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REVIEW

Imino sugar glucosidase inhibitors as broadly active anti-filovirus agents

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Ebola virus and Marburg virus are members of the family of *Filoviridae* and are etiological agents of a deadly hemorrhagic fever disease. The clinical symptoms of Ebola and Marburg hemorrhagic fevers are difficult to distinguish and there are currently no specific antiviral therapies against either of the viruses. Therefore, a drug that is safe and effective against both would be an enormous breakthrough. We and others have shown that the folding of the glycoproteins of many enveloped viruses, including the filoviruses, is far more dependent upon the calnexin pathway of protein folding than are most host glycoproteins. Drugs that inhibit this pathway would be expected to be selectively antiviral. Indeed, as we summarize in this review, imino sugars that are competitive inhibitors of the host endoplasmic reticular α-glucosidases I and II, which are enzymes that process *N*-glycan on nascent glycoproteins and thereby inhibit calnexin binding to the nascent glycoproteins, have been shown to have antiviral activity against a number of enveloped viruses including filoviruses. In this review, we describe the state of development of imino sugars for use against the filoviruses, and provide an explanation for the basis of their antiviral activity as well as limitations.
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INTRODUCTION

Filoviral hemorrhagic fevers are rare but highly lethal diseases associated with outbreaks in developing countries.[1,2] The causative agents, Ebola virus and Marburg virus, are considered to be high level biothreat agents by the United States Centers for Disease Control and Prevention and the National Institutes of Health.[3] There are no effective vaccines to be used as prophylactics and no effective antiviral interventions to manage the diseases. Because the clinical symptoms of Marburg and Ebola hemorrhagic fevers are difficult to distinguish, a drug that is effective against either would be a first, and one that is effective against both the viruses would be an enormous breakthrough.

Filoviruses are non-segmented negative-strand RNA viruses that produce filamentous enveloped virions.[4] There are currently four known clinically relevant species of Ebola virus[4] and a single species of Marburg virus.[5] Initial virus replication occurs in mononuclear cells and viremia is usually apparent within 2 days after infection.[6–9] Death can occur in up to 90% of the infections after 7–10 days of symptoms, usually due to hemorrhagic fevers.[5]

Although the cell receptors for either Ebola virus or Marburg virus have not been fully characterized, the broad cell tropism of the viruses suggests a wide distribution of their receptors. In any regard, the trimeric envelope glycoprotein (GP) spikes of the filoviruses are believed to mediate their entry into host cells via endocytic pathways. Within endo/lysosomal compartments, host endosomal cysteine proteases (cathepsins) cleave the filoviral GP1 protein to generate an entry intermediate comprising an N-terminal GP1 fragment and GP2. Recent work indicates that a cleaved form of Ebola virus GP subsequently interacts with Niemann-Pick C1, an endo/lysosomal cholesterol transporter, to trigger membrane fusion.[9]

As illustrated in Table 1, the pipeline of candidate anti-filovirus therapeutics is limited. However, promising work on inhibition of the virus entry into their host cells with small molecules,[21] recombinant C type lectins[13,22] or immuno-adhesion technology,[23] disruption of viral RNA capping[24] and directly targeting viral RNA with antisense oligos or siRNAs[10,11,25,26] have recently been reported.

As Table 1 also indicates, the oligonucleotide-based therapeutics appear to be the farthest along in development, reaching Phase I clinical trials, but challenges remain and the extent to which these approaches will progress is uncertain. Some of these challenges are inherent to the oligonucleotide approach and the lack of efficient cell-specific delivery technologies.[27] Although a morpholino modification or a lipid nanoparticle formulation has been used for these approaches in their anti-filovirus applications, specific targeted delivery of oligonucleotide into disease-relevant tissues and cells remains a major issue to address.[28]

