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Sialidases: structures, biological significance and therapeutic potential

[Review article]
Garry Taylor
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Abstract

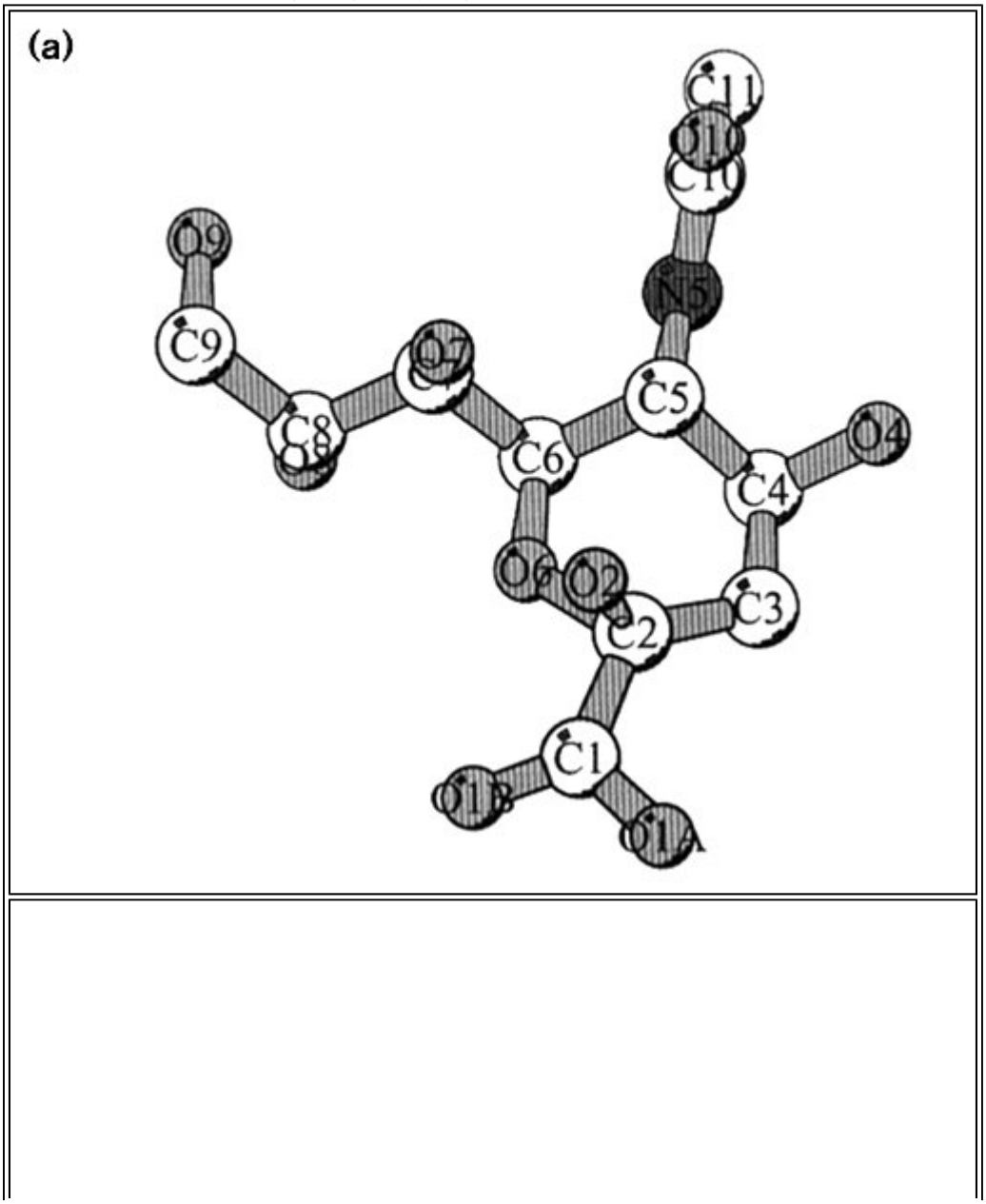
The structure-based design of a potent inhibitor of the influenza-virus neuraminidase (sialidase) is one of the outstanding successes of rational drug design. Recent clinical trials of the drug have stimulated many companies to seek a share of the potentially huge flu market. Sialidases, however, are involved in the pathogenesis of a whole range of other diseases, so perhaps the knowledge and expertise gained from the influenza story can be used in the design of other drugs, given that they all share certain structural features.

