Sialic acid glycoengineering using N-acetylmannosamine and sialic acid analogues

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Abstract

Sialic acids cap the glycans of cell surface glycoproteins and glycolipids. They are involved in a multitude of biological processes and aberrant sialic acid expression is associated with several pathologies. Sialic acids modulate the characteristics and functions of glycoproteins and regulate cell-cell as well as cell-extracellular matrix interactions. Pathogens such as influenza virus use sialic acids to infect host cells and cancer cells exploit sialic acids to escape from the host's immune system. The introduction of unnatural sialic acids with different functionalities into surface glycans enables the study of the broad biological functions of these sugars and presents a therapeutic option to intervene with pathological processes involving sialic acids. Multiple chemically modified sialic acid analogues can be directly utilized by cells for sialoglycan synthesis. Alternatively, analogues of the natural sialic acid precursor sugar N-Acetylmannosamine (ManNAc) can be introduced into the sialic acid biosynthesis pathway resulting in the intracellular conversion into the corresponding sialic acid analogue. Both, ManNAc and sialic acid analogues, have been employed successfully for a large variety of glycoengineering applications such as glycan imaging, targeting toxins to tumor cells, inhibiting pathogen binding, or altering immune cell activity. However, there are significant differences between ManNAc and sialic acid analogues with respect to their chemical modification potential and cellular metabolism that should be considered in sialic acid glycoengineering experiments.

Introduction

Glycans of most vertebrate cells and several microorganisms are frequently terminated by sialic acids, a family of negatively charged sugars derived from 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN) that share a nine-carbon backbone. The most abundant sialic acid family member in humans and other mammals is N-Acetylneuraminic acid (Neu5Ac) that carries an N-acetyl group present on C-5 of the sialic acid backbone (**Figure 1**) (Inoue, S. and Kitajima, K. 2006, Varki, A., Schnaar, R.L., et al. 2015b). Other natural modifications of the sialic acid backbone give rise to family members such as N-Glycolylneuraminic acid (Neu5Gc), 9-O-acetyl (Neu5Ac9Ac) or 9-lactosyl (Neu5Ac9Lt) sialic acid and also *O*-sulfation and *O*-methylation of sialic acids occur (Chen, X. and Varki, A. 2010, Schauer, R. 2009, Varki, A., Schnaar, R.L., et al. 2015b). Strikingly, the biological functions of many of these sialic acid family members are poorly understood. The different sialic acid types are linked via three linkage types (α2,3, α2,6, or α2,8) by twenty sialyltransferase isoenzymes to glycans. This combinatorial diversity enables cells to produce a highly diverse repertoire of sialoglycans referred to as the *sialome* (Cohen, M. and Varki, A. 2010, Varki, A., Schnaar, R.L., et al. 2015b).

Located at the outer end of glycans, sialic acids are at the center of numerous molecular interactions at the cell surface. For example, sialic acids influence the biochemical properties and functions of glycoproteins and lipids, mask underlying glycans, mediate cell adhesion and migration and serve as ligands for sialic acid-binding proteins like factor H, selectins and the Siglecs (Schauer, R. 2009, Varki, A., Schnaar, R.L., et al. 2015b). Factor H can bind sialic acids on the surface of host cells to avoid complement activation and the selectin family interacts with sialic acids during lymphocyte trafficking and extravasation to sites of inflammation (Varki, A. 2007, Varki, A. and Gagneux, P. 2012). Sialic acids are also important immune regulators by interacting with sialic acid-binding immunoglobulin-like lectins (Siglecs), a family of immunomodulatory receptors widely expressed throughout the immune system (Büll, C., Heise, T., et al. 2016, Macauley, M.S., Crocker, P.R., et al. 2014, Varki, A., Schnaar, R.L., et al. 2015a). Next to their vital role in physiological processes, a number of pathologies are associated with sialic acids and their biosynthesis. Many human pathogens use sialic acids on host cells as attachment site or decorate their surface with host sialic acids as molecular mimicry to evade the immune system (Chang, Y.C. and Nizet, V. 2014, Heise, T.,

Langereis, J.D., et al. 2018, Stencel-Baerenwald, J.E., Reiss, K., et al. 2014, Wasik, B.R., Barnard, K.N., et al. 2016). Aberrant expression of sialoglycans supports tumor growth, metastasis and limits anti-tumor immune responses (Boligan, K.F., Mesa, C., et al. 2015, Büll, C., Boltje, T.J., et al. 2018, Büll, C., den Brok, M.H., et al. 2014, Büll, C., Stoel, M.A., et al. 2014, Pinho, S.S. and Reis, C.A. 2015, Schultz, M.J., Swindall, A.F., et al. 2012). Furthermore, genetic defects in sialic acid biosynthesis have been identified and may result in clinical phenotypes such as myopathy, sialuria or abnormal neuronal development (Aula, N., Salomaki, P., et al. 2000, van Karnebeek, C.D., Bonafe, L., et al. 2016, Varki, A. 2008, Willems, A.P., van Engelen, B.G., et al. 2016).

The importance of the expression and recognition of sialic acids in human biology and pathology makes them potential therapeutic targets. Therefore, approaches to track and alter sialic acid expression or to change their biochemical properties of sialic acids are not only essential to study their biological functions, but are also key to their application in biotechnological processes or as therapy in the future. Sialic acid glycoengineering, the introduction of unnatural sialic acids with different chemical properties into sialoglycans, is such an approach that is increasingly applied in the field of glycoscience and beyond. Chemically modified analogues of N-acetylmannosamine, the biological precursor of sialic acid, or sialic acid analogues can be introduced into the metabolic sialic acid biosynthesis pathway resulting in their incorporation into sialoglycans (**Figure 2**). This approach also allows to image sialoglycans in living organisms or to alter their binding to sialic acid-binding lectins. Although ManNAc analogues and sialic acid analogues both are useful tools for sialic acid glycoengineering, there are significant differences in the chemical modification potential and cellular metabolism of both sugars that have important implications for their use in glycoengineering experiments.

