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# REVIEW

# Metabolic glycoengineering: Sialic acid and beyond

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**This report provides a perspective on metabolic glycoengineering methodology developed over the past two decades that allows natural sialic acids to be replaced with chemical variants in living cells and animals. Examples are given demonstrating how this technology provides the glycoscientist with chemical tools that are beginning to reproduce Mother Nature's control over complex biological systems – such as the human brain – through subtle modifications in sialic acid chemistry. Several metabolic substrates (e.g., ManNAc, Neu5Ac, and CMP-Neu5Ac analogs) can be used to feed flux into the sialic acid biosynthetic pathway resulting in numerous – and sometime quite unexpected – biological repercussions upon nonnatural sialoside display in cellular glycans. Once on the cell surface, ketone-, azide-, thiol-, or alkyne-modified glycans can be transformed with numerous ligands via bioorthogonal chemoselective ligation reactions, greatly increasing the versatility and potential application of this technology. Recently, sialic acid glycoengineering methodology has been extended to other pathways with analog incorporation now possible in surface-displayed GalNAc and fucose residues as well as nucleocytoplasmic *O*-GlcNAc-modified proteins. Finally, recent efforts to increase the "druggability" of sugar analogs used in metabolic glycoengineering, which have resulted in unanticipated "scaffold-dependent" activities, are summarized.**

*Keywords:* carbohydrate-based drugs/glycosylation/metabolic glycoengineering/sialic acid

## Overview and introduction

Metabolic glycoengineering has become the premier tactic for incorporating nonnatural "building-blocks" into the cellular architecture (Campbell et al. 2007; Aich and Yarema 2008). In this technique, a synthetic monosaccharide similar in structure to a natural precursor in a biosynthesis pathway for a cell-surface glycan, but bearing an unnatural chemical group, is incubated with cells or introduced into live animals (Kayser et al. 1992a). The modified monosaccharide enters a cell and is processed by the biosynthetic enzymes in a manner similar to the natural precursor, and the resulting cell-surface glycan bears the unnatural functional group (Figure 1). The sialic acid pathway pioneered – and subsequently has served as the workhorse – for metabolic glycoengineering because of its remarkable tolerance for both neuraminic acid (Oetke et al. 2001, 2002) and mannosamine (Kayser et al. 1992b; Keppler et al. 2001) precursors bearing nonnatural chemical substituents.

In theory, metabolic glycoengineering is a very straightforward technique. The sugar analogs can simply be added to the culture medium of a cell and the glycosylation machinery—exemplified by the sialic acid biosynthetic pathway (Figure 1)—functions as a "black box" that performs the synthetically challenging task of creating modified surface glycans. In practice, sialic acid glycoengineering gains incredible complexity by impinging on the underlying biology of this fascinating sugar. To introduce "sialobiology," a brief overview of this topic is given in the next section of this article with an emphasis on complications (e.g., serious human disease) that arise from natural aberrations in sialic acid metabolism—clearly, the metabolic glycoengineer should keep these in mind to avoid doing harm. The chemical complexity of sialic acid, which has long been exploited by nature to modulate cellular functions in ways that glycoscientists are increasingly replicating and extending, is then described followed by a discussion of a significant advance in metabolic glycoengineering realized when chemical function groups appropriate for bioorthogonal chemistries were incorporated into monosaccharide analog design. This article concludes by describing how, in recent years, metabolic glycoengineering has been extended from sialic acid to additional glycosylation pathways thereby greatly increasing the versatility of this technology and in concert, analogs have been designed for greater efficiency, which has led to unexpected new activities.

## An introduction to sialic acid

### *History*

Terminology used by glycobiologists in general, and to describe sialic acids in particular, can be confusing to the nonspecialist; for example, these sugars are often referred to by a second name: neuraminic acids. The reason why there are two names in common – and often interchangeable – usage is historical in nature; the story begins in the mid 1930s when this sugar was isolated from submaxillary mucin by Gunnar Blix (Blix 1936) leading to the name "sialic acid" for this saliva-derived acidic compound. Shortly thereafter, by the early 1940s, Ernst Klenk independently isolated acidic glycosphingolipids (GSLs (Klenk 1941)), which were named gangliosides because they were abundant in brain gray matter and ganglial cells. Klenk identified sphingosine, fatty acid, hexoses – as well as a substance that

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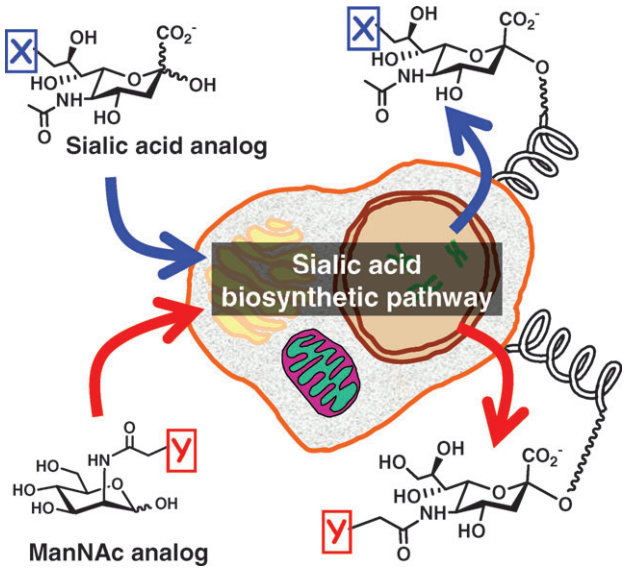


**Fig. 1.** Metabolic sialic acid glycoengineering. A schematic view of chemically modified sialic acid (top) or ManNAc (bottom) analogs that intercept the sialic acid biosynthetic pathway of a mammalian cell and appear on the cell surface as the corresponding "**X**" or "**Y**" modified sialosides.

gave a purple color with Bial's reagent that he called neuraminic acid – as constituents of the brain-derived glycolipids. Klenk's neuraminic acid was later found to be the same compound isolated from saliva by Blix and the nomenclature was clarified in 1957 (Blix et al. 1957) with the name "sialic acid" now collectively referring to a family of over 50 naturally occurring sugars (Angata and Varki 2002) as well as a growing number of synthetic sugars achieved through metabolic substrate-based sialic acid engineering methods (Keppler et al. 2001; Yarema 2001). Individual sugars, however, largely have retained neural-based nomenclature; for instance, the most common form of sialic acid in humans is *N*-acetylneuraminic acid (Neu5Ac) whose chemical structure was reported half a century ago by Saul Roseman's Laboratory (Comb and Roseman 1960).

*Sialic acid bioprocessing – A complex, energetically expensive, and dangerous endeavor*

*Biosynthesis and Recycling.* In the past two decades, the biosynthetic machinery for sialic acid in mammalian cells has been unraveled in considerable detail (Figure 2) and, in common with all facets of glycosylation, found to be complex and energetically expensive. To begin, sialic acid biosynthesis in mammalian cells is supported by flux through the hexosamine pathway, which produces UDP-GlcNAc (Figure 2A) (Hanover 2001). A relatively minor proportion of the millimolar levels of this cytosolic nucleotide sugar (Lau et al. 2007) is converted to ManNAc (Figure 2B), a dedicated precursor for sialic acid biosynthesis with no other roles in a cell (except in specialized situations when it can be epimerized back to GlcNAc (Luchansky et al. 2003)). Considering the other important metabolic fates for UDP-GlcNAc – this versatile intermediate is also used for *O*-GlcNAc protein modification, employed for glycoconjugate biosynthesis, or converted to UDP-GalNAc and used for the same purpose – it is not surprising that ManNAc production is kept under tight control. This regulation occurs through allosteric feedback inhibition of GNE, the bifunctional enzyme that controls flux into the sialic acid pathway (Keppler et al. 1999) through binding of the down-stream metabolite CMP-Neu5Ac (Seppala et al. 1991, 1999; Yarema et al. 2001). The pathway then moves from the cytosol into the nucleus where CMP-Neu5Ac is made and then into the lumen of the Golgi via CMP-Neu5Ac transporter (CMPNT, an anti-port transporter (Eckhardt et al. 1996)) where the activated nucleotide sugar is used to install α2,3-, α2,6-, or α2,8-linked sialosides into glycosphingolipids or glycoproteins by a suite of (in humans) 20 different sialyltransferases (Figure 2C). Finally, glycoconjugate-displayed sialic acids are hydrolyzed by one of several mammalian neuraminidases during glycan recycling (Figure 2D); the liberated sialic acids can be salvaged and reused by the cells.

*Sialobiology.* In addition to being energetically expensive (involving not the usual one, but rather two nucleotide sugars), sialoglycoconjugate biosynthesis is – as the disease-related abnormalities mentioned throughout this article testify – fraught with danger when biochemical events go awry. Moreover, even when nothing goes amiss with the host's metabolism, numerous pathogens exploit this sugar as an entry point to the human body. These pitfalls lead to the question of "Why does nature bother with sialylation?" The answer, of course, is that sialic acid plays immensely important roles in biological recognition by virtue of being situated at the outer periphery of the cell surface where it participates in numerous interactions that a cell makes with its microenvironment. Efforts to study and understand these functions have spawned the field of sialobiology, which seeks to uncover the role of this sugar in intimate relationships between a cell and its surroundings ranging from the nanometer scale (e.g., cell adhesion molecules) up to the whole organism level (e.g., the immune system and tissue-wide and system-level control through hormones).

*Multiple Levels of Biological Control.* Sialic acid exemplifies the several hierarchical levels through which sugars influence biological activity and engineer multi-layered regulation over complex systems. A brief discussion of these levels is now provided to introduce the idea that if similar control could be reproduced in the laboratory or clinically – perhaps through the metabolic glycoengineering methods described in this article – one could gain exquisite control over cells, tissues, and organisms in both health and disease. On one level, the simple presence or absence of sialic acid in a critical macromolecular context can profoundly influence biological activity. For example, the conversion of LacCer to GM3, or GM3 to GD3 by the sequential addition of sialic acid can alter the pro-apoptotic propensities of these glycoshingolipids (GSL) (Figure 3A). Showing similar influence over activity, the sialic acid residue of the sialyl Lewis X ($sLe^X$) tetrasaccharide is required for mucins to support selectin-mediated tethering and rolling (Figure 3B) (McCarty et al. 2000). On a second level, the type of glycosidic linkage (e.g., α2,3-, α2,6-, or α2,8- as shown in Figure 2C) through which sialic acid is attached to the underlying glycoconjugate can profoundly influence activity; for example, the influenza virus discriminates between α2,3- and α-2,6-linked sialosides (Suzuki et al. 2000; Shinya et al. 2006).

