Mechanisms of Antiviral Activity of Iminosugars Against Dengue Virus

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Abstract

The antiviral mechanism of action of iminosugars against many enveloped viruses, including dengue virus (DENV), HIV, influenza and hepatitis C virus, is believed to be mediated by inducing misfolding of viral *N*-linked glycoproteins through inhibition of host endoplasmic reticulum-resident α-glucosidase enzymes. This leads to reduced secretion and/or infectivity of virions and hence lower viral titres, both *in vitro* and *in vivo*. Free oligosaccharide analysis from iminosugar-treated cells shows that antiviral activity correlates with production of mono- and tri-glucosylated sugars, indicative of inhibition of ER α-glucosidases. We demonstrate that glucose-mimicking iminosugars inhibit isolated glycoprotein and glycolipid processing enzymes and that this inhibition also occurs in primary cells treated with these drugs. Galactose-mimicking iminosugars that have been tested do not inhibit glycoprotein processing but do inhibit glycolipid processing, and are not antiviral against DENV. By comparison, the antiviral activity of glucose-mimetic iminosugars that inhibit endoplasmic reticulum-resident α-glucosidases, but not glycolipid processing, demonstrates that inhibition of α-glucosidases is responsible for iminosugar antiviral activity against DENV. This monograph will review the investigations of many researchers into the mechanisms of action of iminosugars and the contribution of our current understanding of these mechanisms for optimising clinical delivery of iminosugars. The effects of iminosugars on enzymes other than glucosidases, the induction of ER stress and viral receptors will be also put into context. Data suggest that inhibition of α-glucosidases results in inhibited release of virus and is the primary antiviral mechanism of action of iminosugars against DENV.

Keywords

N-linked glycoproteins · ER α-glucosidases · Glucose-mimicking iminosugars · Galactose-mimicking iminosugars · ER-associated degradation · Dengue virus

20.1 Introduction

Transmitted by female *Aedes* mosquitoes, dengue virus (DENV) infects almost 400 million people each year [5], and is a growing global

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health problem. Despite the licensure of the first dengue vaccine, Dengvaxia® (CYD-TDV) in 2015, the development and clinical testing of novel antiviral therapies against dengue virus remains imperative. Dengvaxia® displays differential efficacy against the four dengue serotypes and it is not licenced for people in non- or low-endemicity areas (e.g. travellers and military personnel), due to reduced efficacy in seronegative recipients and an increased risk of antibody-dependent enchantment (ADE). A licenced antiviral therapy to treat dengue disease is thus still of vital importance for reducing morbidity and mortality.

20.1.1 *N*-Linked Glycoprotein Production and Processing

Inhibition of biosynthesis of *N*-linked oligosaccharides, involving both glycosylation and glycoprotein processing, has been targeted as an antiviral approach for a number of decades. *N*-linked glycans are added to proteins at specific amino acid sequences, initially by addition of $Glc_3Man_9GlcNAc_2$, where Glc is glucose, Man is mannose and GlcNAc is *N*-acetyl glucosamine (Fig. 20.1). Once this precursor oligosaccharide is transferred to a glycoprotein, the carbohydrate chain is subjected to a variety of processing reactions, including both removal

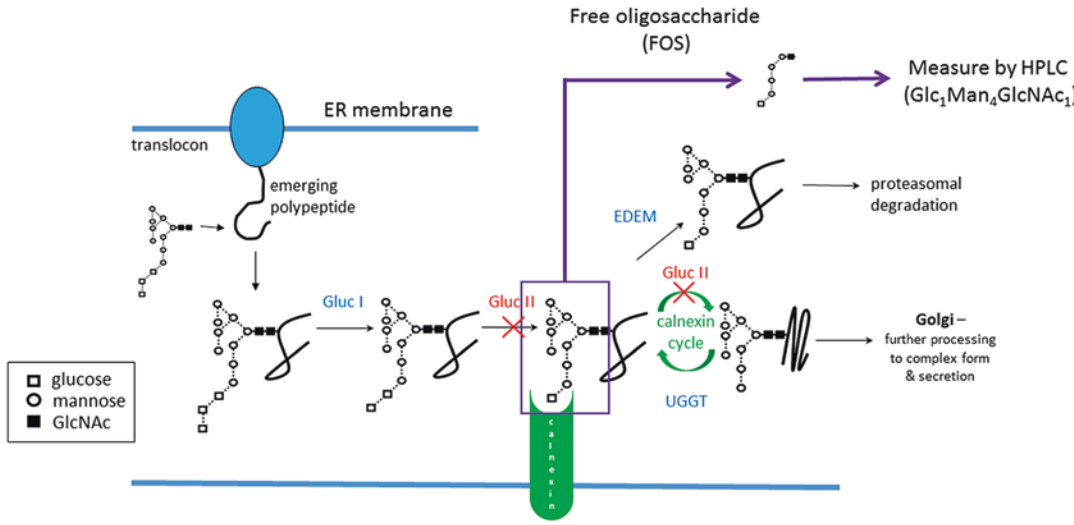

Fig. 20.1 Trimming of *N*-linked glycans in the ER and production of FOS. Following translocation of a newly transcribed peptide into the ER, a preassembled precursor oligosaccharide, consisting of 3 glucose (Glc), 9 mannose (Man) and 2 N-acetylglucosamine (GlcNAc) residues ($Glc_3Man_9GLcNAc_2$) is transferred to the nascent polypeptide. Stepwise removal of first the outer α1,2-linked glucose residue by α-glucosidase I (GluI), then the next two α1,3-linked glucose residues by α-glucosidase II (GluII) occurs. The lectin chaperones calnexin and calreticulin can bind to the monoglucosylated oligosaccharide intermediate ($Glc_1Man_9GLcNAc_2$) to slow glycoprotein progression through the ER and allow for correct folding. UDP-glucose glycoprotein:glycoslytransferase (UGGT) detects misfolded proteins and reglucosylates the oligosaccharides to allow for repeated interaction with calnexin and other folding chaperones and enzymes (calnexin cycle). Correctly folded proteins can bud from the ER for transport to the Golgi and further processing, or alternatively undergo additional processing by ER degradation-enhancing α-mannosidase I–like protein (EDEM) and other ER mannosidases to be targeted for degradation. Free oligosaccharides (FOS) are generated during protein *N*-glycosylation in mammalian cells and specific species can be detected by HPLC in cell lysates. For example, inhibition of GluII results in a monoglucosylated glycoprotein. After trimming of the glycan precursor by mannosidases and recognition of the glycoprotein as terminally misfolded, a $Glc_1Man_4GlcNAc_1$ FOS species is cleaved from the peptide during ER-associated degradation (ERAD). In the case of inhibition of GluI, (not shown) the $Glc_3Man_5GlcNAc_1$ FOS species can be detected (Adapted from [67])

and addition of sugar residues in the endoplasmic reticulum (ER) and Golgi to produce the typical high-mannose, hybrid, and complex types of oligosaccharides. Glycans play a specific role in glycoprotein folding through the calnexin/calreticulin cycle. α-glucosidase I (GluI) and α-glucosidase II (GluII) sequentially trim the terminal glucoses from the precursor oligosaccharide, resulting in the monoglucosylated $Glc_1Man_9GlcNAc_2$ which is bound by calnexin [81]. Through interactions with various protein disulphide isomerases and other chaperones, the glycoprotein has the chance to fold correctly while being held in the ER, before progressing to the Golgi. The glycoprotein eventually plays a role in the viral life cycle or is incorporated into a new virion. These host cell glycosylation processes are required by viruses expressing glycoproteins as no virus has been identified to encode the enzymes required for biosynthesis of *N*-linked oligosaccharides. DENV possesses four *N*-linked glycoproteins: envelope, premembrane, non-structural protein 1 (NS1), and non-structural protein 4B.

20.1.2 Iminosugars

Iminosugars are so named due to their resemblance to monosaccharides, differing where the ring oxygen has been replaced with a nitrogen atom. Replacement of the oxygen reduces iminosugar susceptibility to cleavage by glucosidases and allows derivatization at the nitrogen atom. Several iminosugars are inhibitors of cellular glycosidase enzymes in the *N*-linked glycan processing pathway. In 1982 deoxynojirimycin (DNJ) was first shown to inhibit the formation of complex glycans in *Saccharomyces cerevisiae* [66] by inhibiting the action of the ER GluI and GluII, the earliest enzymes in the *N*-glycan processing pathway (Fig. 20.1). Subsequently, their antiviral potential was realised when castanospermine and *N*-alkyl derivatives of DNJ were shown to inhibit HIV [28, 84].

