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New antiviral approaches for human parainfluenza: Inhibiting the haemagglutinin-neuraminidase

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ABSTRACT

Human parainfluenza viruses cause acute respiratory tract infections and disease predominantly in young children and immunocompromised individuals. Currently, there are no vaccines to prevent hPIV infections, nor licensed anti-hPIV drugs. There is therefore a need for specific antiviral therapies to decrease the morbidity and mortality associated with hPIV diseases. Haemagglutinin-neuraminidase (HN) is one of two hPIV surface proteins with critical roles in host receptor recognition, binding and cleavage; it has been explored as a key drug development target for the past few decades with variable success. Recent advancements in computational modelling and the availability of the X-ray crystal structure of hPIV3 HN have improved our understanding of the structural and mechanistic features of HN. This review explores structural features of the HN protein that are being exploited for structure-guided inhibitor design. We describe past and present hPIV HN inhibition strategies based on sialic acid scaffolds, together with other novel approaches that decrease hPIV infectivity. Although many HN inhibitors have been developed and evaluated as anti-hPIV agents, currently only a host-directed therapy (DAS181) has succeeded in phase II clinical drug trials. Hence, the review concludes with future considerations for targeting the specific function(s) of hPIV HN and suggestions for antiviral drug design.

1. Introduction

Despite over six decades of research since the discovery of the human parainfluenza viruses (hPIVs), there are currently no licensed hPIV-specific antivirals or vaccines for treatment or prevention of hPIV infections. Given the significant health and medical burden associated with hPIV infections, there is need for development of therapeutic interventions. In reviewing the search for targeted hPIV antivirals, we focus on haemagglutinin-neuraminidase (HN), one of two hPIV surface proteins which has multiple roles in the viral lifecycle. We discuss functional and structural properties of HN that make it an interesting target for antiviral drug design. Past approaches to developing hPIV inhibitors have provided a foundation for current drug designs. Present strategies include HN mechanism-based inhibitors, selective targeting of the HN active site, repurposing of approved drugs and host-directed therapies. In the concluding section, we discuss considerations and approaches for future antivirals targeting hPIV HN.

2. Background

2.1. Parainfluenza virus infection and disease

hPIVs cause a wide spectrum of mild to severe clinical respiratory symptoms predominantly in young children under five years of age and are second to respiratory syncytial virus (RSV) as the leading cause of hospitalisations of young children due to bronchiolitis and pneumonia (Hall, 2001). Clinical symptoms of hPIV infection include wheezing, coryza, rhonchi, acute otitis, acute laryngotracheobronchitis (croup) and signs of rales. Over 1.5 million hPIV infection cases are estimated per annum in the United States alone, with up to 100 000 reported hospitalisations (Henrickson, 2003). hPIVs also cause a significant health burden to immunocompromised individuals. hPIV infections are frequently reported in transplant patients, with the mortality rate as high as 30% in hematopoietic stem cell transplant patients (Seo et al., 2014). In the elderly population, morbidity due to hPIV infections is high and outbreaks are occasionally observed in aged care residences. Although humoral and cellular immune components contribute to protection against hPIV infection, primary infection does not confer

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durable immunity (Moscona, 2005). This lack of long-lived immunity occurs even in the absence of significant viral antigenic drift. Supportive care and treatment of secondary infections are the main therapeutic interventions for infected individuals. Hospitalised patients with severe hPIV diseases are often treated with aerosolized ribavirin (Ustun et al., 2012; Falsey, 2012), corticosteroids or nebulized with epinephrine (Schmidt et al., 2011). However, these treatments have an insignificant effect on the overall patient mortality rate. Hence, the development of novel therapeutic drugs is essential to decrease the severity of hPIV diseases, reduce hospitalisation costs and decrease morbidity and mortality in high risk population groups.

2.2. Viral genome and structure

hPIVs belong to the order *Mononegavirales* and are members of the family *Paramyxoviridae* (Amarasinghe et al., 2018). Based on the differences in their genetic sequences and antigenic characteristics, the four hPIV serotypes are divided into two distinct genera: *Respirovirus* (hPIV1 and hPIV3) and *Rubulavirus* (hPIV2, hPIV4a and hPIV4b). The genome of hPIVs is a linear, non-segmented, negative single-stranded RNA between 15 kb and 18 kb long. It encodes at least six structural proteins: large RNA polymerase (L), phosphoprotein (P), nucleoprotein (N), fusion protein (F), matrix protein (M) and haemagglutinin-neuraminidase (HN) (Henrickson, 2003). Each hPIV serotype also encodes at least one non-structural protein. These proteins include the V protein encoded by hPIV2 and hPIV3, C protein encoded by hPIV1, hPIV2 and hPIV3, and the D protein which is unique to hPIV3 (Henrickson, 2003). These accessory proteins enhance hPIV replication (Boonyaratanakornkit et al., 2011) and regulate host interferon responses (Schomacker et al., 2012; Roth et al., 2013), amongst other functions. Current hPIV antiviral research has extensively focussed on understanding the structural and functional properties of HN due to the multiple roles of HN in the hPIV lifecycle (described later).

3. The haemagglutinin-neuraminidase

3.1. Structure of HN

HN is a type-II integral membrane glycoprotein and assembles in the lipid membrane as homotetramers (dimers of dimers). Each HN monomer consists of a single transmembrane domain with a short cytoplasmic N-terminal peptide linked to a flexible helical coiled coil stalk. The extrinsic globular head domain with receptor binding/cleaving activity is formed by the C-terminal region of the protein (Villar and Barroso, 2006). Of the four hPIVs, the three-dimensional crystal structure of only hPIV3 HN (head domain) has been described to date (Lawrence et al., 2004; Dirr et al., 2015; Xu et al., 2013). Crystal structures of HN from two other paramyxoviruses [Parainfluenza virus type 5 (PIV5) and Newcastle disease virus (NDV)] have been determined (Welch et al., 2013; Crennell et al., 2000). Each monomer within the globular head domain of hPIV3 HN has a six bladed beta-propeller fold (Fig. 1A), with an overall mass of 56 kDa. As predicted by sequence analysis, it is structurally similar to the corresponding domain of NDV (Crennell et al., 2000; Colman et al., 1993).

