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Iminosugars: Promising therapeutics for influenza infection

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ABSTRACT

Influenza virus causes three to five million severe respiratory infections per year in seasonal epidemics, and sporadic pandemics, three of which occurred in the twentieth century and are a continuing global threat. Currently licensed antivirals exclusively target the viral neuraminidase or M2 ion channel, and emerging drug resistance necessitates the development of novel therapeutics. It is believed that a host-targeted strategy may combat the development of antiviral drug resistance. To this end, a class of molecules known as iminosugars, hydroxylated carbohydrate mimics with the endocyclic oxygen atom replaced by a nitrogen atom, are being investigated for their broad-spectrum antiviral potential. The influenza virus glycoproteins, hemagglutinin and neuraminidase, are susceptible to inhibition of endoplasmic reticulum α -glucosidases by certain iminosugars, leading to reduced virion production or infectivity, demonstrated by *in vitro* and *in vivo* studies. In some experiments, viral strain-specific effects are observed. Iminosugars may also inhibit other host and virus targets with antiviral consequences. While investigations of anti-influenza iminosugar activities have been conducted since the 1980s, recent successes of nojirimycin derivatives have re-invigorated investigation of the therapeutic potential of iminosugars as orally available, low cytotoxicity, effective anti-influenza drugs.

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Introduction

Influenza viruses (INVs) are negative-sense RNA viruses of the Orthomyxoviridae family that can be classified as INV A, B, or C on the basis of nucleoprotein (NP) antigenic specificity (WHO, 1980). INV A is further classified by hemagglutinin (HA) and neuraminidase (NA) subtype. 18 HA and 11 NA antigenic types are currently described, although only H1N1 and H3N2 INV As are currently in general circulation in the human population (Centers for Disease Control and Prevention, 2013; Zhang et al., 2015). INV strain nomenclature follows the conventions of the World Health Organization throughout (WHO, 1980). Birds constitute the major animal reservoir for INV, as all known INV A subtypes infect birds except for H17N10 and H18N11, which are solely found in bats (Centers for Disease Control and Prevention, 2013). In contrast, INV B subtypes are classified based on the derivation from Victoria or Yamagata strains (Rota et al., 1990). INV A and B cause the majority of human infections during annual epidemics, while INV A can also cause pandemics. Pandemic viruses originate through

antigenic shift, which can occur during co-infection of intermediate hosts, such as pigs, with viruses generated in susceptible species, allowing reassortment between the eight single-stranded RNA segments of each virion (discussed in Kawaoka & Neumann, 2012). INV C infections are typically asymptomatic (Kawaoka & Neumann, 2012) and will not be considered here. INV infection of respiratory epithelial cells results in transient tracheo-bronchitis, whilst alveolar viral replication may lead to severe pneumonia and respiratory distress syndrome. Occasionally, complications such as myopathy, myocarditis, and encephalopathy may arise (Kuiken & Taubenberger, 2008). The annual global incidence of infection is estimated at 5–10% in adults and 20–30% in children (WHO, 2014). Hospitalization and deaths are most likely in the very young, the elderly, and patients with comorbidities, with the total burden of epidemic disease estimated at three to five million severe cases and 250,000–500,000 deaths per year (WHO 2014). This broad profile of infection, morbidity, and mortality exists despite prophylactic vaccination and current therapeutics.

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Influenza A and B viruses share a similar structure, both possessing the major surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) which are instrumental in the viral replication cycle. HA initiates cellular infection through binding sialic acid (N-acetylneuraminic acid) residues on host cell glycoproteins and glycolipids. Following receptor binding, the virion is endocytosed. Endosomal acidification triggers exposure of the HA fusion peptide and membrane fusion, alongside disruption of protein–protein interactions, releasing ribonucleoprotein (RNP) complexes to the cytosol. Nuclear import of RNPs is followed by viral RNA polymerase-mediated production of mature vRNA and mRNA. HA and NA are translated using host machinery in the endoplasmic reticulum (ER) and modified in the Golgi apparatus, before being directed to the cell membrane for virion packaging and budding. HA tethers the virion at the cell surface until NA sialidase activity cleaves terminal sialic acid residues from cell-surface molecules, mediating virion release. NA sialidase activity also opposes virion aggregation and may enhance infectivity by cleaving mucins, improving access to respiratory epithelia (reviewed in Bouvier & Palese, 2008). Thus HA and NA play a central role in INFLUENZA infection.

Influenza infections are targeted using vaccination and antiviral drugs. Vaccine formulations must be updated annually before the flu season due to changes in circulating INFLUENZAS. HA is often the most immunogenic vaccine component but is also the most antigenically variable due to the diversity present in the animal reservoir (Ellebedy & Webby, 2009). Incorrect predictions can adversely affect vaccine efficacy, as seen in the Northern hemisphere 2014/2015 winter where vaccine efficacy was lower and the burden of disease higher than expected, with mismatched H3N2 viruses predominating (Centers for Disease Control and Prevention, 2015). A universal INFLUENZA vaccine remains elusive, although multiple approaches are being taken towards achieving cross-protective immune responses (Zhang et al., 2015). Even with an effective broadly protective INFLUENZA vaccine, a complementary repertoire of therapeutics would be required and particularly important for treating individuals with poor responses to vaccination.

Therapeutics currently in use and under development for influenza infection interfere with multiple stages of the viral life cycle, predominantly directly targeting the virus (Figure 1) and some targeting the host (Figure 2). Given the segmented RNA genome of INFLUENZA, and its reliance on the viral RNA-dependent RNA-polymerase which facilitates genomic mutation (Aggarwal et al., 2010), the development of resistance against any direct-acting antiviral will remain a constant challenge.

Iminosugars, discussed in this review, target the host glycosylation machinery to give rise to antiviral effects. Their indirect mechanism of action both allows broad-spectrum antiviral activity and provides protection against the development of antiviral drug resistance in circulating viruses. This parallels the aims of vaccine development, inducing broad and robust protection against INFLUENZAS. The reliance of INFLUENZA on the host glycosylation machinery will be discussed before iminosugars are introduced.

Cellular glycosylation pathways: the role of ER α -glucosidases I and II

Glycosylation is a fundamental cellular process: most ER-derived proteins are glycosylated (Chang et al., 2013a). The most common form, N-linked glycosylation, is initiated by the addition of the oligosaccharide $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ (Glc, glucose; Man, mannose; GlcNAc, N-acetylglucosamine) to an asparagine residue of the nascent polypeptide in the ER lumen (Kornfeld & Kornfeld, 1985). The asparagine is usually found in the sequon asparagine-X-serine/threonine, where X represents any amino acid except proline (Kasturi et al., 1997). Sequons differ in glycosylation efficiency, with those containing threonine being efficiently glycosylated, and serine, inefficiently so (Kasturi et al., 1997). Sequential processing of the oligosaccharide by α -glucosidases I and II (Figure 3) determines polypeptide interaction with ER chaperones required for correct protein folding. α -Glucosidase I cleaves the α -1,2-glycosidic bond to remove the outermost glucose residue, followed by α -glucosidase II-mediated removal of the two inner α -1,3-linked glucoses (Kornfeld & Kornfeld, 1985). The chaperones calnexin (CNX) (Hammond et al., 1994) and calreticulin (CRT) (Hebert et al., 1995) associate with monoglucosylated glycans, promoting correct disulfide bond formation through interaction with ERp57 (Molinari & Helenius, 1999). Certain iminosugars inhibit ER α -glucosidases, preventing glucose trimming and interaction with CNX/CRT, thus representing a therapeutic target in infections with viruses that require interaction with CNX/CRT for the folding of functional glycoproteins (Norton et al., 2007). If folding is incomplete, UDP-Glc:glycoprotein glucosyltransferase (UGGT) reglucosylates the glycoprotein, enabling cyclical interaction with chaperones until the native conformation is achieved (D'Alessio et al., 2010; Hammond et al., 1994). Alternatively, persistently misfolded proteins enter the ER-associated degradation (ERAD) pathway for proteasomal degradation (reviewed in Benyair et al., 2015). However, the detection of tri-glucosylated viral glycoproteins, produced *in vitro* and *in vivo* in the presence

