Early Career Research Award

Development of inhibitors as research tools for carbohydrate-processing enzymes

Tracey M. Gloster[1]
Biomedical Sciences Research Complex, University of St. Andrews, North Haugh, St. Andrews, Fife KY16 9ST, U.K.

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Tracey Gloster

Abstract

Carbohydrates, which are present in all domains of life, play important roles in a host of cellular processes. These ubiquitous biomolecules form highly diverse and often complex glycan structures without the aid of a template. The carbohydrate structures are regulated solely by the location and specificity of the enzymes responsible for their synthesis and degradation. These enzymes, glycosyltransferases and glycoside hydrolases, need to be functionally well characterized in order to investigate the structure and function of glycans. The use of enzyme inhibitors, which target a particular enzyme, can significantly aid this understanding, and may also provide insights into therapeutic applications. The present article describes some of the approaches used to design and develop enzyme inhibitors as tools for investigating carbohydrate-processing enzymes.

Introduction

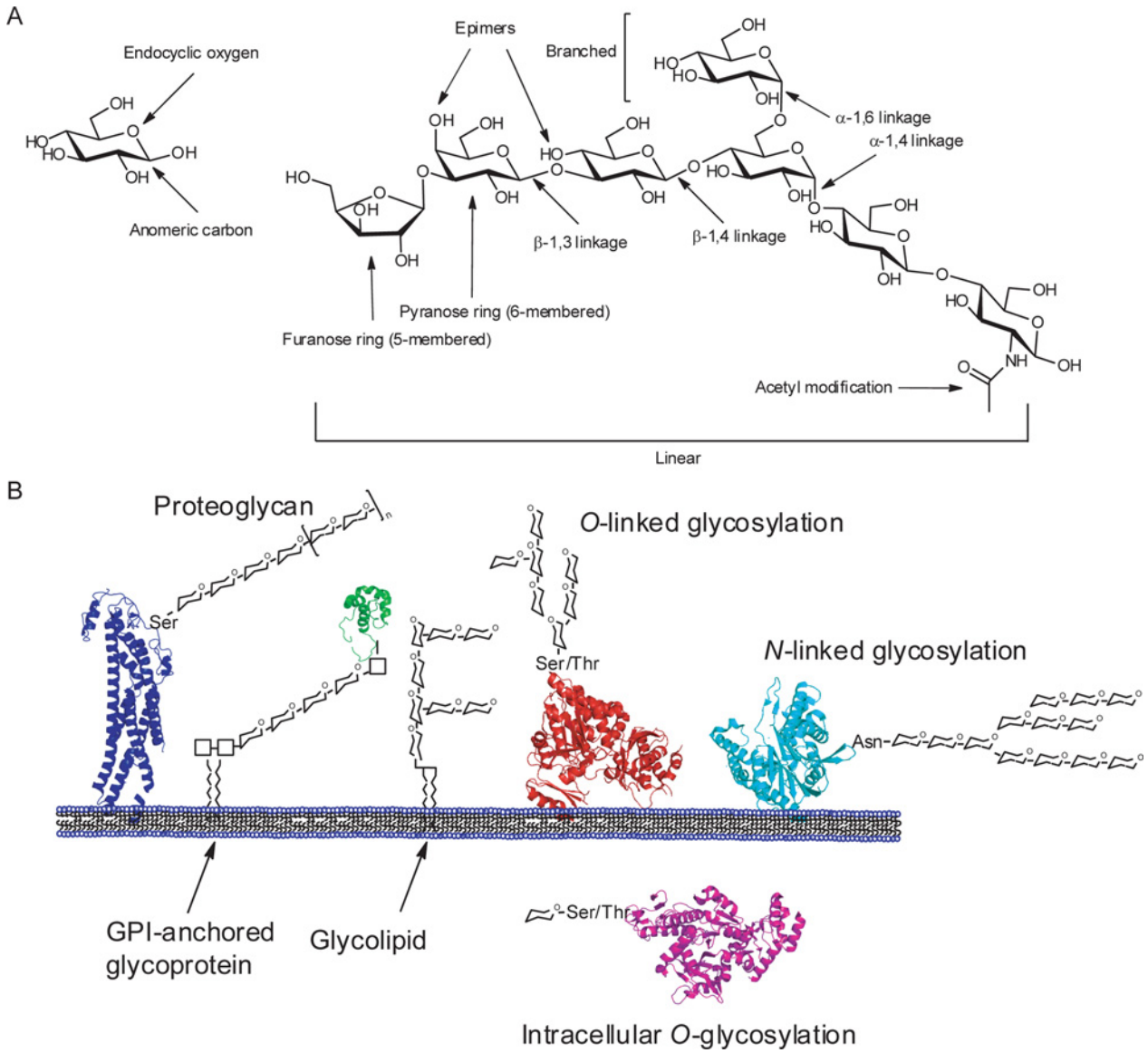
Carbohydrates, described as the "last frontier of molecular and cell biology" [1], are ubiquitous biomolecules found throughout all kingdoms of life. Although carbohydrates are commonly perceived as being simply a source of nutrition, they actually perform a vast number of roles in biological processes. Glycans, oligo- or poly-saccharides that are conjugated to proteins or lipids, decorate the surface of cells and comprise much of the extracellular matrix. Glycans are important structurally to aid tissue integrity and organization, as well as to play a protective role. Perhaps less considered is the role that glycans play in communication, binding and the immune response. Many different glycans on the cell surface facilitate cell–cell interactions, recognition and adhesion, whereas others act as receptors for various viruses, bacteria, pathogens and toxins [2]. Such an assortment of properties for glycans means that they are involved in biological processes as diverse as signalling during fertilization and development [3,4], maintaining protein quality control and localization [5], and aiding biomechanical strength in cartilage [6].

One of the most striking features of glycans is their diversity, not only in function, but also in composition. At the molecular level, it has been calculated that a hexasaccharide has, theoretically, 10^{12} different isomeric possibilities [7]. This can be introduced not only from chemical diversity, but also from different epimers at each chiral centre, ring size, anomeric configuration and linkage position, as well as the degree of branching (Figure 1A). Although, in reality, the number of glycans found to date in Nature does not extend as far as the theoretical values, it is worth considering the different types and localization of glycosylation observed in eukaryotic systems. Glycosylation of proteins is the most common form of a post-translational modification. This occurs most often on asparagine residues (N-glycosylation, found at the consensus sequence Asn-Xaa-Ser/Thr) or serine or threonine residues (O-glycosylation), although other rarer forms on other residues have been observed. Glycans can also be conjugated to lipids and can form part of glycophosphatidylinositol anchors (Figure 1B). The majority of glycoconjugates are extracellular, with synthesis originating in the endoplasmic reticulum or Golgi apparatus, before being exported to the cell surface or secreted. There are exceptions, however, where glycosylation is intracellular; one example is the O-linked modification of nuclear and

Key words: carbohydrate-processing enzyme, glycan, glycoside hydrolase, glycosyltransferase, inhibition.
Abbreviations used: GH, glycoside hydrolase; GT, glycosyltransferase; ITC, isothermal titration calorimetry; MS, mass spectrometry; OGT, O-linked *N*-acetylglucosamine transferase.
[1]**email tmg@st-andrews.ac.uk**

Figure 1 | **The diversity of glycans**

(**A**) Illustration of the diversity in glycan structure at the molecular level using a fictitious glycan. The meaning of various terms used in the text is highlighted. (**B**) Types of glycosylation with the various linkages to proteins and lipids.



cytoplasmic proteins with a GlcNAc (*N*-acetylglucosamine) residue.

Achieving such levels of glycan complexity and diversity, however, is not trivial. Unlike DNA or proteins, glycan sequences are not genetically encoded and hence there is no 'template' that can be read. The structure of glycans is therefore controlled solely by the specificities and localization of the enzymes responsible for their synthesis and degradation, which typically make up 1–3 % of the genome of organisms [8]. GHs (glycoside hydrolases), the enzymes responsible for the degradation of glycoconjugates, catalyse hydrolysis of the glycosidic bond between a mono-, oligo- or poly-saccharide and a leaving group (which may be another saccharide, protein, lipid or small molecule). GTs (glycosyltransferases), on the other hand, are responsible for the assembly of glycoconjugates. These enzymes most commonly employ a sugar nucleotide as the substrate donor, and, in the presence of an acceptor, which may be a saccharide, protein, lipid or small molecule, catalyse transfer of the sugar to the acceptor. Deficiencies in or malfunction of many eukaryotic GHs or GTs have been implicated in a host of diseases [9]. As the function of individual carbohydrate-processing enzymes become established, and it is determined how the structures of glycans are thus influenced, the relationship with enzyme malfunction and disease can be validated and perhaps reveal further diseases or disorders correlated to malfunction of these enzymes.