3.2. The role of HN in replication

HN has a central role in the replication of hPIVs, with key functions in both the early and late stages of the hPIV lifecycle. During the early stage, the haemagglutinin activity of HN is essential for host sialoglycoconjugate receptor recognition and binding to respiratory epithelial cells. Receptors bound by HN have been identified as sialoglycoconjugates terminated by the sialic acid *N*-acetylneuraminic acid (Neu5Ac). Glycan binding studies including glycan array screening have identified minimum glycan motifs required for hPIV binding comprising of Neu5Ac linked to Galβ(1,4)GlcNAc (Amonsen et al., 2007; Tappert et al., 2011; Tappert et al., 2013; Suzuki et al., 2001). The binding specificity of HN likely influences host tissue tropism due to differences in the distribution of sialoglycans in the human respiratory tract (Nicholls et al., 2007).

Following receptor recognition and engagement, a conformational change in HN activates the fusion protein resulting in virus-host membrane fusion and subsequent entry of the viral nucleocapsid (Porotto et al., 2012). The nucleocapsid disassembles and releases the RNA genome, N, P and L proteins into the cytoplasm where viral genome replication and transcription occur. Newly synthesized viral surface glycoproteins (HN and F) are glycosylated, exported to the plasma membrane of infected cells and assembled onto the surface of budding virions (Moscona, 2005). At the end of the replication cycle, the neuraminidase activity of HN cleaves terminal sialic acid residues from host sialoglycoconjugates. Sialic acid cleavage contributes to progeny virion release (Huberman et al., 1995) and prevents auto-agglutination of viral particles (Mahon et al., 1995). The neuraminidase activity of HN is important for viral interference by decreasing hPIV receptor availability in infected cells. Consequently, infected epithelial cells become less susceptible to reinfection (Horga et al., 2000).

3.3. Receptor binding, cleavage and catalytic activity

The combined receptor binding and cleavage site of hPIV3 HN is at the centre of the β- propeller fold within the globular head domain (Chang and Dutch, 2012). The first hPIV3 HN *apo* crystal structures of the active site were obtained at pH 6.5 and 7.5 (Lawrence et al., 2004). Subsequently, the *apo* crystal structure of HN has been produced at pH 4.2 (Xu et al., 2013) and pH 4.6 (Dirr et al., 2015). Seven conserved amino acid residues (Arg192, Arg424, Arg502, Tyr530, Glu409, Glu549, Asp216) (Fig. 1B) are important for engaging the terminal Neu5Ac residue of the natural substrate. A cluster of three arginine residues (Arg192, Arg424 and Arg502) provides a positively charged region within the HN active site (Lawrence et al., 2004). The tri-arginyl cluster is present in neuraminidases from other organisms (Welch et al., 2013; Crennell et al., 2000; Taylor, 1996; Colman et al., 1993) and is known to form strong ionic interactions with the natural ligand's negatively charged carboxyl group. Other active site residues are two glutamic acids, Glu549 and Glu409, that interact with and stabilise the side chains of Arg192 and Tyr530 respectively (Lawrence et al., 2004). A conserved tyrosine residue, Tyr530, has a key role in the catalytic mechanism of HN (Streltsov et al., 2015; Dirr et al., 2015). Another conserved active site residue is Asp216 which is important for the neuraminidase activity of HN. Although the exact mechanism of Asp216 has not yet been established, decreased neuraminidase activity has been observed in D216N mutants (Prince et al., 2001; Porotto et al., 2003). The engagement of Asp216 with the substrate is influenced by the flexibility of the 216-loop, described later in this section.

Structural changes in the catalytic site induced by sialic acid binding, and changes in pH and halide concentration, have been proposed to affect the balance between the receptor binding and receptor cleavage activities of HN (Merz et al., 1981). Furthermore, sequencing of HN from hPIV3 clinical isolates revealed a key mutation, D556N, that is important for neuraminidase activity. D556N mutants were observed to have at least a four-fold increase in neuraminidase activity compared to prototype strains (Palermo et al., 2016). The mechanism by which D556N enhances the neuraminidase activity of hPIV3 HN and the effect of this mutation in hPIV3 pathogenicity remains elusive.

The neuraminidase catalytic mechanism of hPIV3 HN was revealed using derivatives of 2,3-difluorosialic acid (Dirr et al., 2015; Streltsov et al., 2015). Similar to other neuraminidases, hPIV3 HN is a retaining glycohydrolase that maintains substrate configuration during catalysis. Tyr530 was identified as a key catalytic amino acid; the phenolic oxygen acting as a nucleophile to attack the anomeric (C-2) centre of the bound sialoside substrate, promoting hydrolysis. Substrate hydrolysis, which requires reorientation of the side chain of Tyr530 to point