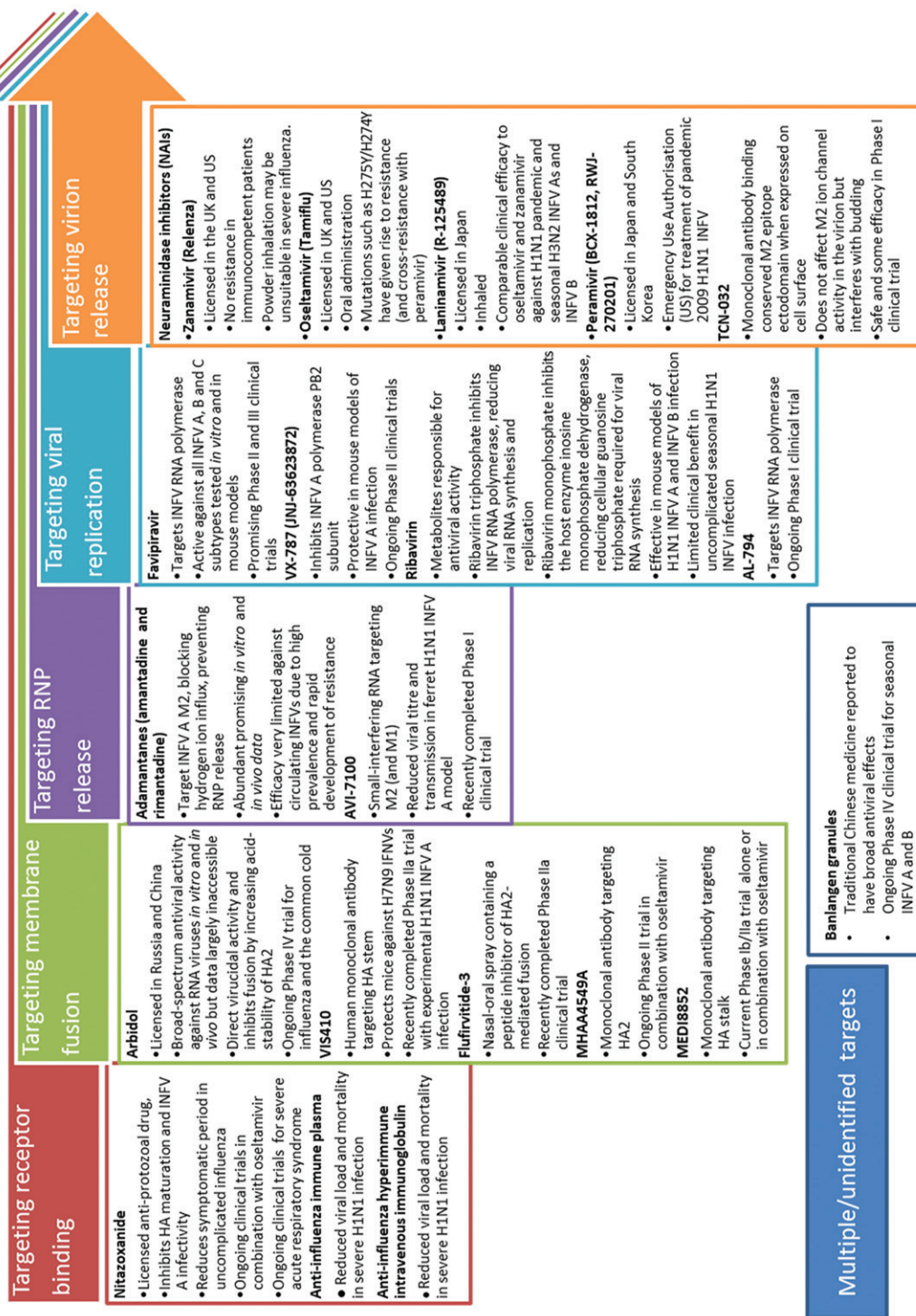


Figure 1. Licensed and prospective antiviral therapeutics in clinical trials for influenza virus infection. The continual rise of resistance to current therapeutics necessitates the development of further anti-influenza drugs, targeted at multiple stages of the infection and replication cycle. Therapeutics described in [Figure 1](#) are currently or have recently been under investigation in clinical trials registered with Clinicaltrials.gov. Therapeutics targeting receptor binding include nitazoxanide (Rossignol et al., 2009; reviewed in Rossignol, 2014), anti-influenza immune plasma (Luke et al., 2006; NIAID, 2010; Zhou et al., 2007; reviewed in Hui & Lee 2013) and anti-influenza hyperimmune intravenous immunoglobulin (Hung et al., 2013; NIAID, 2014). Therapeutics targeting membrane fusion include arbidol (Gagarinova et al., 1993; reviewed in Blaising et al., 2014), VIS410 (Tharakaraman et al., 2015; Visterra Inc, 2015), Fluifirvidide-3 (Autoimmune Technologies LLC [date unknown]; Autoimmune Technologies LLC, 2015), MHAA4549A (Genentech Inc., 2014; Lim et al., 2016; Nakamura et al., 2013), and MEDI8852 (Kallewaard et al., 2016; MedImmune LLC, 2015). Therapeutics targeting ribonucleoprotein release include the adamantanes, amantadine, and rimantadine (Davies et al., 1964; Wingfield et al., 1969; reviewed in Alves Galvão et al., 2014), and AVI-7100 (NIAID, 2012; reviewed in Dunning et al., 2014). Therapeutics targeting viral replication include favipiravir (Furuta et al., 2002; reviewed in Furuta et al., 2013), VX-787 (Clark et al., 2014; reviewed in Stevaert & Naesens, 2016), ribavirin (Durr et al., 1975; Eriksson et al., 1977; Smith et al., 1980; Smeets et al., 2006) and AL-794 (Allos Biopharma Inc., 2015; reviewed in Blair & Cox, 2016). Therapeutics targeting virion release include neuraminidase inhibitors (Babu et al., 2000; Kim et al., 1997; von Itzstein et al., 1993; Yamashita et al., 2009; reviewed in Kamali & Holodny 2013) and TCN-032 (Ramos et al., 2015). Therapeutics with unidentified mechanisms include banlangen granules (Hutchison Whampoa Guangzhou Baiyunshan Chinese Medicine Company Limited, 2012).

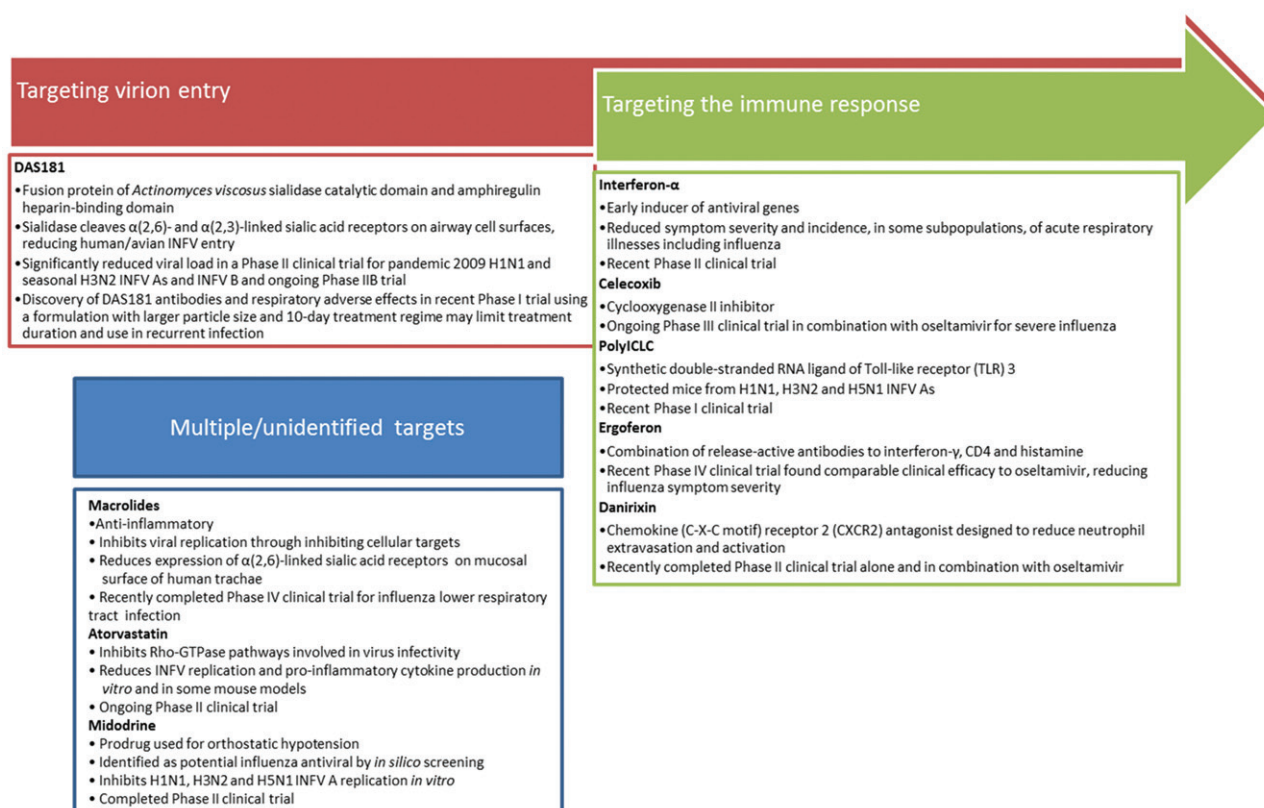


Figure 2. Host-targeted therapeutics in clinical trials for influenza virus infection. In addition to therapeutics directly targeting the INFV virion, there are many host-targeting drugs in development to combat influenza infection. Therapeutics summarized below are currently or have recently been under investigation in clinical trials registered with Clinicaltrials.gov. DAS181 is a prospective therapeutic targeting virion entry (Ansun Biopharma Inc., 2012; Malakhov et al., 2006; Zenilman et al., 2015; reviewed in Nicholls et al., 2013). Therapeutics targeting the immune response to antiviral effect include interferon- α (Amarillo Biosciences Inc, 2010; Bennett et al., 2013; Solov'ev, 1969), celecoxib (University of Hong Kong, 2014; Zheng et al., 2008; reviewed in Hui & Lee 2013), polyICLC (Wong et al., 1995; reviewed in Wong et al., 2009), ergoferon (Aver'ianov et al., 2012; Verevshchikov et al., 2011), and danirixin (GlaxoSmithKline, 2015). Therapeutics with multiple or unidentified targets include macrolides (Chinese University of Hong Kong, 2013; Cronk & Naumann, 1954; reviewed in Min & Jang, 2012), atorvastatin (Beth Israel Deaconess Medical Center, 2013; Haidari et al., 2007; reviewed in Mehrbod et al., 2014), and midodrine (Hospices Civils de Lyon, 2012; Josset et al., 2010).

of iminosugars, demonstrates a pathway whereby mis- or partially folded glycoproteins can be produced, due to lack of interaction with CNX/CRT (Block et al., 1998; Hussain et al., 2015). In addition, certain iminosugars can enhance secretion of high-mannose glycoproteins (Marcus & Perlmutter, 2000), indicating that ER quality control may be bypassed, such as by Golgi-resident endo- α -D-mannosidase, which cleaves the bond between glucose residues and the polymannose chain of the oligosaccharide (Moore & Spiro, 1990). However, the utilization of this pathway is cell-type specific, and is completely absent in the processing of INFV A/Puerto Rico/8/34 (PR8, H1N1) HA in Chinese hamster ovary (CHO) and Madin-Darby canine kidney (MDCK) cells (Karaivanova et al., 1998). Differential utilization of this pathway complicates the results of iminosugar-mediated ER α -glucosidase inhibition and indicates the importance of using physiologically relevant cell types.

The importance of N-linked glycosylation for HA and NA

HA

HA determines initial receptor binding and endosomal fusion, thus underlies virion infectivity. The HA precursor, HA0, trimerizes in the ER, and is later enzymatically cleaved to form functional HA1 and HA2, exposing the HA2 fusion peptide (reviewed in Skehel & Wiley, 2000). Membrane-distal residues of HA1 (Tyr98, Trp153, His183, and Tyr195) and secondary structural elements (130-loop, 220-loop, and 190- α -helix) contribute to the receptor binding site (Gamblin & Skehel, 2010). Different HA subtypes vary considerably in both the number and location of N-linked glycosylation sites. An amino acid sequence analysis in 1991 found four glycosylation sites in H4, seven in H9, H11 and H13, eight in H6, nine in H12,

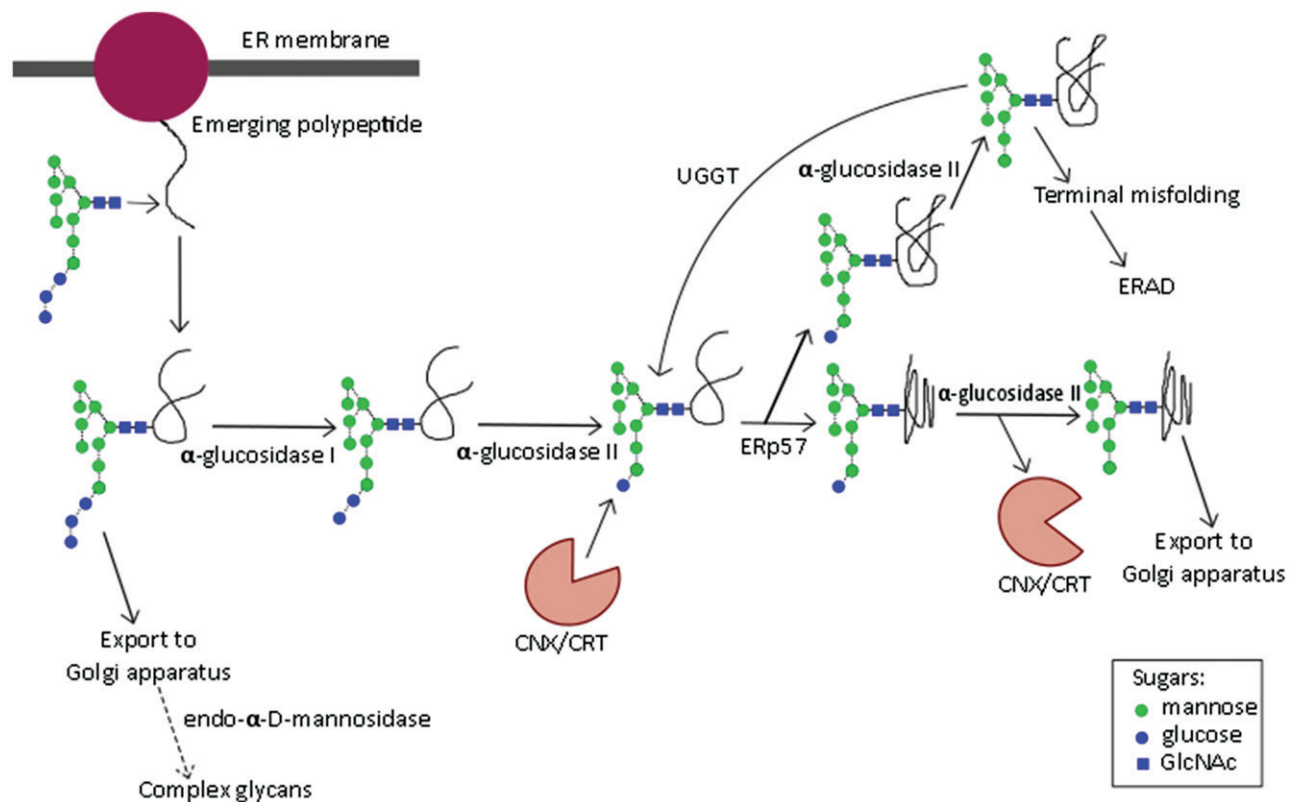


Figure 3. N-linked oligosaccharide processing in the endoplasmic reticulum. Glycan structure nomenclature follows the recommendations of the Consortium for Functional Genomics (Consortium for Functional Genomics, 2012).