Zeroing in to the submolecular level, subtle (or not so subtle!) changes in the chemical structure of sialic acid can also

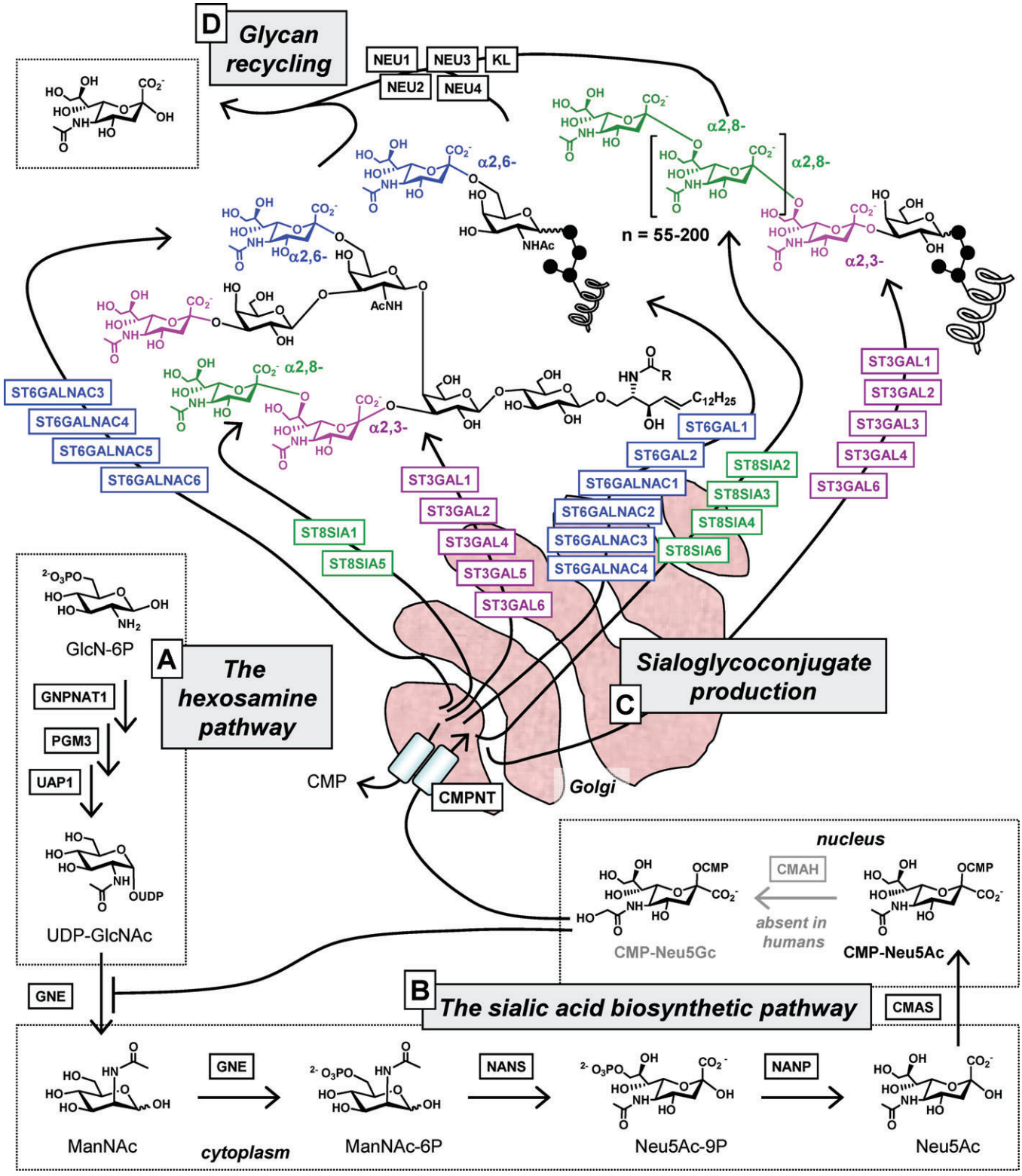


**Fig. 2.** Overview of mammalian sialic acid metabolism. (**A**) The hexosamine pathway converts glucosamine-6-phosphate (GlcN-6P) into the nucleotide sugar UDP-GlcNAc, which in turn is converted into ManNAc by GNE (pathway details are provided elsewhere (Hanover 2001)). (**B**) ManNAc is the dedicated metabolic precursor for the sialic acid biosynthetic pathway that produces Neu5Ac in the cytosol and then enters the nucleus where CMP-Neu5Ac is synthesized (in most nonhuman species, a proportion of CMP-Neu5Ac is converted to CMP-Neu5Gc). (**C**) These two nucleotide sugars are transported into lumen of the Golgi where they are used by several sialyltransferases (up to 20 in human cells, dependent on cell- and tissue-dependent expression patterns) to produce α2,3-, α2,6-, or α2,8-linked sialoglycoproteins or gangliosides. (**D**) Finally, sialosides are recycled by one of four neuraminidases, thereby regenerating sialic acid monomers that can be re-used by a cell. The full names of enzymes are given in Table S1 available online in the *Supplementary Data*.

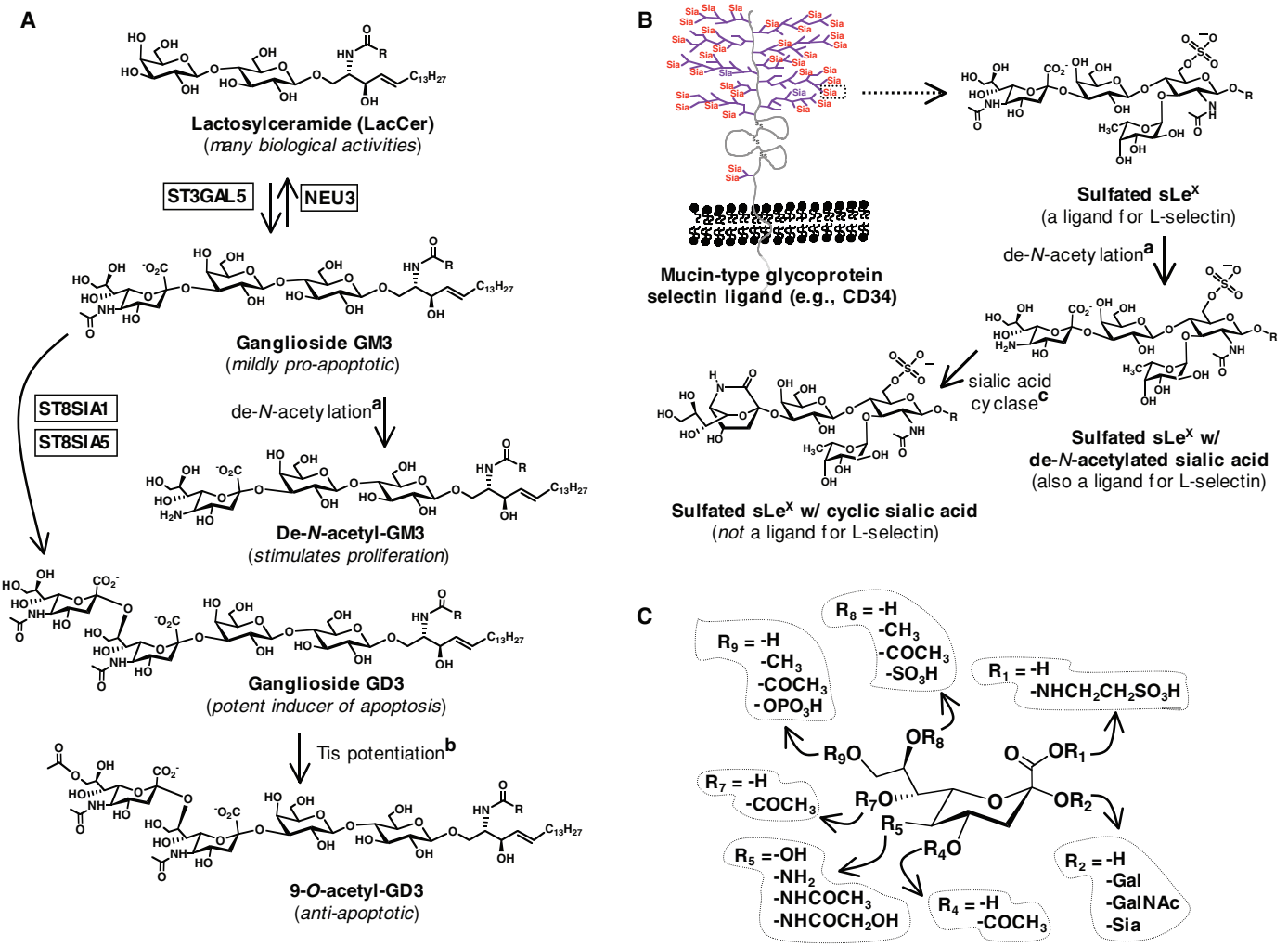


**Fig. 3.** Sialic acid: a template for modulating biological activity. (**A**) Changes to sialic acid – either by the addition (or loss) of the entire monosaccharide or through submolecular chemical alterations – dramatically modulate the impact of gangliosides GM3 and GD3 on proliferation and apoptosis. (**B**) Sialic acid, as part of the $sLe^X$ tetrasaccharide that is multivalently displayed on mucin ligands for selectins, has a large impact on adhesion interactions exemplified here through a hypothesized cyclic form of sialic acid that abrogates binding interactions. (**C**) Sialic acid is a template for modulating biological activity with various "R" groups naturally found at virtually every position (Schauer 2000); to date, nonnatural substitutions (see Figure 4) have predominantly been situated at $R_5$ and $R_9$. The full names for enzymes are given in Table S1 (in the *Supplementary Data* available online) with more information for the conversion indicated by **a** sialic acid de-*N*-acetylation (Hanai et al. 1998), **b** Tis potentiation (Chen et al. 2006), and **c** sialic acid cyclase (Mitsuoka et al. 1999; Kannagi 2002) provided in the references given.

have a dramatic impact on biological activity. For example, again illustrated in Figure 3A, de-acetylation of this sugar when found on GM3 converts this mildly pro-apoptotic GSL into a molecule that stimulates proliferation. Equally consequential is 9-*O*-acetylation of GD3, which transforms the potently pro-apoptotic parent molecule into an anti-apoptotic entity. Another example of the impact of submolecular changes to sialic acid on biological activity is provided by $sLe^X$ (Figure 3B) where the carboxylic acid group of this residue is sufficient to support tethering and rolling behavior under laboratory conditions (Yarema and Bertozzi 1998; Magnani 2004). Additional chemical features of $sLe^X$, however – such as a hypothesized bicyclic form of sialic acid ((Mitsuoka et al. 1999) and sulfation of the 6-position of GlcNAc (Bistrup et al. 1999) or at the 6′-position of galactose (Bochner et al. 2005)) – determine the physiological relevance of $sLe^X$-mediated recognition events (Varki 1997).

With numerous other natural modifications to the core structure of sialic acid now documented (see Figure 3C) (Angata and Varki 2002), the just-discussed examples of biological activities influenced by chemical features of sialic acid are merely the tip of the iceberg. For instance, the "nonhuman" glycolyl-modified form of Neu5Ac (i.e., Neu5Gc where $R_5 = -NHCOCH_2OH$, Figure 3C) provides another window, which is focused on the brain in the discussion below, into the manifold biological roles of sialic acid and how the chemical structure of this sugar influences function. Interestingly, Neu5Gc can be produced both enzymatically (e.g., by CMAH, Figure 2 and Table S1 in the *Supplementary Data*) and artificially through incubation of cells with the appropriate ManNAc analog (e.g., $Ac_5ManNGc$, (Collins et al. 2000) presaging the manipulation of sialic acid through the metabolic glycoengineering methods described later in this article.

*Sialic acid: A sugar that engineers the evolution, function, and pathology of the brain*

*Sialic Acid and Human Brain Evolution.* Compared to sialic acid *research,* which sprung to life about three-quarters of a century ago, the *biological effects* of this sugar reach much farther into the past. The Varki group has proposed that the functional loss of CMP-*N*-acetylneuraminate monooxygenase (CMAH, the enzyme that converts Neu5Ac to the Neu5Gc "non-human" form of this sugar, Figure 2B (Irie et al. 1998)) a few million years ago played a role in human brain evolution (Varki 2002). This premise is supported by evidence that the mutation that abrogated the activity of CMAH transcripts found in present-day humans occurred after the Homo-Pan divergence (Chou et al. 1998) but prior to brain expansion during human evolution (Chou et al. 2002). Unlike most primate brains that stop growing relatively soon after birth, human brains continue to grow postnatally at a rate similar to the whole body, presumably because they were freed from the constraints imposed by Neu5Gc in other mammals that limit brain expansion after birth (Chou et al. 2002).