20.1.3 Iminosugars as Anti-Dengue Compounds

ER chaperones such as BiP and heat shock protein 90 (HSP90) can assist protein folding in a carbohydrate-independent manner, unlike calnexin/calreticulin. DENV E protein binds to BiP in infected cells and knockdown of BiP, calnexin or calreticulin resulted in reduced production of infectious virus, indicating that these chaperones all play a role in folding and assembly of dengue proteins [39]. Interestingly, both HSP90 [61] and BiP [34] have been proposed to act as attachment or entry receptors for DENV in certain cells. Despite this chaperone redundancy, ER α-glucosidase inhibitors were first shown to have antiviral effects on DENV by Courageot et al. [17] via a mechanism that did not affect viral protein synthesis, but appeared to reduce prME heterodimer formation and stability. They concluded that the formation of properly folded DENV envelope complexes requires a lectin chaperone pathway.

Since then many studies have shown that iminosugars are antiviral against DENV [67]. Here we summarise the published antiviral data of iminosugars against DENV *in vitro* (Table 20.1) and *in vivo* (Table 20.2), to highlight the progress in this field. Many different DNJ-derivative and bicyclic iminosugars have now demonstrated antiviral effects against DENV. The number of studies since we last reviewed the field in 2010 has more than tripled, with particular progress in *in vivo* studies, an important step for progression to clinical trials.

20.1.4 Clinical Trials of Iminosugars Against Dengue

Two lead therapeutic candidate iminosugars, methoxy-*N*-nonyl deoxynojirimycin (M*O*N-DNJ, UV-4B) and celgosivir (the prodrug of castanospermine) have progressed to Phase I and Ib clinical trials, respectively. Both appear very safe with no serious adverse events reported.

Table 20.1 Antiviral efficacy of six-membered ring iminosugars against DENV in *in vitro* experiments

Iminosugar	DENV serotype (strain)	Cell type (MOI)	Effect on			Reference
			Viral glycoproteins	Viral replication	Infectious virus secretion	
DNJ-derivative iminosugars						
DNJ	1 (FGA/89)	Neuro 2a (400)	prME dimer formation impaired	ND	Reduced to 20% control at 500 μM	[17]
	2 (16681)	MDMΦ (1)	ND	ND	EC_{50} 308 μM	[68]
*N*B-DNJ	2 (16681)	MDMΦ (1)	ND	ND	IC_{50} 6 ± 7.31 μM; IC_{90} 62.1 ± 60.7 μM	[45]
	2 (NGC)	Vero	ND	ND	IC_{50} 162 μM	[51]
	2 (16681)	MDMΦ (1)	ND	Secreted DENV reduced in 1:1 ratio with infectious DENV	EC_{50} 10.6 μM	[68]
*N*N-DNJ	2 (PL046)	BHK-21 (0.1)	Dose-dependent reduced intracellular E and NS1, and reduced secretion	Reduced RNA replication (16-fold at 100 μM)	Reduced to plaque assay limit of detection with 5 μM; only significantly antiviral when drug added post-infection	[94]
	2	BHK-21 (0.05)	ND	ND	EC_{50} 1.1 μM; EC_{90} 3.3 μM	[13]
			ND	ND	IC_{50} 1 μM	[29]
	2 (16681)	MDMΦ (1)	ND	ND	IC_{50} 0.91 ± 0.40 μM; IC_{90} 8.02 ± 4.14 μM	[45]
	2 (NGC)	Vero	ND	ND	IC_{50} 9 μM	[14]
	2 (16681)	MDMΦ (1)	ND	ND	EC_{50} 1.25 μM	[68]
N-7-oxadecyl-DNJ	2 (NGC)	Vero	ND	ND	IC_{50} 41 μM	[51]
M*O*N-DNJ (UV-4)	1 (779,172)	Vero (0.01)	ND	ND	IC_{50} 5.15 ± 3.85 μM	[89]
	1 (SH 29177)	Vero (0.01)	ND	ND	IC_{50} 2.10 ± 2.50 μM	[89]
	1 (PRS 41393)	Vero (0.01)	ND	ND	IC_{50} 37.69 ± 10.95 μM	[89]
	2 (16681)	MDMΦ (1)	ND	ND	IC_{50} 3.09 ± 3.93 μM; IC_{90} 7.74 ± 3.63 μM	[45]
			ND	1:1 ratio in reduction of secreted total and infectious virus		[89]
	2 (NGC)	Vero	ND	ND	IC_{50} 17 μM	[51]
		Vero (0.01)	ND	ND	IC_{50} 6.49 ± 1.65 μM	[89]
	2 (SL 5–17-04)	Vero (0.01)	ND	ND	IC_{50} 22.34 ± 16.36 μM	[89]
	2 (UIS 1288)	Vero (0.01)	ND	ND	IC_{50} 18.69 ± 7.21 μM	[89]
	3 (SL 5–29-04)	Vero (0.01)	ND	ND	IC_{50} 3.64 ± 1.39 μM	[89]

(continued)

Table 20.1 (continued)

Iminosugar	DENV serotype (strain)	Cell type (MOI)	Effect on			Reference
			Viral glycoproteins	Viral replication	Infectious virus secretion	
	3 (UIS 776)	Vero (0.01)	ND	ND	IC_{50} 6.56 ± 2.80 μM	[89]
	3 (H87)	Vero (0.01)	ND	ND	IC_{50} 86.49 ± 1.58 μM	[89]
	4 (779,157)	Vero (0.01)	ND	ND	IC_{50} 18.18 ± 24.44 μM	[89]
	4 (C258/97)	Vero (0.01)	ND	ND	IC_{50} 8.95 ± 1.25 μM	[89]
	4 (H241)	Vero (0.01)	ND	ND	IC_{50} 2.78 ± 1.42 μM	[89]
*N*AP-DNJ	2 (16681)	MDMΦ (1)	ND	ND	IC_{50} 0.04 ± 0.01 μM; IC_{90} 0.28 ± 0.14 μM	[45]
	2 (NGC)	Vero	ND	ND	IC_{50} 2 μM	[14]
2THO-DNJ (UV-12)	2 (NGC)	Vero (0.01)	ND	ND	IC_{50} 21.71 μM	[87]
CM-9-78	2 (TSV01)	A549 (0.3)	ND	EC_{50} 1.5 μM	ND	[14]
	2	BHK-21 (0.05)	ND	ND	EC_{50} 6.75 μM; EC_{90} 13 μM	[13]
CM-10-18	2 (TSV01)	A549 (0.3)	ND	EC_{50} 1.1 μM	ND	[14]
	2 (NGC)	BHK-21 (0.01)	ND	ND	EC_{50} 4.5 ± 2.0 μM; EC_{90} 47.2 ± 27.6 μM	[16]
CM-10-18 plus ribavirin	2 (TSV01)	A549	ND	Synergistic antiviral effect	ND	[14]
IVHR11029	2 (NGC)	BHK-21 (0.01)	ND	ND	EC_{50} 0.75 ± 0.06 μM; EC_{90} 6.3 ± 3.5 μM	[16]
IVHR17028	2 (NGC)	BHK-21 (0.01)	ND	ND	EC_{50} 0.3 ± 0.03 μM; EC_{90} 1.7 ± 0.8 μM	[16]
IVHR19029	2 (NGC)	BHK-21 (0.01)	ND	ND	EC_{50} 1.25 ± 1.1 μM; EC_{90} 22.5 ± 10.6 μM	[16]
OSL95-ii	2	BHK-21 (0.05)	ND	ND	EC_{50} 4 μM; EC_{90} 8.7 μM	[13]
	2	BHK-21 (0.05)	ND	ND	IC_{50} 2 μM	[29]
PBDNJ0801	2	BHK-21 (0.05)	ND	ND	EC_{50} 0.1 μM; EC_{90} 0.2 μM	[13]
PBDNJ0803	2	BHK-21 (0.05)	ND	ND	EC_{50} 0.1 μM; EC_{90} 0.6 μM	[13]
PBDNJ0804	2	BHK-21 (0.05)	ND	ND	EC_{50} 0.075 μM; EC_{90} 0.6 μM	[13]
N-butyl-cyclohexyl-DNJ	2	BHK-21 (0.05)	ND	ND	IC_{50} 3 μM	[29]
N-propyl-cyclohexyl-DNJ	2	BHK-21 (0.05)	ND	ND	IC_{50} 1.5 μM	[29]
Bicyclic iminosugars						
CAST	1 (Brazil)	BHK-21 (0.01)	ND	ND	$IC_{90} < 50$ μM	[92]