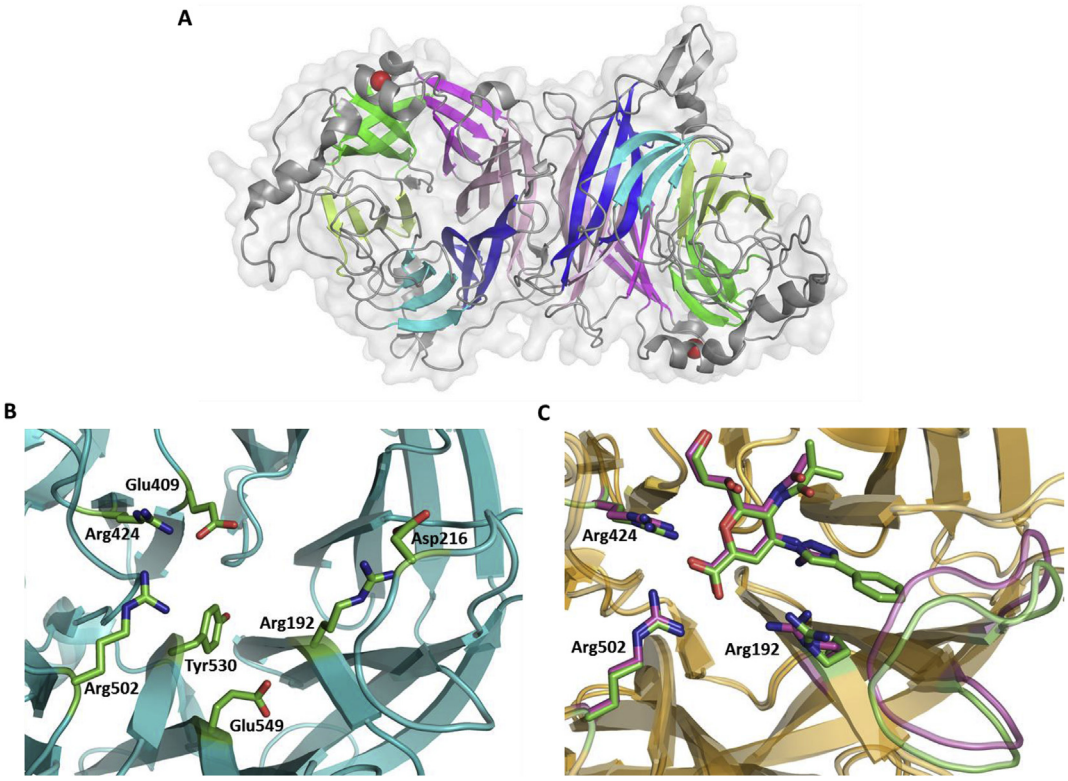


Fig. 1. X-ray structures of hPIV3 HN. A. HN dimer with six bladed β-propeller folds. The active site is at the centre of the β-bladed sheets. Red sphere: Ca^{2+}. (Dirr et al., 2015) (PDB: 4XJQ). **B.** Arg192, Arg424, Arg502, Tyr530, Glu409, Glu549 and Asp216 are conserved active site residues present in other neuraminidases (PDB: 4XJQ). **C.** Superimposed structures of the flexible 216-loop showing the open (green) and closed (magenta) conformations, relative to the tri-arginyl cluster. Compound **8** (green) and Neu5Ac2en (magenta) are shown in ball-and-stick representation, bound to the active site of HN. (PDB: 1V3E, 5KV9). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

towards the bound substrate, is proposed to proceed via a covalent sialosyl-enzyme intermediate (Dirr et al., 2015) and involves an oxocarbenium ion transition state (Streltsov et al., 2015). Understanding the interactions between HN active site residues and ligands is key in the development of potent and specific inhibitors. Inhibitors that target Try530 may prove valuable in decreasing hPIV infectivity.

A flexible protein loop, the 216-loop (residues 210-221), containing the important residue Asp216 is in close proximity to the hPIV3 HN active site (Fig. 1C) (Winger and von Itzstein, 2012). The 216-loop may influence hPIV3 HN interactions with glycan receptors and has been exploited in the development of HN inhibitors (El-Deeb et al., 2017; Winger and von Itzstein, 2012). A study examining the extent of loop flexibility using molecular dynamic simulations proposed that ligand binding regulates the motion of the 216-loop. The 216-loop has greater flexibility in the absence of a bound ligand and closes upon ligand engagement (Winger and von Itzstein, 2012). Thus, in addition to targeting key actives site residues, the large cavity formed by movement of the 216-loop is also being exploited in current hPIV inhibitor designs to further exploit interactions between HN and ligands (El-Deeb et al., 2014; Guillon et al., 2014; Pascolutti et al., 2018; Dirr et al., 2017).

3.4. Glycosylation

The hPIV3 HN contains three *N*-glycosylation sites (N308, N351 and N523) that are thought to contain a mixture of high mannose and complex *N*-glycans (Tanaka et al., 2006) (Fig. 2). Although the role of hPIV HN *N*-glycosylation has not been explored extensively, *N*-linked glycans have been shown to be important in host-receptor interactions (Chu et al., 2013; Gorman et al., 1991) and immune recognition (Ewasyshyn et al., 1993). Site-directed mutagenesis of all three hPIV3 HN *N*-glycosylation residues, followed by transient cell surface expression of these HN mutants resulted in decreased haemagglutinin activity of HN (Chu et al., 2013). Pre-treatment of hPIV3 virions with α-glucosidase inhibitors also decreased hPIV3 infectivity; possibly due to misfolding of HN leading to structural changes in the globular head domain and decreased receptor interactions (Tanaka et al., 2006). Although deglycosylation of whole hPIV1 virions altered receptor recognition, minimal effects on antibody recognition epitopes and neuraminidase activity of HN were observed (Gorman et al., 1991). This suggests that *N*-linked glycans are primarily essential in the haemagglutinin, but not in the neuraminidase activity of HN. In hPIV3, a complete loss of *N*-linked glycans on copurified hPIV3 HN and F proteins decreased the magnitude of the primary immune response in hamsters, with a very limited effect on the secondary antibody response (Ewasyshyn et al., 1993). However, the relevance of these findings to human infection needs to be investigated using whole hPIV virions in a more suitable model. In conclusion, our knowledge concerning the role of HN *N*-glycosylation in viral infectivity is very limited. Additional studies are required to evaluate the interactions between HN *N*-glycosylation mutants and specific sialoglycans. Identification of glycan residues present on the surface of HN could provide useful insights for understanding HN receptor recognition mechanisms and potential drug targets.