Here we review the differences between ManNAc and sialic acid analogues and the consequences hereof on labeling efficiency, selectivity for the sialic acid biosynthesis pathway and effects on biological processes in the cell. Finally, we discuss the implications of these differences for the use of ManNAc and sialic acid analogues in sialic acid glycoengineering.

Sialic acid glycoengineering using ManNAc and sialic acid analogues

Two approaches are used in sialic acid glycoengineering, either selective exo-enzymatic labeling of sialic acids or metabolic labeling via the incorporation of unnatural ManNAc or sialic acids. In this review, we focus on the more commonly used metabolic incorporation approach and the reader is referred to other timely publications covering selective exo-enzymatic labeling (Briard, J.G., Jiang, H., et al. 2018, Mbua, N.E., Li, X.R., et al. 2013, Nischan, N. and Kohler, J.J. 2016, Sun, T.T., Yu, S.H., et al. 2016). Metabolic labeling of sialic acids can be achieved by the incorporation of unnatural biosynthetic precursors into the cell's sialoglycans (see **Figure 2** for a detailed description of the sialic acid biosynthesis). Two precursors to infiltrate the sialic acid biosynthesis are typically utilized, ManNAc and Neu5Ac. ManNAc is converted into Neu5Ac by three enzymes, UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE) that produces ManNAc-6-phosphate, N-acetylneuraminate synthase (NANS) which produces Neu5Ac-9-phosphate, and finally Neu5Ac-9-P-phosphatase (NANP) which produces Neu5Ac. In addition to the *de novo* biosynthesis, vertebrate cells can salvage exogenous sialic acids from dietary sources, such as the non-human Neu5Gc, and certain pathogenic microorganisms can utilize sialic acids derived from host cells (Freeze, H.H., Hart, G.W., et al. 2015, Heise, T., Langereis, J.D., et al. 2018, Severi, E., Hood, D.W., et al. 2007, Varki, A., Schnaar, R.L., et al. 2015b). Neu5Ac is activated with cytidine monophosphate (CMP) in the nucleus by the CMP sialic acid synthase (CMAS) to produce CMP-sialic acid which is transported to the Golgi system and incorporated into sialoglycans by the sialyltransferases (Kajihara, Y., Nishigaki, S., et al. 2011). Cellular uptake of unnatural ManNAc and sialic acid analogues by endogenous transport mechanisms is poor due to their polar nature and the lack of efficient uptake mechanisms in mammalian cells. Studies using radioactive-labeled sugars and other reporter groups showed that concentrations in the range of 1-20 mM need to be added to cell cultures *in vitro* to achieve robust incorporation into glycans (Bardor, M., Nguyen, D.H., et al. 2005, Diaz, S. and Varki, A. 1985). Analogues are therefore often acetylated to facilitate passive diffusion through the plasma membrane and allow working concentrations of 1-200 μM (Wang, Z., Du, J., et al. 2009). The acetyl esters are removed intracellularly by esterases as only the unprotected sugar can be used by the sialylation machinery (Jacobs, C.L., Yarema, K.J., et al. 2000, Mathew, M.P., Tan, E., et al. 2017). The carbon backbone of

ManNAc and sialic acid can further be modified to obtain sugar analogues with different functionalities. Depending on the modification, analogues can be produced that inhibit enzymes involved in sialylation and therefore act as metabolic inhibitor of sialic acid expression. For instance, sialic acid with an axial fluorine on C-3 is a potent sialyltransferase inhibitor that was applied to enhance anti-tumor immunity or to reduce metastasis in mouse models (Büll, C., Boltje, T.J., et al. 2018, Büll, C., Boltje, T.J., et al. 2015, Büll, C., Boltje, T.J., et al. 2013, Macauley, M.S., Arlian, B.M., et al. 2014, Rillahan, C.D., Antonopoulos, A., et al. 2012). On the contrary, modifications to ManNAc or sialic acid that are tolerated by the sialylation enzymes enable the metabolic introduction of sialic acid analogues into glycans. Currently, ManNAc and sialic acid analogues with different functionalities have been developed for sialic acid glycoengineering (**Figure 3**) (Cheng, B., Xie, R., et al. 2016, Sminia, T.J., Zuilhof, H., et al. 2016, Wratil, P.R., Horstkorte, R., et al. 2016).

Initially, ManNAc derivatives with increasingly aliphatic C-2 acyl chains were used to probe the promiscuity of the sialic acid biosynthesis pathway (Wratil, P.R., Horstkorte, R., et al. 2016). Several ManNAc analogues are tolerated by the sialylation machinery and hence are converted into the corresponding sialic acid analogues inside the cell leading to their presentation on cell surface glycans. For instance, Reutter and colleagues generated acyl-chain modified ManNAc analogues that can be transformed into the corresponding sialic acid analogues which either enhanced or abrogated recognition by polyoma viruses (Keppler, O.T., Stehling, P., et al. 1995). Bertozzi and co-workers showed that small chemical reporter groups could also be added to the C-2 acyl chain of ManNAc derivatives resulting in sialic acid analogue carrying the modification on C-5 (Sletten, E.M. and Bertozzi, C.R. 2009). A prime example is the azidoacetyl functionality which can undergo a so-called bioorthogonal reaction to introduce a fluorescent label onto sialic acids (Saxon, E. and Bertozzi, C.R. 2000). Using this approach sialic acid localization and turnover can be imaged in cell lines, zebrafish embryos or tumors in mouse models (Belardi, B., de la Zerda, A., et al. 2013, Dehnert, K.W., Baskin, J.M., et al. 2012, Neves, A.A., Stockmann, H., et al. 2011, Prescher, J.A., Dube, D.H., et al. 2004, Rong, J., Han, J., et al. 2014, Xie, R., Dong, L., et al. 2016). Moreover, introducing ManNAc or sialic acid analogues into the glycans of living cells enables purification of sialylated proteins, targeted delivery of toxins to tumor cells or to alter the interaction with sialic acid-binding proteins such as

