including various H1N1, H2N2, H3N2, H5N1, H5N9, H7N3, and influenza B, a potent inhibition of red blood cell agglutination was found. It was demonstrated that this peptide is targeting HA and blocks its binding to host cells. A series of HA-binding peptides was identified by phage display. The most suitable 15-mer peptides were resynthesized and modified by a fatty acid chain to induce micelle formation, which present multiple ligands on their surface. After several rounds of optimization, the length of the peptides was strongly reduced to a simple pentapeptide (stearoyl-ARLPR-amide), which inhibits the replication of H1N1 and H3N2 strains in cell culture with IC_{50} values of 1.9 and 1.6 μM, respectively [43].

An additional HA-binding compound is the 101-mer protein cyanovirin-N (CV-N), derived from the cyanobacterium *Nostoc ellipsosporum*. It was originally identified as inhibitor of HIV, but it was later discovered to inhibit other enveloped viruses such as influenza and Ebola [44]. It blocks virus attachment by binding to specific high-mannose oligosaccharides at glycosylation sites on the viral HA. Therefore, resistance against CV-N may be caused by modified glycosylation patterns of the individual strains. For instance, due to relatively low glycosylation of the HA from the pandemic H1N1 strain, it is only poorly inhibited by CV-N [45].

The virus attachment can also be inhibited by blocking the terminal sugar moieties on the host cell receptors. Similar as described above, Matsubara et al. have used a phage display library to identify a set of peptides with affinity to these SA residues. The best peptides were resynthesized and modified with a stearoyl residue to induce multivalent binding through micelle formation, where a critical micelle concentration (CMC) between 0.8 and 0.9 μM was found. The most suitable derivative stearoyl-GWWYKGRARPVSAVA-amide inhibits the replication of an A/H1N1 strain in cell culture with an IC_{50} value of 3.2 μM [46].

An alternative strategy to interfere with the virus attachment is the removal of the terminal SA residues on host cell receptors. For that purpose, a fusion protein, composed of a sialidase from *Actinomyces viscosus* and a cell surface-anchoring

protein derived from the heparin-binding sequence of the human protein amphiregulin, was developed and named as DAS181 [47]. Its heparin-binding domain improves the retention of the construct within the respiratory tract leading to an enhanced desialylation. DAS181 exhibits low nanomolar antiviral activity against various seasonal influenza A and B strains grown in MDCK cells, including the pandemic 2009 H1N1 virus or the highly pathogenic H5N1 strain, and was also effective against oseltamivir-resistant viruses [48–50]. These results were confirmed by in vivo studies in mice and ferrets. In clinical phase II studies, DAS181 was well tolerated and significantly reduced viral load in participants infected with influenza [51]. Moreover, DAS181 is presently investigated in phase II studies against parainfluenza infections [52].

4.2 Inhibitors of the Fusion and Uncoating Process

After binding to host cell receptors, the virus enters the cell through endocytosis. During maturation, the formed endosomes are acidified through the activity of a V-ATPase, a vacuolar proton pump that resides in the endosomal membrane [53]. This acidification triggers a conformational change of the HA trimers from a non-fusogenic to a fusogenic conformation. This rearrangement liberates a hydrophobic fusion peptide on the HA2 domain, which is buried before in the interface of the native HA trimer. The formation of a fusogenic HA conformation can be blocked by several strategies.

4.2.1 V-ATPase Inhibitors

One method is the inhibition of the V-ATPase to prevent a sufficient acidification, e.g., by macrolide antibiotics, such as concanamycin (**38**) or bafilomycin (**39**) (Fig. 11) [54]. It was demonstrated in a recent study that a combination of the V-ATPase inhibitor diphyllin (**40**) with oseltamivir effectively inhibits virus propagation in MDCK cells [55].

4.2.2 Inhibitors of HA-Activating Host Proteases

The conformational change of the HA and subsequent fusion depends on the correctly cleaved HA0 precursor into the domains HA1 and HA2, whereby HA2 contains the hydrophobic fusion peptide. Although the HA cleavage is an essential step in virus replication, the influenza genome does not encode for any protease. Therefore, host proteases or possibly proteases from concomitant bacterial infections are employed for HA processing, making them potential drug targets [56, 57].

Numerous studies revealed that the HAs of the subtypes H1, H2, and H3, which are found in the human seasonal influenza strains, are cleaved by trypsin-like serine

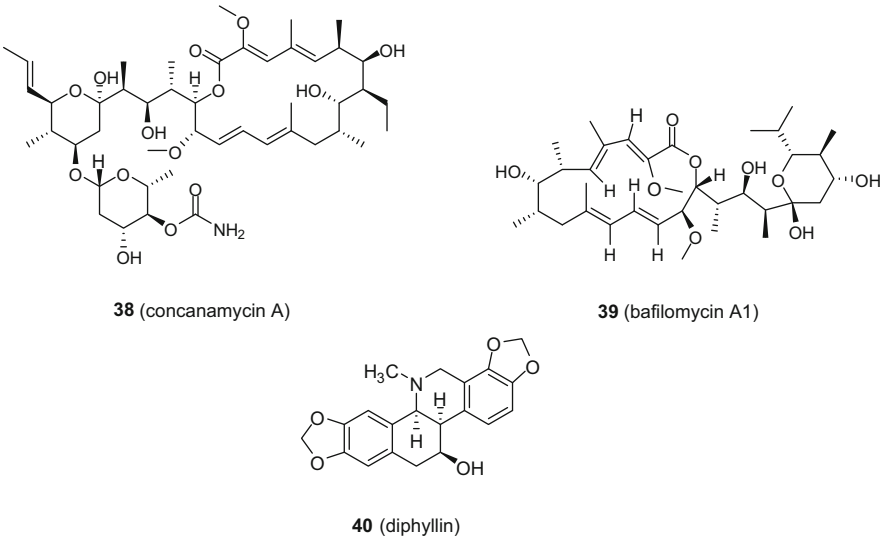


Fig. 11 Inhibitors of the vacuolar proton pump V-ATPase

proteases after a single arginine. In contrast, the HAs of the H5 and H7 HPAIV strains are processed at a multibasic sequence (R-X-R/K-R↓G) by ubiquitously expressed proprotein convertases, such as furin or PC5/6, supporting a systemic infection with often fatal outcome. However, not all H5 and H7 viruses are highly pathogenic and recognition of HA by furin or trypsin-like proteases, respectively, can be affected by the exact amino acid sequence, structure, and size of the cleavable loop connecting HA1 and HA2 as well as adjacent oligosaccharide side chains [58]. In principle, the human genome encodes for approximately 70 trypsin-like and 7 furin-like serine proteases, which cleave after a basic arginine. However, due to differences in their substrate specificity and expression pattern, only a few of them contribute to HA activation in vivo. For the H1, H2, and H3 subtypes with monobasic cleavage site, it is still not absolutely clear which proteases are the major players in the field. Meanwhile, it was shown that knockout mice that do not express the HA-activating type II transmembrane serine protease TMPRSS2 (also designated as epitheliasin) are resistant to pulmonary disease with lethal outcome, when infected with influenza A viruses of subtypes H1N1 or with the recently circulating H7N9, whereas they are not protected from lethal H3N2 infections [59, 60]. Therefore, TMPRSS2 is essential for activation of H1N1 and H7N9 virus, but different trypsin-like serine proteases have to be involved in the processing of the H3 and maybe also of other subtypes. Although the digestive protease trypsin cleaves all of these HAs in vitro, it is not present in the respiratory tract and cannot activate HA in vivo. During the last years, a few additional proteases have been identified by in vitro studies. Among the suggested human candidates are the kallikreins KLK-5 and KLK-12 [61] or the type II membrane-bound serine proteases HAT (human airway trypsin-like protease or TMPRSS11D) [62], TMPRSS4 [63], DESC1, and

MSPL (TMPRSS13) [64]. Despite controversy results, a few H1 subtypes might be also activated by matriptase in addition to TMPRSS2 [65, 66]. The contribution of these proteases to HA cleavage in human airways, however, remains to be investigated. Moreover, a number of soluble trypsin-like proteases purified from lung tissue of rat and swine, including tryptase Clara, mini-plasmin, and tryptase TC30, have been shown to activate influenza virus HA with a monobasic cleavage site in vitro [67]. So far, the HA cleavage by these soluble proteases was not confirmed in human.

The first host protease inhibition studies in mice for influenza treatment have been performed with an aerosol formulation of aprotinin, a 58-mer Kunitz-type serine protease inhibitor isolated from bovine lung [68]. Meanwhile, a small-particle aerosol formulation of aprotinin has been approved in Russia for local respiratory application in mild-to-moderate influenza [69]. The larger hepatocyte growth factor activator inhibitor 2 (HAI-2), which contains two Kunitz-type inhibitor domains, has been recently suggested as alternative to aprotinin and inhibited the virus propagation of H1 and H3 subtypes in mice [70].

Meanwhile, a few small-molecule protease inhibitors have been described, which inhibit the in vitro activity of TMPRSS2, HAT, and matriptase in the nanomolar range. For the substrate-analogue inhibitor (**41**), a nearly identical K_i value of 19 nM against TMPRSS2 and HAT has been determined [71, 72], whereas the 3-amidinophenylalanine derivative (**42**) is a more potent TMPRSS2 inhibitor ($K_i = 0.93$ nM) and has only poor affinity against HAT ($K_i = 1.7$ μM) (Fig. 12). However, both compounds possess similar potency as inhibitors of multicyle replication and HA cleavage of H1 and H3 strains, in which micromolar inhibitor concentration is required in these cell culture studies [72, 73]. Besides TMPRSS2 and HAT, also endogenous matriptase activates certain H9 HAs, which exhibit the di- and tribasic recognition motifs R-S-S-R↓G or R-S-R-R↓G. When MDCK-II cells, which are known to express significant levels of matriptase, were infected by these H9 influenza strains, the virus growth was suppressed by treatment with the matriptase inhibitor (**43**) ($K_i = 3.8$ nM) [66].

Moreover, the furin-catalyzed HA activation of H5 and H7 HPAIV strains can be effectively inhibited by the substrate-analogue inhibitor (**44**) (K_i for furin 16 pM) [74]. Its C-terminal 4-amidinobenzylamide residue (Fig. 12) fits perfectly into the deep S1 pocket of furin and therefore strongly contributes to the picomolar in vitro potency [75]. However, in all of these cell culture studies, micromolar inhibitor concentrations have been required, which are much higher than the determined inhibition constants with the purified enzyme. Most likely, the highly polar and charged inhibitor structures possess limited cell permeability and bioavailability, and therefore, only small amounts can reach their subcellular targets, which activate the HAs mainly within the secretory pathway. A first crystal structure of human furin in complex with a close analogue of inhibitor (**44**), containing the N-terminal guanidinomethyl substitution at the meta-position of the phenyl ring, was recently published (Fig. 13) [75].

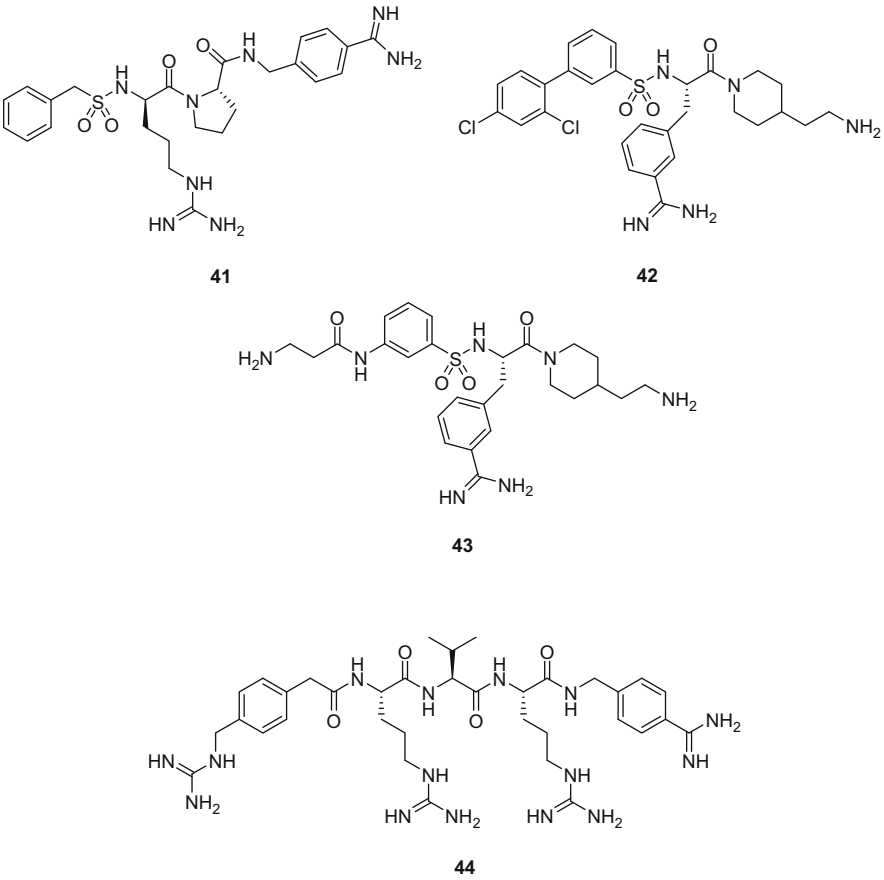


Fig. 12 Inhibitors of host proteases involved in HA activation. Compounds (**41**–**43**) are inhibitors of trypsin-like serine proteases, whereas derivative (**44**) is a highly potent furin inhibitor

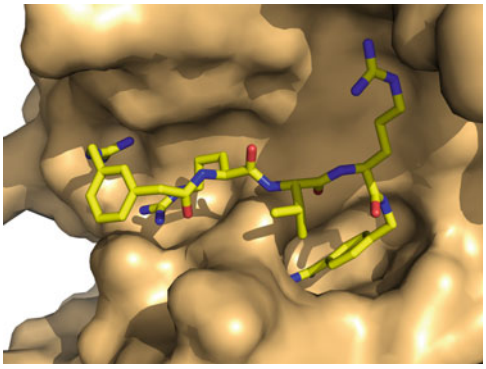


Fig. 13 Crystal structure of human furin (4omc.pdb) shown with its active site surface, in complex with inhibitor 3-guanidinomethyl-phenylacetyl-Arg-Val-Arg-4-amidinobenzylamide, a close analogue of inhibitor (**44**) [75]. The inhibitor is provided as stick model, whereas carbon, nitrogen, and oxygen atoms are shown in *yellow*, *blue*, and *red*, respectively

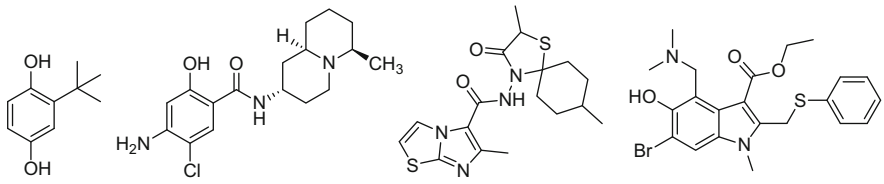


Fig. 14 Compounds, supposed to stabilize the non-fusogenic conformation of hemagglutinins

4.2.3 Stabilizers of the Non-fusogenic HA Conformation

A further approach to inhibit virus propagation is the stabilization of the non-fusogenic HA conformation by small HA-binding molecules [3]. Based on available crystal structures of HAs, a potential surface-exposed binding site in the vicinity of a fusion peptide of one monomer was defined that was located close to the interface of a second HA monomer. A computer-assisted docking approach provided the relatively simple hydroquinone structure (**45**), which should bind to this region (Fig. 14). Compound (**45**) inhibits H3N2 infectivity in MDCK-II cells with an IC_{50} value of 20 μM [76]. However, it should be noted that such hydroquinones are often susceptible to oxidation, whereas the formed benzoquinones can be also reduced back to the appropriate hydroquinones. This redox cycling activity is a limitation of this substance class for drug development. A specific binding to HA that represses the low-pH-induced conformational change of HA was also found for BMY-27709 (**46**). It inhibits H1 and H2 subtypes, but has poor activity on the tested H3 or influenza B strains [77, 78]. In contrast, the spiro compound (**47**) has significant affinity against the tested H3N2 strain, but is inactive against the used H1N1, H5N1, H7N2, and B subtypes [79]. The indole derivative arbidol (**48**) has broad activity against various influenza strains, as well as many other viruses. It is approved as an anti-influenza drug in Russia and China. Mutation and docking studies suggest that arbidol also binds to and stabilizes the non-fusogenic HA state [80, 81]. Moreover, it is described that arbidol stimulates a humoral immune response and induces interferon-production contributing to its antiviral effect.