3.5. Second receptor binding site?

The presence of a secondary binding site(s) in HN of hPIVs is controversial. Some authors propose that only one site exists (Tappert et al., 2011; Tappert et al., 2013; Yewdell and Gerhard, 1982). Indeed, monoclonal antibodies known to block hPIV3 HN haemagglutinin and neuraminidase individual functions bind to a single site and inhibit both activities, revealing the structural connectivity of these functions

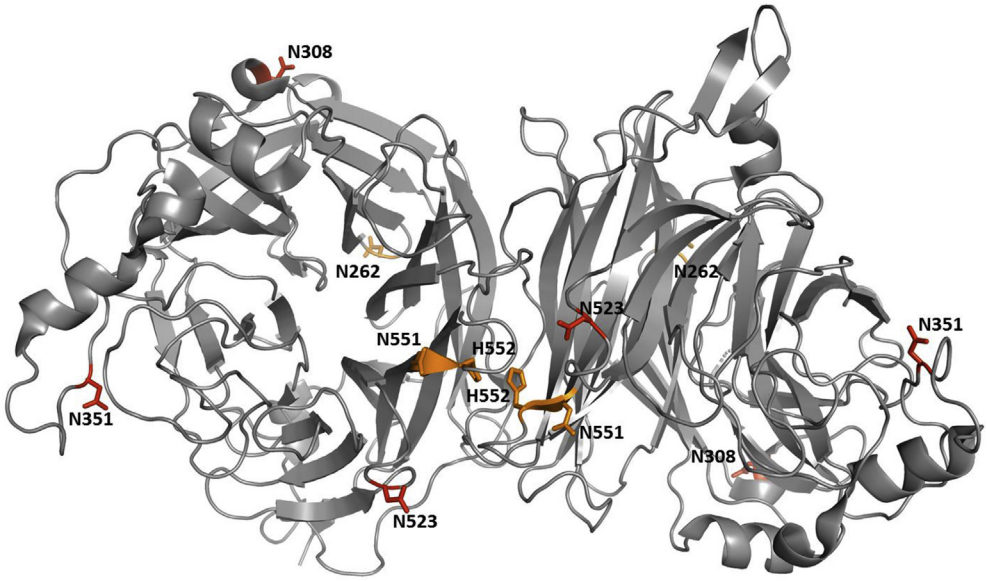


Fig. 2. hPIV3 HN glycosylation sites (red) and regions of proposed secondary binding sites (orange) (Mishin et al., 2010; Porotto et al., 2007; Streltsov et al., 2015). N523 is both an *N*-glycosylation site and proposed secondary binding site (PDB: 4XJQ). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Yewdell and Gerhard, 1982). Enzyme kinetic analyses (Tappert et al., 2013) and the crystal structure of the hPIV3 HN extracellular domain (Lawrence et al., 2004) also support the existence of a single binding site. A separate study also found that zanamivir (4-guanidino-Neu5Ac2en) inhibits receptor binding, membrane fusion and plaque formation in hPIV3; suggesting that an alternative site does not exist to compensate for the inhibition of the primary binding site (Greengard et al., 2000).

On the other hand, other authors have proposed that secondary binding sites exist within hPIV HN; either completely apart from the catalytic site or within close proximity to the active site (Fig. 2, Streltsov et al., 2015; Porotto et al., 2007; Palermo et al., 2009; Alymova et al., 2008; Mishin et al., 2010). The existence of the second binding site in hPIV1 and hPIV3 HN is plausible considering that a second site was discovered in the closely related NDV HN. Crystallisation of NDV HN with a non-hydrolysable thiosialoside substrate revealed a second binding site on the dimer interface at the membrane distal end (Zaitsev et al., 2004). Similar to NDV HN (Takimoto et al., 2002), the proposed second site in hPIV3 HN is described as being important in membrane fusion through activation of the F protein (Porotto et al., 2007). Three additional putative secondary binding sites were identified on hPIV3 HN (Streltsov et al., 2015). Secondary site I was previously identified by Mishin et al. (2010) and is located near residue Asn551 which is close to the primary binding site. It is thought that secondary site I stabilises ligand interactions since stacking interactions were observed between difluorosialic acid and His552. Secondary site II and site III are proposed to be located near residue Asn262 or in the groove of the dimer interface, respectively.

In another study, Neu5Ac was successfully modelled into a putative second site in hPIV3 HN H552Q mutants with increased receptor avidity (Porotto et al., 2007). The second binding site was mapped to the dimer interface near residue 552, and only exposed in the presence of inhibitors, suggesting that selective binding to the primary binding site increases the avidity of the second site (Porotto et al., 2007; Palermo et al., 2009). Mishin et al. (2010) alternatively proposed that the second site is located near amino acid residue 523 instead of 552. Experiments conducted with hPIV3 HN N523D mutants (which lack *N*-glycosylation at this site) revealed a high affinity of the alternative second site for α2-3-linked sialoglycans. The authors conclude that this second site increases the receptor specificity of hPIV3 HN. Additional interactions between the second binding site and host cell receptors lead to inefficient virus release from infected cells, resulting in slow growth of the N523D mutants. Interestingly, only four clinical samples were observed to possess either an asparagine to serine or asparagine to glutamine mutation at the 523 position (Mishin et al., 2010). The clinical relevance of this mutation requires further investigation.

Similarly to hPIV3 (Mishin et al., 2010), an *N*-linked glycan possibly masks the second binding site in hPIV1 HN (Alymova et al., 2008). Loss of the *N*-linked glycan at residue 173 decreased viral infectivity compared to wild type hPIV1, possibly due to unbalanced haemagglutinin and neuraminidase activities caused by the exposure of an additional binding site (Alymova et al., 2008). Further structural studies using hPIV1 HN mutants are required to substantiate these findings. The different structural features of hPIV HN and its multiple roles in the viral lifecycle make it a complicated, but interesting drug target to block host cell infection, viral progeny release and possibly improve disease outcomes within clinical settings.

4. Inhibitor and drug design

Sialidases of pathogens causing different diseases are targets of interest for inhibitor development (Taylor, 1996). Sialidase inhibitors have been successfully developed and licensed for the treatment of influenza. However, the absence of licensed drugs targeting other known sialidases, including hPIV HN, necessitates the design and development of novel potent inhibitors. Structure-informed understanding of sialidases and rational drug design (Taylor and von Itzstein, 1994; Taylor, 1996) has certainly been aided by advancements in molecular modelling methodologies; leading in turn to the development of potent hPIV neuraminidase inhibitors (Pascolutti et al., 2018; Guillon et al., 2014; El-Deeb et al., 2017; Alymova et al., 2005; Dirr et al., 2015). The hPIV HN is an ideal drug target due to its multiple regulatory roles at different stages of the hPIV lifecycle. These stages include host sialoglycoconjugate receptor recognition and interaction, triggering fusion of virus and host lipid membranes and release of progeny virions from infected cells (Moscona, 2005). Interfering with these processes by targeting the HN protein could potentially decrease viral infectivity and improve the disease outcome.

