Metabolic Glycoengineering

International Edition: DOI: 10.1002/anie.201601123
German Edition: DOI: 10.1002/ange.201601123

Metabolic Glycoengineering with *N*-Acyl Side Chain Modified Mannosamines

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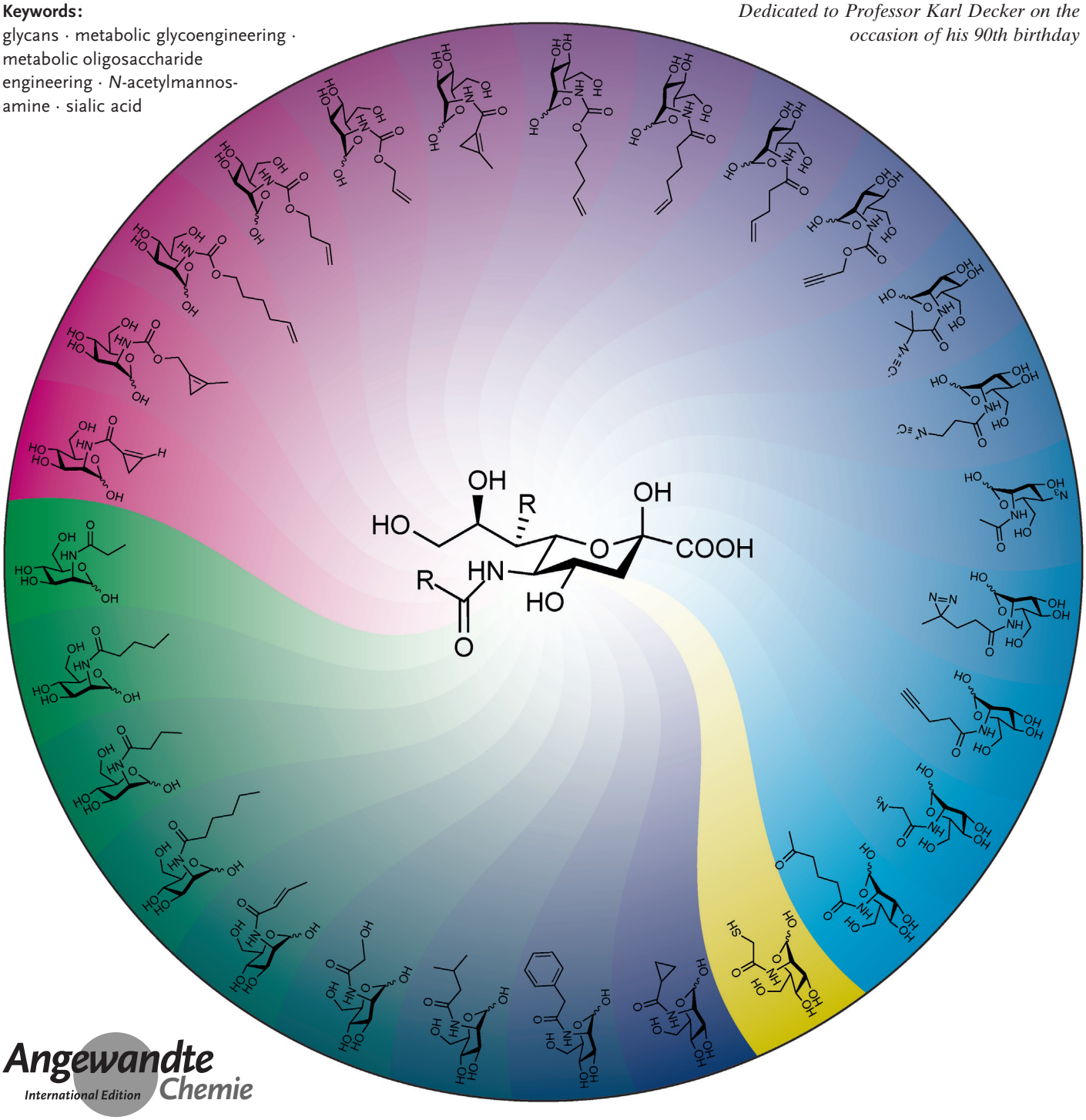
Keywords:
glycans · metabolic glycoengineering · metabolic oligosaccharide engineering · *N*-acetylmannosamine · sialic acid

Dedicated to Professor Karl Decker on the occasion of his 90th birthday



In metabolic glycoengineering (MGE), cells or animals are treated with unnatural derivatives of monosaccharides. After entering the cytosol, these sugar analogues are metabolized and subsequently expressed on newly synthesized glycoconjugates. The feasibility of MGE was first discovered for sialylated glycans, by using N-acyl-modified mannosamines as precursor molecules for unnatural sialic acids. Prerequisite is the promiscuity of the enzymes of the Roseman–Warren biosynthetic pathway. These enzymes were shown to tolerate specific modifications of the N-acyl side chain of mannosamine analogues, for example, elongation by one or more methylene groups (aliphatic modifications) or by insertion of reactive groups (bioorthogonal modifications). Unnatural sialic acids are incorporated into glycoconjugates of cells and organs. MGE has intriguing biological consequences for treated cells (aliphatic MGE) and offers the opportunity to visualize the topography and dynamics of sialylated glycans in vitro, ex vivo, and in vivo (bioorthogonal MGE).

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1. Introduction

Glycans are ubiquitous and essential in nature. As a moiety of glycoconjugates, glycans can be found, for example, in glycoproteins, proteoglycans, and glycolipids. They consist of one or more monosaccharide units that are covalently attached to the underlying chemical species. Linear and branched geometries are observed, and some of the sugar chains can consist of more than a thousand units. A variety of monosaccharides, types of linkages, and branching structures exist, which contribute to the high degree of structural complexity of glycoconjugates. Glycans are involved in a plethora of biological events, principally in cell–cell functions and cell–pathogen interactions. The terminal monosaccharide moieties within glycoconjugates are particularly important for glycan-mediated processes. Sialic acid, L-fucose (Fuc), or D-galactose (Gal) units are typically found at the nonreducing termini and branched points of glycans in vertebrates.[1–3]

Modifying the structure of glycans consistently leads to changes in their biological properties. Several methods can be applied to introduce structural changes in glycans, including enzyme inhibition (e.g. of glycosyltransferases), enzymatic treatment, or genetic modifications of the enzymes involved in glycan metabolism. Another technique that offers the possibility to influence the structure of glycans in vitro and in vivo is metabolic glycoengineering (MGE, also known as metabolic oligosaccharide engineering, MOE).[4,5]

In this case, cells or even entire organisms are treated with unnatural monosaccharides that are subsequently metabolized and incorporated into glycoconjugates. Therefore, the enzymes of the respective anabolic pathway must be promiscuous, that is, they must tolerate chemical modifications of their substrates. The first metabolic pathway to which MGE was successfully applied was the Roseman–Warren pathway, which describes the de novo biosynthesis of *N*-acetylneuraminic acid (Neu5Ac). The precursor molecule for this pathway is D-*N*-acetylmannosamine (ManNAc). The enzymes of the Roseman–Warren pathway are promiscuous, especially for accommodating changes at the *N*-acyl side chain of their substrates. Consequently, if a mannosamine with a modified *N*-acyl side chain is applied to cells, it will be metabolized to the respective unnatural sialic acid and expressed on the cell surface.

Over the last two decades more than 20 unnatural mannosamines have been introduced as suitable substances for MGE. These ManNAc analogues can be divided into two groups: aliphatic and bioorthogonal analogues. In aliphatic analogues, the *N*-acyl function is elongated by one or more methylene groups. MGE with aliphatic ManNAc analogues introduces novel biological characteristics to cells and consequently influences their behavior in multiple ways. Bioorthogonal analogues carry modifications of the *N*-acyl side chain that are both absent in living systems and utilizable for further chemical reactions. These reactive groups can be applied, for example, to label sialylated glycans with a fluorescent dye.

On the basis of this strategy, MGE has been successfully applied to other monosaccharides, including D-*N*-acetylgalactosamine (GalNAc),[6–10] D-*N*-acetylglucosamine

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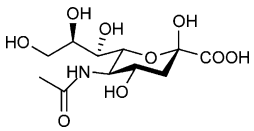
(GlcNAc),[11–13] as well as 3-deoxy-D-manno-oct-2-ulosonic acid (KDO),[14] and the sugar alcohol myo-inositol.[15–17]

This Review describes the uses and capabilities of MGE with *N*-acyl side chain modified mannosamines. In this context, understanding the biochemistry and biology of Neu5Ac is, therefore, a prerequisite.

2. Biochemistry and Biology of N-Acetylneuraminic Acid

N-Acetylneuraminic acid (Neu5Ac), or 5-acetylamino-3,5-dideoxy-D-glycero-D-galacto-non-2-ulopyranosonic acid (Scheme 1), is a crucial structural and functional component



Scheme 1. *N*-Acetylneuraminic acid (Neu5Ac). In solution, Neu5Ac exists mostly in the β-anomeric form (ratio of α/β = 8:92; the β anomer is shown).[23]

of many glycoconjugates expressed on the surface of bacterial and mammalian cells and in many secretory glycoproteins. Neu5Ac is a member of the sialic acid family, which comprises over 50 physiologically relevant compounds and an increasing number of nonphysiological compounds formed by MGE, as described herein.[2,18–21] As a consequence of its terminal position, the negatively charged Neu5Ac is closely associated with intermolecular and intercellular interactions. Neu5Ac is required at an early stage during mammalian development, as shown in mice, where the key enzyme of Neu5Ac biosynthesis was knocked out by site-directed mutagenesis. The embryos died after 8.5 days of development.[22]

The following describes examples of the biological role of Neu5Ac. During the specific immune response, for example, highly sialylated antibodies display reduced affinity for cellular Fc receptors. Accordingly, Neu5Ac regulates the antibody activity in vivo, thus reducing inflammatory reactions. In several autoimmune diseases, antibodies frequently lack Neu5Ac and Gal.[2,24–26] It has been shown that the ability of polymorphonuclear leukocytes (PMN) to adhere to endothelial cells is significantly influenced by their level of cell-surface sialylation. Stronger adhesion is promoted by cleavage of Neu5Ac after activation by an endogenous sialidase. Inhibition of the endogenous sialidase leads to reduced adhesion of PMN, and subsequently reduces the recruitment of PMN to inflammatory sites.[27–29] Moreover, activated PMN desialylates the surface of human pulmonary arterial endothelial cells, which consequently increases PMN migration across the endothelial cell monolayer.[30]

The involvement of Neu5Ac in the regulation of growth processes is demonstrated in many aspects. With respect to nerve growth, it has been shown that the Trk tyrosine kinase receptor (which is physiologically masked by Neu5Ac) is activated after removal of the α2,3-linked Neu5Ac, thus enabling neurotrophin to stimulate nerve growth.[31] This finding is in accordance with the increased axon sprouting induced by intrathecal injection of sialidase in rats with injured spinal cords.[32]

Recently, it was shown that Neu5Ac acts as an intrinsic antioxidant that consumes toxic hydrogen peroxide under physiological conditions in vivo. The reaction between Neu5Ac and H_2O_2 creates a nontoxic carboxylic acid.[33] This previously neglected property of Neu5Ac was underscored by the findings that Neu5Ac counteracts LPS-enhanced acute endotoxemia and oxidative injury and serves as a scavenger for reactive oxygen species.[34,35]

A unique sialylated membrane-associated glycoconjugate that is expressed on the surface of both neurotrophic bacteria (e.g. *Escherichia coli* K1 and *Neisseria meningitides* Gp. B) and in the mammalian neural cell adhesion molecule (NCAM) is the internally α2,8-linked polysialic acid (polySia) glycan. This molecule has been challenging researchers over the past four decades. The degree of polymerization of a respective polysialylated substrate can extend to chain lengths greater than 400 Neu5Ac residues.[36–37] PolySia was first identified in NCAM (Scheme 2).[38] Subsequent studies showed that this unique glycan was also expressed on a subset of other mammalian glycoproteins, including the human milk scavenger receptor CD36,[39] the α subunit of the voltage-gated sodium channel in adult rat brains,[40] on neuropilin-2 in human dendritic cells,[41] and on the synaptic cell adhesion molecule 1 (SynCAM1),[42] which is expressed on NG2 glia cells. Both of the two structurally distinct mammalian

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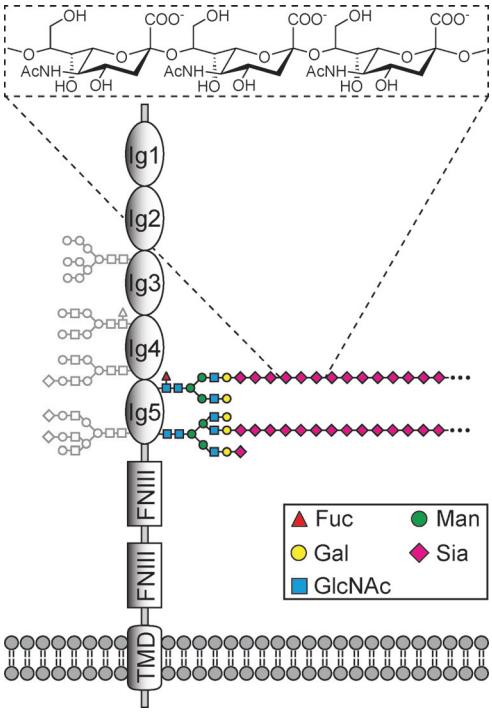
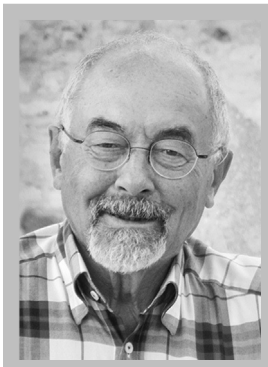
Rüdiger Horstkorte, born in Remscheid, studied biology at the Johannes Gutenberg University Mainz and the Ruprecht Karl University in Heidelberg. His PhD was completed in the group of Melita Schachner at the ETH Zürich in 1993. After research with Ten Feizi at the MRC-Clinical Research Center in London with an EMBO fellowship, he joined the group of Werner Reutter at the Freie Universität Berlin. In 2001 he habilitated as a biochemist. He became a professor of physiological chemistry at the Martin-Luther-University of Halle in 2006.



Scheme 2. Structural representation of the polysialylated neural cell adhesion molecule (NCAM). The extracellular domain of NCAM consists of five Ig domains (Ig1–Ig5) followed by two fibronectin type III repeats (FNIII). Six *N*-glycosylation sites are located in the Ig domains Ig3–Ig5. The last two of these *N*-glycosylation sites (shown in color) are attachment sites for one or more chains of polySia. The degree of polymerization (DP) of polySia can extend to over 400 α2,8-linked *N*-acetylneuraminic acid residues. The illustrated glycans serve as general examples of *N*-gylcans. Fuc: L-fucose, Gal: D-galactose, GlcNAc: D-*N*-acetylglucosamine, Man: D-mannose, Sia: sialic acid, TMD: transmembrane domain. A part of this scheme is modified from Ref. [70].

polysialyltransferases, designated ST8Sia II[43,44] and ST8Sia IV,[45–47] have also been reported to be auto-polysialylated. Interestingly, and in a paradigm shift from mouse studies, the cellular abundance of ST8Sia II and ST8Sia IV are regulated posttranslationally, not transcriptionally, in neonatal piglets.[48] As a member of the immunoglobulin superfamily, NCAM is the major carrier protein for polySia, which is attached posttranslationally. PolySia-NCAM is expressed principally in the central nervous system during embryogenesis, and its expression decreases rapidly during postnatal development. It remains persistently expressed, however, in selected regions of the adult brain showing neuronal plasticity.[49,50] To a lesser extent, NCAM is expressed in skeletal myocytes, natural killer cells, as well as epididymal and pancreatic endocrine cells.[51–54] NCAM is known to modulate cell–cell interactions with counter-adhesion molecules by building homophilic and/or heterophilic interactions with other cell-adhesion molecules, for example, the neural cell adhesion molecule L1 or extracellular matrices.[55] As a large "space-filling" cell-surface molecule, the expression of polySia reduces NCAM-mediated adhesive processes and NCAM-independent cell interactions.[56–59] Polysialylation of NCAM is closely related to the malignancy of many human tumors, including undifferentiated neuroblastoma, Wilms tumor, colon cancer, pancreatic cancer, and small-cell lung carcinoma.[60–66] It is noteworthy that ST8Sia II is the key enzyme during development of these polySia-expressing tumors.[67] Accordingly, the availability of membrane-permeable inhibitors of (poly)sialylation is a challenge (see Section 4.4).