The fusion step can be also blocked by neutralizing antibodies (nAbs) directed against the HAs, which can be phylogenetically divided into groups 1 and 2 [82]. Crystal structure analysis of a H5 in complex with an antibody possessing broad activity against all group 1 influenza viruses, including the H1N1 Spanish flu and H5N1 bird flu, revealed that the nAb binds with its heavy chain to a conserved stem region of HA. Thus, the nAb locks the fusion peptide in place and prevents membrane fusion [83]. Further development provided nAbs, which recognize all 16 group 1 and 2 HAs. Their passive transfer to mice and ferrets provided broad protection against the tested influenza strains. X-ray analysis showed that the antibody is targeting and stabilizing a conserved F subdomain of HA. In addition, it makes a kind of cross-linking between the fusion peptide and the C-terminal HA1 residues of a neighboring HA1 subunit, thereby preventing the conformational change [84].

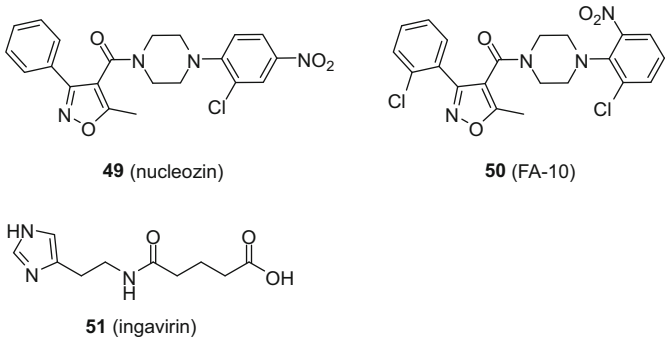


Fig. 15 Compounds supposed to target the viral NP

4.3 Inhibition of Nuclear Import and Export, Replication, Transcription, and Translation

The influenza genome consists of eight minus-stranded RNA segments. After transport of the RNP into the nucleus, two different mechanisms are required for viral propagation. Firstly, during transcription, the vRNA has to be translated into mRNA to enable the synthesis of new viral proteins, and secondly during replication, the generated full-length complementary RNA intermediate serves as a template for the production of new ssRNA copies for incorporation into progeny viruses. Both steps are catalyzed by the viral RNA-dependent RNA polymerase (RdRp). A complete influenza A polymerase structure in complex with a viral RNA promoter was very recently solved [4, 85]. For the beginning of the transcription, a 10–13-nucleotide-long 5′ cap RNA primer is required, which is excised from host pre-mRNA. This "cap snatching" is catalyzed by an intrinsic endonuclease activity of the RdRp. The binding of NP to the RdRp induces a switch from transcription to replication, which is independent from a primer [2, 86].

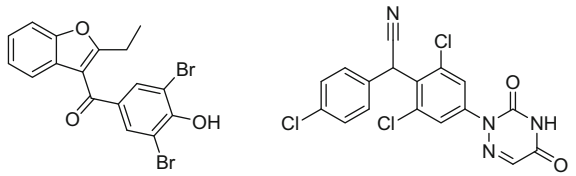
Two regions of influenza NP have been proposed as potential drug targets by interrupting protein/protein or protein/RNA interactions. X-ray analysis revealed that NP forms trimers, whereby the oligomerization is mediated by a flexible tail loop that is inserted into a pocket of a neighboring molecule. Single mutations in this loop prevented oligomerization and subsequent replication. The second target region at NP could be its RNA-binding groove, which contains a large number of basic residues [87]. Meanwhile, first NP-targeting compounds have been identified, although their exact mode of action is not yet completely clear. A screening of a library with approximately 50,000 compounds using an influenza infection assay in MDCK cells provided the piperazine derivative nucleozin (**49**), which inhibits the replication of H1N1 and H5N1 strains in cell culture with a nanomolar concentration ($EC_{50} = 0.07–0.16$ μM) and protects mice infected with lethal doses of H5N1. It is assumed that nucleozin (Fig. 15) induces the formation of larger NP complexes that aggregate with RNA. This blocks the essential transport of NP into the nucleus

and prevents replication [88]. However, nucleozin is inactive against NP containing a common Y298H mutation, which is found in most pandemic 2009 H1N1 strains. In contrast, the close analogue FA-10 (**50**) was active against this Y298H mutant of NP, although it possesses reduced efficacy ($EC_{50} = 5$ μM). Meanwhile, the imidazole derivative ingavirin (**51**) is an approved anti-influenza drug in Russia [89]. Although its mechanism of action is not absolutely clear, it was suggested that ingavirin also interacts with the influenza virus NP, thus preventing the NP oligomerization necessary for viral replication [90].

A promising influenza target is the heterotrimeric RdRp complex, which consists of the proteins PB1, PB2, and PA, whereby the polymerase activity is located on the central PB1 protein. Solved crystal structures revealed numerous hydrophobic contacts and hydrogen bonds between the N-terminal sequence of PB1 and the C-terminal segment of PA [4, 85, 91]. The residues contributing to these interactions are highly conserved among all influenza strains [2]; therefore, blocking this protein/protein interaction could be a strategy for influenza treatment. Indeed, transfection of cells with plasmids encoding for 25-mer peptides derived from the N-terminal PA-binding domain of PB1 fused to GFP blocks the polymerase activity and inhibits viral spread of influenza A virus, but was lacking activity against influenza B due to differences between their PA sequences [92]. Moreover, an inhibition of virus spread was observed, when infected cells were treated with this peptide coupled to a 13-amino-acid-long cell-penetrating TAT sequence. Cutting the size of the influenza A PB1 sequence to a 15-mer peptide and replacement of the original Thr in position 6 by Tyr from influenza B PB1 provided a first chimeric peptide MDVNPYLLFLKVPAQ, which effectively binds to the influenza A and B PAs with IC_{50} values of 22 and 107 nM, respectively [93]. Further optimization resulted in the identification of the related peptide MDYNPYLLFLKVKIF with improved binding properties, although no antiviral activity was demonstrated [94]. The destabilization of protein/protein interactions is nowadays an often used approach for drug design, whereby pure peptides have limited potential as intracellular drugs [95].

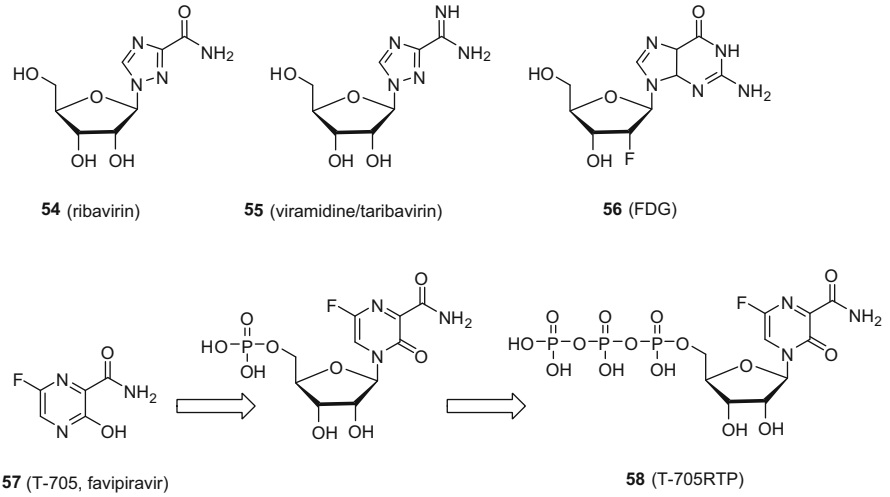
A docking approach was used to target the PA/PB1 interface by small molecules, whereby several compounds with anti-influenza A activity were identified, including the clinically used uricosuric agent benzbromarone (**52**) and the antiprotozoal drug diclazuril (**53**) (Fig. 16). For the binding of both compounds to PA, K_D values of 48 and 211 μM were determined using surface plasmon resonance measurements; moreover, in a plaque inhibition assay, EC_{50} values of 39 and 31 μM were observed, respectively. Both compounds also inhibit the polymerase activity in a replicon assay [96].

A main focus is also directed on the development of inhibitors against the enzymatic polymerase activity based on nucleoside and non-nucleoside derivatives. This strategy was successfully used for the design of antiviral drugs against many other viruses such as HIV, hepatitis C and herpesviruses. Already in 1972, the nucleoside ribavirin (**54**) has been described as a broad-spectrum antiviral drug (Fig. 17) [97]. Although it is mainly used for the treatment of hepatitis C infections, it is also approved as a flu drug in Mexico. Ribavirin can be orally applied or in



52 (benzbromarone) **53** (diclazuril)

Fig. 16 Compounds supposed to inhibit the RdRp by targeting the interface between PA and PB1

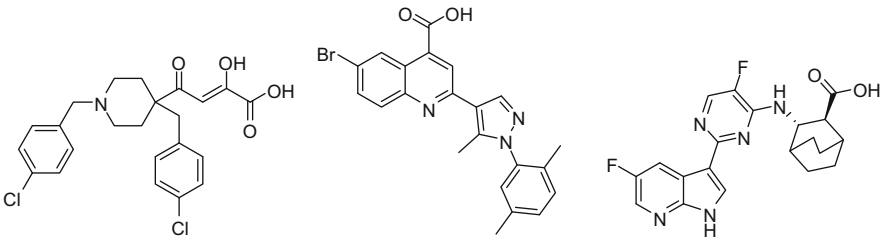


54 (ribavirin) **55** (viramidine/taribavirin) **56** (FDG)

57 (T-705, favipiravir) **58** (T-705RTP)

Fig. 17 Polymerase inhibitors

aerosol form for inhalation, but its application is limited because of many side effects including severe anemia. Ribavirin is a prodrug that is converted to the monophosphate and triphosphate analogues by host kinases. Various mechanisms have been described, which contribute to its antiviral efficacy, including inosine monophosphate dehydrogenase inhibition by the monophosphorylated form of ribavirin resulting in reduced GTP levels, immunomodulatory effects by enhancing host T-cell response, interference with RNA capping, induction of lethal point mutations, and polymerase inhibition [98]. A further development is the prodrug viramidine, also named as taribavirin (**55**), which is converted to ribavirin in the liver. Compared with ribavirin, it possesses similar activity as anti-influenza drug, but has less toxic side effects due to reduced penetration into red blood cells and a shorter half-life in the body [99]. The nucleoside 2′-deoxy-2′-fluoro-guanosine (**56**, FDG) has been described as an alternative derivative. It is intracellularly converted into the triphosphate (FDG-TP) by host kinases and its incorporation results in chain termination as well as subsequent blockage of further virus transcription [100]. FDG has broad activity against influenza A and B strains in mouse and ferret



59 **60** (BPR3P0128) **61** (VX-787)

Fig. 18 Inhibitors of the endonuclease activities of RdRp required for cap snatching

models. In vitro, the triphosphate of (**56**) inhibits the viral transcriptase activity with a K_i of 1 μM and possesses negligible activity against host polymerases.

The most advanced drug candidate with broad activity against numerous RNA viruses including influenza is the orally available pyrazinecarboxamide derivative favipiravir (**57**), which is converted by phosphoribosylation and subsequent phosphorylation to the active triphosphate (**58**) [101]. Competition experiments with purine and pyrimidine nucleosides and bases revealed that only the addition of purine derivatives reduced the antiviral activity suggesting that favipiravir acts as a pseudo purine base. Favipiravir is currently in a late-stage clinical development for influenza treatment; one phase III study has been completed in Japan and two phase II studies have been finished in the USA [102]. So far, very limited resistance to favipiravir (**57**) has been reported. Due to its broad activity against many other RNA viruses such as the negative-stranded arena- and bunyaviruses or positive-stranded noro- and flaviviruses, it was recently successfully tested against Ebola virus infections in a small animal model [103]. In contrast, favipiravir is inactive against DNA viruses.

The intrinsic endonuclease activity necessary for cap snatching from host pre-mRNA is located on PA, whereby the PB2 subunit contributes to the binding to the 5′ cap through its cap-binding domain [86, 104]. The depletion of host mRNA results in a downregulation of translation and defense mechanisms in the host cell. The first inhibitors of this cap-dependent endonuclease function were identified among a series of 4-substituted 2,4-dioxobutanoic acid derivatives such as compound (**59**) [105] (Fig. 18). Moreover, measurements of the cytopathic effects revealed a strong inhibition of influenza A and B replication in MDCK cells (IC_{50} values between 50 and 190 nM) by the quinolone derivative BPR3P0128 (**60**) [106]. Treatment with compound (**60**) reduced the levels of mRNA; further studies suggested that this compound inhibits the cap-dependent endoribonuclease (cap-snatching) activities, although the exact mechanism is still under debate. Very recently, a series of new azaindole-based inhibitors of the cap-snatching step has been described [107]. The most promising compound VX-787 (**61**) blocks the binding domain for 7-methyl GTP on PB2 ($K_d < 0.003$ μM), which was confirmed by X-ray structure analysis of PB2 in complex with a close analogue (4ncm.pdb).

The orally available VX-787 has a strong potency versus various influenza A strains, including pandemic 2009 H1N1 and H5N1 bird flu viruses, and is also active in an influenza mouse model. Moreover, a clinical phase 2a study was finished in 2013. It was reported that the treatment with VX-787 provided statistically significant improvements in viral and clinical measurements of influenza infection (http://www.vrtx.com/search/gss/VX-787).

The influenza genome encodes for additional proteins, which could serve as drug targets. One of these virulence factors is the dimeric NS1 protein of influenza, which reduces the interferon α/β-mediated antiviral answer of the infected host cell. Normally, interferon (IFN) induces the production of RNase L, which initially exists in an inactive form. In the presence of double-stranded RNA (dsRNA), it comes to a stimulation of a 2′-5′-oligo(A)synthetase and subsequent production of unusual 2′-5′-connected strands of adenine nucleotides. Binding of these strands leads to an activation of RNase L, which degrades intracellular RNA in an unspecific manner, thereby also inhibiting viral replication. NS1 blocks this mechanism by binding of its *N*-terminal RNA-binding domain (RBD) to dsRNA; in consequence, this prevents the IFN-induced degradation of viral RNA [2]. Mutation studies revealed that replacement of Arg38 in the N-terminal RBD by Ala blocks binding of the dimeric NS1 to dsRNA. This enables activation of RNase L and subsequent depletion of viral RNA. NMR and X-ray studies have been used to identify the RBD segments that bind to the dsRNA. Meanwhile, the first small molecules have been identified from screening of a library of the National Cancer Institute, which neutralize the function of NS1. Among them are the compounds NSC128164 (**62**) and NSC125044 (**63**) [108], whereby the latter analogue served as lead for the synthesis of the related derivative JJ3297 (**64**) [109, 110] (Fig. 19). Various experiments support a model, in which inhibition of NS1 function by JJ3297 results in a restoration of the IFN-induced antiviral state including inhibition of virus replication and spread. A newly developed fluorescence polarization-based high-throughput assay was used to identify several quinoxaline derivatives such as (**65**), which also target the NS1 binding to dsRNA. Moreover, a similar mechanism was described as reason for the antiviral activity of epigallocatechin gallate (EGCG, **66**), the most abundant catechin in tea [111, 112].