Modulation of the activity of individual carbohydrate-processing enzymes can provide insightful information into its role in glycan processing and possible biological function. One way to do this is to affect protein expression of the enzyme at the DNA level, using gene-knockout or gene-overexpression methods, or at the RNA level using RNA interference technology, and the overall phenotypic effect on cells or organism can be observed. These methods tend to be an 'all-or-nothing' approach, which can be useful at aiding elucidation of the function of an enzyme and its role in the synthesis or degradation of various glycans, but are more limited when the absence of an enzyme is lethal to organism development. Small-molecule inhibitors of carbohydrate-processing enzymes are powerful complementary tools in this respect. Inhibitors are able to modulate the activity of an individual enzyme rather than the protein levels, and, with careful fine-tuning, can be carried out in a dose- and time-dependent manner, allowing enzyme function to be fully probed.

As with any approach, there are, of course, caveats to the use of small-molecule inhibitors [10]. Compounds need to be both potent and selective for the carbohydrate-processing enzyme being targeted. Inhibitors that have off-target effects on other enzymes or proteins are undesirable, and could complicate the interpretation of data resulting from supposed inhibition of the enzyme target. The greatest risk of off-target effects from rationally designed inhibitors almost certainly comes from inhibiting other carbohydrate-processing enzymes. As mentioned above, the structural diversity of glycans is vast, but arises from very subtle differences in, for example, configuration at chiral centres, linkages and/or branching. Carbohydrate-processing enzymes have thus evolved to handle these small variations in glycan structure with an impressive degree of specificity. Compounds that are used to inhibit these enzymes, however, need to be developed to achieve the same amount of specificity. Ensuring that a small-molecule inhibitor is taken up sufficiently into cells or an organism is another important consideration. The penetrance of the compound through cell membranes can be limited with highly polar or charged molecules. The accessibility of an inhibitor to the enzyme target thus needs to be established, and the potency in a cell or organism may not necessarily mirror *in vitro* measurements.

Inhibitors of carbohydrate-processing enzymes are not only invaluable as tools for probing enzyme function, but, in a number of cases, may be developed further therapeutically. Malfunction of GHs and GTs have been implicated in variety of diseases, including cancer [11], diabetes [12], lysosomal storage diseases [13], chronic inflammatory diseases [14], viral infections [15] and neurodegenerative disorders [16]. Specific enzymes can therefore be targets for drug design, and, in a small number of cases, this has successfully led to therapeutics in use or in clinical trials.

The creation and development of inhibitors of carbohydrate-processing enzymes is not trivial, and often the process relies on input from a number of different areas of expertise and techniques. Visualization of the overall architecture of the enzyme and fine details of the active site, using X-ray crystallography or NMR, can be useful for designing inhibitors that take advantage of optimal interactions with enzymic residues. Characterization of putative inhibitors *in vitro*, using techniques such as enzyme kinetics with an appropriate assay read-out or ITC (isothermal titration calorimetry), is required to assess binding potential. ITC can additionally be used to dissect the thermodynamic contributions to binding. It is important to establish potency against both the enzyme target of interest and related enzymes (or indeed unrelated enzymes) to ensure specificity. However, it is often the effect of the inhibitor on glycan structure and/or biological function that ultimately needs to be determined, which requires the use of cells and/or *in vivo* experiments. These have associated difficulties to ensure an appropriate and relevant dose is given, which is often a balance between evoking a response and ensuring no off-target effects. Effects on glycan structure can be monitored in a number of ways. Antibodies or lectins, which recognize specific glycan structures, can be used to analyse glycan content by immunoblots, immunocytochemistry or flow cytometry. MS (mass spectrometry) can indicate the composition of the glycan following enzymatic or chemical digestion. Labelling techniques, through the use of radioactively labelled substrates or modified substrates that are amenable to chemical ligation strategies [17], can also be used to monitor glycan composition *in situ.*

The present article explores the various approaches to design and analyse small-molecule inhibitors of both GHs and GTs. This is illustrated with examples of *in vitro* and *in vivo* methods for characterization of inhibitors of carbohydrate-processing enzymes, and how some of these inhibitors have been used to understand biological processes or developed therapeutically.

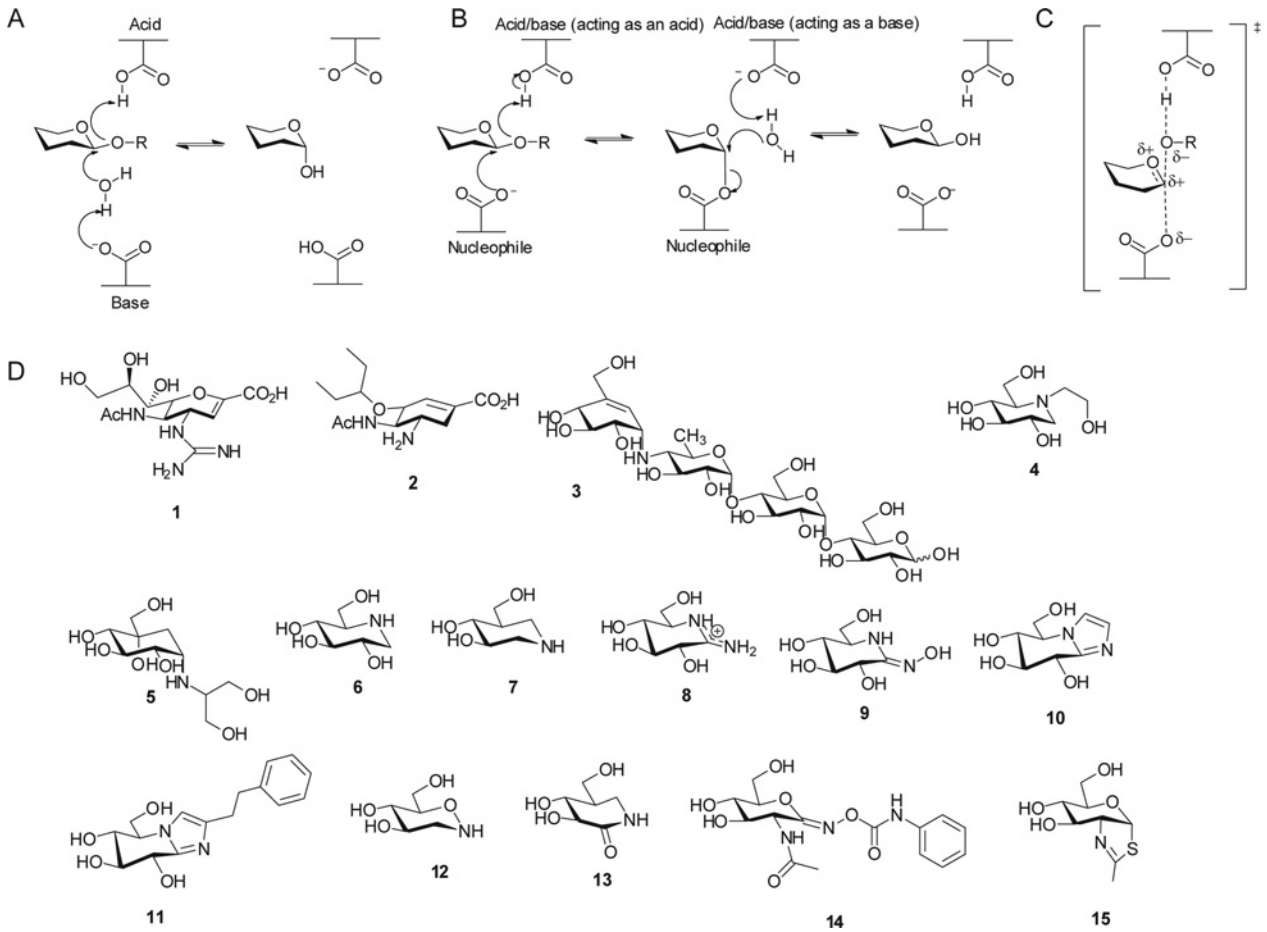
GHs from a mechanistic viewpoint

Rational design of enzyme inhibitors is largely dependent on knowledge of the substrate specificity and enzyme mechanism. As a greater understanding of the subtleties of various GH mechanisms has been gained, there has been a corresponding growth in the design and development of small-molecule inhibitors. The mechanisms for glycoside hydrolysis have been eloquently reviewed previously [18,19], and are described only briefly in the present paper.