Abbreviations

F—fusion protein;
HN—hemagglutinin-neuraminidase;
Neu5Ac—*N*-acetylneuraminic acid;
Neu5Ac2en—2-deoxy-2,3-dehydro-Neu5Ac;
TCTS—*Trypanosoma cruzi* TS;
TS—trans-sialidase.

Introduction

Sialidases, or neuraminidases (the terms are equivalent) catalyze the removal of sialic acid from various glycoconjugates. In animals, sialidases have been located in several tissues, where the enzymes fulfil various roles by regulating the surface sialic acid profile of cells [1]. Such regulation is required for various functions, such as the immune system [2], dictating the half lifes of circulating cell [3], and apoptosis [4•]. The term 'sialic acid' is a generic term for a large family (~40 members) of naturally occurring analogues of *N*-acetyl neuraminic acid (Fig. 1). The appearance of various analogues is correlated with cell type, cell age, tissue type and species, with some analogues protecting glycoconjugates from attack by sialidases [5]. Thus the balance of sialic acids and sialidases is used to tune finely many biological phenomena in animal cells.



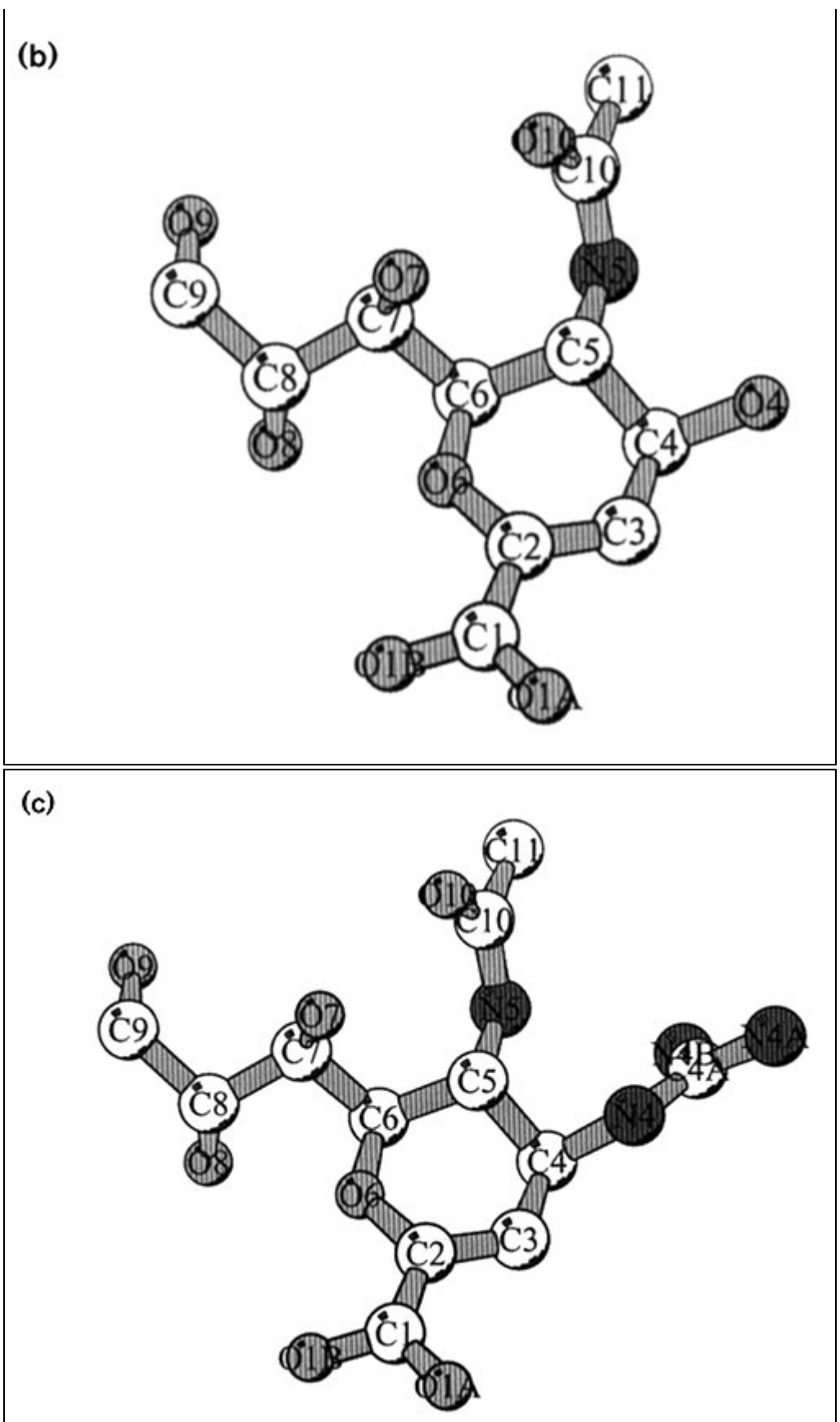


Figure 1 Ball and stick models of **(a)** Neu5Ac, **(b)** Neu5Ac2en, and **(c)** 4-guanidino-Neu5Ac2en

(GG167).