Siglecs or pathogenic lectins (Büll, C., Heise, T., et al. 2016, Büll, C., Heise, T., et al. 2017, Heise, T., Büll, C., et al. 2017, Spiciarich, D.R., Nolley, R., et al. 2017, Wang, H., Wang, R., et al. 2017). The versatile applications of sialic acid glycoengineering with ManNAc or sialic acid analogues have been reviewed by others (Badr, H.A., AlSadek, D.M.M., et al. 2017, Cheng, B., Xie, R., et al. 2016, Du, J., Meledeo, M.A., et al. 2009, Rouhanifard, S.H., Nordstrom, L.U., et al. 2013, Sminia, T.J., Zuilhof, H., et al. 2016, Wratil, P.R., Horstkorte, R., et al. 2016). Although ManNAc and sialic acid analogues are both used for a wide range of applications, little attention has been paid to key differences in their chemical modification potential and metabolic fate that co-determine their incorporation efficiency into sialoglycans, selectivity for the sialic acid pathway and effects on biological processes (**Table I**). These differences have important implications for the use of ManNAc and sialic acid analogues and should be taken into consideration for sialic acid glycoengineering.

Modification potential and synthesis of ManNAc and sialic acid analogues

The differences in structure and cellular metabolism between ManNAc and sialic acid define the spectrum of analogues that can be developed for sialic acid glycoengineering (**Figure 3**). Modifications to the ManNAc backbone need to be tolerated by GNE, NANS and NANP in order to yield the respective sialic acid analogue. The C-2 position of ManNAc can be modified in one step by performing an acylation reaction and C-2 substituted ManNAc will result in the formation of the corresponding C-5 modified sialic acid (Wratil, P.R., Horstkorte, R., et al. 2016). This makes ManNAc an attractive choice if modifications on this position need to be incorporated. In addition, the C-4 position of ManNAc can be modified leading to C-7 modified sialic acid presentation in the cell. The chemical synthesis of C-4 modified ManNAc derivatives can be achieved in five steps and various substituents have been introduced already at C-4 (Thomson, R. and von Itzstein, M. 1995). From a synthesis perspective, ManNAc analogues are readily available and therefore many sialic acid glycoengineering reagents used so far are based on ManNAc precursors. However, there are three major limitations for the development of ManNAc analogues for sialic acid engineering (**Table I**). First, modifications at certain positions on ManNAc are not tolerated by the three enzymes converting it into sialic acids. For example, the C-1 position of ManNAc cannot be modified, because it is a

reactive site in the conversion to sialic acid by NANS. Furthermore, the C-6 position of ManNAc is phosphorylated by GNE and hence modifications at this site would interfere with the production of ManNAc-6-phosphate, the substrate for NANS (Blume, A., Benie, A.J., et al. 2004, Lawrence, S.M., Huddleston, K.A., et al. 2000, Oetke, C., Brossmer, R., et al. 2002, Tanner, M.E. 2005). Potentially, unphosphorylated ManNAc may be converted into sialic acid by sialic acid aldolase or GlcNAc 6-kinase but both are likely very inefficient processes (Hinderlich, S., Berger, M., et al. 2000, Sparks, M.A., Williams, K.W., et al. 1993). Although not directly utilized in sialic acid synthesis, the C-3 hydroxyl of ManNAc can also not be modified since it will be converted into the endocyclic oxygen in sialic acid. Second, the size of the modifications that can be carried through the sialic acid biosynthesis pathway is especially restricted by GNE which carries out the phosphorylation of ManNAc on the C-6 position to yield ManNAc-6-phosphate. Acyl modifications larger than five carbon atoms or branched structures on the C-2 position of ManNAc impede the enzymatic conversion by GNE (Jacobs, C.L., Goon, S., et al. 2001, Viswanathan, K., Lawrence, S., et al. 2003). Third, not all potential sialic acid analogues can be generated utilizing ManNAc precursors since it undergoes an aldol reaction with phosphoenolpyruvate catalyzed by NANS to form sialic acid. Hence, the C-3 and C-4 position of sialic acid cannot be modified starting from ManNAc since it is derived from phosphoenolpyruvate or is a reactive center in the aldol condensation, respectively. These three factors largely limit the development of ManNAc analogues for incorporation into cell surface sialic acids, but can be explored alternatively for the development of inhibitors of ManNAc conversion to sialic acids (Nieto-Garcia, O., Wratil, P.R., et al. 2016, Wratil, P.R., Rigol, S., et al. 2014).