The majority of GHs catalyse using one of two 'classical' mechanisms, which were first proposed by Koshland in the 1950s [20], and have been refined by others since [21,22]. So-called inverting enzymes catalyse with an inversion of stereochemical configuration at the anomeric centre (Figure 2A), and retaining enzymes catalyse with an overall retention of stereochemistry (Figure 2B). Both mechanisms typically involve two carboxylate-containing residues in the enzyme active site. Inversion of stereochemistry proceeds via a one-step mechanism, where one enzymic residue acts

Figure 2 | **GH mechanisms and examples of inhibitors**

(**A**) Glycoside hydrolysis with inversion of stereochemistry. (**B**) Glycoside hydrolysis with retention of stereochemistry. (**C**) Oxocarbenium ion-like transition state formed during glycoside hydrolysis. (**D**) GH inhibitors described in the text.



as a general acid and the other as a general base. During hydrolysis, there is concomitant protonation of the leaving group by the acid residue to aid leaving group departure and attack of a water molecule at the anomeric centre. The base acts to deprotonate the water molecule to provide a highly nucleophilic species. Retaining enzymes hydrolyse substrates using a two-step mechanism, consisting of two serial inverting steps, via a covalent glycosyl–enzyme intermediate. One enzymic residue acts as an general acid/base, and the other acts as a nucleophile. In the first step, the acid/base (acting as an acid) protonates the leaving group, and a nucleophile attacks at the anomeric carbon to form a glycosyl–enzyme intermediate. During the second step, the acid/base residue (now acting as a base) deprotonates a water molecule which attacks the intermediate and causes displacement of the glycoside. Some *exo*-acting (where only the terminal residue is cleaved) retaining sialidases, which hydrolyse sialic acid-containing substrates, use the conventional mechanism for hydrolysis, but with a tyrosine residue acting as the nucleophile in place of the traditional acidic residue. The tyrosine residue is able to relay charge from a nearby carboxy-group-containing residue in order to gain its nucleophilicity [23].

Over the last decade or so, a number of other mechanisms have been proposed for subsets of GHs which deviate from the classical mechanisms. Some enzymes that process substrates containing *N*-acetyl groups with retention of anomeric configuration use a substrate-assisted mechanism [24,25]. In these enzymes the acetamido group at the C2 position of the substrate can act as the nucleophile in place of a residue in the enzyme active site. Typically, two carboxy groups are conserved in the active site; one of these acts as the general acid/base to aid leaving group departure and deprotonate a water molecule, and the other orients and polarizes the acetamido group to facilitate nucleophilic attack. This leads to the formation of an oxazoline intermediate, which is broken down by the deprotonated water molecule. More recently, it has been proposed that *endo*-acting (where cleavage occurs within a oligo- or poly-saccharide chain) sialidases that hydrolyse with inversion of configuration may employ a novel substrate-assisted mechanism where the carboxy group in the sialic acid substrate acts as the general

base instead of an enzymic residue [26]. A few GHs evoke more unusual mechanisms. For example, some enzymes rely on NAD^+ as a cofactor, and proceed via a ketone intermediate [27], and very recently it has been suggested that certain *endo*-mannosidases may utilize their own substrate-assisted mechanism via an epoxide intermediate [10].

GHs can be classified into families by sequence similarity, and details of the members of each family can be viewed online in the CAZy database [28]. This database provides an excellent resource for the carbohydrate community, and serves as a useful prediction tool for mechanism and structure for related sequences. These families do not, however, classify enzymes by the substrate on which they act, and often a range of different substrates are found to be processed within any given family. There are currently 130 families, and, in almost all cases, the mechanism and overall three-dimensional fold of the enzymes within a family are conserved. There are, however, a couple of anomalies. For example, it has been shown that family GH97 contains members with primary sequence and three-dimensional structural homology, but which are capable of hydrolysing with either retention or inversion of stereochemistry [29–31]. GH97 enzymes have an unusual Ca^{2+} ion-dependence which may provide the environment to allow the required plasticity to accommodate both mechanisms, and evolutionary pressures have presumably meant that this is advantageous. An understanding of enzyme mechanism coupled with substrate specificity is an important influence in the rational design of inhibitors.

Transition-state mimicry in inhibitor design

Linus Pauling first proposed in the 1940s that, as an enzyme binds the transition state very tightly, then inhibitors which mimic features of the transition state should be highly potent [32,33]. Indeed, insightful experiments by Wolfenden et al. [34,35] have estimated the dissociation constant for the transition state formed during glycoside hydrolysis could be as low as 10^{-22} M. Huge efforts have therefore been made to incorporate features of the transition state into the development of GH inhibitors. It is highly debated whether a number of these inhibitors are 'true' transition-state mimics, whether they mimic the ground-state substrate or whether they form fortuitous interactions with the enzyme [36]. In most cases, if a potent and specific compound against the target enzyme has been identified, it does not necessarily matter. However, if the compound is being used as a tool to study enzyme mechanism, or as the basis for the development of better inhibitors, it is useful to know which features are important.

Great efforts have been made to deduce the finest details of the transition-state structure formed during glycoside hydrolysis, using a range of physical-organic techniques such as kinetic isotope effects and linear free energy relationships [37,38]. The transition state formed fleetingly during enzyme-catalysed hydrolysis possesses oxacarbenium ion character, with partially formed or broken bonds between the anomeric carbon and the glycosidic oxygen/attacking water molecule, and between the anomeric carbon and attacking nucleophile (Figure 2C). This means that the anomeric carbon has trigonal character, causing sp^2 hybridization predominately along the bond between the anomeric carbon and endocyclic oxygen. The imposition of the double bond character in the pyranose ring means that there is distortion from the relaxed chair conformation to a half-chair or boat conformation, and there is positive charge accumulation along the bond between the anomeric carbon and endocyclic oxygen, at, or close to, the transition state [39,40]. GH inhibitors tend to mimic either the transition-state charge or conformation, or both, and in addition may have appendages to aid binding to the active site and/or to mimic the leaving group.

Despite the number of processes that involve GHs *in vivo*, there are actually relatively few examples of inhibitors that are used as therapeutic agents. Examples of GH inhibitors used in the clinic demonstrate the power of mimicking features of the transition state to gain potency. Great efforts in recent years have been targeted towards the inhibition of viral sialidases, given the widely hyped outbreaks of various influenza strains. Sialidases play an important role in the life cycle of the influenza virus and assist in the spread of virus particles and progeny release. Structure-based drug design led to the development of Relenza (**1**; Figure 2D), a nasally inhaled subnanomolar inhibitor of the viral sialidase which reached the clinic [41]. Relenza possesses an endocyclic double bond, causing distortion towards the energetically unfavourable conformation found at the transition state. Following further development, the orally bioavailable drug Tamiflu (**2**; Figure 2D) became available in the clinic [42], and has been used worldwide during outbreaks of influenza. However, the emergence of viral strains with resistance to Tamiflu has necessitated the need for a continued drive for new sialidase inhibitors. Structural studies are again proving important in defining the molecular basis for the drug resistance [43], and will hopefully aid the design of new compounds.