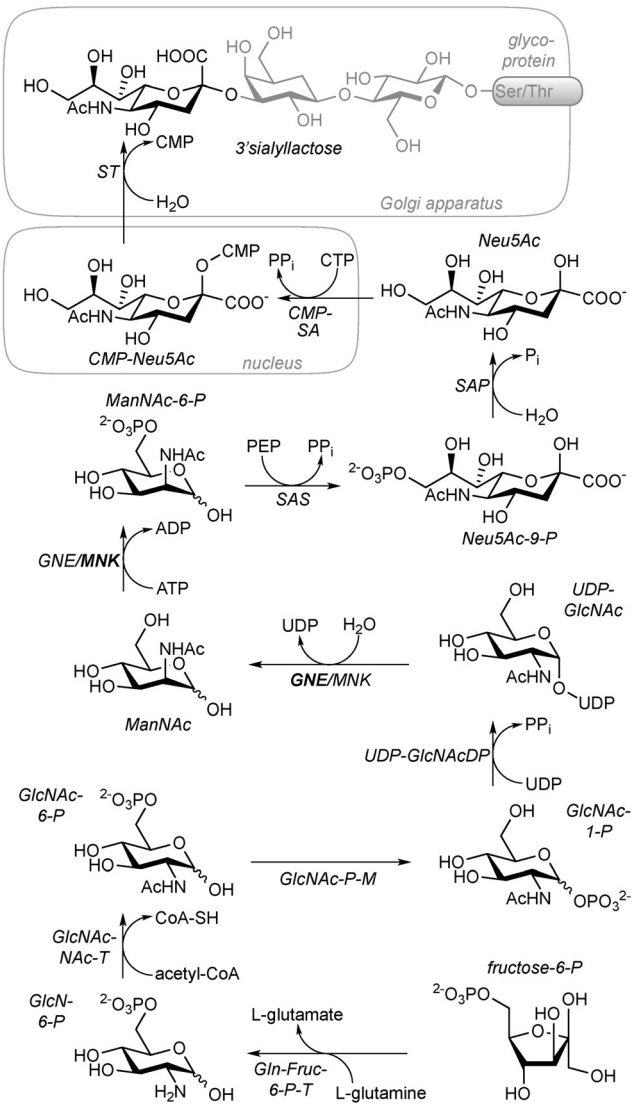
Converse to the role of Neu5Ac as a masking glycotope, it is also essential for the mediation of recognition processes, and is a crucial determinant for interaction with neighboring cells, extracellular matrices, soluble growth factors, and differentiation factors (ligand or counterligand concept).[68,69] For example, in many tumor cells, Neu5Ac is closely associated with altered cell adhesion, invasiveness, and metastasis. In infectious diseases, Neu5Ac can function as an anchor for binding pathogens, including the bacterial toxins cholera, tetanus, and diphtheria. This binding is frequently mediated by the Neu5Ac moiety in gangliosides. Furthermore, Neu5Ac is important for cell–virus interactions, notably for influenza A and C,[71] polyoma,[72] adenoma, and the human immunodeficiency virus (HIV).[73–75] Several bacterial species express carbohydrate-specific adhesins that mediate the attachment of Neu5Ac to endothelial cells, including *Helicobacter pylori*, *Streptococcus* spp., and *E. coli*.[76] Parasites such as *Trypanosoma cruzi* use their *trans*-sialidase to recognize Neu5Ac on host cells and subsequently transfer the sialyl moiety to their own cell surface, thus protecting them against immunological recognition by the host organism.[68,77–81]



Werner Reutter, born in Hornberg, studied physics and medicine in Freiburg and München. He joined the group of Karl Decker, working on the galactosamine hepatitis and on the biosynthesis of N-acetylneuraminic acid as the basis of his habilitation. In 1979 he moved to the Freie Universität Berlin as head of the Department of Biochemistry and Molecular Biology. He was speaker of the Sonderforschungsbereich 366 of the German Research Foundation until 2005 and vice president of the Freie Universität Berlin 1999–2003. In May 2016, Werner Reutter died after a short, but severe illness.

2.1. Biosynthesis of N-Acetylneuraminic Acid

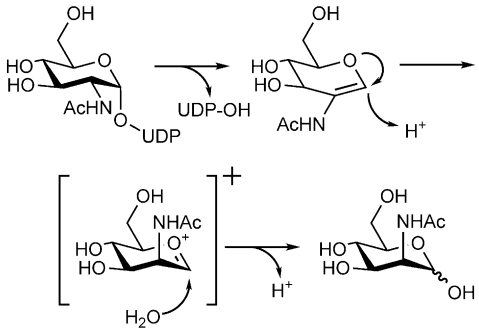
Approximately 2–5% of fructose-6-phosphate (fructose-6-P) branches off from glycolysis and enters the metabolism of amino sugars (Scheme 3).[82] After amination to glucosamine-6-P (GlcN-6-P), which is catalyzed by the fructose-6-P-amidotransferase (Gln-Fruc-6-P-T), *N*-acetylglucosamine (GlcNAc) is formed using acetyl-CoA as a coenzyme. An additional source of GlcNAc originates from endogenous degraded glycoconjugates or from digestion of nutrients. After phosphorylation, GlcNAc-6-P is further metabolized to UDP-*N*-acetylglucosamine (UDP-GlcNAc), the key metabolite of the Roseman–Warren biosynthetic pathway.[83–88] The



Scheme 3. De novo biosynthesis of *N*-acetylneuraminic acid. The 3′-sialyllactose glycan is representative of sialylated glycans in general. GlcNAc-NAc-T: glucosamine-1-phosphate *N*-acetyltransferase, GlcNAc-P-M: *N*-acetylglucosamine phosphomutase, CMP-SA: CMP-*N*-acetylneuraminic acid synthetase, Gln-Fruc-6-P-T: glutamine-fructose-6-phosphate transaminase, GNE/MNK: UDP-*N*-acetylglucsamine-2-epimerase/*N*-acetylmannosamine kinase, SAP: *N*-acetylneuraminic acid 9-phosphatase, SAS: *N*-acetylneuraminic acid 9-phosphate synthase, ST: sialyltransferase, UDP-GlcNAcDP: UDP-*N*-acetylglucosamine diphosphorylase.

bifunctional UDP-GlcNAc 2-epimerase/ManNAc-kinase (GNE/MNK) is the key enzyme for the de novo biosynthesis of Neu5Ac.[89–91] This enzyme catalyzes the 2-epimerization of UDP-GlcNAc to *N*-acetylmannosamine (ManNAc) and the phosphorylation of ManNAc to ManNAc-6-P. 2-Acetamidoglucal is an intermediate formed after *anti*-elimination of UDP (Scheme 4).[92]

The expression of GNE/MNK is ubiquitous,[93,94] and its level of gene expression is responsive to dietary sialic acid intervention in neonatal piglets.[95] The 2-epimerase activity is localized in the N terminus and the kinase activity in the C terminus, as proven by site-directed mutagenesis of the



Scheme 4. The reaction catalyzed by the UDP-*N*-acetylglucosamine-2-epimerase (GNE). GNE first catalyzes an *anti*-elimination of UDP, with 2-acetamidoglucal as an intermediate. This step is mechanistically similar to a glycosyltranferase-catalyzed reaction.

bifunctional enzyme.[91] GNE/MNK exists as a tetrameric enzyme in the cytosol.[89,90,96] The tetrameric state is maintained by UDP-GlcNAc, which shows the highest cytosolic concentration of any sugar nucleotide in a living system.[97–100] In the absence of UDP-GlcNAc, the tetrameric enzyme instead forms a dimer that shows only kinase activity, as observed in vitro.[89,96] As the key enzyme of Neu5Ac biosynthesis, the GNE domain is subjected to feedback inhibition by CMP-Neu5Ac, the end product of the biosynthetic pathway.[100] Total inhibition of GNE enzyme activity in vitro is observed at 60 μM CMP-Neu5Ac, which is approximately twice the concentration that is normally present in the cytosol.[101,102] The enzymatic activity of GNE can be increased by protein kinase C mediated phosphorylation. Eight phosphorylation sites have been described.[94,103] The activity of GNE is epigenetically regulated by methylation of the promoter region that decreases the expression of GNE/MNK-mRNA and, thus, dramatically inhibits cellular sialylation, as shown in several cell lines.[104] Furthermore, GNE activity is influenced by metal ions. The divalent cations Zn^{2+}, Cd^{2+}, and Hg^{2+} (Group IIB) inhibit the enzyme, whereas Na^+, K^+, Rb^+, and Cs^+ (Group IA) enhance GNE activity up to fivefold.[105,106] This finding should stimulate research on the role of Neu5Ac biosynthesis for the treatment of cerebral impairment after intoxication by Hg^{2+} or methyl-Hg^+.[107] Since MNK is not influenced or regulated by these mechanisms, GNE activity is of seminal importance for the regulation of cell-surface sialylation.[108–110]

The kinase activity of GNE/MNK reflects only the level of ManNAc. Therefore, the availability of ManNAc is closely related to the extent of Neu5Ac expression on the cell surface. A dose-dependent increase in Neu5Ac expression was observed after feeding ManNAc to rats.[111] Six hours after treatment, the concentration of Neu5Ac and CMP-Neu5Ac, as measured in the liver, was elevated tenfold. This prompt metabolism of ManNAc is one of the major reasons for the success of MGE with ManNAc and its analogues.

Following the Roseman–Warren pathway, ManNAc-6-P is condensed with phosphoenolpyruvate in the presence of the Neu5Ac-9-P synthase (SAS) to form Neu5Ac-9-P.[86] After dephosphorylation, the resulting Neu5Ac follows a unique step in sugar biochemistry: it is activated in the nucleus by the

CMP-Neu5Ac synthase (CMP-SA).[112–114] This stands in marked contrast to all other monosaccharides that are activated in the cytosol, either by GTP, UTP, or ATP. The biological significance of this unique nuclear localization remains unknown. It is postulated that the negatively charged CMP-Neu5Ac may interact with nuclear proteins involved in gene expression, thereby reducing their binding to DNA. Another hypothesis is that CMP-Neu5Ac binds nuclear proteins, thereby facilitating their transport out of the nucleus when moving to the Golgi apparatus.

The activated CMP-Neu5Ac is transported to the Golgi compartment via vesicles. Specific antiporters balanced by the export of CMP maintain a high concentration of CMP-Neu5Ac within the Golgi lumen.[115] In the Golgi apparatus, specific sialyltransferases catalyze the transfer of Neu5Ac to glycoconjugate acceptors. These reactions are catalyzed by the family of mono-, di-, tri-, oligo-, and polysialyltransferases (STs), designated ST8Sia I–IV. More than 20 different STs have been characterized to date.[116]

The structural diversity of sialic acid arises from the different amino substituents as well as from the type and number of hydroxy substituents at positions C4, C7, C8, and C9. Potential hydroxy substituents can be acetyl, lactoyl, sulfonyl, phosphonyl, and methyl groups.[18] The attachment of hydroxy modifications presumably takes place in the Golgi apparatus onto either free CMP-Neu5Ac or Neu5Ac residues present in glycoconjugates.[117] All types of *O*-modifications are present in the phylum of Deuterostomia. The subphylum of vertebrates usually express *O*-acetylated, or rarely *O*-lactoylated, sialic acids.[20] Although over 50 naturally occurring derivatives of sialic acid have been identified, human tissues express five principal types of sialic acids at any appreciable level: mainly Neu5Ac, small amounts of 9-*O*-acetylated *N*-acetylneuraminic acid (Neu5,9Ac$_2$), 9-*O*-lactosylated *N*-acetylneuraminic acid (Neu5Ac9Lt), and *N*-glycolylneuraminic acid (Neu5Gc), in which a hydroxy group replaces one of the three protons in the C5-position of Neu5Ac, and the newest member of the family, KDN (2-keto-3-deoxy-D-glycero-D-galacto-nononic acid). In mammalian species the level of KDN expression often exceeds that of Neu5Gc.[118]

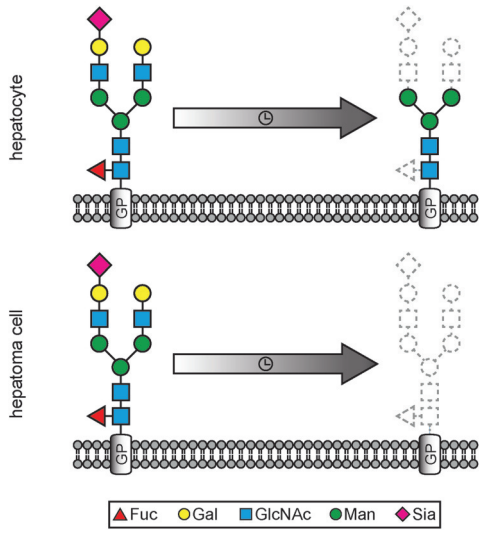
With respect to the amino substituents, both Neu5Ac and Neu5Gc can be expressed in mammalian tissues. However, because of a mutation in the gene coding the synthesis of CMP-Neu5Ac-hydroxylase, human cells are not able to synthesize Neu5Gc or catalyze its covalent modifications.[119,120] The presence of Neu5Gc, originally thought to represent an oncofetal antigen, is now known to be present in human tissues as a result of a diet rich in dairy products and red meat. Renewed interest in this finding has arisen because of the health risks of Neu5Gc associated with inflammatory diseases, including cardiovascular illnesses and cancer.[121]

2.2. Biological Characteristics of N-Acetylneuraminic Acid

Nearly half a century ago the pioneering studies of Ashwell and Morell inspired research on Neu5Ac.[122–124] They demonstrated that terminal Neu5Ac plays a crucial role in determining the survival time of serum glycoproteins by protecting/masking underlying Gal residues. Unprotected galactosyl residues are recognized by the asialoglycoprotein receptor—the Ashwell–Morell receptor—which is located on the cell surface of hepatocytes. The Ashwell–Morell receptor was indeed the first mammalian lectin described.[124] The resulting receptor–ligand concept substantially contributed to our present understanding of the receptor-mediated endocytic mechanism, and demonstrated the central and pivotal role of Neu5Ac in a myriad of recognition and anti-recognition events. In biological interactions, Neu5Ac residues on glycoconjugates are either binding partners for specific receptors (recognition) or they shield underlying sugar residues and thus hinder receptor–ligand-mediated interactions (anti-recognition).[2] The recognition of Neu5Ac is of special clinical importance in the influenza virus infection (see Section 4.1). Examples of the anti-recognition effect are low-density lipoproteins and chylomicron remnants, which are removed from the serum by the Ashwell–Morell receptor after desialylation.[125] Erythrocytes, lymphocytes, and thrombocytes with reduced cell-surface sialylation are eliminated from the circulation.[2,73,126–129] Successive degradation of cell-surface Neu5Ac is a common sign of aging in these cells. More recently, the pathophysiological importance of Neu5Ac has been exemplified by the role of the Ashwell–Morell receptor during *Streptococcus pneumoniae* induced sepsis. The streptococcal sialidase desialylates platelets, which are then recognized by the Ashwell–Morell receptor and sequestered into hepatocytes. As a consequence, the fatal disseminated intravascular coagulopathy occurring during sepsis is attenuated.[130,131]

The anti-recognition effect mediated by Neu5Ac is of particular importance in many human cancers, which show considerable over-sialylation and an aberrant, nonphysiological sialylation pattern. By these means, over-sialylation acts as a "magic cloak" protecting tumor cells from being recognized by the immune system. This anti-recognition phenomenon can facilitate malignancy and the metastatic potential of many types of human cancers.[3,101,132,133] A fateful characteristic of metastasis is the increased sialylation of β1 integrin, which blocks cell adhesion to galectin-3 and prevents apoptosis.[134,135] Surprisingly, elevated levels of free KDN, but not free Neu5Ac or Neu5Gc, were observed in a number of human cancers not yet showing lymphatic metastasis, and that are poorly to moderately differentiated. These findings suggest that the level of free KDN may be a potential biomarker for detecting early stage tumors at biopsy, and be of potential prognostic value in determining the degree of malignancy.[118]

The structural role of Neu5Ac expression on cell-surface glycoconjugates has been widely reviewed,[2,3] particularly in regard to the aberrant glycosylation pattern of tumor cells.[136] An additional characteristic that is underreported is the turnover rates of membrane-bound *N*-glycans. With respect to individual membrane glycoproteins, it has been shown that Neu5Ac and other peripheral sugar residues, for example, Gal and GlcNAc, show a faster rate of turnover than the core sugars mannose and the chitobiose moiety (Scheme 5). The half-life of the core sugars and the polypeptide of all the



Scheme 5. Turnover of membrane-bound *N*-glycans in hepatocytes versus hepatoma cells. In hepatocytes, the core sugars of *N*-glycans are as stable as the polypeptide to which they are attached, whereas the peripheral sugar residues show a half-life between 12 h and 33 h. In hepatoma cells, the entire *N*-glycan chain has a shorter half-life. GP: glycoprotein.

glycoproteins studied to date are 60 to 80 h. By contrast, the half-lives of the peripheral sugar residues range from 12 h (Fuc) to 33 h (Neu5Ac).[137–139] An even shorter half-live of less than 2 h was measured for the unnatural sialic acid analogue *N*-levulinoylneuraminic acid (Neu5Lev).[140] This characteristic, which apparently is not present in serum glycoproteins, for example, transferrin,[141,142] could be a general characteristic of peripheral sugar residues in membrane glycoproteins. It is postulated that the biological function for such microdynamics may be that these different half-lives of the sugar moieties reflect differences in receptor and/or membrane recycling. This presumption is supported by the finding that distinct cell-surface glycoproteins undergo partial resialylation after desialylation, thus indicating recycling from the cell surface to the Golgi apparatus or the trans-Golgi network.[139,143] This rapid turnover of peripheral sugar residues was observed to be domain-specific on the cell surface of liver cells.[144] A striking difference between hepatoma and host liver tissue was observed: Not only the peripheral sugar residues, but the entire *N*-glycan chains had a shorter half-life on the cell surface of hepatoma cells.[145] This finding indicated differences in the glycan composition and turnover between normal and tumor cells.