Only a few small molecule antivirals have been shown to have *in vivo* efficacy against filoviruses. FGI-103, 104, 106 and NSC62914 were discovered using cell-based high-throughput screening, and their antiviral mechanisms are largely unknown. The other two families of small molecules target host enzymes, the *S*-adenosyl-*L*-homocysteine hydrolase and endoplasmic reticulum (ER) α-glucosidases I and II. The ER α-glucosidases are especially interesting because the broad-spectrum antiviral activities of their inhibitors have been extensively demonstrated with a variety of enveloped viruses from many families (Table 1). In addition, more than 1000 US FDA-approved drugs were

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Table 1 Antiviral therapeutics in clinical and preclinical development for the management of human pathogenic *filoviruses*

Name	Type	Virus	Target	Animal model	Efficacy	Reference	Note
AVI-6002	PMOs	EBOV	VP24, VP35	Rhesus macaques	½–1 h post-exposure, 60% protection	10	Phase I clinical trial
AVI-6003	PMOs	MARV	VP24, NP	Cynomolgus macaques	½–1 h post-exposure, 100% protection	10	Phase I clinical trail
TKM-100201	SNALP-siRNAs	EBOV	L polymerase, VP24, VP35	Rhesus macaques	½ h post-exposure, 67%–100% protection	11	Phase I clinical trial
MB-003	Mouse/human chimeric mAbs	EBOV	GP epitopes	Rhesus macaques	1–2 day post-exposure, 67% protection	12	
rhMBL	Recombinant mannose-binding lectin	EBOV	GPs	C57BL/6 mice	12 h post-exposure, 40% protection	13	
C-c^{3}Ado	Adenosine analog	EBOV	*S*-adenosyl-*L*-homocysteine hydrolase	BALB/c mice	2 days post-exposure, >80% protection	14	
c^3-Npc A	Adenosine analog	EBOV	*S*-adenosyl-*L*-homocysteine hydrolase	BALB/c mice	3 days post-exposure, 100% protection	14	
FGI-103	Small molecule	EBOV	Unknown	C57BL/6 mice	1 day post-exposure, 60% protection	15	
		MARV		BALB/c mice	1 day post-exposure, 100% protection		
FGI-104	Small molecule	EBOV	Unknown	C57BL/6 mice	Post-exposure, 100% protection	16	Broad-spectrum, *in vitro*
FGI-106	Small molecule	EBOV	Unknown	C57BL/6 mice	1 day post-exposure, 90% protection	17	Broad-spectrum, *in vitro*
Chloro-quine	Small molecule	EBOV	Unknown	BALB/c mice	80% protection	18	Broad-spectrum, *in vitro*
NSC62914	Small molecule antioxidant	EBOV	Unknown	C57BL/6 mice	1 day post-exposure, 50% protection	19	Broad-spectrum, *in vitro*
		MARV		BALB/c mice	1 h pre-exposure, 90% protection		
IHVR11029	Small molecule imino sugar	EBOV	ER α-glucosidases	C57BL/6 mice	4 h post-exposure, 60% protection	20	Broad-spectrum, *in vitro*
IHVR17028	Small molecule imino sugar	EBOV	ER α-glucosidases	C57BL/6 mice	4 h post exposure, 50% protection	20	Broad-spectrum, *in vitro*
		MARV		BALB/c mice	1 day pre-exposure, 70% protection		
IHVR19029	Small molecule imino sugar	EBOV	ER α-glucosidases	C57BL/6 mice	4 h post-exposure, 80% protection	20	Broad-spectrum, *in vitro*
		MARV		BALB/c mice	4 h post-exposure, 70% protection		

Abbreviations: EBOV, Ebola virus; mAb, monoclonal antibody; MARV, Marburg virus; PMO, phosphorodiamidate morpholino oligomer; SNALP-siRNA, stable nucleic acid lipid particle-small intefering RNA.

tested for new antiviral indications.[18] This effort resulted in the discovery that chloroquine could disrupt entry and replication of two or more viruses *in vitro* and protect mice against Ebola virus challenge *in vivo*.