Perhaps the most obvious disease to target by GH inhibition is Type 2 diabetes, especially given its increasing incidence in developed countries. Type 2 diabetes is characterized by poor absorption of glucose from blood, causing unhealthily high circulating blood glucose levels. One way to prevent these high glucose levels is to limit glucose uptake from the gut, which can be done by administering compounds that inhibit the GHs responsible for degrading ingested starch into glucose. Acarbose (**3**; Figure 2D), a natural product first isolated from a *Streptomyces* species, potently inhibits the pancreatic amylase and gut α-glucosidases and has been approved for the treatment of Type 2 diabetes for several decades [44]. Acarbose is a pseudo-tetrasaccharide containing a valienamine moiety at the non-reducing end. This group possesses endocyclic sp^2 hybridization which may mimic the distortion at the transition state. In addition, the presence of an exocyclic nitrogen atom may mimic the charge element of the transition state if protonated. The additional sugar units attached to the valienamine moiety promote binding with

other residues in the active site to improve potency. Structural studies have revealed that acarbose undergoes rearrangement and transglycosylation, and the true inhibitory species is in fact a pseudo-pentasaccharide [45]. Miglitol (**4**; Figure 2D) and Voglibose (**5**; Figure 2D) are pseudo-monosaccharides that target gut α-glucosidases, and are also widely in use as treatments for Type 2 diabetes [46,47]. Both contain a nitrogen atom that may be protonated; in Miglitol this is endocyclic, and in Voglibose this is exocyclic.

Assessing transition-state mimicry

A variety of complementary techniques can be used to establish which classes of compounds are good inhibitors of different GHs and for determining which features are important for potency. Others have gone a step further and have used different methods for determining whether certain inhibitors are true mimics of the transition state [36]. Structures of enzymes in complex with substrates and inhibitors can provide insights into the likely transition-state structure, as well as demonstrating important interactions with active-site residues and water molecules. Mechanistic and kinetic studies using various assaying methods can be used to assess enzyme activity or inhibition at different pH values or in ways to establish linear free energy relationships. Additionally, ITC is useful for determining the thermodynamic contributions to binding, and whether this can be optimized to increase potency.

The structural diversity of GH inhibitors has been reviewed in depth elsewhere [36,48–50], and in the present article only inhibitors used to illustrate particular studies or applications of different techniques are described. Rationally designed inhibitors of carbohydrate-processing enzymes nearly always incorporate features of the natural substrate, most commonly a number of polar hydroxy group substituents to maximize hydrogen-bonding interactions in the active site. Such compounds are often synthetically challenging to make, and can be difficult to manipulate in ways to introduce variation. Many inhibitors that have been studied are, or are based upon, natural products. These compounds have frequently provided a template for chemists, or in other cases have been discovered in Nature after being created in the laboratory first.

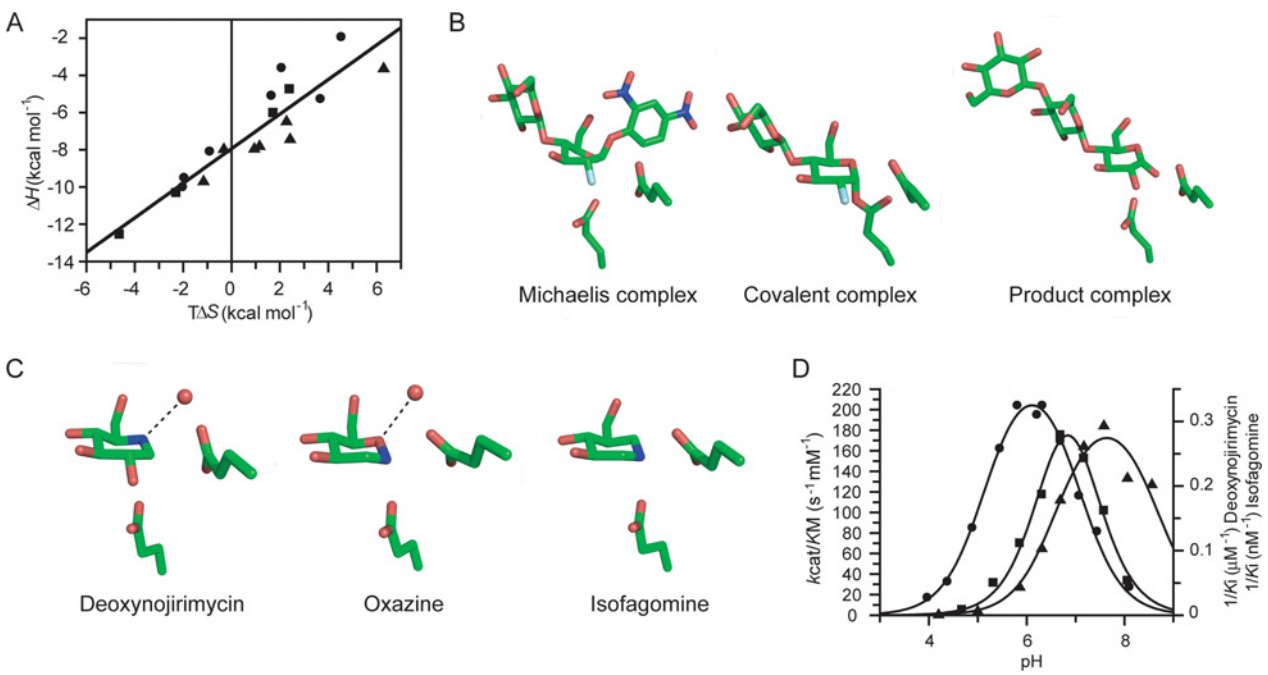
Compounds that mimic the charge at the transition state often have a nitrogen atom introduced into the pyranose ring at either the position of the anomeric carbon or endocyclic oxygen in the native glycoside, which may be protonated under physiological conditions. Examples of inhibitors in this class include deoxynojirimcin (**6**; Figure 2D), a natural product first synthesized in the 1960s [51], and isofagomine (**7**; Figure 2D) [52]. The shape of the transition state can be mimicked by introducing sp^2 hybridization into compounds, either within the pyranose ring or in a group appended at the anomeric carbon, endocyclic oxygen, or both, to cause distortion. Examples of such inhibitors include various derivatives of glycoamidines, such as glucoamidine (**8**; Figure 2D), and glycohydroximolactams, such as glucohydroximolactam (**9**; Figure 2D) [53,54]. Furthermore, elements of both the charge and shape from the transition state can be mimicked in glycoimidazoles, such as glucoimidazole (**10**; Figure 2D), where an imidazole group is fused to a pseudo-glycoside [55]. With each class of inhibitor, substituents can be incorporated to mimic the substrate leaving group and thus promote further interactions in the active site.

Assessing binding of inhibitors to GHs using ITC can be useful as it negates the need for synthetic substrates or kinetic assays, and additionally provides quantification of the thermodynamic parameters for the interaction. Wolfenden et al. [56] proposed that an enzyme's increased affinity for the transition state over the ground state is enthalpic in origin, and thus inhibitors mimicking the transition state should perhaps also bind with large enthalpic contributions. A study examining the thermodynamic properties of binding of 18 inhibitors to a β-glucosidase demonstrated that all bound with a favourable enthalpic contribution [57]. Further analysis of the binding characteristics of the inhibitors revealed, however, that there was no noticeable correlation between the potency and the thermodynamic parameters, and indeed inhibitors with similar properties, such as glucoimidazole (**10**; Figure 2D) and the same compound with a phenethyl substituent (**11**; Figure 2D), bound with very different enthalpic and entropic contributions to binding. The lack of predictability between related inhibitors is most likely to be a consequence of the effect of solvation. Although ITC quantifies the thermodynamic parameters upon binding, the number of water molecules co-ordinated to both the inhibitor and enzyme alone in solution which are displaced upon binding is also an important factor [58]. This is extremely difficult to determine experimentally, and insights into the solvation free energy term can only be gained using modelling approaches [59,60].