(continued)

Table 20.1 (continued)

Iminosugar	DENV serotype (strain)	Cell type (MOI)	Effect on			Reference
			Viral glycoproteins	Viral replication	Infectious virus secretion	
	1 (FGA/89)	Neuro 2a (400)	E protein misfolded; prME dimer formation impaired	ND	Reduced to 5% control at 500 μM	[17]
	2	BHK-21 (0.05)	ND	ND	IC_{50} 6 μM	[29]
	2 (16681)	BHK-21 (0.1–10)	prM glycosylation affected	Replicon expression reduced by <40%	IC_{50} 1 μM; IC_{90} < 50 μM	[92]
		Huh-7 (0.1–10)	ND	ND	IC_{50} 85.7 μM	[92]
		MDMΦ (1)	ND	ND	EC_{50} 36.4 μM	[68]
	2 (N1042)	BHK-21	ND	ND	IC_{90} < 50 μM	[92]
	3 (Sri Lanka)	BHK-21	ND	ND	IC_{90} < 50 μM	[92]
	4 (Tahiti)	BHK-21	ND	ND	IC_{90} < 50 μM	[92]
Celgosivir	1 (2402)	BHK-21 (0.3)	ND	EC_{50} 0.65 ± 0.16 μM	ND	[58]
			ND	ND	EC_{50} 0.105 ± 0.059 μM	[91]
		BHK-21 (0.01)	ND	ND	EC_{50} 0.066 ± 0.019 μM	[91]
		Huh-7 (0.3)	ND	ND	EC_{50} 17.430 ± 4.921 μM	[91]
		Huh-7 (0.01)	ND	ND	EC_{50} 5.961 ± 1.258 μM	[91]
		Vero (0.3)	ND	ND	EC_{50} 51.035 ± 14.47 μM	[91]
		Vero (0.01)	ND	ND	EC_{50} 13.805 ± 1.902 μM	[91]
		THP-1 (2)	ND	ND	EC_{50} 3.236 μM	[91]
	2 (3295)	BHK-21 (0.3)	E transport to Golgi blocked. NS1 in cells reduced (immuno-fluorescence) and colocalises with ER not Golgi.	EC_{50} 0.22 ± 0.01 μM	ND	[58]
			ND	ND	EC_{50} 0.061 ± 0.003 μM	[91]
		Huh-7 (0.3)	ND	ND	EC_{50} 0.824 ± 0.109 μM	[91]
		Vero (0.3)	ND	ND	EC_{50} 2.434 ± 0.773 μM	[91]
		THP-1 (50)	ND	ND	EC_{50} 0.756 μM	[91]

(continued)

Table 20.1 (continued)

Iminosugar	DENV serotype (strain)	Cell type (MOI)	Effect on			Reference
			Viral glycoproteins	Viral replication	Infectious virus secretion	
	2 (S221)	BHK-21 (0.3)	ND	ND	EC_{50} 0.119 ± 0.000 μM	[91]
		Huh-7 (0.3)	ND	ND	EC_{50} 5.093 ± 1.036 μM	[91]
		Vero (0.3)	ND	ND	EC_{50} 8.336 ± 0.773 μM	[91]
		THP-1 (2)	ND	ND	EC_{50} 2.135 μM	[91]
	16 DENV-1 and −2 isolates from CELADEN trial	Huh-7 (various)	ND	ND	Only one strain less sensitive to 3 μM celgosivir than DENV-1 (2402)	[91]
	2 (16681)	MDMΦ (1)	ND	Secreted DENV reduced in 1:1 ratio with infectious DENV	EC_{50} 5.17 μM	[68]
	3 (863)	BHK-21 (0.3)	ND	EC_{50} 0.68 ± 0.02 μM	ND	[58]
	4 (2270)	BHK-21 (0.3)	ND	EC_{50} 0.31 ± 0.12 μM	ND	[58]
DGJ-derivative iminosugars						
*N*B-DGJ	2 (16681)	MDMΦ (1)	ND	ND	No inhibition	[68]
*N*N-DGJ	2 (16681)	MDMΦ (1)	ND	ND	No inhibition	[68]
M*O*N-6d-DGJ	2 (16681)	MDMΦ (1)	ND	ND	No inhibition	[89]

Abbreviations: *DNJ deoxynojirimycin, DGJ deoxygalactonojirimycin MON-DNJ methoxy-nonyl-DNJ, MON-6d-DGJ methoxy-nonyl-6-deoxy-DGJ, NAP-DNJ* N-(6'-4"- azido-2"-nitrophenylamino) hexyl-1-DNJ, *2THO-DNJ* N-8'-(2"-tetrahydrofuranyl)-octyl-DNJ, *CAST* castanospermine, *NB*- N-butyl-, *NN*- N-nonyl-

Celgosivir (BuCAST) is the butylated prodrug cleaved in cells to produce castanospermine, a bicyclic iminosugar. It has submicromolar activity against DENV *in vitro* and in an *in vivo* mouse model [58, 90]. These results, combined with encouraging pre-clinical pharmacology results and human safety data obtained from clinical trials of celgosivir against HIV and hepatitis C virus (HCV) [20, 35], where it had modest antiviral effects, supported a Phase 1b randomised, double-blind, placebo-controlled clinical trial in 50 adult dengue patients (CELADEN, NCT01619969). This trial recruited patients with a fever (≥38 °C) for less than 48 h and dosed celgosivir at an initial 400 mg loading dose, followed by 200 mg every 12 h for a total of nine doses. While this study failed to show a decrease in fever duration or viral load [40, 76], the authors have subsequently investigated optimisation of dosing to give higher minimum concentrations [91] and will perform a Phase IIa clinical trial with four-times daily treatment (NCT02569827). In the first trial, a more rapid clearance of NS1 antigen was observed in patients treated with celgosivir compared to the placebo: an effect that was more prominent in patients with secondary dengue infection. They also highlighted the possibility of a therapeutic difference between patients with primary or secondary infections, indicating that future trials should be powered to investigate this.

Table 20.2 Iminosugar antiviral efficacy against DENV in *in vivo* experiments

Iminosugar	DENV infection	Animal model	Outcome	Reference
*N*B-DNJ	10^5 p.f.u. i/v DENV-2 (D2S10) with ADE (4G2 anti-E antibody)	AG129 mice; n = 5–18/group	PBS or PBS-containing PERLs: death at day 4–5 p.i. Non-significant reduction in liver and spleen viral titres.	[45]
			0.088 mg/kg/day: no effect on survival. Non-significant reduction in liver and spleen virus titres.	
			250 mg/kg/day: 20% survival	
			1000 mg/kg/day: 90% survival. Significant viral load reduction in liver, small intestine, serum and spleen at day 3.5 p.i.	
			0.094 mg/kg/day encapsulated in PERLs: 20% survival, with encapsulation providing >1900-reduction in dose able to increase survival. Non-significant reduction in liver and spleen viral titres.	
	2×10^6 p.f.u. i/v DENV-2 (S221)	AG129 mice; n = 23 (water), 10 (drug), 5 (ribavirin)	Water or ribavirin 100 mg/kg day: euthanised day 4–6 p.i., median survival 4 days	[51]
			100 mg/kg BID orally for 7 days: no significant difference	
*N*N-DNJ	2×10^6 p.f.u. i/p DENV-2 (TSV01)	7–9-week old AG129 mice; n = 8/group	75 mg/kg orally BID for 3 days: 93% reduced viraemia, 68% reduced splenomegaly; significantly reduced pro-inflammatory cytokines and chemokines (TNF-α, IL-6, IL-12, IFN-γ, MCP-1)	[71]
	2×10^6 p.f.u. i/v DENV-2 (S221)	AG129 mice; n = 23 (water), 13 (drug), 5 (ribavirin)	Water or ribavirin 100 mg/kg day: euthanised day 4–6 p.i., median survival 4 days	[51]
			100 mg/kg BID orally for 7 days: median survival 8 days, gradual decrease in mean group weight	
N-7-oxadecyl-DNJ	2×10^6 p.f.u. i/v DENV-2 (S221)	AG129 mice; n = 23 (water), 5 (drug or ribavirin)	Water or ribavirin 100 mg/kg day: euthanised day 4–6 p.i., median survival 4 days	[51]
			100 mg/kg BID orally for 7 days: no significant difference	