Microdynamics during endocytosis may contribute to the remodeling of glycoconjugates. To investigate further the half-lives of cell-surface glycoconjugates, the responsible glycosidases have to be identified. Recently, the membrane-associated sialidases NEU3 and NEU1 were shown to remove terminal Neu5Ac from cell-surface sialosides.[146–148] The existence of a membrane-associated α-mannosidase was also shown using hepatoma cells decorated with high mannose-type *N*-glycans.[149]

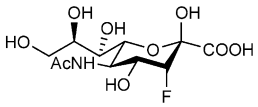
2.3. Inhibition of N-Acetylneuraminic Acid Biosynthesis and Cell-Surface Sialylation

The availability of inhibitors of sialylation is a challenge of clinical and biological importance. Substances were developed that either target the key enzyme of sialic acid biosynthesis, the GNE/MNK, or the group of sialyltransferases (STs), which catalyze the last step in the biosynthetic pathway of sialylated glycans.

The GNE/MNK inhibitors available to date mimic the natural substrates of the enzyme, UDP-GlcNAc, and ManNAc. The ligand-binding epitopes in the active centers of both domains were determined by saturation transfer difference NMR spectroscopy. UDP showed the highest binding affinity in GNE. The presence of at least one phosphate group was necessary for the interaction.[150,151] In this regard, UDP-*exo*-glycal derivatives have been synthesized and proven to inhibit GNE with an affinity that was comparable to the natural substrate, UDP-GlcNAc (K_M(UDP-GlcNAc) = 11 μM).[89,152] Recently, it was shown that 2′,3′-dialdehydo-UDP-*N*-acetylglucosamine irreversibly inhibited the GNE by covalently binding amino acids in the active center of the enzyme. Unfortunately, this inhibitor also reacted with amino acids in other proteins and, therefore, was nonspecific and cytotoxic.[153] Furthermore, GNE activity was shown to be inhibited by analogues of the transition states between UDP-GlcNAc and ManNAc in the GNE-catalyzed reaction.[154,155]

Crystallographic studies suggested the synthesis of C6-modified analogues of ManNAc as possible inhibitors for the MNK domain of the bifunctional enzyme.[156] A C6-acetylated compound was tested as an effective inhibitor of MNK activity in vitro. However, this inhibitor was not tested in cells because the *O*-acetyl group is likely cleaved off by cytosolic esterases.[157–160] Expression of sialic acid on the cell surface was successfully inhibited using a peracetylated C6-ManNAc diselenide dimer.[161] The C3-position of ManNAc has been described as another important binding epitope,[151] and cell-surface sialylation was reduced after applying a peracetylated C3-modified ManNAc analogue.[162] In terms of mannosamines with modified *N*-acyl side chains, it was shown that *N*-propionylmannosamine (ManNProp) inhibits the biosynthesis of Neu5Ac in a cell-free system from rat liver.[163] In a different approach, picolinic acid derivatives were identified that inhibit MNK activity in vitro.[164]

The first potential inhibitors of α2,6- and α2,3-STs were based on neuraminyl substitution by heteroaryl rings as transition-state analogues of CMP-Neu5Ac.[165] By using high-throughput screening, a subset of ST inhibitors was identified that shared no common structural features with CMP-Neu5Ac.[166] This study showed that it was possible to selectively inhibit specific STs selectively from the family of STs. A C3-fluorinated analogue of Neu5Ac was synthesized as a potent ST inhibitor (Scheme 6).[167] This inhibitor was able to reduce surface sialylation in cultured cells. Moreover, it was capable of blocking sialylation in vivo.[168] Mice treated with the C3-fluorinated derivative, however, showed liver impairment, irreversible kidney dysfunction, and failure to thrive. These results confirm the crucial role of Neu5Ac in



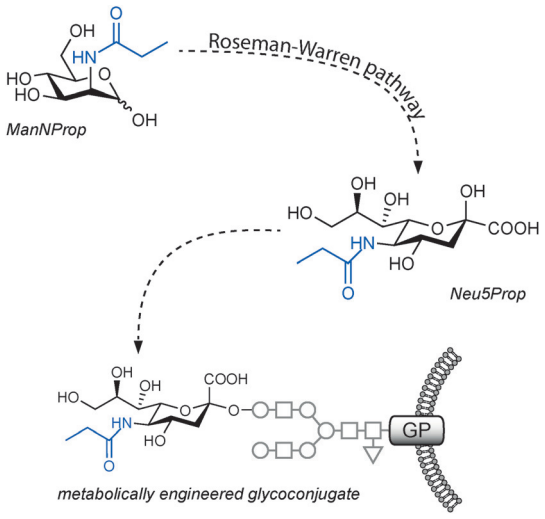
Scheme 6. Sialyltransferase inhibitor. This analogue of *N*-acetylneuraminic acid carries a C3-fluoro substitution.

liver and kidney function and further indicate the limits for pharmacological use of sialylation inhibitors. Recently, the first nanomolar ST inhibitor was published.[169] This compound mimics the structure of CMP-Neu5Ac. It contains a fluorescein moiety that allows monitoring of its cellular uptake and was shown to inhibit both bacterial and mammalian STs.

3. Metabolic Glycoengineering of the N-Acyl Side Chain of Sialic Acid

3.1. Aliphatic Modifications

As part of the biochemical characterization of the enzymes involved in the Neu5Ac biosynthesis (the Roseman–Warren pathway), it was important to investigate the specificity of these enzymes towards modifications of the *N*-acyl side chains of the natural precursor ManNAc. In an initial attempt, ManNAc was replaced by chemically synthesized *N*-propionylmannosamine (ManNProp). Results from in vitro analysis in a cell-free liver system using conventional paper and thin-layer chromatography showed that ManNProp was metabolized to the respective *N*-propionylneuraminic acid (Neu5Prop, Scheme 7).[163,170] Gas-liquid chromatography and mass spectrometry were used to prove the biosynthesis of this novel type of sialic acid.[171,172] These results revealed that the



Scheme 7. Metabolic glycoengineering using *N*-propionylmannosamine (ManNProp). The unnatural ManNProp was the first compound to reveal the promiscuity of the enzymes in the Roseman–Warren pathway. In cells, ManNProp is unidirectionally metabolized to *N*-propionylneuraminic acid (Neu5Prop). Neu5Prop is activated with CTP and expressed on cell-surface sialosides.

enzymes of the Roseman–Warren biosynthetic pathway were promiscuous, thus allowing metabolism of unnatural *N*-acyl-modified mannosamines—the major requirement for successful glycoengineering. Furthermore, cell culture and in vivo experiments showed that the activated Neu5Prop was transferred to asialoglycans, which were functional and structural components of the cell surface.[173,174]

Related homologues including *N*-butanoylmannosamine (ManNBut), *N*-isobutanoylmannosamine (ManNiBut), *N*-pentanoylmannosamine (ManNPent), *N*-cyclopropylcarbamylmannosamine (ManNCycloProp), *N*-crotonylmannosamine (ManNCrot), and *N*-glycolylmannosamine (ManNGc) were subsequently synthesized and shown to be metabolized to the respective Neu5Acyl sugars and incorporated into cell-surface sialosides.[79,172,175,176] Dafik et al. introduced *N*-acyl side chain elongated mannosamines with terminal fluorine groups that were also demonstrated to be metabolized to the respective Neu5Ac analogues in cells.[177]

N-Acyl-modified glucosamines are also metabolized to unnatural sialic acids.[172,178,179] In cells, however, these analogues contribute to the large pool of natural UDP-GlcNAc and, therefore, are significantly diluted. Moreover, *N*-acyl-modified glucosamine analogues are partially converted into galactosamine analogues.[180,181]

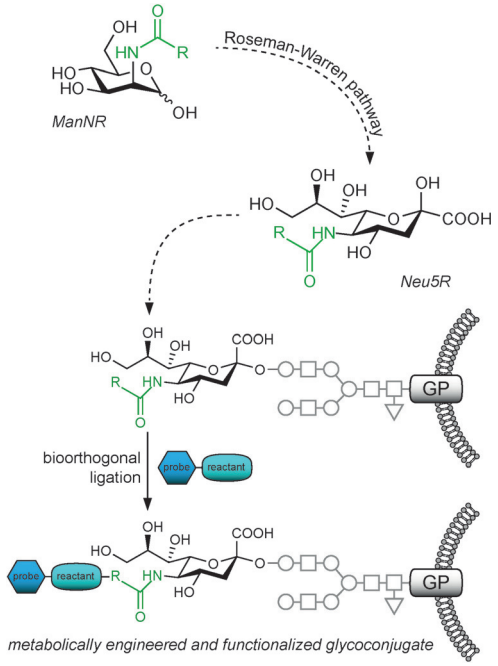
In contrast to the *N*-acyl-modified glucosamines, ManNAc analogues are directly metabolized to the respective Neu5Ac analogues.[182,183] Similar to Neu5Ac, the unnatural *N*-acylneuraminic acids are activated with CTP and incorporated into glycoconjugates in the Golgi apparatus.[171,172,184] The promiscuity of the sialyltranferases (STs) was shown by Brossmer and co-workers by using bulky, unnatural CMP-sialic acids.[185] STs tolerate extension of the *N*-acyl side chain in sialic acid by up to a total of six carbon atoms. This nonspecificity of STs has also been shown for several CMP-sialic acids modified at the C5- or C9-positions by azido, amino, acetamido, benzamido, or hexanoyl groups.[185–187] Fluorophores conjugated with the *N*-acyl side chain of sialic acid are even transferred to glycoconjugates in cell-free systems.[188] Cells are not permeable, however, to these bulky CMP-sialic acid analogues.[175,189]

A further example of *N*-acyl-modified sialic acids is *N*-(1-oxohex-5-ynyl)neuraminic acid (Neu5Hex). After being incorporated into cells and subsequently expressed on cell-surface glycoconjugates, Neu5Hex can be labeled with a fluorescent tag through "click" chemistry (see Section 3.2). Analogues such as Neu5Hex are a promising starting point for future clinical and diagnostic applications.[190]

Modification of the *N*-acyl side chain of sialic acid in cell-surface glycoconjugates has been demonstrated in a variety of cell lines and in animals.[171,173,191–194] These results steadily advance our knowledge on the biological importance of sialic acids bearing *N*-acyl side chain modifications in cells and organs, and thus continue to contribute to the field of metabolic glycoengineering (MGE).

3.2. Bioorthogonal Modifications

Metabolic glycoengineering (MGE) studies using *N*-acyl-modified mannosamines were greatly advanced in a series of seminal studies carried out by Bertozzi and co-workers,[178,192,193,195–198] who introduced functional groups to the *N*-acyl side chain of ManNAc analogues. These functional groups were expressed on cell-surface sialic acids after treating cells with the respective ManNAc analogue. The first compound of this type was *N*-levulinoylmannosamine (ManNLev), which bears a ketone group on the *N*-acyl side chain. This compound was metabolized to *N*-levulinoylneuraminic acid (Neu5Lev) and expressed on cell-surface glycoconjugates in treated cells. ManLev and related analogues (see Section 6) were designated "biorestricted chemical reporters".[199] Chemically orthogonal to native cell-surface components, the ketone is able to react selectively with externally delivered aminooxy- or hydrazide-functionalized probes to form oximes or hydrazones, thereby building stable covalent adducts (Scheme 8).[198] This strategy was used to attach fluorophores to the modified sialic acids, and thereby visualize cellular glycoconjugates. However, metabolites with keto groups are abundant within cells as intermediates in glycolysis, the citric acid cycle, and in pyridoxal phosphate. Therefore, ketones are not satisfactory as chemical reporters for specific MGE of the *N*-acyl side chain in sugars. Moreover, this application is limited, since the reaction is slow at physiological pH values.



Scheme 8. Metabolic glycoengineering using *N*-acetylmannosamine (ManNAc) analogues carrying biorthogonal modifications. Mannosamines with modified *N*-acyl side chains are metabolized by cells to the corresponding sialic acid analogues. A biorthogonal reactant can be covalently attached to the biorestricted chemical reporter (R). Modified sialic acids can be functionalized with probes, for example, fluorophores by utilizing this technique.

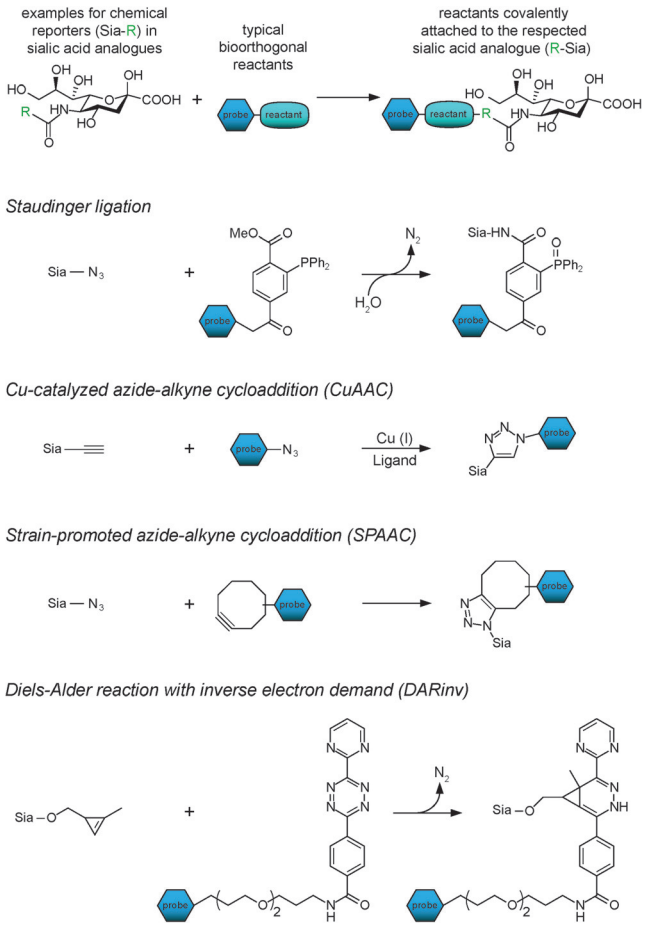
MGE with bioorthogonal reporters was further improved by the use of azides, terminal and strained alkenes, or alkynes as modifications of *N*-acylmannosamines (or *N*-acylgalactosamine, *N*-acylglucosamine, and Fuc). These chemical reporter groups are small and absent in living systems, a prerequisite for effective bioorthogonality. Furthermore, azides represent ideal ligands for functionalized phosphines in the Staudinger ligation and cycloaddition with an activated alkyne. The Staudinger ligation can be used to covalently attach probes to azide-bearing biomolecules. Neither azides nor phosphines react appreciably with biological functional groups and, therefore, are bioorthogonal reactants, although it was shown that endogenous thiols, for example, in glutathione, were able to reduce azides to the corresponding amines.[200] They are stable at physiological temperatures and possess little toxicity.[198,201–204] The Staudinger ligation is a modification of the classic Staudinger reduction of azides using triphenylphosphine. It is essential that the ester group on one of the aryl substituents of the phosphine is replaced, thus allowing the intermediate aza-ylide to undergo intramolecular formation of an amide bond.[198,199,204–207] The Staudinger ligation is a relatively slow reaction and, therefore, not suitable for experiments in which glycan turnover and/or distribution is to be visualized in a relatively short period of time.