An enthalpy–entropy compensation plot with points representing each of the 18 compounds studied with a β-glucosidase showed a strong correlation with the line of best fit having a gradient close to 1, suggesting a large degree of compensation [57] (Figure 3A). Considering the laws of thermodynamics, the plot essentially shows that the most potent inhibitors lie below the best fit line, meaning they benefit from either a more favourable enthalpy or entropy term than the 'average', whereas the least potent inhibitors lie above the best fit line and display a more unfavourable enthalpic or entropic contribution to binding than 'average'. Enthalpy–entropy compensation is not unusual, although debate exists as to the origin of the effect [61]. It may be an effect derived from the intrinsic properties of water, where, for example, upon ligand binding, a favourable enthalpy suggests that a number of intermolecular interactions are formed, but with an associated entropic cost due to loss of configurational freedom in the system. Conversely, loss of bound water molecules contributes favourably to entropy due to disorder, but unfavourably to enthalpy as there is a loss of intermolecular interactions [62]. Such a strong compensation between thermodynamic parameters for the

Figure 3 | **Studying inhibition of GHs**

(**A**) Enthalpy–entropy compensation plot for 18 inhibitors with a single β-glucosidase [57] (●, charge-mimicking inhibitors; ■, shape-mimicking inhibitors; ▲, charge- and shape-mimicking inhibitors). The line of best fit through the points has a slope close to 1; the section above the line indicates inhibitors that are worse than the average, whereas those below are better than average. (**B**) Snapshots along the conformational itinerary of a cellulase to demonstrate the various conformations adopted by the glycoside, which can be used to inform about transition-state structure and subsequently guide inhibitor design [69]. The Michaelis (substrate-distorted) complex, covalent intermediate and product complex are shown. (**C**) Active-site representations of a β-glucosidase bound to deoxynojirimycin, oxazine and isofagomine [57,71]. Isofagomine is unable to co-ordinate a water molecule. (**D**) The profile for pH-dependence of catalysis for a β-glucosidase (●) differs from that of inhibition by deoxynojirimycin (■) and isofagomine (▲) [57,71].



compounds studied, however, means that there are few generalizations that can be made to predict which features can be incorporated into inhibitors to increase potency. However, as illustrated with examples below, small subtleties in thermodynamic parameters between related compounds, in conjunction with information from other techniques, can be used to inform the design of more potent inhibitors.

Structural studies of inhibitors bound to an enzyme of interest can be extremely powerful in establishing the important interactions that are or could be made with active-site residues and water molecules. There are numerous examples of GH structures bound to various compounds, and a number of these have provided very insightful observations about the design of inhibitors. High-resolution X-ray crystallographic data have provided valuable information about the protonation states of the inhibitor and the catalytic residues in the active site, which would not have necessarily been predicted on the basis of our knowledge of the catalytic mechanism [63,64]. Crystallographic studies, coupled with modified substrates and/or mutant enzymes, have also provided insights into the conformational path adopted by certain GHs, from binding of substrate through transition state(s) and intermediate(s) to the final products [65–67] (Figure 3B). These 'snapshot' studies, coupled with insightful interpretations of conformational itineraries for carbohydrates, allow prediction of the reaction co-ordinate, and likely transition-state structure [68,69].

Studies where a large number of inhibitors that differ structurally (but based on a carbohydrate scaffold) have been examined in complex with the same enzyme have, interestingly, shown that the interactions with active-site residues are nearly always conserved [57,70]. Three-dimensional structures can, however, reveal small differences to rationalize other observations. For example, comparison of the inhibitors deoxynojirimycin (**6**), isofagomine (**7**) and oxazine (**12**; Figure 2D) with a β-glucosidase shows that isofagomine is approximately 10-fold more potent than oxazine and 25-fold more potent than deoxynojirimycin [71,72]. Analysis of the thermodynamic parameters shows that oxazine has the most favourable enthalpy, whereas isofagomine has the most favourable entropic contribution, suggesting that it is the more important factor for binding. Examination of the active-site environment for each of the inhibitors in complex with the enzyme shows that

deoxynojirimycin and oxazine co-ordinate a water molecule with the nitrogen and oxygen atoms respectively found at the position of the endocyclic oxygen in a native glycoside (Figure 3C). The presence of the methylene group at the same position in isofagomine, however, means that water is excluded at this position, contributing to the higher entropic contribution to binding, and most probably the higher potency [71,72]. Observations of such subtleties can influence the design of more potent inhibitors. One surprising observation revealed by structures of inhibitor complexes was that the addition of functional groups, such as the phenethyl functional group to the glucoimidazole scaffold (**11**: Figure 2D), did not necessarily promote further interactions with active-site residues, despite significantly increasing the potency [73]. In some cases, these appendages show considerable flexibility, and the binding mode does not appear to be consistent even between related enzymes [57,73,74]. It is possible that the high potency derived from these compounds arises purely from the presence of a bulky group on the inhibitor, which displaces water molecules in the active site and contributes favourably to the entropy of binding (as reflected in the thermodynamic parameters by ITC) [57,73].

Analysis of enzyme activity and inhibition at different pH values can provide an indication of transition-state mimicry, although the interpretation is debated. A difference in the pH-dependence for catalysis and inhibition may suggest that an inhibitor cannot be a transition-state mimic, as, by definition, a catalytically inactive enzyme cannot bind the transition state [75]. Comparison of a number of inhibitors with a β-glucosidase showed that the pH profile for inhibition with very few inhibitors was mirrored in catalysis [57] (and only tended to occur when the pK_a of the inhibitor was below that of the acidic catalytic residue of the enzyme). It could be argued, however, that at the transition state, the nucleophile and acid/base residues would both be partially deionized, meaning that inhibitors with high potency at the pH where the enzyme is doubly deionized may be a better reflection of transition-state mimicry [76]. This was, in fact, the profile observed with a number of inhibitors (both for those which mimicked the charge and shape of the transition state) when tested with a β-glucosidase [57] (Figure 3D). Analysis of pH profiles for catalysis and inhibition, however, can be difficult to interpret [77] and should be made with caution. Atomic resolution crystallography has demonstrated unusually configured protonation states of catalytic residues upon inhibitor binding [63,64], and NMR measurements have shown how the pK_a of the catalytic residues can change during catalysis [78]. pH profiles for inhibition are therefore likely to reflect composites of the free enzyme, free inhibitor and the enzyme–inhibitor complex [77].

One quantitative way of investigating the ability of an inhibitor to mimic the transition state is through the use of linear free energy relationships, where a change in the chemical structure of a substrate and inhibitor or the environment in the enzyme should have an equal effect on the enzyme rate enhancement and the affinity of the inhibitor relative to the substrate [79]. Such studies tend to be conducted in 'model' systems, which are usually bacterial enzymes that can be obtained at high yields and are well characterized mechanistically. Wicki et al. [80] examined five inhibitors with a xylanase. In their hands, the charge-mimicking inhibitors isofagomine (**7**) and deoxynojirimycin (**6**) (in this case the xylobio-derived versions were tested) were poor mimics of the transition state, whereas the shape-mimicking inhibitors lactam oxime (**9**), imidazole (**10**) and isofagomine lactam (**13**; Figure 2D) demonstrated a good correlation between rate enhancement (as determined by k_{cat}/K_m) and inhibition (as determined by K_i), with a slope of 1, suggesting they were good mimics [80]. Along similar lines, Tailford et al. [70] conducted a study with a mannosidase and tested a number of shape-mimicking manno-derived imidazole (**10**) and amidine (**8**) compounds, which possessed a variety of appendages to mimic the leaving group. Interestingly, the imidazole compounds with functional groups showed a strong and unitary correlation between rate enhancement and inhibition, suggesting that they were good mimics of the transition state; the unsubstituted imidazole also showed a good correlation, but with a poorer fit. All amidine compounds and other charge-mimicking inhibitors did not correlate, suggesting that these were poor mimics of the transition state. Although these studies suggest that inhibitors containing a distortion of the pseudo-glycoside ring tend to be better mimics of the transition state, a different outcome was observed with an *N*-acetylglucosaminidase which hydrolyses using a substrate-assisted mechanism. Whitworth et al. [81] tested two inhibitors, PUGNAc (**14**; Figure 2D), which contains a distortion of the pyranose ring, and NAG-thiazoline (**15**; Figure 2D), which resembles the oxazoline intermediate formed during hydrolysis. In that study, NAG-thiazoline was demonstrated to be a good mimic of the transition state, whereas PUGNAc was not, which was perhaps a little surprising given the resemblance of NAG-thiazoline to the intermediate. These examples also illustrate that the same features of inhibitors do not necessarily translate into being transition-state mimics with different enzymes. Two groups have also examined the pseudo-tetrasaccharide inhibitor acarbose (**3**) using kinetic methods [82] or ITC [83] to assess mimicry of the transition state. Whereas both methods showed a reasonable linear correlation between log K_i and log K_m/k_{cat}, the correlation was greater than unity. Whereas Mosi et al. [82] interpreted the correlation to mean acarbose was a true transition-state mimic, Berland et al. [83] concluded the correlation should have a slope of 1 and thus acarbose is not a transition-state mimic. The latter interpretation is consistent with the equation derivation which shows that the correlation between enzyme catalysis and inhibition should be equal [79], and thus acarbose is most probably not a true transition-state mimic.