As for many other enveloped viruses,[29–36] the morphogenesis of filoviruses appears to depend upon ER α-glucosidases mediated processing of envelope glycoproteins. Therefore, the amplification and propagation of filoviruses are sensitive to imino sugar derivatives that inhibit the ER α-glucosidases I and II.[37] Due to their unusual antiviral mechanism, imino sugars should be complementary to the other antiviral approaches and have the advantage of being broadly active.

TARGETING HOST FUNCTIONS TO SUPPRESS VIRAL INFECTION

Medical management of virus infection can, in theory and has in practice, involve targeting either virus or host functions. Targeting virus specified functions such as viral enzymes (DNA or RNA polymerases, proteases, helicases, neuraminidases) has been enormously effective and offered opportunities for great selectivity.[38–40] On the other hand, drugs targeting host functions, upon which the viruses rely, have been approached with more skepticism because of concerns regarding selectivity and toxicity. Table 1 shows a few examples that have been attempted for filoviruses, and discovery of the virus receptor may lead to a few more. The success of the interferon therapy makes the point that it is possible to identify host functions that can serve as targets of broad spectrum antivirals.[41,42] However, there are, to date, very few examples of small molecule antivirals that target host functions and retain broad activity. It is a short list, including inhibitors of host cellular cyclophilins,[43,44] inosine 5-monophosphate dehydrogenase,[45] 3-hydroxy-3-methylglutaryl-coenzyme A reductase,[46] *S*-adenosyl-*L*-homocysteine hydrolase[14,47] and ER α-glucosidases. The ER α-glucosidase inhibitors are thus in a rare group, having been shown to have selective antiviral activity for multiple enveloped viruses in tissue cultures and, in several cases, in animal models

Table 2 Broad-spectrum antiviral activity of imino sugar derivatives *in vitro* and *in vivo*

Virus family	Efficacy *in vitro*	Efficacy *in vivo*	Reference
Herpesviridae	Herpes simplex virus-2	Herpes simplex virus-1, efficacy in mouse	48
	Cytomegalovirus		49
Hepadnaviridae	Hepatitis B virus	Woodchuck hepatitis virus, in woodchucks	30,31,50,51
Retroviridae	Human immunodeficiency virus	Human phase II clinical trials, limited efficacy	52–55
Togaviridae	Sindbis virus		33
	Semliki forest virus		56
Flaviviridae	Hepatitis C virus	Phase II clinical trials, limited efficacy; synergy with interferon and ribavirin	35,57
	Dengue virus		58–67
	Japanese encephalitis virus	Efficacy in several mouse models; phase II clinical trial, ongoing	60,68
	West Nile virus	Efficacy in mouse model	34,64
Coronaviridae	Severe acute respiratory syndrome coronavirus		69
Paramyxoviridae	Measles virus		70
Rhabdoviridae	Vesicular stomatitis virus		71
Filoviridae	Ebola virus	Ebola and Marburg virus in mouse model	20
Arenaviridae	Lassa fever virus		20
	Junin virus		72
Bunyaviridae	Rift Valley fever virus		20
Orthomyxoviridae	Influenza A virus	Efficacy in mouse model	33,73 (Ramstedt *et al.*, patent 20110065752)

(Table 2). Why viral functions are more sensitive to glucosidase inhibition than are host functions is becoming appreciated and touched on briefly below.

FUNCTION OF ER α-GLUCOSIDASES AND CONSEQUENCES OF INHIBITION

Briefly, as illustrated in Figure 1, the *N*-linked glycans of mammalian glycoproteins are 'processed' with the sequential removal of their terminal glucose residues by the ER-resident glucosidases I and II, shortly after becoming glycosylated at specific asparagine residues. Remarkably, cells in culture can tolerate complete shutdown of these ER α-glucosidases.[74–78] Gucosidase I and II knockout mice have limited life spans.[79–81] People can tolerate long-term (months and years) treatment with glucosidase inhibitors under conditions where there is substantial repression of the ER enzymes. However, there are troubling adverse effects (i.e., gastric distress) which must be taken into consideration.[36,82]