M*ON*-DNJ (UV-4)	2×10^6 p.f.u. i/v DENV-2 (S221)	AG129 mice; n = 23 (water), 18 (drug), 5 (ribavirin)	Water or ribavirin 100 mg/kg daily: euthanised day 4–6 p.i., median survival 4 days	[51]
			100 mg/kg BID orally for 7 days: median survival 7.5 days, mean weight significantly higher than control group throughout	
	2×10^4 p.f.u. i/v DENV-2 (S221) with ADE (2H2 anti-prM antibody)	AG129 mice; n = 33 (water), 29 (drug)	Water: 10% survival at day 9 p.i., median survival 4 days	
			100 mg/kg TID orally for 7 days: 89% survival at day 9 p.i. with no symptoms. Serum viral RNA and titres reduced 4-fold at 48 h p.i. equivalent at 72 h p.i., and 100-fold lower at 96 h p.i. Viral RNA levels reduced 100–1000-fold in liver, small intestine and kidney. Lower but significant reduction in viral titre in liver and kidney. No effect on DENV-specific IgM or IgG.	
	2×10^4 p.f.u. i/v DENV-2 (S221) with ADE (2H2 anti-prM antibody)	AG129 mice; n = 11 (water), 10/drug group	Water: 0% survival at day 5 p.i.	
			2.5 mg/kg TID orally for 7 days: 30% survival at day 9 p.i.	
			5 mg/kg TID orally for 7 days: 50% survival at day 9 p.i.	
			10 mg/kg TID orally for 7 days: 90% survival at day 9 p.i.	
			100 mg/kg TID orally for 7 days: 100% survival at day 9 p.i.	
	2×10^4 p.f.u. i/v DENV-2 (S221) with ADE (2H2 anti-prM antibody)	AG129 mice; n = 10 (water), 8/drug group	Water: 0% survival at day 9 p.i.	
			Drug dosing 100 mg/kg TID orally for 7 days.	
			From time of infection: 90% survival at day 12 p.i.	
			Beginning 24 h p.i.: 100% survival at day 12 p.i.	
			Beginning 48 h p.i.: 40% survival at day 12 p.i., median survival 11 days	
			Beginning 72 h p.i.: 0% survival at day 10 p.i., no significant difference from control	
	1×10^{10} GEs in 1st passage, 1×10^8 GEs for 2nd-4th passage	STAT1$^{-/-}$/2$^{-/-}$ 129/Sv mice	100 mg/kg orally TID for 72 h beginning -1 h from infection. 19 nonsynonymous mutations identified in glycoproteins after four serial passages in mice, none of which provided evidence of a true escape mutant.	[52]

(continued)

Table 20.2 (continued)

Iminosugar	DENV infection	Animal model	Outcome	Reference
	10^9 GEs DENV-2 (S221) with ADE (2H2 anti-prM antibody)	AG129 mice; n = 10/group	Vehicle: 10–20% survival, significantly worse clinical scores and weight loss than drug-treated.	[89]
			10 mg/kg TID orally for 7 days: starting -1 h relative to infection, 60% survival; starting 24 h p.i., 56% survival; starting 48 h p.i., 36% survival (not significant).	
			20 mg/kg TID orally for 7 days: starting -1 h relative to infection, 85% survival; starting 24 h p.i., 100% survival; starting 48 h p.i., 70% survival.	
			40 mg/kg TID orally for 7 days: starting -1 h relative to infection, 100% survival; starting 24 h p.i., 100% survival; starting 48 h p.i., 90% survival	
			100 mg/kg TID orally for 7 days: starting -1 h relative to infection, 90% survival; starting 24 h p.i., 90% survival; starting 48 h p.i., 100% survival.	
			100 mg/kg M*O*N-6d-DGJ TID orally for 7 days: No protection	
*N*AP-DNJ	2×10^6 p.f.u. i/v DENV-2 (S221)	AG129 mice; n = 23 (water), 10 (drug), 5 (ribavirin)	Water or ribavirin 100 mg/kg daily: euthanised day 4–6 p.i., MSD 4 days	[51]
			100 mg/kg BID orally for 7 days: no significant difference from water.	
2THO-DNJ (UV-12)	1×10^4 p.f.u. i/v DENV-2 (S221) with ADE (2H2 anti-prM antibody)	5–6 week old AG129 mice	Vehicle: 0% survival, MSD 5 days	[87]
			20 mg/kg TID for 7 days, starting 1 h pre-infection: 100% survival to day 9 p.i.	
			100 mg/kg TID for 7 days, starting 1 h pre-infection: 100% survival to day 9 p.i. Viral loads reduced in kidney (12.9-fold at 72 h p.i., 5.23-fold at 96 h p.i.), small intestine (6.1-fold at 72 h p.i.), but not in serum or liver at 72 or 96 h p.i. Spleen viral load increased 5-fold at 72 h p.i. but no difference at 96 h p.i.	
CAST	10^5 p.f.u. i/c DENV-2 (mouse-adapted NGC)	4-week old A/J mice; n = 30–45/ group	Vehicle: 0% survival	[92]
			10 mg/kg (10 days i/p): 20% survival	
			50 mg/kg (10 days i/p): 90% survival	
			250 mg/kg (10 days i/p): 85% survival	
	2×10^5 p.f.u. i/p DENV-2 (S221) with ADE (4G2 anti-E antibody)	AG129 mice; n = 8 (vehicle), 10 (drug)	Vehicle: 0% survival at day 5 p.i.	[90]
			50 mg/kg BID for 5 days: 60% survival at day 10 p.i.	

Celgosivir	2×10^6 p.f.u. i/p DENV-2 (TSV01)	7–9-week old AG129 mice; n = 8/group	7.5 mg/kg orally BID for 3 days: 62% reduced viraemia	[71]
			75 mg/kg orally BID for 3 days: 88% reduced viraemia	
			1 day delay then 75 mg/kg orally BID for 2 days: 55% reduced viraemia	
	2×10^5 p.f.u. i/p DENV-2 (S221)	AG129 mice; n = 8/group	Vehicle: 75% survival at day 10 p.i.	[58]
			50 mg/kg i/p BID for 5 days: 100% survival at day 10 p.i.	
	2×10^5 p.f.u. i/p DENV-2 (S221) with ADE (4G2 anti-E antibody)	AG129 mice; n = 8/group	Vehicle: 0% survival at day 5 p.i.	[58]
			50 mg/kg i/p BID for 5 days: 100% survival at day 10 p.i., reduced to 50% survival if administered from day 2 p.i.	
	2×10^5 p.f.u. i/p DENV-2 (S221) with ADE (4G2 anti-E antibody)	AG129 mice; n = 7 (50 mg/kg) or 8/group	Vehicle: 0% survival at day 5 p.i.	[90]
			10 mg/kg BID for 5 days: 13% survival at day 10 p.i.	
			25 mg/kg BID for 5 days: 63% survival at day 10 p.i., reduced viraemia at day 3 p.i.	
			50 mg/kg BID for 5 days: 100% survival at day 10 p.i., reduced viraemia at day 3 p.i.	
			100 mg/kg daily for 5 days: 0% survival at day 6 p.i., no viraemia reduction	
	10^5 p.f.u. i/v DENV-2 (D2S10) with ADE (4G2 anti-E antibody)	AG129 mice	33.3 mg/kg every 8 h until sacrifice at 80 h p.i. Viral RNA load significantly reduced, trend towards reduced circulating infectious virus and viral RNA in kidney, parenteral lymph nodes, liver and small intestine. Enhanced viral RNA levels in spleen.	[68]
	7×10^7 p.f.u. i/v DENV-1 (2402) with ADE (4G2 antibody)	AG129 mice; n = 5–6/group	Vehicle: 0% survival at day 5 p.i.	[91]
			10 mg/kg orally BID: 0% survival at day 6 p.i., 1.8-fold viraemia reduction at day 3 p.i.	
			50 mg/kg orally BID: 100% survival at day 10 p.i., viraemia reduced 4.3-fold. No additional reduction in viraemia if treatment started at peak viraemia.	
	1×10^8 p.f.u. i/v DENV-2 (3295) with ADE (4G2 antibody)	AG129 mice; n = 5–6/group	Vehicle: 0% survival at day 5 p.i.	[91]
			10 mg/kg orally BID: 100% survival at day 10 p.i., 3.7-fold viraemia reduction at day 3 p.i.	
			50 mg/kg orally BID: 100% survival at day 10 p.i., viraemia reduced 16.5-fold. No additional reduction in viraemia if treatment started at peak viraemia.	
	2×10^4 p.f.u. i/v DENV-2 (S221) with ADE (4G2 antibody)	AG129 mice; n = 5–6/group	Vehicle: 0% survival at day 5 p.i.	[91]
			10 mg/kg orally BID: 0% survival at day 6 p.i., 1.4-fold viraemia reduction at day 3 p.i.	
			50 mg/kg orally BID: 100% survival at day 10 p.i., viraemia reduced 2.4-fold	