The accumulation of knowledge using *in vitro* studies, such as those touched upon in the present article, is insightful for establishing the important properties of compounds to inhibit GHs. Although the aim for inhibiting a particular enzyme target for use as a research tool or therapeutically is

to identify a compound displaying high potency, specificity and the ability to reach the target in an organism, it is notable that the majority of compounds that show promise or are used as therapeutic agents are based on either natural products that mimic aspects of the transition state, and/or from knowledge gleaned from mechanistic or structural studies. In addition to the compounds that are used in the treatment of diabetes and influenza virus infection described above, isofagomine (**7**) has shown promise as a 'molecular chaperone' in the treatment of the lysosomal storage disorder, Gaucher's disease [84,85]. In this case, it is not so much the inhibitory properties of the compound, but the ability of the molecule (which is dosed at sub-inhibitory concentrations) to bind and stabilize the enzyme and aid trafficking to the lysosome to enable some enzyme activity as required for normally functioning of the cell. In addition, rationally designed inhibitors, based on mechanistic and structural information, against the nucleocytoplasmic *N*-acetylglucosaminidase, *O*-GlcNAcase, show promise in slowing progression of Alzheimer's disease in a transgenic mouse model [86,87], rendering it an exciting target for development of potential therapeutics.

GTs from a mechanistic viewpoint

Knowledge of GTs from a mechanistic and structural viewpoint lags somewhat behind that of GHs, which stems in part from the difficulties associated with obtaining proteins, that are often membrane associated, in high yield and soluble form. An added complication arises from the fact that GTs catalyse bimolecular reactions, and determining the substrate specificity for both the donor and acceptor for a GT of unknown function can be challenging. These factors have had a knock-on effect for the rational design of GT inhibitors and subsequently their limited use both *in vitro* and *in vivo* for analysis of GT function.

GTs catalyse glycosidic bond formation with overall inversion or retention of anomeric configuration. Most often the substrate donor is a nucleotide sugar, and GTs can transfer to a range of biomolecules, including proteins, lipids, saccharides and small molecules. They have been classified into 94 families by sequence homology in the CAZy database [28], which can be a useful predictive tool assuming conservation of structure and mechanism within a family. This classification does not, however, predict substrate specificity.

The structures of all GTs solved to date adopt one of three folds and have been correspondingly classified into three superfamilies: GT-A, GT-B and GT-C. There is limited molecular detail known about GT-C enzymes, since the first structure for this family was only solved recently, but enzymes predicted to adopt this fold are believed to be integral membrane proteins [88,89]. A greater mechanistic understanding for GT-A and GT-B enzymes has been gained over the last decade or so, and this has been reviewed in some depth [90]. GT-A enzymes are generally divalent-metal-ion-dependent, which is co-ordinated by a conserved DXD motif in the protein. The metal ion stabilizes the negatively charged phosphate groups to aid leaving group departure. GT-B enzymes often utilize a positively charged active-site residue or helix dipole to achieve the same purpose. Inverting GTs are proposed to employ a direct displacement S_N2 mechanism with simultaneous leaving group departure and nucleophilic attack, which is facilitated by proton transfer to the catalytic base (Figure 4A). The catalytic base is often a carboxy-group-containing residue, but can also be a histidine residue that interacts with a nearby carboxy group. The mechanism for retaining GTs is less well defined and much debated in the field, and may not even be consistent for all enzymes [91]. It is possible these enzymes use a double-displacement mechanism via a covalent intermediate analogous to GHs [92], although there has been no structural evidence of this to date. Alternatively, an S_Ni or S_Ni-like mechanism has been proposed, and this has been supported by a recent study using a number of physical organic approaches to analyse the likely structure of the transition state [93] and by computational approaches [91].

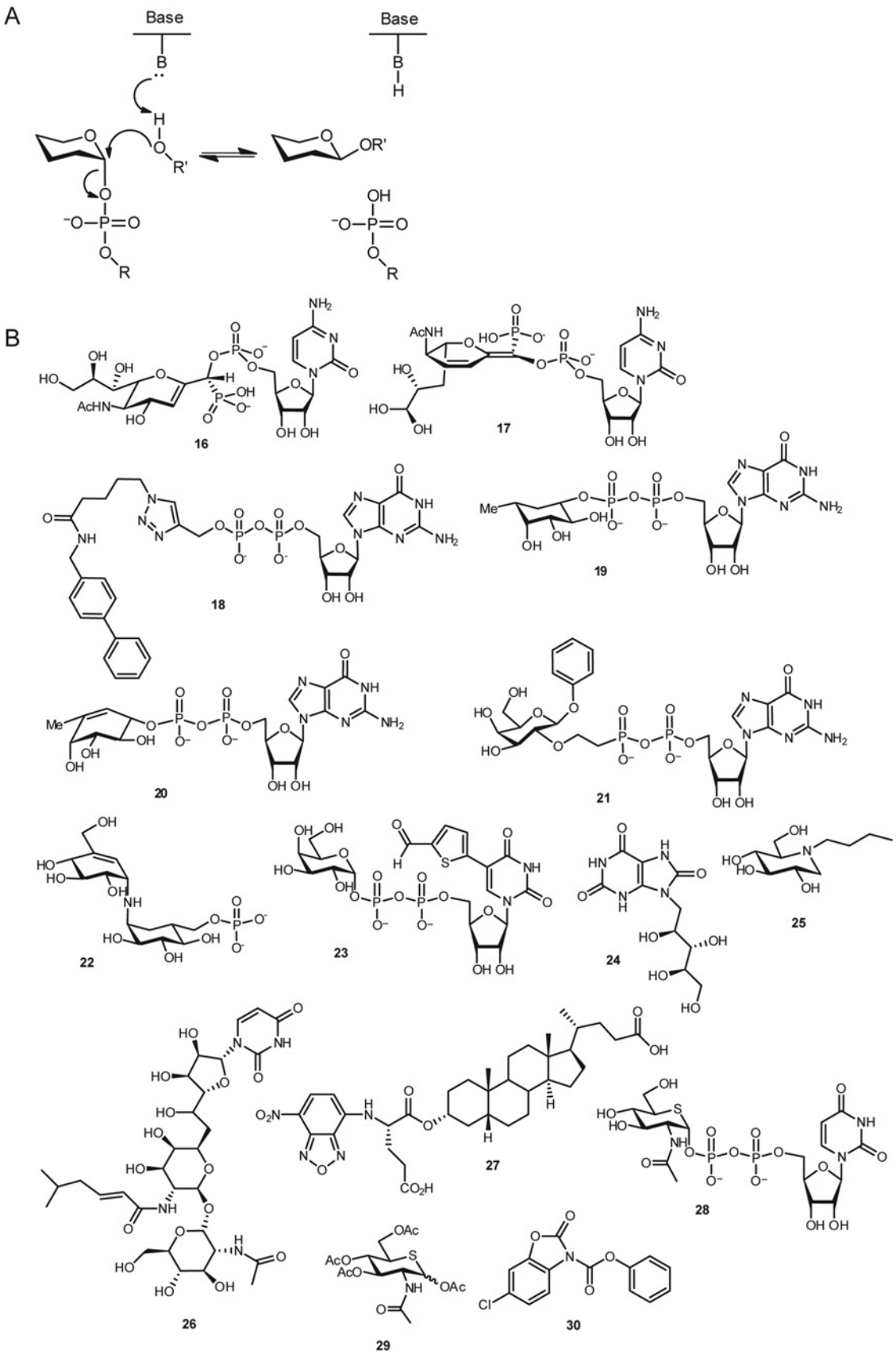
Development of GT inhibitors *in vitro*

Fewer studies have been conducted to define the transition-state structures formed during GT catalysis, but, as for GHs, they are thought to be dissociative with oxocarbenium ion character [90]. Many of the same principles to designing GT inhibitors can therefore be applied as described above for GH inhibitors. GT catalysis differs, however, as it involves two substrates: the donor, commonly a nucleotide sugar, and the acceptor. Although the configuration of the carbohydrate moiety is important for binding the correct substrate donor or a specific inhibitor, significant binding potential and specificity is also derived from the nucleotide, in particular the base and phosphate groups. This means mimicry of most or all of the features of the entire substrate donor is important, and is highlighted by the fact that most GH inhibitors do not inhibit GTs. The difficulties associated with synthesis of these complex molecules, as well as the lack of a natural product pool that has proven a useful starting point for development of GH inhibitors, has meant progress in identifying and developing molecules of GTs is significantly distant from that of GHs. In addition, GTs are often difficult to obtain recombinantly, hence there is limited structural knowledge, and the need for a read-out enzyme assay is often difficult to develop. Assays rely on the identification of both substrate donor and acceptor for a GT, and there is no simple way to obtain a read-out by spectroscopic or fluorimetric techniques using synthetic substrates. Some interesting methods to overcome these problems have been developed in this regard, however, and will hopefully inspire and help to move the field forward.