An additional approach to utilize azides in modified sialic acids expressed by cells is copper-catalyzed azide-alkyne click cycloaddition (CuAAC). Unfortunately, this method requires Cu^{I}-containing reaction buffers that are cytotoxic. To address this issue, a robust and general method for the chemoselective labeling of glycoconjugates was introduced by Finn and co-workers. The reaction time was shortened by the use of the accelerating ligand tris(hydroxypropyltriazol)methylamine (THPTA), which eliminated the cytotoxicity of copper and the accruing sodium ascorbate during the click reaction. Therefore, this reaction preserved intact cell viability.[208,209] Similarly, Wu and co-workers developed a tris(triazolylmethyl)amine-based accelerating ligand for CuAAC.[210,211]

Copper-free click chemistry, also known as strain-promoted azide-alkyne cycloaddition (SPAAC), was developed based on the 1,3-dipolar cycloaddition of Huisgen.[212] The use of fluorinated cyclooctynes resulted in the reaction time of the azide-alkine-reaction being dramatically shortened. The azide-alkyne reaction was completed within minutes at room temperature, without a bioorthogonal catalyst.[213–215]

Recently, it was shown that the Diels–Alder reaction with inverse electron demand (DARinv) between 1,2,4,5-tetrazines and strained dienophiles (e.g. *trans*-cyclooctenes) fulfills the requirements for being a bioorthogonal ligation reaction.[428] Moreover, the Diels–Alder reaction is orthogonal to the azide-alkyne cycloaddition. However, cyclic alkenes or kinetically stable tetrazines are expected to be too large to be metabolized in the Roseman–Warren pathway. In the search for smaller dienophiles suitable for MGE, monosubstituted (terminal) alkenes were identified as a new class of chemical reporters.[216,217] For this purpose, the DARinv between terminal alkenes and 1,2,4,5-tetrazines was established. Since terminal alkenes are not present in biological systems, they are considered to be a promising reporter group. It was

shown that ManNAc analogues containing a terminal alkene in their *N*-acyl side chain were metabolized through the Roseman–Warren pathway, thereby delivering a modified sialic acid to the cell surface. Subsequently, the newly formed alkene bearing sialic acids were labeled using DARinv. DARinv does not require catalysis by toxic Cu^{I}. Moreover, the DARinv is carried out in the presence of azides, thus allowing the simultaneous visualization of two different modified monosaccharides in one cell and in a single experiment.[216,218–221] Double labeling of two different modified sugar residues in glycoconjugates was shown using mannosamine and galactosamine analogues as well as by combining DARinv with SPAAC.[216] The different bioorthogonal reactions used today to functionalize *N*-acyl side chain modified sialic acids are summarized in Scheme 9.



Scheme 9. Bioorthogonal reactions used to functionalize *N*-acyl side chain modified sialic acids. Typical examples of chemical reporters in sialic acid analogues and their bioorthogonal reactions partners are illustrated. In the Staudinger ligation, terminal azides are covalently attached to triaryl phosphines, thereby resulting in the formation of an amide linkage between the sialic acid analogue and the reactant. An azide is reacted with an alkyne in the Cu-catalyzed azide-alkyne cycloaddition (CuAAC), thereby forming a triazole linkage. Terminal azides can also be detected without utilizing cytotoxic metal catalysts in the strain-promoted azide-alkyne cycloaddition (SPAAC). In the Diels–Alder reaction with inverse electron demand (DARinv), terminal dienophiles, for example, alkenes, isonitriles, or cyclopropenes (as shown here) are ligated with 1,2,4,5-tetrazines. A portion of this scheme was modified from Ref. [222].

There still remains a demand for further bioorthogonal reporter groups. Functionalized cyclopropenes,[218–220,223–226] isonitriles,[221,227] and terminal alkenes have been introduced[216,223] which are able to undergo cycloaddition reactions with tetrazine scaffolds. Reaction partners for these scaffolds, including *trans*-cyclooctene, norborene, and bicyclononyne, are abiotic and stable in the cellular environment.[228,229] They react rapidly with electron-deficient tetrazines through DARinv. A small strained *N*-olefin-cyclopropenemannosamine was established as a chemical reporter that reacted faster than strained alkenes and alkynes. Additionally, methylcyclopropenes were applied in tandem with organic azides to target multiple classes of biomolecules.[225]

The bioorthogonal chemical strategies were extended by the introduction of diazirine-containing ManNAc analogues, for example, *N*-acyldiazirinemannosamine (ManNDAz), with various linkers between the pyranose ring and diazirine. These analogues were also metabolized through the Roseman–Warren pathway. The respective modified sialic acids were incorporated into glycoconjugates in α2,3- or α2,6-linkages. The size of the *N*-acyl side chain modification was inversely correlated with metabolic efficiency.[230,231] Larger diazirine-containing sugar analogues did not react through the Roseman–Warren pathway to form diazirine-sialic acid.[232]

The bioorthogonal "decaging" strategy offers an approach to generate deacetylated sialic acid on the cell surface. Therefore, cells are treated with *N*-(propargyloxycarbonyl)-mannosamine (ManNProc), which is metabolized to the corresponding *N*-(propargyloxycarbonyl)neuraminic acid (Neu5Proc) and incorporated into cell-surface sialosides.[183] De-*N*-propargylation was performed using Pd catalysis.[233] This bioorthogonal "decaging" strategy allows deacetylated neuraminic acid (Neu) to be established on the surface of living cells. It is of note that receptors bearing Neu are partially resistant against recognition by Neu5Ac-dependent viruses, because the *N*-acetyl group of Neu5Ac is often a crucial binding determinant for these viruses (see Section 4.1).

A further contribution to MGE was made by establishing C4-modified ManNAc analogues, including *N*-acetyl-4-deoxymannosamine or *N*-acetyl-4-azido-4-deoxymannosamine (4-AzManNAc).[234,235] 4-AzManNAc is metabolized to the corresponding C7-azido-Neu5Acyl in mammalian cells. Hence, the C7-position of sialic acid is accessible for bioorthogonal functionalization and subsequent labeling with biophysical probes.

Chen and co-workers introduced 9-azidosialic acid for the metabolic labeling of sialoglycans.[236] Whereas the known sialic acid analogues could modify sialoglycans with only one functional group, Chen and co-workers succeeded in incorporating two distinct functionalities at the *N*-acyl and at the C9-positions. These *N*-modified 9-azidosialic acid analogues were incorporated into cellular glycans, where the two chemical reporters exerted distinct functions.[237]

In summary, after the Staudinger ligation and CuAAC were introduced, the field of bioorthogonal ligation was

complemented by the use of SPAAC and DARinv.[238,239] SPAAC and DARinv are advantageous because they are rapid, noncytotoxic, and the smaller reporter groups are metabolized through the Roseman–Warren pathway to the respective sialic acids, which are then expressed on cell-surface glycoconjugates.[216]

3.3. Modifications Bearing Thiols

After treating cells with *N*-thioglycolylmannosamine (ManNTGc), the glycocalyx was redecorated with *N*-thioglycolylneuraminic acid (Neu5TGc). Since it is a sialylated glycoconjugate, Neu5TGc influences interactions between cells and the extracellular matrix. It may also promote the differentiation of stem cells. Treating non-adhesive human T-cell leukemia cells with Neu5TGc induced spontaneous cell–cell clustering, expression of ß-catenin, and changes in cell morphology.[240–242]

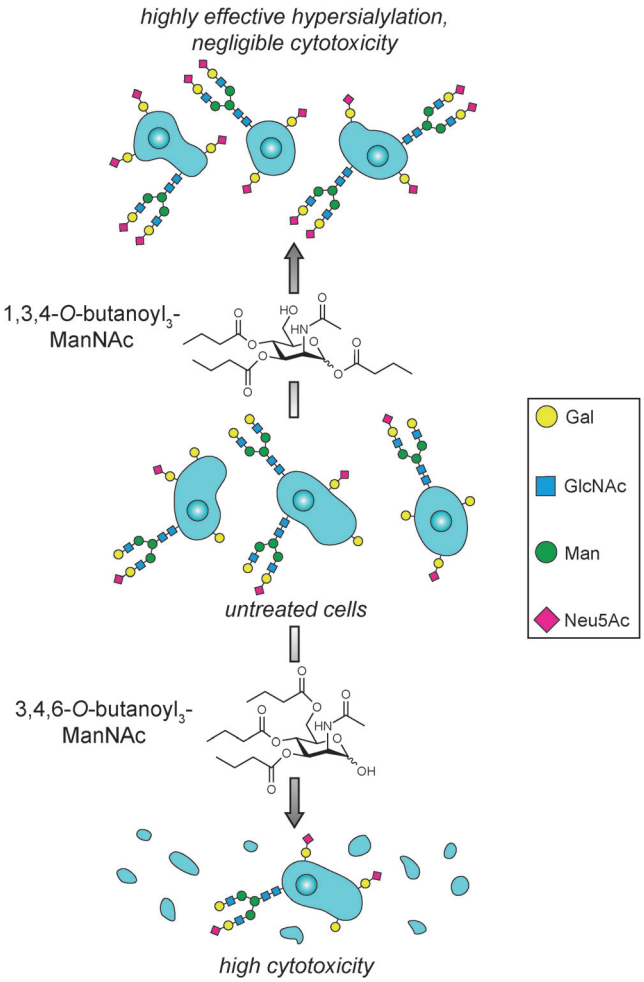
In contrast to azides and diazirines, thiols are not abiotic. However, thiols are not found in the glycocalyx of untreated cells. Thiolylated sialosides are able to form *cis*-disulfide linkages. Furthermore, they provide the capability to bind gold, which can be used to visualize sialosides in cell compartments.[21,240,243] These findings stimulated Howarth and Ting to describe their findings as "*giving cells a new sugar coating*".[244]

In another approach, it was shown that voltage-gated potassium channels (Kv) expressing Neu5TGc could be irreversibly inhibited with a biotin-labeled scorpion toxin that binds to Kv and subsequently reacts with thiol groups.[245] Cleavage of the formed disulfide bond not only restored function, but delivered a biotin moiety to the modified Kv subunit, thereby giving the opportunity to label wild-type Kv complexes functioning in cells.

3.4. Distribution of ManNAc Analogues and Modified Sialic Acids

The high specificity of the cellular monosaccharide transporters results in ManNAc, Neu5Ac, and their analogues being poorly taken up by cells and organs.[246] Therefore, it is advantageous to use analogues with protected hydroxy groups (e.g. peracetylated analogues) for cell culture and in vivo experiments, as they possess increased membrane permeability. In the cytoplasm, the protecting groups are removed by cytoplasmic esterases, thereby releasing the active monosaccharides.[79,157–160,247] The uptake of unnatural ManNAc and Neu5Ac analogues, their metabolism, and incorporation into sialoglycoconjugates have been shown in a myriad of different cell lines. However, the effectiveness of this application varies between different cell lines.[67,173,184,248,249] This may result from variable levels of expression of the enzymes of the Roseman–Warren pathway, particularly the differences in the expression levels of sialyltransferases (STs) that are known to be of critical importance.

Yarema and co-workers extended the use of peracetylation by replacing the *O*-acetyl functions in unnatural monosaccharides with short-chain fatty acid groups. In the case of ManNAc analogues, increasing the carbon chain length attached to the hydroxy group led to increased metabolic efficiency of sialic acid production.[160,250,251] Surprisingly, the activity and cytotoxicity of these ManNAc analogues varied, depending on the number and position of the *O*-hydroxy modifications.[252–255] 3,4,6-Tri-*O*-butanoylated ManNAc analogues, for example, promote growth inhibition of their respective cell lines and suppressed the expression of MUC1 (Scheme 10). Some of the 3,4,6-tri-*O*-butanoylated



Scheme 10. Biological activities of different *n*-butanoylated ManNAc analogues. The triacetlyated derivatives 1,3,4-*O*-(butanoyl)$_3$-ManNAc and 3,4,6-*O*-(butanoyl)$_3$-ManNAc have strikingly different bioactivities. Cells treated with 1,3,4-*O*-(butanoyl)$_3$-ManNAc are characterized by high metabolic efficiency, while treatment with 3,4,6-*O*-(butanoyl)$_3$-ManNAc promotes apoptosis.

ManNAc analogues were shown to be pro-apoptotic, particularly the ketone-containing 3,4,6-tri-*O*-butanoylated ManNLev. In contrast, 1,3,4-tri-*O*-butanoylated ManNAc analogues had a negligible effect on cell viability and showed higher metabolic efficiency.[253,254] It is of note that the monosaccharide analogue 3,4,6-tri-*O*-butanoyl-GlcNAc was proven to reduce the activity of the transcription factor NFκB.

Treatment of IL-1β-stimulated chondrocytes with this hexosamine analogue resulted in an increased expression of extracellular matrix (ECM) molecules, and a corresponding improvement in cartilage-specific ECM accumulation. The corresponding 1,3,4-tri-*O*-butanoyl-GlcNAc derivative, however, had no effect on ECM production.[256,257]

4. Applications of Unnatural Sialic Acids Bearing Aliphatic Modifications of the N-Acyl Side Chain

Metabolic glycoengineering (MGE) by unnatural mannosamines influences the behavior of cells and, therefore, has a myriad of biological and biomedical consequences. MGE supports the hypothesis of glycans being a "third language of life".[68,173,184,198,199,258]

Chemically, cells with modified sialic acids bearing aliphatic *N*-acyl side chains are more hydrophobic than control cells. This modification induces novel biological characteristics to treated cells. Most ManNAc analogues with an aliphatic modification of the *N*-acyl side chain showed little cytotoxicity. Therefore, cells can be treated with relatively high concentrations of these sugar analogues, thereby leading to a pronounced replacement of cellular Neu5Ac by the respective unnatural sialic acids. As a multitude of different biological processes are affected after such a replacement, MGE by ManNAc analogues with aliphatic modified *N*-acyl side chains is a useful tool to study specific sialic acid dependent cell functions in detail.

Section 4 highlights the capabilities of MGE by ManNAc with an aliphatic modified *N*-acyl side chain to modulate and investigate different biological processes, including viral and bacterial infections, the physiology and behavior of tumor cells, cell adhesion, neuronal growth and differentiation, as well as vascularization.

4.1. Aliphatic Modifications and Viral Infection

A crucial step in viral infection is the recognition of appropriate cellular receptors by the virus, followed by stable binding to host cells. Neu5Ac is an essential recognition determinant for several viruses, including influenza A and C, the Newcastle disease virus, *Reoviridae*, the encephalomyocarditis virus, and *Polyomaviridae*.[259] The binding of Neu5Ac to viral proteins is a well-characterized phenomenon that has been widely reviewed.[2,68,260–267]

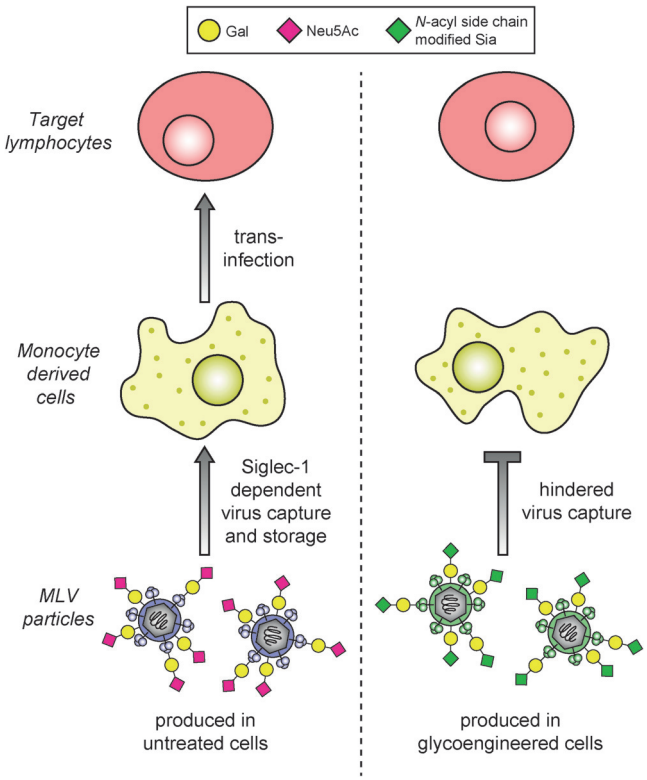
The contribution of the *N*-acetyl side chain in mediating the binding of Neu5Ac to the influenza hemagglutinin was shown using sialic acid analogues, in which the *N*-acetyl group was replaced by a hydroxy, azido, or amino function.[268]

The family of *Polyomaviridae* recognizes host-cell sialic acids on distinct gangliosides via the capsular viral protein 1 (VP1). In the case of the murine polyomavirus infection, Neu5Ac α-linkaged to Gal was identified as an essential receptor component.[269–271] It was shown that the infectivity of the B-lymphotropic polyomavirus (LPV) and the human *Polyomavirus* 1 (BKV) was reduced by nearly 80% after treating cells with sialidase.[272,273] The infection of lymphoma cells with LPV was greatly reduced after pretreatment with ManNProp. The use of ManNBut, ManNPent, and ManNHex also reduced infectivity.[184,274] Surprisingly, the uptake of BKV was dramatically enhanced after pretreating the cells with ManNProp and to a lesser extent by use of ManNBut. However, pretreatment with ManNPent rendered the cells essentially resistant to BKV infection.[184] The sialidase-resistant infection of the closely related Simian virus 40 was unaffected by MGE using ManNAc analogues. These results showed the crucial role of the *N*-acyl side chain of sialic acid for infection with *Polyomaviridae*. Elongation of the *N*-acyl side chain of sialic acid was postulated to result in a conformational change in the sialic acid–VP1 interaction.[270] This interpretation was supported by energy calculations with empirical molecular mechanics.[274] The free energy (ΔG) of all complexes of *N*-acyl side chain elongated sialic acids with VP1 appears to be reduced by about 20 kcal mol^{-1} compared to the Neu5Ac–VP1 complex. It is estimated that the affinity of VP1 for sialic acids with elongated *N*-acyl side chains was several orders of magnitude lower than that for the physiological Neu5Ac.

