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Nucleoside analogs as a rich source of antiviral agents active against arthropod-borne flaviviruses

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Abstract

Nucleoside analogs represent the largest class of small molecule-based antivirals, which currently form the backbone of chemotherapy of chronic infections caused by HIV, hepatitis B or C viruses, and herpes viruses. High antiviral potency and favorable pharmacokinetics parameters make some nucleoside analogs suitable also for the treatment of acute infections caused by other medically important RNA and DNA viruses. This review summarizes available information on antiviral research of nucleoside analogs against arthropod-borne members of the genus *Flavivirus* within the family Flaviviridae, being primarily focused on description of nucleoside inhibitors of flaviviral RNA-dependent RNA polymerase, methyltransferase, and helicase/NTPase. Inhibitors of intracellular nucleoside synthesis and newly discovered nucleoside derivatives with high antiflavivirus potency, whose modes of action are currently not completely understood, have drawn attention. Moreover, this review highlights important challenges and complications in nucleoside analog development and suggests possible strategies to overcome these limitations.

Keywords

Nucleoside analog, antiviral agent, arthropod-borne flavivirus, antiviral therapy, inhibitor

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Introduction

The genus *Flavivirus* belongs to the Flaviviridae family and includes more than 70 single-stranded plus-sense RNA viral species. Flaviviruses of human medical importance are tick- or mosquito-transmitted viruses with typical representatives being tick-borne encephalitis virus (TBEV), Omsk hemorrhagic fever virus (OHFV), Kyasanur Forest disease virus (KFDV), Alkhurma hemorrhagic fever virus (AHFV), Powassan virus (POWV), West Nile virus (WNV), dengue virus (DENV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), or Zika virus (ZIKV).[1,2] The Flaviviridae family also includes some less known or neglected viruses, such as louping ill virus (LIV), Usutu virus, Langat virus, or Wesselsbron virus.[3–6] The flaviviral genome is a single-stranded, plus-sense RNA of about 11 kb in length that encodes a single polyprotein, which is co- and posttranslationally processed into three structural (capsid, premembrane or membrane, and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).[7] Both NS3 and NS5 proteins possess enzymatic activities reported to be important targets for antiviral development. Whereas NS3 acts as a serine protease, a 5′-RNA triphosphatase, a nucleoside triphosphatase (NTPase), and a helicase,[8,9] NS5 consists of a complex containing the RNA-dependent RNA polymerase (RdRp) and the methyltransferase (MTase) activities.[10,11]

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Flaviviral infections are accompanied by a wide spectrum of distinct clinical manifestations, ranging from relatively mild fevers and arthralgia to severe viscerotropic symptoms (YFV and DENV), hemorrhagic fevers (KFDV and OHFV), encephalitis/myelitis (JEV, WNV, and TBEV), and neuropathic or teratogenic manifestations (ZIKV). More than 200 million clinical cases of flaviviral infections, including numerous deaths, are reported annually worldwide.[12] Currently no specific antiviral therapies are available to treat patients with flaviviral infections, thus the search for safe and effective small-molecule inhibitors that would be active against these viruses represents a high research priority.[13]

Nucleoside analog inhibitors have figured prominently in the search for effective antiviral agents.[14] Nucleoside analogs are synthetic, chemically modified nucleosides that mimic their physiological counterparts (endogenous nucleosides) and block cellular division or viral replication by impairment DNA/RNA synthesis or by inhibition of cellular or viral enzymes involved in nucleoside/tide metabolism (Figure 1).[15] The first antiviral analogs were developed in the late 1960s and currently there are over 25 approved therapeutic nucleosides used for the therapy of viral infections of high medical importance, such as HIV/AIDS (tenofovir),[16,17] hepatitis B (lamivudine/entecavir),[18,19] hepatitis C (sofosbuvir),[20] or herpes infections (acyclovir).[21] So far, numerous nucleoside analogs have been described to inhibit arthropod-transmitted flaviviruses. Since these viruses are closely related to the hepatitis C virus (HCV), for which many potent inhibitors are being currently developed, anti-HCV nucleoside analogs represent promising tools to be repurposed against other viruses within the Flaviviridae family.[12]

The aim of this review is to provide an overview of known antiviral agents targeting selected arthropod-borne flaviviruses and to discuss their characteristic properties, modes of action, and advantages or limitations of their therapeutic use. Moreover, the important challenges and complications in antiflavivirus nucleoside analog development are highlighted and possible strategies to overcome their shortcomings are suggested.

Major classes of antiflavivirus nucleosides

Nucleoside analogs active against arthropod-borne flaviviruses are usually classified according to their targets in the viral life cycle. Such antiviral molecules act as inhibitors of flaviviral RdRp,[22–26] MTase,[27,28] and helicase/NTPase.[29,30] Other nucleoside scaffolds suppress host cell enzymes involved particularly in nucleoside biosynthetic pathways[31–34] or in some cases, exhibit multiple modes of action.[32,35] In vitro antiviral effects and cytotoxicity profiles of the most important antiflavivirus nucleoside analogs are summarized in Table 1. An overview of in vivo antiflaviviral activities of selected nucleosides as evaluated in different animal models is presented in Table 2.

Inhibitors of flaviviral NS5 RdRp

The flavivirus NS5 protein is approximately 900 amino acids in length and consists of the NH_2-terminal MTase domain required for the 5′-RNA capping process and the COOH-terminal RdRp domain responsible for the replication of the viral RNA genome.[10,36] Flaviviral RdRp is a right hand-shaped structure with fingers, palm, and thumb domains; the palm domain is the catalytic domain carrying the polymerase active site that coordinates two Mg^{2+} ions essential for catalyzing the polymerization reaction.[37] Nucleoside inhibitors of flaviviral RdRp are the most attractive targets for antiviral drug design; as human replication/transcription enzymes lack RdRp activity, such compounds are expected to show fewer deleterious side effects and favorable safety profiles.[12,15,38,39]

The mode of action for nucleoside RdRp inhibitors is based on the premature termination of viral nucleic acid synthesis.[40] Following the intracellular phosphorylation, the 5′-triphosphate metabolites are competitively incorporated into the flaviviral RNA nascent chains (Figure 1). This prevents further extension of the incorporated analog by addition of the next nucleoside triphosphate resulting in formation of incomplete (nonfunctional) viral RNA chains.[41] Nucleoside inhibitors of flaviviral RdRp act as "nonobligate chain terminators," as their 3′-hydroxyl group is conformationally constrained or sterically/electronically hindered, thus decreasing the potency to form a phosphodiester linkage with the incoming nucleoside triphosphate.[40] The nonobligate terminators differ from "obligate chain terminators," in which the 3′-hydroxy group is completely missing, as exemplified by numerous nucleoside reverse transcriptase inhibitors for the treatment of HIV infections.[42] Chemical modifications of the heterobase moiety, different types of glycosidic bonds, and substitutions at different positions of the sugar ring, which are typical modifications for nucleoside inhibitors of flaviviral RdRps, are shown in Table 3.

1′-Cyano substituted nucleosides

GS-441524, a 1′-cyano substituted *C*-nucleoside derived from 4-aza-7,9-dideazaadenosine,[43] was developed by Gilead Sciences, Inc. as a treatment for filovirus infections also showing reasonable antiviral activity against paramyxo- and pneumoviruses.[44] Within the