(continued)

Table 20.2 (continued)

Iminosugar	DENV infection	Animal model	Outcome	Reference
	2×10^7 p.f.u. i/v DENV-2 DENV-2 (#013)	AG129 mice; n = 6/group	50 mg/kg orally BID: from infection, viraemia on day 3 p.i. reduced 6.8-fold; from 3 days p.i., VLR from day 3 to day 6 not significantly different from control	[91]
	1×10^7 p.f.u. i/v DENV-2 (#031)	AG129 mice; n = 6/group	50 mg/kg orally BID: from infection, viraemia on day 3 p.i. reduced 7.8-fold; from 3 days p.i., VLR from day 3 to day 6 not significantly different from control	[91]
	2×10^7 p.f.u. i/v DENV-2 (#036)	AG129 mice; n = 6/group	50 mg/kg orally BID: from infection, viraemia on day 3 p.i. reduced 12.5-fold; from 3 days p.i., VLR from day 3 to day 6 not significantly different from control	[91]
CM-9-78	5×10^6 p.f.u. i/p DENV-2 (TSV01)	7–8 week old AG129 mice; n = 6/group	75 mg/kg orally BID for 3 days: 2.3-fold viraemia reduction at 3 days p.i.	[14]
			25 and 10 mg/kg orally BID for 3 days: no significant effects on viraemia	
CM-10-18	5×10^6 p.f.u. i/p ENV-2 (TSV01)	7–8 week old AG129 mice; n = 6/group	75 mg/kg orally BID for 3 days: 1.8-fold viraemia reduction at 3 days p.i.	[14]
	2×10^7 p.f.u. i/v DENV-2 (mouse-adapted D2S10)	AG129 mice; n = 5/group	PBS: euthanised day 6 p.i.	[15]
			40 mg/kg/day ribavirin: euthanised day 5 p.i.	
			75 mg/kg or 150 mg/kg orally BID for 3 days: 100% survival to day 15	
	10^7 p.f.u. i/p DENV-2 (D2Y98P-rc)	AG129 mice; n = 5/group	PBS: MSD 9 ± 2.2	[15]
			25 mg/kg BID NITD008: 100% survival at day 24 p.i.	
			3 mg/kg orally BID for 3 days: MSD 12 ± 2.0	
			10 mg/kg orally BID for 3 days: MSD 14 ± 1.1	
			25 mg/kg orally BID for 3 days: MSD 17 ± 2.3	
			75 mg/kg orally BID for 3 days: 40% survival at day 24 p.i.	
CM-10-18 plus ribavirin	5×10^6 p.f.u. i/p DENV-2 (TSV01)	7–8 week old AG129 mice; n = 6/group	CM-10-18 75 mg/kg orally BID for 3 days p.i.: 1.9-fold viraemia reduction at day 3 p.i.	[14]
			Ribavirin 40 mg/kg daily for 3 days p.i.: no effect on viraemia at day 3 p.i.	
			Combination: 4.7-fold viraemia reduction at day 3 p.i.	

Abbreviations: *CAST* castanospermine, *DNJ* deoxynojirimycin, *NAP-DNJ* N-(6′-4″- azido-2″-nitrophenylamino) hexyl-1-DNJ, *NB-* N-butyl-, *NN-* N-nonyl-, *BID* bis in die (twice daily), *i/p* intraperitoneal, *i/v* intravenous, *MSD* mean survival days, *PERL* polyunsaturated endoplasmic reticulum-targeting liposome, *PBS* phosphate buffered saline, *p.f.u.* plaque forming units, *p.i.* post-infection, *TID* ter in die (three times daily), *VLR* virological log reduction

M*O*N-DNJ was developed to be a more potent yet similarly non-toxic derivative of *N*-butyl-DNJ (*N*B-DNJ) through alkyl chain elongation and oxygenation [44, 45] and has demonstrated more potent *in vivo* antiviral effects than *N*B-DNJ against dengue virus. M*O*N-DNJ has antiviral activity against a range of viruses *in vitro,* and *in vivo* efficacy in animal models against dengue [51, 89] and influenza virus [74, 88]. A Phase I single-ascending dose clinical trial of M*O*N-DNJ in humans (NCT02061358) has recently been completed, in which 64 volunteers received a single oral dose ranging from 3–1000 mg, with no serious adverse events reported. Even the highest dose of 1000 mg was overall well tolerated. Multiple-ascending dose studies are currently underway in preparation for efficacy testing against DENV in humans.

20.1.5 Iminosugars Are Broad Spectrum Antivirals

Several members of the class of small molecules known as iminosugars have broad-spectrum antiviral activity *in vitro* against both DNA and RNA viruses and against viruses that bud from either the ER or the plasma membrane (Table 20.3). Furthermore, iminosugars have demonstrated promising *in vivo* results against influenza, Ebola, Marburg, dengue and woodchuck hepatitis (a model for hepatitis B) [8] viruses (Table 20.3). With respect to understanding mechanism of action, it is informative to ask what susceptible viruses have in common, and of equal interest to define what determines lack of susceptibility to iminosugars. Theoretically any virus that depends non-redundantly upon the calnexin/calreticulin pathway for glycoprotein folding would be sensitive to glucosidase inhibitors. This requires at least one *N*-linked glycan on a (viral, but in some cases host [98]) glycoprotein essential for viral infectivity. Interestingly, a single glycan can be sufficient to confer susceptibility to glucosidase inhibition, as is demonstrated in the case of the glycosylation sequon in the pre-S2 domain of M protein of hepatitis B [43]. However, currently it is not possible to predict which if any *N*-glycan may be utilized to engage with the calnexin cycle, and which proteins may depend on it for proper folding. The degree of *N*-glycosylation and number of disulphide bonds, the complexity of folding required for oligomerisation and co-translational cleavage events, amongst other factors, have been proposed to contribute to sensitivity to iminosugars. Ongoing and future studies will continue to elucidate the relationship between glucosidase inhibition and antiviral action.

The formative paper by Hammond, Braakman and Helenius [30] over 20 years ago on the role the calnexin cycle, and specifically the monoglucosylated glycan, played in correct glycoprotein folding was critical in the development of our perception of how iminosugars are antiviral. While research in the last decade has significantly progressed our understanding, the mechanism is not fully elucidated. Iminosugars are known to inhibit α-glucosidases, enzymes that trim terminal glucose residues from nascent glycoproteins in the ER, controlling interaction with the calnexin cycle and hence proper glycoprotein folding and transport. Evidence suggests that by preventing the appropriate folding of viral glycoproteins, iminosugars prevent the formation of infectious viral particles. How has our understanding of the mechanism/s of antiviral action of iminosugars progressed in the last decade? We shall put new findings into context within the field.

20.2 Investigations into Mechanism of Action of Iminosugars against DENV

20.2.1 Reduced Virus Secretion

Experiments published in 2015 have clarified that treatment of DENV-infected cells with a range of iminosugars results in reduced secretion of DENV, rather than a reduction in virion infectivity [68, 89]. Iminosugars have demonstrated antiviral activity against all four serotypes of DENV [92] (Table 20.1) with IC_{50} values falling within a tenfold range across the four serotypes [51].