Furthermore, NS1 inhibits the 3′-end processing of cellular pre-mRNAs by binding two cellular host proteins, the 30-kDa subunit of CPSF (cleavage and polyadenylation specificity factor) and PABII (poly(A)-binding protein II) [113]. The binding to CPSF30 is mediated via a C-terminal effector domain of NS1, which recognizes its second and third zinc finger domains (F2F3) [114]. Binding to CPSF30 results in the inhibition of the production of functional mRNA during virus infection, including mRNA for intracellular proteins with antiviral activity. Blocking of this CPSF30 binding site on NS1, e.g., by constitutively expressed F2F3, leads to an inhibition of influenza A virus replication coupled with an increased production of IFN-β mRNA [2, 113]. Although the first small molecules targeting the CPSF30 binding site of NS1 have been proposed based on a virtual screening of a TCM (traditional Chinese medicine) database, no experimental proof for their NS1A affinity or antiviral activity has been provided [115].

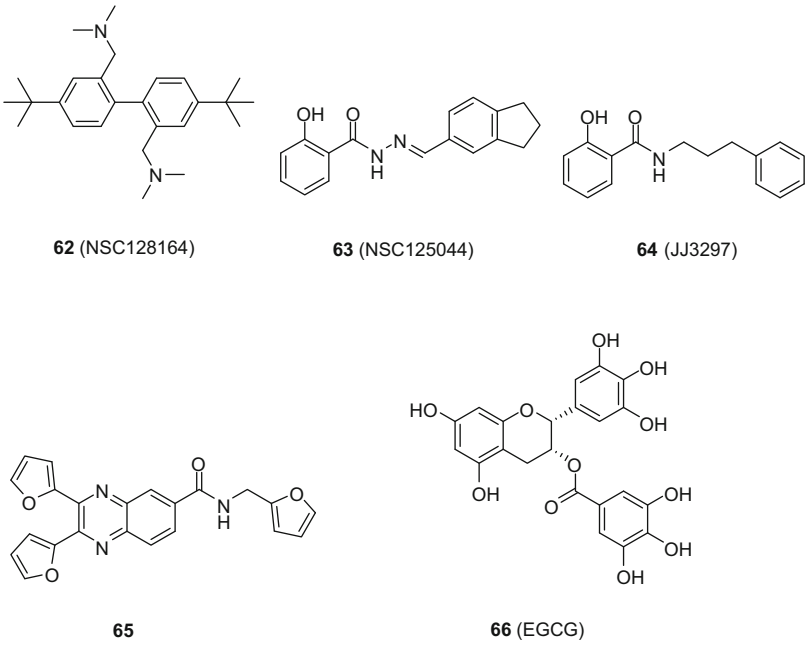
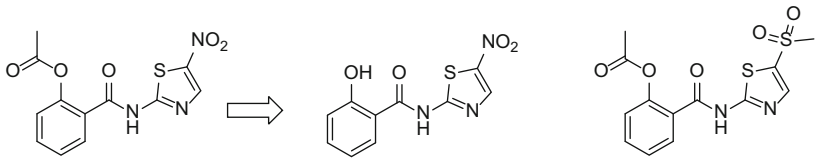


Fig. 19 Inhibitors of NS1A functions

The newly formed nucleocapsid, containing the RNA segments, the nucleoprotein, and the polymerase complex consisting of the PB1, PB2, and PA subunits, is translocated from the nucleus into the cytoplasm, where it is further transported to the cell membrane for assembly of progeny viruses. This transport into the cytoplasm occurs via the Crm1 (chromosome region maintenance 1; also referred to as exportin1 or Xpo1) pathway [116]. It is mediated by the viral protein NEP (previously also named as NS2), which directly binds to Crm1 and furthermore acts as a kind of adapter for the vRNPs through the matrix protein M1, which is believed to bind NP. Meanwhile, it is known that the N-terminal part of NEP mediates binding to Crm1, whereas its C-terminal domain interacts with M1/vRNP. The interruption of these protein/protein interactions could be a further strategy for blocking virus replication. Moreover, recent results suggest that NEP can also bind directly to the vRNA polymerase and thereby contributing to nuclear export of the vRNPs [86, 117].

An additional target could be the viral protein PB1-F2, which is encoded by an alternative (+1) reading frame of the PB1 gene segment. PB1-F2 has variable sizes with truncations at its N- or C-terminal side and is absent in influenza B strains [118]. Interestingly, the 2009 pandemic H1N1 virus contains only an 11-amino-acid C-terminal-truncated PB1-F2 segment, which is thought to be nonfunctional. This suggests that PB1-F2 is not essential for the fitness of the presently circulating H1N1 strains, and therefore, it is under debate if it could be a real target for anti-influenza drugs. In contrast, the PB1-F2 of the devastating 1918 Spanish flu H1N1



67 (nitazoxanide) **68** (tizoxanide) **69** (RM5014)

Fig. 20 Inhibitors of glycosylation and transport of HA to the cell membrane

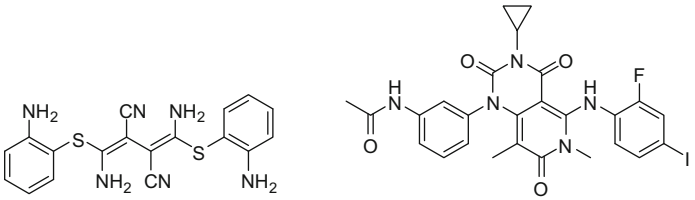
virus contained a complete PB1-F2 protein [119]. It has been reported that functional PB1-F2 can enter mitochondria of infected cells and thereby induce apoptosis, especially in immune cells. It might also contribute to the pathogenesis by dysregulation of cytokine levels, inducing inflammation and by supporting simultaneous secondary bacterial infections, including pneumonia [118]. Furthermore, it was demonstrated that PB1-F2 can directly bind to PB1 of the polymerase and alters their localization and activity [120]. So far, no antagonists or inhibitors of PB1-F2 functions have been described.

4.4 Inhibition of HA Glycosylation and Its Transport to the Cell Surface

In several countries, nitazoxanide (**67**) (Fig. 20) is approved as an antiprotozoal agent; however, it inhibits also several DNA and RNA viruses [121], including influenza A and B and viruses that are resistant against neuraminidase inhibitors. The prodrug nitazoxanide is rapidly hydrolyzed to its active form tizoxanide (**68**) after oral administration. A combination therapy with oseltamivir is presently in phase 3 clinical development [122]. It was shown that nitazoxanide blocks the maturation of HA at the posttranslational stage by interfering with its glycosylation and thereby blocking the proper transport of the HA from ER via Golgi to the cell surface. The close derivative (**69**) is approximately tenfold more active than tizoxanide (**68**) in an infection assay in MDCK cells ($EC_{50} = 0.3$ μM) [121].

5 Additional Strategies That Interfere with Host Mechanisms

The frequency of resistances against the approved inhibitors targeting the M2 channel and NA of the virus stimulated the search for alternative treatment strategies. It is known that multiple interactions between viral and host proteins contribute to virus replication [123]. On one side, influenza virus infections induce various intracellular signaling cascades to defend the cells against these pathogens, and in



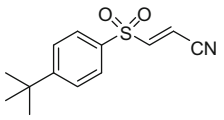
70 (U0126) **71** (trametinib)

Fig. 21 Structures of the MEK-inhibitors U0126 and trametinib

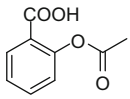
contrast, they also exploit cellular factors, which support virus replication [124]. In two genome-wide RNAi screens, nearly 300 host factors have been identified, which contribute to virus replication [125, 126]. Not all of them will be druggable targets, but in general, inhibitors of cellular components should be less susceptible to the rapid formation of escape mutants.

Mouse models revealed that the Raf/MEK/ERK mitogenic kinase cascade and the IKK/NF-κB module could be suitable antiviral targets [127, 128]. During influenza A and B infections, it comes to an activation of the Raf/MEK/ERK kinase cascade, which belongs to the so-called mitogen-activated protein kinase (MAPK) cascades. Activation of this cascade is essential for effective virus growth; its blocking leads to a nuclear retention of the vRNP complexes during the late-stage replication cycle, most likely due to interference with the viral export factor NEP. Successful proof of concept studies have been performed with the MEK inhibitor U0126 (**70**) (Fig. 21), which was applied as an aerosol to mice. Treatment with this inhibitor was well tolerated and significantly reduced virus titers [129]. Already approved MEK inhibitors with anticancer activity such as trametinib (**71**) or other already launched kinase inhibitors [130] could be alternative candidates as anti-influenza drugs.

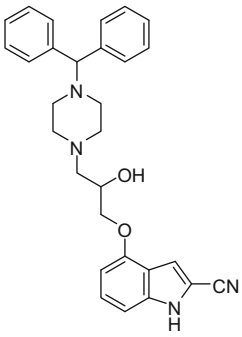
During influenza infections, it comes to an activation of the transcription factor NF-κB, which enhances the expression of various antiviral cytokines including IFN-β. Therefore, NF-κB and its activator kinase IκB (IKK) were considered as important factors of an innate immune response to virus infections [124]. Surprisingly, a stronger virus replication was found in cells with pre-activated NF-κB and reduced virus propagation was observed after treatment with inhibitors of NF-κB activation such as BAY11-7085 (**72**) (Fig. 22). In contrast, no effect was seen with LY294002 targeting the phosphatidylinositol 3-kinase, which is not involved in NF-κB activation [131]. Various mechanisms have been suggested to explain this unexpected virus-supportive function of NF-κB. Among others, induction of apoptosis and activation of caspases, especially caspase 3, were found to be crucial for efficient virus replication. Treatment of cells with caspase inhibitors or with siRNA against caspase 3 strongly reduced virus propagation. Interestingly, inhibition of caspase 3 caused retention of the vRNP complexes in the nucleus and consequently prevented the formation of progeny viruses. Additional potential mechanisms for



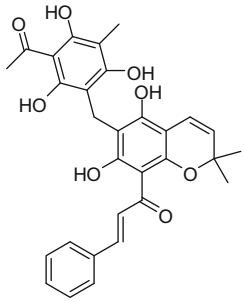
72 (BAY 11-7085)



73 (ASA)



74 (SDZ-201106)



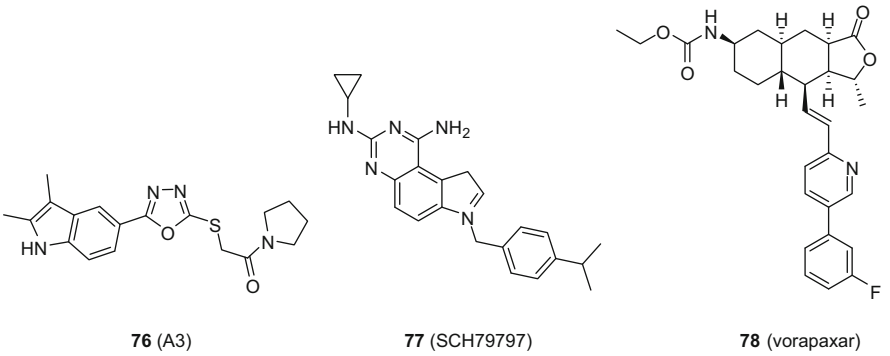
75 (rottlerin)

Fig. 22 Compounds targeting various host pathways by inhibition of NF-κB activation, opening of sodium channels, and PKC inhibition

the virus-supportive functions of the IKK/NF-κB pathway have been recently reviewed in detail [124, 132].

Interestingly, the NF-κB effects could be also inhibited by treatment with the well-known cyclooxygenase inhibitor ASA, also known as aspirin (**73**). Despite its well-known analgesic, antipyretic, and antiinflammatory activities, it is a relatively efficient inhibitor of IKK2; correspondingly, it inhibits the replication of various influenza strains in cell culture. Moreover, ASA treatment did not lead to resistant virus strains in multi-passaging experiments [133]. The efficacy of lysine acetylsalicylate glycine (LASAG), an easily water-soluble form of ASA, inhaled three times daily, is presently investigated in clinical phase II studies (http://www.activaero.de/en/clinical-pipeline/influenza-program/). During the last years, a tremendous number of IKK inhibitors has been developed, which could be also tested as potential anti-influenza drugs [134, 135].

Moreover, an inhibition of influenza replication in cell culture was also found for the sodium channel opener SDZ-201106 (**74**, $IC_{50} = 4.1$ μM) and for the potassium channel opener rottlerin, which also possesses PKC inhibitory activity (**75**, $IC_{50} = 0.46$ μM, Fig. 22) [136]. It was argued that increased intracellular Na^+ concentration caused by opening the sodium channels leads to a decline in influenza virus titers. However, it has to be considered that rottlerin is a typical promiscuous binder, often identified among screening hits from HTS campaigns. Such compounds have a strong tendency to form aggregates at micromolar concentrations in aqueous solution leading to a nonspecific inhibition of the tested targets and resulting in false-positive hits [137]. Despite these questionable results with rottlerin, it was recently demonstrated that PKC and other kinases such as



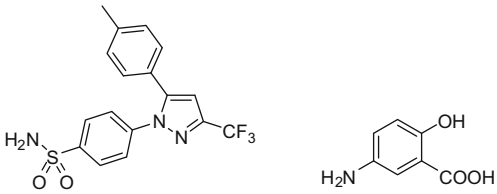
76 (A3) **77** (SCH79797) **78** (vorapaxar)

Fig. 23 Structures of compound A3 targeting the pyrimidine de novo biosynthesis pathway and of PAR-1 antagonists

Rho/Rock and p38MAPKK contribute to influenza-induced cytoskeletal changes and permeability increases in pulmonary microvascular endothelial cells via phosphorylating ezrin-radixin-moesin (ERM) family of membrane-cytoskeletal linker proteins. Therefore, it was suggested that inhibition of these phosphorylation pathways should block influenza virus replication [138]. These data confirm previous results where PKC inhibitors have been identified to reduce influenza virus replication [139].

A strong antiviral activity against various negative- and positive-sense RNA viruses, DNA viruses, and retroviruses was found for the oxadiazole derivative A3 (**76**), which was identified by an HTS of approximately 61,600 small molecules (Fig. 23) [140]. Further experiments revealed that A3 targets the pyrimidine de novo biosynthesis pathway most likely by addressing the dihydroorotate dehydrogenase (DHODH), thereby also inhibiting the synthesis of uridine monophosphate (UMP). UMP is the precursor for all pyrimidine nucleotides required for new RNA and DNA synthesis.

An additional target could be the protease-activated receptor 1 (PAR1), which was originally discovered as thrombin receptor and belongs to the family of G-protein-coupled receptors. Treatment with the PAR-1 antagonist SCH79797 (**77**) widely protected mice from infection with HPAIV H5N1 and pandemic H1N1 strains, whereby a PAR-1 agonist decreased survival and increased lung inflammation [141]. Among other functions, PAR-1 activation triggers various processes, including plasminogen activation and proinflammatory processes, which both might contribute to an enhanced virus propagation. It should be noted that vorapaxar (**78**), a synthetic derivative of the alkaloid himbacine, is the first PAR-1 antagonist, which was recently approved by the FDA for the treatment of acute coronary syndromes [142]. However, during the clinical development, increased brain bleedings have been observed, which suggests a narrow therapeutic window for this type of antiplatelet drugs. This could be a hurdle to use such compounds in other applications.



79 (celecoxib) **80** (mesalazine)

Fig. 24 Structures of the specific COX-2-inhibitor celecoxib and of the nonspecific COX-inhibitor mesalazine

Severe influenza infections induce strong immune responses and systemic inflammation; this can result in an uncontrolled cytokine storm with subsequent acute lung injury. This suggests that also treatments with immunomodulatory agents and cyclooxygenase inhibitors other than ASA, possessing anti-inflammatory activities, could be beneficial [143]. In studies with mice, which were treated 48-h post-H5N1 infection with a combination of the COX-2 selective inhibitor celecoxib (**79**) (Fig. 24), the nonselective COX-inhibitor mesalazine (**80**) and the NA-inhibitor zanamivir (**2**) demonstrated an increased survival to 53%, compared to 13% for mice treated with zanamivir alone [144].