Return to text reference [1] [2] [3]

The location of sialic acids at the termini of various carbohydrate complexes associated with animal cells is exploited by a broad spectrum of microbial pathogens. Certain pathogens have proteins that recognize sialic acid for cell attachment, and many of these pathogens have acquired sialidases to aid in their pathogenesis and/or nutritional requirements.

This review considers the range of diseases in which sialidases are virulence factors, recent structural studies on bacterial sialidases, progress with the influenza-virus neuraminidase drug, and the potential for structure-based drug design based on sialidases to aid the treatment of other diseases.

Sialidases in disease

Table 1 shows the range of diseases in which sialidases (NAs), trans-sialidases (TSs) or hemagglutinin-neuraminidases (HNs) are implicated as virulence factors.

Table 1. Disease and pathogens for which sialidases (NAs), TSs or HNs have been implicated as virulence factors.

Microorganism	Disease	Virulence factor
Orthomyxoviruses		
Influenza virus type A	Influenza of humans, birds, horses, seals, pigs, whales	NA
Influenza virus type B	Influenza of humans	NA
Paramyxoviruses		
Parainfluenza viruses	Respiratory disease of humans	HN
Newcastle disease virus	Respiratory disease of chickens and other birds	HN
Mumps virus	Mumps	HN
Sendai virus	Murine parainfluenza	HN
SV5 virus	Canine parainfluenza	HN
Bacteria		
Clostridia	Gas gangrene, peritonitis	NA
Streptococcus	Septicaemia, pneumonia, meningitis, glomerulonephritis, periodontal disease	NA
Pneumococcus	Septicaemia, haemolytic-uraemic syndrome	NA
Bacteroides	Septicaemia, peritonitis	NA
Actinomyces	Periodontal disease	NA
Corynebacteria	Septicaemia	NA
Enterococcus	Peritonitis	NA
Escherichia	Peritonitis	NA
Vibrio cholerae	Cholera	NA
Pasteurella	Septicaemia, respiratory disease	NA
Pseudomonas aeruginosa	Cystic fibrosis	NA
Helicobacter pylori	Gastritis	NA
Bacteriophage		
K1E and K1F	'Meningitis' (see text)	NA
Parasites		
Trypanosoma cruzi	Chagas disease	TS
Trypanosoma brucei	African sleeping sickness	TS
Trypanosoma congolense	African disease of animals	TS
Trypanosoma rangeli	Non-pathogenic in vertebrates	NA
Trypanosoma vivax	African disease of animals	NA
Cryptosporidium parvum	Human gastrointenstinal disease	TS

Eimeria tenella	Diarrhoeal disease of chickens	TS
Pneumocystis carinii	Pneumonia	TS
Tritrichomonas mobilensis	Colonic parasite of squirrel monkeys	NA

Return to table reference [1]

Sialidases transfer carbohydrate-linked sialic acid to water, but certain parasites possess TSs that transfer carbohydrate-linked sialic acid to other carbohydrates. These TSs also tend to act as poor sialidases, but are distinct from the sialyl transferases found in the Golgi, which transfer nucleotide-linked sialic acids to growing glycoconjugates. Paramyxoviruses possess sialidase activity in the dual-function HN molecule.

Viruses

The influenza type A and B viruses have two surface glycoproteins: hemagglutinin, which recognizes sialic acid for attachment but is also involved in the fusion of viral and cell membranes, and neuraminidase. The role of the neuraminidase is to process progeny virus particles when they bud from an infected cell, removing viral sialic acids to halt self-agglutination of viruses. The neuraminidase forms tetramers on the viral surface, anchored by N-terminal transmembrane regions.