The influenza virus recognizes sialylated glycoconugates using the capsular protein hemagglutinin.[275,276] The ability of different unnatural sialosides to bind hemagglutinin and thus to inhibit hemagglutinin binding to erythrocytes has been studied in detail.[277–280] Compounds without the *N*-acetyl group or *N*-acyl modifications are weak binders of hemagglutinin. Presumably, the *N*-acetyl group of Neu5Ac is a crucial binding determinant for this interaction.[268,281] Elongation of the *N*-acyl side chain of sialic acid in cells using MGE, similar to pretreatment with ManNProp, ManNBut, and ManNPent, drastically inhibited the infection by the influenza A virus.[282] Molecular modeling studies suggested that the increased lipophilicity of *N*-acyl side chain elongated sialic acids caused an enhancement in the total energy of the sialic acid/hemagglutinin complex by over 20 kcal mol^{-1}, thus leading to a greatly reduced binding affinity.[282]

This is a further example of the utility of MGE as a useful and novel strategy to investigate Neu5Ac-dependent virus–receptor interactions. In the case of *Polyomaviridae* and influenza A virus, the presence of the intact *N*-acetyl group of Neu5Ac is a prerequisite for binding.

Siglec-1—a member of the family of sialic acid binding immunoglobulin-like lectins (siglecs)—and the sialyllactose-containing ganglioside GM3—a part of the viral membrane—are critical determinants for retroviral particle infection and storage by monocyte-derived cells (MDCs), and for MDC-mediated transinfection of T cells. In these cases, retroviral particles, for example, from human immunodeficiency virus 1 (HIV-1) or murine leukemia virus (MLV),[283,284] are captured by the low or nonpermissive MDCs that retain the virus for a certain period and then mediate viral transmission to neighboring permissive T cells, and thus promote vigorous infection and spread.[285] The importance of the *N*-acyl side chain of sialic acid for siglec-1-dependent MDC infection, storage, and T-cell transinfection was demonstrated using glycoengineered MLV particles derived from cells pretreated with various ManNAc analogues (Scheme 11).[286] *N*-Buta-



Scheme 11. Role of sialic acid and glycoengineering in retroviral transinfection. Siglec-1 expressed on monocyte-derived cells, for example, primary macrophages, captures retroviruses and mediates their transfer to proliferating lymphocytes. This transinfection was shown using murine leukemia virus (MLV) particles. If monocyte-derived cells are exposed to such MLV particles, the cells capture and store the particles, and further facilitate transinfection of target lymphocytes—all in a siglec-1-dependent manner. If MLV particles that express *N*-isobutanoyl-, *N*-glycolyl-, or *N*-pentanoylsialic acid were used, virus capture and, consequently, transinfection were strongly inhibited.

noyl, *N*-isobutanoyl, *N*-glycolyl, and *N*-pentanoyl side chain modifications resulted in reductions of virus particle capture and transinfection of up to 92% and 80%, respectively, whereas *N*-propanoyl or *N*-cyclopropylcarbamyl modifications had no effect.

4.2. Aliphatic Modifications and Bacterial Infection

After its first detection as a component of the homopolymeric α2,8-linked polysialylated (polySia) capsule in the culture filtrate of neuroinvasive *E. coli* K1, Neu5Ac was found in many bacterial species, including the capsules of *Haemophilus influenzae*, *Pasteurella multocida*, *Neisseria meningitides* serogroups B and C, *Campylobacter jejuni*, *Streptococcus agalactiae*,[287,288] *Moraxella nonliquefaciens*,[289] and *Mannheimia haemolytica*.[66,290] In particular, α2,8-linked polySia capsules are poorly immunogenic in humans (see Section 2).[291]

Pathogenic bacteria use sialic acid to evade immune surveillance, thus advancing their ability to colonize, persist, and cause diseases in mammals.[292] After successfully establishing MGE with *N*-acyl side chain modified mannosamines in mammals, it was reasonable for us to study bacteria as a novel target. Bacteria have two routes to obtain Neu5Ac: de novo biosynthesis and acquisition from the surrounding host environment.[292,293] The biosynthetic machinery of bacteria is tolerant to even longer *N*-acyl side chain modifications of mannosamines.[194] The corresponding unnatural sialic acids are incorporated into *O*-glycan structures, for example, lipopolysaccharide (LPS). Expectedly, relevant pathophysiological functions, including virulence, adhesion, and protection from the host immune system, are all influenced after applying MGE to bacteria.[294]

It was shown that the Gram-negative bacterium *Haemophilus ducreyi* is able to metabolize and incorporate synthetic Neu5Ac analogues, but not their ManNAc precursors.[295] Evidently, *H. ducreyi* cannot synthesize Neu5Ac de novo, but scavenges Neu5Ac from the surrounding.[296–298] *H. ducreyi* causes chancroid, a genital ulcer disease that significantly increases the risk for transmission of the human immunodeficiency virus (HIV).[299] The LPS of *H. ducreyi* is a major virulence factor and has been implicated in the adherence of these bacterial species to fibroblasts and keratinocytes.[300,301] The cell-surface glycoform of *H. ducreyi* LPS typically contains *N*-acetyllactosamine units, which are often highly sialylated.[302,303] It was postulated that *H. ducreyi* uses these sialyl-*N*-acetyllactosamine units to mimic host-cell glycoconujugates and thus escape from immune surveillance.[304] Unnatural sialic acids were incorporated into *H. ducreyi* LPS by MGE with aliphatic *N*-acyl side chain modified Neu5Ac analogues.[194] Analogues with longer *N*-acyl groups diminished the sialylation of *H. ducreyi* LPS. The sialylation of LPS was completely abolished after pretreatment with *N*-octanoylsialic acid.

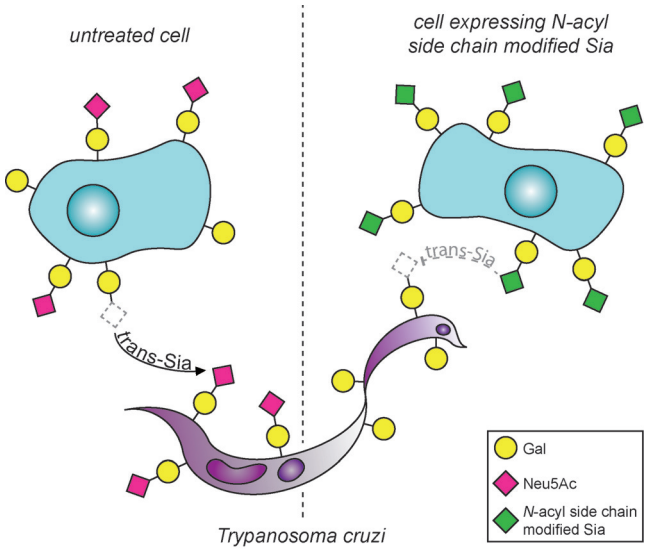
As noted above, several species of pathogenic bacteria, for example, *E. coli* K1 and *N. meningitides* Gp. B express capsular α2,8-linked polySia on their cell surface which mimics host-cell NCAM polysialylation. The neurovirulent capsular polySia chains are structurally and immunological identical to the α2,8-linked polySia chains that covalently modify NCAM in mammalian cells. This "self-recognition" means there are no effective vaccines against pathogenic bacteria bearing the α2,8-linked polySia capsule.[66] The capsule protects these bacteria from immune recognition and is also required for crossing the blood–brain barrier, where the cells colonize the meninges, principally in neonates, causing spinal meningitis.[305,306] As a consequence, these neurovirulent bacteria expressing polySia capsules often show neurotropism, which is associated with their pathogenisis. *Neisseria meningitidis* serogroup C synthesizes a homopolymeric capsule of internally linked α2,9-polySia,[307] which is a potent immunogen;[308] effective vaccines against these strains have thus been developed.[309] Interestingly, bacteria expressing a capsule with alternating α2,8-/α2,9-Neu5Ac linkages that are found in *E. coli* K92[310] appear less antigenic than those expressing the α2,9-Neu5Ac capsule. α2,8-Linked Neu5Ac expressed by *N. meningitides* serogroup B,[66,307] *E. coli* K1,[311] *Moraxella nonliquefaciens*,[289] and *Mannheimia haemolytica*[312] are poorly immunogenic in humans.[291] The antigenicity of α2-8-linked polySia can be increased, however,

by chemical de-*N*-acetylation of the Neu5Ac residues or replacement of the *N*-acetyl groups by *N*-propionyl groups before conjugation to tetanus toxoid.[313–315] After administration to mice, these synthetic polySia glycoconjugates can induce bactericidal antibodies in high yields.[316–318] Furthermore, the immunogenicity against *N. menigitidis* of serogroup B could be augmented by introducing *N*-glycolyl, *N*-propyl, *N*-propionyl, or *N*-butanoyl analogues to trisaccharide fragments of the polysialylated *O* antigen.[319,320]

4.3. Aliphatic Modifications and Infection with Trypanosoma cruzi

Infection with the parasite *Trypanosoma cruzi* may cause Chagas disease, a severe illness that is epidemic in Latin America. Among other symptoms, the acute phase of this disease presents fever, inflammatory nodules or ulcers, as well as hepato- and splenomegalia. Chagas disease often becomes chronic, with infection typically affecting the central nervous system, the gastrointestinal tract, and the heart, causing sleep disorders, and may finally lead to coma and death.[321] An important finding in *T. cruzi* research was that this parasite scavenges Neu5Ac from host sialoglycoconjugates by a unique type of sialidase, the *trans*-sialidase, which acts both as an enzyme and a binding protein.[77,322,323] As noted, *trans*-sialidase is a glycosylphosphatidylinositol (GPI) anchored enzyme that catalyzes the transfer of Neu5Ac residues from host glycoconjugates to galactosyl units on parasite cell-surface mucins.[324] *T. cruzi* is able to evade immune detection because it is covered with sialylated mucins.[325] During infection, the *trans*-sialidase is shed from the parasite surface into the bloodstream, thereby affecting the spleen, thymus, and peripheral ganglia.[326–328] Free *trans*-sialidase can induce thrombocytopenia by desialylation of thrombocytes.[328] It was also shown to interfere with the sialylation of CD45 isoforms in lymphocytes.[324] Patients suffering from Chagas disease often have cardiac symptoms, including conduction anomalies, cardiac arrhythmias, and heart failure.[329] There is evidence that these symptoms may be caused in part by aberrant sialylation of cardiac channel proteins, which can be related to *trans*-sialidase activity during *T. cruzi* infection.[330] More recently, *trans*-sialidase was designated a "parasite-derived neurotrophic factor" because of its role in the neuropathology of Chagas disease.[331]

Unnatural sialic acids can inhibit the activity of *trans*-sialidase and thus reduce the infectivity of *T. cruzi*. Different sialic acid analogues were introduced as potent *trans*-sialidase inhibitors.[327,332,333] Cells modified by MGE using ManNAc analogues with elongated *N*-acyl side chains are more resistant to *T. cruzi* infection (Scheme 12).[334] Inhibition of *trans*-sialidase by MGE is a promising starting point for the development of novel drugs for the treatment of Chagas disease.



Scheme 12. Inhibition of *trans*-sialidase (*trans*-Sia) by sialic acids modified with *N*-acyl side chains. Lacking an engogenous biosynthesis of sialic acid, the parasite *Trypanosoma cruzi* scavenges sialic acid from host sialoglycoconjugates by the unique *trans*-Sia. *Trans*-Sia transfers Neu5Ac residues from host glycoconjugates to galactosyl units on parasite cell-surface mucins. The infectivity of *T. cruzi* and some of the symptoms of Chagas disease are closely related to *trans*-Sia activity. Cells modified by MGE using ManNAc analogues with elongated *N*-acyl side chains were shown to be more resistant to *T. cruzi* infection, most likely because of inhibition of *trans*-Sia by *N*-acyl side chain modified sialic acids.

4.4. Glycoengineering in Tumor Biology

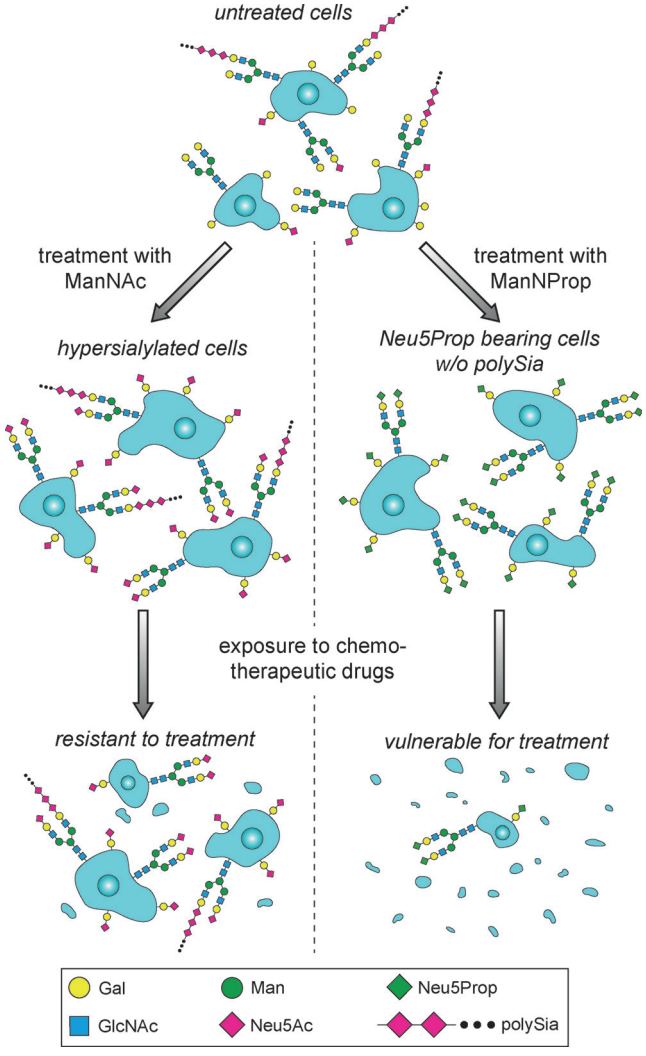
Malignant tumors have to maintain a certain level of proliferation, inflammation, and angiogenesis to progress and successfully invade other tissues. Thus, the interaction of cells and the extracellular matrix is of great importance. Carbohydrates are well-known to play an integral role in these cellular processes and interactions. Many tumor cells demonstrate hypersialylation and an alteration in their sialic acid pattern that often protects these tumors from immune surveillance.[131,335,336] Modification of sialylic acids in tumor cells by using MGE affects the biology of tumor cells in many and various ways.

Gangliosides, by definition, are sialylated glycosphingolipids. Some are highly expressed in malignant tumors and are postulated to play an important role in carcinogenesis. Several gangliosides have been identified as potential targets for immunotherapy with monoclonal antibodies because of their predominant expression on the surface of tumor cells.[337] Unfortunately, poor immune response was achieved against GD3, the dominant ganglioside of melanoma cells,[248,338,339] and GM3, expressed by various tumors.[337,340,341] Surprisingly, studies to modulate the immune response to GD3 and its precursor GM3 by using MGE were successful. After pretreating the respective tumor cells with ManNAc analogues, the immunogenicity of both gangliosides was increased. ManNProp- and ManNBut-modified conjugates were effective in generating an immune response against GD3. GM3–protein conjugates, generated after pretreatment

of cells with *N*-phenylacetylmannosamine (ManNPAc), were successfully utilized to produce antibodies against GM3.[176,342,343] Such protein conjugates with gangliosides modified by MGE were not only useful to obtain antibodies for immunohistologic studies but also to have the potential for development of antitumor vaccines. The application of this in vitro model to tumor-bearing mice resulted in tumor growth and metastasis being significantly inhibited.[344]

PolySia not only fulfills critical functions during development in normal cells and tissues, but is also an important cancer-associated antigen involved in tumor metastasis.[66,345–350] Since polySia is rarely found in healthy adult humans, other than in selected regions of the brain that show persistent expression,[118] the development of antibodies against this epitope is an attractive and relevant area for cancer research. In contrast to the physiological polySia glycan, *N*-propionylated and *N*-butanoylated polySia glycans are highly antigenic.[313,351] When leukemia and myeloma cells were treated with ManNProp, antibodies made against *N*-propionylated polySia induced apoptosis in the respective tumors and facilitated tumor lysis.[352,353] This example shows again the remarkable synergism and capability of combining MGE and immune therapy.

Experimental hypersialylation of tumor cells by treatment with ManNAc rendered these cells more resistant to radiation and various anticancer drugs (Scheme 13).[354] MGE using ManNProp, and in particular ManNPent, reduced the overall cell-surface sialylation and completely inhibited the expression of polySia in neuroblastoma cells.[355] Such engineered neuroblastoma cells lacking polySia also showed increased sensitivity to treatment with radiation and anticancer drugs. These results emphasize again the importance of polySia for the potential inhibition of tumor progression.



