Review

The use of sialidase therapy for respiratory viral infections

John M. Nicholls [a,*], Ronald B. Moss [b], Stuart M. Haslam [c]

[a] Department of Pathology, The University of Hong Kong, Pok Fu Lam, Hong Kong
[b] Ansun BioPharma, San Diego, CA 92121, United States
[c] Division of Molecular Biosciences, Faculty of Natural Sciences, Biochemistry Building, Imperial College London, South Kensington Campus, London SW7 2AZ, United Kingdom

ABSTRACT

DAS181 is an inhaled bacterial sialidase which functions by removing sialic acid (Sia) from the surface of epithelial cells, preventing attachment and subsequent infection by respiratory viruses that utilize Sia as a receptor. DAS181 is typical of bacterial sialidases in cleaving Sia α2-3 and Sia α2-6 linkages, and it also has a demonstrated effect against acetylated and hydroxylated forms of Sia. The potency of the compound has been enhanced by coupling the active sialidase with an amphiregulin tag, allowing a longer duration of action and minimizing spread to the systemic circulation. DAS181 is now in Phase II development for the treatment of influenza, and it has also demonstrated activity in individual cases of parainfluenza in immunosuppressed patients. Continued evaluation of the roles and activities of bacterial sialidases is required to expand the range of successful antiviral therapies targeting Sia or its derivatives.
© 2013 Elsevier B.V. All rights reserved.

Contents

* Corresponding author. Address: Department of Pathology, University Pathology Building, Queen Mary Hospital, Pok Fu Lam, Hong Kong. Tel.: +852 22554883; fax: +852 28725197.
E-mail address: nicholls@pathology.hku.hk (J.M. Nicholls).

1. DAS181: a bacterial sialidase for influenza prevention and therapy

Influenza is an infection of the respiratory tract caused by influenza A and B viruses. To infect epithelial cells, the virus must first penetrate the overlying mucus barrier, then bind to the cell surface

– an interaction involving viral polypeptides or attachment proteins and cognate cell-surface receptors. DAS181 is the first antiviral compound in Phase II development that functions by blocking this pathogen–host interaction, by destroying the influenza host-cell receptor, sialic acid (Sia), on the surface of respiratory epithelial cells. In this paper, we provide background information on Sia and sialidases; discuss the potential role of bacterial sialidases as antiviral agents; review the *in vitro* and Phase II evaluation of DAS181 for the treatment of influenza; and note evidence that the drug would also be useful against parainfluenza virus infections.

Current antiviral strategies focus on prevention of viral replication within the cell or inhibition of release of newly formed virions. Neuraminidase (NA) inhibitors primarily act at this step, but they play a minor role in modulating viral entry (Matrosovich et al., 2004). The first step of viral binding often involves close contact between the viral attachment protein and host receptor. Before the development of DAS181, studies in the 1980s demonstrated that natural compounds present in serum, in particular alpha 2-macroglobulin, inhibited virus adsorption to surfaces (Hanaoka et al., 1989; Rogers et al., 1983). Later studies identified Sia as being the key determinant of binding for these compounds (Pritchett et al., 1987; Pritchett and Paulson, 1989). Lentz proposed a number of strategies to prevent cell entry by blocking the host receptor (Lentz, 1988, 1990), but these were mainly directed at developing an antibody-mediated approach, rather than receptor destruction.

In 1988, Weis and colleague used X-ray crystallography to map the haemagglutinin–Sia interaction (Weis et al., 1988). With this structure solved, it was envisioned that it might be possible to develop synthetic analogs of Sia to block binding (Pritchett et al., 1987; Pritchett and Paulson, 1989), though it was acknowledged that such drug design would have a number of structural obstacles (Weis et al., 1988). This route of anti-influenza therapy was not pursued in as much detail as blocking the viral neuraminidase site, but there have been two recent reports on developing drug design by blocking the HA binding site, using databases of commercially available compounds (ZINC) (Li et al., 2011; Nandi, 2008) and other techniques (Al-qattan and Mordi, 2010a,b; Blessia et al., 2010).

2. Evidence that influenza virus uses sialic acid as a receptor

2.1. An introduction to sialic acid

In 1958, Springer and Ansell found that influenza viruses and bacteria had a common enzyme property: the ability to remove receptors from red blood cells and prevent haemagglutination (Springer and Ansell, 1958). To investigate this requires an understanding of the nature of Sia, its presence in the respiratory tract and the structure and function of bacterial sialidase. Sialic acid is the term used for a family of nine-carbon monoscaccharides found in animals and certain bacteria (Chen and Varki, 2010). They are typically found at the terminal portions of oligosaccharide chains attached to proteins and lipids to form glycoproteins and glycolipids, respectively. There are over 40 naturally occurring variants of these nine-carbon keto-sugars based on a neuraminic acid (Neu) or a 2-keto-3-deoxynononic acid (Kdn) backbone (Schauer, 1973; Varki, 1997). The discovery of neuraminic acid by Klenk and colleagues has been reviewed by Schaeur (Schauer, 1973) with the term sialic acid proposed by Blix, Gottschalk and Klenk based on the Greek word sialion (salivea) as these carbohydrates were isolated from the mucin of submaxillary glands.
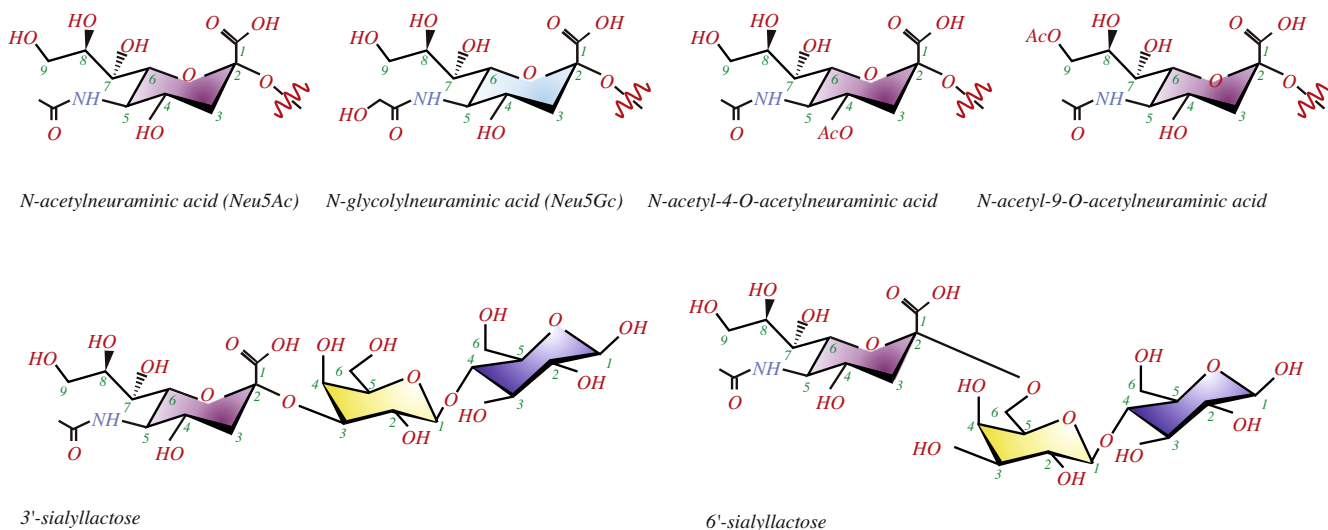
2.2. Sialic acid: structure and function

2.2.1. Sialic acid structure

The diversity of Sia present in nature is due to the substitution at the 4–9 carbons by O-acetyl, sulfate, methyl and lactate groups and by glycosidic linkage by the anomeric C_2 to the 3 or 6 hydroxyl group of galactose (Gal) (referred to as the α2-3 or α2-6), N-acetyl galactosamine (GalNAc) or N-acetyl glucosamine (GlcNAc) (Varki and Varki, 2007) (Fig. 1). Eleven different types of sialic acid have been identified on the human erythrocyte membrane, using gas chromatography and mass spectrometry (Bulai et al., 2003). GalNAc is typically present on O-glycans, and GlcNAc is normally a component of N-glycans. The binding of Sia to Gal or GalNAc requires a family of enzymes called sialyltransferases (ST), with each ST producing a specific linkage (Harduin-Lepers et al., 2005; Paulson and Rademacher, 2009; Xia et al., 2005). In some instances, multiple Sia may be linked together to form polysialic acid, most commonly in a α2-8 configuration (Hildebrandt et al., 2010). The linkage of Sia to the adjacent sugar is in an α configuration, which means the bond is acid-labile and is readily destroyed by enzymes known as sialidases. It should be noted that in solution, unbound Sia is mainly in the ß-configuration, with the C2 hydroxyl positioned axial to the ring and in equilibrium with the minor α-form (Vimr, 1994).