Moreover, it was shown that antecedent influenza infections strongly increase the risk for death following subsequent pneumococcal pneumonia [145], and it is assumed that secondary bacterial pneumonia with subsequent lung injury was the primary cause for the mortality in the 1918 pandemic [146]. Interestingly, experimental and clinical evidence suggest that also statins, PPARα agonists (fibrates), and PPARγ agonists (glitazones), three classes of drugs that were developed for other applications, might individually or in combination prevent H5N1-associated acute lung injury [143, 147, 148].

6 Summary

In addition to the established influenza drugs against the viral NA and the M2 channel, which possess only very limited efficacy and are susceptible to resistance developments, many alternative treatment strategies have been investigated during the last decades. Several compounds against other viral targets are in clinical development. Due to the long and laborious way of a drug development, it is difficult to assess whether and when such new antivirals will be approved. The alternative targeting of host structures should circumvent the resistance problem. For instance, it might be possible that approved drugs, previously developed for other applications including kinase or COX inhibitors, could be beneficial for the treatment of acute influenza infections by addressing signaling pathways and inflammatory processes. However, despite many promising targets for the treatment of influenza infections, which in principle can be all addressed by a specific compound, a single-drug treatment most likely will not be sufficient for an efficient

therapy. Similar to the treatment of HIV or HCV, a combination of various drugs should provide synergistic effects and reduce resistances. Combinations of NA inhibitors with adamantanes, favipiravir, or the nucleoside analogue ribavirin have already been demonstrated to cause additive to synergistic inhibition of influenza virus infection in vitro and to enhance survival of infected mice more efficiently than either drug used alone, even in the case of amantadine- or oseltamivir-resistant viruses [149–152]. However, the clinical benefits of such combinatorial treatments have to be demonstrated in controlled trials in man; a number of studies are ongoing [36].

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Targeting Cellular Cofactors in HIV Therapy

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Abstract Besides viral proteins cellular factors play a key role in the replication of the human immunodeficiency virus HIV-1. The outcome of virus replication is determined by the balance between the activity of a number of cellular dependency factors and restriction factors. Whereas the first are essential cofactors for diverse steps in the viral replication cycle, the latter counteract virus replication by sensing particular viral components as non-self, often as mediators of the innate immune system. Cellular cofactors include receptors for HIV-1 entry, LEDGF as cofactor for the viral integrase, the RNA helicase DDX3 involved in the nuclear export of unspliced viral RNAs, and diverse cellular kinases that promote viral replication. Cellular restriction factors are often antagonized by HIV-1 accessory proteins in order to counteract their restrictive function on viral replication. Although cellular cofactors in the HIV field are understood as factors promoting viral replication, we add a subchapter on the most important restriction factors (Trim5α, APOBEC3G, SAMHD1, and tetherin/BST-2). Today highly active antiretroviral therapy (HAART) mostly targets HIV proteins like reverse transcriptase, protease, or integrase to specifically interfere with virus replication. However, the identification of cellular cofactors and the increasing knowledge on their mode of action at

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defined steps in the HIV-1 replication cycle have opened new avenues towards the development of HIV-1 inhibitors. Here we summarize the most important cellular factors involved in HIV-1 replication along with therapeutic approaches developed to target them, preferentially without harming their normal cellular function.

Keywords Antiviral therapy, APOBEC3G, CCR5, Cellular cofactors, DDX3, HIV-1, Kinases, LEDGF, Restriction factors, SAMHD1, Tetherin/BST-2, Trim5α

Contents

Abbreviations

β-TrCP	β-transducin repeat containing protein
APOBEC3G	Apolipoprotein B mRNA editing enzyme 3G
BRD4	BET bromodomain protein 4
BST-2	Bone stromal protein 2
CADA	Cyclotriazadisulfonamide
CCD	Catalytic core domain
CCR5	Chemokine receptor 5
CD4	Cluster of differentiation 4
CD4bs	CD4 binding site
CD4i	CD4 induced
CHR	C-heptad repeat
CRL	Cullin ring E3 ligases
CRM1	Chromosome region maintenance 1
CypA	Cyclophylin A
DCAF	Ddb1-and Cul4 associated factor
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DDX3	X-linked DEAD-box polypeptide 3
DFS	Dense fine speckles
ECL	Extracellular loop
Env	Envelope
HAART	Highly active antiretroviral therapy
HDACI	Histon deacetylase inhibitors

HDGF	Hepatoma-derived growth factor
HCV	Hepatitis C virus
HCK	Hematopoietic cell kinase
HIV	Human immunodeficiency virus
HRP	Hepatoma-derived growth factor-related proteins
IBD	Integrase binding domain
IFN	Interferon
IN	Integrase
JAK1	Janus kinase 1
LEDGF	Lens epithelium-derived growth factor
mAb	Monoclonal antibody
MLL	Mixed-lineage leukemia
MPER	Membrane proximal external region
Nef	Viral protein Nef (originally negative factor)
NES	Nuclear export signal
NHR	N-heptad repeat
NLS	Nuclear localization signal
NV	Norovirus
PAMP	Pathogen-associated molecular patterns
PIC	Preintegration complex
PKC	Protein kinase C
P-TEFb	Positive Transcription Elongation Factor b
PSIP1	PC4- and SFRS-interacting protein
RIG	RIG-like helicases
RRE	Rev responsive element
sCD4	Soluble CD4
SAMHD1	Sterile alpha motif (SAM) and Histidine/aspartate (HD) residues
SDR	Supercoiled DNA recognition domain
Talens	Transcription activator-like effector nucleases
TLR	Toll-like receptor
TNPO3	Transportin 3
Trim5 α	Tripartite motif 5α
Vif	Viral infectivity factor
VIRIP	Virus inhibitory peptide
Vpr	Viral protein R
Vpx	Viral protein X

1 Introduction

Viruses are obligate intracellular parasites and as such they exploit the metabolic mechanisms of their hosts in order to replicate. This extraordinary ability relies on specific interactions between the virus and key components of the cellular machinery. A number of genome-wide screenings based on siRNAs have been performed

to identify host cell factors involved in HIV-1 replication [1–3]. The outcome of these studies revealed about 2,400 protein-coding genes or close to 10% of all human genes to affect HIV-1 replication. Although there was only partial overlap between these studies, already known cofactors for HIV-1 replication like CD4, CXCR4, or JAK1 could be confirmed and interesting new candidates for potential cellular cofactors could be identified. A detailed meta-analysis of nine genome-wide screens identified that networks consistently targeted by HIV-1 are involved in RNA metabolism, transport between cellular compartments, the proteasome and the innate immune response [4]. In addition, systematic proteomic approaches have been used to identify protein complexes composed of HIV-1 proteins and cellular factors [5].

Many cellular proteins influence HIV-1 replication in a positive manner and these are understood sensu stricto as cellular cofactors in the HIV field. These proteins are being usurped by viral components to shift their activity in favor of enhanced viral replication. Consequently, knockdown of these genes results in reduced virus replication. Some replication-enhancing proteins are absolutely essential for virus replication. These so-called dependency factors include the cellular receptors for HIV-1 entry, CD4, and CCR5/CXCR4 (see Sect. 2.1) and the cofactor for integration, LEDGF (see Sect. 2.2). Several cellular proteins further promote virus replication in between virus entry and genome integration, i.e. during traveling of the incoming viral capsid into the nucleus. These proteins include the peptidyl-prolyl isomerase cyclophylin A (CypA) and the importin β family member transportin 3 (TNPO3), which are involved in the modulation of the stability of the incoming capsid [6, 7]. CypA binding to the viral capsid also allows escape from the cellular restriction factor TRIM5α (see Sect. 2.5) [8]. Another set of cellular factors is involved in postintegration latency, which is a major issue in terms of viral eradication or an HIV cure. Viral reactivation in these reservoirs occurs after treatment interruption and is the major barrier for virus eradication and the major reason for the need of lifelong continous antiviral treatment. Therefore cellular factors involved in postintegration latency are currently being explored therapeutically. The major idea here is to purge these viral reservoirs by inducing HIV expression in order to kill the cells by direct cytopathic effects of the virus or by the immune system, which would now be able to recognize and kill the virus-expressing cells. New antiviral strategies in this context include histone deacetylase inhibitors (HDACI), some of which are already licensed for the treatment of cutaneous T cell lymphoma, protein kinase C (PKC) inducers, and BET bromodomain 4 (BRD4) inhibitors. As the cellular factors involved in postintegration latency and their inhibitors have been nicely reviewed in a very recent publication, they will not be further addressed here [9]. We have concentrated in this review on those cellular cofactors, which are most advanced or promising as potential drug targets and we therefore also include cellular RNA helicases (see Sect. 2.3) and some kinases (Sect. 2.4), which promote HIV-1 replication, without being absolutely essential.

A minority of cellular factors, called restriction factors, instead counteract viral replication. These proteins often are part of intrinsic antiviral responses belonging

to the innate immune system, which recognizes certain pathogen-associated molecular patterns (PAMP) as foreign. The most important restriction factors for HIV-1 replication are APOBEC3G, SAMHD1, Trim5α, and tetherin/BST2 with the very recent addition of the MxB protein (see Sect. 2.5).

Understanding the complex network of host–pathogen interactions can provide new avenues for the treatment of viral infections. First, characterization of the interactome can identify the specific domains of viral factors that are essential for their interaction with cellular proteins, thus providing information on potential druggable sites. Second, the identification of cellular factors essential for viral replication provides entirely new targets for alternative approaches to treat viral infections. Targeting cellular cofactors of viral infection limits the occurrence of drug resistance, as cellular proteins are by far less prone to mutate than viral proteins. However, a clear *caveat* of this approach is the possibility of serious side effects due to the inhibition of normal cellular functions of the target protein. Some considerations can avoid or limit such adverse effects. First, the cellular target should be carefully selected to exploit a protein, which is absolutely essential for the virus, but not for the cell. An example for this is the chemokine receptor CCR5, which is essential for entry of primary HIV-1 strains into cells (see Sect. 2.1). However it is not essential for humans, as carriers of a homozygous 32 basepair deletion in the human *ccr5* gene leading to a premature stop codon and consequently the absence of the receptor on the cell surface are perfectly healthy [10, 11]. Second, inhibitors should be developed that target specific functions of the cellular protein in the HIV-1 life cycle or directly its viral interaction interface. Here, interesting small molecule inhibitors are being developed that target the interaction of the viral restriction factor APOBEC3G and the counteracting viral antagonist Vif or the interaction between the cellular factor LEDGF and the viral integrase (see Sects. 2.5, [12, 13] and 2.2, [14, 15]). Third, the timing and dosage of the drug has to be calibrated carefully in order to achieve maximal viral suppression with minimal cellular toxicity. Such strategies therefore require: (1) a detailed knowledge of the functions of the target protein in the cellular and viral life cycle; (2) the availability of selective inhibitors; (3) the availability of cellular and animal models to study the mechanism of action and the pharmacokinetic properties of the experimental compounds including their interaction with the antiviral drugs contained in the HAART regimens.

2 Cellular Cofactors in HIV-1 Replication

Due to its small genome size (<10 kb) and its complex life cycle, which includes decisions about latency versus active expression of the integrated proviral genomes, the crosstalk between virus and cell is particularly intense for the lentivirus HIV-1. For this purpose, besides the retroviral proteins Gag, Pol, and Env, HIV-1 encodes two regulatory proteins, Tat and Rev, and four accessory proteins, Vif, Vpu, Vpr, and Nef (Fig. 1). Each of these proteins interacts with cellular factors, either to

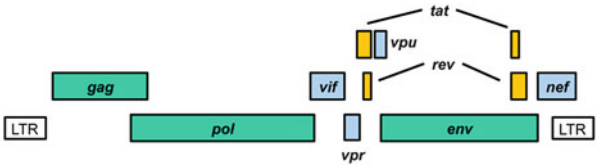

Fig. 1 HIV-1 genome. Besides the typical retroviral genes *gag*, *pol*, and *env* the HIV-1 genome contains two regulatory genes *tat* and *rev* as well as four accesory genes *vif*, *vpu*, *vpr*, and *nef*

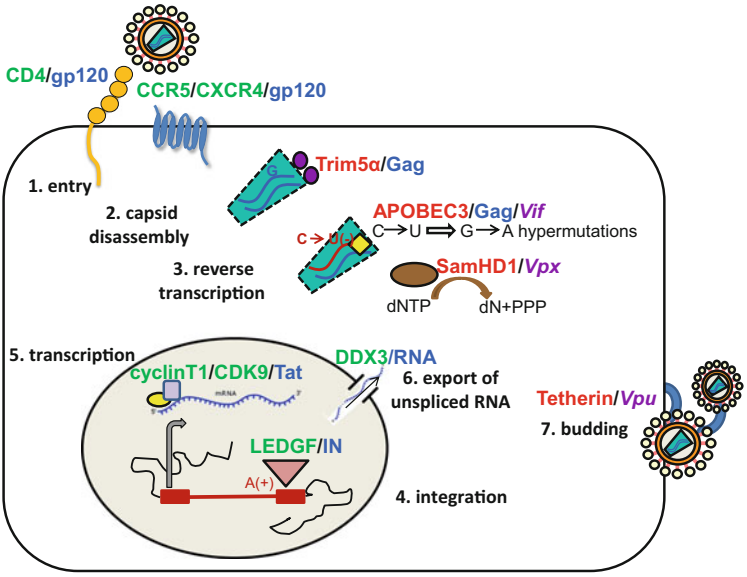

Fig. 2 Cellular proteins involved at different steps in the HIV-1 replication cycle. Cellular proteins acting positively on HIV-1 replication (including dependency factors) are shown in *green*, those acting negatively (restriction factors) are in *red*. The corresponding viral binding partners are indicated in *blue*. Viral proteins counteracting cellular restriction factors are in *purple*

promote/enhance HIV-1 replication or to antagonize cellular restriction factors that have evolved to counteract viral infections (Fig. 2). The species specificity of these cellular factors is one of the major reasons that HIV-1 replication is restricted to humans, as transmission between species requires adaptation to the respective cellular cofactors.

2.1 Cellular Cofactors for HIV Entry

The initial event in the HIV replication cycle is the entry of the virus into a target cell that depends on the interplay between the HIV envelope protein (Env) and different

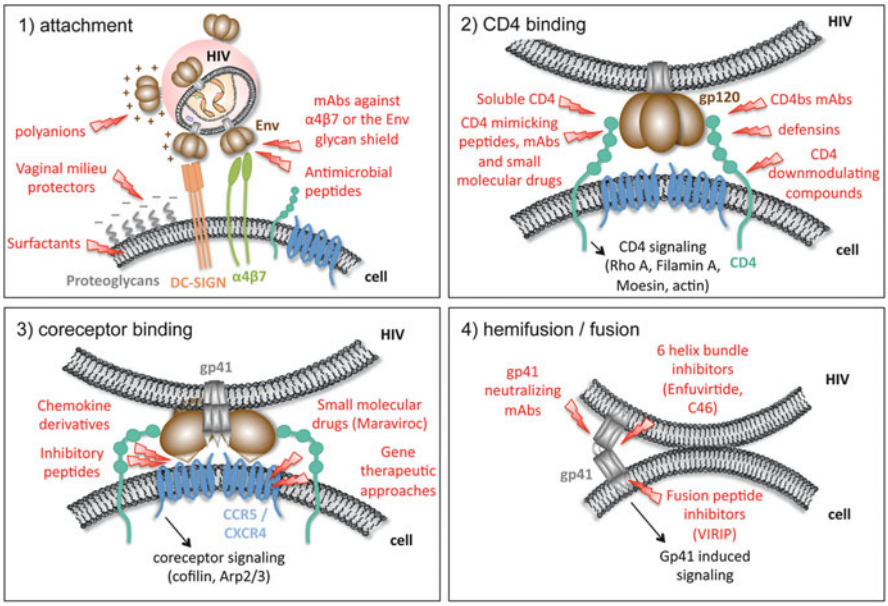

Fig. 3 HIV-1 entry and its inhibition. The important molecules mediating HIV-1 entry are indicated for the four entry steps (1–4) mentioned in the text. Inhibitors for the four entry steps are shown in *red* with the respective inhibition sites indicated by *red arrows*

cellular receptors. The multifaceted process of HIV entry requires sequential conformational changes of Env that are induced upon consecutive receptor interactions. Env originates from gp160 precursor glycoproteins that are cleaved into the surface glycoprotein gp120 and the transmembrane glycoprotein gp41 by cellular proteases. Both non-covalently linked proteins undergo extensive N-linked glycosylation and trimerization of the heterodimers on their way to functional surface expression [16, 17]. Although trimeric Env interacts with a broad array of cellular proteins, the essential receptors for primary HIV-1 infection in vivo are the CD4 receptor and the chemokine receptor CCR5 on hematopoietic cells, in particular on T4 helper cells. The entire HIV-1 entry event can be subdivided into four major steps: (1) viral attachment, (2) CD4 binding, (3) coreceptor binding, and (4) fusion (Fig. 3). Each step comprises the interaction of Env proteins with one or more cellular proteins or other components like the membrane or the extracellular matrix. These diverse interactions between viral and cellular components potentially enable the derivation of a broad spectrum of inhibitors targeting the individual entry phases. Despite the description of a number of compounds targeting these individual entry steps in vitro, only one coreceptor antagonist, *Maraviroc*, and one fusion inhibitor, *Enfuvirtide*, have been clinically approved so far (see reviews [18–20]). We review here the development of compounds targeting HIV-1 entry from attachment to fusion. The broad field of HIV neutralizing antibodies is only touched superficially, as these are covered by a number of excellent recent reviews [21–24].