The paramyxoviruses also have two surface glycoproteins: the fusion protein (F), which is involved in the fusion of viral and host cell membranes, and HN, which both recognizes sialic acid for cell attachment and also cleaves sialic acid. There is evidence that both F and HN are required for fusion [6] [7]. Human parainfluenza viruses, one of the major causes of infant respiratory disease, have several serotypes, making vaccination an unattractive route, in contrast to the mumps virus, for which an effective vaccine exists. The HN molecule also forms on the viral surface tetramers comprising two disulphide-linked dimers (in most strains), anchored via N-terminal transmembrane segments [8]. No sequence similarity between the influenza and paramyxovirus enzymes is evident, although modeling has suggested that they share a common fold [9].

Bacteria

Sialidases are produced by a wide range of bacteria, and are often one of several virulence factors secreted by bacteria [10]. Many pathogenic and nonpathogenic sialidase-producing bacteria can use sialic acid as a carbon and energy source, and possess both permeases to transport the sugar inside the cell, and a cascade of enzymes for its catabolism. In certain cases the enzyme also has a defined role in disease. For example, *Vibrio cholerae* sialidase removes sialic acid from higher order gangliosides to create G_{M1}, the binding site for cholera toxin [11].

Corfield [10] has provided an excellent review of bacterial sialidases, and recent papers have reported sialidases as virulence factors in important diseases [12•] [13]. Bacterial sialidases vary in size from 40 kDa to 120 kDa. Most exist as monomers, but higher oligomeric states have been reported. Most are secreted as soluble proteins, others are tethered to the bacterial surface (e.g. in *Streptococcus pneumoniae* [14]), and some are not secreted (e.g. the small sialidase of *Clostridium perfringens*, which also secretes a larger, quite distinct sialidase [15]). The bacterial sialidases share little sequence identity, typically 30%, but contain two conserved sequence motifs: the first is the RIP/RLP motif (Arg–Ile/Leu–Pro), the second is the Asp–box motif (Ser/Thr–X–Asp–[X]–Gly-X–Thr–Trp/Phe; where X represents any amino acid), which can occur several times along the chain [16].

Parasites

Many trypanosome species possess TSs on their surfaces. The appearance of the enzyme is developmentally regulated, such that in *Trypanosoma cruzi* the enzyme shows highest activity in the trypomastigote stage of the parasite — the infective host form [17]. In contrast, for *Trypanosoma brucei*, the TS is more active in the insect form of the parasite [18]. The *T. cruzi* TS (TCTS) is a fascinating molecule. TCTS is part of a large gene family, some members of which do not have any enzyme activity [19]. TCTS is involved in cell attachment, which may be the role of enzymatically inactive members [20]. The enzymatically active members transfer sialic acid from host cells to the parasite's own surface glycoproteins, the parasite being unable to synthesize sialic acid itself. This sialylation appears to be important for cell invasion and evasion of the immune system.

TCTS is a multidomain protein: a catalytic N-terminal domain (containing an RIP motif and two Asp

boxes) is followed by one or two other domains, one of which may have a fibronectic type III fold, which in turn is/are followed by a 12-amino-acid motif that is repeated 44 times before a GPI anchor links the molecule to the parasite's surface [21]. In the trypomastigote form, TCTS appears to form a trimeric structure through association of the repeat sequences. TCTS has a high substrate specificity for sialic acid linked α 2 → 3 to β-galactose [22]. Finally, TCTS is also shed from the parasite surface in a soluble form, and recent studies have shown that this form enhances virulence via inflammatory cells [23•]. Trans-sialidases and sialidases have been found in several *Trypanosoma* species, but not in many other parasites, such as *Leishmania* [24•] and *Plasmodium falciparum* [25].

Bacteriophage

Several pathogenic bacteria and various tumour cells express poly-α 2 → 8-linked sialic acid on their surfaces. One such bacterium is *Escherichia coli* K1, which can cause high mortality rates in cases of neonatal meningitis. An endosialidase that binds to and hydrolyzes such polysialic acid substrates has been isolated from bacteriophage K1E [26] and K1F [27]. The enzyme is a trimer of 74 kDa monomers that contain two Asp boxes. The enzyme may find a use in the diagnosis and therapy of K1 meningitis.

Sialidase structures: the sialidase superfamily

The structure of the influenza-virus neuraminidase was elucidated in 1983 [28]. This structure revealed a tetrameric association of identical monomers whose fold has been described as a superbarrel or β propeller. Each monomer is made from six four-stranded antiparallel β sheets arranged as the blades of a propeller around a pseudo sixfold axis (Fig. 2). Structural studies on enzymes from influenza A virus N2, N6, N8 and N9 subtypes, as well as from two influenza B viruses, have revealed a structure that is well conserved, despite sequence identities down to 40%. The active site is highly conserved, and presents a rigid catalytic centre [29].