2.2.2. Sialic acid function

The acidic nature of Sia is due to the carboxylate at C_1, which is ionized at physiological pH and has a pK of 1.8–2.6, giving the Sia a number of useful features, as highlighted by Schauer (Schauer,

N-acetylneuraminic acid (Neu5Ac) *N-glycolylneuraminic acid (Neu5Gc)* *N-acetyl-4-O-acetylneuraminic acid* *N-acetyl-9-O-acetylneuraminic acid*

3'-sialyllactose *6'-sialyllactose*

Fig. 1. Structures of different sialic acid derivatives. The numbering of carbons is listed in green; AcO refers to acetlylation. Two common linkages of Sia with galactose to form 3′sialyllactose (Siaα2-3Galß1-4Glu) and 6′sialyllactose (Siaα2-6Galß1-4Glu) are shown.

2000) and Vimr (Vimr et al., 2004). The negatively-charged glyco-proteins will mutually repel each other, allowing trapping of water molecules to produce a highly viscous aqueous solution that comprises the glycocalyx of many epithelial surfaces. The importance of this shield was highlighted by Varki and Varki, who indicated that >1% of the mammalian genome encodes proteins involved in this glycosylation pathway (Lehmann et al., 2006; Varki and Varki, 2007). The high Sia content of mucin acts as a lubricant for epithelial surfaces in the cornea, nasal mucosa, respiratory and gastrointestinal tracts and other tissues in contact with the environment. This mucin protects the cells against mechanical and chemical damage, such as gastric acid and digestive enzymes. The Sia content of mucin also functions as a decoy for pathogens that bind to Sia, such as respiratory viruses (Lehmann et al., 2006). The mucin is produced by goblet cells and normally is a benefit to the host (Nicholls et al., 2007). However, in certain diseases such as cystic fibrosis, changes in mucin composition allow increased bacterial growth and pathogenesis (Xia et al., 2005).

The second main function of Sia is to enable host proteins avoid and evade recognition by the body's defense mechanisms. The capping of terminal galactose by Sia masks the recognition of certain oligosaccharides by the asialoglycoprotein receptor of the liver (Grewal et al., 2008) or similar receptors present on macrophages. The sialylation of compounds allows plasma macromolecules to have a longer period of time in the circulation; the number of glycosylation sites is related to biological functions (Banks, 2011; Sun et al., 2011). Conversely, the half-life of many serum glycoproteins such as thyrotropin, erythropoietin, and follicle stimulating hormone is decreased upon desialylation. Glycosylation of proteins as a shield to prevent recognition is not limited to host factors, but is also employed by pathogens such as the influenza viruses, leading to the development of antigenic variation (Sun et al., 2011). Finally, the presence of Sia on glycans may also modulate the immune response, through recognition by Sia-binding lectins, now known as Siglecs. The first Siglec to be identified, sialoadhesin, was found on macrophages (O'Neill et al., 2013), but more than 10 different Siglec molecules have since been discovered (reviewed in Varki and Gagneux (2012)).

2.2.3. The diversity of sialic acids in nature

As mentioned previously, Sia can undergo modification, and the two most common changes are acetylation and hydroxylation to glycolylneuraminic acid (Neu5Gc), a variant present in many animal species but not in humans. In the former situation, this occurs at C4 and C6–9 (Cao et al., 2009; Schauer et al., 2011) with identification of the enzyme sialate-O-acetyltransferase as a candidate recently identified (Arming et al., 2011). Modifications of Sia are important to consider, as these changes, in particular O-acetylation, may have an effect on enzymatic cleavage by sialidases. Schauer demonstrated that the position of acetylation has an effect on sialidase activity: an O-acetyl group on C_8 reduces cleavage by 40% and at C_4 by about 80% (Schauer, 1973). Bacteria and some viruses (notably influenza C virus and coronaviruses) have circumvented this protective mechanism through the development of esterases located in the cytosol or in lysozymes. Although influenza C virus appeared to lack sialidase activity, it was still able to agglutinate red blood cells from certain species, which led to the finding of an esterase combined with the haemagglutinin and fusion protein to form the HEF that is present on influenza C and some coronaviruses (Martin et al., 2003).

O-acetylation is one of the most common modification of sialic acids, and it was initially thought to be a species-specific determinant (Klein and Roussel, 1998). The tissue localization of the 9-O-acetylation variant has been investigated by the use of specific monoclonal antibodies (Arguexo and Sumiyoshi, 2006), or binding by influenza C virus or its recombinant soluble form, coupled with the Fc portion of human immunoglobulin (Che-FcD) (Mandal et al.,

2000). These studies showed that O-acetylation of sialic acid in humans appears localized to the suprabasal plasma membranes of cells exposed to a "wet" surface, such as the cornea, conjunctiva, laryngeal and vaginal epithelium. There is a variation in the degree of O-acetylation between ethnic backgrounds, depending on whether individuals are slow or fast acetylators. Apart from the sialidase from *Arthrobacter urefaciens*, *Actinomyces viscosus* and to a lesser extent the sialidase from Newcastle disease virus, other sialidases are apparently not effective at cleaving the terminal sialic acid. The O-acetylation inhibits sialidase sterically, and is not competitive. Thus, O-acetylation of mucins can protect against the action of bacterial sialidases. This O-acetlylation is conspicuous on the terminal aspect of MUC16, a glycoprotein with a long extracellular domain that prevents infection of the intact epithelium.

The other common modification is the addition of an oxygen to the C5 to form N-glycolylneuraminic acid (Neu5Gc), through the action of the enzyme cytidine monophosphate N-acetylneuraminic hydroxylase (CMAH). Many studies by Ajit Varki and colleagues have shown a high expression of this enzyme in many animal species. Humans lack CMAH activity and the only source of Neu5Gc is from the diet. This additional oxygen is important from a sialidase point of view in that many bacterial and viral sialidases preferably cleave Neu5Ac over Neu5Gc in the α2-3 and α2-6 configuration (Davies et al., 2012). Viral sialidases from Newcastle disease virus, fowl plague and most influenza viruses also show poor cleavage of Neu5Gc over Neu5Ac. Molecular dynamic studies suggest that there is a conformational change induced by the extra oxygen that may explain this decreased activity.

If bacterial sialidases are to be used as potential therapeutic agents, these two changes of O-acetylation and Neu5Gc will have practical implications, as the agent will have to be one that is able to break through the mucin barrier to cleave sialic acid on the surface of epithelial cells. In the case of enzymatic cleavage of Neu5Gc, this enzymatic cleavage is unlikely to be a major concern, because of the lack of CMAH enzyme in humans, and thus the mucin is exclusively Neu5Ac. However, with O-acetylation, this enzymatic cleavage may be of concern if topical therapy is to be used in regions of the body where there is a high degree of O-acetylation of non-keratinized stratified squamous epithelium, as is present in the eye and larynx.

2.2.4. Interaction of sialic acid with respiratory pathogens

In addition to recognition by Siglecs, Sia also functions as a receptor for a number of respiratory viral agents, some of which were discovered by their ability to cause clumping or agglutination of red blood cells in vitro (haemagglutination). As viruses replicate intracellularly, it follows logically that successful attachment to the cell requires a matching up of the receptor with the pathogen. This is often a two-step process: first, a low-affinity "browsing", followed by a higher-affinity interaction with the virus, and more specific receptor interactions (Helander et al., 2003). An extensive list of viruses that utilize Sia and which may infect the respiratory tract has been reviewed by Lehmann and colleagues (Lehmann et al., 2006). Even though influenza virus has been the most well characterized of the pathogens studied, it must be noted that other viruses, including cytomegalovirus (Taylor and Cooper, 1989), rhinovirus 87 (Blomqvist et al., 2002), mumps Urabe AM9 (Reyes-Leyva et al., 2007) and the paramyxoviruses all utilize Sia (Suzuki et al., 2001) (Paulson et al., 1979), suggesting that sialidase treatment may potentially be useful for these infections.