Glucosidase inhibitors have been approved for the management of type II diabetes, Gaucher's disease. Glucosidase inhibitors had also advanced to phase II human trials for management of hepatitis C virus and human immunodeficiency virus infection.[82,83] Thus, although mammalian cells and animals tolerate significant, and even total, repression of ER glucosidases, many viruses cannot. Indeed, completion of the

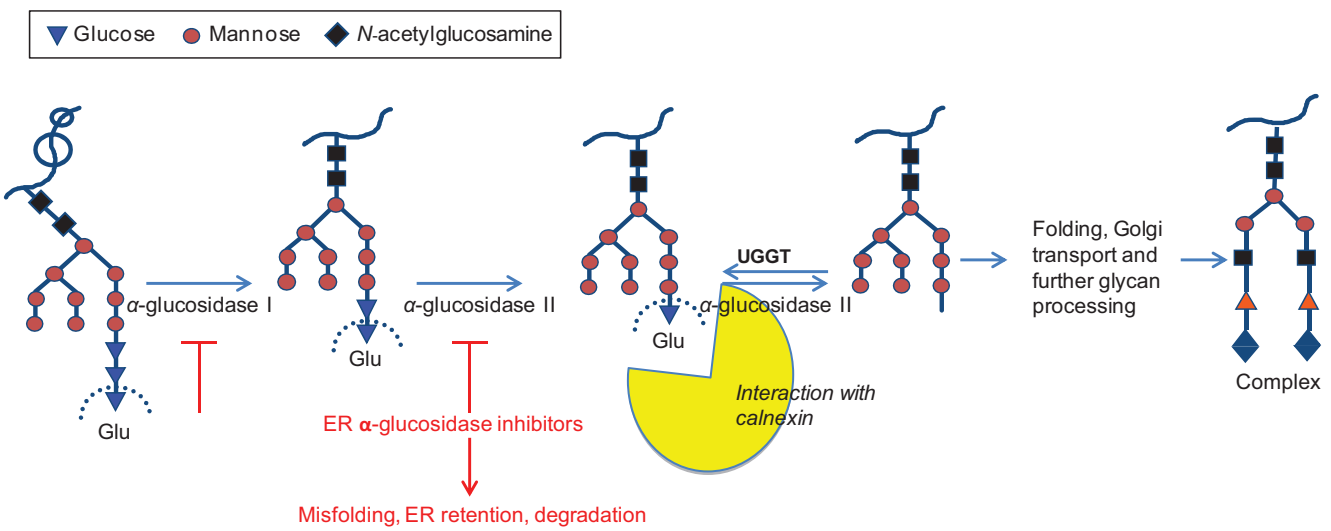

Figure 1 Glucosidase mediated steps in the *N*-linked glycosylation pathway. The pathway of *N*-linked glycosylation at asparagine of nascent polypeptides is shown. Polypeptides synthesized by translation in the ER are shown as the ribbon line (black) as unfolded, with the unprocessed 3-glucose terminal containing 'lollipop' oligosaccharide structure, attached at an asparagine. The terminal glucose and second to terminal glucoses (Glu) of this oligosaccharide is removed processively by ER resident, membrane bound, glucosidases I and II. Following removal of these glucose molecules, the protein chaperon, Calnexin, mediates folding of the polypeptide, which is then transported to the Golgi and further processed for secretion. Inhibition of ER glucosidases prevents polypeptide interaction with Calnexin and results in polypeptide misfolding, retention and/or degradation.

life cycle of many enveloped viruses requires functional glucosidases.[29] For example, viruses such as the hepadnaviruses, flaviviruses, filoviruses and influenza virus are significantly repressed by imino sugars under conditions where there is no detectable affect upon the host cells, or in the cases of animal experiments, only limited affect upon the host animals[36,74,84] (Table 2). The sensitivity of these viruses to glucosidase inhibitors is presumably because they possess envelope proteins that must oligomerize, and they have an apparent requirement for glucosidase processing of their glycoproteins presumably to enable time-sensitive proper calnexin mediated folding for their oligomerization.[35,49,52,58,85]

Taken together, the trend is clear and has not been broken for any of the viruses that have been tested so far: enveloped viruses that bud from intracellular membranes and/or use calnexin dependent pathways are selectively sensitive to glucosidase inhibitors. This has been expanded to viruses from four families causing hemorrhagic fevers (*Filoviridae, Bunyaviridae, Arenaviridae and Flaviviridae*) (Table 2).