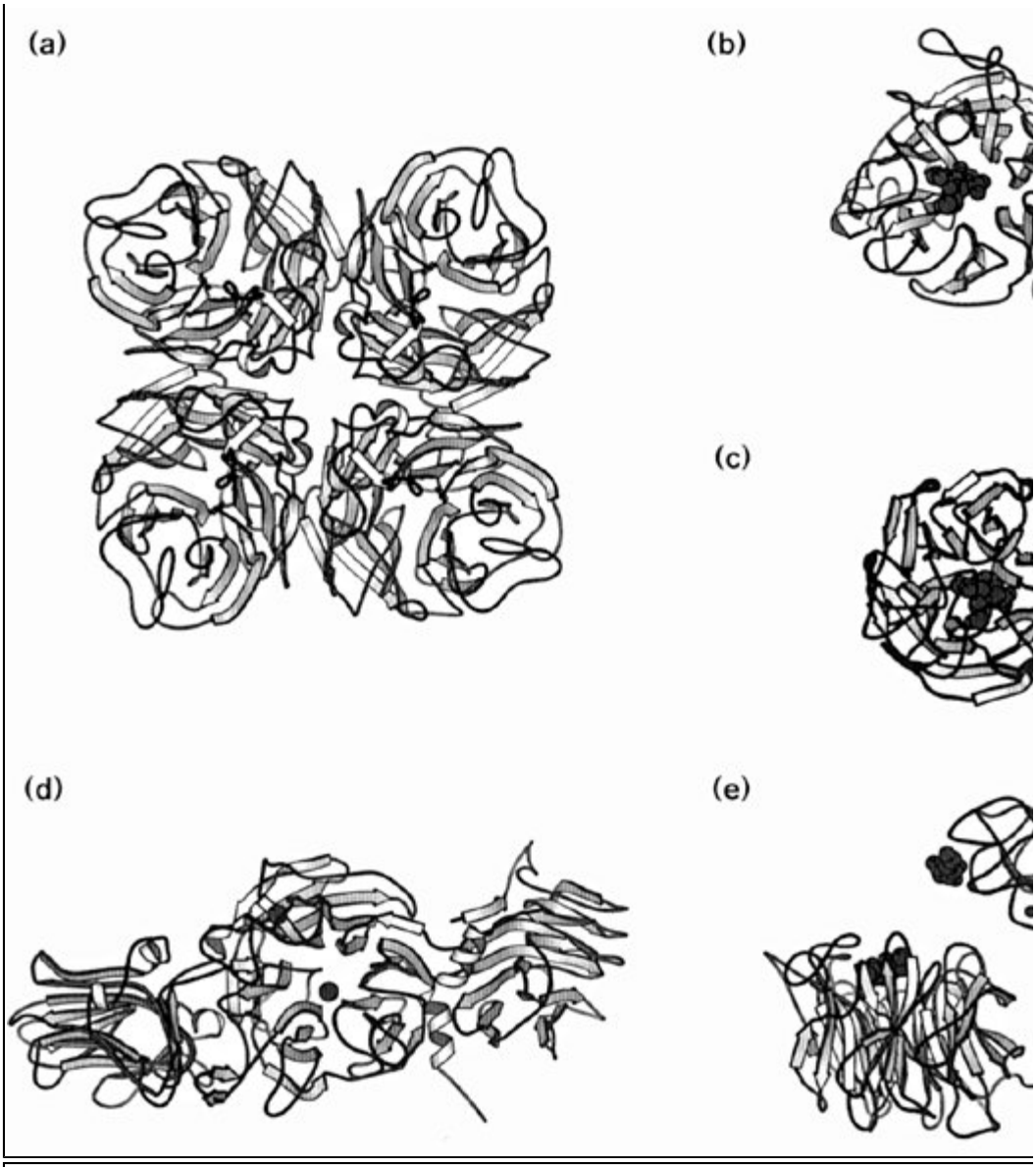


Figure 2 The sialidase superfamily. **(a)** Influenza virus neuraminidase. **(b)** *S. typhimurium* sialidase wi bound [30] [53]. **(c)** *Micromonospora viridifaciens* sialidase, 42 kDa form with Neu5Ac2en bound [32•• *cholerae* sialidase, showing the two lectin-like domains flanking the catalytic domain [31]. **(e)** Side view to the other sialidases, of *Micromonospora viridifaciens* sialidase 68 kDa form, showing the two addition one with an immunoglobulin-fold (right) which connects to a galactose-binding domain (above). Neu5Ac galactose are shown bound to the protein [32••].