4.1. Chronology of HN inhibitor discoveries

4.1.1. Neu5Ac2en and zanamivir

Neu5Ac2en (2-deoxy-2,3-dehydro-D-*N*-acetylneuraminic acid) (**1**) is an unsaturated derivative of *N*-acetylneuraminic acid (Perlman et al., 1999) (Fig. 3). Structure-based studies of the influenza virus neuraminidase with Neu5Ac2en led to the development of zanamivir (4-

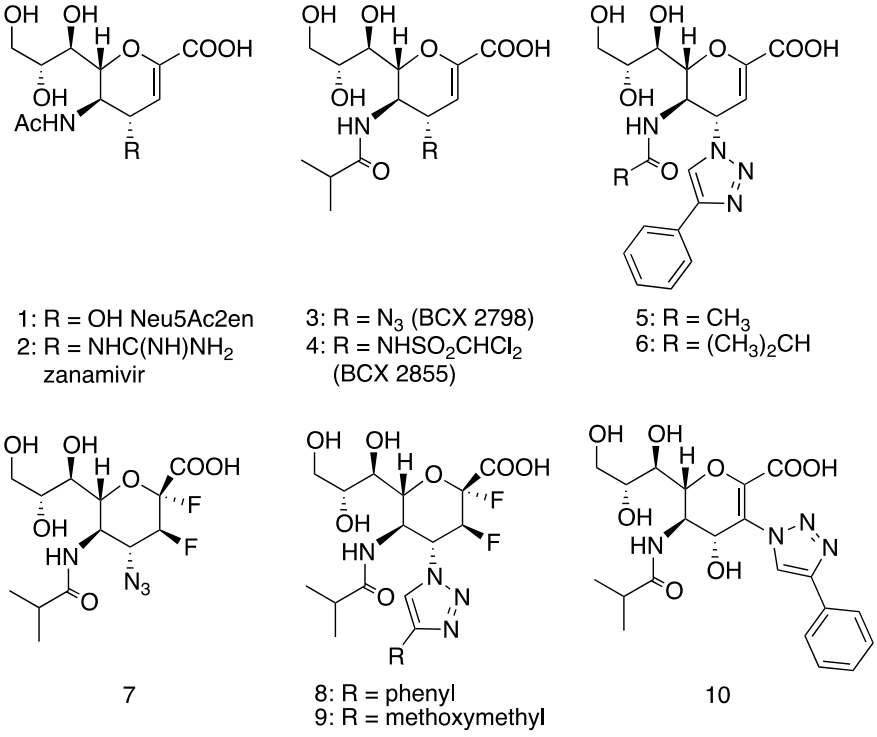
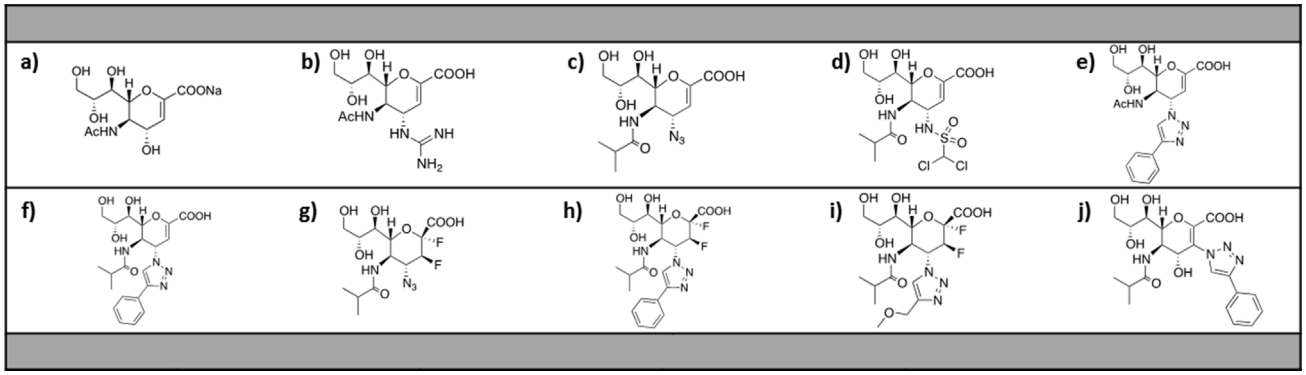


Fig. 3. Structures of Neu5Ac2en-derived hPIV3 HN inhibitors. Structures of Neu5Ac2en (**1**), Zanamivir (**2**), BCX2798 (**3**), BCX2855 (**4**), bulky C-4 substituted derivatives (**5** and **6**) (Guillon et al., 2014), sialosyl fluoride derivatives (**7**, **8** and **9**) (Dirr et al. 2015, 2017), bulky C-3 substituted derivative (**10**) (Pascolutti et al., 2018).

Table 1
The *in vitro* potency of sialic acid-based inhibitors targeting hPIV3. Inhibitors were evaluated using functional assays (neuraminidase inhibition assay, haemagglutination assay) and virus growth inhibition assay.