CURRENT USES AND LIMITATIONS OF GLUCOSIDASE INHIBITORS

A wide variety of structurally diverse compounds, from both nature and synthesis, have been identified as glucosidase inhibitors.[86,87] Some of them have been successfully used to treat human diseases.[82,83] For example, inhibition of intestinal glucosidases digesting carbohydrates is therapeutic for the management of type II diabetes.[88] The imino sugar *N*-butyl-deoxynorjirimycin (NBDNJ) is currently approved for the treatment of Gaucher's disease, with patients taking near gram amounts a day for many years.[89,90] However, the molecular target of the imino sugar in Gaucher's disease is a glucocerebroside transferase, which is highly sensitive to NBDNJ, and not ER α-glucosidases. Nevertheless, these successes demonstrate the principle of tolerability of this category of drugs, as well as their practical therapeutic benefits.

The benefits of imino sugar glucosidase inhibitors in the treatment of viral infections have been less clear. The currently available and human-tested glucosidase inhibitors are all limited by side effects and poor pharmacokinetic properties that make them less appealing for controlling chronic diseases. For example, although glucosidase inhibitor celgosivir met the viral reduction milestone for the treatment of chronic hepatitis C in human studies, the drug was not advanced presumably because of gastritis and, frankly, the emergence of more potent direct acting antivirals. Thus, a consistent problem has been maintaining serum concentrations of compounds that are antiviral without causing the intestinal upsets, due to the inhibition of intestinal glucosidases.[30,91]

ANTIVIRAL ACTIVITY OF IMINO SUGARS AGAINST HEMORRHAGIC FEVER VIRUSES

There are now several reports showing that orally administrated imino sugar derivatives inhibited dengue virus[59–62] and Japanese encephalitis virus [67] in mice, as well as woodchuck hepatitis virus in woodchucks.[30] Although the reduction of viremia level was limited, the results were encouraging. We note, however, that reduction of viremia by even 1–2 logs has been anticipated to significantly improve clinical outcomes by reducing the severity of disease and increasing survival rates.[39,92] Along these lines, we recently reported imino sugar compounds that significantly reduced mortality in mouse model of lethal derivatives inhibited dengue virus infection.[63] Taken together, these results make it reasonable to consider finding broadly active glucosidase inhibitors reaching therapeutic levels in people for multiple viral indications.

However, as indicated, one major limitation for developing imino sugar antivirals has been the lack of potency and/or poor pharmacokinetic properties, which lead to difficulty in maintaining therapeutic drug concentrations *in vivo*. We have improved the antiviral potency of imino sugars from the platform (NBDNJ) by more than 500-fold (Figure 2). For example, based upon the encouraging *in vivo* efficacy