Scheme 13. Polysialic acid (polySia) and the *N*-acyl side chain in sialic acid play important roles in facilitating resistance and vulnerability against chemotherapeutic drugs. Hypersialylated cells that were treated with ManNAc were shown to be more resistant to various anticancer drugs, especially against 5-fluorouracil. In contrast, cells treated with ManNProp were more vulnerable to treatment with chemotherapeutic drugs compared to untreated cells. These findings can be related to the loss of polySia in Neu5Prop-expressing cells.

4.5. Aliphatic Modifications and Neuronal Growth and Differentiation

MGE by *N*-acyl side chain modified ManNAc analogues in neuronal cell cultures was first established using cerebellar microexplants from rats. When such explants were treated with ManNProp, they showed an increased number of early oligodendrocyte progenitor cells. These results were not due to enhanced proliferation, but more likely caused by differentiation of stem cells.[356]

It was shown that the application of ManNProp to oligodendrocyte cultures increased the calcium spiking in these cells. This result indicates that MGE interfered with cellular signal transduction in the central nervous system.[357]

Voltage-gated potassium channels (Kv) are critical determinants for control and modulation of the action-potential repolarization in neurons, as well as in cardiac and skeletal myocytes. Neuronal Kv channels have two highly conserved sialylated *N*-glycans.[358–360] Cleavage of these sialylated *N*-glycans leads to decreased activation, inactivation, and deactivation kinetics of the ionic currents.[359] The sialic acids in these *N*-glycans can be modulated by MGE. Replacement of the *N*-acetyl side chain by *N*-propionyl or *N*-pentanoyl groups decreased both the rates of activation and inactivation.[360] Thus, the *N*-acetyl side chain appears to account for the defined biological characteristics of the Kv voltage-gated channel.

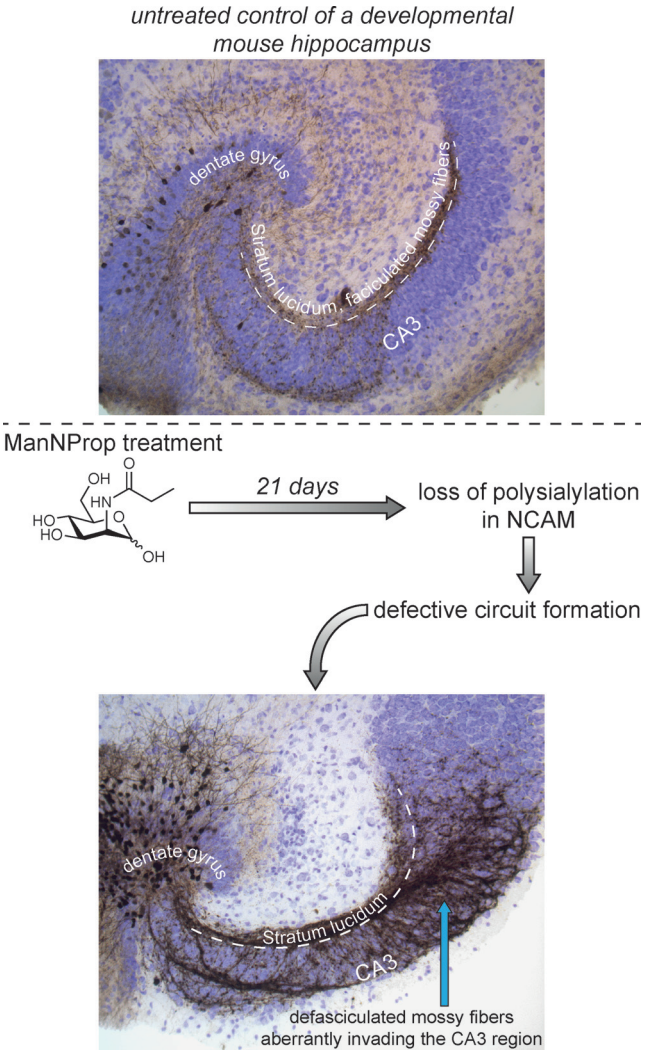
The capability of MGE to stimulate neuronal cells has been studied extensively. Examples of how ManNProp can modulate specific neuronal cell functions follow.

It was shown that treatment of neuronal cells with ManNProp enhanced neurite outgrowth, which represented axonal growth in vitro.[361] Furthermore, 2D gel electrophoresis and proteomic analyses provided evidence that ManNProp regulates the expression of several proteins involved in neurite outgrowth.[362] After treatment of PC12 cells with ManNProp, the extracellular-signal-regulated kinases 1 and 2 (erk1/2) were activated by phosphorylation before being translocated to the nucleus. Thus, Neu5Ac metabolism appears to be involved in the differentiation of neuronal

cells. This hypothesis is supported by the finding that neuronal cells lacking sufficient de novo biosynthesis of Neu5Ac have increased neurite outgrowth.[362]

Polysialylated NCAM (Scheme 2) is crucial for neuronal growth and differentiation,[363] and the activity of ST8Sia II was shown to be critical for neurite outgrowth.[364] ManNProp, with its negligible cytotoxicity, can be applied to cells and animals in relatively high concentrations. Long-term treatment of rats with ManNProp did not affect the viability of the animals.[365] However, detailed toxicological studies on ManNAc analogues with modified *N*-acyl side chains are still lacking. As described in Section 4.4, the application of ManNProp decreased the polysialylation of NCAM, likely through inhibition of ST8Sia II.[365,366] MGE can be used as a template for in vivo experiments, and has advantages over other approaches, for example, knock-out experiments of polysialyltranserases in mice. Recently, it was shown that inhibition of NCAM polysialylation by ManNProp induces aberrant border formation and fiber tract outgrowth in the hippocampus of newborn mice (Scheme 14).[367] Presumably, homeostatic regulation of the quantity of polySia at restricted stages of development is essential for correct synaptic targeting and circuit formation during hippocampal development. MGE by ManNProp is a useful method to interfere with polysialylation and signal transduction in vivo.

In vivo nerve regeneration can also be modulated by treatment with ManNProp.[368] The systemic injection of peracetylated ManNProp, but not of peracetylated ManNAc, increased the extent of axonal elongation, the amount of arborizing axons, and the branches per regenerating axon. No stimulation was observed, however, in mice with a knock-down in ST8Sia II. Moreover, it has been shown that the application of ManNProp resulted in an increased secretion of dopamine, presumably because of activation of the tyrosine-3-monooxygenase.[369]



Scheme 14. Polysialylated NCAM plays an important role in hippocampal circuit formation. In histologic sections of untreated mice, mossy fibers typically converge in the *stratum lucidum* of the hippocampus. However, treatment of new-born mice with ManNProp for 21 days led to a loss of polysialylation in NCAM and consequently caused a defective circuit formation in their hippocampi. The mossy fibers in Neu5Prop-expressing animals were observed to defasciculate and aberrantly invade the *Cornu amonis* region 3 (CA3). A part of this scheme is modified from Ref. [367].

4.6. Aliphatic Modifications and Angiogenesis

The formation of a lumen within developing blood vessels is a crucial step in angiogenesis. Endothelial cells often express highly sialylated glycoconjugates, for example, mucins and proteoglycans. The negative charge of Neu5Ac creates repelling electrostatic fields that are required for the formation of the endothelial lumen.[370]

Capillary-like sprouting of human umbilical vein endothelial cells on gel matrices is a common experimental design that is postulated to mimic angiogenesis in vitro. Highly sialylated endothelial cells show less sprouting than cells with highly exposed galactosyl residues.[371] This can be explained by electrostatic repulsion and also by binding to galectin-1 (Gal1). Gal1, a protein expressed by many cell lines, primarily binds to Galβ1-4GlcNAc residues on glycoconjugates. In desialylated endothelial cells, the binding of Gal1 activated the vascular endothelial growth factor receptor 2 (VEGFR-2) and thus induced proliferation and angiogenesis. The importance of the Gal1 pathway, particularly in tumor biology, has been highlighted by the studies of Rabinovich and co-workers.[371]

An additional experimental approach was to treat endothelial cell spheroids with ManNAc analogues, transfer the cells to a gel matrix, and subsequently stimulate them with basic fibroblast growth factor (bFGF). Oversialylated cells that received a pretreatment with ManNAc showed reduced sprouting, whereas cells expressing the unnatural sialic acids Neu5Prop and Neu5But showed enhanced sprouting after stimulation with bFGF.[372] A possible explanation for this effect of MGE on angiogenesis could be the altered turnover of unnatural sialic acids or, alternatively, changes in the binding affinity for selective sialic acid dependent receptors.

4.7. Aliphatic Modifications and the Immune System

Sialylation and desialylation of cells is important for the immune system, particularly for the class of selectins and siglecs that bind cellular Neu5Ac, and thus regulate leukocyte functions.[373,374]

There is evidence that Neu5Ac is involved in the differentiation and maturation of lymphocytes. Treatment of human peripheral blood mononuclear cells with ManNProp, for example, stimulated cell proliferation in a dose-dependent manner. The response to ManNAc under the same experimental conditions is weaker by far. It was demonstrated that treatment of mononuclear cells with ManNProp showed an increased expression of proliferation markers, including the transferrin receptor (CD71) and the interleukin-2 receptor (CD25).[375] Furthermore, ManNProp-treated cells secreted higher levels of the cytokine interleukin-2 (IL-2). After binding its receptor, IL-2 stimulated the activation of the transcription factor, NFκB. A doubling of the NFκB p65 subunit in nuclear extracts of ManNProp- and ManNAc-treated mononuclear cells was a consistent finding compared to untreated cells. To explain these results, it was postulated that the negatively charged CMP-Neu5Ac analogue, CMP-Neu5Prop, may interact with nuclear proteins and thus influence gene expression. These results emphasize the crucial role of the *N*-acyl side chain of Neu5Ac in cells related to the immune system.

4.8. Aliphatic Modifications and Glycoprotein Stability and Turnover

The biological stability of glycoproteins depends on their terminal sugars, particularly on the penultimate galactosyl and the terminal Neu5Ac residues. The level of sialylation is an essential determinant for the stability and biological activity of hormones in vivo,[376] and hypersialylation has an antiproteolytic effect.[73] The peripheral sugar moieties of membrane-bound glycoproteins reveals their own dynamics. As noted in Section 2.2, the half-life or turnover of the peripheral sugars is much shorter than the turnover of the core sugars and their polypeptide backbone.[137,138,377] After feeding cells with ManNProp, the biological half-life of two highly sialylated glycoproteins—the plasma membrane associated cell adhesion molecule CEACAM1 (a member of the immunoglobulin superfamily) and the growth hormone erythropoietin—was measured independently. In the case of CEACAM1, metabolic replacement of only 35% of the glycoconjugate-bound Neu5Ac by Neu5Prop increased the biological half-life of the protein by more than 40%. With erythropoietin, partial replacement of Neu5Ac by Neu5Prop reduced sialidase-mediated desialylation, and consequently protected the hormone from recognition by the Ashwell–Morell receptor.[378] By this means, the biological half-life of the MGE-modified erythropoietin was significantly prolonged. The incorporation of even longer *N*-acyl side chains into unnatural sialic acids of erythropoietin, for example, by treating cells with ManNPent, resulted in a further increase in the biological stability of the hormone. Thus, recombinant expression of glycoproteins, for example, growth hormones or clotting factors, bearing unnatural sialic acid residues can modulate their pharmacokinetic properties in a beneficial way.

4.9. Aliphatic Modifications and Gene Expression

As noted in Section 2.1, the negatively charged CMP-Neu5Ac, which is synthesized in the nucleus, may itself interact with proteins involved in gene regulation. An excess of CMP-Neu5Ac or the presence of unnatural CMP-sialic acids may potentially have a direct effect on proteins regulating gene expression.

The first systematic study showing that ManNProp treatment could significantly influence gene expression was demonstrated in neuronal cells. In the presence of Neu5Prop, for example, 69 of 13778 genes in the probe set were differentially expressed. Fifteen of these were upregulated and 54 were downregulated.[362] One of the upregulated genes encoded for thioredoxin, which is directly involved in cell differentiation and neurite outgrowth.[362] Treatment with ManNAc, which leads to an increase in CMP-Neu5Ac levels in the nucleus, was also shown to influence the expression of 1410 out of 20862 genes tested. 150 of these genes in the probe set were upregulated, while 1260 of them were downregulated.[379]

4.10. Aliphatic Modifications and Stem Cells

Increased expression of the sialyl Lewis^X (sLe^X) epitope confers mesenchymal stromal cells (MSCs) with bone marrow tropism[380] which, among other effects, is postulated to be beneficial for bone healing. sLe^X is a sialylated tetrasaccharide that is usually attached to *O*-glycans. To enter inflammatory tissues by transendothelial migration, a high expression of sLe^X on the cell surfaces of MSCs is required.[381] The level of cell surface associated sLe^X can be increased by ex vivo fucosylation.[380] These results show that MGE is an efficient method to influence cellular trafficking, which is fundamental for a multitude of physiological and pathophysiological processes.

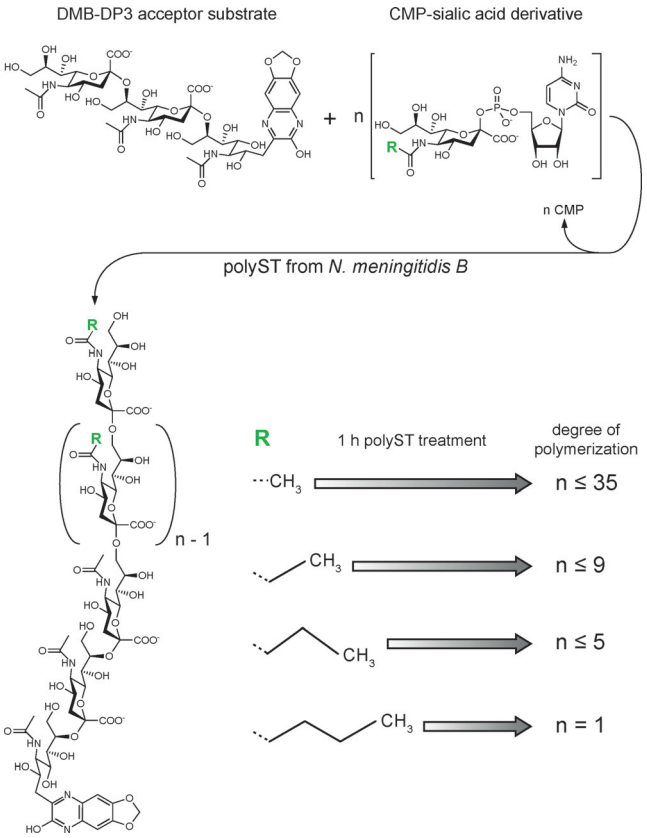
After growth of MSC in the presence of ManNProp, more than half of their natural Neu5Ac was replaced by Neu5Prop.[382] The glycan structures in the treated cells were altered following incorporation of Neu5Prop. In particular, an increase in fucosylated glycan species compared to untreated cells was observed. Interestingly, most of the fucosylated glycans were of the Lewis^X or blood group H epitopes, but not sLe^X (sLe^X). The expressions of tri- and tetra-antennary glycans and polylactosamine-containing *N*-glycans were also increased in MSCs treated with ManNProp. Polylactosamines are a typical feature of MSC *N*-glycosylation. Their expression level decreases during the differentiation of these cells.[383] Polylactosamines are also the preferred ligands for some members of the galectin group. For example, the affinity of galectin-3 (Gal3) for its ligands increases if multiple consecutive lactosamine units are present, as is the case in

polylactosamine.[384] The biological functions of galectins cover a wide variety of cell types and functions, including the role of galectin-1 in angiogenesis, as noted in Section 4.6.[385] Furthermore, the high expression levels of Gal1 and Gal3 are postulated to be responsible for the immunosuppressive properties of MSC, and it is notable that neuronal cells pretreated with ManNProp overexpress Gal3.[366] Altering the properties of stem cells by growth in the presence of the ManNAc analogue ManNTGc leads to the biosynthesis of Neu5TGc (see Section 3.3), which can induce neuronal development.[240] In contrast to the results obtained using MSCs, increased expression of sLe^X has been reported in ManNProp-treated promyeloblasts.[386] This finding can be attributed to either enhanced affinity of the respective sialyltransferase for Neu5Prop or to a decreased activity of sialidases towards Neu5Prop-containing sLe^x-glycans. Furthermore, treatment of neuronal stem cells with ManNTGc can induce neuronal development.[240]

4.11. N-Acyl Side Chain Specific Metabolism of N-Acylmannosamines

From the findings summarized herein, it is evident that the type of modification introduced to the *N*-acyl side chain of sialic acids can induce multiple biological consequences. In some cases, different aliphatic *N*-acyl side chain modifications can even mediate opposite effects, depending on the length of the acyl chain. As noted in Section 4, such opposing effects can be observed during polyomavirus infection,[184] neuronal differentiation,[375] or capillary-like sprouting.[372] These results highlight the need to characterize the specificity of the different sialyltransferases (STs) towards the sialic acids with modified *N*-acyl side chains. Different glycoconjugate structures may be affected if such unnatural sialic acids are metabolized differently by specific STs. This may, in part, explain the contradictory results obtained after treating cells with mannosamines with modified aliphatic *N*-acyl side chains of various chain lengths.