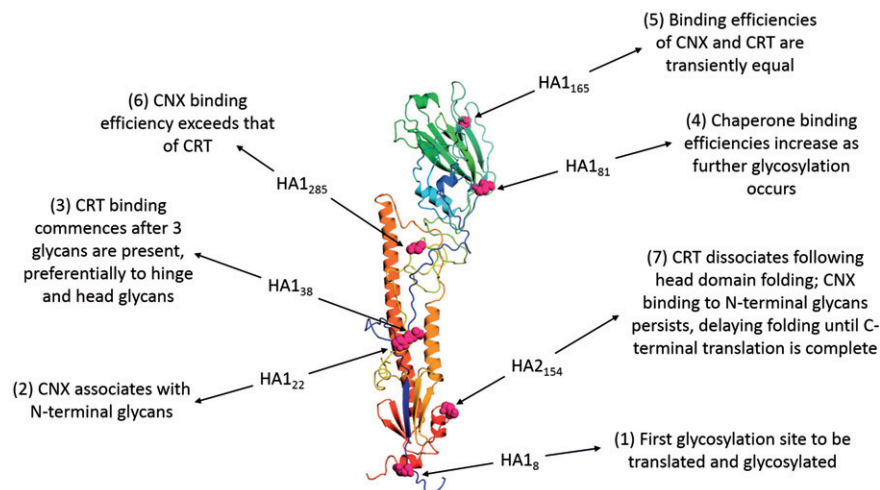


Figure 4. The role of N-linked glycosylation in the folding of INFLUENZA A VIRUS (INFLUENZA) HA. The figure shows HA of the INFLUENZA A/Aichi/68-derived X31 strain (H3N2) with data derived from PDB ID: 1HGF (Sauter et al., 1992b). The polypeptide chain is colored in an N- to C-terminal blue-to-red gradient. The asparagine residues of the seven N-linked glycosylation sites are highlighted in magenta spheres and numbered according to position within mature HA1 or HA2. Labels indicate how the binding of CNX and CRT varies during cotranslational glycosylation.

and 10 in H8 subtype HA (Nobusawa et al., 1991). However, this represents a snapshot view of the glycosylation status of INFLUENZA A VIRUS (INFLUENZA) HA molecules, since the number of glycosylation sites varies both within subtypes and over time, as exemplified by H1 (Sun et al.,

2011), H3 (Skehel & Wiley, 2000), H5 (Chen et al., 2012), and H7 (Lebarbenchon & Stallknecht, 2011) INFLUENZAS. Despite the extensive variation in glycosylation, iminosugars retain their potential as antivirals for INFLUENZA A VIRUS (INFLUENZA) as even a single N-linked glycan can be sufficient to

render a glycoprotein susceptible to iminosugar activity (Block et al., 1994).

The receptor linkage specificity of HA determines the host range of the virus. $\alpha(2,6)$ -linked sialic acid is abundant in the human respiratory tract and this is reflected in the binding preference of human INFLUENZA A virus (HA), while $\alpha(2,3)$ -linkages are more common in the avian intestine and are preferentially bound by avian INFLUENZA A virus (HA) (Baum & Paulson, 1990; Rogers & D'Souza, 1989). In contrast, the porcine respiratory tract contains both linkages, reflected in the promiscuous receptor binding of porcine INFLUENZA A viruses (Ito et al., 1998). Despite the glycosylation of sialic acid-linked receptors for INFLUENZA A virus, iminosugars are not expected or intended to affect their sialic acid linkages, and thus not impact INFLUENZA A virus tropism. Glycosylation is implicated in the determination of host range since HA is divergently glycosylated in INFLUENZA A viruses from different species. An analysis of H1 INFLUENZA A viruses from ducks, swine and humans (Inkster et al., 1993) found that human viruses contained at least four additional glycosylation sites, some located at the HA head (compared with four and five membrane-proximal sites, respectively). Furthermore, mutations affecting N-glycosylation sites influence the receptor binding specificity and affinity of HA. Comparison of the parental INFLUENZA A virus isolate A/USSR/90/77 (H1N1), with HA Asn131, to the MDCK-adapted strain, with HA Asp131 (non-glycosylated), showed that glycosylation at residue 131 interfered with binding to soluble $\alpha(2,6)$ -linked sialic acid-containing receptors, but not to those with an $\alpha(2,3)$ -linkage (Gambaryan et al., 1998). Replication of the parent virus in mice led to loss of the HA glycosylation site at Asn131, or in the presence of mouse serum, at Asn158 (Marinina et al., 2003). The loss of either glycosylation site increased HA affinity for $\alpha(2,6)$ -linked sialic acid, and reduced affinity for the $\alpha(2,3)$ -linkage (Marinina et al., 2003). In contrast, enhanced binding to $\alpha(2,3)$ -linked receptors was observed for chicken egg-adapted H1 INFLUENZA A virus A and INFLUENZA B virus, with a loss of glycosylation sites at HA Asn163 and Asn187, respectively (Gambaryan et al., 1999). As such, it appears that different N-linked glycans differentially promote binding to the two sialic acid linkages. Consequently, perturbation of HA glycosylation profiles can have significant consequences for receptor binding and tissue tropism. To clarify, while the passage history of an INFLUENZA A virus is known to affect glycosylation, there is no precedent for this changing CNX/CRT dependency and, therefore, iminosugar susceptibility.

Glycosylation is important for HA folding, providing binding sites for the lectin-like chaperones CNX and CRT in the ER (Daniels et al., 2003). The nascent polypeptide chain is targeted to the ER by the N-terminal signal sequence, which is cleaved as translation

commences, allowing cotranslational folding to occur. Chaperone association with HA is dependent on the translation of glycosylation sites and is dynamic (Figure 4).

As translation proceeds, folding commences from the most luminal region of HA0 and once the top domain is folded, CRT dissociates, potentially due to glycan inaccessibility to UGGT-mediated reglucosylation. CNX binding persists and delays N-terminal processing until the C-terminus is translated, allowing assembly of the HA stem domain. Chaperone binding recruits ERp57 and disulfide bond formation occurs once HA Cys76 enters the ER lumen (Daniels et al., 2003). Thus glycosylation of HA facilitates correct folding, which enables protein progression along the secretory pathway. This is demonstrated by the requirement for at least five of the seven glycosylation sites, in both head and stem regions, of the HA from INFLUENZA A virus A/Aichi/68-derived X31 strain (H3N2) for transport of functional HA to the plasma membrane (Gallagher et al., 1992). Binding immunoglobulin protein (BiP), an HSP70-family chaperone, also has a role in HA folding, binding the N-terminal stalk region and helping to prevent the formation of non-native disulfide bonds (Segal et al., 1992). However, the lack of co-immunoprecipitation of BiP with normal X31 HA folding intermediates has led to the suggestion that it may not play a major role in normal HA maturation (Braakman et al., 1992), instead predominantly associating with and retaining misfolded proteins in the ER prior to ERAD (Gallagher et al., 1992; Hurtley et al., 1989).

Folding of HA is clearly glycosylation dependent, but what impact does glycosylation have on INFLUENZA A virus virulence? The majority of glycosylation sites are located on the membrane-distal globular head of HA (Vigerust & Shepherd, 2007). Antibodies targeted to this antigenically important site would reduce virulence, but this is prevented by immune evasion mechanisms, such as those contributed by N-glycosylation. Non-immunogenic host-derived oligosaccharides used in glycosylation mask immunogenic viral epitopes, preventing their recognition by the host immune system (Skehel & Wiley, 2000), illustrated by the 1968–1979 invariance of the A/Hong Kong/68 (H3N2) HA in the region masked by the oligosaccharide attached at Asn165 (Wiley & Skehel, 1987). Second, antigenic drift creates new glycosylation sites, generating resistance to antibody binding (Skehel & Wiley, 2000). This is demonstrated by the reversion of 1969 and 1975 INFLUENZA A virus A/Hong Kong isolates, which are glycosylated at Asn63, to recognition by an A/Hong Kong/68-targeted antibody when produced in the presence of a glycosylation inhibitor (Skehel et al., 1984). These mechanisms provide a rationale for the

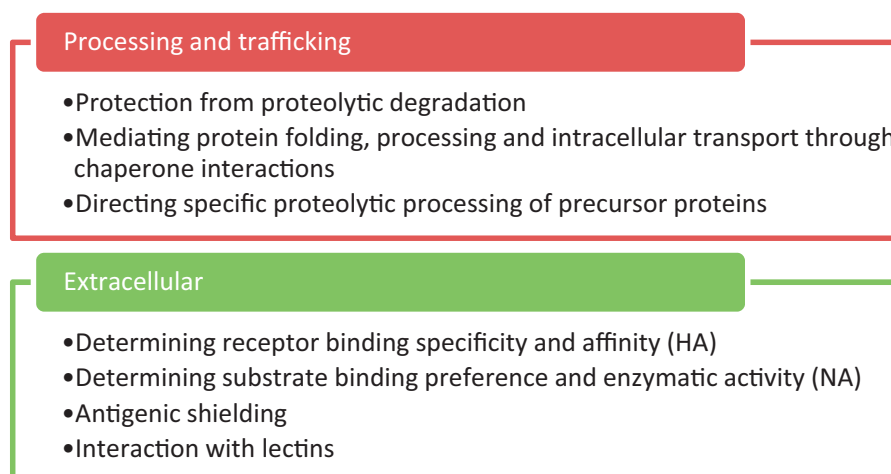


Figure 5. Summary of the roles of glycosylation in HA and NA function.

increase in HA glycosylation over time, which is seen in the H3N2 Hong Kong INFLVs, where the number of glycosylation sites has increased from 6 to 10, with three and seven of these in the antigenically important head region (Skehel & Wiley, 2000; Verhoeven et al., 1980). It must also be considered that the potential tri-glucosylated HA and NA produced following iminosugar treatment (Block et al., 1998; Hussain et al., 2015) may interact differently with soluble and cell-associated lectins important in INFLV interaction with the host (Tate et al., 2014). Such mechanisms indicate that the glycosylation status of HA is an important determinant of INFLV virulence. Thus N-linked glycosylation is important for HA production, viral tissue tropism, and virulence.