*Sialic Acid and Brain Development – Is It a Glyconeutraceutical?* Ganglioside levels vary significantly among mammals; interestingly, humans – a life form self-regarded as being highly intelligent – have over twice the amount found in any other species (Wang et al. 1998). Moreover, cerebral gray matter contains ~900 μg/(g wet wt) of GSL-bound Neu5Ac, which is strikingly higher than the ≤30 μg/(g wet wt) found in most tissues and organs (Wang and Brand-Miller 2003; Schnaar 2004). Sialylated molecules play many roles in the brain beginning with the simple gangliosides GM3 and GD3 (Figure 3A) that influence early development by impacting cell proliferation and apoptosis (Bieberich et al. 2001). After birth, the GSL composition of the CNS undergoes dramatic changes (Yu et al. 2004) with close to 90% of the dozens of different gangliosides found in the mature brain limited to only four structures (GM1, GD1a, GD1b, GT1b).

In addition to their GSL-mediated contributions, sialic acids attached to proteins in each of the three glycosidic linkages (i.e., α2,3-, α2,6-, and α2,8-) common in mammals play key roles during brain development and then facilitate neuronal plasticity in the adult (Breen and Georgopoulou 2003). To provide representative examples of each linkage, CD34 is a stem cell marker that proliferating resident microglia express in response to acute neural injury (Ladeby et al. 2005) that predominantly displays α2,3-linked sialic acid in the form of $sLe^X$ (Lanza et al. 2001). Integrin function is modulated by α2,6-sialic acids (Yamamoto et al. 2001) in turn influencing adhesion, migration, and differentiation of neural progenitor cells (Tate et al. 2004). Finally, the neural cell adhesion molecule (NCAM) is modified by polysialic acid (PSA), a highly hydrated, negatively charged homopolymer of α2,8-linked sialic acids 55 or more residues in length that facilitates cell–cell interactions during cell migration, axonal pathfinding, axon branching, fasciculation, and learning (Cremer et al. 1994; Brusés and Rutishauser 2001). PSA endows the normally pro-adhesive NCAM with anti-adhesive characteristics that provide neural plasticity in many tissues during fetal development and uniquely in the brain in the adult (Mühlenhoff et al. 1998; Angata and Fukuda 2003).

Based on the multiple roles of sialic acid in CNS development, it intuitively follows that nervous tissue requires a copious supply of this sugar during growth. Evidence linking sialic acid intake with optimal brain development and cognitive abilities was reported for rat pups over 25 years ago (Morgan and Winick 1980). Since then several corroborating studies – reviewed by Wang and Brand-Miller (2003) – showed that dietary supplementation or intraperitoneal administration of Neu5Ac in newborn rodents increased cerebral and cerebellar glycoprotein and ganglioside sialic acid content; beneficial effects persisted into adulthood and occurred without affecting brain weight, cell size, cell number, or DNA, RNA, and protein content, suggesting that a sugar-specific mechanism was responsible. The analysis of a small number (~25) of human infants who died of sudden infant death syndrome provides evidence that dietary intake of sialic acid also benefits human brain development as breast-fed infants had 32% higher ganglioside-bound and 22% higher protein-bound sialic acids compared to formula-fed infants consistent with the higher levels of sialic acids in breast milk (Wang and Brand-Miller 2003; Wang et al. 2003). All though far from conclusive, and by no means meant to promote unfounded claims from glyconutrient sham artists (Schnaar and Freeze 2008), these studies do suggest that exogenous sialic acids (i) can be utilized by cellular systems and (ii) have an impact on their biology; these two features comprise a recurring theme in metabolic glycoengineering.

*Sialic Acid and Brain Disease.* Sialic acid has been implicated in many neurological disorders ranging from inborn errors of metabolism (such as sialuria, Salla Disease, or infantile sialic acid storage disease) to the late onset degenerative disorders of the brain including schizophrenia, Parkinson's and Alzheimer's diseases; these diseases have been reviewed extensively elsewhere (Sampathkumar et al. 2006a) and only a few highlights are presented here. Not surprisingly considering their overriding importance in brain development, congenitial defects in ganglioside biosynthesis are particularly prone to result in neurologic dysfunction. GM3 alone is implicated in several such diseases that include infantile-onset symptomatic epilepsy syndrome caused by a homozygous loss-of-function mutation of GM3 synthase (Simpson et al. 2004). In mice, a lack of GM3 synthase activity results in complete hearing loss due to degeneration of the organ of Corti (Yoshikawa et al. 2009).

Moving to GSL of greater complexity, Tay-Sachs disease is a classic example of an inborn lysosomal storage disease characterized by massive accumulation of ganglioside GM2 (Fernandes Filho and Shapiro 2004). Although not usually regarded as a primary factor in the etiology of Tay-Sachs, sialic acid has been implicated in disease progression when neural cells respond to massively elevated levels of GM2 as a quasi-xenobiotic and undertake detoxification efforts. To rid themselves of GM2, cells employ taurine conjugation (a well-known method for mammals to facilitate the clearance of xenobiotics by increasing their polarity and aqueous solubility) of the sialic acid moiety of this ganglioside. In effect, the taurine-conjugated form of GM2 becomes an activated xenobiotic with potent surfactant properties that contribute to the pathophysiological symptoms of Tay-Sachs disease (Li et al. 2003). This example is germane to metabolic glycoengineering as a cautionary note because some of the more flamboyant nonnatural sialosides discussed later in this paper have the potential to change the biophysical properties of the sugars dramatically and lead to pathologies.

Conversely, in cases where sugar analogs alter the profile of biosynthetic enzymes responsible for sialylation (Wang et al. 2006; Sampathkumar et al. 2006b) in potentially beneficial manners, an enticing possibility is that these agents could be exploited to correct aberrant sialylation.

*Sialic acid – A metabolic engineering opportunity exploited by nature*

To evaluate the prospect of manipulating sialylation for therapeutic purposes, we note that specific patterns of sialylation associated with health and disease are determined by two factors: (i) the rate of flux through the sialic acid pathway and (ii) the expression of an appropriate complement of sialyltransferases in a particular cell or tissue. Unfortunately, neither flux nor sialyltranferase expression is readily manipulated with sufficient precision to achieve specific endpoints. Surface sialylation patterns, for example, typically are insensitive to changes in metabolic flux because the Golgi concentration of CMP-Neu5Ac exceeds the $K_m$ for sialyltransferases (Monica et al. 1997); as a consequence, enhanced CMP-Neu5Ac levels do not translate into increased sialylation of glycoconjugates (conversely, a similar buffer exists against moderate decreases in flux). Exceptions may exist in specialized cases, such as the "high demand" imposed by the production of polysialic acids (Bork et al. 2005), or in hereditary inclusion body myopathy (HIBM), a disease where flux is compromised due to mutations in GNE (Eisenberg et al. 2001); this latter situation can be remedied in mice by supplementation with exogenous ManNAc (Galeano et al. 2007; Malicdan et al. 2009).

Manipulating sialylation by modulating sialyltranferases is also problematic (once again, specialized exceptions may exist) because of redundancy in enzymatic activity, highly specific small molecule inhibitors are lacking, and genetic methods – even siRNA approaches – remain beset by technical issues (such as off-target effects and a lack of efficient in vivo delivery methods). Fortunately, a third level of control over sialic acid display exists that avoids many of the difficulties inherent in controlling the rate of flux or the expression of the biosynthetic (or recycling) enzymes. As introduced above, this ability lays in the "submolecular biochemical engineering" approach where a specific portion of the sialic acid molecule is subject to a chemical modification (Figure 3C) (Keppler et al. 2001). This age-old strategy is exemplified by the postulated impact of Neu5Gc on human brain evolution; this molecule, when compared to Neu5Ac, amply demonstrates the critical influence of a single atom – in this case an added oxygen – in specifying the activity of much larger sialoglycoconjugates.

## Modified sialic acid – Building on nature's metabolic glycoengineering

*Sialic acid – A molecular template for chemical diversity*

Striking examples beyond Neu5Gc where nature modulates biological function through the submolecular biochemical engineering of sialic acid include the influence of acetylation over cell growth and death (Figure 3A) and $sLe^X$ on leukocyte recruitment (Panel B). Comparing the de-*N*-acetyl form of sialic acid in these two settings vividly illustrates the nuanced and context-dependent nature of sialic acid-dependent responses; the absence of the *N*-acetyl group from the sialic acid moiety of $sLe^X$ has no impact on the selectin binding activity of this tetrasaccharide while loss from GM3 converts a mildly pro-apoptotic GSL to a growth-stimulatory entity. These examples, and many more yet to be discovered (at present the known diversity in natural sialic acid structures – over 50 (Angata and Varki 2002) – greatly outpaces insight into their individual biological functions), make it clear that nature has long known that a monosaccharide is a versatile template for modifying biological activity. Based on a desire to mimic nature's ability to modulate biological activity by using sialic acid as a chemical template – a concept now embraced for many monosaccharides in drug discovery efforts (Meutermans et al. 2006) – metabolic sialic acid engineering was born almost two decades ago.

Considering how chemical engineering of sialic acid occurs naturally, through enzymatic methods, this approach may appear at first to be as intransient as the strategies based on modulating the rate of flux or manipulating biosynthetic enzymes considered above. Insight gained from the study of "nonhuman" Neu5Gc in tumors (Malykh et al. 2001), however, offered a possible solution. Specifically, Neu5Gc found in cancerous tissue in humans was initially puzzling because the inactivating mutation of CMAH, which was the loss of an entire exon, could not plausibly have been subject to a reciprocal activating mutation even in the mutagenic milieu of a tumor.

The mystery of Neu5Gc display in human tissues was solved by the discovery that dietary Neu5Gc – which is richly supplied by "red meat" and dairy products – could be taken up by cells, intercept the sialic acid pathway, and be incorporated into the host's glycans (Tangvoranuntakul et al. 2003). This observation decisively showed that the biosynthetic machinery for sialic acid was permissive to nonnatural metabolites, thus laying a conceptual foundation for sialic acid glycoengineering (in reality, the pioneers of this technology were not privy to these more recent insights provided by Neu5Gc, making the early experiments performed almost two decades ago all that much more remarkable). In the next sections of this report, a retrospective look at various supplementation strategies used to display nonnatural sialosides in living cells – organized by various classes of metabolites used to intercept the sialic acid pathway – is given.

*The sialic acid pathway can be intercepted at multiple points*

*CMP-Sialic Acid Analogs.* The first conclusive reports of the incorporation of nonnatural sialic acids into cellular glycoconjugates date from the late 1980s (Gross and Brossmer 1988) and were based on discovery that sialyltransferases were remarkably permissive for substrates bearing certain bulky functional groups (Figure 4). Remarkably, even bulky fluorophores appended to CMP-Neu5Ac (Gross and Brossmer 1988; Gross 1992; Brossmer and Gross 1994) could be biosynthetically incorporated into glycans (Gross et al. 1989; Gross and Brossmer 1995). The downside to this early nucleotide sugar-based approach was that the multiply charged analogs were not membrane permeable imposing a severe obstacle to their use in living cells where two sets of membranes had to be crossed for colocalization of the substrate with sialyltransferase inside the Golgi lumen. Consequently, a nucleotide sugar donor approach was only useful in semi-permeabilized cells or for cell-free chemoenzymatic synthesis.