2.1.1 Attachment Cofactors and Inhibitors

The initial contact of the virus with the cell is mediated via unspecific electrostatic interactions between positively charged residues on viral Env and negatively charged proteoglycans on the cell surface [25, 26]. Furthermore, HIV Env is attracted by lectins and integrins [27–29] that also bridge the distance to the obligatory HIV receptors, CD4 and CCR5, on a cell or allow the virus to travel to more distant anatomical sites in lymphatic tissues. Targeting the unspecific virus-cell interactions was attempted with the development of surfactants like Nonoxynol-9 (N9) or C31G (SAVVY) interfering with membrane integrity. Despite some efficiency in vitro and in animal studies [30, 31], clinical trials were stopped due to the lack of beneficial effects [32–35].

Polyanionic compounds were developed to shield cationic Env from the negatively charged surface of the cell. Several candidates like the cotton derivative cellulose sulfate Carraguard from red algae, bacterial Cyanovirin N and the polynaphtalene sulfonate PRO 2000 exhibited HIV inhibitory effects in vitro and ex vivo; however, they also failed in clinical trials [19, 36]. Interestingly, the only microbicidal formulation showing partial protection (39%) in a clinical study (CAPRISA 004) did not contain an entry inhibitor, but was based on a reverse transcriptase inhibitor (tenofovir, see Fig. A1) gel formulation. Based on these results, further tenofovir regimens including coadministration with the clinically tolerable polyanion Carraguard are currently under investigation [37, 38].

A more specific attachment of HIV to target cells is conferred via α4β7 integrin, a homing receptor for CD4 T cells to the gut-associated lymphatic tissues (GALT). HIV uses this interaction to travel from the primary infection sites (mostly genital mucosae) to the GALT, where it finds densely packed CD4 T cells to boost its replication via efficient cell to cell spread [27, 39]. Interestingly, the most promising human HIV-1 vaccine trial (RV144) conducted in Thailand and showing roughly 30% of protection from HIV acquisition [40] revealed that the observed protective effect could partially be explained by antibodies against the second variable loop V2 of Env, which is responsible for α4β7 binding [41].

Another important attachment factor for HIV as well as other viruses is the C-type lectin DC-SIGN expressed on dendritic cells. HIV hijacks the non-susceptible dendritic cells to shuttle as internalized DC-SIGN/HIV complexes to lymphatic tissues, where adjacent CD4 T cells can be *trans*-infected through virological synapses (for review see [42]).

2.1.2 The Primary HIV Receptor CD4 and CD4-gp120 Inhibitors

Binding of HIV Env to its primary receptor CD4 occurs via its highly conserved CD4 binding site (CD4bs), localized in a cavity between the inner and outer domain of gp120 [43]. Upon binding to CD4, gp120 undergoes large conformational rearrangements leading to the formation of the bridging sheet and the exposure of

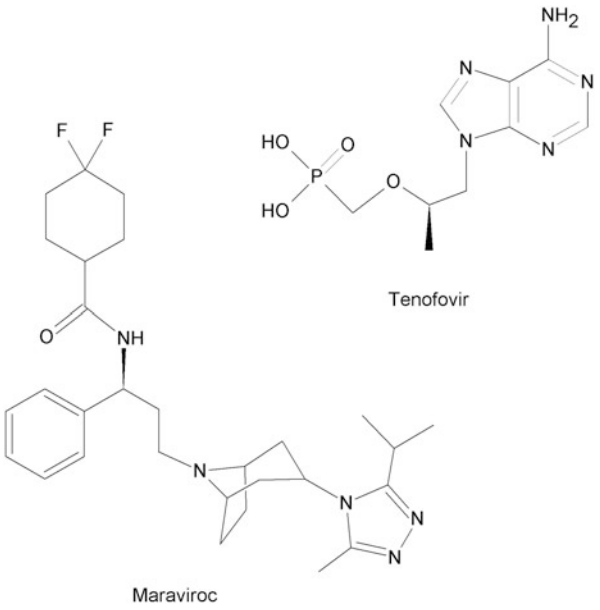

Fig. A1 Chemical structures of Maraviroc and Tenofovir

the CD4-induced epitopes (CD4i) required for the subsequent obligatory coreceptor interaction. CD4-gp120 interaction further promotes the exposure of the third variable loop V3, which is the principal determinant of coreceptor usage [44]. CD4 binding also induces signaling and cytoskeleton rearrangements leading to receptor clustering ([45], see also Sect. 2.4). Attempted strategies to target CD4 binding comprise soluble forms of CD4 (sCD4), CD4 mimetics, small molecular drugs against CD4 or the CD4bs on gp120, specific antibodies against CD4 or the CD4bs and CD4 downmodulators (for review see [46]). Interestingly, some mediators of innate immunity like defensins, small cationic peptides secreted from epithelial cells and neutrophils, also operate by interfering mainly with the gp120-CD4 interaction [47–49].

Early studies reported that high levels of sCD4 are able to block HIV infection in vitro [50]. Clinical trials only demonstrated a low response to sCD4 probably due to a lower sensitivity for sCD4 in primary HIV-1 isolates [51]. Multimeric CD4 constructs were developed to improve pharmacokinetic properties. A tetrameric fusion construct containing the Fc part of an IgG with the D1 and D2 domain of CD4 (PRO 542) was also effective against primary isolates and showed modest efficacy in clinical phase I and II trials [52]. However Fc receptor-mediated uptake led to rapid clearance from the circulation and nonlinear pharmacokinetics [53]. A CD4 mimicking peptide rationally designed based on structural information (CD4 M33) was able to both bind and inhibit different HIV-1 strains in the nanomolar range [54]. The peptide was further optimized via multimerization with efficient inhibition of HIV-1 subtypes B, A, and E, but limitations in neutralization of subtype C variants [55]. Further, small molecular drugs were derived to

compete for CD4 binding on gp120, however with limited efficacy, probably due to inadequate occupation of the relatively large interaction surface [43]. More promising results were obtained with neutralizing antibodies against the CD4bs. The first candidate, mAb b12, neutralized 40% of HIV-1 isolates and was able to protect rhesus macaques from HIV infection [56, 57]. Recently, several classes of much more potent broadly neutralizing antibodies against the CD4bs were identified [23], which were able to neutralize more than 90% of HIV-1 strains [58, 59]. On the other side, small molecular drugs [60] and monoclonal antibodies (Ibalizumab, [61]) against CD4 were developed with the CD4 antibody advancing to phase II clinical studies. A novel class of inhibitors is the group of CADA compounds that specifically decrease surface and intracellular CD4 levels. Promising results with inhibition of several primary and lab adapted HIV-1 and -2 subtypes, including drug-resistant strains on different cells, are still regarded with criticism due to the fact that a critical cellular receptor is downmodulated [62, 63].

2.1.3 HIV-1 Coreceptors CCR5 and CXCR4 and Inhibitors of Coreceptor–gp120 Interaction

Binding to coreceptors occurs between the HIV gp120 CD4i epitopes in the bridging sheet in concert with gp120 V3 and the coreceptor N-terminus as well as the extracellular loop (ECL) 2, respectively [64]. In vivo HIV-1 mainly interacts with the chemokine receptors CCR5 or CXCR4, V3 being the major determinant for coreceptor choice. Transmission and initial stages of HIV infection are dominated by CCR5 tropic (R5) viral strains, while CXCR4 tropic (X4) strains appear in about 50% of individuals at later stages of the disease and usually come along with a faster progression towards AIDS [65, 66]. Besides being the gatekeeper of HIV-1 entry, coreceptor signaling seems to play an important role in promoting further steps for productive infection [67, 68]. HIV coreceptor signaling in T lymphocytes includes activation of cofilin and Arp2/3 that locally loosen the cortical actin cytoskeleton and promote the release of the viral core into the cytoplasm and transport through the cortical actin [69] (see also Sect. 2.4).

Strategies to target the HIV-coreceptor interaction are promising, especially against the dispensable CCR5 receptor, while approaches against the physiologically important CXCR4 receptor are much more critical [18]. The identification of chemokine receptors as essential HIV receptors was based on the initial finding that certain chemokines provoke antiviral activity (for review see [70]). Of interest, the CCR5 and CXCR4 chemokines show structural homologies with V3 loops of R5 or X4 HIV gp120, respectively [71]. Based on that, CCL5 chemokine derivatives were developed, including AOP-RANTES, NNY-RANTES, and PSC-RANTES acting by receptor blockade and receptor downmodulation. Subnanomolar affinities in entry inhibition made these compounds promising leads for further microbicide applications [72, 73].

Inhibitors could also be derived from extracellular sequences of the chemokine receptors themselves. The importance of the N-terminal domain, especially from the

CCR5 receptor, for gp120 binding led to the derivation of synthetic peptides inhibiting HIV entry in the micromolar range [74–76]. We could select an entry inhibitory peptide (XD3) with remarkable homology to the N-terminus of CCR5 and CXCR4 by phage display using CD4i epitopes on native dualtropic Env as target [77]. For CXCR4, which is less dependent on its N-terminus for HIV entry, we further designed a peptide comprising ECL1 to 3 linked to short spacers, which showed selective CXCR4 binding and entry inhibition in the low micromolar range [78].

For an enhanced affinity and entry inhibition, monoclonal antibodies and small molecular drugs had to be developed. The antibody PRO 140 recognizing epitopes at the N-terminus and ECL2 of CCR5 advanced to clinical trials and achieved dose-dependent HIV RNA suppression over a prolonged time when administered weekly [79, 80]. Several low molecular weight drugs targeting CCR5 were clinically examined (TAK-779, TAK-220, Vicriviroc, Aplaviroc, Maraviroc), of which Maraviroc opened a new era in 2007, being the first FDA approved CCR5 antagonist and the first drug for HIV treatment directed against a cellular target (for review see [81, 82]). Maraviroc (Selzentry) is an allosteric modulator of CCR5 and used as a second-line drug in treatment-experienced or drug-resistant patients after an obligatory tropism test to exclude X4 variants.

The success of the first functional HIV cure in the "Berlin patient," an HIV-infected leukemia patient who received stem cell transplantation with cells from a CCR5Δ32 homozygous donor [11], directed many efforts towards gene therapeutic approaches against CCR5. Zinc finger nucleases and Talens are tools for an incomplete knockdown of CCR5 on CD4 T cells. In vitro, CCR5 gene modified CD4 cells were reported to be resistant to HIV-1 infection and were also safe after transplantation into humans in clinical studies. Critical points remain the incomplete CCR5 ablation coming along with the difficulty to achieve long-term protection as well as the possibility to induce a coreceptor switch to X4 variants [18, 83]. Interestingly, recent sporadic reports from HIV conferences also indicate control of HIV infection after bone marrow transplantations for the treatment of lymphomas, even if these are not derived from donors homozygous for the CCR5Δ32 deletion (Henrich and Kuritzkes, IAS Conference, Kuala Lumpur 2013).

CXCR4 is more difficult to target. Clinical tests of CXCR4 antagonists (AMD3100, AMD070) for the treatment of HIV were halted due to liver or cardiac toxicities besides their effect on stem cell mobilization. The latter fact led to the approval of AMD3100 at low doses for hematological purposes in cancer patients [84]. A new CXCR4 targeting small molecular drug GSK812397 as well as peptides derived from the innate immune defense from horseshoe crabs (T22, POL3026) inhibit X4 and dualtropic R5X4 strains in vitro in the nanomolar range and are promising tools against X4 variants [85–87].

2.1.4 Fusion Inhibitors

The fusion process is dominated by the transition of the gp41 protein from an unstable state fixed by gp120 to a liberated stable conformation with low entropy.

First, the gp41 N-terminal hydrophobic fusion peptide penetrates into the cellular membrane, thereby linking the virus with the cell (Fig. 3). The extracellular part of the trimeric gp41 forms a prebundle intermediate that is sensitive to N-helix and C-helix inhibitors, until the helices finally conglomerate to their highly stable 6-helix bundle made up of 3 N-heptad repeats (NHR) and 3 C-heptad repeats (CHR). This leads to the approximation of cellular and viral membranes allowing membrane mixing and fusion pore formation.

The fusion process allows to interfere with virus entry at different steps. Most advanced is the development of fusion inhibitors corresponding to viral peptides derived from the N- and C-terminal heptad repeats (for review see [88]). CHR-derived peptides show efficient antiviral activity in vitro and in vivo. In 2003, T20 (Enfuvirtide, Fuzeon) was the first clinically approved HIV-1 entry inhibitor. Due to the short half-life of the peptidic drug, T20 has to be administered twice daily and has to be given in combination with other HIV drugs due to rapid development of resistance. Second-generation fusion inhibitors are derived from the T20 peptide and extend N-terminally in order to additionally target a highly conserved gp41 pocket. Candidates like T1249, C34, Sifuvirtide, and C46 exhibited longer half-lives and were also effective against T20-resistant strains supporting their ongoing clinical development. For C46, efficient inhibition of viral entry as well as selection of cells expressing the membrane-bound peptide was observed [89–91]. Further peptidic and small molecule candidates are under development to directly target the critical hydrophobic gp41 pocket. The 20 aa peptide VIRIP (virus-inhibitory peptide) was identified as an effective fusion inhibitor of HIV by screening a human hemofiltrate for antiviral peptides [92]. The peptide corresponding to a fragment of the human protease inhibitor α1-antitrypsin acts by binding to the gp41 fusion peptide. The optimized peptide variant VIR-576 had promising results in phase I/II clinical studies, which were save and revealed a dose-dependent decrease in viral load [92, 93]. In a different approach the fusion peptide could be effectively inhibited in vitro by a variant of the natural fusion peptide containing four inserted D-amino acids [94].

Further, gp41 antibodies were described to mediate broad neutralization, most of them located in the membrane proximal external region (MPER). These antibodies represented by mAbs 2F5, 4E10, and Z13 are rarely found in patients due to their crossreactivity with autoantigens (for review see [95]). More recently, the antibodies m44, 10E8, and antibodies targeting the EC26-2A4 epitope combined broad neutralization capacity with lack of autoreactivity [96–98]. This may direct new strategies towards the development of more effective antibodies for prevention of HIV-1 entry.