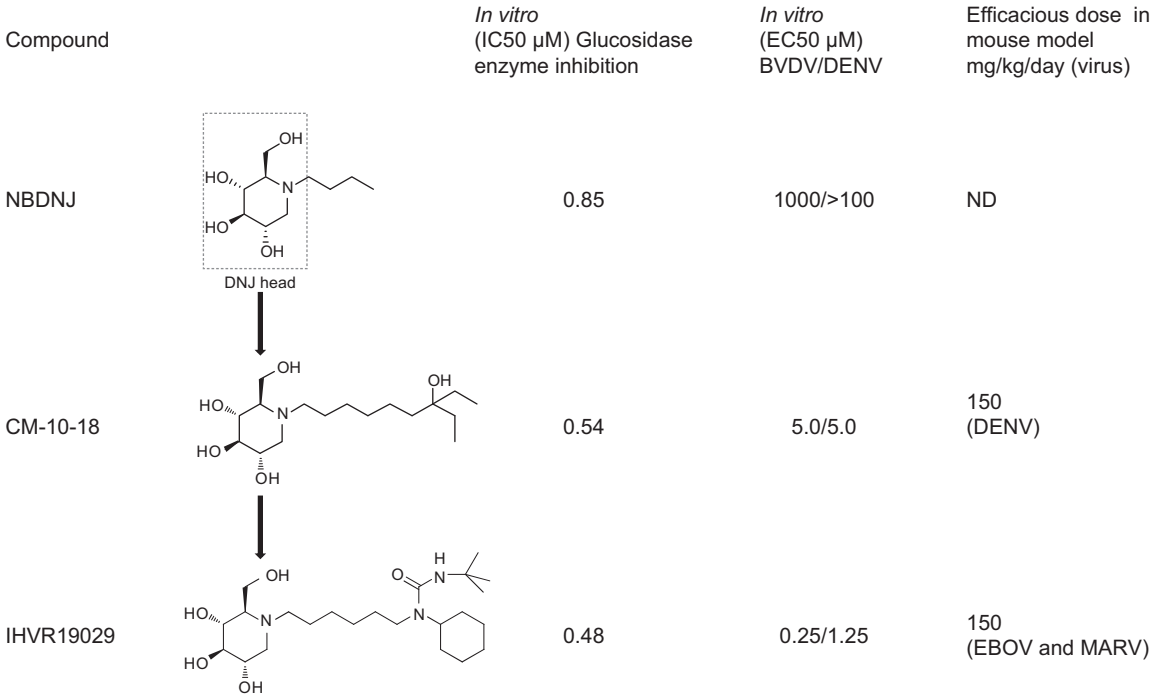

Figure 2 Modifications of the imino sugar NBDNJ that greatly improve antiviral activity but not enzyme inhibitory activity. The imino sugar NBDNJ is a butylated DNJ with millimolar antiviral activity *in vitro*. Alterations of its side chain as represented in compounds CM-10-18 and IHVR-19029, improve antiviral activity by up to 1000-fold, but only modestly improve enzyme inhibition. This is presumably because the improvements are largely related to cell access. See text. ND, not done.

results achieved with Dengue mouse models, we have synthesized imino sugar glucosidase inhibitors, represented by tert butyl urea (u) DNJ (19029), with submicromolar antiviral activity against four families of hemorrhagic fever viruses in cultured cells.[37] In addition, significant *in vivo* efficacy in Ebola and Marburg virus infected animals has also been achieved (70%–80% protection).

MOLECULAR BASIS OF THE ANTIVIRAL SELECTIVITY OF GLUCOSIDASE INHIBITORS

Imino sugars, such as those with DNJ head groups, are glucose mimetics competitive inhibitors of the *N*-glycan processing enzymes glucosidase I and II (Figure 2). As stated and shown in Figure 1, all *N*-linked glycans, following transfer to acceptor asparagine amino acids on nascent glycoproteins, are 'trimmed' in the ER by a series of sequentially active glycoprocessing enzymes. ER α-glucosidases I and II are the first enzymes to function in this pathway.[77,78] Specifically, all nascent *N*-linked glycoproteins contain three terminal glucose residues at the distal termini of their *N*-glycans following transfer of the oligosaccharide to the protein by way of the enzyme oligosaccharyltransferase. Immediately following this transfer, the terminal glucoses are removed, sequentially, by the action of ER resident enzymes glucosidases I and then II. The ER chaperons, calnexin and calreticulin, recognize monoglucosylated *N*-glycans and then 'fold' the nascent glycoprotein after which it is further processed by mannosidases and transferred to the Golgi where it is further modified into its characteristic complex carbohydrate structures. Underglucosylated polypeptides that have not been folded and have not transferred to the Golgi may get second chances following reglucosylation and the possibility of calnexin and calreticulin interactions by the enzyme UDP-glucose glycoprotein: glucosyltransferase. However, ultimately, misfolded or unfolded polypeptides are sent to the proteasomes where they are degraded. Thus, glucosidase inhibitors prevent the interaction of nascent *N*-glycoprotiens with calnexin and calreticulin and cause their misfolding and degradation. Misfolded or unfolded polypeptides may be sent to the proteasomes for degradation. It has been known for more than 20 years that many viruses are sensitive to glucosidase inhibition, and the sensitivity of Sindbis virus, influenza virus and fowl plague virus envelope proteins was reported in the 1980s.[33]