Inhibitor	Neuraminidase inhibition (NI) IC_{50}	Haemagglutinin inhibition (HAI) IC_{50}	Virus growth inhibition assay EC_{50}	References
a) Neu5Ac2en	2.1 mM[1]	1.4 mM[2]	–	[1](Greengard et al., 2000) [2](Guillon et al., 2014)
b) Zanamivir	0.25 mM	–	–	Greengard et al. (2000)
c) BCX 2798	20.0 ± 1.7 μM	4.8 ± 0.1 μM	11.3 ± 1.1 μM	Alymova et al. (2004)
d) BCX 2855	4.3 ± 0.2 μM	2.0 ± 0.5 μM	2.4 ± 0.7 μM	Alymova et al. (2004)
e) Inhibitor 5	6.5 ± 0.6 μM	4.6 ± 0.7 μM	–	Guillon et al. (2014)
f) Inhibitor 6	2.7 ± 0.2 μM	1.5 ± 0.3 μM	2.1–13.9 μM*	Guillon et al. (2014)
g) Inhibitor 7	4.8 ± 1.8 μM	–	12.3 ± 1.3 μM	Dirr et al. (2015)
h) Inhibitor 8	55.3 ± 8.9 μM	775 ± 35.3 μM	40.1 ± 7.0 μM	Dirr et al. (2017)
i) Inhibitor 9	6.7 ± 1.4 μM	57 ± 9.9 μM	14.3 ± 0.3 μM	Dirr et al. (2017)
j) Inhibitor 10	31.8 ± 1.2 μM	275 ± 35.5 μM	> 1000 μM	Pascolutti et al. (2018)

-Published data unavailable; *IC_{50} range evaluated in different cell lines.

guanidino-Neu5Ac2en) (von Itzstein et al., 1993) and oseltamivir (Whitley et al., 2001; Li et al., 1998; Kim et al., 1997; Nicholson et al., 2000). Both inhibitors have potent activity against influenza A virus and influenza B virus neuraminidase. Zanamivir, marketed as Relenza, was the first structure-based anti-influenza drug licenced by the FDA. Oseltamivir, marketed as Tamiflu, was also licensed shortly thereafter. Influenza virus neuramindase-targeting inhibitors have since been evaluated as possible hPIV HN inhibitors due to structural similarities and the presence of neuraminidase activity in both viral species. Zanamivir (**2**) mimics the transition state of Neu5Ac during substrate hydrolysis and interferes with hPIV3 HN receptor binding, membrane fusion and neuraminidase activity. However, the high *in vitro* neuraminidase half inhibitory concentration (IC_{50}) of 0.25 mM limits zanamivir as a clinical drug against hPIV3 (Greengard et al., 2000)

(Table 1).

4.1.2. BCX 2798 and BCX 2855

Using the crystal structure of NDV HN as a template, BCX 2798 and BCX 2855 were developed as potent hPIV inhibitors due to sequence conservation of amino acid residues within the active site of paramyxovirus HN (Alymova et al., 2004). BCX 2798 (**3**) and BCX 2855 (**4**) are derivatives of Neu5Ac2en that inhibit hPIVs specifically, with limited activity against influenza viruses (Fig. 3). BCX 2798 was further studied due to its broader activity against different hPIV strains (Alymova et al. 2008, 2009). BCX 2798 has a greater inhibitory effect against hPIV1 haemagglutinin and neuraminidase activity compared to BCX 2855. Possibly, the C-4 azido group in BCX 2798 is better accommodated into the active site of hPIV1 HN. In comparison, the bulkier C-4 dichloromethanesulfonylamino moiety present in BCX 2855 resulted in better inhibition of hPIV3 HN. As a result of the limited *in vivo* efficacy of both BCX 2798 and BCX 2855, being useful only as prophylactic, there is still need for effective treatments that limit/prevent hPIV infections, especially in transplant patients and immunocompromised individuals.

4.1.3. Targeting the 216-loop

The development of antiviral drugs against hPIV3 has been advanced by the availability of the crystal structure of hPIV3 HN (Lawrence et al., 2004; Dirr et al., 2015). Moreover, advancements in computational modelling tools have enhanced our ability to investigate and synthesize potent, rationally designed inhibitors. An important consideration in current hPIV3 HN drug designs is the flexible 216-loop (Fig. 1C). Molecular dynamic simulations proposed that the 216-loop has flexible conformations which influence the cavity size and consequently the capacity of ligands to interact with HN (Winger and von Itzstein, 2012). This flexibility could have other important physiological functions including glycan receptor binding.

Recent structure-guided inhibitors based on the Neu5Ac2en scaffold explored the effect of functionalisation at different positions of the Neu5Ac2en skeleton, in an effort to lock open the 216-loop and inhibit the haemagglutinin and/or neuraminidase activity of hPIV3 HN. A similar approach has been previously used to explore influenza A virus inhibitors that lock open the 150-loop (Rudrawar et al. 2010, 2012). Different chemical substituents (including amides, ureas, triazoles and sulfonamides) can be accommodated into the cavities surrounding active site residues, resulting in differential inhibition of hPIV3 HN activity (Fig. 4). Neu5Ac2en-based inhibitors with bulky C-4 substituents (5 and 6, Fig. 3) were found to efficiently occupy the HN active site by locking open the 216-loop. Neu5Ac2en derivatives with bulky and hydrophobic C-4 substituents have improved *in vitro* potency against hPIV3 HN neuraminidase activity and exhibit extra hydrophobic interactions with active site residues, making them potent against hPIV3 HN haemagglutinin and neuraminidase activities (El-Deeb et al., 2014; Guillon et al., 2014; Tindal et al., 2007). The size of the C-4 substituent in these hPIV3 HN inhibitors is important for engagement of the 216-loop and structural rearrangement of active site residues (Dirr et al., 2017). The flexibility of the loop in hPIV1 has not been proven due to the lack of availability of the crystal structure of hPIV1 HN.

C-5 functionalisation of Neu5Ac2en derivatives has been explored to enhance hPIV HN inhibition (El-Deeb et al., 2014; Guillon et al., 2014). Modification of the C-5 acetamido moiety in Neu5Ac2en-based inhibitors to a bulkier isobutyramido group slightly enhanced *in vitro* inhibition of both hPIV3 and hPIV1 HN. Thus, hydrophobic C-5 modifications are important in increasing the potency of Neu5Ac2en-based inhibitors. We extended our studies on hPIV3 to also explore inhibitors against hPIV1 (El-Deeb et al., 2014). Although the three-dimensional crystal structure of hPIV1 HN is not yet available, studies on hPIV3 have been used to infer the molecular interactions that could occur in the active site of hPIV1 HN. In previous reports (Ikeda et al. 2006, 2008; Tindal et al., 2007), different C-4 substituents of Neu2en were synthesized and evaluated for their antiviral activity against hPIV1. However, BCX 2798 remains the most potent anti-hPIV1 inhibitor synthesized to date.