Consideration of the predicted transition-state structure for an α-2,6-sialyltransferase led to the design of compound **16** (Figure 4B). Given the CMP-Neu5Ac substrate donor for the sialyltransferase, an extra bond between the anomeric carbon and the CMP leaving group was incorporated to

Figure 4 | **GT mechanisms and examples of inhibitors**

(**A**) Proposed mechanism for glycosyl transfer with inversion of stereochemistry. The mechanism for retention of stereochemistry is less well understood and not shown. (**B**) GT inhibitors described in the text.



mimic the partially formed bond at the transition state. In addition, sp^2 hybridization was introduced in the pyranose ring to mimic the distorted conformation at the transition state. Whereas the chemistry called for removal of the carboxy group found at the anomeric carbon, an additional phosphate group was incorporated in a similar spatial position as found in CMP-Neu5Ac, to maintain the overall net charge, which resulted in a nanomolar inhibitor [94]. Further exploration was carried out to replace the sialyl group with simpler aryl groups, and there was no apparent loss in potency. After more refinement, the most potent inhibitor (**17**; Figure 4B) contained a flattened pyranosyl ring mimicking the sialyl group but containing two double bonds, one endocyclic and one exocyclic [94]. The groups on the sugar moiety could also tolerate modifications and, in some cases, aided potency [95]. In order to determine inhibition, a novel non-radioactive assay was developed using HPLC separation of the UV-labelled acceptor [96]. Others have similarly demonstrated the importance of inhibitors containing the nucleotide moiety, whereas more flexibility is tolerated in the sugar region. For example, microarrays identified a GDP-triazole inhibitor (**18**; Figure 4B) for a fucosyltransferase which also inhibited with nanomolar potency [97].

MS has been a useful tool in the development of assays to examine GT activity and inhibition. It was employed to measure the potency of rationally designed carba-analogues of GDP-fucose with a fucosyltransferase [98]. The carba-fucose was saturated in one analogue (**19**; Figure 4B), and unsaturated in another (**20**; Figure 4B), which introduced elements of the distortion at the transition state. Interestingly, the unsaturated analogue has slightly higher potency [98,99].

Bisubstrate inhibitors of GTs were a concept first postulated in 1989, where a single compound contains elements of the substrate donor and acceptor and allows specificity for both to be introduced. A bisubstrate compound (**21**; Figure 4B) was first synthesized and shown to be a micromolar inhibitor for a fucosyltransferase [100]. Other analogues have since been developed for enzymes including sialyltransferases [101] and *N*-acetylglucosaminyltransferases [102].

The availability of structural information to influence the design of GT inhibitors is rare, but extremely powerful. Inhibitors mimicking the disaccharide reaction product were designed for a trehalose 6-phosphate synthase. Although kinetics showed them to be relatively poor inhibitors, the structure of the ternary complex with UDP and a bisubstrate analogue (**22**; Figure 4B) bound gave important insights into the mechanism for retaining GTs, which supported an S_Ni-like mechanism [103]. Inhibition using a different mode of action, which could not have been predicted in other ways, was eloquently demonstrated with the human galactotransferase AA(Gly)B, which synthesizes blood group antigens. Inhibitors against this enzyme were developed by adding functional groups to the base of the nucleotide. Attachment of a 5-formylthien-2-yl group to the uridine of UDP-galactose (**23**; Figure 4B) produced a compound that, surprisingly, acted as a poor substrate, but as a good inhibitor [104]. Structural determination of AA(Gly)B with and without the inhibitor bound indicated that, upon binding the substrate donor, an internal loop in the active site moved towards a closed conformation (Figure 5A). The addition of the bulky group to the uridine hindered the loop closing completely, however, thus preventing catalysis [104]. This approach for inhibition could be applicable to other GTs that use a similar loop-locking mechanism to secure the substrate donor in the active site.

One approach to GT inhibition was described very recently, also using the human blood group B galacto-transferase [105]. The compounds examined were based on the observation that UDP is important for binding to the GT, but the sugar to a lesser extent. Known non-ionic compounds which mimicked UDP were screened by *in silico* docking using the three-dimensional structure to identify molecules capable of binding in the active site of the enzyme. Hits from the screening, such as compound **24** (Figure 4B), were synthesized, tested and resulted in low millimolar inhibition against the galactosyltransferase, but are yet to be tested in cells. Further refinement of the structure could, however, allow for increases in potency. The study highlighted novel approaches to studying GT inhibitor potency, and an interesting comparison was made between inhibition obtained using an activity assay, saturation transfer difference NMR and surface plasmon resonance [105].

The understanding gained, albeit at a relatively early stage, of the elements involved in designing GT inhibitors and testing them *in vitro* is needed to appreciate which features of the compounds are important. This should guide future efforts in this area and provide insights into GT mechanisms.

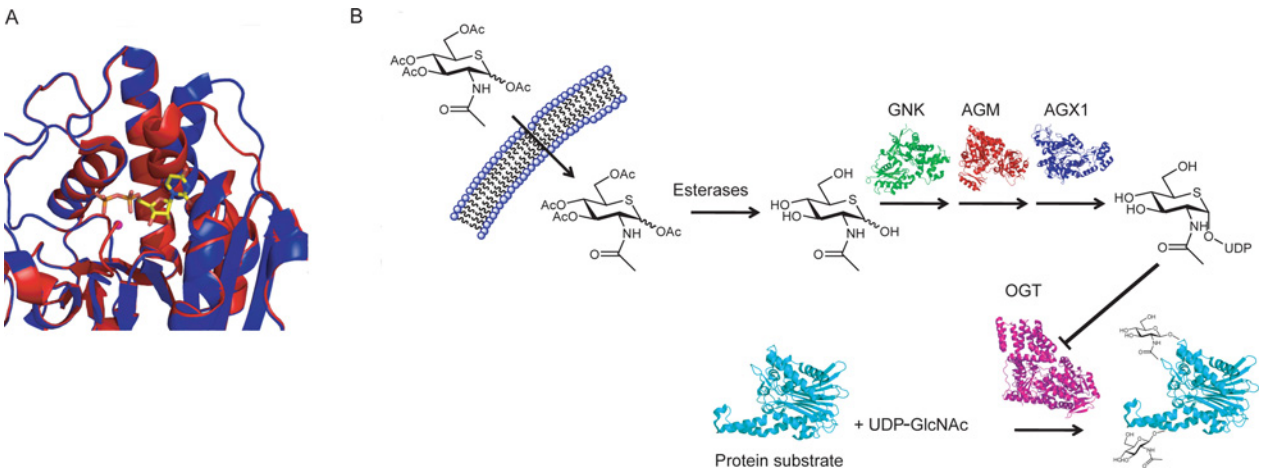
Using GT inhibitors in cells or *in vivo*

There are relatively few examples of GT inhibitors that work in cells or *in vivo*. Visual inspection of those that are promising demonstrates they are widely diverse in structure. In addition, it is worth noting that they have mostly been discovered fortuitously or from high-throughput screening approaches, and not from rational design.