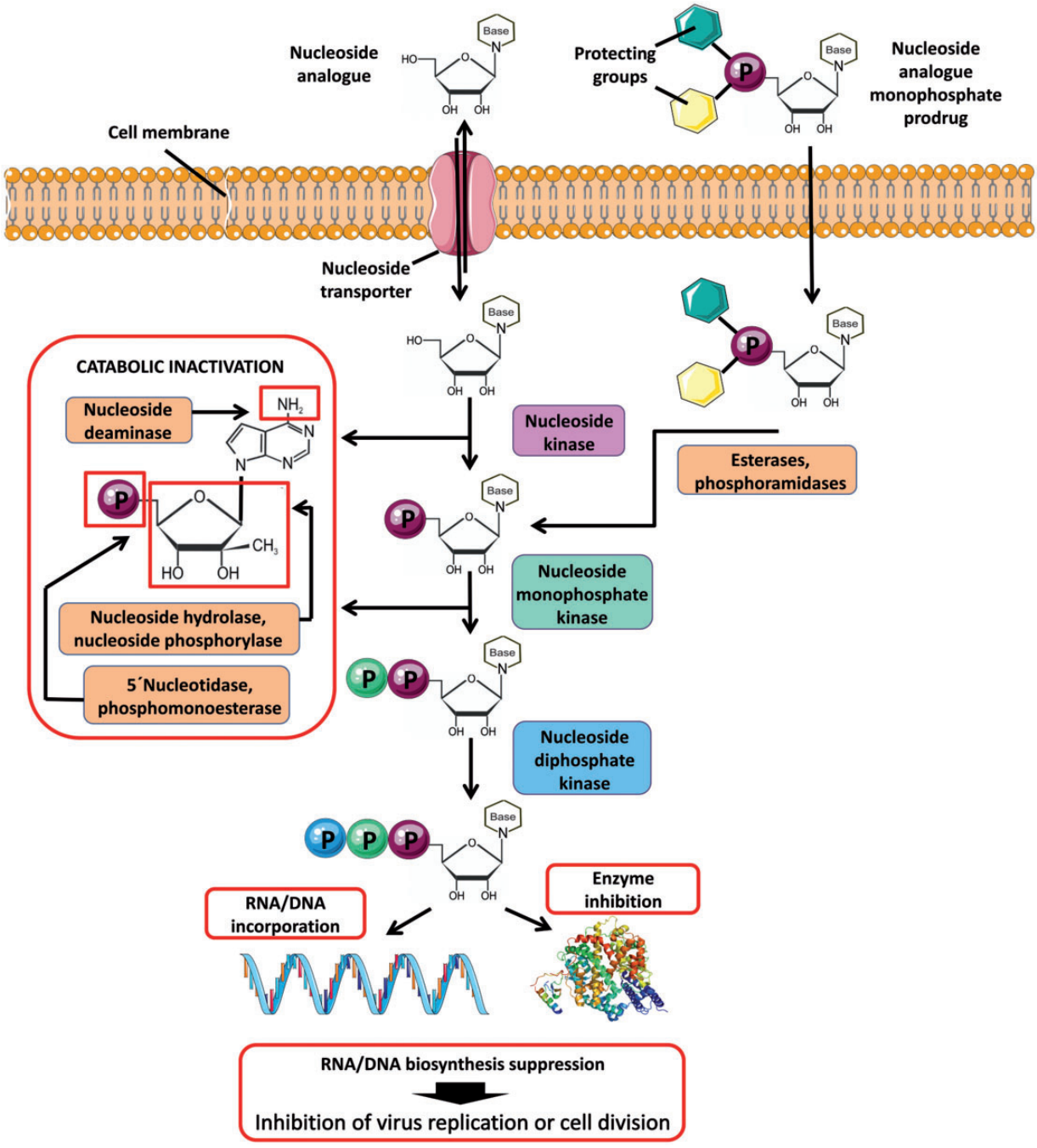


Figure 1. Intracellular uptake and metabolism of nucleoside analogs and nucleoside analog prodrugs. Nucleoside analogs enter cells through specific plasma membrane nucleoside transporters. Inside the cell, the compounds are phosphorylated by cellular nucleoside kinases resulting in formation of nucleoside mono-, di-, and triphosphates. The first kinase phosphorylation is the rate-limiting step of the triphosphate conversion, which can be overcome by the monophosphate prodrug approach based on the introduction of a phosphorylated group into the 5′ nucleoside position. The phosphorylated group includes protecting moieties to increase hydrophobicity and facilitate the cellular uptake of the prodrug. Monophosphate prodrugs enter cells independently of membrane transporters and the protecting groups are removed by intracellular esterases or phosphoramidases after cell penetration. The triphosphates of nucleoside species represent the active forms of nucleoside analogs that act by inhibiting cellular or viral enzymes, such as DNA/RNA polymerases. During DNA/RNA replication, nucleoside analogs are incorporated into nascent DNA or RNA chains resulting in termination of nucleic acid synthesis or in accumulation of mutations in viral genomes to suppress viral replication due to error catastrophe. At normal physiological conditions, intracellular nucleoside concentrations are maintained at low levels due to nucleoside/nucleotide catabolic pathways, such as deamination (oxidation) of heterocyclic base, hydrolysis or phosphorolysis of heterocyclic base, and hydrolysis of phosphomonoester bonds. These catabolic reactions also concern most nucleoside analogs containing the natural *N*-glycosidic bond and/or the degradable functional groups of the heterocyclic base. Figure created using Servier Medical Art available on www.servier.com.

Table 1. In vitro antiviral activities and cytotoxicity profiles of selected nucleoside inhibitors of flaviviral replication.

Structure	Virus	Strain	Cell line	Assay	EC_{50} (μM)	CC_{50} (μM)	References
Inhibitors of flaviviral RdRp							
GS-441524	AHFV	200300001	Vero	CPE	49.9	ND	Lo et al.[45]
	KFDV	P9605	Vero	CPE	46.3	ND	Lo et al.[45]
	TBEV	Hypr	Vero	CPE	51.2	ND	Lo et al.[45]
	OHFV	Bogoluvovska	Vero	CPE	50.6	ND	Lo et al.[45]
	YFV	ND	ND	Cell based	11	>30	Cho et al.[43]
	DENV-2	ND	ND	Cell based	9.46	>30	Cho et al.[43]
	WNV	ND	ND	Cell based	>30	>30	Cho et al.[43]
GS-5734	AHFV	200300001	Vero	CPE	4.2	ND	Lo et al.[45]
	KFDV	P9605	Vero	CPE	1.8	ND	Lo et al.[45]
	TBEV	Hypr	Vero	CPE	2.1	ND	Lo et al.[45]
	OHFV	Bogoluvovska	Vero	CPE	1.2	ND	Lo et al.[45]
2′-C-methyladenosine	TBEV	Hypr	PS	VTR	1.4	>50	Eyer et al.[54]
	WNV	New York isol.	Vero	CPE	5.1	25	Migliaccio et al.[49]
	DENV-2	New Guinea C	Vero	CPE	4	18	Migliaccio et al.[49]
	YFV	17-D	Vero	CPE	3.2	13	Migliaccio et al.[49]
	ZIKV	MR766	Vero	VTR	5.26	>100	Eyer et al.[23]
7-Deaza-2′-C-methyl-adenosine	TBEV	Hypr	PS	VTR	1.1	>50	Eyer et al.[54]
	WNV	New York isol.	Vero	CPE	4.5	250	Olsen et al.[50]
	DENV-2	New Guinea C	Vero	CPE	15	>320	Olsen et al.[50]
	YFV	17-D	Vero	CPE	15	>320	Olsen et al.[50]
	ZIKV	MR766	Vero	CPE	20	>357	Zmurko et al.[62]
	ZIKV	MR766	Vero	VYR	9.6	>357	Zmurko et al.[62]
	ZIKV	MR766	Vero	PA	1.3	>357	Zmurko et al.[62]
	ZIKV	MR766	Vero	IFA	5.7	>357	Zmurko et al.[62]

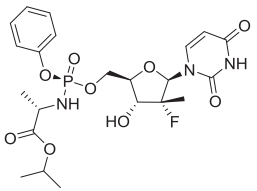
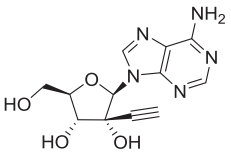
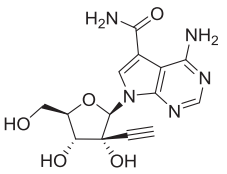
(continued)

Table 1. Continued

Structure	Virus	Strain	Cell line	Assay	EC_{50} (μM)	CC_{50} (μM)	References
2′-C-methylguanosine	TBEV	Hypr	PS	VTR	1.4	>50	Eyer et al.[54]
	WNV	New York isol.	Vero	CPE	30	>100	Migliaccio et al.[49]
	DENV-2	New Guinea C	Vero	CPE	13.6	>60	Migliaccio et al.[49]
	YFV	17-D	Vero	CPE	17	>50	Migliaccio et al.[49]
	ZIKV	MR766	Vero	VTR	22.25	>100	Eyer et al.[23]
INX-08189	DENV-2	ND	Huh-7	Replicon	0.0142	>1	Yeo et al.[58]
2′-C-methylcytidine	TBEV	Hypr	PS	VTR	1.8	>50	Eyer et al.[54]
	AHFV	200300001	A549	qRT-PCR	2.5		Flint et al.[55]
	AHFV	200300001	A549	CPE	15.3		
	KDFV	P9605	A549	CPE	7.2		
	OHFV	Bogoluvovska	A549	CPE	3.2		
	POWV	Byers	A549	CPE	5.5		
	DENV	ND	Huh-7	Replicon	11.2	–	Lee et al.[56]
	YFV	17-D	Vero	Visual inspection	2.5[a]	22[a]	Julander et al.[63]
	YFV	17-D	Vero	Neutral red uptake	2.1[a]	22[a]	Julander et al.[63]
	YFV	17-D	Vero	VYR	0.7[a]	22[a]	Julander et al.[63]
	ZIKV	MR766	Vero	VTR	10.51	>100	Eyer et al.[23]
2′-C-methyluridine	TBEV	Hypr	PS	VTR	11.1	>50	Eyer et al.[54]
	ZIKV	MR776	Vero	VTR	45.45	>100	Eyer et al.[23]

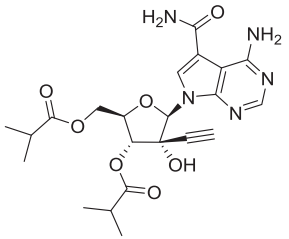
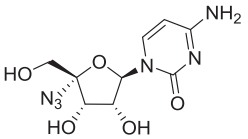
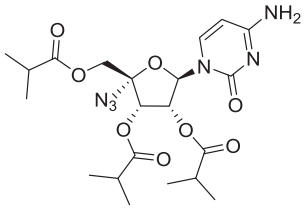
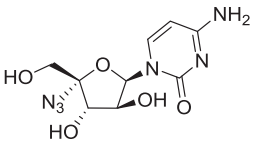
(continued)

Table 1. Continued

Structure	Virus	Strain	Cell line	Assay	EC_{50} (μM)	CC_{50} (μM)	References
Sofosbuvir	ZIKV	ND	BHK-21, SH-sy5y, Huh-7	CPE	0.12–1.9	>300	Sacramento et al.[25]
	ZIKV	ND	-	RdRp inhibition	0.38	ND	Sacramento et al.[25]
	ZIKV	PRVABC59	Huh-7, Jan	PA	1–5	>200	Bullard-Feibelman et al.[69]
	ZIKV	Paraiba	Huh-7, Jan	qRT-PCR	1–5	>200	
	ZIKV	Dakar 41519	Huh-7, Jan		1–5	>200	
2′-C-ethynyladenosine	DENV-2	ND	A549	CFI	1.41	40	Chen et al.[22]
							