3. Bacteria and sialic acid

Bacteria are able to produce Sia both through *de novo* synthesis and by using sialyl precursors scavenged from animal hosts. The syn-

thesis of Sia in bacteria is limited to a few, mostly pathogenic species that incorporate Sia into surface structures, such as the capsular polysialic acid found in *Neisseria meningitidis* and *Escherichia coli K1* (Plumbridge and Vimr, 1999). The Sia that is incorporated or synthesized is used as a growth factor or a source of carbon (Kim et al., 2011; Stafford et al., 2012; Vimr et al., 2004). Bacteria that utilize Sia include *Neisseria, Haemophilus, Bacteroides, Fusobacteria* and *Streptococci*. To achieve this scavenger effect, a bacterium first require a sialidase to remove the Sia from the host environment, then enzymes for catabolism, followed by uptake into the bacterium for utilization. The latter two steps will not be elucidated further, but an excellent review on these processes is available (Vimr et al., 2004). Another outcome of the sialylation of bacterial surfaces is similar to that mentioned above for viruses, in that they are able to avoid recognition by the host defense mechanisms, as sialylation results in recognition as "self" rather than "foreign".

Over 70 microorganisms have sialidase activity, and several bacteria such as *Streptococcus pneumoniae* produce more than one sialidase (NanA, NanB, and NanC) (Gut et al., 2008). The sialidases of *S. pneumoniae* may expose host receptors and desialylate host glycoproteins such as lactoferrin and immunoglobulin that clear bacteria. They may also lead to the desialylation of the lipopolysaccharides of other bacteria such as *N. meningitidis* and *Haemophilus influenza*, giving the pneumococcus a competitive growth advantage (Pettigrew et al., 2006). The NanA is cell surface-anchored, and functions in adherence. It may also provide a carbon source for the bacterium. Similar to many other bacterial sialidases, NanB is secreted as an extracellular enzyme and implicated in the severity of pneumococcal infection (Manco et al., 2006).

Desialylation may also involve the respiratory epithelium, and there have been previous concerns as to whether the desialylation of an epithelium by bacterial or viral sialidases may lead to increased bacterial adherence and superinfection. It has been well recognized that patients with influenza are susceptible to secondary bacterial infection (van der Sluijs et al., 2006), and the high mortality of the 1918 influenza pandemic has been attributed to secondary infections (Brundage and Shanks, 2008). If sialidase is to be used as a prophylactic or therapeutic strategy, there have therefore been concerns that this may lead to increased bacterial adherence and secondary bacterial pneumonia (Zhang, 2008). In reality, increased adherence is due to epithelial necrosis and exposure of the basement membranes as a result of viral-induced cell death (Plotkowski et al., 1986), rather than surface-induced desialylation, which was not associated with epithelial cell death (Nicholls et al., 2008).

Although sequence homologies among the different bacterial sialidases are less than 30%, there are unique motifs and conserved regions in the sialidase domains. The catalytic domain of *Clostridium perfringens* sialidase has been determined by NMR, and shares key features with other bacterial sialidases (Newstead et al., 2008). These include a cluster of three arginines that interact with the carboxylate of C1, an acid/base catabolic Asp and a hydrophobic pocket to accommodate the N-acetyl group. Three water molecules are involved to bridge the side chains and ligands. Despite many overall similarities in structure, each bacterial sialidase displays a preference for a different linkage, in a manner that is not clearly related to the bacterial species.

The range and diversity of bacterial sialidase specificities was initially studied and characterized by Schauer in the early 1980s (Corfield et al., 1981; Corfield et al., 1983). For instance, *Bacteroides fragilis* STB3182 prefers the α2-8 to the α2-3/6 linkage, but *B. fragilis* 4852 appears quite flexible in its preference. It is even possible for the same bacteria to have different specificities: the NanA of *S. pneumonia* can cleave α2-3, α2-6 or α2-8 linkages, but the extracellular NanB prefers a substrate with the α2-3 linkage. The broad range of specificities has been attributed to a number of mechanisms, including the configuration of the active site and the role

of water molecules as nucleophiles. The crystal structure of NanB has been used to demonstrate α2-3 cleavage specificity, proposing that an additional rim of residues above the arginine triad may only allow an α2-3 configuration to bind (Gut et al., 2008).

4. Bacterial sialidases as antiviral agents

Because Sia are the primary recognition sites for many viral respiratory pathogens, it would seem appropriate that a bacterial sialidase could be used as an inhibitory agent to cleave Sia from the surface receptor, and thus block binding. Achieving this would require a number of properties. The first is that an effective sialidase would need to have a broad catalytic activity, because with agents such as influenza virus, binding is dependent on the linkage between the Sia and galactose. The first evidence of this in influenza was demonstrated by Rogers and Paulson, who desialylated red blood cells, then re-sialylated them using specific sialyltransferases that were either α2-3 or α2-6 linked (Rogers and Paulson, 1983). Avian or equine viruses agglutinated the former, while human and swine viruses agglutinated the latter. From these studies the paradigm emerged that avian viruses were primarily α2-3 binding and human and swine viruses were α2-6 binding (Wilks et al., 2012). As ducks and waterfowl contain mainly α2-3 terminated Sia in their intestines, there would be little selection pressure for avian viruses to acquire α2-6 binding, and cross-species transmission would require a number of mutations.

In ferrets, for example, up to nine passages with a high inoculum has been required for adaptation of an H9N2 virus to the avian configuration (Sorrell et al., 2009). Recent studies in the USA (Imai et al., 2012) and the Netherlands (Herfst et al., 2012) have shown that H5N1 undergoes natural purifying or negative selection, rather than a diversifying positive selection, and that multiple introduced mutations are required for avian viruses to develop greater affinity for α2-6 receptors and to replicate in the ferret upper respiratory tract, leading to transmission. Lectin binding studies have shown that α2-6 and α2-3 N-linked sialic acids are distributed throughout the upper and lower respiratory tract in humans, with an increased distribution of α2-3 O-linked sialic acids in the lower respiratory tract (Nicholls et al., 2007). If a sialidase was to be used to prevent influenza in humans, it would therefore have to be one that cleaves both types of linkages present in the upper and lower airways.

To be used as therapy for respiratory tract infections, a sialidase also have to be effective as a topical or inhalational agent. It was mentioned previously that one of the properties of Sia is to protect "self" glycoproteins from being recognized as "foreign". If a sialidase were administered systemically, this could lead to widespread desialylation of glycoproteins and suppression of the Siglec pathways. For instance, since erythrocytes are removed from the circulation once they are desialylated, it has been proposed that systemic sialidase treatment might result in widespread erythrophagocytosis (Biondi et al., 2002; Bratosin et al., 1998). Furthermore, as many viral respiratory pathogens infect both the upper and lower respiratory tract, including the alveoli, the sialidase would need to be in an aerosolized or nebulized form, to allow even distribution throughout the airway. Finally, a mechanism for retention in the extracellular environment, avoiding rapid catabolism, would be advantageous for effective pharmacokinetics.

5. DAS181 as an antiviral agent

5.1. Structural basis for the design of DAS181

Sialidases have been demonstrated to inhibit influenza virus infection in a number of *in vitro* experiments. Even before Sia were

proven to be the receptors for influenza viruses, it was observed that when Sia was enzymatically removed from cell surfaces, the cells were less susceptible to infection (Gottschalk et al., 1960). Further work using bacterial sialidases and cell lines showed a reduction in influenza virus infection (Bergelson et al., 1982; Griffin et al., 1983). The sialidase of *Micromonospora viridifaciens* was used in 1995 to destroy influenza receptors (Air and Laver, 1995) but these findings were not translated into a practical approach until 2006, when there were two reports of using sialidase to successfully block respiratory virus infection.