Return to text reference [1] [2]

Recent studies on several bacterial sialidases have revealed a superfamily of multidomain enzymes built around the canonical catalytic β-propeller fold (Fig. 2) [30] [31] [32••]. Where additional domains are present, these appear to be involved in carbohydrate recognition, and suggest methods of targeting the enzyme to specific environments and substrates.

The sequence identity between the influenza enzymes and the bacterial enzymes is very low, at 15%, and even between the nonviral enzymes the sequence identities are only 30%. Nevertheless, the topology of the catalytic domain is conserved, and the active sites share many features (Fig. 3). The structural studies on the bacterial sialidases have revealed the location of sequence fingerprints that allow identification and modeling of other nonviral sialidases. The RIP/RLP motif contains one of the arginines that interact with the sialic acid carboxylate group. The Asp-box motif appears in topologically identical positions in the β propeller, and may be involved in secretion, as regularly spaced acidic residues have been implicated in secretion in other systems [33]. Influenza virus neuraminidases do not possess Asp boxes, but have an REP motif that corresponds to the RIP motif. All nonviral sialidase sequences reported possess one or more Asp boxes, which allows location of the

β propeller in the various sequences. However, the low sequence identities and relatively high structural deviations of the sialidases (root mean square deviation of C_αs from 1.7 Å to 3.8 Å) makes homology modeling of other sialidases quite challenging.