NA

As described for HA, glycosylation of NA is of multifactorial importance. NA is a tetrameric-stalked molecule with a membrane-distal sialidase active site which binds sialic acid in a different conformation to HA (Gamblin & Skehel, 2010). NA sialidase activity enables virion release from cells and prevents virion aggregation (Seto & Chang, 1969). NA does not affect receptor binding specificity (Rogers & Paulson, 1983), but the cleavage sequence specificity of NA and binding preferences of HA are interlinked (Wagner et al., 2002). Sialidase sequence specificity and activity, at least in N2 subtype NA, have changed over time to reflect adaptation to the human host and the HA binding preference for $\alpha(2,6)$ -linked sialic acid (Baum & Paulson, 1991; Matrosovich et al., 2000). Interestingly, increased HA glycosylation may reduce receptor-binding activity, resulting in inefficient replication if NA receptor-destroying activity remains high (Tsuchiya et al., 2002). Therefore, while NA does not directly affect host and tissue tropism through

receptor binding specificity, matching HA and NA sialic acid-linkage specificities are crucial for optimal virulence.

NA subtypes vary in numbers of glycosylation sites, in a manner similar to HA. NA subtypes possess two to four highly conserved glycosylation sites in the stem domain (Asn42, Asn52, Asn63, and Asn66), two conserved and additional middle-low conserved sites in the globular region (Asn87, Asn147, and Asn202, both by N2 numbering), and further sites at the junction of these domains (Chen et al., 2012; Wang et al., 2008). NA glycosylation is a determinant of correct folding. NA derived from the 1918 pandemic INFLV is additionally glycosylated on the stalk domain in the tetrameric form, compared with inactive monomeric or dimeric NA, enabling the formation of the higher-order structure. This additional glycosylation also endows resistance to trypsin digestion, which has been hypothesized to increase virulence through contributing to the diverse tissue distribution of this pandemic virus (Wu et al., 2009). Conversely, glycosylation may also reduce virulence. The introduction of Asn130 into the NA of INFLV A/WSN/33 (H1N1) reduced NA enzymatic activity 20-fold and attenuated neurovirulence in mice (Li et al., 1993). This glycosylation site (Asn146 by N2 numbering) is conserved across all NA subtypes (Chen et al., 2012), so might represent a virulence determinant of wider significance. Glycosylation of NA is also important for its maturation. Globular head rather than stem domain glycans are essential for maturation of NA from INFLV A/tern/Australia/G70C/75 (H1N9) (Wang et al., 2008) and INFLV A/duck/Ukraine/1/63 (H3N8) (Saito & Kawano, 1997). Glycosylation can affect NA substrate preference, with mutation of NA Asn130 of INFLV A/duck/Ukraine/63 (H3N8) reducing binding to small but not large substrates (Saito & Kawano, 1997). Glycosylation of NA also

functions in viral immune evasion as discussed for HA, with multiple glycosylation sites in the N1 and N2 globular domains implicated (Chen et al., 2012). Thus glycosylation impacts NA folding, maturation, and virulence.

Dependency on N-glycosylation differs between HA and NA: MDCK cells infected with the reassortant virus NWS-duck/Ukraine/1/63 (H1N8) and treated with the glycosylation inhibitor tunicamycin showed an 80% and 97% reduction in HA and NA transport to the cell surface, respectively (Saito & Yamaguchi, 2000). Therefore, N-linked glycosylation is important for both HA and NA function, to different extents (Figure 5).

The importance of glycosylation for structure and function of HA and NA indicates that inhibition of ER α -glucosidases might be an effective therapeutic strategy against INFLUENZA VIRUS (INFLU). Since both endogenous and viral glycoproteins utilize the ER folding apparatus, one might anticipate detrimental effects on host cell protein homeostasis. However, glycoprotein folding is not eliminated by glucosidase inhibitors with antiviral efficacy (Braakman & van Anken, 2000), indicating redundancy in the folding apparatus. Since binding mechanisms to CNX/CRT are the same for host and viral glycoproteins, this suggests that there could be an intrinsic difference in folding requirements. The oligomeric structures of HA and NA necessitate correct folding of multiple interfaces for functional glycoprotein production. Such stringency may confer an amplification effect of susceptibility to iminosugar-mediated glycoprotein misfolding. Furthermore, glycoproteins including INFLU HA fold in domains formed from non-continuous regions of the polypeptide chain, requiring a delay in N-terminal folding until the C-terminus has been translated (Braakman & van Anken, 2000), potentially conferring greater reliance on ER chaperones than proteins folding in an N-to-C-terminal fashion. Thus, viral glycoproteins may be more dependent on ER folding machinery than those of the host.

Iminosugars: potential broad-spectrum antivirals

Iminosugars are a structurally diverse class of molecules defined as hydroxylated carbohydrate mimics where the endocyclic oxygen atom is replaced by a nitrogen atom (Nash et al., 2011). Modifications, such as alkyl chain addition, affect biological properties such as uptake by cells and organelles thereby impacting antiviral efficacy and cytotoxicity (Norton et al., 2007; Sayce et al., 2016). Structural mimicry of terminal sugar moieties in natural substrates underlies iminosugar biological activity. Namely, glucose mimics competitively inhibit ER

α -glucosidases causing misfolding of viral glycoproteins that may lead to retention or degradation of these products (Asano, 2007). Iminosugars have additional targets: N-alkylated deoxynojirimycin (DNJ) and deoxygalactonojirimycin (DGJ) derivatives inhibit ceramide-specific glucosyltransferase, preventing glycosphingolipid accumulation in lysosomal storage disorders (Dwek et al., 2002). There are other off-target effects of iminosugars, such as inhibition of glucosidases in the gastrointestinal tract, which must be considered for clinical application (Andersson et al., 2000). Despite such activity, iminosugars are already well tolerated in the clinic, with type I Gaucher and Niemann-Pick type C diseases treated with *N*-butyl-deoxynojirimycin (NB-DNJ, Miglustat), and non-insulin-dependent diabetes with *N*-hydroxyethyldeoxynojirimycin (Miglitol) (Dwek et al., 2002).

Iminosugar antiviral activity has been demonstrated for a range of viruses. 6-O-Butanoyl castanospermine (BuCAST) reduced murine brain viral load of herpes simplex virus type 1 (HSV-1) following cutaneous infection (Bridges et al., 1995). In studies of bovine viral diarrhoea virus (BVDV), DNJ derivatives reduced virion production and infectivity, impairing E1 and E2 glycoprotein folding and heterodimerization (Durantel et al., 2001). DGJ and DNJ derivatives with long alkyl chains additionally increased E2 homodimerization and reduced virion infectivity (Durantel et al., 2001). In HCV-like particles, DNJ derivatives impaired glycoprotein processing and folding (Chapel et al., 2006). DNJ derivatives provided some protection in lethal murine infection models with Ebola and Marburg filoviruses (Chang et al., 2013b). Iminosugar activity against flaviviruses has been demonstrated: *N*-nonyl-deoxynojirimycin (NN-DNJ) reduced secretion of the glycoproteins E and NS1 and virion production in dengue virus (DENV) and Japanese encephalitis virus (JEV), and reduced mortality rate in a JEV mouse lethal challenge model (Wu et al., 2002). Multiple iminosugars have demonstrated antiviral efficacy against DENV *in vitro* and *in vivo* (Chang et al., 2011; Perry et al., 2013; Sayce et al., 2010; Warfield et al., 2015), and celgosivir has recently completed Phase IB clinical trial in DENV infection (Low et al., 2014; Sung et al., 2016). It has been suggested that iminosugars might impact ER-budding viruses, like DENV, and plasma membrane-budding viruses, such as INFLU, differently (Norton et al., 2007). However, NB-DNJ reduced glycoprotein maturation, secretion, and function of the human immunodeficiency virus (HIV) gp120 (Fischer et al., 1996), which, like INFLU, buds from the plasma membrane. Thus, it is the dependence of viral glycoproteins on CNX/CRT for virion morphogenesis, rather than the cellular structure from which the virus buds, that is hypothesized to underlie antiviral activity mediated by

Table 1. Iminosugars targeting ER α -glucosidases have been tested *in vitro* against a range of INFLUENZA A VIRUS (INFLUENZA A VIRUS) strains, resulting in effects on viral glycoproteins and virion production.

Iminosugar; concentration	INFLUENZA A VIRUS strain	Cell line	Viral glycoprotein processing inhibition Inhibition of virion production or infectivity	Reference
<i>Swainsonine</i> 1 μ g/ml	A/fowl plague virus/ Rostock/34 (H7N1)	Primary calf kidney	Complex glycan formation (10% of control) and high-mannose oligosaccharide processing	Elbein et al. (1982)
0.005–5 μ g/ml	A/NWS/33 (H1N1)	MDCK	No effect on infectivity or hemagglutination Glucose trimming	Merkle et al. (1985)
25, 100 ng/ml	A/NWS/33 (H1N1)	MDCK	No effect on hemagglutination Fucosylated hybrid oligosaccharides produced; no effect on sulfation	Schwarz & Elbein (1985)
			Not tested	
<i>Castanospermine (CAST)</i> 10 μ g/ml	A/NWS/33 (H1N1)	MDCK	Complex glycan formation None	Pan et al. (1983)
10–500 μ g/ml	A/NWS/33 (H1N1)	MDCK	Glucose trimming and sulfation No effect on hemagglutination	Merkle et al. (1985)
25, 100 μ g/ml	A/NWS/33 (H1N1)	MDCK	Glucose trimming and sulfation	Schwarz & Elbein (1985)
			Not tested	
1 mM	A/HKx31 (H3N2)	CHO 15B	HA glucose trimming and CNX binding	Hammond et al. (1994)
			Not tested	
200 μ g/ml	A/NWS/33 (H1N1)	MDCK	HA glucose trimming	Kaushal et al. (1988)
			Not tested	
200 μ g/ml	A/HKx31 (H3N2)	CI42	HA glucose trimming and CNX binding	Ermonval et al. (2000)
			Not tested	
200 μ g/ml	A/HKx31 (H3N2)	B3F7 AP2-1	HA glucose trimming and CNX binding	
			Not tested	
200 μ g/ml	A/HKx31 (H3N2)	Mad1 A214	No inhibition of HA glucose trimming or CNX binding	
			Not tested	
1 mM	Reassortant virus NWS-duck/Ukraine/ 1/63 (H1N8)	MDCK	NA secretion 50% of control; HA unaffected. NA activity 50% and HA titer >50% of control	Saito & Yamaguchi (2000)
			PFU 30% of control	
12, 25, 50, 100, 200 μ M	A/Hong Kong/11/88 (sic)	MDCK	Not tested	Tyms & Virogen Ltd (2003)
			IC ₅₀ 15 μ M	
<i>6-O-butanoyl-castanospermine (BuCAST)</i> 0.2 mM	A/Puerto Rico/8/34 (H1N1)	MDCK	HA processing by endomannosidase	Karaivanova et al. (1998)
			No effect on production	
6, 12, 25, 50, 100 μ M	A/Hong Kong/11/88	MDCK	Not tested	Tyms and Virogen Ltd (2003)
			IC ₅₀ <6 μ M	
<i>2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (DMDP)</i> 5, 50, 250 μ g/ml	A/NWS/33 (H1N1)	MDCK	Glucose trimming Not tested	Elbein et al. (1984b)
<i>1-deoxynojirimycin (DNJ)^a</i> 2 mM	A/chick/Germany/49 (H10N7)	MDCK	Surface HA expression unaffected	Burke et al. (1984)
			Infectivity by approximately 30%. No effect on production	
10 mM	A/fowl plague virus/ Rostock/34 (H7N1)	MDCK	Not tested	Huang et al. (1991)
			Hemagglutination and CPE (complete)	
1 mM	A/HKx31 (H3N2)	CHO 15B	Glucose trimming and CNX binding	Hammond et al. (1994)
			Not tested	
1 mM	Reassortant virus NWS-duck/Ukraine/ 1/63 (H1N8)	MDCK	NA activity >40% of control and HA titer >40% of control	Saito & Yamaguchi (2000)
			PFU 30% of control	
<i>N-methyl-1-deoxynojirimycin (NM-DNJ)</i> 0.5, 1 mM	A/fowl plague virus/ Rostock/34 (H7N1)	CEC	HA cleaved	Romero et al. (1983)
			No effect on virion release	