The first approach used the sialidase from *Vibrio cholera* to treat influenza and parainfluenza virus three infections (Thompson et al., 2006), while the second used a bacterial sialidase that was coupled to amphiregulin and tested for anti-influenza activity (Malakhov et al., 2006). This second compound, called DAS181, used the sialidase from *A. viscosus*. The coupling of sialidase to amphiregulin allowed it to bind to the respiratory epithelium, thus prolonging the duration of drug effect. *A. viscosus* is one of the first bacteria to colonize the surface of teeth. It is abundantly present in patients with gingivitis (Haffajee et al., 1997), and has been implicated in the immunopathogenic role of periodontal disease (Mahanonda et al., 1989). It has been proposed that continuous swallowing of this bacterium leads to a low-grade immunological response and the induction of oral bacterial tolerance (Sosroseno et al., 2006).

The sialidase from *A. viscosus* was chosen for DAS181 three reasons. Like many bacterial sialidases, it was effective at cleaving many types of Sia; it had a high specific activity; and as it was a normal commensal of the oral cavity, there would not be natural antibodies against it, as a result of oral tolerance. The sialidase from *A. viscosus* had been studied in 1989 by Teufel and colleagues who found that it was not primarily secreted, but cell-bound (Teufel et al., 1989). Though it had a higher hydrolysis rate against the α2-6 linkage, it was also effective at cleaving α2-3 linkages, and was also able to cleave the ganglioside GD1a, an uncommon property among bacterial sialidases. Acetylation at C5,7 and 9 of Sia still allowed cleavage, but 4-O-acetylation, GM1 and Neu5Gc GM3 were not attacked at all.

Amphiregulin is an 84-amino acid glycoprotein that was discovered in 1988 (Shoyab et al., 1988), as reviewed in (Busser et al., 2011). One of its features is an epidermal-growth factor (EGF)-like domain, which is homologous with other members of the EGF-like growth factor family, meaning that it can bind to the EGF receptor present on epithelial cells. Unlike many other members of the EGF family, amphiregulin binding does not lead to EGFR degradation, but to recycling of the receptor and continued accumulation at the surface (Willmarth et al., 2009). At the other end of the protein is a heparin- and glycosaminoglycan-binding domain, which is involved in the inhibition or transformation of this glycoprotein (hence the name *amphi*-regulin). In the case of DAS181, the sialidase was combined with the GAG-binding portion of human AR. By binding to the negatively-charged glycosaminoglycans on the surface of airway epithelial cells, the cationic C-terminal amphiregulin tag anchors DAS181 on the respiratory epithelium, improving its potency. This net effect of combining the sialidase with amphiregulin means that the sialidase will be targeted to epithelial cells lining the respiratory tract, but without activating the EGFR pathway.

5.2. Preclinical evaluation

DAS181 was tested in ferret and human airway epithelium models and was shown to have activity against a wide range of influenza A and B viruses (Malakhov et al., 2006). Compared to other sialidases, DAS181 had higher activity and was stable over a wide range of pH values that would be present in the respiratory tract. After a single treatment, there was effective desialylation of MDCK cells for 2 days and recovery to 80% after 4 days; however, later treatment regimens adopted a daily dosing schedule. The amphiregulin attachment increased the potency 5–30-fold (Malakhov et al., 2006). There was no appreciable cell toxicity in a number of established cell lines, and no endogenous production of interferon or TNFα was seen, indicating that the amphiregulin tag was not detrimental to the host cells.

Inhalation toxicity was evaluated in Sprague–Dawley rats. Repeated daily exposures of inhaled DAS181 for 28 days at achieved doses of up to 3.00 mg/kg/day were well tolerated, and clinical signs were not affected by treatment with DAS181. Minimal but significant decreases in red blood cells, haematocrit and hemoglobin were identified, together with an increased reticulocyte count, in keeping with a mild anemia; systemic absorption from the respiratory tract was estimated at 2% (Larson et al., 2011). In this
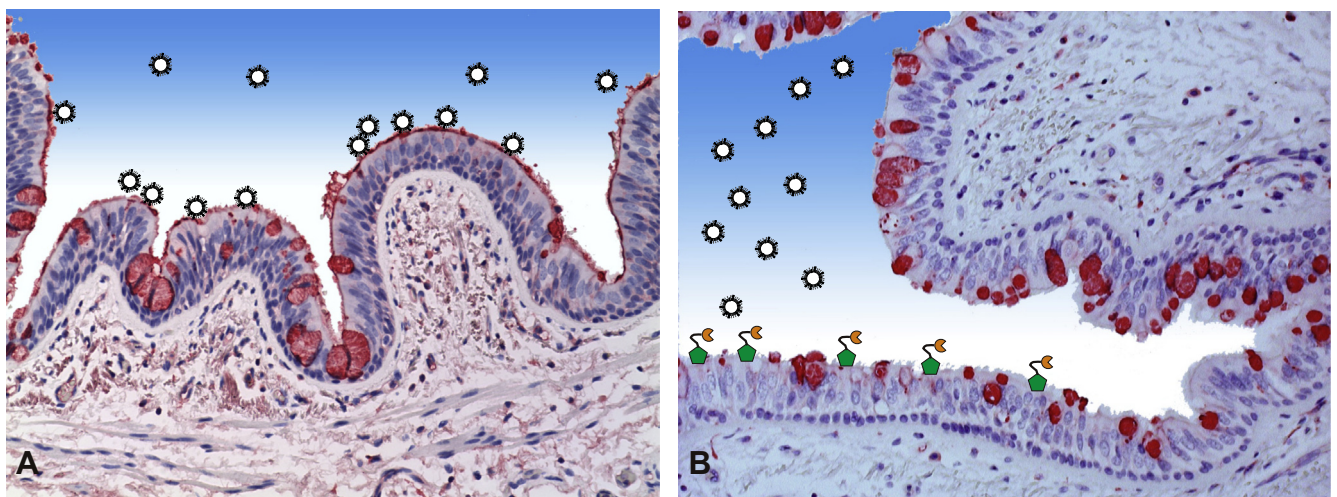
Fig. 2. Mechanism of action of DAS181, representing lectin-binding histochemistry of normal human bronchial mucosa before (A), and after 2 h of incubation with topical DAS181 (10 mg/cm^2). In the control panel (A), there is surface binding to ciliated cells and within goblet cells by the lectin *Sambucus nigra* agglutinin (SNA), which binds Siaα2-6 linkages. The red color indicates the presence of Siaα2-6, which allows binding and infection by influenza viruses. In panel B the amphiregulin component of DAS181 (green polygon) binds to glycosaminoglycans present on epithelial cells, and the functional sialidase (orange circle) cleaves Sia from the surface, preventing infection by influenza viruses.

study there was also a 3-fold increase in alkaline phosphatase (ALP) from the liver, which was attributed to diminished clearance, owing to competition for the asialoglycoprotein receptor. The rise in ALP was not associated with any evidence of hepatotoxicity. It is thought that desialylation of the serum proteins may lead to saturation of the asialoglycoprotein receptors (ASGR) in the liver and spleen, and reduced clearance of certain glycoproteins, including ALP.

Further studies were done on human airway epithelial cells (Triana-Baltzer et al., 2010). In this study, a 90% desialylation effect was seen after 15 min, and the presence of mucus did not interfere with desialylation (Fig. 2). Similar changes were also seen in freshly isolated human bronchial *ex vivo* cultures (Triana-Baltzer et al., 2009). In the HAE model, re-sialylation was achieved by 10 days, whereas without the amphiregulin tag re-sialylation was much faster. A 24 h postexposure regimen also produced a reduction in viral titre in H1N1 infection, thus demonstrating that DAS181 could have a therapeutic was well as a prophylactic role. This was also demonstrated in an animal model, in which treatment at 48 h postinfection reduced mortality and viral load in mice infected with A/PR8/34(H1N1) and A/Victoria/3/75(H3N2)(Hedlund et al., 2010). Additional studies were done on lung *ex-vivo* infections, using H5N1 (Chan et al., 2009) and H1N1pdm viruses (Triana-Baltzer et al., 2009), showing a similar reduction in viral infection without toxicity.

DAS181 was formulated into dry-powder microparticles with a diameter ranging from 3 to 6 μm using a proprietary Temperature-controlled organic solvent assisted precipitation (TOSAP) technology, and drug deposition was tested using a cast of the human airway. As expected, smaller particles (3 μm) were associated with increased deposition in the deep lungs (43%) and higher systemic exposure, compared to 8.6% for 10 μm particles. Using the same airway model, Relenza® had a 13.2% deposition in the distal bronchi and lungs (data on Company file). In the Phase 2A trial, the 6 μm size was used, but current Phase 2B clinical studies are comparing 6 μm and 10 μm particles.