4.1.4. Sialosyl fluoride compounds

Fluorine-containing drugs have been studied and developed for the treatment of various infectious diseases, cancers and respiratory illness over the past six decades. Fluorination of compounds is known to alter chemical reactivity, binding affinity and electrostatic interactions with ligands; influencing the pharmacokinetic properties of drug candidates (Wang et al., 2013). Sialosyl fluorides in particular, were previously synthesized and successfully used to describe the catalytic mechanisms of human neuraminidase 2 (Buchini et al., 2014), trypanosomal sialidase (Watts et al., 2006) and influenza A virus neuraminidase (Vavricka et al., 2013; Kim et al., 2013); and have now been evaluated as potential inhibitors of hPIV3 HN (Dirr et al., 2015; Streltsov et al., 2015).

Following the synthesis of multiple 2,3 difluorosialic acid analogues of BCX2798, one of the evaluated compounds (**7**) effectively inhibited hPIV3 HN neuraminidase activity *in vitro* with prolonged inhibition (Dirr et al., 2015) (Fig. 3). Crystallisation and nuclear magnetic resonance (NMR) spectroscopy studies of hPIV3 HN in complex with these sialosyl fluoride compounds revealed the catalytic mechanism of HN, demonstrating that hPIV3 HN is a retaining glycohydrolase which catalyses substrate hydrolysis via a covalent intermediate formed between the phenolic oxygen of Tyr530 and the C-2 carbon of the Neu5Ac glycoside (Dirr et al., 2015). A separate study confirmed these novel findings and deduced that an additional oxocarbocation transition state analogue was key to the catalytic activity of hPIV3 HN (Streltsov et al., 2015). Blocking the enzymatic activity of HN through further chemical modification of these 2,3 difluorosialic acid analogues could provide promising drug candidates for the treatment of hPIV infections. The size of the chemical substituent, particularly in the C-4 position, requires careful consideration. 2,3-difluoro derivatives of Neu2en with larger C-4 substituents (8) have reduced hPIV3 HN neuraminidase inhibition through loss of important interactions with Tyr530 (Dirr et al., 2017). Replacing the large C-4 phenyl triazole in 8 with a smaller C-4 methoxymethyl triazole in 9 increased inhibition potency against hPIV3 HN neuraminidase activity (Fig. 3). However, haemagglutinin inhibition was still weak as a result of the lost interactions with Arg192, a key residue in the tri-arginyl cluster. Thus, computational studies need to account for the structural flexibility of HN which influences inhibitor interactions and potency (Dirr et al., 2017).

4.2. Other approaches for limiting hPIV infection

4.2.1. Repurposing licensed drugs

Most of the hPIV HN inhibitors that have been evaluated to date are *de novo* compounds based on a Neu5Ac2en scaffold. However, repurposing of licensed drugs could potentially decrease the drug discovery timeline and associated costs; providing healthcare providers with multifunctional broad-spectrum drugs. Suramin, a drug approved for the treatment of sleeping sickness, was recently proven to have *in vitro* potency and synergistic activity when combined with zanamivir and recently developed hPIV3 HN inhibitors. This synergistic effect could be attributed to the dose-dependant, non-competitive inhibition of the HN neuraminidase activity by suramin (Bailly et al., 2016). Although suramin has high *in vivo* toxicity, non-competitive hPIV HN sialidase inhibitors should be explored for the development of synergistic drug treatments. Alternatively, the discovery of compounds that are structurally similar to suramin or the discovery of other novel non-sialic acid-derived inhibitors could prove worthwhile in the clinical fight against hPIV.

4.2.2. DAS181

DAS181 is a fusion protein with broad sialidase activity against Neu5Acα2-3Gal and Neu5Acα2-6-Gal-linked glycans on human