Initial studies that first showed a difference in the specificity of a bacterial polysialyltransferase were carried out using CMP-Neu5Prop, CMP-Neu5But, CMP-Neu5Pent, and CMP-cyclopropylcarbonylneuraminic acid (CMP-Neu5CycloProp).[387] The *N*-propionyl, *N*-butanoyl, and *N*-pentanoyl derivatives were efficiently utilized by the polysialyltransferase for the initial transfer of a Sia moiety to a triSia acceptor. However, elongation of a tetraSia decreased progressively with the size of the *N*-acyl side chain (Scheme 15). Although the CMP-Neu5Pent nucleotide catalyzed only the transfer of a single Sia residue to a tetraSia acceptor, CMP-Neu5Cyclo was not utilized by the bacterial polyST to catalyze the transfer of Sia to the tetraSia acceptor.[387]



Scheme 15. Investigating the specificity of bacterial polysialyl-transferase (polyST) for *N*-acyl side chain modified CMP-sialic acid analogues. Bacterial polyST from *Neisseria meningitides* B was used to transfer different *N*-acyl side chain modified CMP-sialic acid derivatives to a fluorescence-labeled triSia acceptor (DMB-DP3). Elongation of the tetraSia decreased progressively with the size of the *N*-acyl side chain, thus indicating high specificity of the polyST for CMP-Neu5Ac.

5. Applications of Unnatural Sialic Acids Bearing Bioorthogonal N-Acyl Side Chain Modifications

The application of bioorthogonal chemistry was once described by Sletten and Bertozzi as "*fishing for selectivity in a sea of functionality*".[204] After incorporation into biomolecules, bioorthogonal reporter groups allow chemoselective ligation. Azides, for example, can be reacted with phosphines in the Staudinger ligation and with linear alkynes by use of CuAAC or SPAAC (see Section 3.2).[222,388,389] Bioorthogonal reactions provided new opportunities for biological investigation that led to fundamental discoveries in areas as diverse as protein/membrane biophysics, neurophysiology, developmental and stem cell biology, and cancer research. The development of such bioorthogonal reactions as well as the advantages and disadvantages of specific reporter groups and coupling agents was expressively reviewed by Bertozzi and co-workers.[199,204,222,390,391]

Combining bioorthogonal chemistry and MGE by introducing bioorthogonal functional groups to the *N*-acyl side chain of ManNAc analogues and other monosaccharides provided heretofore novel capabilities to explore uncharted areas. Coupling of fluorophores to such bioorthogonal reporter groups was used to visualize the glycoconjugate topology and turnover in vitro, ex vivo, and in vivo.

Section 5 provides a summary of the findings of the application of MGE using bioorthogonal ManNAc analogues with modified *N*-acyl side chains to investigate several

relevant biological processes and to develop strategies for the potential treatment of diseases, for example, through targeted drug delivery.

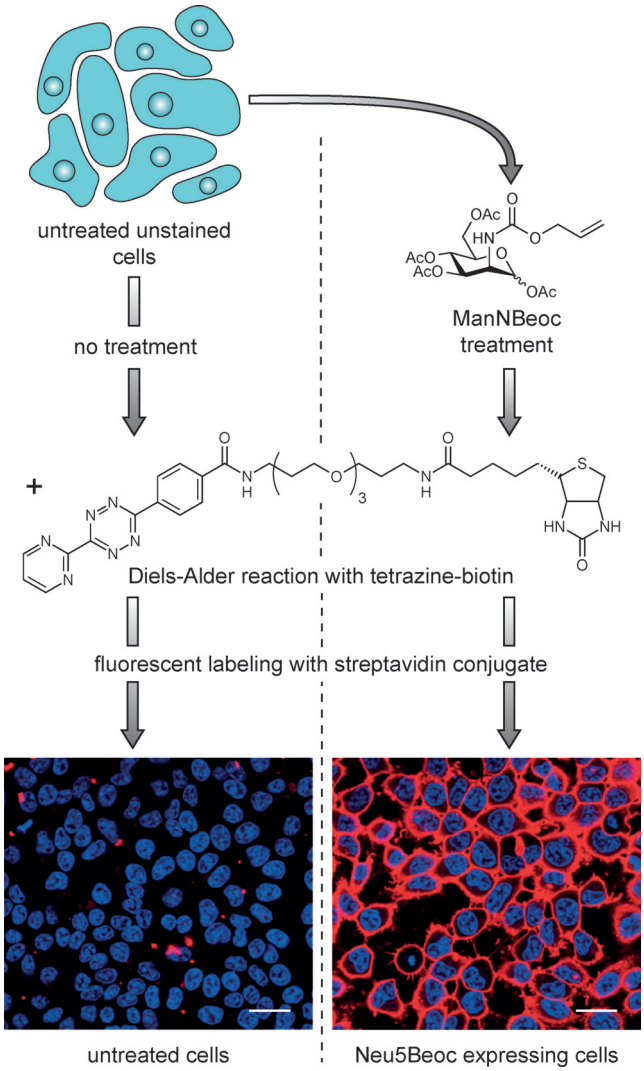
5.1. Visualizing Sialylated Glycoconjugates In Vitro by Metabolic Glycoengineering

The first successful studies to utilize MGE with bioorthogonal reporters for visualizing glycoconjugates were carried out in experiments using cell cultures. Cells were first treated with ManNLev and later *N*-azidoacetylmannosamine (ManNAz), which are metabolized to the corresponding Neu5Lev and *N*-azidoacetylneuraminic acid (Neu5Az), respectively. Subsequently, the glycoconjugate-bound unnatural sialic acids were labeled with fluorophore-conjugated phosphine reagents through the Staudinger ligation.[15,198] Early reagents, for example, fluorescein or rhodamine conjugates, had the disadvantage of considerable background fluorescence, which was due to nonspecific binding that led to poor clearance of the unreacted probe. To increase the sensitivity, conjugates that only became fluorescent after reaction with their target were used.[205,392–394] Such "smart" probes included, for example, a coumarin scaffold. The phosphine quenches the fluorescence of the coumarin in its reduced, unreacted state with its lone pair of electrons. If the phosphine reacts through the Staudinger ligation, the resulting oxide eliminates the quenching, thereby yielding a fluorescent product.[205]

Cell-surface glycoconjugates were also visualized by coupling fluorescent probes with CuAAC after pretreating cells with *N*-alkynyl or *N*-azidoacetyl-modified monosaccharide analogues. Experiments using CuAAC in mammalian cells revealed that it takes about 30 to 45 min for ManNAz to be metabolized and expressed as the corresponding Neu5Az on the cell surface.[395] CuAAC "smart" probes have been synthesized by using a 1,8-naphthalamide scaffold.[396] As noted in Section 3.2, attempts were made to eliminate the cytotoxicity of the required copper by adding ligands, for example, THPTA to the reaction, thus making CuAAC applicable for in vivo experiments.[208,209]

Fast-reacting, nontoxic conjugates that react with their target through SPAAC can be used to monitor glycan turnover in living cells. For example, a difluorinated cyclooctyne conjugate (DIFO, Scheme 17 in Section 5.2) was shown to effectively stain glycoconjugates containing Neu5Az and *N*-azidoacetylgalacosamine (GalNAz).[397,398]

Furthermore, cellular glycoconjugates were also labeled using DARinv. In such experiments, cells were pretreated with monosaccharide analogues bearing small alkenes, for example, *N*-butenyloxycarbonylmannosamine (ManNBeoc), which was metabolized to the corresponding *N*-butenyloxycarbonylneuraminic acid (Neu5Beoc).[216,217,223] Subsequent labeling with a fluorescent tetrazine conjugate allowed visualization of the modified glycocojugates in cells (Scheme 16). Cyclopropene-containing analogues, for example, 9-cyclopropene-*N*-acetylneuraminic acid (9-CpNeu5Ac), were also visualized on the cell surface by using the same labeling strategy.[225]



Scheme 16. In vitro labeling of *N*-acyl side chain modified sialic acids using the Diels–Alder reaction with inverse electron demand (DARinv). Hek293 cells expressing Neu5Beoc were ligated with a biorthogonal tetrazine-biotin reactant. Modified cell-surface sialic acids could be visualized by fluorescent labeling using a streptavidin conjugate. Scale bar = 35 μm. A portion of this scheme is modified from Ref. [223].

Notably, labeling strategies using SPAAC and DARinv can, for example, be combined to visualize different modified sugar moieties simultaneously in the same cell.[216]

MGE with bioorthogonal reporters can also be used to visualize bacterial glycocojungates, thereby providing new insight into bacterial growth parameters, glycoconjugate turnover, and enzyme activity on the cell surface.[399] This technique is particularly relevant for the detection of bacteria, and may be used, for example, to screen for contamination in samples such as drinking water. LPS from *E. coli* was visualized after pretreatment with 8-azido-8-deoxy-KDO.[400] By pretreatment with 8-azido-3,8-dideoxy-KDO and subsequent ligation through SPAAC, culturable *E. coli* was made detectable in bacterial mixtures also containing either dead *E. coli* or live *Bacillus subtilis* (as a model of microorganism not containing KDO).[14] C6-modified Fuc analogues were utilized to label Fuc-containing bacterial polysaccharides.[401]

Recently, it was shown that the time to identify *Legionella pneumophila* from contaminated samples could be drastically shortened by MGE with bioorthogonal reporters and subsequent fluorescent labeling.[402] The cell surface of the Gram-positive bacterium *Staphylococcus aureus*, could be visualized using MGE with *N*-azidoacetylglucosamine (GlcNAz).[403] After labeling with a fluorescent dye, the adherence of the labeled bacteria to cultured cells was reduced.

Diazirine-containing ManNAc analogues, for example, ManNDAz, are metabolized to the corresponding unnatural sialic acids (see Section 3.2). The diazirine moiety in these analogues is available for photo-cross-linking. Gangliosides in cell lysates were covalently sequestered by using this experimental strategy, thus providing the opportunity to further analyze the resulting ganglioside–protein complexes by blotting or mass spectrometry.[231,404,405]

The glycosylation in the Golgi apparatus and in early endosomes was visualized after treating fibroblasts with *N*-(5-pentynoyl)neuraminic acid (Neu5Al) and subsequent fluorescent labeling through CuAAC.[406] By applying this method, it was shown that fibroblasts of patients suffering from congenital disorders of glycosylation (CDG) presented decreased alkyne-tagged bound sialic acid in the investigated cellular compartments. Today, methods based on HPLC and mass spectrometry (MS) are used to identify and quantify glycosylation deficiencies.[407,408] Techniques involving glycoengineering with bioorthogonal reporters may become a fast and easy alternative to screen for such diseases.

Recently, an investigation of the cellular metabolism of peracetylated ManNAz showed that the efficiency of ManNAz and Neu5Az metabolism varied between different cell lines.[409] Furthermore, the cellular uptake mechanisms and trafficking of both ManNAc and sialic acid were analyzed in vitro by using CuAAC.[410] Herein, cells were treated with *N*-(4-pentynoyl)mannosamine (ManNAlk), or the corresponding *N*-(4-pentynoyl)neuraminic acid (Neu5Alk), which were subsequently labeled with a fluorescent dye. Neu5Alk was shown to be taken up by endocytosis. The release of Neu5Alk from lysosomes into the cytosol was mediated by the lysosomal protein sialin. In contrast, ManNAlk was shown to enter the cell by an as yet unknown plasma membrane transporter.

There has been considerable effort directed towards the development of different applications to visualize glycoconjugates in cells. Some of these methods were developed for future in vivo experiments (see Section 5.2), while others were directed at understanding the biology of glycoconjugates, particularly their turnover and distribution, in the cellular environment.

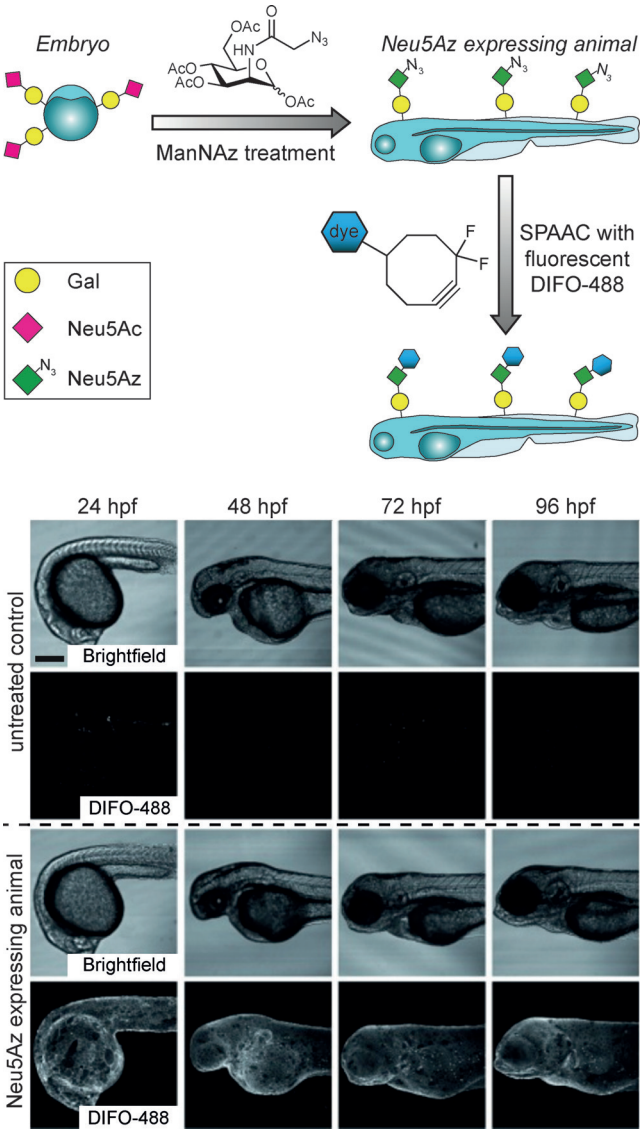
5.2. Metabolic Glycoengineering To Visualize Sialylated Glycoconjugates In Vivo and Ex Vivo

The first studies to validate MGE with bioorthogonal reporter groups as suitable for in vivo experiments were carried out in mice.[202] After pretreatment with peracetylated ManNAz, the corresponding Neu5Az was detected ex vivo by utilizing the Staudinger ligation to stain splenocytes isolated from the euthanized mice. The same method was used to prove that GalNAz was incorporated into glycoconjugates in mice.[411] An additional ManNAc analogue used for bioorthogonal imaging of siaylated glycoconjugates in cells and ex vivo was *N*-(cycloprop-2-ene-1-ylcarbonyl)mannosamine (ManNCp).[224] The fact that bioorthogonal GDP-Fuc analogues were metabolized in animals was shown using early stage zebrafish embryos.[412] In this study, fucosylated glycoconjugates were reacted with fluorescent probes by CuAAC ex vivo in paraformaldehyde-fixed embryos.

Rats were treated with ManNAz to study the distribution of sialylated glycoconjugates in their hearts, and to compare sialylation between healthy cardiac tissue and tissue of animals suffering from cardiac hypertrophy.[413] The hearts of pretreated rats were extracted and perfused with a nutrient-rich, oxygenated solution (Langendorff perfusion) containing a fluorescent dye that was coupled by SPAAC. Histological sections revealed that the overall extent of sialic acid expression was increased in cardiac tissue of rats suffering from hypertrophy.[413] These findings were related to an up-regulation of sialylated glycoproteins, including NCAM, T-kininogens, and α2-macroglobulin.

Scherer and co-workers used MGE with bioorthogonal reporters to investigate protein turnover in the cupula (unpublished studies). The cupula is a highly conserved organ that senses torsional movements of the head. The major component of the gelatinous cupula is cupulin, a glycoprotein with a mass of 45 kDa.[414] Zebrafish were treated with unnatural monosaccharides bearing bioorthogonal modifications. Ex vivo analysis of CuAAC-stained histological sections from the inner ear after various time-points suggested a baso-apical turnover of the cupula-associated glycoproteins, and furthermore, a rapid turnover of the corresponding sugar analogues in vivo.