5.3. Clinical studies

A single Phase II study of DAS181 has been completed, which comprised 177 participants with PCR-confirmed influenza. The inclusion criteria were: male and female participants age 18–70 who gave informed consent, were in generally good health, were febrile with oral temperature >37.8 °C or reported temperature >37.8 °C, or had felt "feverish" in the past 24 h, and who had one or more respiratory (cough, sore throat, nasal symptoms) or constitutional symptoms (headache, myalgia, sweat/chills, prostration). There were two treatment arms: one with a single dose of 10 mg, and the second using 10 mg/day over 3 days. The mean age of the patients in the multiple-dose arm was 31.7 years, in the single-dose arm 32.2 years and in the placebo arm 33 years, with a slight female predominance in the treatment arms. Thirty-seven percent of the participants had confirmed infection with influenza B, 33% with seasonal H3N2 and 29% with H1N1pdm. No H274Y mutations were identified that would confirm the oseltamivir-resistant phenotype in the H1N1 subtype of influenza viruses.

The trial was conducted over many geographical areas, thus allowing an evaluation of a broad range of commonly circulating influenza viruses. The study demonstrated a significant reduction in viral load over 5 days, for the 3 day dosing group, and a significantly shorter time to sustained decrease in viral shedding in this group, compared to the placebo group. The 3 day dose schedule was more effective than the single-day dose regimen, but no change in flu-like symptoms was seen between the control or treatment groups. However, the study lacked statistical power and was not designed to assess clinical differences among the treatment groups. There was a low incidence of serious adverse events, and only a mild increase in serum ALP, possibly a desialylation-mediated effect (Moss et al., 2012). There did not appear to be a reported increase in secondary bacterial pneumonia.

5.4. Potential unwanted effects of DAS181 therapy

There are a number of potential concerns with sialidase therapy, including systemic dissemination of the drug, development of antibodies and the role of secondary bacterial infection. It was mentioned earlier that A. viscosus was chosen because it is a natural commensal of the oral cavity, and there have been no reported changes of immunity with exposure to DAS181. A mild increase in ALP was seen in the Phase II study, which may be due to systemic desialylation, but no changes in hematological parameters were identified (Moss et al., 2012).

It was mentioned previously that patients with influenza had increased bacterial infection with Pneumococcus, and there were concerns that the same may be found with DAS181 treatment (Zhang, 2008). The interaction between influenza virus infection and secondary pneumonia is a complex one that has been investigated by McCullers and others (Grijalva and Griffin, 2012; Iverson et al., 2011; Karlstrom et al., 2011). Since the 1918 H1N1 pandemic, there has been an association of increased bacterial infection following influenza infection. The recent 2009 H1N1 pandemic was associated with an increase in invasive pneumococcal infection (Nelson et al., 2012), suggesting that pneumococcal vaccination should be included as a vaccine strategy in future pandemics. Indeed, vaccination strategies using pneumococcal vaccination reduced the incidence of influenza-associated pneumonia. However, the link between influenza and pneumococcal infection has been complicated by the finding that not all pneumococcal serotypes are associated with secondary pneumonia, and that influenza virus infection may select out and favor the growth of particular serotypes (McCullers et al., 2010). It has been proposed that the desialylation produced by the viral neuraminidase exposes receptors that allow increased binding by pneumococci, followed by invasion. As we pointed out previously, a clear distinction needs to be made between desialylation in viral infection and that induced by topical therapy (Nicholls et al., 2008).

It is therefore important to study whether sialidase therapy will lead to an increased risk of secondary pneumonia. Studies by Hedlund and colleagues using DAS181 showed that, in contrast to expectations, desialylation appeared to protect mice against secondary bacterial colonization (Hedlund et al., 2010). Also, in a study previously cited, there was no evidence of increased adherence of pneumococci to desialylated tracheal epithelial specimens (Nicholls et al., 2008). The contrast between changes induced by influenza virus infection and by topical desialylation can be explained by the different pathologies: influenza virus infection of an epithelial surface causes necrosis and desialylation, while the changes induced by bacterial sialidase consist only of desialylation. In a virus infection, there is acute epithelial denudation and exposure of the basement membrane, allowing adherence of S. pneumoniae. With sialidase treatment alone, because there is no cell cytotoxicity or epithelial loss, there is no increased adherence of pneumococi. As detailed earlier, the Phase II clinical study did not document any increase in pneumococcal infection.

6. Bacterial sialidases and parainfluenza

6.1. Parainfluenza virus interaction with sialic acids

Even though influenza viruses are one of the leading pathogens causing acute respiratory illness, infections with parainfluenza

virus 1, 2 and 3 make up a significant proportion of the causative agents isolated during these illnesses, especially in the pediatric population (Weigl et al., 2007). Although parainfluenza viruses typically cause bronchitis and bronchiolitis, they are increasingly recognized as agents of lower respiratory tract disease, where they have a similar clinical presentation to infections by other respiratory viruses. Parainfluenza viruses are of increasing concern in pediatric and immunocompromised populations, in which they may cause severe pulmonary disease.

PIV1-3 resemble influenza viruses in using Sia as a receptor, but binding differs among strains (Villar and Barroso, 2006). Use of a sialylated microarray has recently demonstrated that hPIV1 has the narrowest specificity, binding to Neu5Ac, but stronger binding to Neu5Gc with a methylated derivative. In contrast, hPIV2 showed a broader range of binding to Neu5Ac and Neu5Gc derivatives, while hPIV3 showed a similar range of binding, but at a much lower magnitude than hPIV2. All three strains preferentially bound glycans with an α2-3, rather than an α2-6 linkage (Song et al., 2011).

6.2. Sialidase treatment of parainfluenza

The mechanism of binding and release of PIV differs from influenza viruses, as they have a different replication pathway, and thus may not be amenable to standard anti-influenza therapy. Thus, although zanamivir inhibited PIV neuraminidase activity, it did not prevent release of virus from the cell (Moscona, 2005; Porotto et al., 2001). As the parainfluenza viruses use α2-3 and α2-6 bound Sia for attachment, infection with these viruses could be amenable to sialidase treatment. Even though most studies utilizing DAS181 have used influenza A as a target, it has also been used in patients with parainfluenza. In an experimental setting, DAS181 treatment of human parainfluenza virus-permissive cell lines and of infected cotton rats led to effective desialylation and reduced infection (Moscona et al., 2010). DAS181 has also been used on a compassionate, emergency basis in immunocompromised patients infected with parainfluenza virus, with a beneficial effect. The first case was a 63 year old female stem cell transplant recipient who developed nasal congestion and cough, followed by clinical and radiological features of viral bronchiolitis and worsening respiratory function. She received three daily inhaled administrations of DAS181 using a dry powder inhaler, and showed improvement in clinical, radiological and pulmonary function parameters after treatment. PIV3 infection was confirmed, and there was a reduction in viral load after treatment (Chen et al., 2011). In another report, two immunocompromised lung or stem cell transplant recipients who developed PIV3 infection were successfully treated after developing lower respiratory tract disease. In these cases, there was a 5 day treatment with improvement in clinical symptoms and respiratory function, though changes in quantitative viral load did not match the clinical findings (Guzman-Suarez et al., 2012). However, a more recent case of DAS181 treatment of PIV3 pneumonia in a lung transplant patient was associated with both clinical improvement and reduction in viral load (Drozd et al., 2012).

7. Conclusion

A novel bacterial sialidase, DAS181, has been developed over the past six years from *in vitro* testing to Phase II studies for influenza virus infection. This agent shows promise in the repertoire of antivirals for influenza, and it should also show potential benefit against other respiratory viruses that use Sia as a receptor, such as severe parainfluenza virus infection.

References

Air, G.M., Laver, W.G., 1995. Red cells bound to influenza virus N9 neuraminidase are not released by the N9 neuraminidase activity. Virology 211, 278–284.

Al-qattan, M.N., Mordi, M.N., 2010a. Docking of sialic acid analogues against influenza A hemagglutinin: a correlational study between experimentally measured and computationally estimated affinities. J. Mol. Model. 16, 1047–1058.

Al-qattan, M.N., Mordi, M.N., 2010b. Site-directed fragment-based generation of virtual sialic acid databases against influenza A hemagglutinin. J. Mol. Model. 16, 975–991.