NITD008	DENV-2	New Guinea C	A549	CFI	0.46–2.61	>100	Chen et al.[72]
	DENV-2	MY10245	PBMC	CFI	0.58	>100	Chen et al.[72]
	DENV-2	MY10340	PBMC	CFI	0.58	>100	Chen et al.[72]
	DENV-2	MY22366	PBMC	CFI	0.58	>100	Chen et al.[72]
	DENV-2	MY22713	PBMC	CFI	0.58	>100	Chen et al.[72]
	ZIKV	GZ01/2016	Vero	VTR	0.241	ND	Deng et al.[76]
	ZIKV	GZ01/2016	Vero	qRT-PCR	0.137	ND	Deng et al.[76]
	ZIKV	FSS13025/2010	Vero	VTR	0.950	ND	Deng et al.[76]
	ZIKV	FSS13025/2010	Vero	qRT-PCR	0.283	ND	Deng et al.[76]
	TBEV	Hypr	A549	CPE, CFI, VTR	0.9–2.99	>100	Lo et al.[77]
	AHFV	200300001	A549	CPE, CFI, VTR	1.51–9.29	>100	Lo et al.[77]
	KDFV	P9605	A549	CPE, CFI, VTR	1.42–4.01	>100	Lo et al.[77]
	OHFV	Bogoluvovska	A549	CPE, CFI, VTR	0.61–3.04	>100	Lo et al.[77]
NITD449	DENV (1–4)	New Guinea C	A549	CFI	1.62–6.99	ND	Chen et al.[72]
		MY10245	PBMC	CFI	5	ND	Chen et al.[72]
		MY10340	PBMC	CFI	5	ND	Chen et al.[72]
		MY22366	PBMC	CFI	5	ND	Chen et al.[72]
		MY22713	PBMC	CFI	5	ND	Chen et al.[72]

(continued)

Table 1. Continued

Structure	Virus	Strain	Cell line	Assay	EC_{50} (μM)	CC_{50} (μM)	References
NITD203	DENV (1–4)	New Guinea C	A549	CFI	0.54–0.71	ND	Chen et al.[72]
		MY10245	PBMC	CFI	<0.1	ND	Chen et al.[72]
		MY10340	PBMC	CFI	<0.1	ND	Chen et al.[72]
		MY22366	PBMC	CFI	<0.1	ND	Chen et al.[72]
		MY22713	PBMC	CFI	<0.1	ND	Chen et al.[72]
							
4′-C-azidocytidine	TBEV	Hypr	PS	VTR	2.7	>50	Eyer et al.[54]
							
Balapiravir	DENV (1–4)	Various	DC	qRT-PCR	5.2–6	ND	Nguyen et al.[83]
			Huh-7	qRT-PCR	1.9–11	ND	Nguyen et al.[83]
			PHM	qRT-PCR	1.3–3.2	ND	Nguyen et al.[83]
							
RO-9187	TBEV	Hypr	PS	VTR	0.3	>50	Eyer et al.[54]
							

(continued)

Table 1. Continued

Structure	Virus	Strain	Cell line	Assay	EC_{50} (μM)	CC_{50} (μM)	References
BCX4430	TBEV	Hypr	PS	VTR	1.48	>100	Eyer et al.[90]
	LIV	LI/31	PS	VTR	12.33	>100	Eyer et al.[90]
	KFDV	W-377	PS	VTR	11.37	>100	Eyer et al.[90]
	WNV	Eg101	PS	VTR	2.33	>100	Eyer et al.[90]
	YFV	17-D	ND	ND	14.1	>100	Warren et al.[89]
	JEV	SA14			43.6	>100	Warren et al.[89]
	DENV-2	New Guinea C			32.8	>296	Warren et al.[89]
	ZIKV	Various	Vero, Huh-7, RD	CPE	3.8–11.7[a]	>100[a]	Julander et al.[91]
	ZIKV	Various	Vero, Huh-7, RD	VYR	5.4–18.2[b]	>100[a]	Julander et al.[91]
T-1106	YFV	17-D	Vero	Neutral red uptake	1800	>4000	Julander et al.[94]
	YFV	17-D	Vero	CPE	2630	>4000	Julander et al.[94]
	YFV	17-D	Vero	Luciferase based	1080	>4000	Julander et al.[94]
6-Methyl-7-deazaadenosine	DENV-2	ND	Vero	PA	0.062	ND	Wu et al.[96]
	DENV-2	ND	Vero	qRT-PCR	0.039	ND	Wu et al.[96]
N6-(9-antranylmethyl) adenosine	TBEV	Absettarov	PEK	PA	15	>50[c]	Orlov et al.[35]

(continued)

Table 1. Continued

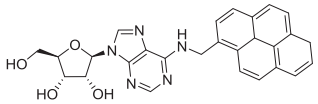
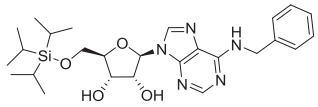
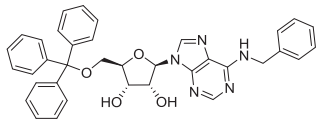
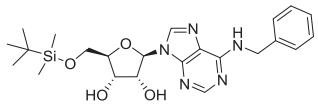
Structure	Virus	Strain	Cell line	Assay	EC_{50} (μM)	CC_{50} (μM)	References
N6-(1-pyrenylmethyl) adenosine	TBEV	Absettarov	PEK	PA	6	>50[c]	
							
N6-benzyl-5′-O-triisopropylsilyl adenosine	TBEV	Absettarov	PEK	PA	5	>50[c]	
							
N6-benzyl-5′-O-trityl adenosine	TBEV	Absettarov	PEK	PA	2	>50[c]	
							
N6-benzyl-5′-O-tert-butyldimethylsilyl-adenosine	TBEV	Absettarov	PEK	PA	20	>50[c]	
							

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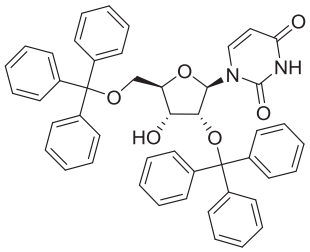
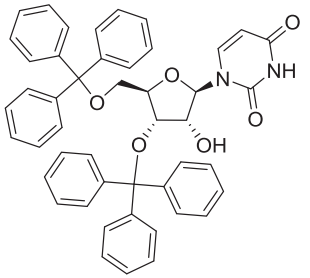
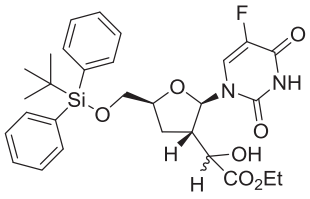
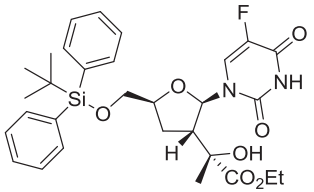
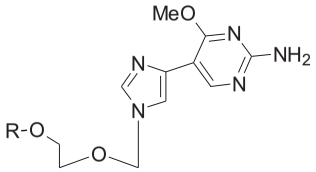
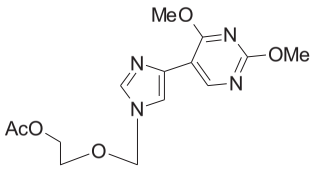
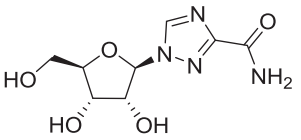
Table 1. Continued

Structure	Virus	Strain	Cell line	Assay	EC_{50} (μM)	CC_{50} (μM)	References
2′,5′Di-O-trityluridine	DENV-2	New Guinea C	Vero	CPE	30[a]	>100[a]	Saudi et al.[104]
	YFV	17-D	Vero	CPE	1.2[a]	>100[a]	Saudi et al.[104]
							
3′,5′Di-O-trityluridine	DENV-2	New Guinea C	Vero	CPE	1.75[a]	>10[a]	
	YFV	17-D	Vero	CPE	1[a]	>85[a]	
							
Inhibitors of flaviviral methyltransferase							
GRL-002	WNV	NY99	–	N-7 methylation inhibition	33.9[d]	–	Chen et al.[111]
	WMV	NY99	–	2′-O-methylation inhibition	5.5[d]	–	Chen et al.[111]
	WMV	NY99	A549	VTR	52	48	Chen et al.[111]

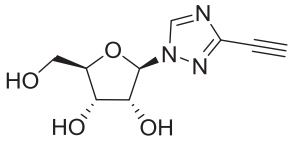
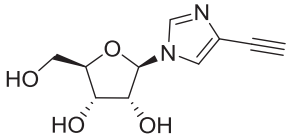
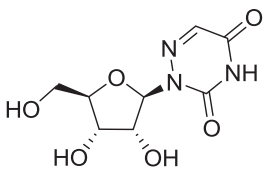
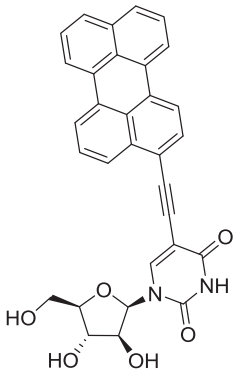
(continued)