(continued)

Table 1. Continued

Iminosugar; concentration	INFLV strain	Cell line	Viral glycoprotein processing inhibition Inhibition of virion production or infectivity	Reference
2 mM	H7 strains	CEC	Glucose trimming No effect on infectivity	Bosch et al. (1984)
<i>N</i> -butyl-deoxynojirimycin (NB-DNJ) Titration	A/Udorn/307/72 (H3N2)	MDCK	Not tested	Hussain et al. (2015)
			HA titer: IC ₅₀ 21.7 ± 15.9 µM, IC ₉₀ 280.0 ± 23.6 µM. Infectivity: IC ₅₀ 34.7 ± 11.2 µM, IC ₉₀ 296.1 ± 16.1 µM.	
Titration	A/Brisbane/10/2007 (H3N2)	MDCK	Not tested	
			HA titer: IC ₅₀ 43.8 ± 6.5 µM, IC ₉₀ 207.0 ± 95.3 µM. Infectivity: IC ₅₀ 43.6 ± 11.8 µM, IC ₉₀ 250.0 ± 10.4 µM.	
Titration	A/Lviv/N6/2009 (H1N1)	MDCK	Not tested	
			HA titer: IC ₅₀ 51.3 ± 11.3 µM, IC ₉₀ >312.5 µM. Infectivity: IC ₅₀ 46.5 ± 12.2 µM, IC ₉₀ >312.5 µM.	
<i>N</i> -nonyl-deoxynojirimycin (NN-DNJ) Titration for IC ₅₀ and IC ₉₀ determination, otherwise 62.5 µM	A/Udorn/307/72 (H3N2)	MDCK	HA secretion. NA sialidase activity (by 35–45%). 26.4% triglycosylated HA glycans HA titer 6–8% of control. Infectivity: IC ₅₀ 0.4 ± 0.2 µM, IC ₉₀ 16.2 ± 4.7 µM. Plaque number: IC ₅₀ >62.5 µM. Plaque size: IC ₅₀ 6.6 ± 5.5 µM, IC ₉₀ >62.5 µM, not restored with exogenous NA	Hussain et al. (2015)
Titration for IC ₅₀ and IC ₉₀ determination, otherwise 62.5 µM	A/Brisbane/10/2007 (H3N2)	MDCK	HA secretion. NA sialidase activity (by 30–40%). 21.8% triglycosylated HA glycans HA titer 0% of control. Infectivity: IC ₅₀ 1.73 ± 0.3 µM, IC ₉₀ 10.3 ± 0.3 µM. Plaque number: IC ₅₀ 8.2 ± 2.4 µM, IC ₉₀ 22.0 ± 9.5 µM. Plaque size: IC ₅₀ 4.1 ± 1.2 µM, IC ₉₀ 10.9 ± 0.3 µM, not restored with exogenous NA	
Titration for IC ₅₀ and IC ₉₀ determination, otherwise 62.5 µM	A/Lviv/N6/2009 (H1N1)	MDCK	No effect on surface HA or NA. NA sialidase activity (by 45–60%). 37.3% triglycosylated HA glycans HA titer 13–25% of control. Infectivity: IC ₅₀ 1.9 ± 0.8 µM, IC ₉₀ >62.5 µM. Plaque number: IC ₅₀ >62.5 µM. Plaque size: IC ₅₀ 1.8 ± 0.3 µM, IC ₉₀ >62.5 µM, not restored with exogenous NA	
Titration	Reassortant X-181 (H1N1)	MDCK	Not tested	
Titration	Reassortant X-171b (H3N2)	MDCK	IC ₅₀ >62.5 µM Not tested	
Titration	Reassortant A/ Brisbane/10/2007 (H3N1) with A/Lviv/ N6/2009 NA	MDCK	IC ₅₀ 0.4 ± 0.1 µM, IC ₉₀ 2.4 ± 0.5 µM Not tested	
			Plaque number: IC ₅₀ 9.2 ± 1.7 µM, IC ₉₀ 55.0 ± 6.8 µM. Plaque size: IC ₅₀ 5.8 ± 1.2 µM, IC ₉₀ 14.3 ± 2.7 µM. Effects greater relative to A/Lviv/N6/2009 and comparable to A/Brisbane/10/2007	
<i>N</i> -8'-(2'-tetrahydrofuranyl)-octyl-deoxynojirimycin (2THO-DNJ, UV-12) Titration <250 µM	A/Texas/36/91 (H1N1)	MDCK	Not tested Infectivity: IC ₅₀ >250 µM	Warfield et al. (2015)
<i>N</i> -(9-methoxynonyl)-1-deoxynojirimycin (MON-DNJ, UV-4) Titration	A/Texas/36/91 (H1N1)	MDCK	Not tested IC ₅₀ >125 µM	Warfield et al. (2016)
Titration	A/California/07/2009 (H1N1)	MDCK	Not tested IC ₅₀ >125 µM	

(continued)

Table 1. Continued

Iminosugar; concentration	INFLV strain	Cell line	Viral glycoprotein processing inhibition Inhibition of virion production or infectivity	Reference
Titration	A/Mississippi/3/2001 (H1N1)	MDCK	Not tested	
			IC ₅₀ >125 µM	
Titration	A/Mississippi/3/2001 H275Y (H1N1)	MDCK	Not tested	
			IC ₅₀ >125 µM	
Titration	A/Hong Kong/68 (H3N2)	MDCK	Not tested	
			IC ₅₀ 6.01 µM	
Titration	A/Perth/16/2009 (H3N2)	MDCK	Not tested	
			IC ₅₀ 63.9 µM	
Titration	A/Victoria/361/2011 (H3N2)	MDCK	Not tested	
			IC ₅₀ 3.75 µM	
Titration	A/Victoria/3/75 (H3N2)	MDCK	Not tested	
			IC ₅₀ >84.9 µM	
Titration	A/Philippines/2/82 (H3N2)	MDCK	Not tested	
			IC ₅₀ >250 µM	
Titration	B/Lee/40	MDCK	Not tested	
			IC ₅₀ >125 µM	
Titration	B/Brisbane/60/2008	MDCK	Not tested	
			IC ₅₀ >125 µM	
Titration	B/Wisconsin/01/2010	MDCK	Not tested	
			IC ₅₀ >125 µM	
Titration	A/California/07/2009 (H1N1)	dNHBE	Not tested	
			IC ₉₀ >320 µM	
Titration	A/California/12/2012 (H1N1)	dNHBE	Not tested	
			IC ₉₀ 320 µM; 219 µM	
Titration	A/Victoria/3/75 (H3N2)	dNHBE	Not tested	
			IC ₉₀ 440 µM; 483 µM	
Titration	A/Texas/50/2012 (H3N2)	dNHBE	Not tested	
			IC ₉₀ 82 µM	
Titration	B/Brisbane/60/2008	dNHBE	Not tested	
			IC ₉₀ 200 µM	
Titration	B/Florida/4/2006	dNHBE	Not tested	
			IC ₉₀ 150 µM	
Titration	B/Massachusetts/2/ 2012	dNHBE	Not tested	
			IC ₉₀ 209 µM; 245 µM	
Titration	B/Malaysia/2506/2004	dNHBE	Not tested	
			IC ₉₀ >500 µM	
<i>N</i> -benzyl-1,5-dideoxy-1,5-imino- <i>D</i> -glucitol 10 mM	A/fowl plague virus/ Rostock/34 (H7N1)	MDCK	Not tested	Huang et al. (1991)
			Hemagglutination (partial)	
<i>N</i> ,2- <i>O</i> -dibenzyl-1,5-dideoxy-1,5-imino- <i>D</i> -glucitol 10 mM	A/fowl plague virus/ Rostock/34 (H7N1)	MDCK	Not tested	Huang et al. (1991)
			Hemagglutination and CPE (complete)	
<i>Homonojirimycin</i> (HNJ) 100, 200 µg/ml	A/NWS/33 (H1N1)	MDCK	HA high-mannose oligosaccharide processing Not tested	Zeng et al. (1997)
Titration	A/Puerto Rico/8/34 (H1N1)	MDCK	Not tested	Zhang, et al. (2013)
			Infectivity: IC ₅₀ 10.4 µg/ml in CPE reduction assay	
<i>N</i> -methyl- α - <i>homonojirimycin</i> (NM-HNJ) 25, 100 µg/ml	A/NWS/33 (H1N1)	MDCK	25 µg/ml: HA high-mannose oligosaccharide processing (5-fold) 100 µg/ml: HA high-mannose oligosac- charide processing (complete) Not tested	Zeng et al. (1997)

B3F7AP2–1 and MadIA214: glycosylation-defective CHO cells; CEC: chicken-embryo cell; C142: parental CHO cell; CPE: cytopathic effect; dNHBE: differentiated normal human bronchial epithelial; HA titer: haemagglutination titer; IC₅₀ or IC₉₀: drug concentration required to inhibit by 50% or 90%, respectively. ^aDNJ cannot be considered a specific inhibitor of α -glucosidases since it also inhibits the formation of dolichol-linked oligosaccharides required for N-linked glycosylation (Datema et al., 1984).

Table 2. Antiviral activity of UV-4B in a virus yield reduction assay format varies with INFLV strain in MDCK cells.

Influenza strain	IC ₅₀ UV-4B (μM) ^a				IC ₅₀ oseltamivir (μM) ^a			
	A	B	C	Average	A	B	C	Average
A/Texas/36/91 (H1N1)	>125	>125	–	>125	0.63	1.32	–	0.975
A/California/07/09 (H1N1)	>125	>125	–	>125	3.99	1.91	–	2.95
A/Mississippi/3/2001 (H1N1)	>125	>125	–	>125	0.173	0.069	–	0.121
A/Mississippi/3/2001 H275Y (H1N1)	>125	>125	–	>125	>500	>500	–	>500
A/Hong Kong/68 (H3N2)	5.98	6.04	–	6.01	0.673	0.423	–	0.548
A/Perth/16/2009 (H3N2)	60.5	67.2	–	63.9	1.55	3.83	–	2.69
A/Victoria/361/2011 (H3N2)	2.45	5.05	–	3.75	1.93	2.06	–	1.995
A/Victoria/3/75 (H3N2)	>250	90.9	79.0	>84.9	0.151	0.137	0.131	0.140
A/Philippines/2/82 (H3N2)	>250	>250	>250	>250	7.27	31.3	6.22	14.9
B/Lee/40	>125	>125	–	>125	16.4	17.2	–	16.8
B/Brisbane/60/2008	>125	>125	–	>125	0.657	0.391	–	0.524
B/Wisconsin/01/2010	>125	>125	–	>125	3.20	0.98	–	2.09

Cells were seeded in 24-well plates and incubated with two-fold serial dilutions of UV-4B, starting at 125 μM for 1 h. Cells were infected with INFLV for 1 h after which medium was added and cells were incubated for 4 d. Harvested supernatants were stored at –80 °C and thawed for titer evaluation in MDCK cells using a TCID₅₀ assay. Titers for each dilution were plotted against the UV-4B concentration and data points were fitted using a 4-PL algorithm.