Argueso, P., Sumiyoshi, M., 2006. Characterization of a carbohydrate epitope defined by the monoclonal antibody H185: sialic acid O-acetylation on epithelial cell-surface mucins. Glycobiology 16, 1219–1228.

Arming, S., Wipfler, D., Mayr, J., Merling, A., Vilas, U., Schauer, R., Schwartz-Albiez, R., Vlasak, R., 2011. The human Cas1 protein: a sialic acid-specific O-acetyltransferase? Glycobiology 21, 553–564.

Banks, D.D., 2011. The effect of glycosylation on the folding kinetics of erythropoietin. J. Mol. Biol. 412, 536–550.

Bergelson, L.D., Bukrinskaya, A.G., Prokazova, N.V., Shaposhnikova, G.I., Kocharov, S.L., Shevchenko, V.P., Kornilaeva, G.V., Fomina-Ageeva, E.V., 1982. Role of gangliosides in reception of influenza virus. Eur. J. Biochem. 128, 467–474.

Biondi, C., Cotorruelo, C., Ensinck, A., Garcia Borras, S., Racca, L., Racca, A., 2002. Senescent erythrocytes: factors affecting the aging of red blood cells. Immunol. Invest. 31, 41–50.

Blessia, T.F., Rapheal, V.S., Sharmila, D.J., 2010. Molecular dynamics of sialic acid analogues and their interaction with influenza hemagglutinin. Indian J. Pharm. Sci. 72, 449–457.

Blomqvist, S., Savolainen, C., Raman, L., Roivainen, M., Hovi, T., 2002. Human rhinovirus 87 and enterovirus 68 represent a unique serotype with rhinovirus and enterovirus features. J. Clin. Microbiol. 40, 4218–4223.

Bratosin, D., Mazurier, J., Tissier, J.P., Estaquier, J., Huart, J.J., Ameisen, J.C., Aminoff, D., Montreuil, J., 1998. Cellular and molecular mechanisms of senescent erythrocyte phagocytosis by macrophages. A review. Biochimie 80, 173–195.

Brundage, J.F., Shanks, G.D., 2008. Deaths from bacterial pneumonia during 1918–19 influenza pandemic. Emerg. Infect. Dis. 14, 1193–1199.

Bulai, T., Bratosin, D., Pons, A., Montreuil, J., Zanetta, J.P., 2003. Diversity of the human erythrocyte membrane sialic acids in relation with blood groups. FEBS Lett. 534, 185–189.

Busser, B., Sancey, L., Brambilla, E., Coll, J.L., Hurbin, A., 2011. The multiple roles of amphiregulin in human cancer. Biochim. Biophys. Acta 1816, 119–131.

Cao, H., Li, Y., Lau, K., Muthana, S., Yu, H., Cheng, J., Chokhawala, H.A., Sugiarto, G., Zhang, L., Chen, X., 2009. Sialidase substrate specificity studies using chemoenzymatically synthesized sialosides containing C5-modified sialic acids. Org. Biomol. Chem. 7, 5137–5145.

Chan, R.W., Chan, M.C., Wong, A.C., Karamanska, R., Dell, A., Haslam, S.M., Sihoe, A.D., Chui, W.H., Triana-Baltzer, G., Li, Q., Peiris, J.S., Fang, F., Nicholls, J.M., 2009. DAS181 inhibits H5N1 influenza virus infection of human lung tissues. Antimicrob. Agents Chemother. 53, 3935–3941.

Chen, X., Varki, A., 2010. Advances in the biology and chemistry of sialic acids. ACS Chem. Biol. 5, 163–176.

Chen, Y.B., Driscoll, J.P., McAfee, S.L., Spitzer, T.R., Rosenberg, E.S., Sanders, R., Moss, R.B., Fang, F., Marty, F.M., 2011. Treatment of parainfluenza 3 infection with DAS181 in a patient after allogeneic stem cell transplantation. Clin. Infect. Dis. 53, e77–80.

Corfield, A.P., Veh, R.W., Wember, M., Michalski, J.C., Schauer, R., 1981. The release of N-acetyl- and N-glycolloyl-neuraminic acid from soluble complex carbohydrates and erythrocytes by bacterial, viral and mammalian sialidases. Biochem. J. 197, 293–299.

Corfield, A.P., Higa, H., Paulson, J.C., Schauer, R., 1983. The specificity of viral and bacterial sialidases for alpha(2–3)- and alpha(2–6)-linked sialic acids in glycoproteins. Biochim. Biophys. Acta 744, 121–126.

Davies, L.R., Pearce, O.M., Tessier, M.B., Assar, S., Smutova, V., Pajunen, M., Sumida, M., Sato, C., Kitajima, K., Finne, J., Gagneux, P., Pshezhetsky, A., Woods, R., Varki, A., 2012. Metabolism of vertebrate amino sugars with N-glycolyl groups: resistance of alpha2-8-linked N-glycolylneuraminic acid to enzymatic cleavage. J. Biol. Chem. 287, 28917–28931.

Drozd, D.R., Limaye, A.P., Moss, R.B., Sanders, R.L., Hansen, C., Edelman, J.D., Raghu, G., Boeckh, M., Rakita, R.M., 2012. DAS181 treatment of severe parainfluenza type 3 pneumonia in a lung transplant recipient. Transpl. Infect. Dis. 15, E28–32.

Gottschalk, A., De St, Fezekas., Groth, S., 1960. On the relationship between the indicator profile and prosthetic group of mucoproteins inhibitory for influenza virus haemagglutinin. J. Gen. Microbiol. 22, 690–697.

Grewal, P.K., Uchiyama, S., Ditto, D., Varki, N., Le, D.T., Nizet, V., Marth, J.D., 2008. The Ashwell receptor mitigates the lethal coagulopathy of sepsis. Nat. Med. 14, 648–655.

Griffin, J.A., Basak, S., Compans, R.W., 1983. Effects of hexose starvation and the role of sialic acid in influenza virus release. Virology 125, 324–334.

Grijalva, C.G., Griffin, M.R., 2012. Unveiling the burden of influenza-associated pneumococcal pneumonia. J. Infect. Dis. 205, 355–357.

Gut, H., King, S.J., Walsh, M.A., 2008. Structural and functional studies of Streptococcus pneumoniae neuraminidase B: an intramolecular trans-sialidase. FEBS Lett. 582, 3348–3352.

Guzman-Suarez, B.B., Buckley, M.W., Gilmore, E.T., Vocca, E., Moss, R., Marty, F.M., Sanders, R., Baden, L.R., Wurtman, D., Issa, N.C., Fang, F., Koo, S., 2012. Clinical

potential of DAS181 for treatment of parainfluenza-3 infections in transplant recipients. Transpl. Infect. Dis. 14, 427–433.

Haffajee, A.D., Cugini, M.A., Dibart, S., Smith, C., Kent Jr., R.L., Socransky, S.S., 1997. Clinical and microbiological features of subjects with adult periodontitis who responded poorly to scaling and root planing. J. Clin. Periodontol. 24, 767–776.

Hanaoka, K., Pritchett, T.J., Takasaki, S., Kochibe, N., Sabesan, S., Paulson, J.C., Kobata, A., 1989. 4-O-acetyl-N-acetylneuraminic acid in the N-linked carbohydrate structures of equine and guinea pig alpha 2-macroglobulins, potent inhibitors of influenza virus infection. J. Biol. Chem. 264, 9842–9849.

Harduin-Lepers, A., Mollicone, R., Delannoy, P., Oriol, R., 2005. The animal sialyltransferases and sialyltransferase-related genes: a phylogenetic approach. Glycobiology 15, 805–817.

Hedlund, M., Aschenbrenner, L.M., Jensen, K., Larson, J.L., Fang, F., 2010. Sialidase-based anti-influenza virus therapy protects against secondary pneumococcal infection. J. Infect. Dis. 201, 1007–1015.

Helander, A., Silvey, K.J., Mantis, N.J., Hutchings, A.B., Chandran, K., Lucas, W.T., Nibert, M.L., Neutra, M.R., 2003. The viral sigma1 protein and glycoconjugates containing alpha2-3-linked sialic acid are involved in type 1 reovirus adherence to M cell apical surfaces. J. Virol. 77, 7964–7977.