Table 1. Continued

Structure	Virus	Strain	Cell line	Assay	EC_{50} (μM)	CC_{50} (μM)	References
GRL-003 	WMV	NY99	–	N-7 methylation inhibitin	17.3[d]	–	Chen et al.[111]
	WMV	NY99	–	2′-O-methylation inhibition	19.8[d]	–	Chen et al.[111]
	WMV	NY99	A549	VTR	27	236	Chen et al.[111]
Flex 1, R=H, Ac, Flex 1-TP, R=triphosphate 	DENV-3	ND	–	2′-O-methylation inhibition	22[d]	–	K. Seley-Radtke, manuscript in preparation
	ZIKV	French Polynesisa (2013/PF KJ776791.2)	–	2′-O-methylation inhibition	22[d]	–	
Flex 2 	DENV-3	ND	–	2′-O-methylation inhibition	3.2[d]	–	unpublished results, Smee laboratory, Utah
Ribavirin and other nucleoside synthesis inhibitors							
Ribavirin 	DENV (1–4)	Various	Vero	CPE	19.8–41.9[a]	>100[a,e]	Crance et al.[31]
	JEV	Nakayama	Vero	CPE	134.1[a]	>100[a,e]	Crance et al.[31]
	WNV	E101	Vero	CPE	71.2[a]	>100[a,e]	Crance et al.[31]
	USUV	DakArD 19848	Vero	CPE	62.6[a]	>100[a,e]	Crance et al.[31]
	LGTV	ND	Vero	CPE	33.9[a]	>100[a,e]	Crance et al.[31]
	YFV	17D and FNV	Vero	CPE	42.4; 48.2[a]	>100[a,e]	Crance et al.[31]
	WESSV	ND	Vero	CPE	91.7[a]	>100[a,e]	Crance et al.[31]
	ZIKV	Various	Vero, Huh-7, RD	CPE	3.8–142.9[a]	>100[a,e]	Crance et al.[31] and
	ZIKV	Various	Vero, Huh-7, RD	VYR	9.52–281[b]	>100[a,e]	Julander et al.[91]

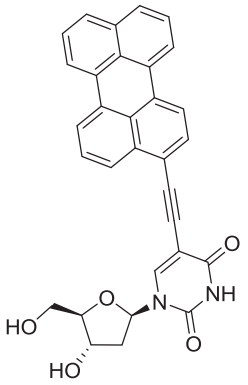
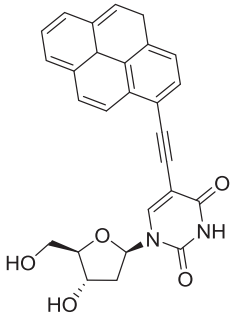
(continued)

Table 1. Continued

Structure	Virus	Strain	Cell line	Assay	EC_{50} (μM)	CC_{50} (μM)	References
ETAR	DENV-2	ND	Vero	ND	9.5	>1000	McDowell et al.[135]
							
IM18	DENV-2	ND	Vero	ND	106.1	ND	McDowell et al.[135]
							
6-Azauridine	DENV (1–4)	Various	Vero	CPE	0.1–0.5[a]	>100[a,e]	Crance et al.[31]
	JEV	Nakayama	Vero	CPE	0.5[a]	>100[a,e]	Crance et al.[31]
	WNV	E101	Vero	CPE	0.2[a]	>100[a,e]	Crance et al.[31]
	USUV	DakArD 19848	Vero	CPE	0.1[a]	>100[a,e]	Crance et al.[31]
	LGTV	ND	Vero	CPE	0.2[a]	>100[a,e]	Crance et al.[31]
	YFV	17D and FNV	Vero	CPE	0.2; 0.2[a]	>100[a,e]	Crance et al.[31]
	WESSV	ND	Vero	CPE	1.3[a]	>100[a,e]	Crance et al.[31]
	ZIKV	DakArB 11514	Vero	CPE	1.5[a]	>100[a,e]	Crance et al.[31]
							
Rigid amphipathic nucleosides							
5-(Perylen-3-yl)ethynyl-arabino-uridine	TBEV	Absettarov	PEK	PA	0.018[f]	>50[c]	Orlov et al.[101]
							

(continued)

Table 1. Continued

Structure	Virus	Strain	Cell line	Assay	EC_{50} (μM)	CC_{50} (μM)	References
5-(Perylen-3-yl)ethynyl-2′-deoxy-uridine	TBEV	Absettarov	PEK	PA	0.024[f]	>50[c]	Orlov et al.[101]
							
5-(Pyren-1-yl)ethynyl-2′-deoxy-uridine	TBEV	Absettarov	PEK	PA	0.98[f]	>50[c]	Orlov et al.[101]
							

AHFV: Alkhurma hemorrhagic fever virus; CFI: cellular flavivirus immunodetection; CPE: cytopathic effect reduction assay; DC: dendritic cells; DENV: dengue virus; JEV: Japanese encephalitis virus; KFDV: Kyasanur Forest disease virus; LGTV: Langat virus; LIV: louping ill virus; ND: not determined; OHFV: Omsk hemorrhagic fever virus; PA: plaque reduction assay; PBMC: peripheral blood mononuclear cells; PHM: primary human macrophages; POWV: Powassan virus; TBEV: tick-borne encephalitis virus; USUV: Usutu virus; VTR: viral titer reduction assay; VYR: viral yield reduction assay; WESSV: Wesselsbron virus; WNV: West Nile virus; YFV: yellow fever virus; ZIKV: Zika virus.

[a]EC_{50} and CC_{50} values are expressed as μg/ml.

[b]EC_{90} values, expressed as μg/ml.

[c]CC_{50} (24 h): the cell culture was treated for 24 h with the appropriate compound to obtain the cytotoxicity data.

[d]IC_{50} values obtained from enzyme-based inhibition assays.

[e]CC_{50} values for confluent compound-treated cells.

[f]EC_{50} (sim): the cell culture was TBEV infected and simultaneously treated with the appropriate compound.

Table 2. Examples of in vivo antiflaviviral activities of selected nucleoside analogs.

Compound	Animal model	Admin. route	Treatment start	Treatment length	Dose	Virus	Infection route	Survival rate (%)	References
7-Deza-2′-*C*-methyladenosine	BALB/c mouse	i.p.	0 dpi	17 days	25 mg/kg/2× day	TBEV	s.c.	60	Eyer et al.[61]
					5–15 mg/kg/2× day			35–50	
	AG129 mouse	p.o.	−1 h	10 days	50 mg/kg/day	ZIKV	i.p.	25	Zmurko et al.[62]
2′-*C*-methylcytidine	ICR suckling mouse	i.c.	1 dpi	5 days	15 or 30 mg/kg/day	DENV	i.c.	60	Lee et al.[56]
	Syrian golden hamster	i.p.	−4 h	4–7 days	120 mg/kg/day	YFV	i.p.	90	Julander et al.[63]
			3 dpi					80	
Sofosbuvir	C57BL/6J mouse	p.o.	1 dpi	7 days	33 mg/kg/day	ZIKV	s.c.	50	Bullard-Feibelman et al.[69]
	Suckling Swiss mouse	i.p.	−24 h	7 days	20 mg/kg/day	ZIKV	i.p.	40	Ferreira et al.[71]
			2 dpi					25	
NITD008	AG129 mouse	p.o.	0 h	ND	25–50 mg/kg/day	DENV	i.v.	100	Yin et al.[26]
			1 dpi		25 mg/kg/day			70	
	A129 mouse	p.o.	ND	5 days	50 mg/kg/day	ZIKV	i.p.	50	Deng et al.[76]
T-1106	Syrian golden hamster	i.p.	−4 h	8 days	100 mg/kg/2× day	YFV	i.p.	100	Julander et al.[93]
			3 dpi					100	
			5 dpi					20	
BCX4430	Syrian golden hamster	i.p.	−4 h	7 days	12.5–125 mg/kg/day	YFV	i.p.	100	Julander et al.[92]
			4 dpi		200 mg/kg/day			80	
	AG129 mouse	i.m.	−4 h	7–8 days	300 mg/kg/day	ZIKV	s.c.	90	Julander et al.[91]
			1 dpi					85	
			3 dpi					10	

DENV: dengue virus; i.c.: intracerebral administration; i.m.: intramuscular administration; i.p.: intraperitoneal administration; i.v.: intravenous administration; ND: not determined; p.o.: *per os* (oral administration); s.c.: subcutaneous administration; TBEV: tick-borne encephalitis virus; YFV: yellow fever virus; ZIKV: Zika virus.

Table 3. Heterobase substitutions and ribose modifications of selected flaviviral RdRp nucleoside inhibitors.

Heterobase identity/ modification	Type of the glycosidic bond	Ribose substitution	Ribose position	Example
4-Aza-7,9-dideazaadenie	*C*-glycosidic	-CN (α)	*C*1′	GS-441524
4-Aza-7,9-dideazaadenie	*C*-glycosidic	-CN (α)	*C*1′	GS-5734
Adenine	*N*-glycosidic	$-CH_3$ (β)	*C*2′	2′-C-methyladenosine
7-Deazaadenine	*N*-glycosidic	$-CH_3$ (β)	*C*2′	7-Deaza-2′-*C*-methyladenosine
Guanine	*N*-glycosidic	$-CH_3$ (β)	*C*2′	2′-*C*-methylguanosine
Cytosine	*N*-glycosidic	$-CH_3$ (β)	*C*2′	2′-*C*-methylcytidine
Uracil	*N*-glycosidic	$-CH_3$ (β)	*C*2′	2′-*C*-methyluridine
6-*O*-methylguanine	*N*-glycosidic	$-CH_3$ (β)	*C*2′	INX-08189
Uracil	*N*-glycosidic	-F (α), CH_3 (β)	*C*2′	Sofosbuvir
Adenine	*N*-glycosidic	-ethynyl (β)	*C*2′	2′-*C*-ethynyladenosine
7-Deazaadenine	*N*-glycosidic	-ethynyl (β)	*C*2′	NITD008
7-Deaza-7-carbamoyladenine	*N*-glycosidic	-ethynyl (β)	*C*2′	NITD449
Cytosine	*N*-glycosidic	-H (α), OH (β) + N_3 (α)	*C*2′+ *C*4′	4′-Azido
Cytosine	*N*-glycosidic	$-N_3$ (α)	*C*4′	4′-Azido-aracytidine
9-Deazaadenine	*C*-glycosidic	O exchanged for N	–	BCX4430
3-Oxopyrazine-2-carboxamide	*N*-glycosidic	No substitution	–	T-1106
6-Methyl-7-deazaadenine	*N*-glycosidic	No substitution	–	6-Methyl-7-deazadenosine
Uracil	*N*-glycosidic	Trityl	*C*2′ and *C*5′	2′,5′di-*O*-trityluridine
Uracil	*N*-glycosidic	Trityl	*C*3′ and *C*5′	3′,5′di-*O*-trityluridine