^aIC₅₀ data shown are the results for individual experiments (A–C), each as the average of 2–3 replicates. Results shown as greater than (>) are the highest concentration tested. –: not determined.

ER α-glucosidase inhibition. Additionally, the host-rather than virus-directed mechanism of action of iminosugars is expected to provide protection against the development of resistance. This has been observed in NB-DNJ treatment of HIV-1 infection *in vitro* (Pollock et al., 2008), and a high genetic barrier to escape mutants was found in DENV treatment with N-(9-methoxynonyl)-1-deoxynojirimycin (MON-DNJ, UV-4) (Plummer et al., 2015). Therefore, investigation of iminosugar-mediated antiviral activity against INFLV is warranted.

Iminosugars: effective therapeutics against INFLV?

INFLV glycoproteins and iminosugars have been used as tools to analyze cellular glycosylation pathways, with clear evidence of iminosugars affecting INFLV glycoproteins in isolation, as summarized in Appendix 1. In some cases, effects on influenza virion production and infectivity were observed, leading to consideration of iminosugar therapeutic application. The following sections illustrate the effects of iminosugars targeting ER α-glucosidases in INFLV infection *in vitro* and *in vivo*. The impact of iminosugars targeting other host enzymes on INFLV infection is detailed in Appendix 2.

Iminosugars have variable efficacy against INFLV *in vitro*

There are variable effects of iminosugars on viral glycoprotein processing and virion release and infectivity (Table 1). In some cases, there are differences in iminosugar antiviral efficacy between INFLV strains, such as with BuCAST (Karaivanova et al., 1998; Tyms & Virogen Ltd, 2003), NB-DNJ (Hussain et al., 2015), NN-DNJ

(Hussain et al., 2015), and MON-DNJ (Table 2). However, MON-DNJ demonstrated antiviral efficacy independently of strain against H1N1 and H3N2 INFLV As and INFLV Bs when primary human bronchial epithelial cells were infected rather than MDCK cells (Warfield et al., 2016). To pursue the determinant of strain-specificity, reassortants derived from INFLV A/Puerto Rico/8/34, X-181 (with HA and NA sequences 99% identical to A/Lviv/N6/2009 (H1N1)) and X-171b (with HA and NA of A/Brisbane/10/2007 (H3N2)) have been compared, demonstrating that HA was likely to be the determinant of iminosugar susceptibility (Hussain et al., 2015). Furthermore, NA was not deemed responsible due to a lack of strain-specificity in reductions in NA sialidase activity, or plaque size restoration with exogenous NA. In addition, comparison of parental strains and a reassortant encoding NA from INFLV A/Lviv/N6/2009 (H1N1) and other RNA segments from INFLV A/Brisbane/10/2007 (H3N2) showed that reduced virion infectivity with NN-DNJ treatment was mediated by effects on HA rather than NA (Hussain et al., 2015). Cell type-dependent differences are observed in iminosugar effects, such as with CAST (Ermonval et al., 2000) or MON-DNJ treatment (Warfield et al., 2016 compared with Table 2), despite the same INFLV strains being used. Potential explanations for the cell type- and INFLV strain-specificity of iminosugar effects are discussed in “Conclusions and perspectives”.

Iminosugars have antiviral efficacy in INFLV mouse models

Following *in vitro* studies, investigations in animal models have been conducted. Iminosugars have been tested in BALB/c mice against a range of INFLV strains (Table 3). MON-DNJ was tested against lethal doses of INFLVs A/Texas/36/91 (H1N1) and A/Perth/261/2009 (H1N1;

Table 3. Iminosugars have been tested *in vivo* against a range of INFLUENZA strains, with reduced pathology and increased survival in murine lethal infection models achieved.

Iminosugar; INFLUENZA	Model	Study size	Dose Response Control(s)	Reference
<i>6-O-butanoyl-castanospermine (BuCAST)</i> 10 ³ –10 ⁴ PFU A/Puerto Rico/8/34 (H1N1), intranasal	Female BALB/c mice weighing 15–20 g	<i>n</i> = 5/group	200 or 400 mg/kg/d orally BID from 2 h p.i. for 72 h 200 mg/kg: 20% less increase in lung tissue mass and 2.8-fold reduction in lung PFU 400 mg/kg: 15% less increase in lung tissue mass and 10-fold reduction in lung PFU PBS	Tyms & Virogen Ltd (2003)
<i>Homonojirimycin (HNJ)</i> 5 ID ₅₀ A/Puerto Rico/34 (H1N1), intranasal	Female BALB/c mice weighing 18–22 g	<i>n</i> = 12/group	0.5, 1, and 2 mg/kg orally BID from 2 d pre-challenge, for 6 d 1 mg/kg: MSD 12.3 ± 1.5 d. 2 mg/kg: MSD 11.7 ± 0.9 d Saline: MSD 9.7 ± 3.2 d. 70 mg/kg ribavirin: 70% survival at day 15	Zhang et al. (2013)
		<i>n</i> = 6/group	1 mg/kg orally BID from 2 d pre-challenge, for 6 d Significant reduction in lung viral titer at days 4 and 6 p.i. Saline. 70 mg/kg ribavirin: significantly reduced lung viral titer	
		<i>n</i> = 18/group	1 mg/kg orally BID beginning 2 d pre-challenge, for 6 d Increased serum IFN-γ increased at days 4 and 6 p.i., and IL-10 at days 2, 4 and 6 p.i. Reduced serum IL-6 at days 4 and 6 p.i., and TNF-α at days 2, 4, and 6 p.i. Similar effects in lung tissue Saline	
<i>N-(9-methoxynonyl)-1-deoxynojirimycin (MON-DNJ, UV-4)</i> 1 LD ₉₀ mouse-adapted A/Texas/36/91 (H1N1), intranasal	6–8 week old female BALB/c mice	<i>n</i> = 10/group	10 or 100 mg/kg orally from 1 h pre-challenge, and for 7 d p.i. BID or TID 10 mg/kg: no effect. 100 mg/kg: BID 60% survival; TID 100% survival Water: 0% survival	Stavale et al. (2015)
		<i>n</i> = 10/group	10, 20, 40, 60, 80, or 100 mg/kg orally TID from 1 h pre-challenge, for 10 d 80 mg/kg: 60% survival at day 14. 100 mg/kg (the MED): 100% survival at day 14 Water: 0% survival. 20 mg/kg oseltamivir phosphate orally BID for 5 d: 100% survival at day 14	
		<i>n</i> = 10/group	100 mg/kg orally TID starting at –1, 24, 48, 72, 96, or 120 h relative to challenge, for 7 or 10 d Significant protection when given 72–96 hp.i. for 7 or 10 d Water: 20% survival. 20 mg/kg oseltamivir phosphate BID for 5 d: protection <120 h p.i.	
		<i>n</i> = 5/group	100 mg/kg orally TID from 1 h pre-challenge, for 10 d No significant increase in lung tissue mass; lower mean lung viral titer per gram by TCID ₅₀ assay at days 2, 4, and 7 p.i. Water: increase in lung tissue mass; approximately 1 log higher lung viral titers per gram than treated mice. 20 mg/kg oseltamivir phosphate: similar mass and titers to MON-DNJ-treated mice	
		<i>n</i> = 20/group, treated; <i>n</i> = 10/group, control	100 mg/kg orally TID from 1 h pre-challenge, for 10 d 100% survival; average serum HAI titers were 62, 43, and 174 on days 15, 30, and 120, respectively Water: 10% survival; similar HAI titers	
1 LD ₉₀ mouse-adapted oseltamivir-resistant A/Perth/261/2009 (H1N1), intranasal	6–8 week old female BALB/c mice	<i>n</i> = 10/group	40, 60, 80, 100, 150, or 200 mg/kg orally TID from 1 h pre-challenge, for 10 d 40 mg/kg: 40% survival. 60 mg/kg: 70% survival. 80, 100, 150, and 200 mg/kg: 100% survival Water: 20% survival. 20 mg/kg oseltamivir phosphate BID for 5 d: 10% survival	
50 µl 2010/2011 Fluvin® INFLUENZA vaccine, intramuscular, on days 0, 14, and	6–8 week old female BALB/c mice	<i>n</i> = 20/group, control; <i>n</i> = 10/group, treated	100 mg/kg orally TID for 10 d post-vaccination No effect on serum HAI titer on days 0, 14, 30, and 42 50 µl PBS intramuscular	

(continued)

Table 3. Continued

Iminosugar; INFV	Model	Study size	Dose Response Control(s)	Reference
28				
1 LD ₉₀ mouse-adapted A/California/04/2009 (H1N1), intranasal	6–8 week old female BALB/c mice	<i>n</i> = 10/group, treated; <i>n</i> = 15/group, control	50, 75, 100, or 150 mg/kg orally TID from 1 h pre-challenge, for 10 d 50 or 150 mg/kg: 80% survival. 75 mg/kg (the MED): 100% survival. 100 mg/kg: 100% survival Water: 7% survival. 20 mg/kg oseltamivir phosphate BID for 5 d: 100% survival	Warfield et al. (2016)
1 LD ₉₀ mouse-adapted A/New Caledonia/99 (H1N1), intranasal	17 g female BALB/c mice	<i>n</i> = 10/group, treated; <i>n</i> = 14/group, control	50, 75, 100, or 150 mg/kg orally TID from 1 h pre-challenge, for 7 d 50 mg/kg: 50% survival. 75 mg/kg: 75% survival. 100 mg/kg: 67% survival. 150 mg/kg: 89% survival Water: 0% survival. 30 mg/kg oseltamivir phosphate: 78% survival	
1 LD ₉₀ A/Pennsylvania/10/2010 (H3N2) swine variant, intranasal	17–20 g female BALB/c mice	<i>n</i> = 10/group, treated; <i>n</i> = 14/group, control	50, 75, 100, or 150 mg/kg orally TID from 1 h pre-challenge, for 7 d 50 mg/kg: 30% survival. 75 mg/kg: 90% survival. 100 mg/kg: 50% survival. 150 mg/kg: 70% survival Water: 7% survival. 20 mg/kg oseltamivir phosphate BID for 5 d: 50% survival	
1 LD ₉₀ B/Sichuan/379/99, intranasal	18 g female BALB/c mice	<i>n</i> = 10/group, treated; <i>n</i> = 15/group, control	50, 75, 100, or 150 mg/kg orally TID from 1 h pre-challenge, for 10 d 50 mg/kg: 0% survival. 75 mg/kg: 20% survival. 100 mg/kg: 50% survival. 150 mg/kg (the MED): 100% survival Water TID for 8 d: 0% survival. 10 mg/kg oseltamivir phosphate TID for 8 d: 100% survival	
<i>N</i> -8'-(2'-tetrahydrofuranyl)-octyl-deoxynojirimycin (2THO-DNJ, UV-12)				
1 LD ₉₀ mouse-adapted A/Texas/36/91 (H1N1), intranasal	6–8 week old female BALB/c mice	<i>n</i> = 10/group	20, 40, 60, 80, or 100 mg/kg orally TID from 1 h pre-challenge, for 10 d 20 mg/kg: 0% survival. 40 mg/kg: 50% survival at day 14. 60–80 mg/kg: 90% survival at day 14. 100 mg/kg (the MED): 100% survival at day 14 Water: 0% survival	Warfield et al. (2015)
		<i>n</i> = 10/group	100 mg/kg orally TID starting at –1, 24, 48, or 72 h relative to challenge, for 10 d 1 h relative to challenge: 100% survival. 24 h p.i.: 70% survival. 48–72 h p.i.: 0% survival Water: 0% survival	
		<i>n</i> = 10/group	60 mg/kg orally TID starting at -1, 24, 48, or 72 h relative to challenge, for 10 d –1 h relative to challenge: 80% survival. 24 h p.i.: 60% survival. 48 h p.i.: 80% survival. 72 h p.i.: 40% survival Water: 0% survival	