2.2 LEDGF/p75, the Cellular Cofactor for Viral Integration

2.2.1 LEDGF and Its Implementation in Human Disease

Integration of the proviral DNA into the cellular chromatin is an essential step in HIV replication. Provirus establishment is needed for productive infection and for

archiving the HIVgenetic information in the host. Due to its limited genome, HIV needs to rely on cellular cofactors for provirus establishment and efficient replication in the host cell [99].

Lens epithelium-derived growth factor (LEDGF), a chromatin-associated cellular protein with multiple functions during embryogenesis, transcriptional regulation and cell survival, has been implicated in different human diseases such as cancer, autoimmunity, and HIV/AIDS (reviewed in [100]). Its key feature underlying these different roles is the ability of LEDGF to interact with other proteins, anchoring them to the cellular chromatin and therefore promoting their interaction with the cellular chromatin. This in turn leads to proviral integration (HIV [101]), transcriptional activation (leukemia [102]), cell survival, and DNA repair [103].

Research in five seemingly unrelated fields led to the independent identification of a protein migrating at the apparent weight of 75 kDa. Initially LEDGF/p75 and LEDGF/p52 were co-purified with the general transcriptional co-activator positive co-factor 4 (PC4) [104]. In 1999 it was reported that a cDNA clone, encoding for p75 and isolated from a mouse lens epithelium library, protects cells against oxidative stress [105], coining p75 ever since as LEDGF. Later screenings of a cDNA library with the nuclear autoantigen DFS70 again led to the identification of LEDGF/p75 implying a role of this chromatin-binding protein in autoimmunity [106, 107]. Coincidental co-immunoprecipitation of HIV-1 integrase (IN) from cells overexpressing a synthetic gene of HIV-1 IN identified LEDGF/p75 as the predominant binding partner of IN implementing a role in HIV replication [108]. Several years later co-immunoprecipitation from leukemic cell lines identified LEDGF/p75 as part of a triple complex with MLL and menin implementing yet another role: leukemic transformation and development of acute lymphoblastic leukemia [102]. LEDGF/p75 is a member of the hepatoma-derived growth factor family (HDGF), composed of chromatin-associated proteins sharing certain structural features.

2.2.2 Genetic Organization of LEDGF/p75

The PSIP1 gene (PC4- and SFRS-interacting protein 1) located on the human chromosome nine encodes for two variants, the p52 (333 amino acids) and the p75 protein (530 amino acids), produced through alternative splicing of the PSIP1 transcript [109] (Fig. 4). Both share their N-terminal regions containing the nuclear localization (NLS, aa 148–156) and chromatin-binding elements defined by the PWWP (Pro–Trp–Trp–Pro) domain [110–112], the A/T hook-like elements (aa 178–198) [113] and 3 of 4 charged regions (CR1-4) [114–116]. Recently it has been shown that CR2-4 are essential for binding supercoiled DNA and therefore this domain is referred to as supercoiled DNA recognition domain (SDR) [115]. Mutations in the NLS relocate newly expressed LEDGF/p75 to the cytoplasm [117]. The PWWP domain also defines LEDGF as a member of the hepatoma-derived growth factor (HDGF)-related protein (HRP) family [110]. Next to p52 and p75 five family members have been described so far, namely

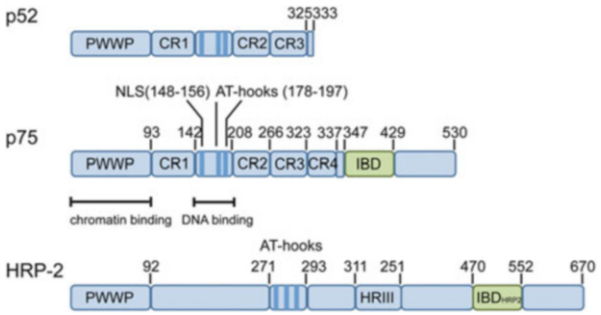

Fig. 4 Integrase interaction partners. Schematic drawing of LEDGF/p52, LEDGF/p75 and HRP-2, the HDGF family members discussed in this chapter. Protein–binding domains are highlighted in *green*. *CR* charged regions, *IBD* integrase binding domain

HDGF, hepatoma-derived growth factor-related proteins HRP-1, HRP-2, HRP-3, HRP-4 [111, 118]. Recently it was shown that in the absence of LEDGF/p75, HRP-2 (Fig. 4) can substitute for its function in HIV replication [119].

Whereas the N-terminal part of LEDGF contains the DNA tethering function, the C-terminal part is different and much extended in p75. It has multiple functions and can be envisioned as a molecular playground for protein–protein interactions. The larger splice variant exclusively contains the integrase binding domain (IBD, aa 347–429), essential for LEDGF/p75's cellular and virological functionalities. Although this domain was originally characterized by its association with HIV IN [120], later studies have shown that different cellular binding partners interact with LEDGF/p75 through this domain [102, 121–123]. LEDGF/p52 contains only eight unique aa at its C-terminus [104] and fails to interact with HIV IN [124, 125].

2.2.3 The Tertiary Structure of LEDGF/p75

The overall structure of LEDGF/p75 has not been determined so far; multiple flexible loops might hamper crystallization of full-length LEDGF/p75. Single domains though have been studied shedding light on their function in HIV replication and chromatin tethering. PWWP belongs to the Tudor (Royal) family of protein domains known to recognize methylated lysine including histones and specifically the H3K36me3 [126–128]. A direct interaction of the LEDGF-PWWP domain with H3K36me3 was demonstrated recently for the p52 splice variant [129, 130]. In addition it was shown that LEDGF/p75 interacts via the PWWP domain with methylated histones associated with active chromatin, but not with markers of inactive chromatin in accordance with the preferred integration sites of HIV [103]. Solution structures and more recently crystal structures of

PWWP domains evidence the presence of two C-terminal α-helices and a five-stranded antiparallel β-barrel responsible for the recognition of methylated histones [112, 131–133].

The structure of the LEDGF/p75-specific part in the C-terminus has been solved in two distinct co-crystal structures of the protein–protein interaction domain (IBD) in complex with HIV IN (PDB ID: 2B4J; [134]) or mixed-lineage leukemia (MLL) methyltransferase and menin (PDB ID: 3U88 [135]). The IBD structure is composed of a right-handed compact bundle of four α-helices [134], locating the amino acid residues contacting HIV IN (Lys364, Ile365, Asp366, Phe406, Val408) on the interhelical loop regions of the structure. In IN two regions of the catalytic core domain are in direct contact with LEDGF/p75: (1) the region around Trp131 and Trp132 and (2) the region stretching from Ile161 to Glu170 [134, 136, 137]. A pocket formed by the two subunits of the IN core dimer carries the interface, namely the α1 and α3 of one monomer and the α4/5 connector in the other monomer. Residues located in the α4/5 connector and a hydrophobic pocket formed by the other subunit engage tightly with the two inter-helical loops of LEDGF/p75–IBD [134]. Mutation of aa Ala128, His170, Thr174, Trp131, Trp132, Gln168, or Glu170 of HIV-1 IN renders the protein defective for LEDGF/p75 interaction [134, 136–138]. Vice versa substitution of aa residues Ile365, Asp366, Phe406, and Val408 decrease binding of LEDGF/p75 to IN [134]. The well-defined nature and small size of the LEDGF/p75-IN interface, consisting of multiple hydrophobic and hydrogen bond interactions, suggested that its disruption by small molecules might be a feasible endeavor. In a later study, the feasibility of this concept was indeed proven by designing and developing LEDGINs, first-in-class small molecule inhibitors binding to the LEDGF/p75 binding pocket of HIV-1 IN and potently blocking HIV replication [14, 139]. A detailed review of the drug development will be provided in Demeulemeester et al. [140].

2.2.4 LEDGF/p75 in HIV Replication

LEDGF/p75 is a hub protein interacting with multiple cellular proteins and HIV IN, tethering these to the chromatin and therefore promoting their biological function. The role of LEDGF/p75 in HIV replication has been studied intensively over the past 10 years and constitutes to date its best understood biological function. IN plays a central role in lentiviral replication as it (1) is responsible for processing the reverse-transcribed DNA of the virus, (2) travels as part of the preintegration complex (PIC) to the cellular nucleus where it (3) catalyzes the integration reaction establishing the provirus in a cellular chromosome. As mentioned above, co-immunoprecipitation of cellular proteins in cell lines expressing a synthetic gene of HIV-1 integrase originally led to the identification of the LEDGF/p75-IN interaction in 2003 [108]. Knockdown of LEDGF/p75 in cell lines expressing IN re-localized IN to the cytoplasm and resulted in its loss of chromosomal association

as demonstrated in mitotic cells, providing first proof that LEDGF/p75 indeed tethers IN to the chromatin and is essential for the karyophilic properties of IN [124]. Additional studies revealed that dependence on LEDGF/p75 is specific for lentiviral but not for other retroviral IN [141–143]. Initial RNAi-based studies led to controversial results concerning the role of LEDGF/p75 in HIV replication [114, 144–147], most likely due to insufficient gene silencing in some studies. However, significant reduction of LEDGF/p75 levels in mouse and human knockout cell lines unambiguously confirmed the important role of p75 in productive HIV-replication [99, 119, 125]. Furthermore, knockdown of LEDGF/p75 disfavored the preferred integration of HIV in active genes [144] and expression of an LEDGF/p75 variant with a replacement of the N-terminal chromatin-binding domain with other chromatin-interacting domains resulted in retargeting of integration sites underlining the chromatin-targeting role of LEDGF/p75 [148, 149]. In vitro studies suggest that LEDGF/p75 not only determines integration sites but also increases the affinity of IN for DNA. The stimulation was outcompeted by LEDGF/p75 lacking the N-terminus but not by an LEDGF/p75D366N mutation, which fails to interact with IN [150]. Furthermore LEDGF/p75 acts as an allosteric stimulator of IN catalytic activity and stimulates integration in chromatinized DNA [108, 120, 150–153]. In accord with these findings small molecules binding to the LEDGF/p75-binding sites and inhibiting LEDGF/p75 interaction act as well as allosteric inhibitors of IN catalytic activity (LEDGINs, [14, 139, 154–156]). Ectopic expression of an LEDGF/p75 deletion mutant lacking the PWWP domain, but containing the IBD, in cell lines depleted of endogenous LEDGF/p75, potently restricts HIV replication to nearly undetectable levels [157]. Furthermore, by repeated passaging of HIV in cells overexpressing the LEDGF/p75 deletion mutant a virus strain could be selected, which was resistant to this phenotype. Interestingly, only two mutations in IN were sufficient to render the virus resistant: A128T and E170G [158]. As seen in the co-crystal structure of IBD in complex with the IN CCD [134] both amino acids are crucial for the LEDGF/p75-IN interaction. The ultimate proof that LEDGF/p75 is pivotal for provirus formation and therefore viral persistence was gained by the development of small molecule inhibitors inhibiting this interaction, coined LEDGINs (for a review on the different classes of LEDGINs see [154]). The best studied class of these inhibitors [14] demonstrates a multimodal mechanism of action, inhibiting the IN-LEDGF/p75 interaction and allosterically the catalytic activities of IN [14, 139, 155, 156]. Most interestingly, next to their function in early steps of the replication cycle prior to integration, this novel class of inhibitors also affects the infectivity of progeny virus produced in their presence [139]. We also observed this late effect for small cyclic peptides inhibiting HIV-1 replication through binding to IBD and therefore blocking LEDGF/p75 interaction with IN [15].

After a decade of intensive research on LEDGF/p75 in HIV replication multiple research groups have studied the pivotal role of this unique cofactor of HIV-1 integration leading to the ultimate cofactor validation: the targeting of the LEDGF/p75-IN interaction by a small molecule. LEDGINs are now in advanced preclinical drug development.

2.3 The Cellular RNA Helicase DDX3 as a Novel Target for HIV-1 Chemotherapy

2.3.1 The RNA Helicase/ATPase DDX3

Recent studies have revealed that the cellular ATPase/RNA helicase X-linked DEAD-box polypeptide 3 (DDX3) is an essential host factor for the replication of different viruses. Particularly, its functions are known to be exploited by at least four viruses belonging to different families: Human Immunodeficiency Virus 1 (HIV-1, Retroviridae), Hepatitis C virus (HCV, Flaviviridae) [159], Vaccinia virus (VACV, Poxviridae), and Norovirus (NV, Caliciviridae) [160].

DDX3 is an ATPase/RNA helicase containing all nine conserved motifs that characterize the members of the RNA helicase superfamily [161]. Also, DDX3 contains a nuclear export signal (NES) at its N-terminus. The crystal structure of DDX3 shows that these conserved motifs are found in two subdomains connected via a short flexible linker. The amino-terminal domain 1 contains the ATP binding motifs Q, I (Walker A), and II (Walker B), and the RNA-binding motifs Ia, Ib, and the motif III, whereas the RNA-binding motifs IV, V, and motif VI, which may coordinate ATPase and unwinding activities, are found in the carboxy-terminal domain 2. Sequence alignment of all different human DExD-box helicases shows a DDX3-specific insertion of ten residues (250–259, E-A-L-R-A-M-K-E-N-G) between motifs I and Ia forming a positively charged loop in close proximity to the putative RNA ligand. A biochemical characterization of this motif in DDX3 showed that it is important for RNA binding and helicase activity and that it could potentially be a target for small molecule inhibitors [162].

DDX3 has been postulated to be involved in many cellular metabolic pathways, even though its precise functions are still unclear in most cases [163].

For example, recent evidence suggests that DDX3 is involved in mRNA nuclear export in association with two other shuttle proteins CRM1 and TAP. The proposed mechanism is that DDX3 binds both, mRNAs and TAP, in the nucleus and subsequently helps to facilitate mRNPs export to the cytoplasm. The interaction with CRM1 seems to be important only for the export of unspliced or incompletely spliced RNAs of HIV-1 (see below). DDX3 also interacts with translation initiation factors eIF4E, eIF4A, eIF4G, PABP, and eIF3. While conflicting results have proposed both a negative and a positive effect of DDX3 on translational initiation, recently a role for DDX3 in enhancing translation of a specific subset of cellular and viral mRNAs carrying specific structural features within their 5′-UTRs has been postulated [164]. These RNA structures must be located immediately adjacent to the cap structure to be unwound by DDX3 in order to prepare the mRNA for ribosome binding.

A role in transcription and cell cycle regulation has been proposed for DDX3, since it also downregulates E-Cadherin, and stimulates interferon (IFN)-β and p21 expression by interacting with their respective promoters. The DDX3 effect on the IFN-β promoter is independent of its ATPase activity or unwinding function, while

the ATPase, but not unwinding activity, is required for p21 promoter stimulation. Knockdown of DDX3, along with overexpression of the oncogene v-Ras, led to premature S-phase entry and enhanced the cellular transformation phenotype of murine fibroblast NIH3T3 cells [165]. The same group found DDX3 expression to be downregulated in hepatocellular carcinoma cells (HCC) derived from hepatitis B virus (HBV), but not hepatitis C virus (HCV)-infected patients. Another study found elevated DDX3 levels to correlate with a more aggressive phenotype of breast cancer cell lines [166]. Thus, whether DDX3 acts as an oncogene or a tumor suppressor is still debated. However, in several independent studies RNAi-mediated inhibition of DDX3 expression in HeLa, HEK293, and PBMC cells consistently failed to reveal any deleterious effect on cell proliferation or viability [161]. These results suggest that DDX3 functions in cell proliferation are either not essential, or they take place only in the context of altered cell metabolism, such as tumor transformation or viral infections.