Flaviviridae family, this compound exerted micromolar inhibitory activity against YFV and DENV-2 (EC_{50} values of 11 and 9.46 μM, respectively) in various cell-based screening systems.[43] Surprisingly, considerably less favorable in vitro activities (>30–51.2 μM) were reported for WNV and tick-borne flaviviruses, such as AHFV, KFDV, TBEV, and OHFV.[43,45]

A phosphoramidate prodrug of GS-441524, referred to as GS-5734, recently entered Phase II clinical trials for treatment of Ebola infections, displayed 10- to 40-fold higher antiviral effect against members of the TBEV serocomplex when compared with its parental analog GS-441524. The increased antiviral potency could be related to an improved conversion of the prodrug to the biologically active form; however, the reported SI values (2.4–8.3) indicate a low therapeutic potential for this nucleoside to treat flaviviral infections.[45] Further substitutions of GS-441524 molecule at the C1′ position with methyl, vinyl, or methyl–ethynyl moieties yielded compounds with considerably reduced potency and a narrower spectrum of antiviral activity.[43]

2′-C-methyl substituted nucleosides

2′-*C*-Methyl-nucleoside scaffolds represent the initial major class of therapeutic nucleosides developed by Merck Research Laboratories to demonstrate potent inhibition of HCV replication.[46–50] Antiviral activity of 2′-C-methylated nucleosides beyond the Flaviviridae family was reported for representatives of Picornaviridae and Caliciviridae families,[51–53] indicating the potential for broad-spectrum inhibitory activity for these compounds within the positive single-stranded RNA viruses.

The 2′-C-methyl substituent introduced at the nucleoside β-face appears to be an important structural element for highly selective micromolar inhibition of tick-borne flaviviruses, particularly for TBEV, AHFV, KDFV, OHFV, and POWV, when assayed in porcine stable kidney cells (PS), human neuroblastoma cells UKF-NB4, or adenocarcinomic human alveolar basal epithelial cells A549.[24,54,55] From mosquito-transmitted flaviviruses, 2′-*C*-methylated nucleosides inhibited WNV, DENV, and YFV in cell-based or cell-free reporter assay systems, showing low micromolar antiviral activities.[50,56,57] A phosphoramidate prodrug of 6-*O*-methyl-2′-*C*-methylguanosine, denoted as INX-08189, exerted nanomolar inhibitory activity against DENV-2, and the combination of INX-08189 with ribavirin resulted in significant synergistic anti-DENV activity in vitro.[58] 7-Deaza-2′-*C*-methyladenosine together with other 2′-*C*-methylated species was the first described nucleoside-based inhibitors of ZIKV, after its epidemiological outbreaks in Oceania and Latin America.[23,59] 7-Deaza-2′-*C*-methyladenosine showed anti-ZIKV potency not only on immortalized cell lines, but also on induced pluripotent stem cell-derived neuronal cell types, such as cortical neurons, motor neurons, and astrocytes.[60] The triphosphate analogs of 2′-C-methylated nucleosides exhibited strong inhibitory activity in a

polymerase-based in vitro assay using an active recombinant ZIKV RdRp.[61]

Strong antiflaviviral activity for several 2′-*C*-methyl modified nucleosides was also demonstrated using numerous in vivo efficacy models. For example, 7-deaza-2′-*C*-methyladenosine substantially improved disease outcome, increased survival, and reduced signs of neuroinfection and viral titers in the brains of BALB/c mice infected with a lethal dose of TBEV.[62] This compound also reduced viremia in AG129 mice infected with the African strain of ZIKV.[59] Moreover, 2′-*C*-methylcytidine protected suckling mice challenged with DENV[56] and hamsters infected with a lethal dose of YFV, even when administered up to three days postinfection.[63]

2′-Fluoro-2′-C-methyl substituted nucleosides

Similar to the 2′-*C*-methylated nucleosides, analogs possessing the 2′-α-fluoro-2′-β-*C*-methyl modification were initially identified as promising inhibitors of HCV polymerase activity.[64] Sofosbuvir, a phosphoramidate prodrug of 2′-fluoro-2′-*C*-methyluridine developed by Gilead Sciences, Inc., is one of the most potent and selective inhibitors in this series and was approved by the Food and Drug Administration (FDA) for the treatment of chronic HCV infection.[65] Sofosbuvir is nontoxic for most human cell lines and is a very poor substrate for human mitochondrial RdRp, resulting in an acceptable safety profile and negligible mitochondrial toxicity.[66,67]

Sofosbuvir was demonstrated to inhibit the ZIKV RdRp in a recombinant polymerase assay[68] and to suppress ZIKV replication in different cell-based systems using U87 glioblastoma cells, baby hamster kidney fibroblasts (BHK-21), SH-sy5y neuroblasts, hepatocarcinoma Huh-7 cells, Jar human placental choriocarcinoma cells, neural stem cells, and brain organoids with nanomolar or low micromolar inhibitory activity.[25,69] Interestingly, no inhibition of ZIKV replication with sofosbuvir even at the 50 μM level was observed in Vero cells,[23] indicating that the sofosbuvir-mediated anti-ZIKV effect is strongly cell-type dependent.[70] Sofosbuvir protected inbred C57BL/6J mice, which were previously immunosuppressed with a single dose of anti-Ifnar1 blocking monoclonal antibody, against ZIKV-induced mortality.[69] Moreover, this compound reduced viral titer in blood plasma, spleen, kidney, and brain in suckling Swiss albino mice and prevented virus-induced neuromotor impairment in ZIKV-infected animals.[71]

Surprisingly however, sofosbuvir was inactive against TBEV, when screened on both PS and UKF-NB4 cells.[54] Another 2′-α-fluoro-2′-β-*C*-methyl modified nucleoside, PSI-6206, and its 3′,5′-diester prodrug mericitabine, also displayed no activity against TBEV replication. The lack of anti-TBEV activity for the 2′-α-fluoro-2′-β-C-methyl modified nucleosides could be ascribed to their inefficient intracellular conversion to their corresponding triphosphates and, moreover, to extensive deamination/demethylation in the tested cell cultures resulting in their conversion to uridine.[46,54]

2′-C-ethynyl substituted nucleosides

2′-*C*-Ethynyl substituted nucleosides have been primarily identified as inhibitors of DENV.[22,72–75] 2′-*C*-Ethynyladenosine represents the lead compound in this series, which inhibited DENV-2 replication with an EC_{50} of 1.41 μM in cell-based assays and with a CC_{50} value of 40 μM.[22] The 7-deaza derivative of 2′-*C*-ethynyladenosine, denoted as NITD008, inhibited DENV of different serotypes at submicromolar or low micromolar concentrations when tested in BHK-21 cells, A549 cells, Huh-7 hepatocarcinoma cells, and human peripheral blood mononuclear cells (PBMCs), showing a significantly improved cytotoxicity profile (CC_{50} of >100 μM) compared with that of 2′-*C*-ethynyladenosine.[26] NITD008 was also assayed against WNV, YFV, and ZIKV and exhibited excellent in vitro inhibitory parameters and a protective effect against WNV and ZIKV infections in mouse efficacy models.[26,76] NITD008 was also reported to effectively inhibit the in vitro replication of TBEV, AHFV, KDFV, OHFV, and POWV, with nanomolar or low micromolar antiviral levels observed in various cell-based screening systems.[77]

Introduction of the C7 carbamoyl moiety to NITD008 molecule provided another 2′-ethynyl modified derivative, referred to as NITD449. Despite its low micromolar anti-DENV efficacy, this nucleoside disappointingly exhibited only low levels in plasma when dosed orally.[72] To increase the oral bioavailability, the isobutyryl ester prodrug of NITD449, designed as NITD203, was synthesized. NITD203 successfully exhibited both nanomolar anti-DENV activity as well as improved pharmacokinetic parameters.[72] Although NITD008 and NITD203 showed anti-DENV potency in rodent models, even when the treatment was delayed up to 48 h after infection, both nucleosides failed in preclinical toxicity studies in rats and dogs due to their insufficient safety profiles.[26,72] NITD008 failed to achieve no-observed-adverse-effect levels (NOAEL) when rats (10 mg/kg/day) and dogs (1 mg/kg/day) were dosed daily for two weeks.[26] Similarly, NOAEL was not achieved for NITD203 in the two-week toxicity test when rats were dosed at 30 and 75 mg/kg/day.[72]

Further substitutions of the C7 position of NITD008 with fluoro or cyano moieties provided compounds with nano- or low micromolar anti-DENV activities and acceptable cytotoxicity profiles. In contrast, 2′-*C*-ethyl, -vinyl, or -methylethynyl substituted derivatives of 2′-*C*-ethynyladenosine were completely inactive when tested against DENV. Similarly, the exchange of the adenine base for cytosine or guanine also yielded inactive compounds.[22,72]

2′-O-substituted nucleosides

2′-*O*-Methyl substituted adenosine, guanosine, cytidine, and uridine were evaluated for their potential anti-TBEV activity; however, no or negligible antiviral effects were observed when tested in both PS and UKF-NB4 cells.[54] Such abrogation of the nucleoside inhibitory activity could be related to the elimination of the 2′-α-hydroxy hydrogen bond donor/acceptor properties when the methyl moiety is introduced at the nucleoside O2′ position.[46,54] The ability of flaviviral RdRp to discriminate among nucleosides modified at the 2′-position on the nucleoside α-face is likely related to the need of the polymerase to avoid incorporation of 2′-α-deoxynucleoside monophosphates into the viral nascent RNA chain.[46]