Sialic acid is a more versatile reagent as it bypasses the metabolic conversion by GNE, NANS and NANP and therefore has a broader modification potential than ManNAc analogues (**Table I**). Except from C-1 and C-2 that are required for CMP activation and glycosidic linkage formation, sialic acid can potentially be modified at positions C-3 to C-9 since these positions are not directly involved in further metabolism (Büll, C., Heise, T., et al. 2016). Indeed, numerous modifications at the C-5 and C-9 of sialic acid are well tolerated and efficiently incorporated (Cheng, B., Xie, R., et al. 2016). Compared to ManNAc, the chemical modification of sialic acid is more challenging as this sugar contains more chemical functionalities. The selective modification of the sialic acid backbone

therefore requires more elaborate protecting group strategies (Hemeon, I. and Bennet, A.J. 2007, Yu, C.C. and Withers, S.G. 2015). However, the more complicated scaffold of sialic acid provides more freedom to introduce unnatural modifications on the positions C-3 to C-9 with exception of the oxygen bound C-6. Access to C-3 modified sialic acids can be achieved by the use of 2-deoxy-2,3-dehydro-N-acetyl-neuraminic acid (DANA) derivatives in which the double bond can undergo electrophilic addition reactions. The C-4 positions can be modified using a nucleophilic addition to a sialic acid 4,5-oxazoline precursor or by using protecting group strategies and utilizing the intrinsic reactivity of the 4-OH. Functionalizing the C-5 position chemically requires cleavage of the acetamide, which requires harsh conditions and protection of the anomeric center to avoid side products (Büll, C., Heise, T., et al. 2015). The C-7 and C-8 hydroxyl groups are of low reactivity and strategies to modify them selectively require multiple protecting group manipulations. The C-9 hydroxyl group is the only primary alcohol in sialic acid and can be modified with good selectivity in the presence of other reactive groups. C-9 azido sialic acid is therefore readily available and a popular labeling reagent (Gross, H.J., Bunsch, A., et al. 1987, Han, S., Collins, B.E., et al. 2005, Xie, R., Dong, L., et al. 2016). In addition to the chemical synthesis of sialic derivatives, a chemo-enzymatic strategy can also be employed. N-acetylneuraminic acid lyase (NAL) is a bacterial aldolase capable of breaking down sialic acid into ManNAc and pyruvate. By using an excess of ManNAc or pyruvate, however, NAL can also catalyze the reverse reaction and hence modified ManNAc and pyruvate derivatives can be utilized to obtain the corresponding sialic acid analogue. For example, the use of fluoro pyruvate leads to the formation of 3-fluoro sialic acid that inhibits sialoglycan biosynthesis (Büll, C., Boltje, T.J., et al. 2013, Burkart, M.D., Vincent, S.P., et al. 1999, Heise, T., Langereis, J.D., et al. 2018, Rillahan, C.D., Antonopoulos, A., et al. 2012). Modifications on the ManNAc scaffold at C-2, C-4 and C-6 can be tolerated by NAL and hence yields C-5, C-7 and C-9 modified sialic acids (Cheng, B., Xie, R., et al. 2016). Out of the modifiable sites present in sialic acid only the C-3, C-5, C-7 and C-9 have been explored for sialic acid glycoengineering to date (Büll, C., Heise, T., et al. 2016) (**Figure 3**). Utilizing other positions (C-4 and C-8) with unnatural moieties for sialic acid glycoengineering is still unexplored. Elegant chemical procedures to make these derivatives available are therefore needed to further expand the scope of sialic acid glycoengineering.

Differences in the cellular utilization of ManNAc and sialic acid analogues

ManNAc and sialic acid, and hence their analogues, follow a different metabolic fate inside the cell, although their metabolism is not understood in all detail. Exogenous ManNAc and its unprotected analogues have been suggested to enter the cell via a so far unknown transporter system and are converted to sialic acids in the cytoplasm as depicted in **Figure 2** (Bardor, M., Nguyen, D.H., et al. 2005). Sialic acids and unprotected analogues enter the cell most likely via pinocytosis and independent from clathrin as suggested by Neu5Gc uptake experiments in the presence of the inhibitors amiloride and genistein, respectively (Bardor, M., Nguyen, D.H., et al. 2005). Further studies have shown that exogenous sialic acids can exit the lysosome via the sialic acid transporter sialin (SLC17A5) and are directly available for CMP activation and sialylation (Gilormini, P.A., Lion, C., et al. 2016, Vanbeselaere, J., Vicogne, D., et al. 2013) (**Table 1**). Foulquier and co-workers illustrated the different cellular fate of ManNAc and sialic acids using unprotected C-5 alkyne-modified versions abbreviated ManNAl and SiaNAl, respectively. SiaNAl was clearly detectable inside the Golgi compartment already two hours after addition to the culture, but labeling derived from ManNAl was detected only after five to seven hours (Gilormini, P.A., Lion, C., et al. 2016). This difference in kinetics supports the idea that sialic acid analogues become readily available for sialoglycan synthesis, whereas ManNAc analogues have to be converted by GNE, NANS or NANP first. Another possibility is that the alkyne reporter group of ManNAl could interfere with the enzymatic conversion into sialic, which should be addressed in future studies. Overall, the different metabolism of ManNAc and sialic acid analogues has important consequences for their incorporation efficiency, selectivity and effects on biological processes.