Table 20.3 Iminosugar antiviral efficacy against viruses relevant for human health

Virus (*N*-linked glycoproteins, where known)	Efficacious iminosugars *in vitro*	Efficacious iminosugars *in vivo*	References
Flaviviridae			
Dengue (E, prM, NS1, NS4b)	See Tables 20.1 and 20.2		
Japanese encephalitis (E, prM, NS1)	*N*N-DNJ	*N*N-DNJ	[94]
West Nile (E, prM, NS1)	*N*N-DNJ, SP169, SP173 OSL-1, OSL-3, OSL95-II, CAST, PBDNJ0801, PBDNJ0803, PBDNJ0804	ND	[13, 29, 92]
Kunjin (E)	*N*N-DNJ	ND	[41]
Hepatitis C (E1, E2, NS4B)	DNJ, *N*B-DNJ, *N*N-DNJ, *N*N-DGJ, OSL-95II, CM-10-18, CM-9-78, PBDNJ0802, PBDNJ0803, PBDNJ0804	ND	[56, 75]
Yellow fever (prM, E, NS1)	CAST	ND	[92]
Bunyaviridae			
Rift valley fever (Gn, Gc, LGp)	*N*B-DNJ, *N*N-DNJ, *N*-7-oxadecyl-DNJ, M*O*N-DNJ, *N*AP-DNJ, IHVR11029, IHVR17028, IHVR19029	ND	[16, 57]
Filoviridae			
Ebola (GP, sGP)	ND	IHVR11029, IHVR17028, IHVR19029, *N*B-DNJ, M*O*N-DNJ	[16, 46]
Marburg (GP)	ND	IHVR11029, IHVR17028, IHVR19029	[16]
Togaviridae			
Sindbis (E1, PE2)	DNJ, *N*M-DNJ, DMJ, CAST	ND	[42, 70]
Semliki forest (E1, E2, E3)	*N*M-DNJ	ND	[36]
Chikungunya (E1, E3E2)	*N*B-DNJ, *N*N-DNJ, *N*-7-oxadecyl-DNJ, M*O*N-DNJ, *N*AP-DNJ	ND	[57]
Orthomyxoviridae			
Influenza A (HA, NA)	DNJ, *N*B-DNJ, *N*N-DNJ, M*O*N-DNJ, 2THO-DNJ, *N*N-DGJ, CAST, celgosivir, HNJ, DMJ, *N*-benzyl-1,5-dideoxy-1,5-imino-D-glucitol, *N*,2-*O*-dibenzyl-1,5-dideoxy-1,5-imino-D-glucitol, *N*-benzyl-1,5-dideoxy-1,5-imino-D-mannitol, *N*-benzyl-1,5-dideoxy-1,5-imino-4,6-O-isopropylidene-D-mannitol, 3-episiastatin B	M*O*N-DNJ, 2THO-DNJ, celgosivir, HNJ	[11, 31, 32, 48, 65, 74, 83, 88, 87, 96, 97]

(continued)

Table 20.3 (continued)

Virus (*N*-linked glycoproteins, where known)	Efficacious iminosugars *in vitro*	Efficacious iminosugars *in vivo*	References
Influenza B (HA, NA)	M*O*N-DNJ	M*O*N-DNJ	[88]
Paramyxoviridae			
Measles (F, H)	*N*AP-DNJ, CAST, DMJ	ND	[9, 57]
Newcastle disease (F, HANA)	DNJ, CAST	ND	[82]
Herpesviridae			
Herpes simplex type 1 (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL)	Celgosivir	Celgosivir	[10]
Herpes simplex type 2 (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL)	*N*M-DNJ, *N*B-DNJ, *N*H-DNJ, CAST, celgosivir	ND	[33, 1]
Cytomegalavirus (gN)	DNJ, *N*B-DNJ, CAST, fagomine	ND	[27, 33, 78]
Retroviridae			
Human immunodeficiency 1 (gp160 → gp120)	DNJ, DNJ derivatives 1–8 & 10–11 [[73]], *N*M-DNJ, *N*E-DNJ, *N*B-DNJ, CAST, celgosivir, MDL 43305, MDL 28653, MDL 29435, MDL 29204, MDL 44370, MDL 29270, DMJ, *N*M-DMJ, L-fuconic-1,5-lactam, *N*-methyl-FT, *N*-acetyl-FT, *N*-(5-carboxy methyl-1-pentyl)-FT, DMDP	ND	[4, 18, 25, 28, 38, 47, 49, 53, 59, 60, 72, 73, 77, 79, 84, 86]
Human immunodeficiency 2 (gp160 → gp120)	*N*B-DNJ	ND	[53]
Hepadnaviridae			
Hepatitis B (S, M, L)	*N*B-DNJ	ND	[7]
Rhabdoviridae			
Vesicular stomatitis (G)	DNJ, CAST, Miglitol	ND	[6, 69]
Coronaviridae			
Severe acute respiratory syndrome (M, S)	Compound 7 & 15	ND	[95]

Iminosugars with six-membered rings have broad-spectrum antiviral activity against viruses infecting humans. Investigations using only native virions are included (pseudotyped viruses and replicon systems are not included). Abbreviations: *DMDP* 2R,5R-dihydroxymethyl-3R,4R-dihydroxypyrrolidin, *FT* 1,5-dideoxy-1,5-imino-L-fucitol, *Miglitol* (2R,3R,4R,5S)-1-(2-hydroxyethyl)-2-(hydroxymethyl) piperidine-3,4,5-triol, *DNJ* deoxynojirimycin, *DGJ* deoxygalactonojirimycin, *HNJ* homonojirimycin, *DMJ* deoxymannojirimycin, *MON-DNJ* methoxy-nonyl-DNJ, *NAP-DNJ* N-(6'-4"- azido-2"-nitrophenylamino) hexyl-1-DNJ, *2THO-DNJ* N-8'-(2"-tetrahydrofuranyl)-octyl-DNJ, *CAST* castanospermine, *NM-* N-methyl-, *NB-* N-butyl-, *NN-* N-nonyl-, *NH-* N-hexyl, *NE-* N-ethyl-, *NN-* N-nonyl.

When supernatant from iminosugar-treated DENV-infected primary human macrophages is assayed for both infectious virus (by plaque assay) and total virus production (by qRT-PCR for DENV RNA), the levels of virus decrease concomitantly. This is consistent with ER α-glucosidase inhibition resulting in sufficient misfolding of viral glycoproteins such that they are targeted for degradation and there is reduced secretion of virus. However, reduced secretion is not the outcome of iminosugar treatment of all viruses. In the case of HIV, iminosugars affect infectivity to a greater degree than secretion: iminosugar treatment alters the glycosylation of gp120 resulting in only modest reductions in virus released from infected cells, but strongly impaired viral entry at a stage post CD4-binding [22, 23]. It is not possible to predict whether iminosugar treatment will result in reduced virion infectivity alone, or also in reduced secretion, and as such this has to be determined on a virus-by-virus basis [38]. The differential effects of iminosugars on

secretion and infectivity seen with different viruses may also be compound or cell type specific, as in baby hamster kidney (BHK)-21 cells *N*-nonyl-DNJ (*N*N-DNJ) reduced DENV RNA replication in addition to effects on the DENV glycoproteins [94]. This is unlike observations for other, and related viruses, such as bovine viral diarrhoea virus (BVDV) [21] and HCV [75] where an absence of direct effect on RNA replication was shown. Additional research monitoring effects of iminosugars on DENV replication in relevant primary cells would be valuable to clarify the relative contributions of inhibition of viral RNA replication and glycoprotein folding on the overall antiviral effect observed.

20.2.2 Inhibition of ER α-Glucosidases

Findings from the only two documented living individuals with a genetic deficiency in GluI provide support for the treatment of viral infections with α-glucosidase inhibitors as the two children seem to be resistant to infection with enveloped viruses [64]. These children have no history of viral disease and despite substantial hypogammaglobulinemia, did not produce immune responses to live viral vaccines while still producing a normal response to protein, polysaccharide and conjugated protein-polysaccharide immunogens. Cells cultured from these children (shown to express GluII) were equally susceptible to infection with HIV in comparison to control cells; however virions produced were less infectious than those produced when the GluI gene was re-complemented back in. Displaying a similarly antiviral phenotype, but manifesting at the initial stage of cell infection, monocyte-derived macrophage cultures from each patient were either only very weakly or not productively infected with influenza virus (and less infectious virus was produced from these cells). These phenotypes are consistent with the hypothesis that inhibition of GluI is sufficient for antiviral activity.