Herfst, S., Schrauwen, E.J., Linster, M., Chutinimitkul, S., de Wit, E., Munster, V.J., Sorrell, E.M., Bestebroer, T.M., Burke, D.F., Smith, D.J., Rimmelzwaan, G.F., Osterhaus, A.D., Fouchier, R.A., 2012. Airborne transmission of influenza A/H5N1 virus between ferrets. Science 336, 1534–1541.

Hildebrandt, H., Muhlenhoff, M., Gerardy-Schahn, R., 2010. Polysialylation of NCAM. Adv Exp. Med. Biol. 663, 95–109.

Imai, M., Watanabe, T., Hatta, M., Das, S.C., Ozawa, M., Shinya, K., Zhong, G., Hanson, A., Katsura, H., Watanabe, S., Li, C., Kawakami, E., Yamada, S., Kiso, M., Suzuki, Y., Maher, E.A., Neumann, G., Kawaoka, Y., 2012. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature 486, 420–428.

Iverson, A.R., Boyd, K.L., McAuley, J.L., Plano, L.R., Hart, M.E., McCullers, J.A., 2011. Influenza virus primes mice for pneumonia from Staphylococcus aureus. J. Infect. Dis. 203, 880–888.

Karlstrom, A., Heston, S.M., Boyd, K.L., Tuomanen, E.I., McCullers, J.A., 2011. Toll-like receptor 2 mediates fatal immunopathology in mice during treatment of secondary pneumococcal pneumonia following influenza. J. Infect. Dis. 204, 1358–1366.

Kim, S., Oh, D.B., Kang, H.A., Kwon, O., 2011. Features and applications of bacterial sialidases. Appl. Microbiol. Biotechnol. 91, 1–15.

Klein, A., Roussel, P., 1998. O-acetylation of sialic acids. Biochimie 80, 49–57.

Larson, J.L., Kang, S.K., Choi, B.I., Hedlund, M., Aschenbrenner, L.M., Cecil, B., Machado, G., Nieder, M., Fang, F., 2011. A safety evaluation of DAS181, a sialidase fusion protein, in rodents. Toxicol. Sci. 122, 567–578.

Lehmann, F., Tiralongo, E., Tiralongo, J., 2006. Sialic acid-specific lectins: occurrence, specificity and function. Cell Mol. Life Sci. 63, 1331–1354.

Lentz, T.L., 1988. Binding of viral attachment protein to host-cell receptor: the Achilles heel of infectious viruses. Trends Pharmacol. Sci. 9, 247–252.

Lentz, T.L., 1990. The recognition event between virus and host cell receptor: a target for antiviral agents. J. Gen. Virol. 71 (Pt 4), 751–766.

Li, X.B., Wang, S.Q., Xu, W.R., Wang, R.L., Chou, K.C., 2011. Novel inhibitor design for hemagglutinin against H1N1 influenza virus by core hopping method. PLoS One 6, e28111.

Mahanonda, R., Seymour, G.J., Powell, L.W., Good, M.F., Halliday, J.W., 1989. Limit dilution analysis of peripheral blood T lymphocytes specific to periodontopathic bacteria. Clin. Exp. Immunol. 75, 245–251.

Malakhov, M.P., Aschenbrenner, L.M., Smee, D.F., Wandersee, M.K., Sidwell, R.W., Gubareva, L.V., Mishin, V.P., Hayden, F.G., Kim, D.H., Ing, A., Campbell, E.R., Yu, M., Fang, F., 2006. Sialidase fusion protein as a novel broad-spectrum inhibitor of influenza virus infection. Antimicrob. Agents Chemother. 50, 1470–1479.

Manco, S., Hernon, F., Yesilkaya, H., Paton, J.C., Andrew, P.W., Kadioglu, A., 2006. Pneumococcal neuraminidases A and B both have essential roles during infection of the respiratory tract and sepsis. Infect Immun 74, 4014–4020.

Mandal, C., Chatterjee, M., Sinha, D., 2000. Investigation of 9-O-acetylated sialoglycoconjugates in childhood acute lymphoblastic leukaemia. Br. J. Haematol. 110, 801–812.

Martin, L.T., Verhagen, A., Varki, A., 2003. Recombinant influenza C hemagglutinin-esterase as a probe for sialic acid 9-O-acetylation. Methods Enzymol. 363, 489–498.

Matrosovich, M.N., Matrosovich, T.Y., Gray, T., Roberts, N.A., Klenk, H.D., 2004. Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. J. Virol. 78, 12665–12667.

McCullers, J.A., McAuley, J.L., Browall, S., Iverson, A.R., Boyd, K.L., Henriques Normark, B., 2010. Influenza enhances susceptibility to natural acquisition of and disease due to Streptococcus pneumoniae in ferrets. J. Infect. Dis. 202, 1287–1295.

Moscona, A., Porotto, M., Palmer, S., Tai, C., Aschenbrenner, L., Triana-Baltzer, G., Li, Q.X., Wurtman, D., Niewiesk, S., Fang, F., 2010. A recombinant sialidase fusion protein effectively inhibits human parainfluenza viral infection in vitro and in vivo. J. Infect. Dis. 202, 234–241.

Moscona, A., 2005. Entry of parainfluenza virus into cells as a target for interrupting childhood respiratory disease. J. Clin. Invest. 115, 1688–1698.

Moss, R.B., Hansen, C., Sanders, R.L., Hawley, S., Li, T., Steigbigel, R.T., 2012. A Phase II study of DAS181, a novel host directed antiviral for the treatment of influenza infection. J. Infect. Dis. 206, 1844–1851.

Nandi, T., 2008. Proposed lead molecules against Hemagglutinin of avian influenza virus (H5N1). Bioinformation 2, 240–244.

Nelson, G.E., Gershman, K.A., Swerdlow, D.L., Beall, B.W., Moore, M.R., 2012. Invasive pneumococcal disease and pandemic (H1N1) 2009, Denver, Colorado, USA. Emerg. Infect. Dis. 18, 208–216.

Newstead, S.L., Potter, J.A., Wilson, J.C., Xu, G., Chien, C.H., Watts, A.G., Withers, S.G., Taylor, G.L., 2008. The structure of Clostridium perfringens NanI sialidase and its catalytic intermediates. J. Biol. Chem. 283, 9080–9088.

Nicholls, J.M., Bourne, A.J., Chen, H., Guan, Y., Peiris, J.S., 2007. Sialic acid receptor detection in the human respiratory tract: evidence for widespread distribution of potential binding sites for human and avian influenza viruses. Respir. Res. 8, 73.

Nicholls, J.M., Aschenbrenner, L.M., Paulson, J.C., Campbell, E.R., Malakhov, M.P., Wurtman, D.F., Yu, M., Fang, F., 2008. Comment on: concerns of using sialidase fusion protein as an experimental drug to combat seasonal and pandemic influenza. J. Antimicrob. Chemother. 62, 426–428 (author reply 428–429).

O'Neill, A.S., van den Berg, T.K., Mullen, G.E., 2013. Sialoadhesin – A macrophage-restricted marker of immunoregulation and inflammation. Immunology 138, 198–207.

Paulson, J.C., Rademacher, C., 2009. Glycan terminator. Nat. Struct. Mol. Biol. 16, 1121–1122.

Paulson, J.C., Sadler, J.E., Hill, R.L., 1979. Restoration of specific myxovirus receptors to asialoerythrocytes by incorporation of sialic acid with pure sialyltransferases. J. Biol. Chem. 254, 2120–2124.

Pettigrew, M.M., Fennie, K.P., York, M.P., Daniels, J., Ghaffar, F., 2006. Variation in the presence of neuraminidase genes among Streptococcus pneumoniae isolates with identical sequence types. Infect. Immun. 74, 3360–3365.

Plotkowski, M.C., Puchelle, E., Beck, G., Jacquot, J., Hannoun, C., 1986. Adherence of type I Streptococcus pneumoniae to tracheal epithelium of mice infected with influenza A/PR8 virus. Am. Rev. Respir. Dis. 134, 1040–1044.

Plumbridge, J., Vimr, E., 1999. Convergent pathways for utilization of the amino sugars N-acetylglucosamine, N-acetylmannosamine, and N-acetylneuraminic acid by Escherichia coli. J. Bacteriol. 181, 47–54.