Incorporation Efficiency

The incorporation of ManNAc and sialic acid analogues into cell surface sialoglycans can be analyzed with several methods. For example, radioactivity, mass spectrometry or HPLC are used to quantify the levels of modified cell surface sialic acids and azide- or alkyne-modified sialic acids can be quantified after biorthogonal reaction to fluorophores (Sminia, T.J., Zuilhof, H., et al. 2016, Wratil, P.R., Horstkorte, R., et al. 2016). The incorporation efficiency can then be calculated as ratio of natural

versus modified surface sialic acids or as relative fluorescent signal, respectively. Next to the differences in ManNAc and sialic acid metabolism, also other factors such as the type and position of the reporter, the expression of genes involved in sialic acid biosynthesis as well as levels of competing endogenous sugars influence incorporation (Badr, H.A., AlSadek, D.M.M., et al. 2017, Pham, N.D., Fermaintt, C.S., et al. 2015). In the case of the frequently used peracetylated analogues that need to be deacetylated by intracellular esterases, the labeling efficiency is also depending on expression levels of esterases and the effect of the modifications on esterase activity (Pham, N.D., Fermaintt, C.S., et al. 2015, Wang, Z., Du, J., et al. 2009). Despite these variables, the incorporation efficiency of ManNAc and sialic acid analogues can be compared when using the same reporter and equimolar concentrations.

In a direct comparison, Luchansky and colleagues showed that various, unprotected C-5 ketone modified ManNAc analogues were incorporated poorly or not at all while the corresponding sialic acid acids showed efficient labeling as detected by flow cytometry after conjugation with fluorescein. For instance N-levulinoyl modified sialic acid (SiaLev) was incorporated three times higher relative to ManLev (Luchansky, S.J., Goon, S., et al. 2004). Interestingly, azide-modified ManNAc and sialic acid showed similar incorporation efficiencies, but when a photocrosslinkable aryl group was used as reporter the ManNAc analogue was barely incorporated whereas high levels of aryl-containing sialic acids were found at the cell surface. Similar results were also obtained with peracetylated analogues that passively diffuse through the cell membrane. In a comparative study, Dafik and colleagues found that various peracetylated sialic acid analogues carrying fluorine modifications on the C-5 position were incorporated with higher efficiency compared to their mannosamine counterparts as measured by HPLC-based detection of fluorine groups in membrane preparations relative to untreated cells (Dafik, L., d'Alarcao, M., et al. 2008). Whereas fluorinated sialic acids analogues replaced up to 90 % of the natural sialic acids at the cell surface, the corresponding fluorinated mannosamines replaced maximum 50 % at equimolar concentrations. In line with these findings, our group found that THP-1, Jurkat, HEK293 and HL-60 cells treated with peracetylated C-5 azido sialic acid (Ac_5SiaNAz) and C-5 propargyloxycarbonyl sialic acid (Ac_5SiaNPoc) had 2-4 times higher fluorescence intensity compared with corresponding Ac_4ManNAz and Ac_4ManNPoc (Büll, C., Heise, T., et al. 2015). At the single

glycoprotein level, we found that human hepatocytes could incorporate Ac_5SiaNPoc, but not Ac_4ManNPoc into the sialic acids of transferrin, supporting the other studies that show higher incorporation efficiency of sialic acid analogue compared to ManNAc analogues.

So far, only few studies have directly compared the incorporation efficiency of ManNAc and SiaNAc analogues *in vivo*. Our group has found that systemically administered Ac_5SiaNPoc resulted in two times or more labeling of mouse organs compared to the ManNAc counterpart after reaction to fluorescent azide biotin (Büll, C., Heise, T., et al. 2015). Similarly, Cheng and co-workers showed that liposome encapsulated C-9-modified azido sialic acid resulted in potent labeling of sialoglycans in the mouse brain whereas azidomannosamine-containing liposomes resulted in no labeling (Xie, R., Dong, L., et al. 2016). Free, peracetylated C-9 azido sialic acid or Ac_4ManNAz were not incorporated into brain sialoglycans, most likely because they are unable to pass the blood-brain barrier. These findings are further supported by experiments showing that incorporation of systemically administered peracetylated N-propanoylmannosamine (Ac_4ManNProp) or Ac_4ManNAz into brain sialoglycans is scarce whereas they are incorporated in most other organs (Gagiannis, D., Gossrau, R., et al. 2007, Shajahan, A., Parashar, S., et al. 2017). The reason why liposome-encapsulated azido sialic acids were incorporated into brain sialoglycans, but not liposome-encapsulated ManNAz remains to be investigated. Noteworthy, labeling of brain sialoglycans with azidomannosamine was recently achieved by intracranial injection or by coupling the ManNAc analogue to neuroactive carriers that pass the blood-brain barrier (Shajahan, A., Parashar, S., et al. 2017). The performance of corresponding sialic acid analogues has not been compared to the ManNAc analogues in this study, and would be interesting to address in further experiments.

Collectively, most *in vitro* studies and few *in vivo* experiments advocate that sialic acid analogues are incorporated with higher efficiency compared to their ManNAc congener (**Table II**). This difference is potentially caused by the more extensive metabolism of ManNAc analogues, whereas sialic acid analogues can directly be used for sialylation. Modifications of ManNAc have to be tolerated by the ManNAc kinase domain of GNE (MNK) as well as NANS and NANP to produce the respective sialic acid analogue. Especially, tolerance of the MNK domain of GNE for the reporter appears to be rate-limiting for the labeling with ManNAc analogues (Benie, A.J., Blume, A., et al. 2004, Jacobs, C.L.,

Goon, S., et al. 2001, Keppler, O.T., Hinderlich, S., et al. 1999). Interestingly, in cells with low or silenced GNE expression, sialic acid engineering with ManNAc analogues is highly efficient compared to control cells, presumably due to the low levels of competing endogenous ManNAc (Mantey, L.R., Keppler, O.T., et al. 2001, Moller, H., Bohrsch, V., et al. 2011, Oetke, C., Hinderlich, S., et al. 2003). The corresponding sialic acid analogues were not tested in this context. Future studies should investigate further details of ManNAc and sialic acid metabolism and more comparative studies *in vitro* and *in vivo* are needed to determine the most potent ManNAc and sialic acid analogues for glycoengineering.