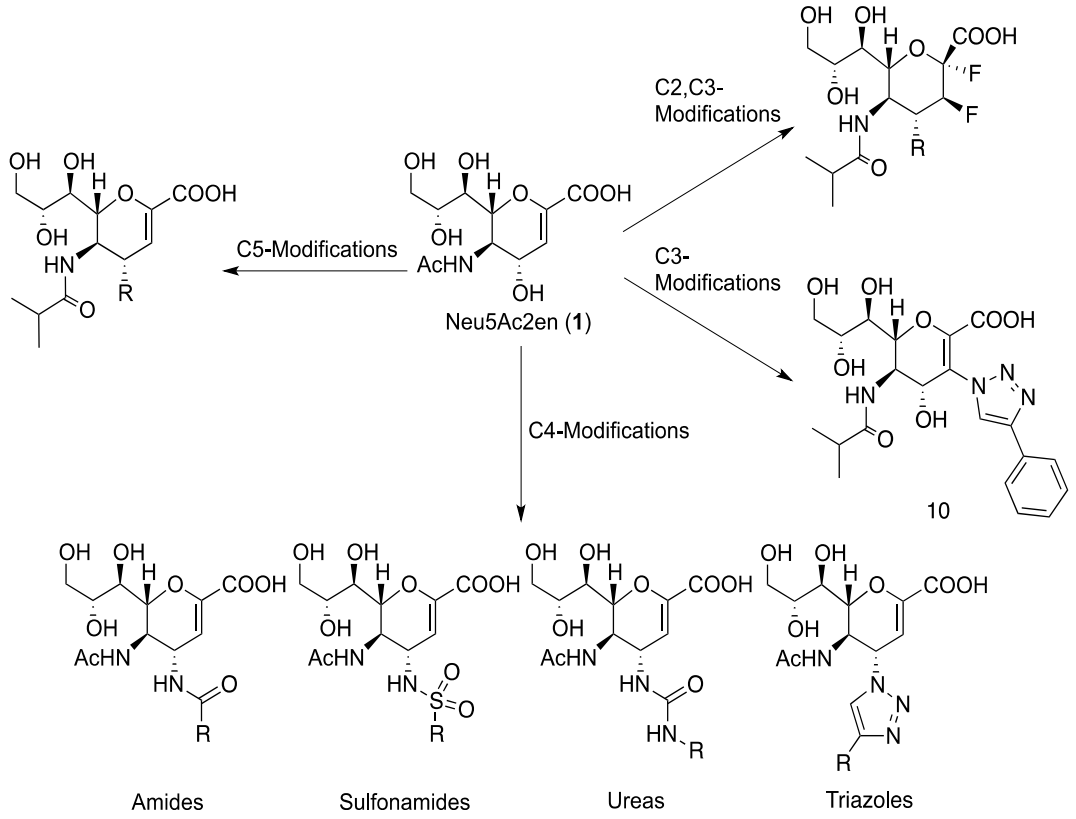


Fig. 4. The mechanism of hPIV3 HN inhibition by Neu5Ac2en derivatives is dependent on the position of the chemical substituent. C-2 and C-3 fluoro derivatives target Tyr530, a critical catalytic amino acid residue, to react and form an adduct that has a prolonged interaction (Dirr et al. 2015, 2017); bulky C-4 substituents differentially lock open the 216-loop (Guillon et al., 2014); C-5 substituents exhibit extra hydrophobic interactions with active site residues (Guillon et al., 2014). The effect of modifying the glycerol side chain (C-6 to C-9) of Neu5Ac2en derivatives is yet to be explored.

respiratory epithelial cells. It contains the catalytic domain of *Actinomyces viscosus* sialidase fused to an epithelium anchorage heparin-binding domain (derived from the human amphiregulin protein) (Malakhov et al., 2006). DAS181, marketed as Fludase®, is an effective host-directed therapeutic treatment for influenza (Triana-Baltzer et al., 2009; Moss et al., 2012; Malakhov et al., 2006); and is now being explored as a treatment for hPIV infections due to the similarities in receptor specificity between hPIVs and human influenza viruses. DAS181 inhibits hPIV3 replication *in vitro* and *in vivo* (Moscona et al., 2010; Jones et al., 2013) and clinical data from case reports of transplant patients suggests that DAS181 could be an effective treatment for hPIV infections (Guzman-Suarez et al., 2012; Dhakal et al., 2016; Salvatore et al., 2016). DAS181 was well tolerated by patients and decreased the viral load, improving disease outcomes (Salvatore et al., 2016). To date, DAS181 is the only hPIV treatment that has succeeded in phase I clinical drug trials, showing limited adverse respiratory effects in healthy adults up to seven days. The effective therapeutic dose of DAS181 in immunocompromised patients requires careful consideration due to immunogenic effects that result from prolonged drug exposure (Zenilman et al., 2015). Subsequently, DAS181 succesfully progressed through a phase II clinical drug trial in hospitalised patients with hPIV induced lower respiratory symptoms (Chemaly et al., 2018). DAS181 had limited adverse effects in immunocompromised adults and a phase III clinical trial has been scheduled.

4.2.3. Targeting antiviral drug resistance

Antiviral drug resistance is a significant problem in immunocompromised individuals. Prolonged viral replication and antiviral treatment increase selective pressure and the risk of developing drug resistant viral mutants (Strasfeld and Chou, 2010; Irwin et al., 2016). Thus, antiviral designs based on ligand/substrate analogues need to consider pathways by which hPIVs can develop drug resistance. For instance, substitutions in amino acid residues 193 and 567 have been shown to induce resistance to zanamivir in hPIV3 by preventing efficient interactions between the inhibitor and HN (Murrell et al., 2001). In addition, ZM1 mutants (T193I and I567V double mutants) were found to be five times less sensitive to zanamivir treatment. Despite the lack of structural evidence, the authors propose that the large side chain of isoleucine on residue 193 decreases the affinity of HN for zanamivir (Murrell et al., 2001). Other amino acid residues that should be considered as key targets for hPIV3 HN inhibition are D216 (Porotto et al., 2003) and D556 (Palermo et al., 2016), which are both important for the neuraminidase activity of HN. Alternative antivirals with activity against resistant hPIV mutants are essential, particularly for immunocompromised individuals experiencing prolonged periods of drug treatment.

5. Future perspectives

Targeting the specific functions of HN is critical in future antiviral designs. Although inhibiting the neuraminidase activity of hPIV3 HN is important, targeting the receptor binding activity appears to be more important in the development of potent antivirals. Addition of nitrogen-based moieties to the unsubstituted C-3 position of Neu2en derivatives (**10**) selectively blocked the neuraminidase activity of hPIV3 HN, but did not correlate positively with enhanced antiviral activity (Fig. 3) (Pascolutti et al., 2018). Hence, inhibitors that block the neuraminidase function of HN alone might not sufficiently block hPIV3 infectivity (Dirr et al., 2017). Novel C-3 substituted Neu2en-based inhibitors provide useful research tools in understanding the molecular requirements for efficiently inhibiting hPIV3 HN and should be explored further. Modification of the glycerol sidechain (C-6 to C-9 position) is currently

being explored as a method to possibly increase the potency of current Neu2en-based inhibitors. Although progress in hPIV1 drug development has been hindered by the lack of a crystal structure for HN, current inhibitor studies suggest that the 216-loop in hPIV1 HN is less flexible than in hPIV3 HN (El-Deeb et al., 2014). The flexibility of the 216-loop should continue to be explored in inhibitor designs. Although significant progress has been achieved in developing potent sialic acid-based hPIV antivirals, novel inhibitor scaffolds, repurposing of licensed drugs and host directed therapies need to be explored further as strategies for developing effective hPIV therapeutics.

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List of abbreviations

F	fusion
HN	haemagglutinin-neuraminidase
hPIV	human parainfluenza virus
NDV	Newcastle disease virus
Neu2en	2,3-dehydro-2-deoxy-neuraminic acid
Neu5Ac	*N*-acetylneuraminic acid
Neu5Ac2en	2-deoxy-2,3-dehydro-D-*N*-acetylneuraminic acid

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.04.001.

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