There is convincing evidence to show that inhibition of α-glucosidases correlates with antiviral activity against DENV, much of which depends on quantification of mono- and tri-glucosylated free oligosaccharide (FOS) species. Generation of these specific FOS as the end products of protein misfolding gives a measure of both accessibility of iminosugars to the ER combined with their ER α-glucosidase inhibition activity. As a consequence of inhibition of ER GluII, a monoglucosylated glycoprotein is produced (Fig. 20.1). After trimming of the glycan precursor by mannosidases and recognition of the glycoprotein as terminally misfolded, $Glc_1Man_4GlcNAc_1$ and $Glc_1Man_6GlcNAc_1$ FOS species are cleaved from the peptide during ER-associated degradation (ERAD). In the case of inhibition of ER GluI, a similar process produces a $Glc_3Man_5GlcNAc_1$ species. Thus, the presence of each species of FOS can be correlated with successful inhibition of the respective cellular α-glucosidase. Addition of 100 μM *N*B-DNJ, *N*N-DNJ, M*O*N-DNJ or celgosivir to macrophages led to the generation of both mono- and tri-glucosylated FOS species, demonstrating inhibition of both α-glucosidases [68, 89]. When the degree of inhibition of just GluII (ie. generation of $Glc_1Man_4GlcNAc_1$ FOS) is plotted against the antiviral activity for a range of iminosugar concentrations a clear correlation is observed between these two parameters (Fig. 20.2), consistent with GluII inhibition being sufficient to achieve an antiviral effect.

20.2.3 Inhibition of Glycoprotein Folding

Interaction of dengue E with ER chaperones facilitates DENV production [39]. Ideally, to confirm that blocking viral glycoprotein entry to the ER calnexin quality control cycle results in the formation of misfolded viral glycoproteins with subsequent antiviral effect, a demonstration that iminosugar treatment of DENV-infected cells results in misfolded DENV glycoprotein(s) would be important. The three studies that monitored the effects of iminosugars on DENV glycoprotein folding and secretion [17, 58, 94] are documented in Table 20.1. Castanospermine treatment of DENV-infected cells reduced levels of immunoprecipitated E to 15–30% compared to that from untreated cells, when a conformation-sensitive monoclonal antibody was used, and the

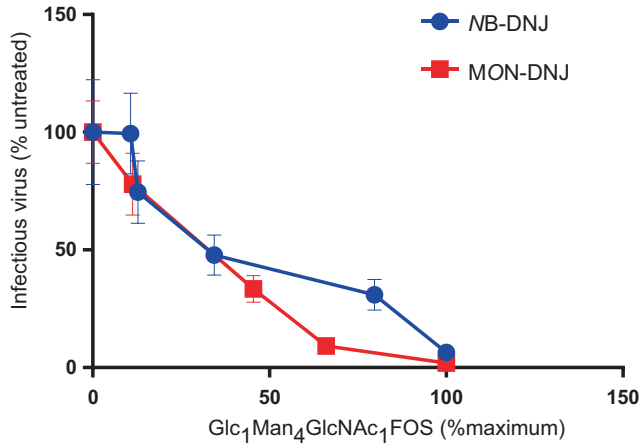

Fig. 20.2 Correlation between the antiviral activity of iminosugars and inhibition of GluII. Infectious titre (% untreated) of DENV is plotted against $Glc_1Man_4GlcNAc_1$ FOS (% maximum) (as a measure of inhibition of α-GluII) for titrations of *N*B-DNJ and M*O*N-DNJ. The viral titres and FOS are means +/− SD of samples generated in at least duplicate from at least 2 donors, at equal concentrations of iminosugar, originally published in [68, 89]

amount of E co-precipitated with prM was about 25% of that from untreated cells, demonstrating that the iminosugar decreased formation of the prME heterodimer, and that inhibition of glucosidases affects the correct folding of DENV E in this system [17]. Such a study monitoring the effects of iminosugars on folding of DENV glycoproteins could be expanded utilizing larger panels of monoclonal antibodies which recognise both conformation-dependent and -independent epitopes to all four DENV glycoproteins. This has been performed for the heavily studied HIV gp120, for which many more validated reagents are available. Studies on the effects of *N*B-DNJ on gp120 folding demonstrated that interaction with the calnexin/calreticulin pathway was critical for correct folding of the V2 loop of HIV gp120, as blocked entry into this folding pathway resulted in conformation defects in this region [23]. A recent study took this observation a step further, assessing regional folding using monoclonal antibodies against conformational epitopes in combination with mathematical modelling, which showed that misfolding of only a portion of the gp120 was sufficient to produce an amplified effect on infectivity (S.G. Spiro, 2016, unpublished results). Additional studies that compare both the level of expression of DENV glycoproteins and their state of folding in the presence and absence of iminosugars, will clarify whether iminosugars can induce misfolding and degradation of DENV glycoproteins.

20.2.4 Consequences of α-Glucosidase Inhibition for Glycans

There are at least two outcomes of α-glucosidase inhibition. By preventing the production of the monoglucosylated glycan it blocks glycoprotein binding to calnexin/calreticulin as has been described above and in Wu et al. [94]. Secondly, the retained terminal glucoses block access of α-mannosidase enzymes to the D1 arm of the glycan, theoretically preventing the formation of complex glycans. The hypothesis that glucosidase inhibitors may be antiviral due to the absence of complex sugars was the basis of the original studies on iminosugars and viruses in the 1980s. In cells with an active Golgi endomannosidase, this can be partially salvaged; however, the activity of endomannosidase is highly cell line dependent [21, 50, 75]. For example, BHK-21 cells and the human hepatoma cell line HepG2 express high levels of endomannosidase [37]

while Chinese hamster ovary (CHO) and Madin-Darby canine kidney (MDCK) cells have no detectable endomannosidase activity [63]. Production of DENV prM and NS1 with hyperglycosylated glycans has been demonstrated by mobility shift of the viral glycoprotein in electrophoresis [58, 92]. This has been confirmed by analysing glycan structures attached to specific glycoproteins for SARS coronavirus [62] and influenza virus [32] though not yet for DENV glycoproteins. Human cells have active endomannosidases so it is important that *in vitro* experiments into the effects of glucosidase inhibition use cell lines that express endomannosidase for relevance. It will be interesting to ascertain whether DENV glycoproteins produced in the presence of iminosugars bear triglucosylated glycans.

20.2.5 Off-Target Effects

Being glucose mimetics, it is not surprising that iminosugars inhibit more than just ER α-glucosidases. Iminosugars also target intestinal digestive enzymes including sucrases and isomaltases, and this interaction is consistent with the mild/moderate, reversible, gastrointestinal symptoms (including flatulence and diarrhoea) seen in some participants in celgosivir and *N*B-DNJ clinical trials. Combinations of low sucrose/starch, high glucose diets and anti-diarrhoea agents can control these symptoms. Of interest, iminosugar inhibition of intestinal α-glucosidases can be used for benefit in patients with non-insulin dependent diabetes. *N*-2′-hydroxyethyl-DNJ is marketed as Miglitol®, and through preventing digestion of carbohydrates lowers the degree of postprandial hyperglycemia to establish greater glycemic control in diabetes mellitus type 2.

Long alkyl chain iminosugars such as *N*N-DNJ and the galactose mimetic *N*N- deoxygalactonojirimycin (*N*N-DGJ) mediate an antiviral effect on BVDV and HCV via inhibiting viral p7 ion channel activity, in a manner independent of inhibiting glucose-recognising host cell enzymes [21, 50, 75]. For the same antiviral mechanism of action to be evoked for DENV, the existence of a dengue ion channel needs to be postulated, as well as its inhibition by long alkyl chain iminosugars. Although some controversy exists in the literature [55, 93], studies by Wong et al. [93] suggesting that neither DENV1 nor DENV2 prM or M proteins show pH-activated ion channel activity when expressed on the surface of *Xenopus* oocytes, in combination with the observation that *N*N-DGJ is not antiviral against DENV *in vitro* at up to 100 μM [68], would indicate that this is an unlikely mechanism of action in the case of DENV.