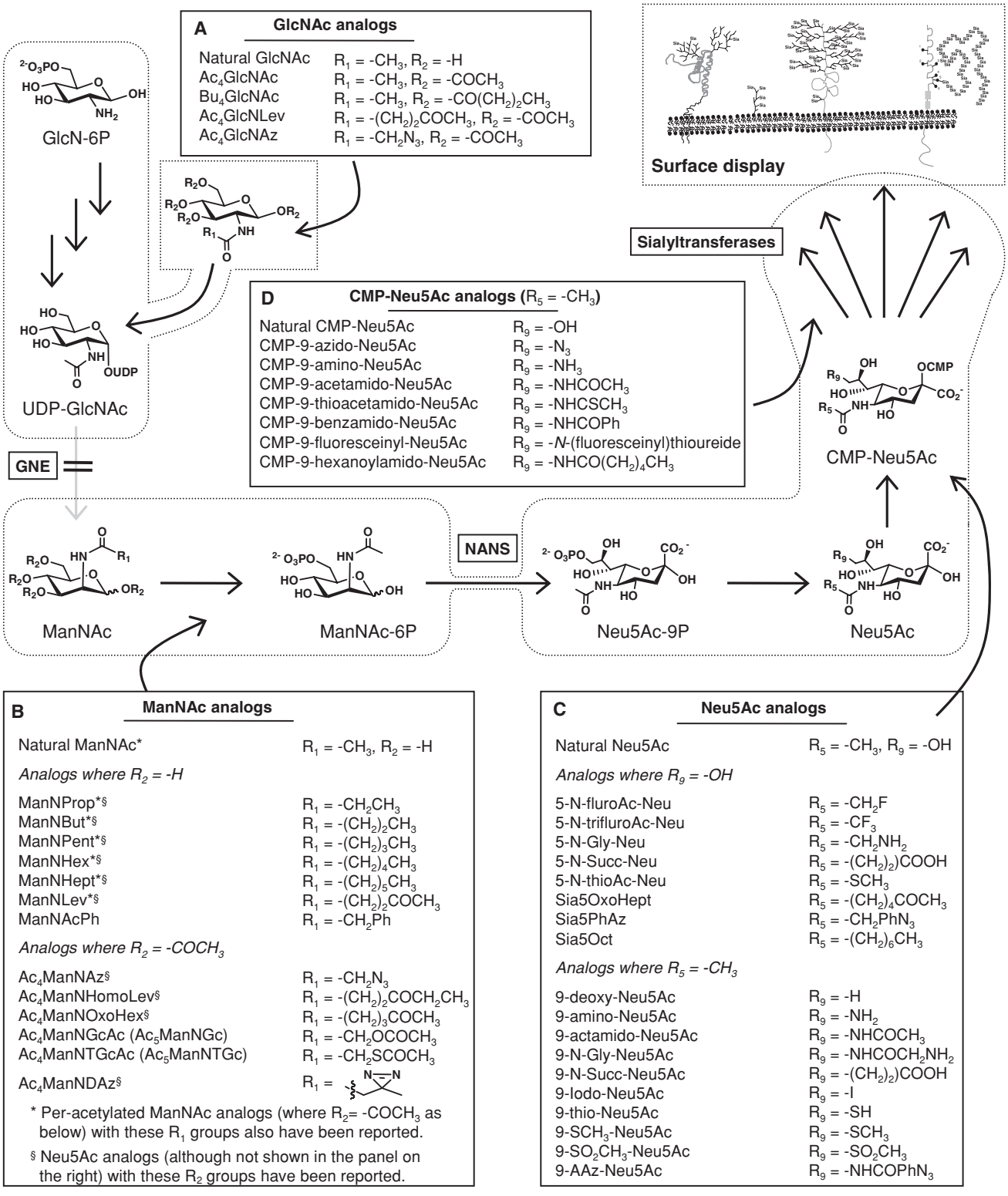


**Fig. 4.** Pathway options for sialic acid glycoengineering. Analogs can be introduced into the sialic acid metabolic machinery (as shown in detail in Figure 2) at several points. (**A**) GlcNAc analogs can enter the hexosamine pathway by salvage mechanisms but subsequent entry into the sialic acid pathway is severely restricted by the strict substrate specificity of GNE. (**B**) ManNAc analogs can feed directly into the canonical sialic acid pathway but constraints on the size and position of nonnatural modifications to this metabolite are posed by the bottleneck enzyme NANS. (**C**) Sialic acid analogs bypass the NANS bottleneck and can be modified at both the C5 (*N*-acyl) and C9 (OH) positions. (**D**) CMP-Neu5Ac analogs enter the pathway at the final stage and need only to be processed by sialyltransferases; however, the membrane impermeability of these compounds severely restricts their intake into the Golgi lumen where they are required for biosynthetic incorporation. Additional analogs, and literature references for the analogs shown in this figure, are provided in Table S2 of the *Supplementary Data* available online.

*ManNAc Analogs.* In a circuitous manner, an attempt at cancer therapy serendipitously provided an alternative strategy for introducing nonnatural monomer building blocks into surface glycans via a cell's biosynthetic machinery. Specifically, fluorinated ManNAc analogs intended to block sialylation in cancer cells were found – instead of inhibiting the biosynthetic pathway – to actually be incorporated into surface components, most plausibly sialic acid (Hadfield et al. 1983; Schwartz et al. 1983). Extending this tentative discovery in a deliberate manner, the Reutter group employed hexosamine analogs to install nonnatural monosaccharides into surface displayed glycans (Kayser et al. 1992a; Kayser et al. 1992b) and, for reasons explained below, ManNAc has remained the monosaccharide vehicle of choice for sialic acid glycoengineering. Dozens of analogs – mainly with the chemical modification intended for cell surface glycan display situated at the *N*-acyl position – have been synthesized and tested in biological assays; a sampling of these compounds is given in Figure 4 and more extensive listings are provided in the *Supplementary Data* to this article and elsewhere (Aich and Yarema 2008).

*Sialic Acid Analogs.* Sialic acid analogs, which are efficiently taken up by cells (Oetke et al. 2001; Bardor et al. 2005), provide a second viable alternative to CMP-Neu5Ac analogs for surface display of nonnatural sialosides. Many of these analogs are modified at the *N*-acyl position (see Figure 4 for representative structures) with the same functional groups found at the *N*-acyl position of ManNAc analogs (Oetke et al. 2002; Saxon et al. 2002), essentially mimicking cell surface sialoside modifications provided by the hexosamine derivatives. An important benefit of the sialic acid analogs, however, is that they bypass the "bottleneck" posed by *N*-actylneuraminic acid synthase (NANS, with the bottleneck indicated schematically in Figure 4) in two important ways. First, NANS is restrictive for long or branched *N*-acyl modifications to ManNAc (Jacobs et al. 2001; Viswanathan et al. 2003) limiting substitutions to three or less additional carbons (or equivalently sized groups such as an azide). Second, the pathway does not readily accommodate substitutions at the C6-position of ManNAc (Lawrence et al. 2000) that ultimately appear at the C9-position of sialic acid (Tanner 2005). While the C6-position *can* be modified in ManNAc analogs, this strategy leads to a large loss in efficiency (∼97% reduction, Lawrence et al. 2000)) because this hydroxyl is usually phosphorylated before ManNAc is converted to sialic acid. Consequently, the accessibility of the 9-position of Neu5Ac analogs without compromising metabolic efficiency positions sialic acid analogs as superior reagents (Oetke et al. 2002; Han et al. 2005) for modulating activities influenced the C9-OH group, such as Siglec binding. Additional scenarios where sialic acid analogs are either preferred or absolutely necessary include organisms such as certain bacteria (and possibly plants (Shah et al. 2003)) where sialic acid biosynthesis does not utilize ManNAc (Schilling et al. 2001; Goon et al. 2003) but rather depends on a direct source of sialic acids.

*GlcNAc Analogs are a Poor Option for Sialic Acid Engineering.* The previous sections describe three options (CMP-Neu5Ac, ManNAc, and Neu5Ac analogs) for cell surface sialic acid replacement. Of these, the Neu5Ac analogs are superior in several respects; in particular, they bypass the NANS bottleneck and provide options to install chemical variation at either the *N*-acyl or 9-OH positions. In reality, practical issues (such as the lower cost and synthetic tractability of ManNAc compared to sialic acid) come into play and the use of ManNAc analogs in sialic acid glycoengineering experiments continues to outpac e sialic acid. Glucosamine analogs continue the trend toward facile synthesis and economy, spurring efforts to evaluate the incorporation of GlcNAc analogs into the hexosamine pathway, which in turn feeds into the sialic acid pathway through GNE (Figure 2A and B). These efforts – exemplified by GlcNLev and GlcNDAz, which failed to be displayed on the cell surface as Sia5Lev (Yarema et al. 1998) or Sia5DAz (Tanaka and Kohler 2008), respectively – presumably were not successful because of the obstacle presented by GNE, an enzyme that appears to have strict specificity for natural UDP-GlcNAc. Similarly, as will be discussed later, GlcNAc analogs are poorly, if at all, incorporated into *N*-glycans, mucins, or GAGs, but are instead selectively partitioned into nucleocytoplasmic *O*-GlcNAc-modified proteins.

Summarizing analog choices for sialic acid glycoengineering, the first decade of work provided several options – coalescing around ManNAc analogs – for modifying sialoside display. By now the reader may be wondering if these efforts were paying off in the quest to complement – or even one-up – natural mechanisms by which cells modulate biology through glycochemistry. Therefore, even though additional exciting chemistry-based developments lay on the horizon (and have been reaching fruition of late), we next outline biological response elicited by early analogs and potential applications for the "first generation" of these compounds.

### *Applications: The impact of N-acyl-modified sialosides on biology*

*Enhancing the Immunogenicity of Carbohydrate Epitopes for Cancer Treatment.* An initial opportunity – or apprehension, depending on one's perspective – of sialic acid engineering was that the modified sugars would constitute immunogenic epitopes when introduced into the body. These concerns were given credence by reports that 9-*O*-acetylation of GD3 (Figure 2A) produced an antigenic form of this ganglioside (Cheresh et al. 1984) as well as studies showing that Neu5Gc was not only immunogenic in humans but also an important factor in the evolution and functioning of the human immune system, especially in the context of Siglec biology (Brinkman-Van der Linden et al. 2000; Crocker and Varki 2001). The demonstration that Sia5Pr (Hayrinen et al. 1995; Liu et al. 2000) and Sia5Lev could be antigenic (Lemieux and Bertozzi 2001) extended the immunogenic precedent set by Neu5Gc and 9-*O*-Neu5Ac to nonnatural sialic acids created by metabolic glycoengineering.

Today, from a perspective compiled from almost two decades of animal testing with multiple analogs, nonnatural sialic acids – despite having antigenic potential in some situations – are generally too weakly immunogenic to raise a red flag for in vivo sialic acid glycoengineering experiments. Similarly, they are typically ineffective for applications where a strong immune response is desired. The concept of deliberately trying to evoke immunogenicity (which may seem counterintuitive on the surface) once again has a natural precedent from Neu5Gc where appearance in human tumors (Malykh et al. 2001) elicits a complex interplay with the host immunity (Hedlund et al. 2008) that may have therapeutic benefits that include "tagging" the cancerous tissue for eradication by the host immune system.

In one of the first experiments where connections between modified sialic acid, tumor growth, and the immune system were explored, the Jennings lab increased the immunogenicity of tumor cells by metabolic incorporation of *N*-propionyl derivatized residues into polysialic acid. By adjusting the dose and timing of administration of the nonnatural sugar, they were able to control the targeting of an antibody specific for the altered polysialic acid (Hayrinen et al. 1995) and selectively kill tumor cells through an innovative passive immunotherapy approach (Liu et al. 2000). The Guo group has methodically extended this strategy by systematically surveying *N*-acyl groups for immunogenicity and has found that most substituents are weakly, if at all, antigenic, consistent with a lack of an overt immune response in animal studies. However, *N*-phenylacetyl analogs – when incorporated into tumor-associated carbohydrate antigens (TACA) such as sTn (Wu and Guo 2006; Wang et al. 2008) or GM3 (Chefalo et al. 2006; Wang et al. 2007) – are sufficiently antigenic to raise antibody titers (Chefalo et al. 2004). In a two-pronged strategy, a tumor bearing animal can be treated with the analog, which selectively partitions into TACA. The animal is also treated with the antibody raised against the modified sialoside, which labels the glycoengineered tumor cells and helps target them for eradication by the host's immune response.