3′-C- and 3′-O-substituted nucleosides

Methylation of the C3′ or O3′ position to generate the corresponding 3′-*C*-methyl or 3′-*O*-methyl modified structures resulted in a complete loss of anti-TBEV activity, regardless of the purine/pyrimidine heterobase identity. These nucleosides exerted no cytotoxic effects and caused no morphological changes in PS or porcine embryo kidney (PEK) cell cultures.[35,54] Similarly, 3′-deoxynucleosides exhibited no detectable inhibitory effect on TBEV replication.[54] In contrast, several nucleosides with a trityl group at the C3′ position showed micromolar inhibitory activity against DENV and YFV (see below).[78] The observed inactivity of 3′-*O*-methylated and 3′-dehydroxylated nucleosides could be related to either a strict requirement of the TBEV RdRp active site for a 3′-hydroxyl group to form the appropriate hydrogen bonding interactions with the nucleoside triphosphate molecule, or, to inefficient cellular uptake and metabolism to convert the nucleoside molecule into the corresponding triphosphate form.[46,54]

4′-Azido substituted nucleosides

Using high-throughput screening of large nucleoside libraries in combination with a rational drug design approach by investigators at Roche, several cytidine analogs with an azido group at the C4′ position were identified as potent inhibitors of HCV replication in subgenomic replicon assays.[79–81] It was later shown that these compounds were also highly active against henipaviruses and other paramyxoviruses.[82] Two 4′-azido modified nucleoside analogs, 4′-azidocytidine (R-1479) and 4′-azido-aracytidine (RO-9187), showed nano- or micromolar in vitro antiviral activity against TBEV.[54] Moreover, both compounds were found to be active also against WNV (L. Eyer, manuscript in preparation).

The anti-TBEV activity of RO-9187 (the arabino-counterpart of 4′-azidocytidine) was unexpected, as this compound lacks the 2′-α-hydroxy moiety, a determinant which was generally considered to be crucial for specific hydrogen-bonding interactions with RdRps during the RNA replication process.[54,80] Some additional interactions of the polymerase active site with both the 2′-β-hydroxy substituent and the 4′-azido substituent are thought to compensate for the loss of the 2′-α-hydroxy interaction, resulting in the strong and selective anti-TBEV activity of RO-9187. Interestingly, the anti-TBEV efficacy of both 4′-azido modified nucleosides was cell-type dependent; the compounds were active only in PS cells, but not in UKF-NB4 cells.[54]

An ester prodrug of 4′-azidocytidine, denoted as balapiravir, was completely inactive against TBEV in vitro, probably because of its poor intracellular uptake or insufficient kinase phosphorylation in the tested host cell lines.[54] In contrast, balapiravir was reported to show strong in vitro antiviral activity against DENV of various serotypes; it was the first direct antiviral agent tested clinically for DENV infection. Unfortunately, this compound failed to achieve antiviral efficacy in DENV patients, which was reflected in a negligible reduction of DENV viremia and persistence of clinical symptoms, even though the plasma concentration of the compound was higher than the 50% effective concentration.[83] One of the possible explanations is that DENV infection stimulates PBMCs to produce cytokines, which are responsible for the decreased efficiency of the conversion of balapiravir to its triphosphate form.[84]

Interestingly, nucleoside analogs combining the 4′-azido moiety with the 2′-*C*-methyl group in one molecule (e.g. 2′-*C*-methyl-4′-azidocytidine) did not exhibit any antiviral activity when tested against HCV; however, the corresponding 5′-monophosphate prodrugs displayed considerably improved virus inhibitory effects (EC_{50} values in the micromolar ranges) without apparent cytotoxicity.[85] Other interesting compounds, 4′-azido-2′-deoxy-2′-*C*-methylcytidine and its ester prodrugs, were found to show nano- or low micromolar antiviral efficacy in vitro.[86] Unfortunately, such nucleoside scaffolds have not been evaluated against

arthropod-borne flaviviruses; the reported results originate from HCV replicon-based assays.[85,86]

Imino-C-nucleoside analog BCX4430

BCX4430, developed by BioCryst Pharmaceuticals Inc., is an adenosine analog with the furanose oxygen on the ribose ring replaced by nitrogen and the heterobase nitrogen 9 replaced by carbon.[87] This interesting nucleoside is classified as imino-*C*-nucleoside.[88] BCX4430 was initially described as an inhibitor of filovirus infections, exerting antiviral activity against a broad spectrum of single-stranded RNA viruses, particularly against members of the Bunyaviridae, Arenaviridae, Picornaviridae, Orthomyxoviridae, Paramyxoviridae, Coronaviridae, and Flaviviridae families.[89] Currently, this compound has entered Phase I clinical trials for Ebola virus disease treatment focused on intramuscular administration of BCX4430 in healthy volunteers and to date has shown promising pharmacokinetics properties and good tolerability.[87]

BCX4430 is active against numerous mosquito-transmitted flaviviruses, such as WNV (EC_{50} of 2.33 μM)[90] and representatives of both the African and Asian lineages of ZIKV (3.8–11.7 μg/ml).[91] For YFV, JEV, and DENV-2, micromolar EC_{50} values were reported.[89] In vivo efficacy of BCX4430 was also demonstrated in a lethal hamster model of YFV infection and in a mouse model of ZIKV infection.[91,92] Low micromolar antiviral activity of BCX4430 was also described for some of the medically important tick-borne flaviviruses, such as TBEV, LIV, and KFDV.[90]

Heterocyclic base-modified nucleosides

Heterocyclic base-modified nucleosides with demonstrated antiflaviviral activities include T-1106,[93–95] 6-methyl-7-deazaadenosine,[96] and numerous *N6*-alkyl or aryl substituted nucleosides.[35,97] T-1106 is a ribosylated analog of the pyrazine derivative T-705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide, favipiravir),[98] which was described to inhibit the HCV RdRp in enzyme assays.[93] Nucleoside inhibitor T-1106 displayed a negligible in vitro activity against YFV in Vero cells, as well as in luciferase-based assays.[94] In contrast, this compound exerted favorable efficacy, bioavailability, and low toxicity in a hamster model of YFV infection, using a hamster-adapted Jimenez YFV strain. After intraperitoneal application of 100 mg/kg/day, T-1106 improved survival rates, serum parameters, weight gain, and mean day to death when administered up to four days after virus challenge.[93,94] In this model, the combination of T-1106 with ribavirin gave superior effects compared to monotherapy (with either T-1106 or ribavirin).[95]

6-Methyl-7-deazaadenosine is a hydrophobic mimic of adenosine showing nanomolar antiviral activity against DENV-2 in a Vero cell-based screening system, as well as in luciferase-driven DENV-2 replicon assay, with no cytotoxic effects noted after 7 h of treatment.[96] Related compounds such as 6-methyl-1-deazaadenosine and 6-methyl-4-deazaadenosine were completely inactive when tested against DENV-2. Mechanistic studies of the 5′-triphosphate of 6-methyl-7-deazaadenosine revealed that this nucleotide is an efficient substrate for viral RdRp (screened against polio RdRp) and is incorporated into nascent viral RNA strains mimicking both ATP and GTP.[96]

N6-Alkyl or aryl substituted nucleosides, originally identified as inhibitors of Lassa fever virus, Marburg virus, and enterovirus A71, showed interesting bioactivity profiles when tested against TBEV.[35,99,100] Whereas *N6*-methyladenosine and *N6*-benzyladenosine were completely inactive, nucleosides with bulky substituents, such as *N6*-(9-anthracenylmethyl)adenosine and *N6*-(1-pyrenylmethyl)adenosine, exerted a micromolar anti-TBEV effect. In contrast, *N2*- and *N4*-substituted analogs showed no antiviral activity. Moderate anti-YFV and anti-DENV activities were also observed in *N6*-subsitituted analogs of 5′,3′-*O*- and 5′,2′-*O*-*tert*-butyldiphenylsilyl-modified adenosine.[97] The mechanism of action of these nucleosides is poorly understood; however, they likely interact with the RdRp or MTase domain of the flaviviral NS5 protein, as demonstrated by docking studies.[35] Bulky aromatic substituents could also play a role in the interaction with the viral membrane resulting in cell entry inhibition.[101]

Tritylated nucleosides

In a large-scale cell-based screening campaign of alkylated, silylated, or acylated pyrimidine nucleosides, 2′,5′di-*O*-trityluridine and 3′,5′di-*O*-trytiluridine were identified as inhibitors of DENV-2 and YFV replication, showing high antiviral potency and favorable cytotoxicity profiles in Vero cells.[78,102,103] Substantial antiviral effect against YFV was observed also in 2′-deoxy-3′,5′-di-*O*-trityluridine and in several 5-halogenated bis-tritylated pyrimidine nucleosides; however, their anti-DENV activity was proven to be weaker.[104] Thymidine or 2′-deoxyuridine congeners of 2′,5′- and 3′,5′-tritylated nucleosides led to the loss of antiflavivirus activity or to increased compound cytotoxicity.