BID: bis in die (twice daily); HAI: hemagglutination inhibition; ID₅₀: dose that is infectious in 50% cases; LD₉₀: lethal dose in 90% cases; MED: minimum effective dose that is 100% protective; MSD: mean survival days; PBS: phosphate buffered saline; PFU: plaque forming units; p.i.: post infection; TCID₅₀: amount of pathogen inducing pathological change in 50% inoculated cell cultures; TID: ter in die (thrice daily).

oseltamivir-resistant) (Stavale et al., 2015). The MEDs (minimum effective dose that is 100% protective) were 100 and 80 mg/kg orally thrice daily, indicating efficacy against both oseltamivir-susceptible and -resistant INFVs. Significant protection was provided when MON-DNJ administration began as late as 72–96 h post-infection (Stavale et al., 2015). In contrast, *N*-(9'-methoxynonyl)-1,6-dideoxygalactonojirimycin (MON-6-deoxy-DGJ) administration did not protect mice from challenge with INFV A/Texas/36/91 (personal communication from KL Warfield to other authors; unreferenced). This supports the hypothesis that antiviral activity of iminosugars depends on inhibition of ER α -glucosidases, exhibited by MON-DNJ but not MON-6-deoxy-DGJ, rather than on their shared inhibition of ceramide-

specific glucosyltransferase. A further study demonstrated the protective effect of MON-DNJ against lethal doses of the H1N1 INFVs A/California/04/2009 and A/New Caledonia/99, the H3N2 INFV A/Pennsylvania/10/2010, and INFV B/Sichuan/379/99 (Warfield et al., 2016). MON-DNJ had no effect on serum haemagglutination inhibition titer following infection with INFV A/Texas/36/91 or vaccination with the 2010/2011 Fluvirin® INFV vaccine, indicating that protective vaccine-induced antibody responses were not disrupted by iminosugar treatment (Stavale et al., 2015). *N*-8'-(2'-tetrahydrofuranyl)-octyl-deoxynojirimycin (2THO-DNJ, UV-12) was also tested for efficacy against INFV A/Texas/36/91 (H1N1) and found to have a MED of 100 mg/kg orally thrice daily, and was protective against lethal infection

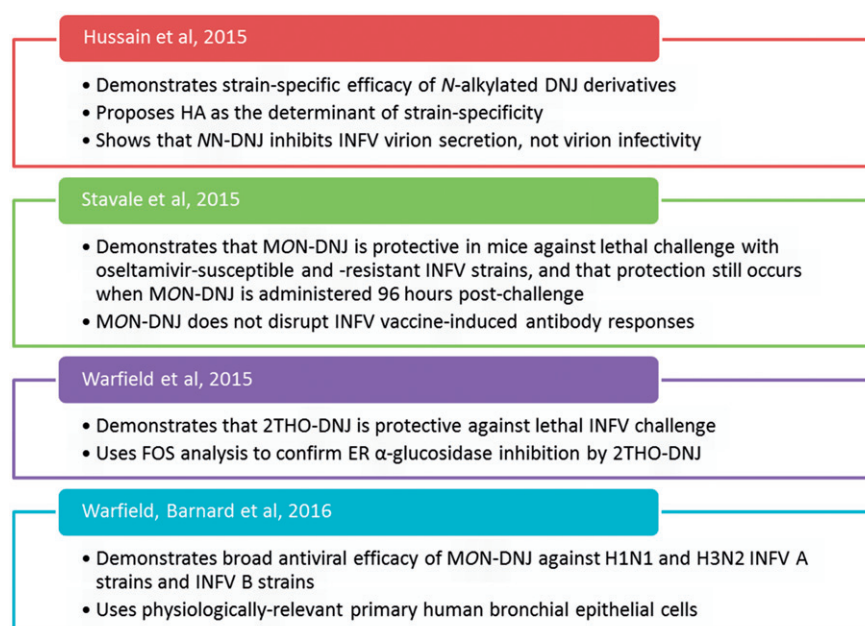


Figure 6. The key findings of recent papers to have advanced the field.

when treatment started 24–48 h post-infection (Warfield et al., 2015). Homonojirimycin (HNJ) has been tested *in vivo* against PR8 (H1N1), with 1 mg/kg twice per day increasing MSD (mean survival days) by 2.6 days relative to a saline control, reducing lung viral titers and modulating cytokine production (Zhang et al., 2013). BuCAST was also tested against the same viral strain and reduced lung viral titers and tissue mass (Tyms & Virogen Ltd, 2003).

Conclusions and perspectives

Iminosugars with glucostereochemistry present the opportunity to target a host process: modifying N-linked glycosylation and thus glycoprotein production, through inhibiting ER α -glucosidases. This may be harnessed as a broad-spectrum antiviral strategy, and antiviral efficacy has previously been shown against viruses including BVDV (Durantel et al., 2001), HCV (Chapel et al., 2006), JEV (Wu et al., 2002), DENV (Chang et al., 2011; Perry et al., 2013; Sayce et al., 2010; Warfield et al., 2015; Wu et al., 2002), and HIV (Fischer et al., 1996). Iminosugar antiviral activity is thought to be mediated by ER α -glucosidase inhibition; however, due to the ubiquity of glucose, iminosugars with glucostereochemistry exhibit off-target effects, which may or may not impact antiviral efficacy. Here, evidence for antiviral efficacy against INFVs has been presented, considering effects on INFVs in cell culture (Tables 1 and 2 and Appendix 2) and in mouse models (Table 3). In summary, iminosugar antiviral efficacy is observed *in vitro* and also in mouse models with MON-DNJ, HNJ and BuCAST, and in mouse

models with 2THO-DNJ (Table 3). In particular, MON-DNJ was protective against lethal doses of H1N1 and H3N2 INFV As and INFV B (Stavale et al., 2015; Warfield et al., 2016), and reduced lung viral titers with both oseltamivir-sensitive and -resistant INFV strains (Stavale et al., 2015), suggesting potential efficacy in difficult-to-treat infections. Recent publications have re-invigorated the investigation of iminosugars as anti-influenza therapeutics, and their contributions are highlighted in Figure 6.

INFV strain-dependent effects

One intriguing observation is that iminosugar efficacy varies between INFV strains. This is repeatedly seen for iminosugars that inhibit ER α -glucosidases, such as DNJ, NB-DNJ, NN-DNJ, and MON-DNJ in INFV-infected MDCK cells (Table 1). The molecular basis for this is poorly understood, but may be composed of cell type-specific and INFV strain-specific effects.

Cell type-dependent iminosugar efficacy may occur for a number of reasons, despite iminosugars having a common target. Iminosugar uptake by different cell types may vary. Iminosugars must cross both the plasma and ER membranes to access their ER α -glucosidase targets, which impose different permeability barriers to iminosugar entry (Tan et al., 1994), and have variable compositions in different cell types. While there is evidence for iminosugars entering cells by non-facilitated diffusion (Bollen & Stalmans, 1989; Neefjes et al., 1989), it is possible that iminosugar uptake may be affected by transporters (Mellor et al., 2004b). Iminosugar design has sought to improve antiviral

potency by increasing cellular uptake, through N-alkylation of DNJ and DGJ derivatives (Zitzmann et al., 1999), although very long N-alkyl chains limit iminosugar accessibility to the ER lumen (Mellor et al., 2004a). Iminosugar uptake can be confirmed through free oligosaccharide (FOS) analysis, whereby inhibition of ER α -glucosidases can be quantified through detecting the accumulation of diagnostic FOS on enzyme inhibition – for ER α -glucosidase I, Glc₃Man₅GlcNAc₁, and for ER α -glucosidase II, Glc₁Man₄GlcNAc₁ (Alonzi et al., 2008). This technique has been used previously in studies of iminosugar efficacy in INFLV infection (Warfield et al., 2015), but could be more widely adopted to confirm that iminosugars have accessed ER α -glucosidases to aid interpretation of antiviral efficacy data. Second, glycosylation pathways vary in different cell types. Expression levels of glycosylation pathway components may vary between cell types, alongside glycoprotein production levels. This means that iminosugars may be able to compete more or less successfully with N-linked glycoproteins for the ER α -glucosidase active site. Iminosugar inhibition of oligosaccharide processing by ER α -glucosidases can be bypassed by endo- α -D-mannosidase, but this pathway is not active in all cell types, and for example, does not contribute to HA processing in MDCK or CHO cells (Karaivanova et al., 1998). Cell types also show variation in glycoprotein trafficking pathways (Zurzolo et al., 1992). These mechanisms indicate that iminosugar antiviral efficacy with the same INFLV strain can be affected by the cell type used in studies. This illustrates the importance of using physiologically relevant cell types when studying glycosylation in viral infection, and of performing experiments in the same cell types. It is encouraging that broad antiviral efficacy was observed with MON-DNJ against H1N1 and H3N2 INFLV As and INFLV Bs using human bronchial epithelial cells (Warfield et al., 2016), a more physiologically relevant system than the historically favored MDCK cell.

However, cell-type differences alone cannot account for the observed variation in antiviral efficacy, since iminosugars tested in the same *in vitro* model against different INFLV strains display divergent efficacy. This implicates a viral determinant. HA has been proposed to underlie this strain-specificity by Hussain et al. (2015), who used reassortant viruses to analyze the strain-dependent effects of NN-DNJ. Since glycoproteins from different INFLV strains have evolved with different glycosylation sites (Chen et al., 2012; Nobusawa et al., 1991), their reliance on glucose trimming and dependence on CNX/CRT for correct folding may vary. While a single N-linked glycan can be sufficient to endow susceptibility to iminosugars (Block et al., 1994), glycoprotein misfolding will only occur with iminosugar-mediated inhibition

of ER α -glucosidases if folding is dependent on the CNX/CRT cycle. Different INFLV glycoprotein subtypes may vary in dependency on CNX/CRT for folding, perhaps relying to a greater or lesser extent on other ER chaperones such as BiP, or requiring less chaperoning to fold correctly. INFLVs may also have strain-specific requirements for the positioning of glycans to determine disulfide bond formation and for the extent of glucose trimming needed for glycoprotein stability and transport, leading to a variable impact of iminosugar treatment on virulence.