*Modulating Viral Binding.* Based on the immune system's ability to distinguish subtle structural differences between natural sialic acids such as Neu5Ac, Neu5Gc, and 9-*O*-AcNeu5Ac (Higa et al. 1985) and to recognize nonnatural forms such as Sia5Pr and Sia5PhAc, it was intriguing to postulate that similar discrimination extended to other biological recognition phenomenon. In this vein, the modulation of viral binding by metabolic glycoengineering was evaluated and used to weaken, rather than strengthen as had been demonstrated for the immunogenicity of TACA, responses. In particular, the binding and infectivity of influenza virus – where the *N*-acyl side chain of sialic acid is in close proximity to the edge of the hemagglutinin (HA) binding pocket – are dampened by sialosides with elongated *N*-acyl chains y (Herrler et al. 1990). As surveyed in a previous review (Keppler et al. 2001), attenuation of viral binding and infectivity is typical but occasionally the opposite response does occur, as shown by the Reutter group who incubated kidney epithelial and B lymphoma cells with ManNProp, ManNBu, and ManNPent. All three precursors were metabolized into the corresponding unnatural cell surface sialic acids by both cell types and influenced polyoma virus binding but infection was either inhibited by up to 95% or – depending on the *N*-acyl group – increased by as much as 7-fold (Keppler et al. 1995). These experiments are typical of metabolic glycoengineering endeavors to date, where the biological response can often be predicted in advance but unanticipated and sometimes difficult-to-explain outcomes are by no means a rarity.

*Altering Cell Adhesion.* Sialic acid is involved in cell–cell and cell–substrate adhesion in numerous ways; well-known examples include the impact of α2,8-linked polysialic acid on NCAM, the importance of the α2,3-linked sialic acid of $sLe^X$ in leukocyte tethering and rolling, and the role of α2,6-linked sialic acids in integrin function. This information, combined with the examples provided above where sialic acid engineering altered the binding of biological molecules (antibodies and viruses) to cells, suggests that ManNAc analogs – by installing nonnatural sialosides that interact differently with their cognate receptors – are fairly general tools for altering cell adhesion. Pursuing this objective by inhibiting adhesive interactions by installing "teflon" on a cell, glycoengineering has been employed to fluorinate mammalian cell surfaces resulting in modestly decreased adhesion of HL-60 cells to fibronectin (Dafik et al. 2008). By contrast, ManNProp *promotes* binding these cells to fibronectin (Villavicencio-Lorini et al. 2002); unexpectedly, however, this gain of binding was not directly correlated with cell surface sialic acid display but rather was linked to transcriptional regulation of integrins.

*Modified Sialosides – Are they Signaling Molecules?* Corroborating evidence that gene expression is influenced by sialic acid glycoengineering has been gleaned from a decade of studies, beginning with experiments where ManNAc analogs altered the fate of embryonic neural cells (Schmidt et al. 1998) and culminating with recent evidence that ManNAc analogs can function as signaling molecules (Kontou et al. 2008), perhaps through the metabolic intermediate CMP-Neu5Ac (or an analog such as CMP-Sia5Pr) during the steps where the biosynthetic pathway transits the nucleus. In a more convoluted explanation, analog incorporation into surface glycans could alter the "sugar code" and subsequently perturb the decoding process by which lectins engage signaling pathways (Dam and Brewer 2002; Gabius et al. 2004; Gabius 2008). Mechanistically, lectin binding relies on the cluster glycoside effect (Lundquist and Toone 2002) and it is plausible that an analog such as ManNPr, which replaces up to 85% (Mantey et al. 2001) but more typically from 30 to 70% of Neu5Ac with Sia5Pr (Gagiannis et al. 2007), could affect the avidity of recognition molecules that employ multivalent sialoside recognition. Another way that nonnatural sialic acids, in particular those containing longer alkyl chains that impair carbohydrate–carbohydrate hydrogen bonding interactions, could indirectly alter gene expression is through their impact on the biophysical properties of lipid rafts (or, "the glycosynapse" (Hakomori 2002)) that "lubricate cell signaling" (Allende and Proia 2002). Clearly, resolving these complex issues will require a considerable future effort, which will be well repaid if the analogs become a new class of reagents for controlling signaling pathways.

As briefly surveyed in this section, two decades of sialic acid metabolic glycoengineering experiments, which have intentionally (or serendipitously) emulated natural mechanisms that employ structurally altered sialosides to modulate biology, have spawned interesting applications (e.g., novel cancer immunotherapy) as well as unsolved msyteries (e.g., the question "how do ManNAc analogs engage transcription?"). We will next shift gears and focus on a development that – depending on one's perspective – either exacerbates the complexity of these challenges or conveniently sidesteps them.

## Introducing "chemical biology" and bioorthogonal chemistries

### *Chemoselective ligation and bioorthogonal chemistry – Exemplified by the ketone*

An important conceptual advance in metabolic glycoengineering occurred when chemical functional groups not normally found in sugars were incorporated into analog design. The

impetus behind efforts to endow sialic acids with bioorthogonal chemical functional groups was the enticing ability to perform chemoselective ligation reactions on the surfaces of living cells and even in vivo (Lemieux and Bertozzi 1998). In these reactions, the two participating functional groups – which ideally are abiotic – have finely tuned reactivity that avoids interference with coexisting functionalities, and they recognize only each other while ignoring their cellular surroundings ultimately forming stable covalent adducts under physiological conditions (Saxon and Bertozzi 2000).

The ketone group—incorporated into the sialic acid pathway using ManNLev (Figure 4B)—introduced the concept of bioorthogonal chemistry to sialic acid glycoengineering (Mahal et al. 1997); ketones were selected for these pilot experiments because they are not found in proteins, lipids, or carbohydrates and are thus absent from the cell surface. Moreover, ketones already had been successfully exploited for drug delivery via chemoselective ligation reactions (Rideout 1986; Rideout et al. 1990) demonstrating the concept of bioorthogonal ketone-based chemistry in living cells. Once a ketone is displayed on the cell surface (Figure 5A), chemical nuances of chemoselective ligation partners open interesting possibilities. For example, conjugation with hydrazide-derivatized ligands – which does not occur rapidly at physiological pH – can be driven forward by an excess of reagent. Once internalized into a low pH vesicle the reaction rate increases substantially but, in the absence of excess unligated ligand, the reverse reaction takes place liberating the ligand inside a cellular compartment. If the cell in question happens to be diseased and in need of detection or eradication, this strategy can be used to load a cell with an MRI contrast agent (Lemieux et al. 1999), a small molecule drug (Nauman and Bertozzi 2001), a gene vector (Lee et al. 1999), or a toxin such as ricin (Mahal et al. 1997). By contrast, aminooxy-derivatized ligands are not pH sensitive and ligands delivered by this route are recycled from internal vesicles back to the cell surface (Nauman and Bertozzi 2001); this strategy is preferred when prolonged surface display of a ligand is beneficial.

Despite its utility in proof-of-concept experiments and interesting chemical properties, the ketone was far from an ideal chemoselective ligation partner. Biologically, the ketone is only bioorthogonal on the cell surface; inside the cell, metabolites with ketones (and their more reactive aldehyde cousins) are common and compete for chemoselective ligation of Sia5Lev with hydrazide or aminooxy groups. From a chemical perspective, the ketone-hydrazide coupling is optimal at a lethal pH of $\leq$5; Sia5Lev labeling, therefore, is typically performed at an intermediate pH of 6.5 where both cell viability and labeling efficiency are compromised by about 75% (Yarema 2002). Although interest in ManNLev continues, with progress being made toward improved bioorthogonal reaction conditions (e.g., aniline-promoted aminooxy coupling (Zeng et al. 2009)) and recently reported applications (e.g., tracking trogocytosis (Daubeuf et al. 2007) and probing the mechanism of gangliogenesis in the enteric nervous system (Faure et al. 2007)), additional options for bioorthogonal metabolic glycoengineering chemistry have now been developed that provide the metabolic glycoengineer with attractive alternatives.

*Additional bioorthogonal functional groups*

*Azides.* Azides became the second (chronologically speaking) bioorthogonal functional group used in sialic acid glycoengineering when $Ac_4$ManNAz was used to install Sia5Az into surface sialosides (Figure 5B). Azides have several advantages over ketones with perhaps the most striking being that they are completely abiotic allowing intracellular as well as cell surface chemoselective ligation strategies to be pursued. From a chemical perspective, the original azide reaction strategy employed an elegant modification of the Staudinger reaction (Saxon and Bertozzi 2000; Saxon et al. 2002). More recently, glycan-displayed azides have been exploited in the increasingly ubiquitous "click" reaction. One pitfall of click chemistry, however, is the copper catalysis, which can be cytotoxic for applications where ongoing cell viability is required; a solution to this problem may lie in a strain-promoted click reaction (Baskin et al. 2007) compatible for use in living cells and in vivo imaging (Laughlin et al. 2008).

*Alkynes and Photoactivated Crosslinking Sugars.* Click chemistry was first used in metabolic glycoengineering by installing the azide group into surface-displayed glycans; the reverse strategy where the alkyne is displayed in the glycan (Figure 5C) has also been demonstrated (Hsu et al. 2007). The alkyne exemplifies how the current modestly sized bevy of bioorthogonal chemical strategies now available has been constantly growing; for example photoactivatable crosslinking sugars have been recently reported. In one manifestation, the Kohler group developed the versatile diazirine-containing ManNAc analog $Ac_4$ManNDAz (Tanaka and Kohler 2008) (Bond et al. 2009) (Figure 5D) and in another, the Paulson group installed the photo-activated aryl azide group into CD22-displayed glycans of CD22 (Han et al. 2005; Yarema and Sun 2005) (Figure 5E). As discussed earlier in the context of pathway bottlenecks, the C9-position of sialic acid was able to accommodate the bulky aryl azide group, while the more restrictive *N*-acyl position was permissive for the diazirine modification.

*Thiols.* The thiol group – installed into the glycocalyx via thioglycolyl analogs (e.g., $Ac_5$ManNTGc, (Sampathkumar, Jones, Yarema 2006), Figure 5F) – possesses its own unique set of pros and cons. Unlike azides or diazirines, thiols are not abiotic but are common within a cell and do not even have the advantage of the ketone of being absent from the cell surface. Thiols, however, are not found in the glycocalyx, the intended destination for modified sialosides, positioning Sia5TGc with a degree of chemical uniqueness. Also, thiols are well tolerated and rather inert within the reducing environment of the nucleocytoplasm (if anything, they may be protective if they function analogously to glutathione, although there is not yet evidence for this premise). Once thiols reach the oxidizing conditions found in the secretory pathway, where protein disulfide bonds are generated (Frand et al. 2000), thiolated sialosides also form *cis*-disulfide linkages that are maintained in the slightly oxidizing cell surface milieu under tissue culture conditions (Sampathkumar, Jones, Yarema 2006). Rather than being an impediment, the "masking" of thiols in this manner provides additional chemical versatility insofar as certain ligation partners, such as gold, bind to both forms of sulfur whereas others, such as maleimide, only react with free thiols. Therefore, differential binding can be achieved depending on the probe used and modulation of oxidizing conditions (e.g., disulfides can be "unmasked" and reactivated with mild TCEP treatment (Sampathkumar, Li, Jones, et al. 2006) or controlling the cysteine/cystine ratio in the culture medium)