*N*B-DNJ, as well as other DNJ- and DGJ-derivative iminosugars with longer alkyl tails, also inhibits glucosyl-ceramide synthase (GCS), a glucosyltransferase: an effect that is not dependent on mimicking glucose stereochemistry, but on mimicking the other substrate of GCS, ceramide. Through a comparison of antiviral activity with inhibition of glycolipid processing using glucose and galactose analogues of iminosugars, we have recently shown that antiviral activity of iminosugars against DENV is a function of inhibition of glycoprotein processing rather than due to any effects on GCS [68, 89].

20.2.6 Induction of ER Stress

Blocking productive folding of viral glycoproteins can alter retention times in the ER lumen followed by accumulation and/or increased ERAD. Accumulation of misfolded DENV glycoproteins induces the unfolded protein response (UPR) as the cell attempts to redress the imbalance in homeostasis. While DENV infection alone induces and regulates UPR pathways in human monocytic cells [85], stimulating BiP and XBP-1 mRNA transcription, addition of celgosivir appeared to reduce downstream effector mechanisms of UPR pathways, as demonstrated by reduced phosphorylation of EIF2α [58]. Celgosivir treatment, alone or in the presence of DENV, upregulated transcription of EDEM-1, an ER chaperone that promotes degradation of unfolded proteins, clearing the ER to reduce stress. Taken together, Vasudevan and colleagues suggest that DENV infection in the presence of celgosivir is

characterised by reduced ER stress and enhanced survival. Modulation of the UPR induced in response to increased viral protein levels in the ER has been proposed as a therapeutic target [19, 26].

20.2.7 Iminosugar Effects on Viral Receptors

Modulation of receptors important in the DENV lifecycle and pathogenesis is an additional potential pathway by which iminosugars may exert their antiviral effect. Many host proteins vital for DENV attachment, uptake, signalling and the immune response are themselves *N*-linked glycoproteins and thus perturbations in their expression and function could have implications for virus growth. As with the effects of iminosugars on secretion of different viruses, their effects on host glycoproteins are predicted to be protein specific. Treatment with IHVR-17028, a DNJ-derivative, altered the *N*-linked glycan structure of angiotensin I-converting enzyme 2 (ACE2) in a manner that did not affect its expression or binding to SARS-CoV spike glycoprotein but disrupted its ability to participate in virus envelope-triggered membrane fusion [98]. Very few host glycoproteins have been examined specifically for effects of iminosugars on expression and function.

20.3 Iminosugars As Pharmaceuticals

A number of challenges lie ahead to optimise clinical delivery of iminosugars for pharmaceutical use. One of the specific difficulties of using iminosugars to treat dengue disease, which is more broadly applicable to its use against any acute viral infection, is the short window available for treatment. By the time a dengue patient presents to the healthcare system, they typically may have had a fever for 2–4 days, at which stage there is only 24–48 h before viral load drops as the immune system controls viral replication. The task for an antiviral to reduce viral load in such a window will require a safe, fast acting, highly potent drug. In considering whether an iminosugar could be administered to people living in an endemic setting who present with fever, independent of the differential diagnosis, a dengue therapeutic would need to have an excellent safety profile. Phase I single-ascending dose trial results recently released for MON-DNJ are promising in this respect (NCT02061358), however recruitment for the clinical trial testing the safety and pharmacokinetics of MON-DNJ administered as multiple ascending doses (NCT02696291) was terminated for business reasons in March 2018. All these challenges will be relevant for the use of iminosugars therapeutically against a number of acute viruses, while treating chronic viral disease will present different challenges.

The rapid clearance of iminosugars *in vivo* makes reaching sufficient concentrations to mediate antiviral effects a specific challenge for these compounds. Following oral administration celgosivir had a plasma half-life of 2.5 h in patients [76], which is similar to 5.14 h in mice given a single dose of M*O*N-DNJ orally at 200 mg/kg [51]. In addition, iminosugars are generally excreted rapidly in the urine [2]. The clinical trial testing *N*B-DNJ against HIV concluded that sufficient plasma concentrations could not be achieved to obtain a convincing antiviral effect [24, 80]. In efforts to maximise the mean trough concentrations, and increase the chance of success in testing celgosivir efficacy against DENV, coordinators of the next celgosivir trial performed pharmacokinetic modelling and propose increasing the number of doses per day [76]. An alternate approach, previously shown to enhance antiviral activity of iminosugars against HIV >100,000-fold *in vitro* [53, 54], is encapsulation of the compounds in liposomes, a system used clinically to mediate intracellular delivery of anti-cancer and anti-fungal treatments. When tested *in vivo* against DENV in an ADE mouse model, liposome-mediated delivery of *N*B-DNJ, in comparison with free *N*B-DNJ, resulted in a 3-$\log_{10}$ reduction in the dose of drug required to enhance animal survival [45]. Although a promising approach, the specific formulation of liposomes tested in this study was costly and not sufficiently stable for liposome-mediated delivery to be investigated further. The availability of iminosugars with generally low toxicity makes the

optimisation of pharmacokinetics and dosing regimens currently a more promising approach to optimising clinical iminosugar delivery.

20.3.1 Selectivity

Because ER glucosidases control glycan processing of both viral and host cellular glycoproteins, it would not necessarily be predicted that inhibition of ER glucosidases selectively suppresses viral replication, and yet, in animals at least, a therapeutic window clearly exists where iminosugars are antiviral and well tolerated, at least for acute treatment. A number of possible explanations exist for this dichotomy but further experiments will need to be performed to determine their relative contributions. When viruses infect cells, their proteins are the predominant proteins being synthesised and hence may be more susceptible to inhibition of ER glucosidases. In addition, the DENV virion is comprised of a closely packed, repetitive and coordinated interaction of E and prM proteins which may increase virion susceptibility to any perturbation. Interestingly, in the case of HIV, very little protein misfolding is required to affect virion infectivity (S.G. Spiro, 2016, unpublished results). When the proportions of misfolded gp120 in the presence of *N*B-DNJ were modelled, HIV infectivity was shown to be highly sensitive to the misfolding of only a small proportion of total gp120, suggesting an amplification effect that may contribute to the selectivity of iminosugars against viruses over the host.

20.4 Conclusions

Against the background of historical findings, we highlight advances made in the last decade in understanding the mechanisms of antiviral activity of iminosugars against DENV. The generally accepted antiviral mechanism of ER glucosidase inhibitors, that inhibition of GluI and/or GluII prevents the removal of the terminal glucose moieties on *N*-linked glycans and results in misfolding and retention of glycoproteins in the ER and ultimate degradation via ERAD is supported by a number of pieces of evidence. These include the observations that iminosugars induce the electrophoretic mobility shift of viral glycoproteins, as well as structural changes of *N*-linked glycans and measurement of FOS consistent with GluI and GluII inhibition correlating with antiviral effect. Substrate flux along the *N*-linked glycosylation pathway makes the correlation between key enzymes and an antiviral effect complex [3]. Though both GluI and GluII are targets of iminosugars, the slower removal of the third glucose residue by GluII, even though iminosugars bind approximately tenfold more avidly to GluI [2], is more sensitive to inhibition by iminosugars. As a result of inhibiting these enzymes, viral envelope glycoproteins cannot interact with ER chaperones such as calnexin and calreticulin, preventing correct glycoprotein folding, oligomerization and assembly of infectious virions.

Uniting the conclusions from these multiple studies also allows us to highlight areas where the mechanism of action of iminosugars against DENV could be understood in greater molecular detail. The use of panels of anti-DENV glycoprotein monoclonal antibodies with known specificity binding to DENV glycoproteins produced in the presence of iminosugars has the potential to enable mapping of regional iminosugar-induced misfolding, which may inform our understanding of, for example, E dimerization. The degree or location of misfolding may be protein dependent, potentially even down to strain-dependent differences. Use of iminosugars with galactose stereochemistry (DGJ compounds) has allowed the conclusion that the antiviral effect of piperidine iminosugars (monocylic iminosugars with an iminopyranose structure) against DENV observed in macrophages is not mediated by effects on enzymes of the glycolipid pathway. Development of selective ER α-glucosidase inhibitors would allow both confirmation that ER α-glucosidase inhibition (and not other enzymes) is responsible for the antiviral effect of iminosugars and avoidance of gastrointestinal side effects due to inhibition of intestinal glucosidases. Recently published structures of GluII [12], alone and in complex with M*O*N-DNJ and castanospermine

(see Chap. 19), provide the opportunity for rational drug design and greater understanding of the biochemical detail underlying the inhibition of this host enzyme.

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