But how does the impact of iminosugars on INFLV compare with other viruses? The consequences of iminosugar-induced glycoprotein misfolding vary between viruses, possibly because misfolding may be detrimental for a certain life-cycle stage, such as virion formation or receptor binding and membrane fusion. Iminosugar treatment can reduce virion formation and secretion rather than infectivity of individual virions, such as with DENV (Sayce et al., 2016; Warfield et al., 2016) and HBV (Block et al., 1994). Alternatively, iminosugars can predominantly affect the infectivity of virions produced rather than total virion secretion, such as with HIV (Fischer et al., 1995). In INFLV infection, NN-DNJ reduces virion secretion rather than specific infectivity, to an extent that is dependent on INFLV strain (Hussain et al., 2015). This suggests that the overarching impact of iminosugars on INFLV is similar to that seen with DENV. However, the striking strain-specificity seen with INFLV is not recapitulated with DENV. DENV has four established serotypes, each expressing antigenically distinct N-linked glycoproteins prM and E on the virion surface, which confer susceptibility to iminosugars (alongside NS1) (reviewed in Sayce et al., 2010). Unlike with INFLV, MON-DNJ treatment of Vero cells infected with several isolates of each DENV serotype resulted in a similar antiviral efficacy between serotypes (Warfield et al., 2016). This suggests that the strain-specificity of iminosugar effects observed with INFLV is not a generalized feature of viruses possessing subtypes with distinct surface glycoproteins. Further work is certainly required to investigate the strain-dependence of iminosugar anti-influenza effects, enabling progress towards clinical development.

***In vitro* data may not predict *in vivo* iminosugar efficacy against INFLVs**

It is notable that iminosugars targeting ER α -glucosidases have consistently weaker effects on viral infection *in vitro* (Table 1) when H1 subtype INFLVs are considered relative to other subtypes, in experiments mainly utilizing MDCK cells. Although HA trimming and processing is affected in some of these experiments, the evidence

points to low efficacy against H1-bearing INFVs. However, in contrast to the MDCK-based *in vitro* data, mouse models consistently indicate potent antiviral efficacy of iminosugars against H1N1 INFVs, alongside a single report of efficacy against an H3N2 INFV (Table 3). Further *in vivo* investigation of iminosugar efficacy against H3N2 INFVs is required to better understand iminosugar strain-specificity, as it is possible that antiviral efficacy could be more pronounced than in H1 INFVs given the *in vitro* results. However, it is also possible that the observed disconnect in antiviral efficacy against H1N1 INFVs may be a function of the difference in the biology of the cells used for *in vitro* studies and mice, reinforcing the importance of using relevant cell types as a foundation for mouse models.

Iminosugars: promising influenza antivirals

Taken together, the data presented in this review indicate promise for iminosugars as future therapeutics against influenza A and B. An antiviral drug active against the diversity of INFVs would be of significant clinical benefit, particularly enabling the treatment of infections resistant to alternative drugs. Targeting a host process, the approach of iminosugars directed against the glycosylation pathway, endows resilience against the development of resistance and enables broad-spectrum antiviral efficacy. Several other host-acting antivirals are currently in development for INFV (Figure 2), largely targeting the immune response or virus entry, including DAS-181, which has sialidase activity, cleaving $\alpha(2,6)$ - and $\alpha(2,3)$ -linked sialic acid receptors for human and avian INFVs (Malakhov et al., 2006), and macrolides, which reduce $\alpha(2,6)$ -linked sialic acid receptor expression (Min & Jang, 2012). Resistance to DAS-181 should prove difficult to generate, as both classical sialic acid linkage chemistries are targeted. However, there have been reports of some INFV strains, including H3N2 clinical isolates, entering, and undergoing multicycle replication in desialylated cells (Stray et al., 2000), as well as the identification of a second ligand-binding site in X31 HA (H3N2) (Sauter et al., 1992a). One could imagine that escape from DAS-181 efficacy, requiring receptor binding properties that have already been identified in some INFV isolates, might occur more readily than escape from iminosugars, which would require loss of the requirement for glycosylation or evolution of a novel glycosylation mechanism.

Despite targeting the host, iminosugars display remarkable non-toxicity, and are already successfully utilized in the clinic in the treatment of lysosomal storage disorders and non-insulin-dependent diabetes (Dwek et al., 2002). This, in combination with the recent

Phase IB clinical trial of celgosivir in DENV infection (Low et al., 2014; Sung et al., 2016), and the recently completed trial of MON-DNJ for safety, tolerability, and pharmacokinetics in healthy individuals (Unither Virology, 2014) indicates the clinical potential of iminosugars for the treatment of viral disease. In addition, the significant protection afforded by MON-DNJ administration up to 96 h post-infection (Stavale et al., 2015) or 2THO-DNJ up to 48 h post-infection (Warfield et al., 2015) with INFV A/Texas/36/91 (H1N1) suggests that as well as being efficacious as prophylaxis, iminosugars have potential as post-infection therapeutics. In summary, the possibilities for iminosugars as antivirals are exciting and timely: in this era of multiple emerging and potentially devastating viruses, broad-spectrum antivirals could prove instrumental in the future arsenal of control strategies for viral disease.

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Appendices

Appendix 1. Iminosugars tested against cell-free INFLV glycoproteins can impact HA glycosylation and NA sialidase activity, providing a rationale for investigation of their antiviral efficacy. GPI: glycosylphosphatidylinositol; WT: wild type.

Iminosugar; concentration	Viral target	Effect on virion component	Reference
3-episiastatin B 100 µM	NA from A/Fort Monmouth/1/47 (H1N1)	53.1% inhibition of enzymatic activity	Nishimura et al. (1993)
100 µM	NA from A/Kayano/57 (H2N2)	25.68% inhibition of enzymatic activity	
100 µM	NA from B/Lee/40	67.2% inhibition of enzymatic activity	
3,4-diepiasiastatin B 100 µM	NA from A/Fort Monmouth/1/47 (H1N1)	No inhibition of enzymatic activity	Nishimura et al. (1993)
100 µM	NA from A/Kayano/57 (H2N2)	2% inhibition of enzymatic activity	
100 µM	NA from B/Lee/40	19.8% inhibition of enzymatic activity	
1-deoxymannojirimycin (DMJ) 0.25 mM	WT and GPI-linked HA from A/HKx31 (H3N2)	High-mannose HA formed; restored erythrocyte-binding ability of GPI-linked HA	Kemble et al. (1993)
Deoxynojirimycin (DNJ) 1 mM	HA from A/HKx31 (H3N2)	Prevented binding to CNX and inhibited glucose trimming	Hebert et al. (1995)
N-methyl-1-deoxynojirimycin (NM-DNJ) 1 mM	HA from A/HKx31 (H3N2)	Prevented binding to CNX and inhibited glucose trimming	Hebert et al. (1995)
Castanospermine (CAST) 1 mM	HA from A/HKx31 (H3N2)	Prevented binding to CNX and inhibited glucose trimming	Hebert et al. (1995)
		Reduced folding efficiency and increased degradation	Hebert et al. (1996)
1 mM	HA truncations derived from A/Aichi/68 (X31, H3N2)	HA secretion reduced to 2% of control with puromycin, increased to 10% with CAST due to reduced HA association with CNX/CRT	Zhang et al. (1997)

Appendix 2. Iminosugars with targets other than ER α -glucosidases have variable effects against INFLV *in vitro*. CEC: chicken-embryo cell; LLC-PK1: pig kidney epithelial cell line.

Iminosugar and target; concentration	INFLV strain	Cell line	Viral glycoprotein processing inhibition	Reference
			Virion production or infectivity inhibition	
1-deoxymannojirimycin (DMJ), targeting mannosidase I 1 mM	A/chick/Germany/49 (H10N7)	MDCK	HA expression unaffected Infectivity (slight). No effect on production	Burke et al. (1984)
2, 10, 25 µg/ml	A/NWS/33 (H1N1)	MDCK	High-mannose oligosaccharide processing and complex chain formation No effect on hemagglutination	Elbein et al. (1984a)
25 µg/ml plus 100 µg/ml CAST	A/NWS/33 (H1N1)	MDCK	No inhibition of oligosaccharide processing (CAST concentration too low to inhibit α -glucosidases) Not tested	
0.25, 0.5, 1, 2, 4 mM	A/Puerto Rico/8/34 (H1N1)	MDCK	Mannose incorporation (50% at >2 mM) Not tested	
0.05, 0.1, 0.25, 0.5, 1, 2, 4 mM	A/Puerto Rico/8/34 (H1N1)	CEC	Mannose incorporation Not tested	
1, 10, 50 µg/ml	Unknown	MDCK	Unaffected Not tested	Elbein et al. (1990)
1,4-dideoxy-1,4-imino-D-mannitol (DIM), targeting mannosidase I and II 10, 100, 250 µg/ml	A/NWS/33 (H1N1)	MDCK	High-mannose oligosaccharide processing Not tested	Palamarczyk et al. (1985)

(continued)

Continued

Iminosugar and target; concentration	INFV strain	Cell line	Viral glycoprotein processing inhibition	Reference
			Virion production or infectivity inhibition	
Kifunensine, targeting mannosidase I 10, 100 ng/ml 1, 10 µg/ml	Unknown	MDCK	High-mannose oligosaccharide processing and complex chain formation	Elbein et al. (1990)
0.18 mM	A/Puerto Rico/8/34/MS (H1N1)	MDCK	Not tested	Karaivanova & Spiro (1998)
			HA sulfation	
0.18 mM	A/Puerto Rico/8/34/MS (H1N1)	LLC-PK ₁	Not tested	
			HA sulfation	
			Not tested	
<i>N</i> -benzyl-1,5-dideoxy-1,5-imino- <i>D</i> -mannitol, targeting mannosidases 10 mM	A/fowl plague virus/Rostock/34 (H7N1)	MDCK	Not tested	Huang et al. (1991)
			Haemagglutination and CPE	
<i>N</i> -benzyl-1,5-dideoxy-1,5-imino-4,6- <i>O</i> -isopropylidene- <i>D</i> -mannitol, targeting mannosidases 10 mM	A/fowl plague virus/Rostock/34 (H7N1)	MDCK	Not tested	Huang et al. (1991)
			Haemagglutination and CPE	
3-episiastatin B, targeting NA 10, 20, 40, 100 µM	A/Fort Monmouth/1/47 (H1N1)	MDCK	IC ₅₀ = 74 µM for NA	Nishimura et al. (1993)
			PFU 11.1% of control at 40 µM	
100 µM	A/Kayano/57 (H2N2)	MDCK	IC ₅₀ > 10 µM for NA	
			Not tested	
100 µM	B/Lee/40	MDCK	IC ₅₀ = 42 µM for NA	
			Not tested	
<i>N</i> -nonyl-deoxygalactonojirimycin (NN-DGJ), targeting ceramide-specific glucosyltransferase Titration	A/Udorn/307/72 (H3N2)	MDCK	Not tested	Hussain et al. (2015)
			HA titer 117 ± 19%, viral titer 97 ± 20%, plaque number 74 ± 16% and plaque size 88 ± 20% of control at 62.5 µM	
Titration	A/Brisbane/10/2007 (H3N2)	MDCK	Not tested	
			HA titer 75 ± 15%, viral titer 71 ± 21%, plaque number 60 ± 10% and plaque size 76 ± 4% of control at 62.5 µM	
Titration	A/Lviv/N6/2009 (H1N1)	MDCK	Not tested	
			HA titer 60 ± 10%, viral titer 85 ± 16% and plaque number 73 ± 8% of control at 62.5 µM. No effect on plaque size	