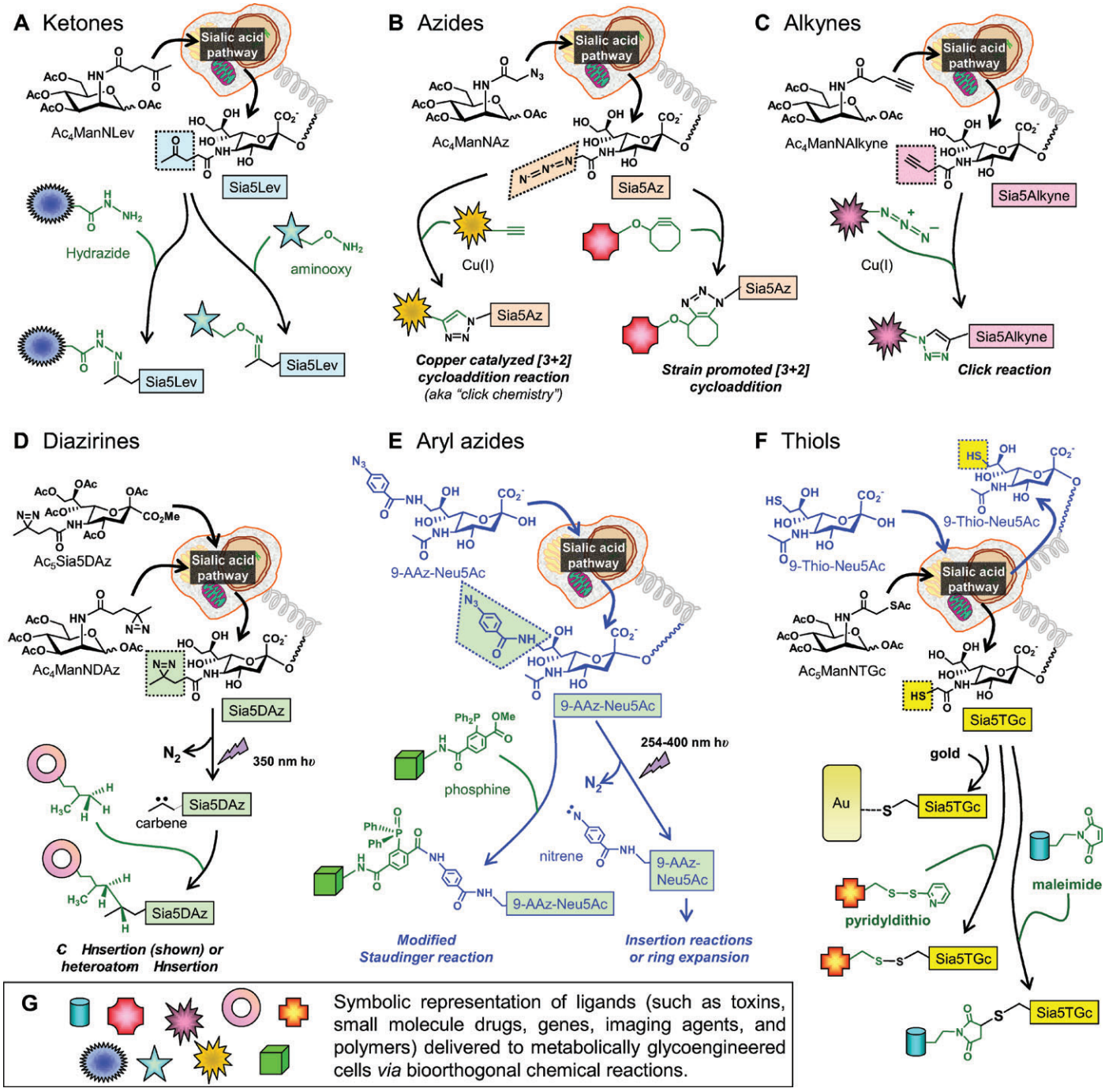


**Fig. 5.** Bioorthogonal ligation reactions, exemplified by glycan-displayed ketones, azides, alkynes, diazirines, aryl azides, and thiols. (**A**) Ketones displayed on cells incubated with ManNLev or $Ac_4$ManNLev (Jacobs et al. 2001) can be ligated with hydrazide in a pH-dependent reaction (Yarema et al. 1998) or with aminooxy in a pH-independent reaction (Nauman and Bertozzi 2001). (**B**) Azides can be displayed in sialosides using $Ac_4$ManNAz and ligated with phosphines using the Bertozzi-modified Staudinger reaction (shown for aryl azides in (E) (Saxon and Bertozzi 2000) or via [3+2] cycloaddition with alkynes using copper-catalyzed click reactions or strain-promoted cyclootyne (Agard et al. 2004). (**C**) Conversely, alkynes can be displayed in glycans (e.g., by using $Ac_4$ManNAlkyne) and ligated with azide-derivatized ligands or fluorophores (Hsu et al. 2007). (**D**) The diazirine-containing analog $Ac_4$ManNDAz (or $Ac_5$Sia5DAz) installs photoactivatable Sia5DAz onto the cell surface where irradiation produces carbenes that undergo insertion reactions with neighboring biological molecules (Tanaka and Kohler 2008), thus crosslinking in situ sialoside ligands. (**E**) Aryl azides can be incorporated into sialic acid via 9-AAZ-Neu5Ac, which can then be subject to the modified Staudinger reaction referenced in (**B**) or irradiated to provide crosslinking reactions similar to those resulting from diazirine in (**D**) (Han et al. 2005). (**F**) Thiols can be incorporated into sialosides using $Ac_5$ManNTGc (Sampathkumar, Li, Jones, et al. 2006) or 9-thio-Neu5Ac (Oetke et al. 2002), which can then bind to gold or undergo the chemoselective reaction with pyridyldithio- or maleimide-derivatized ligands. (**G**) In this figure, ligands being delivered to a cell are represented symbolically with specific examples provided in the main text and references therein.

thereby providing an additional layer of control, on a time scale of seconds to minutes, rather than the multi-day intervals needed for de novo biosynthetic display.

*The second wave of – chemistry-based – applications*

*Tools for Analog Detection and Glycan Imaging.* In tandem with the development of analogs endowed with chemical functionalities not naturally found in sugars, the metabolic glycoengineering community has sought to develop applications for this new technique. The ability to tag sialic acids with unique chemical functionality in living systems was first exploited for targeting with imaging agents, such as fluorophores, for analysis of surface expression by flow cytometry (Mahal et al. 1997) or confocal microscopy (Sampathkumar, Li, Jones, et al. 2006). These methods allowed estimates to be made of the complete transit of nonnatural analogs through the biosynthetic pathway (Yarema et al. 1998) and enabled recycling kinetics to be probed by using chemical ligation reactions that were (or were not) sensitive to the lower pH of the endosomal vesicles (Nauman and Bertozzi 2001). In the past few years, chemoselective labeling has progressed from cell culture experiments and rodent testing where samples are harvested before analysis (Kayser et al. 1992a; Gagiannis et al. 2007) to imaging in living animals such as zebrafish (Laughlin et al. 2008).

*Emerging Glycomics Techniques.* Empowered by the ability to label glycans with unique chemical functional groups that subsequently can be selectively captured and isolated from a complex biological milieu, metabolic glycoengineering is becoming a valued contributor to glycoproteomics. To date, variations of the "tagging-via-substrate" strategy (Sprung et al. 2005; Nandi et al. 2006), which have mostly been applied to sugars other than sialic acid (as discussed below), have been particularly fruitful for identifying *O*-GlcNAc modified proteins. In one approach, an analog with a chemical tag (such as a ketone or azide) is metabolically incorporated directly into the glycoconjugate, replacing a natural *O*-GlcNAc (for example) with a *O*-GlcNLev or *O*-GlcNAz residue (Khidekel et al. 2003, 2004; Vocadlo et al. 2003; Gurcel et al. 2008). In a variation, Qasba and colleagues have developed mutant glycosyltransferases that label glycoconjugates carrying specific sugar moieties, such as *O*-GlcNAc, with monosaccharides endowed with bioorthogonal functional groups (Qasba et al. 2008; Boeggeman et al. 2009). Moving forward, as commercialization of this methodology reaches fruition coupled with analogs that target a greater range of glycosylation pathways, metabolic glycoengineering will undoubtedly gain importance for glycomics efforts (Campbell and Yarema 2005).

*Drug Delivery and Cancer Targeting.* An increasing number of modern drugs consist of large molecules such as viruses, peptides, and nucleic acids. Delivery to a particular organ or cell type and subsequent uptake by the target can be severely hampered by the lack of specific receptors or transport mechanisms for these drug candidates leading to efforts to chemically engineer cell surfaces to enhance their interactions with drugs and drug delivery systems. In one example, synthetic peptide-based receptors that control the selective permeability of the cell membrane to large drug molecules have been inserted into cell surfaces to facilitate the uptake of exogenous proteins (Martin and Peterson 2003). Of relevance to sialic acid glycoengineering, metabolically engineered glycans, such as ketone-bearing Sia5Lev sialosides (Figure 5A), represent new synthetic receptors for drug delivery. Already, ManNLev has been used to install ketones for chemoselective-ligation-based killing of cancer cells via ricin (Mahal et al. 1997, 1999); small molecules, such as the cancer drug doxorubicin, have been delivered by this manner (Nauman and Bertozzi 2001), as they have much larger entities, such as the adenovirus used for gene therapy (Lee et al. 1999).

Moving to a larger size scale, Iwasaki and coworkers decorated 2-methacryloyloxyethyl phosphorylcholine polymer nanoparticles bearing hydrazide groups (PMBH) with anticancer drugs such as doxorubicin or paclitaxel. Exposure of these constructs, which react with the ketone groups of Sia5Lev, to ManLev-treated cells reduced viability while untreated cells remained unaffected indicating that the drugs were selectively delivered to the glycoengineered cells (Iwasaki et al. 2007). Work reported by Iwasaki and colleagues also raised the intriguing possibility that polymers capable of recognizing engineered glycans, such as those bearing Sia5Lev, could be used to adsorb cells and thus function as an adhesive filter to separate cancer cells from their normal counterparts (Iwasaki et al. 2005).

*Polymer-based Adhesion Approaches – Toward Creating Artificial Cell Niches.* An interesting observation from the experiments reported by Iwasaki and coworkers was that after metabolically glycoengineered cells were attached to a polymer, cell viability could be maintained (or diminished) through the chemistries present on the surfaces of both the cell and the material. This finding builds on over a decade of reports – beginning with the abolishment of normal contact-dependent inhibition of cell growth in cell incubated with ManNProp (Wieser et al. 1996) – that sialic acid influences adhesion-dependent cell–cell or cell–ECM interactions. The contributions of chemical functional groups installed into sialisodes via metabolic glycoengineering became apparent when Shakesheff's group demonstrated that aldehyde groups can be effectively introduced into the cell surface glycoconjugates of adherent cell monolayers by mild periodate oxidation and exploited to aggregate cells (De Bank et al. 2003). This technique, which can be mimicked by replacing aldehyde generated through chemical oxidation with ketones derived from ManNLev, demonstrated that metabolic glycoengineering can be valuable for engineering the surface properties of cells to manipulate their adhesion, behavior, and function.

To build on the growing evidence that adhesion plays a major role in cell fate during development, our laboratory used $Ac_5$ManNTGc to install thiols onto the cell surface as the Neu5TGc form of sialic acid (Sampathkumar, Jones, Yarema 2006) (Figure 5F). In contrast to thiols situation in membrane proteins that have only limited accessibility to the microenvironment (due, for example, to formation of disulfide bridges), thiols displayed on sialic acid are at the outer periphery of the glycocalyx and are highly accessible. As a result, normally nonadhesive blood cells expressing Neu5TGc spontaneous undergo cell–cell clustering and readily adhere to a gold matrix (either to a 2D flat surface (Sampathkumar, Li, Jones, et al. 2006) or 3D nanofibers. Interestingly, Neu5TGc triggered the differentiation of a human embryoid body-derived germ cell line grown on gold toward a

neuron lineage, and the growth of normally nonadherent Jurkat cells on the nanofibers led to adhesion and dramatic changes in morphology without compromising viability. These results, combined with earlier reports that metabolic glycoengineering alters the fate of embryonic neural cells (Schmidt et al. 1998) and the subsequent finding by Schnaar's group that Neu5Gc ablates MAG binding and spurs neurite outgrowth (Collins et al. 2000), position metabolic glycoengineering as a fascinating tool for future efforts in tissue engineering and regenerative medicine.