The mechanisms of action of tritylated nucleosides are not completely understood. Based on the observed inhibition of DENV replication in subgenomic replicon assays, it is assumed that these compounds may be

acting as inhibitors of intracellular viral replication events rather than suppressing either early or late processes of viral infection, such as entry or assembly.[103] Although the presence of large, hydrophobic trityl moieties does not make these structures ideal candidates for further drug development, their chemical structures may provide valuable information for advanced mechanistic studies and for further development of related nucleoside scaffolds with improved biological parameters.[104]

Nucleoside inhibitors of flaviviral MTase

The NH_2 domain of flaviviral NS5 protein is associated with the virus's MTase activity, which is involved in methylation of the 5′-cap structure of genomic RNA.[8,105] The flaviviral cap structure is formed by the conserved dinucleotide sequence AG (m7GpppAm) and is crucial for mRNA stability and efficient translation.[106] Two topologically distinct methylation reactions are mediated by the flaviviral NS5 MTase: the *N7* of guanine is methylated by the (guanine-*N*7)-MTase and the first nucleotide of RNA transcript is further methylated at the ribose 2′-hydroxyl by (nucleoside-2′-*O*)-MTase. The resulting product of the methyl donation for both methylation reactions by S-adenosyl-L-methionine is the nucleoside analog S-adenosyl-L-homocysteine (SAH).[107]

SAH and the nucleoside antibiotic sinefungin are natural nonselective inhibitors of many eukaryotic and viral MTases, including those of DENV[108,109] or ZIKV.[61] Chemical derivatization of SAH at the *N6* position provided inhibitors with nano- or low micromolar activity against DENV MTase, which did not inhibit the corresponding human enzymes.[110] Other rationally designed nucleosides with potent inhibitory activity against MTase contain a thymine base with a hydrophobic methyl *tert*-butyl substituent at the 5′ position of the sugar moiety.[111] Two of these nucleosides, GRL-002 and GRL-003, inhibited the *N7* and 2′-*O* MTase activity of WNV in enzyme-based assays and the observed MTase inhibition was in agreement with the micromolar in vitro anti-WNV efficacy.[111] Another class of promising selective antiflavivirus compounds is represented by 5′-silylated 3′-1,2,3-triazole-substituted nucleoside scaffolds derived from 3′-azidothymidine. Similar structures were originally developed for HIV-1 inhibition.[27] Both the 5′-silyl protecting group and the 3′ bulky triazole substituent appeared to be crucial structural elements for low micromolar inhibition of flaviviral MTase in enzyme-based assays, as well as for inhibition of WNV and DENV replication in cell culture. These nucleosides inhibit the methylation reactions through competitive interactions with the substrate binding site and also with the GTP-binding pocket of the flaviviral NS5 MTase.[28]

Recently, a novel series of flexible nucleoside analogs known as "fleximers" have exhibited activity against several hard to treat viruses, including filoviruses such as Ebola and Sudan,[112] coronaviruses including Severe Acute Respiratory Virus and Middle East Respiratory Virus,[113] as well as most recently, flaviviruses including ZIKV and DENV. The fleximers feature a "split" purine nucleobase that has been shown to impart significant activity to the nucleoside scaffold as well as to allow it to overcome resistance related to point mutations.[114–116] The most recent series combined the fleximer approach with the acyclic nucleoside acyclovir, an FDA approved drug for herpes virus. While these analogs inhibited the aforementioned viruses, acyclovir shows no activity against any of those viruses, thereby underscoring the importance of the fleximer approach. Since those initial findings, these compounds have also demonstrated potent activity against DENV and ZIKV (K. Seley-Radtke, manuscript in preparation). Preliminary results indicate that these compounds target the DENV and ZIKV NS5 in at least its cap-MTase activity, with negligible effects on the cognate human *N7*-MTase. In that regard, initial screening revealed promising levels of MTase inhibition, particularly for the triphosphate of the compound (Flex 1-TP), with an IC_{50} of 22 μM for both the DENV and ZIKV 2′-*O*-MTase (K. Seley-Radtke, manuscript in preparation). As a result, Flex 1-TP was further tested against DENV NS5, and while it was not incorporated, it successfully inhibited further incorporations of additional nucleotides, thereby halting replication, however not as a typical chain terminator. In addition, the acetate-protected dimethoxy analog (Flex 2) is a potent DENV inhibitor (3.2 μM, unpublished results, Smee laboratory, Utah); however, more work needs to be done to fully elucidate these novel compounds' mechanism of action. It may well be that these nucleotide analogs target the NS5 protein at both the RdRp and MTase regions, which would make them highly effective viral inhibitors, with low probability of viral resistance developing.

Nucleoside inhibitors of flaviviral NTPase/helicase

Flaviviral NTPase and helicase activities are associated with the COOH-proximal domain of the NS3 protein.[117] Flaviviral helicases are capable of unwinding duplex RNA structures during viral replication by disrupting the hydrogen bonds keeping the two strands together.[118,119] The helicase activity is strictly associated with NTP hydrolysis (NTPase activity); the released

chemical energy is used for the translocation of the enzyme along the double-helix structures, capturing the exposed single strand regions or for a direct disruption of the hydrogen bonds between the two RNA strains.[120]

Specific nucleoside inhibitors of flaviviral NTPase/helicase can interact with dsRNA or DNA resulting in the weakened stability of double-helix structures or by steric hindrance of translocation of the enzyme along the polynucleotide chain. Such a mechanism could modulate the efficacy of the unwinding reaction or NTPase activity of the enzyme and therefore, affect the viral replication process.[117–121] Weak inhibitory effects on flaviviral NTPase/helicases were described for ribavirin triphosphate,[124,125] 5′-*O*-fluorosulfonylbenzoyl esters of purine nucleosides,[126,127] or halogenated benzotriazole-modified nucleosides.[30,122] These compounds were primarily evaluated in enzyme-based assays for their putative anti-HCV activity; however, some of them have been found to inhibit also NTPase/helicases of WNV, JEV, or DENV.[117–127]

Ribavirin and other nucleoside synthesis inhibitors

Ribavirin, a nucleoside analog featuring a [1,2,4]triazole ring for a nucleobase, is a licensed drug against various RNA viruses. The predominant inhibitory mechanism for ribavirin against flaviviral replication is the suppression of de novo biosynthesis of guanine nucleotides through direct inhibition of inosine monophosphate dehydrogenase, an enzyme converting inosine monophosphate to xanthosine monophosphate, a precursor in GTP biosynthesis.[32] Speculation over other modes of action for ribavirin includes specific inhibition of the viral RdRp,[128] accumulation of mutations in viral genomes resulting in error catastrophe,[129,130] interference with mRNA capping guanylation,[131] and immuno-modulation promoting the Th1 antiviral response.[32,132,133]

Ribavirin was shown to exert a moderate inhibitory effect for multiple mosquito-borne flaviviruses in various cell cultures,[31,134,135] often being used as a positive control in many in vitro[25,59,92] and in vivo antiviral studies.[91–94] Ribavirin administered to YVF-infected hamsters challenged intraperitoneally, resulted in significant improvement in survival rates, even if the therapy was started two days post-YFV infection.[93,94] In contrast however, in primates, only a weak prophylactic effect on viremia in rhesus monkeys challenged with DENV was observed.[136] Several ribavirin derivatives were recently synthesized and showed interesting bioactivity profiles: ETAR (1-β-D-ribofuranosyl-3-ethynyl-[1,2,4]triazole) and IM18 (1-β-D-ribofuranosyl-4-ethynyl-[1,3]imidazole) inhibited DENV-2 replication in Vero cells by more than 10-fold compared with ribavirin and showed no detectable cytotoxic effects up to 1000 μM.[135] Another derivative, EICAR (1-β-D-ribofuranosyl-5-ethynyl-imidazole-4-carboxamide), was reported to possess a similar in vitro spectrum of antiviral activity, but lower selectivity compared with those of ribavirin.[137]

Two nucleosides whose antiviral activity is based on the depletion of the intracellular nucleoside pool are 6-azauridine and 5-aza-7-deazaguanosine. 6-Azauridine and its derivatives are inhibitors of orotidine monophosphate decarboxylase blocking cellular de novo pyrimidine biosynthesis.[31,33] 6-Azauridine proved to be active against numerous arthropod-borne flaviviruses,[55] however exhibited slight cytotoxicity with an inhibitory effect on the growth of host cells.[24] A triacetate prodrug of 6-azauridine showed low micromolar activity against AHFV and WNV in vitro[55] and very low toxicity in both animal and human studies.[138] Another derivative, 2-thio-6-azauridine, exerted a moderate inhibitory effect on WNV.[33] Similar results were achieved using 5-aza-7-deazaguanosine (ZX-2401)[139]; this compound exhibited synergistic in vitro anti-YFV activity in combination with interferon. The mechanism of action for 5-aza-7-deazaguanosine is currently unknown; however, it is conceivable that it likely resembles that of ribavirin.[34]

Rigid amphipathic nucleosides

Nucleoside derivatives containing bulky aromatic substituents (perylene or pyrene moieties) attached to the heterocyclic base were originally synthesized as fluorescent nucleoside probes[140–142]; later these structures were identified as inhibitors of herpes simplex virus, type 1 and 2 (HSV-1 and HSV-2), vesicular stomatitis virus, and Sindbis virus replication.[143] Further studies demonstrated their broad-spectrum antiviral activity against other enveloped viruses, such as influenza virus, murine cytomegalovirus, and HCV.[144] The mechanism of action of these rigid amphipathic nucleosides is based on their incorporation into the viral or cellular membranes, preventing fusion.[143,144] Alternatively, these nucleosides may also function by photosensitization of lipid membranes, resulting in irreversible damage of enveloped virion particles.[145]

5-(Perylen-3-yl)ethynyl-arabinouridine and 5-(perylen-3-yl)ethynyl-2′-deoxyuridine were shown to act as strong inhibitors of TBEV in PEK cell culture.[101] The perylene moiety as well as the rigid ethynyl linker appeared as crucial structural elements for nanomolar anti-TBEV activity and low cytotoxicity (>50 μM). Interestingly, uracil nucleosides bearing the pyrene moiety, such as 5-[(pyren-3-yl)methoxypropyn-1-yl]-

2′-deoxyuridine and 5-(pyren-1-yl)ethynyl-2′-deoxyuridine, showed almost a 10-fold lower anti-TBEV potency compared to their perylene-substituted counterparts. Such compounds, if not used as therapeutic agents, could still contribute to a better understanding of different modes of action of various nucleoside scaffolds.[101]

Challenges and complications of antiflavivirus nucleoside analog development

Introduction of various chemical substituents into different positions of the nucleoside scaffold can dramatically affect the physicochemical properties of the compound. Such modifications can also significantly influence the compound's biological/pharmacokinetic parameters, such as cellular uptake,[15,146] the ability of the compound to be activated (phosphorylated) by cellular kinases,[147] degradation by nucleoside catabolic enzymes,[148] or cellular toxicity.[149,150] Use of nucleoside analogs can also result in the undesirable emergence of drug-resistant virus mutants.[151–153] This section highlights the most important challenges and complications toward the development of nucleoside inhibitors of arthropod-transmitted flaviviruses and suggests possible strategies to surmount these difficulties.