Viruses that depend upon calnexin and calreticulin would thus be expected to be the most sensitive to glucosidase inhibition. And we developed a 'biogenesis' theory that viruses that bud from the endoplasmic reticulum would be sensitive to glucosidase inhibitors. This was not to say that viruses that did not bud from the ER would not be sensitive to glucosidase inhibitors, and indeed the infectivity of retroviruses which bud from the plasma membrane is apparently greatly affected by glucosidase inhibitors.[93] We would now offer a refinement of the 'biogenesis' theory, in light of what is now known, to claim that viruses that depend upon the calnexin/calreticulin type pathway for morphogenesis will be sensitive to glucosidase inhibition.

These predictions, for the most part, seem to be supported by experimental results. Most strikingly is the example of hepatitis B virus and bovine viral diarrhea virus which are both sensitive to glucosidase inhibition, and are two completely different viruses.[8,94–96] Hepatitis B virus has a DNA genome and is a human pararetrovirus that primarily infects hepatocytes. Bovine viral diarrhea virus is a flavivirus with an RNA genome and grows primarily in non-liver cells. Both, however, acquire their envelopes by budding into the endoplasmic reticulum.[8,94–96] Both, we now know, also depend upon calnexin for the maturation of specific viral glycoproteins.[97–100] As Table 2 shows, both are also greatly inhibited by glucosidase inhibitors at concentrations that do not apparently affect cell viability.

ADDITIONAL BIOLOGICAL ACTIVITY OF IMINO SUGARS

Although imino sugars have been developed as host glucosidase inhibitors to disrupt viral glycoprotein folding and consequentially, virion morphogenesis, the compounds have additional biological activities due to either on-target suppression of ER α-glucosidases or off-target interference of other cellular components.[101] For example, on-target inhibition of ER glucosidase results in viral glycoprotein degradation has been shown to enhance major histocompatibility complex I presentation of epitopes derived from viral envelope glycoproteins. Moreover, changes in the glycan structures associated with viral glycoproteins may also alter their interaction with C-type lectins and consequentially, inhibit pro-inflammatory cytokine production.[62] In addition, some imino sugars may even have off target activities, repressing the virus by mechanisms that do not involve inhibition of ER glucosidases.[59] Off-target activities are not unusual in compound development, and in the case of extra biological effects upon antigen presentation and cytokine function, could even be beneficial. They may also provide interesting research leads. However, it is essential that such activities be recognized and be part of the decision to either progress or disqualify a compound, and if compounds with off-target activity are advanced, these activities must be monitored.

CONCLUSIONS

The imino sugars have, in the past, been proposed primarily for use in chronic infections such as human immunodeficiency virus and chronic viral hepatitis.[30,82,83] However, they may be best suited to treat acute viral infections, such as filoviral hemorrhagic fevers which involve weeks of therapy, as opposed to chronic infections which may require years of use. This would certainly avoid the complications that sometimes appear with longer-term use. Moreover, the new generation of compounds being brought forward should be designed to avoid or reduce intestinal distress. Finally, used for life-threatening infections such as the hemorrhagic fevers or severe influenza, either alone or in combinations with other management regimes, the glucosidase inhibitors could provide a powerful option in the treatment tool kit.

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