Selectivity

The sialic acid biosynthesis pathway is unique as compared to other carbohydrates used for glycosylation. Sialic acids are produced from their dedicated precursor sugar ManNAc that is first converted to ManNAc-6-P and then condensed with phosphoenolpyruvate to form sialic acid. Furthermore, sialic acids are the only sugar that uses CMP as nucleotide sugar donor and this activation step takes place in the nucleus for unknown reasons (Angata, T. and Varki, A. 2002, Freeze, H.H., Hart, G.W., et al. 2015, Varki, A., Schnaar, R.L., et al. 2015b, Willems, A.P., van Engelen, B.G., et al. 2016). This unique biosynthesis pathway implies that ManNAc and sialic acid analogues are specifically used for sialoglycan synthesis. However, there is evidence that ManNAc analogues and possibly sialic acid analogues can also be converted into carbohydrates other than sialic acid (**Table II**). Early metabolic labeling experiments in cell lines with radioactive ManNAc by Varki and co-workers showed that next to the sialic acids, radioactive label was derived also from other membrane components (Diaz, S. and Varki, A. 2009, Varki, A. 1991). These findings suggest that intracellular ManNAc is not exclusively used for sialic acid biosynthesis, but can be converted into other carbohydrates as well. In line with this observation, others and our own group provided evidence that ManNAc analogues are metabolized into different glycan types. Splenocytes or tumors isolated from mice that were injected with Ac_4ManNAz showed strong labeling of surface glycans. Upon treatment with sialidase, the labeling signal was reduced by only 15 %, indicating that the label was incorporated into glycans more broadly (Dube, D.H., Prescher, J.A., et al. 2006, Neves, A.A.,

Stockmann, H., et al. 2011). Similarly, we observed that Ac_4ManNPoc was still incorporated into glycans in cells treated with the pharmacological sialyltransferase inhibitor $Ac_5$3F_{ax}Neu5Ac and also in cells with complete SLC35A1 knockout (Büll, C., Heise, T., et al. 2015). Combination of the sialyltransferase inhibitor with the UDP-GlcNAc synthesis inhibitor 4-deoxy-GlcNAc abrogated labeling with Ac_4ManNPoc indicating that this ManNAc analogue is converted into UDP-GlcNAc in case that the efflux of sialic acids is blocked. Next to the UDP-N-acetylglucosamine 2-epimerase domain of the bifunctional enzyme GNE, mammalian cells can express another GlcNAc 2-epimerase also known as renin-binding protein (RenBP). This GlcNAc 2-epimerase has been reported to catalyze ManNAc into GlcNAc, thereby deflecting the flux of ManNAc away from sialic acid synthesis (**Figure 2**) (Ghosh, S. and Roseman, S. 1965, Luchansky, S.J., Yarema, K.J., et al. 2003, Maru, I., Ohta, Y., et al. 1996, Takahashi, S., Takahashi, K., et al. 1999). Depending on the expression levels and activity of GlcNAc 2-epimerase, ManNAc analogues could enter the hexosamine biosynthesis pathway via this alternative metabolic route, resulting in the production of modified N-Acetylgalactosamine (GalNAc), GlcNAc or other sugars.

Next to the ManNAc analogues, recent studies indicate that sialic acid analogues can enter the hexosamine pathway under certain circumstance as well. Ahmed and co-workers reported that under starvation conditions, exogenous sialic acid is converted to UDP-GlcNAc to fuel the hexosamine biosynthesis pathway in cells. Presumably, sialic acid is degraded into ManNAc and pyruvate by N-acetylneuraminate lyase and ManNAc is either recycled for sialic acid synthesis or can be converted into GlcNAc and other sugars derived from its metabolism (Badr, H.A., AlSadek, D.M.M., et al. 2017, Badr, H.A., AlSadek, D.M.M., et al. 2015, Varki, A., Schnaar, R.L., et al. 2015b).

Altogether, these studies suggest that ManNAc and possibly sialic acid analogues can be interconverted and hence their modification could basically end up in all products of the hexosamine pathway. So far, it is largely unknown under which circumstances and to what extent ManNAc and sialic acid analogues follow these alternative metabolic routes and should be subject of further investigations.

Effects on biological processes

Natural ManNAc and sialic acid sugars are poorly taken up by cells, and as a consequence can be added to cell cultures at concentrations in the millimolar range without affecting cell proliferation or viability (Bardor, M., Nguyen, D.H., et al. 2005, Bork, K., Reutter, W., et al. 2005, Büll, C., Boltje, T.J., et al. 2013, Ghaderi, D., Taylor, R.E., et al. 2010, Han, S.S., Lee, D.E., et al. 2017, Keppler, O.T., Hinderlich, S., et al. 1999, Oetke, C., Brossmer, R., et al. 2002, Saxon, E., Luchansky, S.J., et al. 2002, Wang, J., Cheng, B., et al. 2015). Major differences exist between peracetylated ManNAc and sialic acid analogues with respect to their effects on biological functions. Generally, the acetyl groups that promote passive diffusion of ManNAc and sialic acid and are released by esterases were recently shown to induce non-enzymatic cysteine *S*-glycosylation at high sugar concentrations (2mM in cell lysate). The cellular effects of this reaction remain to be investigated, but this study indicates that care needs to be taken when using acetylated precursors at millimolar concentrations (Qin, W., Qin, K., et al. 2018). Furthermore, the toxicity profile of ManNAc and sialic acid analogues varies significantly depending on the type of modification (Almaraz, R.T., Mathew, M.P., et al. 2012). Therefore, we focus here only on the characteristics of peracetylated ManNAc (Ac_4ManNAc) and sialic acid (Ac_5Neu5Ac) and their commonly used azido variants Ac_4ManNAz and Ac_5SiaNAz.