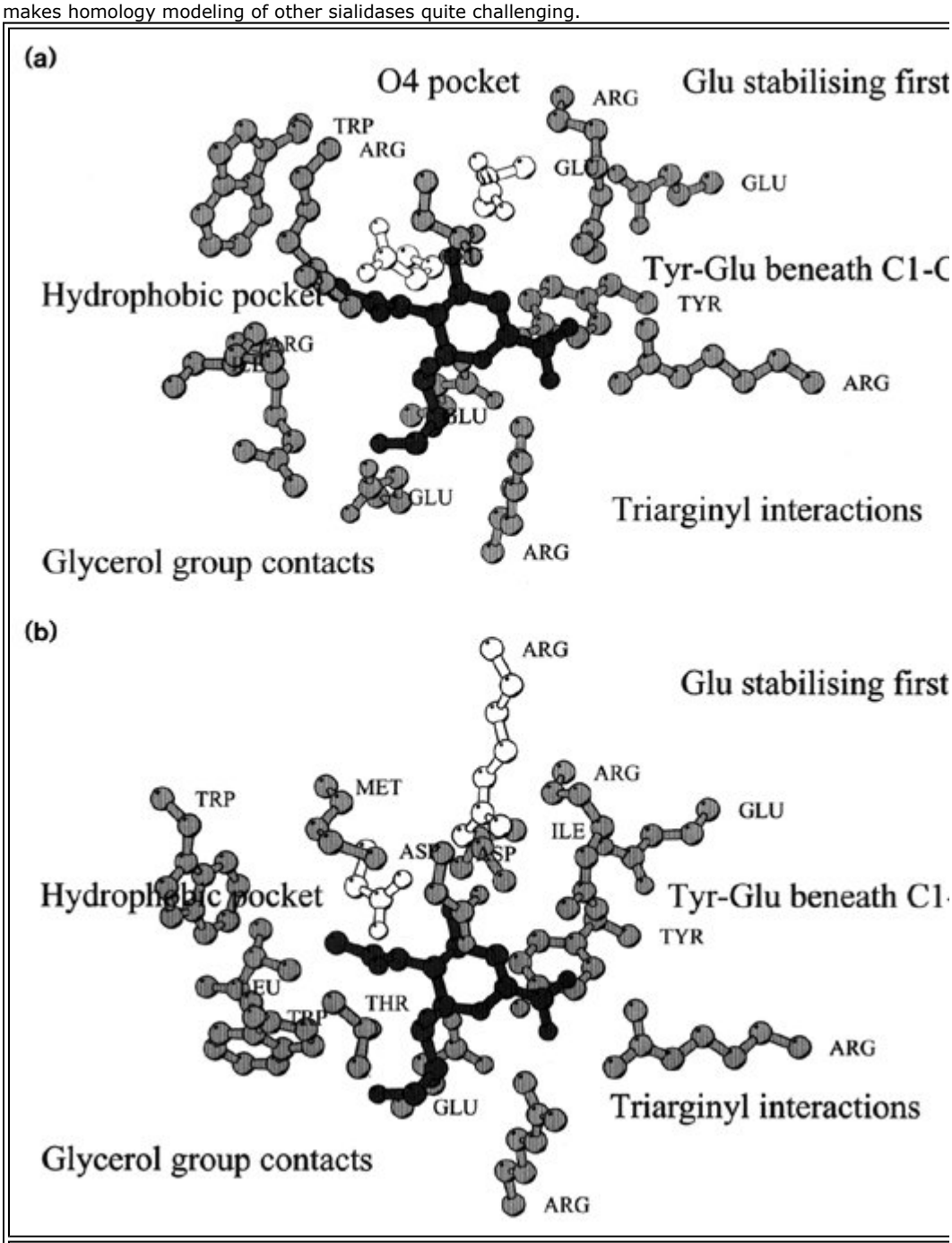


Figure 3 Viral and bacterial active sites with Neu5Ac2en bound (shown in black). **(a)** Influenza virus ne active site. The two conserved glutamic acids that line the pocket close to O4 on the inhibitor are shown *S. typhimurium* sialidase active site. The arginine and aspartic acid that interact with O4 on the inhibito are conserved across many bacterial and mammalian sialidases, are shown in white. There are similarit these structures: (i) the triarginyl cluster that binds to the sialic acid carboxylate group, and a glutamic stabilizes the first arginine; (ii) a tyrosine and glutamic acid under the C1–C2 bond, which are believed oxocarbonium ion intermediate; (iii) an aspartic acid that sits on a loop above the sugar ring, and which stabilize the water of hydrolysis; and (iv) a hydrophobic pocket that accommodates the *N*-acetyl group. this pocket and the extent of interactions with the glycerol group vary among the sialidases.

Return to text reference [1] [2]

The active sites of the sialidases were identified crystallographically through complexes with the inhibitor 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en, Fig. 1), discovered in the 1970s

[34], which inhibits most sialidases with a K_i of ~10^{-6} M. This complex is thought to represent a transition-state analogue, with its ring in a half-chair conformation. *N*-acetylneuraminic acid (Neu5Ac) is also a weak inhibitor of the influenza enzyme with a K_i of ~10^{-3} M, and has been observed crystallographically. The active sites share many similarities, yet also exhibit differences that reflect differences in kinetics (turnover of bacterial sialidases is typically 1000 times that of the influenza enzyme), binding affinities (bacterial enzymes do not bind Neu5Ac) and substrate preferences (many bacterial enzymes are promiscuous in their choice of sialyl conjugate, whereas the flu enzyme prefers α2 → 3 linkages over α2 → 6 linkages).

The bacterial sialidase structures have already provided as explanation as to why a single tyrosine differentiates between the active and inactive members of the *T. cruzi* TSs [35•]. The critical mutation is a Tyr → His: from sequence alignments of the TSs with the bacterial enzymes, the tyrosine is the one associated with a glutamic acid close to the C1–C2 bond of Neu5Ac in the active site.

Drug design

Analysis of the active site of the influenza enzyme, computationally and by eye, revealed a pocket close to O4 of sialic acid lined by two glutamic acid residues, which are conserved across all known strains of influenza A and B viruses. Substitution of the O4 hydroxyl on Neu5Ac2en with a guanidinium group produced an inhibitor, named GG167 by Glaxo (Fig. 1), which has a K_i approaching ~10^{-11} M [36]. GG167 is specific for the influenza enzyme, being a poorer inhibitor of nonviral sialidases and paramyxovirus HNs than Neu5Ac2en [37]. This can be understood in structural terms, as most nonviral sialidases have a conserved arginine and aspartic acid that fill the pocket near O4, and indeed hydrogen bond to O4 (Fig. 3).

GG167 has been shown to be effective in human trials, both as a prophylactic and as an early treatment [38••]. In contrast, the drug is ineffective against a highly pathogenic influenza virus of chickens [39•] [40•]. The kinetics of GG167 binding have revealed a slow 'on' rate [41], which has been interpreted in terms of the need to remove water from the active site. The binding of GG167 to influenza-virus neuraminidase has been studied crystallographically [42••], confirming the predicted binding mode. Details of the use of the program GRID to design inhibitors by probing the active site for ligand-binding 'hot-spots' have been reported [43••]. Variations on GG167 have shown the importance of the *N*-acetyl and glycerol moieties in binding [44] [45]. Other novel ligands, based on benzoic acid, have revealed the importance of the half-chair ring configuration of the sugar and the axial/equatorial positioning of the ring substituents [46••] [47] [48] [49•]. Placing a guanidinium group on the flat chemistry of benzoic acid is simply not subtle enough!