## Extending metabolic glycoengineering to additional pathways

### *The connectness of glycosylation pathways poses potential problems*

Isotope labeling studies have shown that exogenously supplied monosaccharides (such as glucose, which can enter the hexosamine pathway shown in Figure 2A) can broadly partition into many metabolites including other sugars (Jin et al. 2004). Extending this concept to metabolic glycoengineering, the ability of *natural* hexosamines to be interconverted through the connectedness of glycosylation pathways (as briefly outlined in Figure 6 and shown in more detail in other publications (Goon and Bertozzi 2002; Murrell et al. 2004) or in online resources such as the KEGG Pathway database (Hashimoto et al. 2006; Kanehisa et al. 2006)) raises the possibility that the nonnatural monosaccharide analogs could become broadly distributed throughout all glycans. While not hindering some uses of this technology, such as attaching cells to a tissue culture scaffold or directing therapeutic agents to cancer cells experiencing a global increase in glycosylation, this possibility does render certain applications less meaningful. For example, glycomics experiments where the objective is to metabolically label and isolate a specific class of glycans (such as sialic acids targeted by ManNAc analogs, mucins targeted by GalNAc analogs, or *O*-GlcNAcylated proteins targeted by GlcNAc analogs) would suffer.

Empirical observations have largely discounted the possibility of unruly or universal partitioning of monosaccharide analogs into nontargeted glycans thus debunking the theoretical possibility that analog targeting is not feasible. Fortuitously, rigorous substrate discrimination of key enzymes situated at critical junctures between various nucleotide sugars facilitates precise targeting to various pathways, with ManNAc, GlcNAc, and GalNAc all experiencing distinctive metabolic fates and selective incorporation into a restricted set of surface-displayed glycans (Figure 6). The ability of pathways to discriminate between hexosamine isomers has spawned exciting developments in metabolic glycoengineering in recent years as the methods pioneered with sialic acid have been extended to additional glycosylation pathways, as summarized next.

### *Metabolic glycoengineering: Moving beyond sialic acid*

*GlcNAc.* The selective utilization of nonnatural monosaccharide analogs by one (but not by a second or even third) pathway branch is epitomized by GlcNAc. The natural form of this monosaccharide can feed into sialic acid metabolism by one of two mechanisms. First, GlcNAc can be directly converted to ManNAc by GlcNAc 2-epimerase (Luchansky et al. 2003); alternately, it can be phosphorylated and intercept the hexosamine pathway via a salvage mechanism (Hinderlich et al. 2000; Ligos et al. 2002; Weihofen et al. 2006) and subsequently be converted to UDP-GlcNAc, which is the canonical feedstock for sialic acid biosynthesis (see Figure 2). Despite these possible routes, an early experiment showed that GlcNLev – the ketone-derivatized isomer of ManNLev (see Figure 4) – was not incorporated into cell surface elements (Yarema et al. 1998) thereby indicating that this GlcNAc analog was not converted to its ManNAc counterpart and thus was unable to gain access to the sialic acid pathway.

The lack of surface display of ketones in GlcNLev-incubated cells was consistent with a complete inability of these analogs to enter the hexosamine pathway. This possibility, however, was eliminated by a study that showed that GlcNAc analogs *do* partition into *O*-GlcNAcylated nucleocytosolic proteins (Khidekel et al. 2004); this result established that GlcNAc analogs do intercept the hexosamine pathway and are converted into the corresponding nonnatural forms of UDP-GlcNAc required for *O*-GlcNAcylation of proteins (as shown by the OGT-catalyzed reaction in Figure 6). As also outlined in Figure 6, other fates for UDP-GlcNAc besides conversion into ManNAc or use in *O*-GlcNAcylation include initiation of *N*-glycan biosynthesis by DPATG1, serving as a substrate for MGAT1/5 "branching" enzymes, or conversion to UDP-GalNAc by GALE. The lack of surface display of GlcNAc analogs indicates that modified forms of UDP-GlcNAc (such as UDP-GlcNLev or UDP-GlcNDAz) are not used by any of the GlcNAc transferase family members; neither are they converted to ManNAc by GNE, and finally, they are not epimerized to UDP-GalNAc. Instead, of the many enzymes that process natural UDP-GlcNAc, only OGT is highly permissive for nonnatural variants of this versatile metabolite.

*GalNAc.* Unlike GlcNAc analogs that do not gain biosynthetic access to surface glycans, GalNAc analogs are successfully incorporated into cell surface elements via biosynthetic routes. This feat presumably occurs through analog utilization by salvage pathways (e.g., *N*-acetylgalactosamine 1-kinase, GALK2 (Thoden and Holden 2005) and UDP-GalNAc pyrophophorylase (UAP1 or AGX1) (Wang-Gillam et al. 1998; Bourgeaux et al. 2005)). Once a GalNAc analog is converted to the nucleotide sugar (e.g., an UDP-GalNAc analog), it competes for replacement of natural GalNAc found in *O*-linked glycans (Hang et al. 2003) and (most likely) GAGs (Hang and Bertozzi 2001).

*Fucose.* The final monosaccharide that has (so far) shown facile analog incorporation into cell surface glycans in mammals is fucose. On one hand, this ability is not surprising insofar as oral supplementation corrects the metabolic defect found in Type 2 leukocyte adhesion disorder (Marquardt et al. 1999). This result established the ability of exogenous fucose, as well as analogs (Sawa et al. 2006; Hackenberger and Hinderlich 2007), to intercept the biosynthetic pathway and be utilized by the enzymes involved in the installation of fucose into surface glycans (i.e., FUK, FPGT, and a fucosyltransferase, Figure 6). On the other hand, biosynthetic processing of fucose analogs breaks the mold set by amino sugars as exclusive vehicles for metabolic glycoengineering. It is tempting to speculate that the increased biosynthetic permissivity for fucose compared to other hexoses is a consequence of its configuration as a rare L-sugar, compared

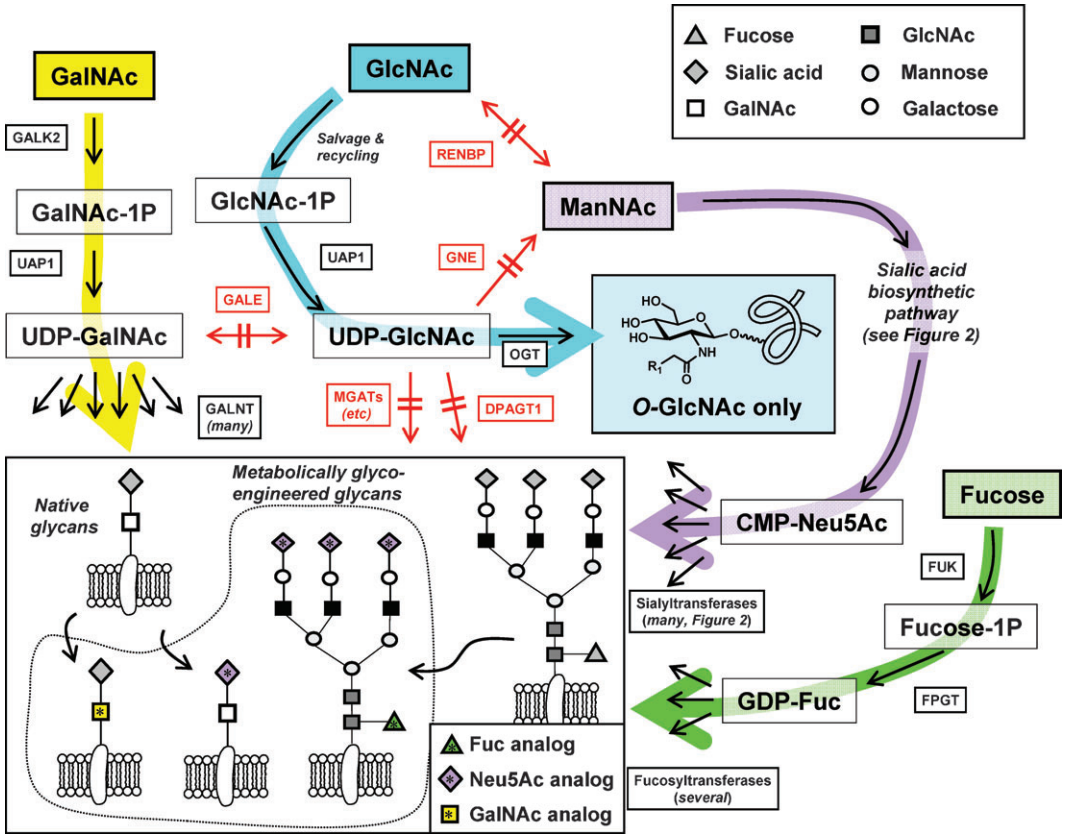


**Fig. 6.** Selective pathway utilization of analogs is ensured by the nonpermissivity of enzymes at critical junctures. The interconnectedness of glycosylation pathways linking hexosamines (and fucose) used in metabolic glycoengineering is diagrammed indicating enzymes that are restrictive to analog utilization (the complete names of the enzymes are provided in Table S1 in the *Supplementary Data* available online). The restrictive nature of key enzymes results in the partitioning of GalNAc, GlcNAc, ManNAc, and fucose analogs into selectively targeted classes of mature glycans (inset, indicated with the starred symbols).

to the more common D-configuration of other mammalian hexoses and hexosamines.

*Precise targeting – Remaining ambiguities*

As outlined above, a considerable amount of information describing the metabolic utilization of monosaccharide analogs and their selective partitioning into targeted classes of glycans has been compiled. Many unanswered questions remain, however; for example, do any of the 20 sialyltransferases shown in Figure 2 – or the similar suites of GalNAc- and fucosyltransferases alluded to in Figure 6 – have unique specificities for analogs? To date, evidence points in both directions. On one hand, when natural flux through the sialic acid pathway is restricted, thus biasing the biosynthetic incorporation of nonnatural sialosides such as Sia5Prop (Mantey et al. 2001), up to 85% of the native sialosides can be replaced with their engineered counterparts. Moreover, nonnatural sialic acids have been detected broadly in *N*-linked and *O*-linked glycans as well as in GSL; similarly, they occur in the entire range of α2,3-, α2,6-, and α2,8-linked sialosides under conditions of high flux. A more interesting, and so far untested, scenario involves what occurs at very low levels – e.g., 1% or 0.1% – of replacement and asking the question "would the nonnatural metabolites selectively partition into certain classes of glycans when the firehose supplying the pathway is replaced with an eyedropper?" To date, preliminary but intriguing evidence of such discrimination comes from studies that show α2,8-sialyltransferases respond selectively to ManNAc analogs (Mahal et al. 2001; Horstkorte et al. 2004), likely through the interaction with downstream CMP-Neu5Ac metabolites (Miyazaki et al. 2008). If family member-specific analog processing proves to be a general feature of glycosyltransferases, an additional layer of control may be possible that entails specifically directing nonnatural moieties to (or away from) α2,3-, α2,6- or α2,8-linked sialosides situated primarily on proteins or lipids.