Peracetylated ManNAc and Ac_4ManNAz affect proliferation and viability at concentrations above 100 µM depending on the cell type (Aich, U., Campbell, C.T., et al. 2008, Almaraz, R.T., Aich, U., et al. 2012, Charter, N.W., Mahal, L.K., et al. 2002, Han, S.S., Lee, D.E., et al. 2017, Hsu, T.L., Hanson, S.R., et al. 2007, Jones, M.B., Teng, H., et al. 2004, Kim, E.J., Jones, M.B., et al. 2004, Rochefort, M.M., Girgis, M.D., et al. 2014). At lower concentrations around 50 µM, Ac_4ManNAz appears to be generally tolerated and allows sufficient incorporation into sialoglycans. Recent studies, however, report that peracetylated ManNAc analogues influence cellular function already at low micromolar concentrations. For instance, Ac_4ManNAz as well as Ac_4ManNAc reduced neurite outgrowth in primary mouse neuron cultures at 5 µM and showed cytotoxicity at 50 µM (Kang, K., Joo, S., et al. 2015). In a sprouting assay using spheroids derived from human umbilical vein endothelial cells, 50 µM ManNAc reduced the total capillary length by 75% compared to control (Bayer, N.B., Schubert, U., et al. 2013). More recently, Kang and co-workers performed more detailed studies on the effects of

Ac_4ManNAz on human adenocarcinoma cells as well as umbilical cord blood-derived endothelial progenitor cells (Han, S.S., Lee, D.E., et al. 2017, Han, S.S., Shim, H.E., et al. 2018). They found that treatment with 10 µM Ac_4ManNAz or higher resulted in the > 7 fold differential expression of more than 2000 genes compared to control cells affecting various cellular pathways. In particular treatment with 50 µM Ac_4ManNAz induced strong changes in the expression of genes involved in the cell cycle, proliferation, adhesion and apoptosis. Accordingly, supplementation with 20 µM Ac_4ManNAz or higher reduced cell growth, invasive properties and cellular respiration. Other tested parameters such as the tube-forming potential of the endothelial progenitor cells and LDL uptake were not significantly altered by Ac_4ManNAz suggesting that this sugar does not generally influence all cellular functions. Altogether, these studies demonstrate that peracetylated ManNAc analogues, although useful for sialoglycan labeling, have a broad impact on the molecular and cellular level (**Table II**). Curiously, the mechanisms by which ManNAc and its analogues affect cellular processes are not known. The release of acetyl groups by esterases in the cytoplasm and the recently discovered non-enzymatic cysteine *S*-glycosylation could contribute to the described effects (Qin, W., Qin, K., et al. 2018). Another possibility is that ManNAc influences cellular processes by altering biochemical pathways or changing gene expression (Du, J., Meledeo, M.A., et al. 2009, Han, S.S., Lee, D.E., et al. 2017). Further studies are needed to unravel the effect of ManNAc and its analogues on these molecular processes.

In comparison, peracetylated sialic acid analogues are less frequently used and only few studies have investigated their effects on biological processes. We have found that peracetylated sialic acid is well tolerated by cell lines even at concentrations above 2 mM without affecting proliferation or viability (van den Bijgaart, R.J.E., Kroesen, M., et al. 2019). Also, pretreatment of mouse melanoma cells with Ac_5Neu5Ac for three days *in vitro* or intratumoral Ac_5Neu5Ac injections for several weeks *in vivo* had no effect on melanoma growth in mice, indicating that peracetylated sialic acid does not alter overall cell behavior (Büll, C., Boltje, T.J., et al. 2018, Büll, C., Boltje, T.J., et al. 2013). In line with these findings, Ac_4ManNAz affects cellular viability *in vitro* at concentrations above 100 µM whereas the corresponding sialic acid analogues Ac_5SiaNAz showed no signs of cytotoxicity even at concentrations above 256 µM (Büll, C., Heise, T., et al. 2015). These studies indicate that

peracetylated sialic acids are less cytotoxic than peracetylated ManNAc analogues and suggest that the effects detected with ManNAc are not solely caused by release of acetyl groups, but mediate by intracellular ManNAc. Clearly more detailed studies are required to assess the effects of both types of sugar analogues at the molecular level.

Despite peracetylated ManNAc analogues affect cell proliferation and viability *in vitro*, no adverse effects have been reported so far for the *in vivo* use of ManNAc analogues. Bertozzi and colleagues have demonstrated the use of Ac_4ManNAz for the labeling of sialoglycans in developing zebrafish embryos as well as in mice (Baskin, J.M., Dehnert, K.W., et al. 2010, Chang, P.V., Chen, X., et al. 2009, Dehnert, K.W., Baskin, J.M., et al. 2012, Prescher, J.A., Dube, D.H., et al. 2004, Saxon, E. and Bertozzi, C.R. 2000). Malicdan and co-workers showed that continuous oral administration of Ac_4ManNAc for more than 40 weeks was tolerated without affecting renal or liver function (Malicdan, M.C., Noguchi, S., et al. 2012). Accordingly, Reutter and co-workers found that injections with N-Propanoylmannosamine (Ac_4ManNProp) twice a day for up to 45 days were tolerated and allowed to replace about 60 % of the natural sialic acids in mice. Although no effects of the injections on the histological and physiological level were found, Ac_4ManNProp injections strongly reduced the expression of polysialic acids (NCAM) in the brain without being incorporated (Gagiannis, D., Gossrau, R., et al. 2007). The consequences hereof for brain function were not assessed so far. We as well have not noticed adverse effects derived from repeated injections with alkyne-modified peracetylated ManNAc as well as sialic acid in mice (Büll, C., Heise, T., et al. 2015). Thus, peracetylated ManNAc and sialic acid analogues appear to be well tolerated *in vivo*, but more detailed studies are required to rule out possible effects on physiological processes.