For most nucleoside analogs, the first kinase phosphorylation is the rate-limiting step for the conversion to the nucleoside triphosphates. This limitation has a major influence on nucleoside analog antiviral activity[147,154] but can be bypassed by the use of a monophosphate prodrug approach based on the introduction of the phosphorylated group into the 5′ nucleoside position. The phosphorylated group includes masking moieties on the charged phosphate leading to a neutral and eventually hydrophobic entity able to deliver the nucleoside 5′-monophosphate into the cells (Figure 1).[15] The monophosphate prodrug approach has been shown to convert some inactive nucleosides into strong inhibitors, or, has improved the kinetics parameters of intracellular nucleoside triphosphate formation.[85] This strategy, together with enantioselective purification, led to the development of the phosphoramidate prodrug sofosbuvir, which exhibited considerably increased phosphorylation efficacy compared to the parent nucleoside, 2′-*C*-fluoro-2′-*C*-methyluridine.[155,156]

Rapid degradation of nucleoside analogs by nucleoside catabolic pathways (Figure 1) is another undesirable phenomenon, which can adversely affect the antiviral potency of some nucleoside analogs.[148] To address this problem, appropriate structural changes can be introduced into the nucleoside scaffolds to protect the nucleosides from metabolic deactivation.[157] Such a strategy was successful for 2′-*C*-methyladenosine, which was found to be rapidly deaminated by cellular adenosine deaminase to the inactive inosine derivative and/or degraded by purine nucleoside phosphorylase, resulting in poor bioavailability and rapid clearance of the nucleoside in plasma.[49,50] Substitution of the adenine *N7* nitrogen for a carbon provided metabolically stable 7-deaza-2′-*C*-methyladenosine, which is a poor substrate for both nucleoside catabolic enzymes. This compound was characterized by excellent bioavailability and half-life in beagle dogs and rhesus monkeys.[50] Another possible strategy to increase the metabolic stability of therapeutic nucleosides is based on the introduction of a *C*-glycosidic bond into the nucleoside scaffold to generate *C*-nucleoside analogs, such as BCX4430[89] or GS-5734.[43] The major advantage of *C*-nucleosides over the canonical *N*-nucleosides lies in their resistance to unwanted phosphorolysis by intracellular phosphorylases, which otherwise would cleave the *N*-glycosidic linkage.[88]

Individual host cellular types can display differences in expression levels of nucleoside kinases and other enzymes/proteins involved in nucleoside metabolism and transport. This can then result in cell-type dependent antiviral activity as manifested by different EC_{50} values for the same inhibitor when assayed on different cell lines.[158,159] Strong anti-TBEV activity for 2′-*C*-methylguanosine, 4′-azidocytidine, and 4′-azido-aracytidine in PS cells was associated with rapid and efficient nucleoside conversion to the corresponding triphosphates. On the other hand, no anti-TBEV effect or phosphorylation products were observed when both compounds were tested in UKF-NB4.[54] Similarly, the loss of anti-ZIKV activity in Vero cells for sofosbuvir is likely related to the increased expression of the multidrug resistance ABC transporter in this cell line, resulting in the efflux of the compound from the cells.[23,25] Clearly, cell-type dependent antiviral activity of some nucleosides can considerably affect the results of antiviral screens and therefore, using multiple clinically relevant cell lines for evaluation of compounds for antiviral activity is important.[70]

Another possible complication in nucleoside drug development is an undesirable toxicity profile for the nucleoside inhibitor, which can be related to poor selectivity between viral and human enzymes.[39,149,150] A typical example of a nonselective nucleoside analog is 7-deazaadenosine (tubercidin), which exhibits high in vitro cytotoxicity. This has been attributed to the incorporation of tubercidin monophosphate into cellular DNA/RNA by human polymerases.[50] In contrast, two derivatives of tubercidin, 7-deaza-2′-*C*-methyladenosine and 6-methyl-7-deazaadenosine, are selectively recognized by flaviviral RdRp and are nontoxic in most mammalian cell lines.[50,96] Some nucleoside analogs

inhibit the mitochondrial DNA or RNA polymerase γ, resulting in mitochondrial toxicity.[149,160] This has been the primary reason for the failure of several promising nucleosides/prodrugs in clinical trials, as has been shown for some 2′-*C*-methyl- and 4′-azido modified nucleosides.[67] Newly developed compounds should also be evaluated for their genotoxicity and mutagenicity, as well as for renal, cardiovascular, or liver toxicity using various biochemical in vitro assays.[161–163] Nevertheless, even if a compound successfully passes through numerous in vitro tests, it can still exhibit harmful side effects when tested in animals, as observed with the adenosine analog NITD008.[26]

In that regard, the availability of suitable animal models is crucial for the successful evaluation of a nucleoside's therapeutic in vivo antiviral efficacy. Some flaviviruses, particularly DEVN and ZIKV, do not readily replicate or cause pathology in immunocompetent mice and, therefore, the use of these rodents as animal infection models is substantially limited.[164–166] To overcome this problem, suckling or young mice,[167] AG129 mice lacking INF-α/β and INF-γ receptors,[168] IFNAR$^{-/-}$ mice lacking only the IFN-α/β receptor,[169] or immunosuppressed mice[170] can be used as appropriate models to evaluate nucleosides in vivo. However, as these animals are defective in an immune response, this model may also underestimate the real efficacy of the test compounds. Rodent-adapted flavivirus strains, such as the hamster-adapted YFV strain Jimenez,[171] mice-adapted DENV strain D2S10,[26] or ZIKV African strain Dakar 41519,[172] represent other possible options for in vivo antiviral studies; the biological properties of such viruses can be, however, considerably different compared with those of the parent human-adapted strains.[169]

Another issue is related to the length of therapeutic treatment. Most tick- and mosquito-borne flaviviruses cause acute infections, in which short-time treatment duration is expected, ranging between several days to weeks.[173] This is in contrast to chronic diseases, such as HCV, HBV, and HIV infections, which require long-lasting, and sometimes lifelong, treatment regimens.[72,173] The differences between the acute and chronic diseases should be considered during preclinical development of antiflaviviral inhibitors. Thus, some compounds that show insufficient safety profiles when tested for treatment of chronic infections can be still suitable and safe for short-term therapy of acute flaviviral diseases.[72]

Antiviral therapies based on chemical inhibitors of viral replication can be accompanied with a rapid emergence of drug-resistant mutants which substantially complicates the course of infection treatment, as seen in HIV, HBV, or HCV infections.[152,153] In flaviviruses, a rapid evolution of resistance to 2′-*C*-methylated nucleoside inhibitors was observed; this resistance was associated with a signature mutation S603T (in AHVF and TBEV)[55,62] or S604T (in ZIKV)[68] within the active site of the viral RdRp. Interestingly, the biological properties of the TBEV mutant viruses were dramatically affected, which was manifested by resistance-associated loss of viral replication efficacy in cell culture and a highly attenuated virulence phenotype in mice. This resulted in an unusually low mortality rate when mice were infected with the mutant strain.[62] As TBEV mutants resistant to 2′-*C*-methylated nucleosides are highly sensitive to 4′-azido substituted nucleosides,[62] a combination treatment based on two or more inhibitors could be a possible strategy in order to minimize the risks for the emergence of viral drug resistance.[174–176]

Conclusions

More than 200 million clinical cases caused by arthropod-borne flaviviruses, including numerous deaths, are reported worldwide annually. So many cases of infection indicate the importance for the pursuit of new small molecule-based therapeutics to combat emerging viral pathogens. Among these, inhibitors of flaviviruses represent a critical unmet medical need. In that regard, nucleoside inhibitors of flaviviral RdRps are the most attractive targets for antiviral drug design. Nucleosides with the methyl- or ethynyl- modification at the C2′ position and their 2′-fluoro derivatives are the best understood antiflavivirus nucleoside analogs, many of which were initially developed for treatment of HCV infections and later reemployed to suppress replication of other non-HCV flaviviruses. Other important antiflavivirus nucleosides are represented by inhibitors of nucleoside biosynthesis, whose mode of action is predominantly based on depletion of the intracellular nucleoside pool. Nucleoside inhibitors of flaviviral MTase and NTPase/helicase, as well as some newly discovered flavivirus inhibitors, such as tritylated nucleosides, rigid amphipathic nucleosides, or *N6*-aryl-substituted adenosine derivatives, whose mechanisms of action are still poorly understood, can be used as initial structures or starting points for further developments of new generations of nucleoside scaffolds with improved biological parameters. Taken together, specific nucleoside analog-based antiviral therapy in combination with effective vaccination strategies could provide potent prophylactic and curative tools to treat human infections caused by flaviviruses.

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