SPAAC glycan turnover was also used to visualize live, developing zebrafish embryos (Scheme 17).[203,415] In this in vivo experiment, the embryos were fed peracetylated ManNAz or GalNAz. The corresponding Neu5Az and GalNAz in mucin-type *O*-linked glycans were stained by bathing the animals in DIFO–fluorophore conjugates. Insights into the expression patterns and trafficking of glycans in vivo were obtained by applying a multicolor labeling strategy. The same strategy was used to label mucin-type *O*-linked glycans in *C. elegans*. This study showed the distinct morphological expression of such mucins during development.[398] Zebrafish or *C. elegans* have the advantage of being transparent and growing ex utero. Labeling *N*-acyl side chain modified sialic acids bearing bioorthogonal reporters in live mammals is an even greater challenge. Chang et al. showed that conjugation with a fluorescent dye by SPAAC was possible in live mice.[213] In these experiments, however, the success of fluorescent labeling was still evaluated ex vivo. Neves et al. injected peracetylated ManNAz into a murine tumor model. The newly formed Neu5Az was subsequently labeled through Staudinger ligation in vivo.[416] Since they found an accelerated sialic acid turnover, tumor tissues could be visualized with high specificity in live mice by single photon emission computed tomography (SPECT). Recently, a double-click approach was established, which combined



Scheme 17. In vivo labeling of sialylated glycans in developing zebrafish. Developing zebrafish express Neu5Az on their cell surface after pretreatment with ManNAz. *N*-Acyl side chain modified sialic acids were visualized in living animals by SPAAC using a fluorophore-conjugated difluorinated cyclooctyne (DIFO-488) reagent. Scale bar = 200 μm; hpf: hours post-fertilization. Parts of this scheme are from Ref. [415].

either the Staudinger ligation or SPAAC with subsequent DARinv labeling to visualize *O*-glycans by SPECT in organs and in tumor tissue.[417] In an alternate approach, culture cells were pretreated with peracetlyated ManNAz and transplanted into the liver of live mice.[418] Subsequently, the cells were labeled with a fluorescent conjugate by SPAAC and visualized in vivo to reveal their localization within the liver.

Visualizing glycoconjugates in vivo and ex vivo is an application of great importance. Not only research will benefit from newly introduced or already well-established methods, but clinical applications, for example, in diagnostics, are also conceivable. Compared to conventional techniques, for example, labeling with lectins or antibodies, MGE with bioorthogonal reporters has the advantage of revealing the entire metabolism of the respective unnatural monosaccharides in both intra- and extracellular studies. As noted in Section 3.2, rapid, nontoxic reaction methods, particularly SPAAC and DARinv, in combination with multicolored staining, can be used to visualize glycoconjugate metabolism with more than one reporter molecule at the same time.

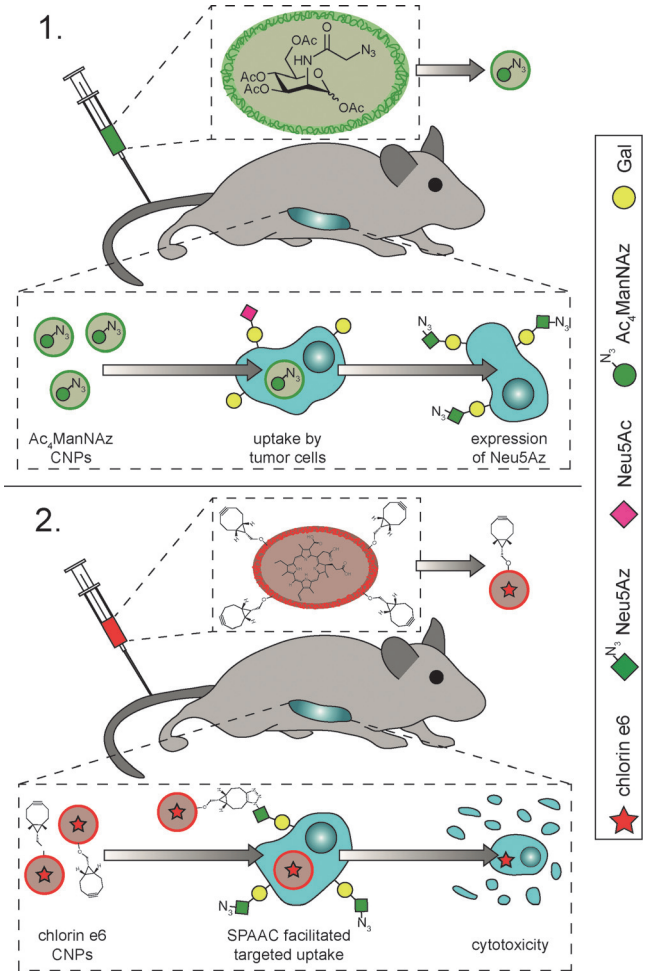
5.3. Further Applications for Metabolic Glycoengineering with Bioorthogonal N-Acyl Side Chain Modified Mannosamines

MGE with bioorthogonal reporters can be used to visualize glycocojungates. The following studies describe further applications of this powerful technique.

Pan et al. utilized MGE to enrich sialylated glycoproteins from different cell lines.[419] Cells were pretreated with peracetylated ManNAz. After labeling with an alkyne-biotin reagent, glycoproteins from the lysed cells containing the corresponding Neu5Az were purified by using streptavidin magnetic beads. Comparison of the isolated proteins from the different cell lines by HPLC and mass spectrometry revealed that cells derived from non-small-cell lung cancer uniquely expressed certain glycoproteins, including NCAM or contactin 1. In a similar approach, Neu5Az-containing glycoproteins from lysates of cells derived from pancreatic cancer were labeled with biotin by the Staudinger ligation, and bound to a streptavidin agarose support.[420] Subsequently, the glycan-linked peptides were released and further analyzed by mass spectrometry. In parallel, the isolated glycans were characterized by lectin microarrays. Unique *N*-glycoside-containing peptides were identified from different metabolically labeled glycoproteins, many of which had been previously linked to cancer. These findings once again highlight the importance of sialylation in cancer and cancer progression.

Boons and co-workers introduced Neu5Az to an *anti*-CD22-antibody by in vitro enzymatic treatment with the corresponding CMP-Neu5Az.[421] By using SPAAC, these antibodies could be labeled with fluorescent dyes and also with chemotherapeutics, for example, the DNA-intercalating drug, doxorubicin. These drug–antibody conjugates were shown to be cytotoxic to CD22-positive B cells.

A combination of treatment with ManNAz and SPAAC was shown to be useful for the delivery of nanoparticles to tumor targets.[422] If pretreated tumor-bearing mice were injected with specially designed nanoparticles that could bind the corresponding Neu5Az by SPAAC, such nanoparticles could be enriched in tumor tissue and other metabolically active organs. Refining this concept, tumor-bearing mice were treated with nanoparticles containing peracetylated ManNAz.[423] The advantage of the ManNAz packaging is that the nanoparticles were accumulated in angiogenic disease sites such as tumors by the enhanced permeation and retention effect.[424] Subsequently, secondary nanoparticles containing chemotherapeutics were used and coupled to the NeuAz-bearing tumor tissue, thereby resulting in considerable inhibition of tumor growth in vivo (Scheme 18). With this type of targeted drug delivery, high concentrations of the corresponding therapeutic can be applied to the desired location with fewer side effects for the whole organism.



Scheme 18. Utilization of MGE in targeted tumor therapy. First, tumor-bearing mice were injected with glycol-chitosan nanoparticles (CNPs) containing peracetylated ManNAz. These CNPs typically enrich in tumor tissue and other metabolically active organs. After the uptake of ManNAz, tumor cells were shown to express Neu5Az. In a second step, the mice were treated with CNPs coupled with a bioorthogonal reagent and loaded with chlorin e6. Pretreated tumor cells bind these nanoparticles through SPAAC and take up the cytotoxic chlorin e6.

The use of *N*-propargylcarbonylmannosamine (ManNProc) or *N*-propagylcarbonyl sialic acid (Neu5Proc) led to the creation of high-affinity siglec ligand-expressing cells (HASLECs).[425] In this study, pretreated cells expressing the *N*-propargylcarbonyl-containing sialic acids were linked through CuAAC with distinct azide groups, which are known to strongly enhance their binding affinity to specific siglec subtypes.[426] HASLECs are useful to investigate siglec-dependent interactions on a cellular level. Approaches such as this show that MGE in combination with bioorthogonal ligation can be applied to chemically reprogram the cell's glycocalyx and modulate its interaction with sialic acid binding receptors such as siglecs in a natural context.

6. Outlook

Currently, mannosamines with modified aliphatic *N*-acyl side chains are primarily used to investigate Neu5Ac-dependent biological processes, whereas mannosamines with bioorthogonal modified *N*-acyl side chains are mainly used to visualize sialylation and sialic acid metabolism in vitro and in vivo. It is evident from the findings described in this Review that the set of unnatural mannosamines available today provide useful tools to reveal as yet potentially unknown characteristics of glycans in future studies. Table 1 contains a compilation of all ManNAc analogues that have been successfully used for MGE to date. Novel ManNAc analogues, for example, could be used to introduce novel chemical, biochemical, or biological characteristics to the respective unnatural sialic acids. Furthermore, glycoengineering of the *N*-acyl side chain offers a creative new way to influence tumor growth or to stimulate the immune system. It may also be possible to extend the successful stimulation of neurite growth in cell cultures to live animals.

Particular attention should be paid to analyzing the specificity of given sialyltransferases (STs) for different *N*-acyl side chain modifications in unnatural sialic acids. There is evidence that the N-modifications used today possess preference for specific STs. As we learn more about the specificity and selectivity of the STs constituting the ST8Sia I–IV family of STs, it will become possible to understand in greater detail the molecular mechanisms regulating the chemistry, biochemistry, and biological properties of the novel mannosamines with modified *N*-acyl side chains, including the turnover of modified sialic acids in cells. Some of these analogues, for example, may be accepted by all STs and, therefore, appear globally on sialylated glycans. In contrast, others may be more highly specific for just a single ST and, thus, appear only on defined sialylated glycoconjugates. It is our view that future studies will provide the opportunity for glycan-specific investigations utilizing MGE in all organ systems.

A further area for investigation would be studies directed at understanding the structure of engineered glycans and moreover the influence of MGE on the glycome of cells, tissues, and organs. Herein, not only does the specificities of individual STs play a crucial role, but also the metabolism of *N*-acyl side chain modified ManNAc analogues in the Roseman–Warren biosynthetic pathway, the catabolism and re-utilization of unnatural sialic acids in glycans, and finally their resistance against cleavage by sialidases.

Research on sialic acid binding immunoglobulin-like lectins (siglecs) could also benefit from the technique of MGE. There are a myriad of biological pathways and interactions that involve the binding of sialylated glycoconjugates to siglecs. Many of them are still unknown or not well understood. First data showed that siglec–sialic acid interactions can be modified using ligands that bear unnatural sialic acids (see Sections 4.1 and 5.3). MGE could be used to elucidate the role of the *N*-acyl side chain of sialic acid in siglec binding, to modulate specific siglec-dependent pathways, or even might help in the discovery of new members of the siglec family.

Given the rapid progress made in this field, we anticipate that MGE with *N*-acyl side chain modified mannosamines will become a common tool in a diverse range of research fields and, furthermore, in clinical diagnostic, and even potential therapies. Therefore, it will be imperative that the toxicity of

Table 1: Compilation of ManNAc analogues that were shown to be applicable for MGE, ordered by type and date of first description. To simplify and unify this presentation, all ManNAc analogues are depicted in their deacetylated, metabolically active forms.

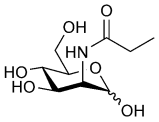
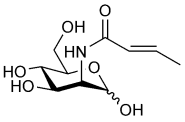
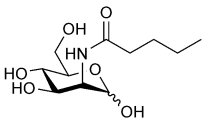
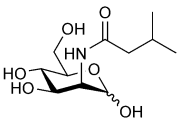
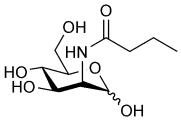
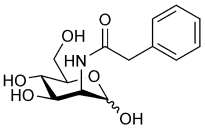
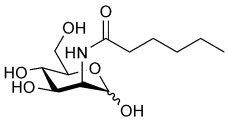
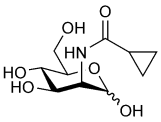
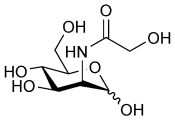
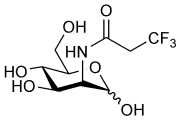
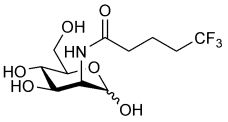
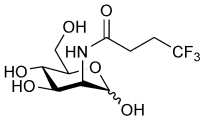
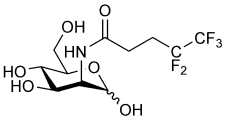
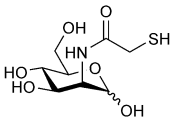
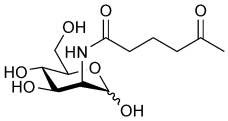
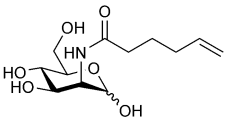
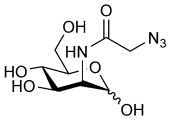
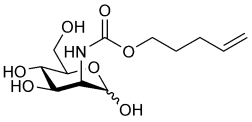
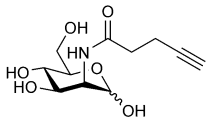
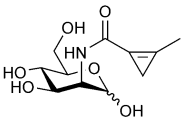
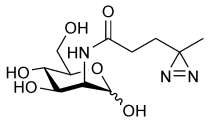
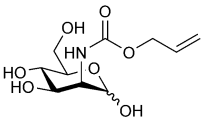
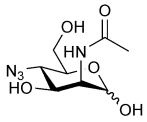
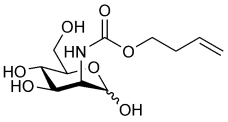
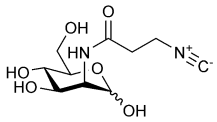
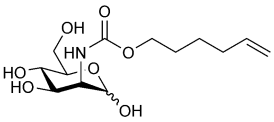
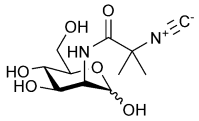
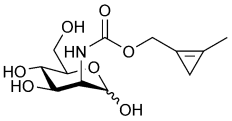
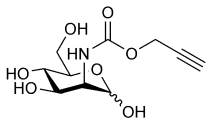
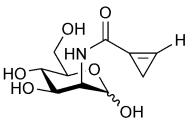
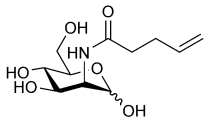
Structure	Abbreviation	Author, year	Structure	Abbreviation	Author, year
Aliphatic Modifications:					
	ManNProp	Kayser et al., 1992[171]		ManNCrot	Keppler et al., 2001[173]
	ManNPent	Kayser et al. 1992[171]		ManN*iso*But	Chefalo et al., 2006[176]
	ManNBut	Keppler et al., 1995[184]		ManNPAc	Chefalo et al., 2006[176]
	ManNHex	Keppler et al., 1995[184]		ManNCycloProp	Wolf et al., 2012[387]
	ManNGc	Collins et al., 2000[79]			
Modifications with Fluorines:					
	ManNPropF_3	Dafik et al., 2008[177]		ManNPentF_3	Dafik et al., 2008[177]
	ManNButF_3	Dafik et al., 2008[177]		ManNPentF_2F_3	Dafik et al., 2008[177]
Modifications with Thiols:					
	ManNTGc	Sampathkumar et al., 2006[241]			
Bioorthogonal Modifications:					
	ManNLev	Mahal et al., 1997[192]		ManNHxl	Niederwieser et al., 2013[216]
	ManNAz	Saxon et al., 2000[198]		ManNPeoc	Niederwieser et al., 2013[216]

Table 1: (Continued)

Structure	Abbreviation	Author, year	Structure	Abbreviation	Author, year
	ManNAlk	Hsu et al., 2007[396]		ManNMCp	Cole et al., 2013[218]
	ManNDAz	Yu et al., 2008[427]		ManNAloc	Späte et al., 2014[223]
	4-AzMan-NAc	Möller et al., 2012[235]		ManNBeoc	Späte et al., 2014[223]
	ManN-n-Iso	Stairs et al., 2013[227]		ManNHeoc	Späte et al., 2014[223]
	ManN-t-Iso	Stairs et al., 2013[227]		ManNCCp	Späte et al., 2014[219] Patterson et al., 2014[220]
	ManNProc	Bateman et al., 2013[183]		ManNCp	Xiong et al., 2015[224]
	ManNPtl	Niederwieser et al., 2013[216]			

given and newly introduced ManNAc analogues will have to be studied in vivo. A standardized, easy to use labeling and visualization procedure that is applicable in vitro, ex vivo, and in vivo will have to be elaborated.

Hopefully, MGE will be recognized by a broader audience as a versatile technique to modify glycoconjugates. Further applications similar to those described, including targeted drug delivery, the design of engineered antibodies, and the enrichment and purification of sialylated glycocojugates, could be established that might lead to the development of novel biotechnological, diagnostic, and even therapeutic approaches as well as new modalities for MGE.

Acknowledgements

We thank the "German Research Foundation" (W.R. and R.H.), the "Sonnenfeld Stiftung" (W.R.), the "Roland und Elfriede Schauer Stiftung" (W.R.), and the "Studienstiftung des Deutschen Volkes" (P.R.W.) for their support. We thank F. A. Troy II for useful discussions and extensive help in refining the text. We also thank A. K. Späte, V. F. Schart, and V. Wittmann for helping us with the organic chemistry part of this manuscript, and F. Kern as well as Y. Song for proofreading.

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, –
Angew. Chem. **2016**, *128*, –

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Received: January 31, 2016
Published online: July 20, 2016