## Solving a practical problem leads to new challenges and opportunities

*Designing analogs for increased efficiency leads to metabolic quirks*

An issue faced by a practical-minded metabolic glycoengineer is the generally modest (or even abysmal) efficiency at which nonnatural analogs enter a cell; levels as high as 30–50 millimolar are often needed to maximize cellular responses. In some cases, the biosynthetic pathway cannot be saturated even at 75 or 100 millimolar, at which point cells cultured in vitro suffer nonspecific cytotoxicity due to osmotic imbalance. Moving to in vivo experiments, costs for medium-sized or large animal testing becomes prohibitive. Exploiting an age-old solution – exemplified by aspirin where cell-impermeable hydrophilic compounds are rendered more lipophilic through

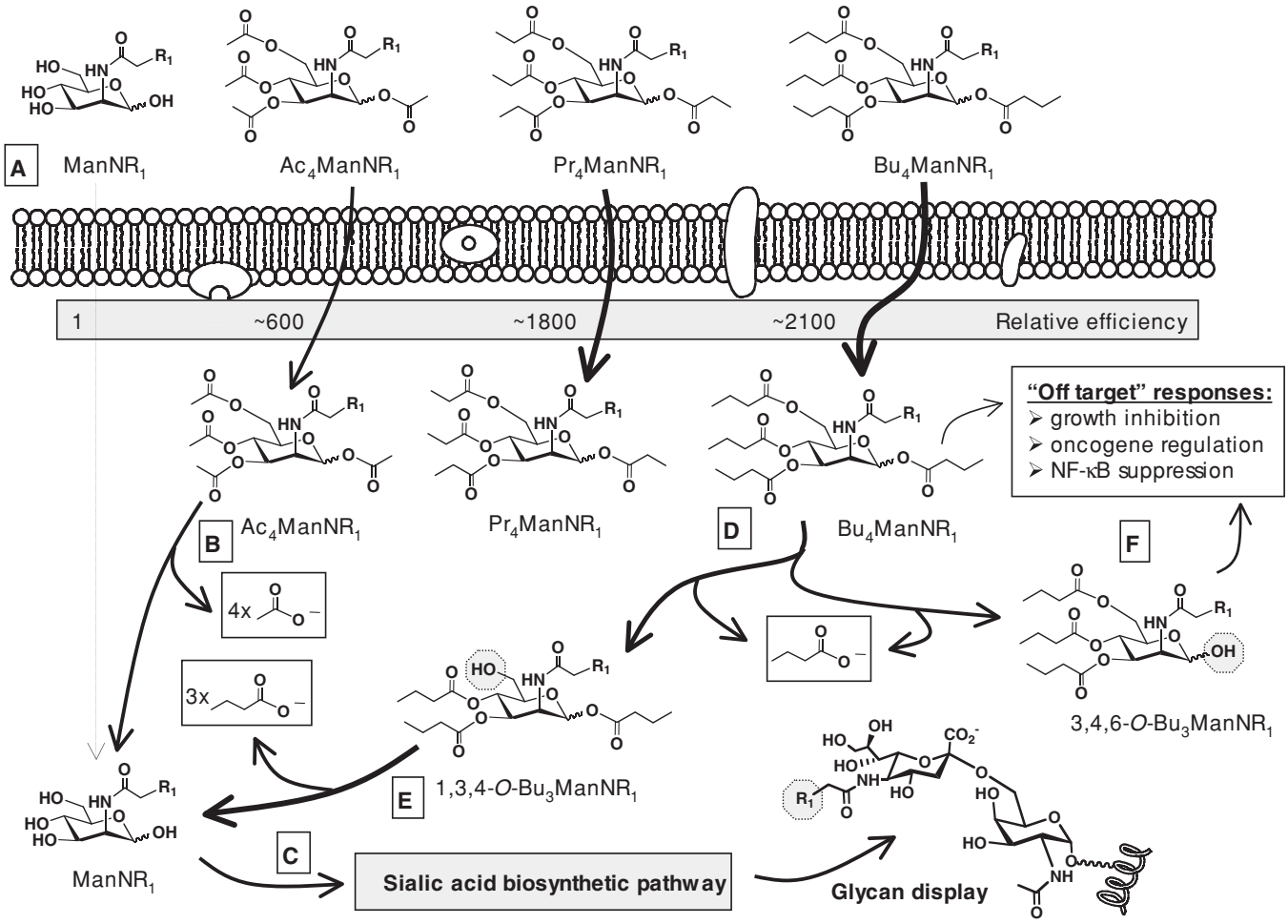


**Fig. 7.** SCFA-analog hybrids enhance cellular uptake and endow analogs with novel activities. (**A**) Peracetylated, perpropionylated, and per*n*-butanoylated analogs are taken up by cells with progressively greater efficiency, presumably due to enhanced lipophilicity that facilitates transporter-independent cellular uptake (i.e., membrane diffusion). (**B**) Once inside a cell, the SCFA groups, such as acetate, are removed by nonspecific esterases regenerating the "core" sugar (**C**) that – in the case of ManNAc – enters the sialic acid pathway en route to surface glycan display ("$R_1$"-modified examples of peracetylated analogs are shown in Figure 5). (**D**) Perbutanolylated hexosamines, represented by $Bu_4ManNR_1$, generate *n*-butyrate upon esterase processing that can modulate transcription through HDACi activity as well as the core sugar that can access the sialic acid pathway (**C**). Along the route to complete SCFA hydrolysis, tributanolyated ManNAc isomers elicit dramatically different cellular effects with (**E**) "1,3,4-*O*-$Bu_3$" analogs supporting high flux with negligible cytotoxicity while (**F**) "3,4,6-*O*-$Bu_3$" isomers trigger a bevy of NF-κB-associated "off-target" responses.

ester derivatization with short chain fatty acids (SCFA) such as acetate (Lavis 2008) – sugar analogs have been peracetylated to increase cellular uptake (Hadfield et al. 1983; Sarkar et al. 1995; Jones et al. 2004). In the case of ManNAc, evidence that increased lipophilicity enhances uptake was provided by an experiment where propionyl- ($Pr_4ManNAc$) and *n*-butanoyl- ($Bu_4ManNAc$) analogs were used with increased efficiency compared to peracetylated ManNAc ($Ac_4ManNAc$, Figure 7A) (Kim, Sampathkumar, et al. 2004). Once inside a cell, the SCFA moieties (e.g., acetate, propionate, or *n*-butyrate, Figure 7B) of *O*-acylated ManNAc analogs are removed by nonspecific esterases generating four equivalents of SCFA and the core sugar, which can then intercept and transit the targeted biosynthetic pathway (Figure 7C).

Although SCFA-monosaccharide hybrid molecules have proved to be valuable agents in dozens of studies, puzzling wrinkles arose that threatened the broad use of these compounds. Peracetylated ManNAc analogs (several examples are shown in Figure 5), for example, proved to be mildly toxic in cell culture whereas their fully unprotected counterparts were not (Kim, Sampathkumar, et al. 2004). While a nagging hindrance, especially when trying to achieve robust labeling with very high analog concentrations, analog cytotoxicity has generally been a manageable issue that can be worked around. As an illustration, fluorinated ManNAc analogs can be highly toxic (Hadfield et al. 1983) but their sialylated counterparts were not (Dafik et al. 2008). Moreover, cytotoxicity reported during in vitro experiments has not extended systematically to in vivo experiments.

Nonetheless, the mechanism behind analog toxicity intrigued our laboratory. Obvious explanations, such as detergent activity that disrupted membranes, have been discounted (this possibility was ruled out, in part, by the development of resistant cell lines (Kim, Jones, et al. 2004) and lack of an effect in liposome leakage assays (our laboratory, unpublished results). Similarly, HDACi activity emanating from hydrolyzed SCFA – while playing a minor role – could not explain the potency of various analogs (Sampathkumar, Jones, Meledeo, et al. 2006; Elmouelhi et al. 2009). For example, despite having equal

equivalents of SCFA, $Ac_4$ManNLev was considerably more toxic than the non-ketone containing counterpart $Ac_4$ManNPent or $Ac_4$ManNOxoHex where the ketone was moved down the chain one unit (Kim, Sampathkumar, et al. 2004). None of these analogs were toxic in the nonacetylated form or when equimolar concentrations of acetate were added to the culture to more accurate mimic the hydrolysis products of the prodrug. These results suggested that SCFA-ManNAc analogs have structure–activity relationships (SAR) that engage cellular receptors and elicit off-target effects through "scaffold-dependent" or "whole-molecule" mechanisms.

*SCFA-monosaccharides exemplify "carbohydrates as a scaffold for drug discovery"*

Over the past year, the "scaffold-depend" activity of SCFA-hexosamine hybrid molecules has come into focus through experiments that were initially inspired by the observation that $Bu_4$ManNAc was more effective at killing cancer cells than perbutanoylated mannose ($Bu_5$Man), a compound that only transiently inhibited the growth of the cells (Sampathkumar, Jones, Meledeo, et al. 2006). Similarly, $Bu_4$ManNAc inhibited the invasive tendencies of metastatic breast cancer cells whereas $Bu_5$Man did not (Campbell et al. 2008). Because the hexosamine analog was superior in both respects to $Bu_5$Man, a clinically tested cancer drug candidate that held modest efficacy (Sampathkumar, Campbell, et al. 2006), we anticipated testing $Bu_4$ManNAc in vivo and noted that a tributanoylated hexosamine would have a favorable Lipinski rule of five properties for oral availability and proceeded to evaluate two isomers: 1,3,4-*O*-$Bu_3$ManNAc (Figure 7E) and 3,4,6-*O*-$Bu_3$ManNAc (Figure 7F).

Based on long-held dogma that the biological activity of SCFA-hexosamines results from their hydrolysis products, we expected that 1,3,4-*O*-$Bu_3$ManNAc and 3,4,6-*O*-$Bu_3$ManNAc would elicit identical cellular responses because they both produce three equivalents of *n*-butyrate and one equivalent of ManNAc upon complete hydrolysis. Therefore, the dramatically different activities of these isomers, where 1,3,4-*O*-$Bu_3$ManNAc lacked the off-target responses of 3,4,6-*O*-$Bu_3$ManNAc but instead provided high flux with negligible toxicity (Aich et al. 2008), were surprising. In relation to metabolic glycoengineering, these SAR will allow researchers to carefully match analog design to an intended application. For example, 1,3,4-*O*-$Bu_3$ManNLev lacks the toxicity of peracylated ManNLev analogs and supports highly efficient installation of Sia5Lev into surface glycans (Aich et al. 2008). In other situations, exemplified by 3,4,6-*O*-$Bu_3$ManNAc, the "off-target" responses (Figure 7F) hold attractive anti-cancer properties that may be valuable of themselves or important complements to toxin- (Mahal et al. 1997) or immune-mediated (Liu et al. 2000), analog-based approaches to cancer treatment. More broadly, the SAR exhibited by 1,3,4-*O*-$Bu_3$ManNAc and 3,4,6-*O*-$Bu_3$ManNAc provide an outstanding example of the rise of carbohydrates as templates for drug discovery, a topic reviewed in detail elsewhere (Meutermans et al. 2006).

## Summary and conclusions

The first two decades of metabolic sialic acid glycoengineering have largely been chemistry driven but with enough biology added to the mix to firmly establish this methodology as a potent new tool for the experimental glycobiologist and pique the interest of the clinician. Moving forward, advances in chemistry are sure to continue as the pace of discovery has accelerated in the synthesis of analogs that target new pathways, enable new bioorthogonal chemical strategies, and endow the analogs with increased efficiency in recent years. Efforts to further understand mechanism are also in full swing as fascinating – albeit vexing – new wrinkles are being thrown to the cell biologist, one of which is the ability of analogs to modulate cell signaling pathways and gene expression by unanticipated mechanisms. Finally, although translation of sialic acid glycoengineering methods to commercial products and the clinic therapies has been painstakingly slow (in part because of ongoing advances in the basic chemistry and the need to understand underlying mechanisms before rationale applications can be pursued) progress is now being made on both fronts and we foresee an optimistic future for this burgeoning technology.

## Supplementary Data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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## Conflict of interest statement

None declared.

## Abbreviation

GSL, glycoshingolipids; NCAM, neural cell adhesion molecule; PSA, polysialic acid; TACA, tumor-associated carbohydrate antigens.

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