Turning to escape mutants, the worry that the slippery influenza virus would mutate its neuraminidase gene to escape the pressure of GG167 has been born out [50••] [51••]. The virus mutates Glu119 (the E of the REP motif), one of the two glutamic acids in the O4 pocket, to a glycine, making the enzyme 200-times less sensitive to GG167. Such a change was anticipated with the structures of the bacterial sialidases, which have an isoleucine or leucine at this position (the RIP/RLP motif). Such a mutation has yet to be seen in human hosts, but remains a concern for the long-term effectiveness of GG167. Why has the virus maintained two glutamic acids, that have not been assigned any catalytic function? Glu119 forms a salt bridge with a conserved arginine in the influenza enzyme, thus suggesting an important structural role. However, the structure of the Glu → Gly mutant has been reported, and shows no significant change in the fold of the enzyme [50••]. In contrast, the mutant neuraminidase has been seen in the electron microscope to preferentially form monomers or dimers, rather than tetramers (WG Laver, personal communication).

Very little has been reported on structure-based ligand design for other sialidases. *Salmonella typhimurium* sialidase, although of no pathogenic interest, has been used as a model for ligand design [52]. Recent high-resolution crystallographic analyses of the binding of Neu5Ac2en and of two phosphonate derivatives of Neu5Ac to the *S. typhimurium* enzyme have been reported [53], complementing similar studies on the influenza enzyme [54].

Other targets for drug design

Do sialidases represent good targets for similar structure-based drug design in other diseases? I believe so.

Parasites

One of the most exciting prospects is the design of an inhibitor for the TCTS. Chagas disease is

endemic in Latin America and is associated with no effective drug therapy. The TS is an excellent target: it is present in greatest amounts in the infective trypomastigote form of the parasite; it appears to be critical for cell attachment and immune system evasion; and it has very specific substrate requirements. The TS is probably not such a good target in other trypanosomes, where the enzyme is more prevalent in the insect form of the parasite. *Cryptosporidium parvum* has a TS and, being a major cause of pneumonia in AIDS sufferers, might elicit support from that perspective.

Viruses

The HN of human parainfluenza viruses is another good target. These viruses are a major cause of respiratory disease in infants, and no effective vaccines are available. In addition, Newcastle disease virus is a devastating pathogen of fowl, and may be an economically viable target.

Bacteria

Bacteria offer, as yet, no clear examples of where inhibition of secreted sialidases would halt a disease. However, bacterial sialidases are clearly important virulence factors and much more analysis needs to be carried out. One obvious requirement is specific, high affinity inhibitors with which to block the sialidases in order to assess their importance. It is wrong to assume that Neu5Ac2en is a good inhibitor (K_i ~10^{-6} M) of all sialidases, as it inhibits *S. typhimurium* sialidase with a K_i of ~10^{-4} M, and indeed no TSs are inhibited by this compound.

Animals

Very little has been said of the human enzymes in this review, as nothing is known of their structures. Many sialidases have been located in various human tissues, however, and some have been sequenced and shown to contain the RIP/Asp-box motifs. As we come to understand better their roles in the control of various functions, they too might become targets for inhibitor design.

Conclusions

The design of GG167 was in some ways wonderfully simple, and benefited from three factors: the availability of an excellent 20-year old lead compound (Neu5Ac2en); the availability of the atomic structure of the 'flu enzyme'; and the conservation of a charged binding pocket. Subsequent attempts by others to design novel compounds have shown how subtle the chemist has to be in design. The marketing of GG167, or its successors, will be watched with interest, as the cost of its use in prophylaxis compared with an annual, one-shot vaccination will be high. It is hard to be sure that the drug will protect against any possible mutant flu virus that comes along, but that will certainly be one of its selling points.

The investment and concentration of effort into the design of influenza-virus neuraminidase inhibitors seems to have paid off, and will be of enormous benefit to the design of inhibitors for other sialidases. The common structural core together with the subtle differences in the active sites gives hope for disease-specific inhibitors. Structural determinations of several of the target sialidases discussed above are in progress in several laboratories, including mine. The hope is that some of the diseases that are not so attractive to drug companies, because they are of the 'developing world' or have too small a market, will benefit from the investment in influenza. Perhaps when the general practitioner prescribes a long, expensive course of treatment for influenza, he or she will see it as investment in ligand design for other sialidase-linked diseases!

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Author Contacts

G Taylor, School of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK; e-mail: g.l.taylor@bath.ac.uk.

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