The innate immune system is responsible for early detection of viral infection by sensing cytoplasmic viral nucleic acids through the endosomal subset of Toll-like receptors (TLRs) and the RIG-like helicases (RLHs). Both receptor systems lead to activation of the transcription factors NF-κB, IRF3, and IRF7, thus stimulating production of antiviral type I interferons. DDX3 has been shown to interact with the protein kinases IKKε and TBK-1, key kinases that phosphorylate and activate IRF3 and IRF7. Interestingly, this function of DDX3 is ATPase-independent. In addition, DDX3 has been found to interact with IPS-1, the adaptor protein that facilitates RLH signaling and to bind directly to the IFN-β promoter following its phosphorylation by TBK1. Thus, while there is strong evidence that human DDX3 contributes to IFN-β induction, its exact placement in the signaling pathways and mechanism of action are unclear. Recent work [167] suggests that DDX3 directly enhances IKKε activation acting as a downstream scaffolding adaptor that mediates the coordinated activation of IKKε and IRF3.

2.3.2 Role of DDX3 in HIV-1 Infection

HIV-1 replication requires the nuclear export and translation of unspliced, singly spliced and multiply spliced derivatives of the full-length RNA transcript. Fully spliced mRNAs encode the viral regulatory proteins Tat, Rev, and Nef (see Fig. 1). Rev is a sequence-specific nuclear mRNA-export factor, which binds to a highly complex RNA structure in long HIV-1 RNAs, the Rev response element (RRE), to mediate nuclear export of the Rev/RNA complex, through interaction with the cellular export receptor CRM1 and the cellular cofactor DDX3 (Fig. 5). DDX3 expression was found to be induced in HIV-1-infected cells by the viral transcriptional activator Tat and DDX3 silencing abrogated the export of unspliced/partially spliced HIV-1 transcripts [168]. However, the molecular details of DDX3 role(s) in this pathway are yet to be fully elucidated. For example, DDX3 was not required for the CRM1-dependent export of cellular endogeneous transcripts, raising the intriguing possibility that this function of DDX3 might be specific for HIV-1 RNAs

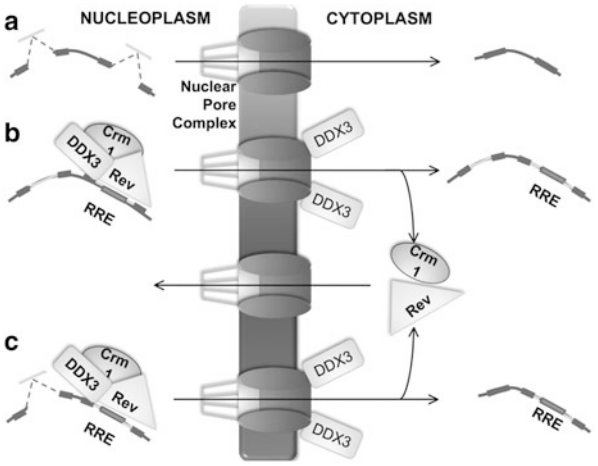

Fig. 5 Role of DDX3 in the nuclear export of unspliced or singly spliced viral RNA transcripts. (**a**) Upon LTR-mediated transcription of proviral DNA, fully spliced mRNAs are generated for the expression of the proteins Tat and Rev. Upon reentering the nucleus, Rev binds the RRE sequence at the 3′-end of unspliced (**b**) or singly spliced (**c**) viral mRNAs. Rev binding recruits the exportin CRM1 and the helicase DDX3, which together cooperatively promote the nuclear export of viral mRNAs. Rev and CRM1 are then re-imported into the nucleus to initiate a new round of nuclear exporting

and thus may represent an attractive drug target to complement the anti-HIV drug arsenal [168].

DDX3 as a Validated Target for HIV-1 Therapy

The recently unveiled roles of DDX3 in HIV-1 replication made this protein an attractive target for the development of an alternative anti-HIV-1 strategy. In particular, the ATPase and helicase activities of DDX3 seemed to be dispensable for most of the cellular functions of DDX3, but essential for its role in HIV-1 replication. Thus, we applied rational drug design to identify specific inhibitors of these activities. As no specific inhibitors of DEAD-box proteins were known, in order to design specific ligands, chemical feature-based pharmacophoric models were generated based on two different crystallographic structures for the human DDX3 helicase (PDB ID 2JGN and 2I4I). Since the 2JGN structure lacks the ATP binding, the 2I4I protein was selected as a starting point for virtual screening protocols, even if a few residues were missing (namely residues 407–410, 535–536, and 581–582). The LigandScout software has been used for the detection and interpretation of the crucial interaction pattern between the DDX3 protein and its cocrystallized ligand (AMP): the chemical features responsible for AMP–enzyme interactions were thus codified into a structure-based pharmacophoric model. This model was used as a three-dimensional query in a virtual screening approach to filter

databases of commercially available compounds from Asinex Gold and Synergy collections (about 250,000 compounds) to identify chemical scaffolds with putative affinity towards the DDX3 ATP binding site. The molecules selected via the in silico screening approach were submitted to geometry optimizations and were then docked into the DDX3 ATP binding site, to analyze their ability to have profitable interactions with the enzyme. Analysis of docking results, on the basis of the consensus scoring, and by visual inspection of the docked poses of each compound, led to select ten compounds, which were submitted to biological tests. Among these, one rhodanine and one pyrazolo derivative were found to bear antienzymatic activity. Starting from these two hits, subsequent hit-to-lead optimization led to the synthesis of nanomolar inhibitors of the ATPase activity of DDX3, which were also able to suppress HIV-1 replication in infected cells at low micromolar concentrations. Toxicity towards different cell lines (HeLa, Molt-4, PBMCs) was 10–50-fold higher than the anitiviral effective concentrations, demonstrating the possibility to target DDX3 in order to suppress HIV-1 replication [169].

A parallel approach was followed to design specific inhibitors of the helicase activity, targeting the RNA-binding site [170]. Since the 3D structure of the hDDX3 in the closed (catalytically active) conformation is unavailable, a model was built by homology modeling for structure-based drug-discovery approach: each individual domain of hDDX3 crystallized with AMP in an open conformation (PDB ID: 2I4I) was aligned with the corresponding domains of the closed conformation of the DEAD-box helicase eIF4AIII (PDB ID: 2J0S), and the resulting structure was subjected to energy minimization. A high-throughput docking approach was then applied to the RNA-binding site of the hDDX3 model in order to identify high affinity hits within the Asinex database collection. Analysis of docking results performed on the basis of the consensus scoring, followed by a visual inspection of the docked poses of best molecules, led us to select 10 compounds (out of the original 220,000 entries of the Asinex database), which were purchased and then submitted to in vitro biological tests. Three compounds were identified, which were able to inhibit the helicase activity with 20–100-fold higher selectivity than the ATPase activity. The two most potent compounds were also able to suppress HIV-1 replication in infected cells at low micromolar concentrations, with cytotoxicity 10–15-fold higher than the antiviral effective doses [169]. Overall, these results provide proof-of-principle for the feasibility to target DDX3 in the context of HIV-1 infection and promising initial results.

2.4 Cellular Protein Kinases as Possible Targets for HIV-1 Therapy

The human kinome (the whole complement of protein kinases in the human cell) comprises more than 500 members, testifying the high complexity of protein phopshorylation pathways [171]. Consequently, protein kinases do not only modulate

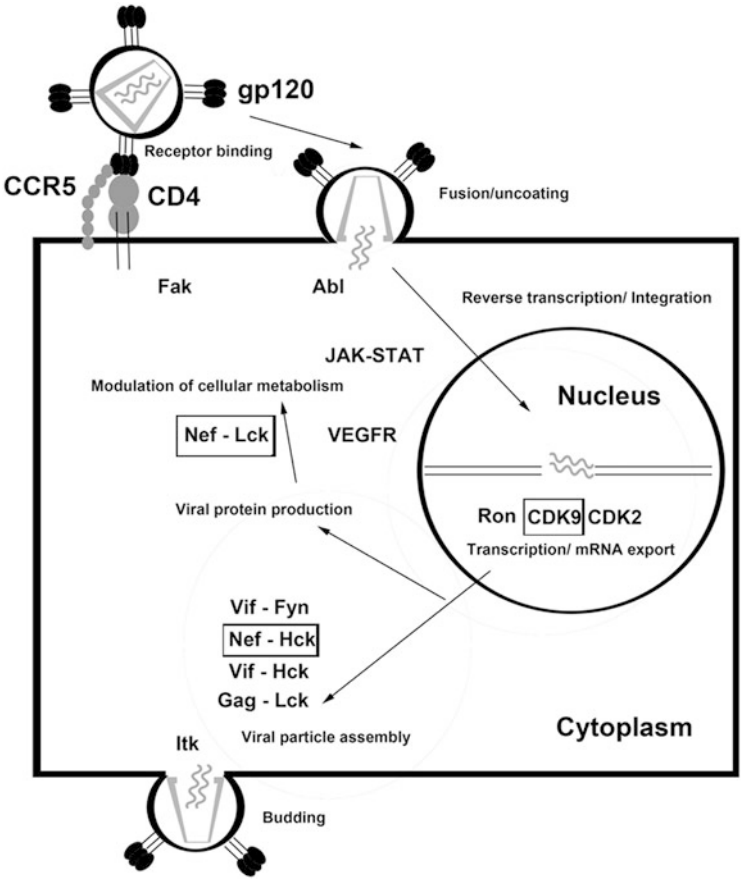

Fig. 6 Roles of cellular protein kinases in the HIV-1 life cycle. Kinases known to participate at different stages of HIV-1 replication are indicated, along with their viral interactors, if known. Boxes highlight those kinases currently exploited as anti-HIV targets. For details see text

the host cell response to viral infections but are also heavily exploited by viral proteins during the virus-mediated subversion of the cell metabolism. Accumulating evidence revealed important roles of different protein kinases in the HIV-1 life cycle. Figure 6 schematically summarizes the stages of the viral cell cycle known to be affected by various protein kinases and the viral interactors identified so far. As therapeutic interventions targeting kinases to interfere with HIV-1 replication are still rudimentary, we refer to these data separately at the end of this subchapter.

2.4.1 Non-Receptor Tyrosine Kinases of the Src Family

The Src protein tyrosine kinase family consists of non-receptor tyrosine kinases Src, Fyn, Yes, and Lyn that are ubiquitously expressed, and Fgr, Hck, Lck, and Blk

which are expressed in a tissue-specific manner [172]. These kinases are composed of distinct functional regions including a unique region at the N-terminal end, the src homoly regions SH1 (catalytic domain), SH2, and SH3 and the negative regulatory tail [173]. The enzymes can exist in a close inactive conformation, in which SH3 binds to an internal PxxP region, while SH2 binds to a negative regulatory phosphotyrosine in the tail, or in an open active conformation [172]. Their functions include cell proliferation, mobility, adhesion, apoptosis, and DNA repair. Due to the important roles of Src kinases within the cell, it is not surprising that some of them resulted to be involved in several steps of the HIV-1 life cycle.

Hematopoietic cell kinase (Hck) is mainly expressed in promonocytic cells and monocyte-derived macrophages. In these cells, Hck mediates several functions including the induction of cytokine production, cell spreading, and phagocytosis. Moreover, Hck participates in the FcγRI receptor signaling pathway [174–176]. Hck was found to interact with the accessory HIV-1 protein Vif [177]. This interaction occurs through the proline-rich motif (PPLP) of Vif and the SH3 domains of Hck. Interestingly it was shown that Hck phosphorylates APOBEC3G and that this results in reduced APOBEC3G packaging in the absence of Vif [178]. The presence of Vif is sufficient to overcome the Hck-dependent inhibition of viral replication [177]. As consequence of Vif expression, Hck autophosphorylation, and thus autoactivation, is dramatically reduced [178]. Interestingly, the Fyn kinase, which is highly expressed in the nervous system and T-cells, secondary and primary targets of HIV-1 infection, can also phosphorylate APOBEC3G, thus possibly substituting for Hck in these cells types [178].

Besides Vif, Hck interacts with another HIV-1 accessory protein, Nef. Nef interacts with a variety of cellular proteins, causing an alteration of signal transduction pathways, i.e. T cell receptor (TCR) signal transduction [179]. Like Vif, Nef interacts with the SH3 domain of Hck through its polyproline motif [180, 181]. In contrast to Vif, Nef–SH3 interaction does not lead to kinase inhibition, but rather to increasing kinase catalytic activity resulting in viral growth pomotion [182]. The interaction of Nef with Hck results in the displacement of the Hck intramolecular SH3/linker domain, which is needed to maintain the kinase inactive form. Thus, Vif and Nef lead to opposite actions on Hck kinase activity, suggesting that the activity of this kinase must be finely tuned within the HIV-1 life cycle.

In T cells, the lymphoid T cell protein kinase Lck is involved in HIV-1 transcription, replication, and pathogenesis [183]. During HIV-1 assembly, Lck regulates Gag trafficking from the intracellular compartments to the plasma membrane and facilitates the virus release by binding of HIV-1 Gag through its unique domain (UD). The UD can undergo palmitoylation in the early exocytic pathway, allowing for subsequent transport of the protein to the plasma membrane; HIV-1 Gag takes advantage of this property of Lck for its targeting to the plasma membrane [183]. Another important role of Lck in HIV-1 replication involves the transduction of TCR signals upon TCR activation Nef counteracts TCR transmitted activation signals, probably by altering the subcellular localization of Lck [184–186].

Still another kinase, Abelson murine leukemia kinase (Abl), is involved in actin cytoskeleton rearrangements induced upon HIV-1 entry receptor signaling (see Sect. 2.1). This is mostly mediated by the Rho family of GTPases, including the Cdc42, Rac, and Rho subfamilies [187]. The role of Rac appears to be of primary importance, since the overexpression of a Rac dominant negative mutant leads to the inhibition of HIV fusion [188]. An essential role in cytoskeleton rearrangements supporting membrane fusion is also played by the Arp2/3 complex that induces actin polymerization and rearrangements necessary for pore formation and virus entry [187]. Using siRNAs or small molecules targeting Abl, it was shown that Abl acts on actin remodeling both upstream and downstream of Rac [189, 190]. Moreover, drugs selectively targeting Abl (like imatinib, nilotinib and dasatinib) have shown to provoke the arrest of the viral fusion process [187].

2.4.2 Tyrosine Kinases Other Than the Src Family

The Tec family of non-receptor tyrosine kinases (TEK) are important mediators of antigen receptor signaling in lymphocytes. This family is composed of five members: Tec, Btk, Itk, Rlk, and Bmx. Among these, Itk plays an important role during HIV-1 infection in T cells. Here, Itk coordinates cytoskeleton reorganization during signaling downstream the TCR and the chemokine receptor cascade, therefore being a critical factor in the regulation of the HIV-1 life cycle in T cells [191]. Besides entry, Itk activity is crucial for a late step in HIV-1 replication, as inhibition of Itk leads to the suppression of HIV assembly and virion release, probably by increasing the intracellular Ca^{2+} concentration [192].

The focal adhesion kinase (FAK) plays a pivotal role in the assembly, signaling and disassembly of focal adhesions influencing processes like migration, adhesion, and survival [193]. FAK colocalizes with the intracellular domains of CD4 and CCR5 receptors upon gp120 interaction and participates in HIV-1 entry (see Sect. 2.1; [194]). Like other tyrosine kinases, FAK is also involved in TCR signaling. Upon TCR complex activation, FAK becomes phosphorylated and by interacting with the SH2 domain of Lck, displaces this kinase from the cytoplasmatic CD4 domain. The FAK domain responsible for CD4 interaction (FAT; [193]) and the mode of interaction resembles the binding surface of CD4 and Nef [195]. However, whereas gp120 FAK-CD4 interaction facilitates viral post-entry stages and triggers HIV envelope-mediated apoptosis in uninfected T cells, binding of Nef to CD4 blocks super infection signaling, impeding the proapoptotic pathway activation [70, 196]. These observations, taken together, suggest that Nef might counteract gp120 signaling via FAK or Lck displacement from CD4, thus avoiding apoptosis and consenting effective virus replication [193].