Porotto, M., Greengard, O., Poltoratskaia, N., Horga, M.A., Moscona, A., 2001. Human parainfluenza virus type 3 HN-receptor interaction: effect of 4-guanidino-Neu5Ac2en on a neuraminidase-deficient variant. J. Virol. 75, 7481–7488.

Pritchett, T.J., Paulson, J.C., 1989. Basis for the potent inhibition of influenza virus infection by equine and guinea pig alpha 2-macroglobulin. J. Biol. Chem. 264, 9850–9858.

Pritchett, T.J., Brossmer, R., Rose, U., Paulson, J.C., 1987. Recognition of monovalent sialosides by influenza virus H3 hemagglutinin. Virology 160, 502–506.

Reyes-Leyva, J., Banos, R., Borraz-Arguello, M., Santos-Lopez, G., Rosas, N., Alvarado, G., Herrera, I., Vallejo, V., Tapia-Ramirez, J., 2007. Amino acid change 335 E to K affects the sialic-acid-binding and neuraminidase activities of Urabe AM9 mumps virus hemagglutinin-neuraminidase glycoprotein. Microbes Infect. 9, 234–240.

Rogers, G.N., Paulson, J.C., 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology 127, 361–373.

Rogers, G.N., Pritchett, T.J., Lane, J.L., Paulson, J.C., 1983. Differential sensitivity of human, avian, and equine influenza A viruses to a glycoprotein inhibitor of infection: selection of receptor specific variants. Virology 131, 394–408.

Schauer, R., Srinivasan, G.V., Wipfler, D., Kniep, B., Schwartz-Albiez, R., 2011. O-Acetylated sialic acids and their role in immune defense. Adv. Exp. Med. Biol. 705, 525–548.

Schauer, R., 1973. Chemistry and biology of the acylneuraminic acids. Angew. Chem. Int. Ed. Engl. 12, 127–138.

Schauer, R., 2000. Achievements and challenges of sialic acid research. Glycoconj. J. 17, 485–499.

Shoyab, M., McDonald, V.L., Bradley, J.G., Todaro, G.J., 1988. Amphiregulin: a bifunctional growth-modulating glycoprotein produced by the phorbol 12-myristate 13-acetate-treated human breast adenocarcinoma cell line MCF-7. Proc. Natl. Acad. Sci. USA 85, 6528–6532.

Song, X., Yu, H., Chen, X., Lasanajak, Y., Tappert, M.M., Air, G.M., Tiwari, V.K., Cao, H., Chokhawala, H.A., Zheng, H., Cummings, R.D., Smith, D.F., 2011. A sialylated glycan microarray reveals novel interactions of modified sialic acids with proteins and viruses. J. Biol. Chem. 286, 31610–31622.

Sorrell, E.M., Wan, H., Araya, Y., Song, H., Perez, D.R., 2009. Minimal molecular constraints for respiratory droplet transmission of an avian-human H9N2 influenza A virus. Proc. Natl. Acad. Sci. USA 106, 7565–7570.

Sosroseno, W., Bird, P.S., Gemmell, E., Seymour, G.J., 2006. The induction of oral tolerance to Actinomyces viscosus in mice. Oral Dis. 12, 387–394.

Springer, G.F., Ansell, N.J., 1958. Inactivation of human erythrocyte agglutinogens M and N by influenza viruses and receptor-destroying enzyme. Proc. Natl. Acad. Sci. USA 44, 182–189.

Stafford, G., Roy, S., Honma, K., Sharma, A., 2012. Sialic acid, periodontal pathogens and Tannerella forsythia: stick around and enjoy the feast! Mol. Oral Microbiol. 27, 11–22.

Sun, S., Wang, Q., Zhao, F., Chen, W., Li, Z., 2011. Glycosylation site alteration in the evolution of influenza A (H1N1) viruses. PLoS One 6, e22844.

Suzuki, T., Portner, A., Scroggs, R.A., Uchikawa, M., Koyama, N., Matsuo, K., Suzuki, Y., Takimoto, T., 2001. Receptor specificities of human respiroviruses. J. Virol. 75, 4604–4613.

Taylor, H.P., Cooper, N.R., 1989. Human cytomegalovirus binding to fibroblasts is receptor mediated. J. Virol. 63, 3991–3998.

Teufel, M., Roggentin, P., Schauer, R., 1989. Properties of sialidase isolated from Actinomyces viscosus DSM 43798. Biol. Chem. Hoppe Seyler 370, 435–443.

Thompson, C.I., Barclay, W.S., Zambon, M.C., Pickles, R.J., 2006. Infection of human airway epithelium by human and avian strains of influenza a virus. J. Virol. 80, 8060–8068.

Triana-Baltzer, G.B., Gubareva, L.V., Nicholls, J.M., Pearce, M.B., Mishin, V.P., Belser, J.A., Chen, L.M., Chan, R.W., Chan, M.C., Hedlund, M., Larson, J.L., Moss, R.B., Katz, J.M., Tumpey, T.M., Fang, F., 2009. Novel pandemic influenza A(H1N1) viruses are potently inhibited by DAS181, a sialidase fusion protein. PLoS One 4, e7788.

Triana-Baltzer, G.B., Babizki, M., Chan, M.C., Wong, A.C., Aschenbrenner, L.M., Campbell, E.R., Li, Q.X., Chan, R.W., Peiris, J.S., Nicholls, J.M., Fang, F., 2010. DAS181, a sialidase fusion protein, protects human airway epithelium against influenza virus infection: an in vitro pharmacodynamic analysis. J. Antimicrob. Chemother. 65, 275–284.

van der Sluijs, K.F., van Elden, L.J., Nijhuis, M., Schuurman, R., Florquin, S., Shimizu, T., Ishii, S., Jansen, H.M., Lutter, R., van der Poll, T., 2006. Involvement of the platelet-activating factor receptor in host defense against Streptococcus pneumoniae during postinfluenza pneumonia. Am. J. Physiol. Lung Cell Mol. Physiol. 290, L194–L199.

Varki, A., Gagneux, P., 2012. Multifarious roles of sialic acids in immunity. Ann. N. Y. Acad. Sci. 1253, 16–36.

Varki, N.M., Varki, A., 2007. Diversity in cell surface sialic acid presentations: implications for biology and disease. Lab. Invest. 87, 851–857.

Varki, A., 1997. Sialic acids as ligands in recognition phenomena. FASEB J. 11, 248–255.

Villar, E., Barroso, I.M., 2006. Role of sialic acid-containing molecules in paramyxovirus entry into the host cell: a minireview. Glycoconj. J. 23, 5–17.

Vimr, E.R., Kalivoda, K.A., Deszo, E.L., Steenbergen, S.M., 2004. Diversity of microbial sialic acid metabolism. Microbiol. Mol. Biol. Rev. 68, 132–153.

Vimr, E.R., 1994. Microbial sialidases: does bigger always mean better? Trends Microbiol. 2, 271–277.

Weigl, J.A., Puppe, W., Meyer, C.U., Berner, R., Forster, J., Schmitt, H.J., Zepp, F., 2007. Ten years' experience with year-round active surveillance of up to 19 respiratory pathogens in children. Eur. J. Pediatr. 166, 957–966.

Weis, W., Brown, J.H., Cusack, S., Paulson, J.C., Skehel, J.J., Wiley, D.C., 1988. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. Nature 333, 426–431.

Wilks, S., de Graaf, M., Smith, D.J., Burke, D.F., 2012. A review of influenza haemagglutinin receptor binding as it relates to pandemic properties. Vaccine 30, 4369–4376.

Willmarth, N.E., Baillo, A., Dziubinski, M.L., Wilson, K., Riese 2nd, D.J., Ethier, S.P., 2009. Altered EGFR localization and degradation in human breast cancer cells with an amphiregulin/EGFR autocrine loop. Cell Signal. 21, 212–219.

Xia, B., Royall, J.A., Damera, G., Sachdev, G.P., Cummings, R.D., 2005. Altered O-glycosylation and sulfation of airway mucins associated with cystic fibrosis. Glycobiology 15, 747–775.

Zhang, H., 2008. Concerns of using sialidase fusion protein as an experimental drug to combat seasonal and pandemic influenza. J. Antimicrob. Chemother. 62, 219–223.