One example of a GT inhibitor used successfully therapeutically is *N*-butyldeoxynojirimycin (**25**; Figure 4B) for the treatment of Gaucher's disease. This is one of the exceptional cases where a compound first identified as a GH inhibitor could be used to inhibit a GT, albeit in an unusual manner. Gaucher's disease is caused by inherited mutations in glucocerebrosidase, the enzyme responsible for degrading glucosylceramide, causing accumulation of the substrate in the lysosome. One effective way to tackle this has been to slow the synthesis of the glucosylceramide substrate by inhibiting the GT (glucosylceramide synthase) responsible for its synthesis, thus negating the need for the GH to degrade it. So-called 'substrate reduction therapy' has successfully been employed using *N*-butyldeoxynojirimycin (or Zavesca) to inhibit glucosylceramide synthase *in vivo* [106]. On the basis of the deoxynojirimycin scaffold, it may mimic aspects of the charge at the transition state. In addition, the butyl

Figure 5 | **Studying inhibition of GTs**

(**A**) The apo structure of human galactotransferase AA(Gly)B (blue) and in complex with a substrate donor where a 5-formylthien-2-yl group was attached to the uridine of UDP-galactose (red) [104]. Upon binding of the substrate donor, a loop in the active site moves to a closed conformation, thus preventing catalysis. (**B**) A novel approach for inhibition of OGT in cells by 'hijacking' the biosynthetic pathway to synthesize an inhibitor inside cells [113]. Ac-5SGlcNAc is dosed to cells, becomes deacetylated by esterases, and is transformed into UDP-5SGlcNAc by the enzymes GNK (GlcNAc kinase), AGM (GlcNAc mutase) and AGX1 (UDP-GlcNAc pyrophosphorylase). UDP-5SGlcNAc is an effective OGT inhibitor and causes a decrease in *O*-GlcNAc levels.



chain presumably harnesses significant binding potential and mimics the glucosylceramide product.

Other compounds which target GT activity include an inhibitor of *N*-glycan biosynthesis, tunicamycin (**26**; Figure 4B), which is a natural product and first investigated for its antibiotic and antiviral properties [107]. Later, this was fortuitously shown to inhibit UDP-GlcNAc dolichol phosphate synthesis, and hence had an impact on one of the crucial steps in *N*-glycan formation [108]. Interestingly, a hexapeptide (GNWWWW) was identified from a synthetic peptide combinatorial library screen to be a potent inhibitor of specific sialyltransferases *in vitro*, and to be effective at decreasing sialic acid expression on cell-surface glycoproteins in mammalian cells [109]. Likewise, screening approaches identified lithocholic acid as an effective α-2,3-sialyltransferase inhibitor, which due to the steroidal moiety it possesses can be taken up by cells [110]. Various analogues were developed, and AL10 (**27**; Figure 4B) was shown to effectively decrease sialic acid levels in cells and *in vivo*, and affect cell migration and invasion in cancer models [111].

Despite the design of some highly potent GT inhibitors that work *in vitro*, it is ironically one of the features that makes them potent that is also their downfall for translating them for use in cells or *in vivo*. Rational design of GT inhibitors often involves mimicry of the nucleotide substrate donor, which demands incorporation of negatively charged groups to emulate the phosphate groups that contributes significantly to binding. However, the charged groups prevent uptake into cells, meaning their use for studying the role of GTs in biological systems is limited. There are few examples of approaches to circumvent this issue, and it certainly is the rate-limiting factor in the development of GT inhibitors.

A novel approach to inhibiting a particular GT circumvents the issue of getting a charged molecule across the cell membrane, and holds promise for inhibiting other classes of GTs. The post-translational modification of nucleocytoplasmic proteins involves the addition of a single GlcNAc residue to serine or threonine residues (*O*-GlcNAc). This is a dynamic modification, which can occur multiple times over the lifetime of the protein, and has been implicated in a number of cellular processes and diseases [112]. The modification is modulated by just two enzymes, a GH responsible for removing *O*-GlcNAc, and a GT [OGT (O-linked *N*-acetylglucosamine transferase)] responsible for its formation. OGT uses the substrate donor UDP-GlcNAc to transfer GlcNAc on to proteins.

The approach to inhibition takes advantage of the UDP-GlcNAc biosynthetic salvage pathway which occurs in cells. Addition of an analogue of GlcNAc, which contained an endocyclic sulfur in place of oxygen (5SGlcNAc), could essentially 'hijack' the biosynthetic pathway, resulting in synthesis of a UDP-GlcNAc analogue, UDP-5SGlcNAc (**28**; Figure 4B) [113] (Figure 5B). The recombinantly expressed enzymes in the biosynthetic pathway responsible for synthesis of UDP-GlcNAc were shown to tolerate 5SGlcNAc *in vitro* and successfully synthesize UDP-5SGlcNAc in a one-pot reaction. UDP-5SGlcNAc was an effective inhibitor of OGT *in vitro*, and a relatively poor substrate donor. The acetylated version (to aid cellular uptake) of 5SGlcNAc, Ac-5SGlcNAc (**29**; Figure 4B), could

be dosed to cells, deacetylated by cellular esterases to 5SGlcNAc and biosynthesized to UDP-5SGlcNAc. Dosing with Ac-5SGlcNAc caused a decrease in *O*-GlcNAc levels in cells in a time- and dose-dependent manner, both globally and on an individual heavily modified protein. Although UDP-GlcNAc levels were reduced to approximately 60 % of the basal levels, there appeared to be no impact on cell-surface glycosylation or cellular proliferation over the timeframes examined. Furthermore, use of a chemical ligation strategy with an azide-labelled 5SGlcNAc analogue demonstrated that there was no transfer of the 5SGlcNAc on to proteins, indicating that UDP-5SGlcNAc acted as an inhibitor and not a substrate donor [113]. The creation of an inhibitor mimicking the substrate donor within cells takes advantage of the binding potential of the negatively charged phosphate groups, but circumvents the need for getting a charged species across the cell membrane.

The roles played by *O*-GlcNAc and OGT are largely unknown, and so a tool such as Ac-5SGlcNAc provides the basis for the start of these investigations. Indeed, preliminary studies with an OGT inhibitor (**30**; Figure 4B) obtained from high-throughput screening have implicated OGT in cell-cycle regulation [114]. Two groups have shown that both *O*-GlcNAc and OGT levels are higher in some cancer cell lines [115,116], and raises the possibility of OGT being a target for inhibition to slow cancer progression. The 'hijacking' strategy provides an exciting starting point for inhibition of other classes of GTs. Indeed, this approach of forming incompetent donor sugars inside cells to inhibit GTs has very recently been demonstrated for a fucosyltransferase and a sialyltransferase [117].

Although there are few examples of GT inhibitors that work in cells or *in vivo* and can thus be used to probe biological function or be used therapeutically, the combination of different techniques and areas of expertise will help to drive this quest forward. Like GHs, GTs are involved in diverse biological processes and their inhibition is relevant to a number of different applications. Although knowledge of enzyme mechanisms, substrate specificity and three-dimensional structure is not as well advanced for GTs, novel approaches are emerging with ways to rationally design inhibitors that work *in vivo*.

Summary

It is an exciting time in the glycobiology and carbohydrate-processing enzyme field. Armed with the information from genomic sequencing and proteomic studies, we are able to tackle gaining a fuller understanding of glycosylation *in vivo*. This is not a trivial task. Given that glycosylation is not sequence-encoded like DNA and protein, each and every glycan in an organism needs to be identified and characterized. Their regulation is primarily governed by the enzymes responsible for their synthesis and degradation, but matching the glycan with the enzymes needed to control it, particularly given the number of unknowns on each side, is a difficult task. Even when this can be deduced, the biological role played by the glycans, or indeed the enzymes themselves, is often an even tougher question to answer.

The carbohydrate community has to exploit the use of tools to enable a greater understanding of carbohydrate-processing enzymes and the processes in which they are involved. Enzyme inhibitors play a significant contribution towards this, and can be explored as ways to aid identification of the role played by the glycan and/or enzymes. They also have therapeutic potential in the treatment of a host of diseases. The rational design of inhibitors is a reasonable way to approach this, as it takes into account enzyme specificity and mechanistic considerations. There are, of course, other ways to discover potential inhibitors; more random screening methods have identified potent inhibitors against both GHs [118,119] and GTs [120,121]. The question of specificity, however, may be more difficult to address for these compounds.

The combined efforts from all areas of expertise of carbohydrate chemistry and biology will be needed to gain a greater understanding of the role of the various glycans and enzymes *in vivo*. The amount still to be understood means that there are many opportunities for novel discoveries, whether it be mechanistically, structurally, biologically or medically.

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