The tyrosine kinase “Recepteur d’origin nantais” (RON) is a member of the MET family of receptor tyrosine kinases. Its activity is linked to inhibition of inflammation and macrophage function [197]. In HIV infection, RON inhibits virus transcription [198] through inhibition of NF-κB [199], a transcription factor

common to proinflammatory responses and HIV transcription. Suppression of HIV-1 transcription is achieved through RON-dependent promotion of the interaction between RNA pol II and negative elongation factors. RON is also involved in nucleosome organization process by negatively regulating histone acetylation, and thus inducing a transcriptionally repressed chromatin organization. Interestingly, Tat counteracts RON activity by specifically targeting this kinase for ubiquitin-mediated proteosome degradation [200].

A further kinase influenced by HIV-1 infection is the Janus kinase and Signal Transducer and Activator of Transcription (Jak/STAT). Interferons and cytokines signal via the JAK/STAT pathway. The majority of HIV-infected individuals shows a constitutive activation of STAT proteins due to deregulated cytokine secretion, especially important for IL-2 and IL-7 [201–203]. Further, T cells from patients show reduced SOCS-1 and SOCS-3 protein levels and increased phosphorylated levels of STAT-1,-3, and -5. The JAK/STAT signaling exerts a pivotal role in the induction of IL-2, which induces T-cell proliferation. In particular, activation of STAT5 protects cells from apoptosis and is important in cell cycle progression. In HIV-infected patients, CD8 T cells are less active and undergo more spontaneous apoptosis than those isolated from healthy individuals. This effect has been correlated with an impaired activation of the STAT5 pathway due to the deregulation of JAK-3. Consistently, during HAART administration, the JAK/STAT pathway is restored with the consequent IL-2 induction [204].

2.4.3 Serine/Threonine Protein Kinases CDK9 and CDK2

Finally, serine/threonine protein kinases CDK9 and CDK2 also have essential functions in the HIV-1 life cycle. Positive Transcription Elongation Factor b (P-TEFb) is a complex formed by T-type cyclins or cyclin K, which interact with the serine/threonine protein kinase CDK9. This complex is required for RNA Polymerase II transcription in vitro and is recruited to several promoters in cells. It is also essential for HIV-1 transcription. HIV-1 Tat recruits the cyclin T1/CDK9 complex to the nascent HIV-1 transcript [205]. This event is crucial for HIV-1 transcription elongation; as virus replication is strongly affected by inhibition of CDK9 activity, either using a dominant-negative form of CDK9 (dnCDK9) or siRNAs directed to either CDK9 or cyclin T1 in transformed cells [206–209]. Furthermore, mimetic peptide inhibitors targeting CDK9 inhibited viral replication in humanized mouse models [210].

As for CDK9, also the knockdown of CDK2, that was found to associate with the HIV-1 promoter in vitro [211] and in vivo [212], leads to the inhibition of HIV-1 transcription and replication [213]. It was recently shown that the siRNA-mediated knockdown of CDK2 led to the inhibition of CDK9 activity by reducing CDK9 phosphorylation. Indeed, CDK2 phosphorylates CDK9 on Ser 90 and this event represents a novel mechanism of HIV-1 transcription regulation [214].

2.4.4 Current Perspectives of Protein Kinases as Anti-HIV Targets

As outlined above, many kinases appear to play a role in the HIV-1 life cycle. However, only few of them have been extensively exploited so far as potential anti-HIV targets. Small molecule inhibitors of the interaction of the Hck kinase with HIV-1 Nef were developed by high-throughput screening of large chemical libraries. A particularly promising class of compounds, diphenylfuropyrimidines, was found to be able to disrupt Nef-dependent Hck activation and to suppress HIV-1 replication [215]. Similarly, guanidine alkaloid analogues were identified as inhibitors of the Nef–Lck interaction [216]. The most studied kinase target is certainly CDK9 and its associated Cyclin T1. Two general CDK inhibitors, Roscovitine and Flavopiridol, were found to be active against CDK9 and to suppress HIV-1 replication, providing the proof-of-principle for considering CDK9 a potential anti-HIV target [206]. Since then, several small molecules, as well as peptide-based CDK9 inhibitors have been shown to suppress HIV-1 replication [210, 217, 218].

Targeting protein kinases in HIV-1 replication/transcription is still at its infancy. However, since many of the kinases playing a role in the HIV-1 life cycle were also being studied as potential anticancer targets, it is entirely possible that among the hundreds of known inhibitors of these kinases developed as antiproliferative compounds, some would also be active against HIV-1 replication. Thus, larger screenings of focused libraries may, in the next future, identify novel promising anti-HIV agents. Also, novel proteomic approaches are being developed to explore, for example, the entire Nef interactome in search of inhibitors [219]. Such approaches are also promising tools to identify novel compounds targeting the interaction between a cellular factor and a viral factor.

2.5 Cellular Restriction Factors of HIV

Besides classical innate and acquired immune responses, mammals have evolved a set of genes that are capable of suppressing or preventing virus replication at the cellular level. Over the past 10 years a number of such host-encoded restriction factors were discovered that can act as potent inhibitors of HIV-1 replication. The most notable members of this group of interferon-induced innate immunity factors are TRIM5α, APOBEC3G, CD317/tetherin, and SAMHD1. HIV encodes a unique set of accessory gene products (Vif, Nef, Vpr, Vpu, Vpx) to optimize its replication in the human host (Fig. 1). Knowledge gained over the last years suggests that this is achieved, in part, by counteracting these host restriction factors (Fig. 2). This section very briefly describes our current understanding of the mode of action of these restriction factors, the means used by HIV-1 to evade their restriction and their potential for the development of innovative antiviral strategies. After submission of this article, a new interferon-induced antiviral factor, MX2/MxB belonging

to the guanosine triphosphatase superfamily, was described independently by two groups [220, 221]. This factor restricts lentiviral replication in a cyclophylin A dependent manner after virus entry and before integration of the proviral genome. The detailed molecular mechanisms have not yet been fully elicited, however.

2.5.1 Trim5α

TRIM5α is a cytoplasmic protein that exerts its antiviral activity following entry of retroviral capsids into the cytoplasm of host cells typically by preventing the accumulation of reverse-transcribed HIV-1 cDNA [222]. TRIM5 is one of a family of approximately 70 tripartite motif (TRIM)-containing proteins. The TRIM domain is composed of amino-terminal RING and B-box type 2 domains linked to a central coiled-coil domain. The range of retroviruses that are inhibited by TRIM5α varies considerably, depending on its species of origin. For instance, the human TRIM5α protein is an effective inhibitor of nonhuman retroviruses, e.g. N-MLV and equine infectious anemia virus (EIAV) [223, 224]; however, it is virtually inactive against HIV-1. To the contrary, TRIM5α proteins from Old World monkeys inhibit HIV-1 infection [222]. As a general rule, TRIM5α proteins are weak or even inactive inhibitors of retroviruses that are found naturally in the same host species, but are frequently active against retroviruses that are found in other species. As such, TRIM5α can impose rather effective barriers to cross-species transmission of primate lentiviruses [225]. The evolution of capsid within a host or related hosts seems to be a key mechanism employed by retroviruses to escape recognition by TRIM5α. Due to the virtual absence of antiviral efficacy against HIV-1 the human TRIM5α–capsid interaction currently plays no role in the development of anti-HIV strategies.

2.5.2 APOBEC3

Members of the human APOBEC family of cellular cytidine deaminases can be potent inhibitors of HIV infection (for review see [226]). In the absence of essential accessory viral protein Vif, members of the APOBEC3 family – in particular the most potent members G and F – are encapsidated by budding particles resulting in cytidine to uridine editing of negative sense reverse transcripts in the newly infected cell. These changes to the viral genome are detrimental to the virus since it translates into guanosine to adenosine hypermutations in the plus-stranded HIV-1 cDNA. In addition, APOBEC3G also appears to impede the translocation of reverse transcriptase along the viral template RNA, and recent studies suggest that it can also exert deamination-independent effects in the early phase of infection [226].

Natural HIV-1 infections are largely spared from APOBEC3 proteins' antiviral activity through the action of the Vif protein. The expression of Vif in a productively infected cell induces the polyubiquitylation and subsequent proteasomal degradation of APOBEC3 proteins, thereby depleting the cytosolic pool of these

restriction factors available for incorporation into budding virus particles [227, 228]. Vif accomplishes this by binding to both APOBEC3 proteins and the cullin5-elongin B/C-Rbx ubiquitin ligase, thus serving as a virus-encoded adaptor that recruits the ligase complex to its substrate [229]. Recently it has been shown that Vif hijacks the transcription cofactor CBF-β and that this interaction is necessary for the Vif-induced degradation of APOBEC3G via the proteasome [230]. Thus, interfering with the Vif/CBF-β interaction could represent a new therapeutic strategy to enhance the antiviral activity of APOBEC3G.

Several lines of evidence suggest that expression levels [231] and function of APOBEC3 family members as well as Vif protein diversity can profoundly influence the natural history of HIV-1 infection and disease outcome, among other mechanisms by inhibiting viral replication or by driving viral sequence diversification. Conceptually, restoring the potent anti-HIV activity of APOBEC3 family members by pharmacological interference with the Vif-APOBEC3 interaction is potentially an attractive therapeutic approach. Along these lines several interesting small molecule inhibitors have been identified. RN-18 [232] acts at the point of Vif-induced recruitment of the E3 ubiquitin ligase complex prior to proteasomal degradation of human APOBEC3G. RN-18 "misdirects" polyubiquitination from the restriction factor onto the antagonizing Vif molecule. As a consequence, Vif is destroyed by the proteasome, which in turn allows APOBEC3G levels to be unharmed and the restriction factor to exert its antiviral function. Two other compounds, IMB26 and IMB35 [13], disrupt the Vif-mediated degradation of human APOBEC3G by directly inhibiting the binding of Vif to the restriction factor, rather than inflicting a negative effect on Vif levels (for review see [233]).

2.5.3 CD317/Tetherin

Discovered in 2008 [234, 235], the innate immunity factor CD317 (Tetherin/BST-2/HM1.24) imposes a potent interferon-inducible barrier to the release of a wide range of enveloped viruses, including HIV-1, at the cell surface (for review see [236]). This activity requires multimerization of CD317, and the current model suggests that dimers of the restriction factor physically bridge the cell's membrane and nascent virions as well as virions among each other [237, 238]. To counteract this activity, several viruses have evolved strategies to antagonize CD317, each apparently using a unique mode of action [236]. Expression of the accessory protein Vpu of HIV-1 results in a reduction of CD317 surface levels, lowering their density at sites of virus budding [239]. To achieve this, Vpu inhibits both the anterograde transport of newly synthesized CD317 and the recycling of the restriction factor to the plasma membrane [240]. Vpu traps CD317 molecules trafficking in either of these pathways at the *trans*-Golgi network, where the two proteins physically interact via their transmembrane domains. On a molecular level, the highly conserved diserine S52/S56 motif of the cytoplasmic tail of Vpu is central to changes induced by the viral protein to intracellular CD317 trafficking, trapping of CD317 at the *trans*-Golgi network, and antagonism of the HIV-1 release restriction.

Intriguingly, the functionality of this motif does not require recruitment of the diserine motif interactor and substrate adaptor of the SCF-E3 ubiquitin ligase complex, β-TrCP [240].

Similar to the Vif-APOBEC3 interaction, the CD317 antagonism by Vpu poses a conceptually interesting interaction for therapeutic intervention [241]. The fact that this interaction maps to the transmembrane domains of both proteins and primarily occurs in the lipid bilayer of the membranes of the *trans*-Golgi network imposes very specific and hard-to-reach requirements on putative inhibitors that could potentially interfere with this protein–protein interaction. Alternatively, a high-throughput screening approach for anti-Vpu compounds could focus on inhibiting the critical diserine motif in Vpu's cytoplasmic tail, thus avoiding the requirement for a transmembrane-targeting inhibitor. Such compounds would be predicted to completely block Vpu's distortion of CD317 trafficking and allow the restriction factor to exert its release inhibiting activity.

2.5.4 SAMHD1

In 2011, SAMHD1 was identified as a restriction factor, the expression of which prevents the completion of HIV-1 reverse transcription in macrophages and dendritic cells [242, 243]. Recently, we and others were able to show that SAMHD1 also acts as a restriction in resting T cells, expanding the relevant cell pool from myeloid to lymphoid cells [244, 245]. The mode of action of this deoxynucleotide triphosphohydrolase appears to be the depletion of intracellular dNTP pools below a threshold required for reverse transcription of the viral RNA into a complementary DNA [244, 246]. In addition, a nuclease activity of SAMHD1 that is able to degrade reverse transcription intermediates has been reported [247]. SAMHD1 imposes a post-entry barrier of variable extent in a number of primary HIV target cells in vivo implying that an ability of HIV to antagonize this factor would be beneficial for viral pathogenesis and prevalence in the human population. The contrary seems to be true. The accesory lentiviral Vpx protein of the less pathogenic HIV-2 and SIV, which bind to SAMHD1 in a CRL4DCAF1-dependent manner to induce proteasomal degradation of the cellular factor [242–244], is absent from the pandemic HIV-1 and more pathogenic SIVs [248]. The answer why HIV-1 may have lost a *vpx* gene function may lie in an advantage provided by avoiding triggering of an antiviral innate immune response. Experimental support for this notion has been obtained in dendritic cells, where the presence of Vpx determined the induction of a potent anti-HIV cytokine response [249]. A lot of work is ahead to decipher the regulation, function and in vivo-relevance of SAMHD1 for HIV in human disease. In the absence of a clear understanding of SAMHD1's net impact on pathogenesis, establishment of latent reservoirs and pandemic spread, it is currently not possible to design an innovative therapeutic strategy based on this cellular protein.

2.5.5 Perspectives to Target Cellular Restriction Factors for HIV Therapy

The concept of exploiting restriction factors for the development of novel antiretroviral or preventive strategies has yet to receive widespread attention in the scientific community. Imprinting an antiviral innate signature on target cells of HIV, e.g. at sites of mucosal transmission, by boosting the expression of several of these potent antiviral proteins is an approach that requires deeper insight into their specific regulation. The potency of Vif, Vpu, and Vpx to overcome replication barriers can be clearly limited by enhanced expression of the respective restriction factor, at least in vitro. Whether the specific stimulation of certain pattern recognition receptors or sensors may be sufficient to induce a general anti-retroviral state of the innate immune system that builds on these restriction factors is an exciting field of research. Alternatively, enhancement of restriction factor activity by pharmacological interference with interactions between virus-encoded antagonists and restriction factors is an attractive approach for which some interesting lead compounds have already been reported. This provides a paradigm for a possible drug-based exploitation and boosting of the intrinsic anti-HIV capacity of the human innate immune system.

3 Summary, Conclusions, Outlook

HIV-1 has evolved sophisticated mechanisms to take advantage of cellular proteins for its own replication. Besides the classical retroviral genes *gag*, *pol*, and *env* encoding essential proteins for virus replication, additional regulatory and accessory genes encode proteins that by interacting with a number of cellular proteins create a huge functional network that allows HIV to take advantage of cellular factors supporting its replication, while restictive cellular factors are being antagonized. Increasing knowledge about cellular factors involved in HIV-1 replication and their mode of action potentially offers new opportunities to interfere with virus replication. In contrast to classical HAART, which essentially targets viral proteins, targeting cellular factors has the advantage to drastically reduce the development of drug resistance. Such approaches therefore would nicely complement HAART therapy by extending the number of potential targets allowing virus inhibition at additional steps of the replication cycle. One *caveat* of course is that the normal cellular functions of the HIV cofactors should not be compromised. Some examples of successful development of drugs targeting cellular cofactors of HIV-1 with the aim to inhibit virus replication like Maraviroc against the coreceptor CCR5 or LEDGINs against the integrase cofactor LEDGF demonstrated the proof of principle of this approach. Genome-wide screens and systems biology recently identified a number of additional cellular proteins involved in HIV-1 replication, which after being validated await further development of new promising drugs against HIV-1.

Further, modulation of the activity of some of the cellular factors involved in postintegration latency may allow to also target latent HIV reservoirs in the body that are not amenable to the currently used drugs mostly focussing on actively replicating HIV-1. This may shift the field of HIV therapy further into the direction of a functional cure.

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