Applications of ManNAc and sialic acid analogues

Both, ManNAc and sialic acid analogues are useful tools to introduce modified sialic acids into cell surface glycans, but their differences in chemical modification potential, incorporation efficiency, selectivity and effects on physiological processes should be taken into account when performing sialic acid glycoengineering experiments (**Table I**). Although, studies directly comparing ManNAc analogues and their corresponding sialic acid analogues are limited, sialic acid analogues appear to

allow for higher labeling efficiencies, have fewer effects on cell biology and are more specific for the sialic acid pathway. Furthermore, labeling of sialylated microorganisms e.g. Nontypeable *Haemophilus influenzae* that utilize ManNAc for energy production, but not for synthesizing sialic acids can only be achieved using sialic acid analogues (Apicella, M.A. 2012, Heise, T., Langereis, J.D., et al. 2018).

However, depending on the application both ManNAc and sialic acid analogues are highly useful. For instance, both ManNAc and sialic acid analogues allow for the detection of and possibly rescue of genetic defects in the sialic acid biosynthesis pathway (Freeze, H.H. 2013). In a recent study, exome sequencing revealed mutations in NANS in a cohort of patients with infantile-onset severe developmental delay and skeletal dysplasia (van Karnebeek, C.D., Bonafe, L., et al. 2016). Accordingly, elevated levels of ManNAc were identified in serum and urine of the patients and fibroblasts cultured from these patients featured increased ManNAc-6-phosphate levels compared to control. Remarkably, no reduction in systemic sialic acid levels and sialylation was found, suggesting residual activity of the NANS mutants or efficient recycling of dietary sialic acids. However, in NANS-deficient zebrafish embryos, addition of sialic acid rescued abnormal skeletal development suggesting that the reduced sialic acid production from ManNAc is causative for the disease phenotype. ManNAl and SiaNAl were used to demonstrate the reduced NANS activity in patient fibroblasts. Whereas ManNAl was not incorporated into membrane sialoglycans, SiaNAl supplementation resulted in clear labeling. Similarly, ManNAl and SiaNAl were employed to demonstrate a genetic defect in the lysosomal sialic acid transporter sialin (SLC17A5) that leads to sialic acid storage disorder. In sialin-deficient patient fibroblasts, the addition of ManNAl, but not SiaNAl resulted in efficient labeling of surface sialoglycans (Gilormini, P.A., Lion, C., et al. 2016).

Next to their potential diagnostic use, ManNAc and sialic acid analogues are also under investigation for the treatment of GNE myopathy, a rare disease resulting in muscular atrophy. GNE myopathy is caused by mutations either in the epimerase (GNE) or kinase (MNK) domain resulting in reduced sialic acid production (Carrillo, N., Malicdan, M.C., et al. 2018). The pathophysiology of GNE myopathy is not understood completely, but (pre-)clinical studies suggest that ManNAc analogues and potentially sialic acid analogues could be applied therapeutically to bypass the genetic defect in GNE

to reduce muscular atrophy (Carrillo, N., Malicdan, M.C., et al. 2018, Malicdan, M.C., Noguchi, S., et al. 2009, Malicdan, M.C., Noguchi, S., et al. 2012).

Despite ManNAc and sialic acid analogs both are valuable probes to identify defects in GNE (MNK domain), NANS, NANP or sialin, defects downstream sialic acid synthesis might not be detected unequivocally. We have shown that defects inhibiting sialylation in the Golgi system that reduce sialic acid efflux can be detected with sialic acid analogues, but not with ManNAc analogues. Complete knockdown of the CMP-sialic acid transporter (SLC35A1) in haploid cells or pharmacological inhibition of sialyltransferases with fluorinated sialic acid reduced labeling of glycans with alkyne sialic acid about 90% of control, but only reduced labeling with alkyne ManNAc by about 50% (Büll, C., Heise, T., et al. 2015, Riemersma, M., Sandrock, J., et al. 2015). In line with the findings, Kohnz et al still observed labeling with ManNAz in CMAS knockdown cells (Kohnz, R.A., Roberts, L.S., et al. 2016). Presumably, blocked sialylation results in the conversion of ManNAc in the hexosamine pathway into monosaccharides other than sialic acid. Overall, these studies are good examples for the effective use of ManNAc and sialic acid analogues to monitor the metabolic flux through the sialic acid pathway and to isolate defects in sialic acids biosynthesis on the biochemical level. ManNAc and sialic acid analogues can thus be applied to explore the interconnection of the sialic acid pathway with other glycosylation pathways. Furthermore, based on the analogues, assays can be developed to determine the severity of genetic effects for enzyme function and sialic acid production and to develop robust screening assays to select pharmaceuticals restoring the biochemical defects in the sialic acid biosynthesis pathway.

Conclusion

Sialic acids are at the center of numerous molecular interactions at the cells surface and associated with several pathologies including infection and cancer. Glycoengineering using ManNAc and sialic acid analogues has therefore a wide range of biological and therapeutic applications, but there are intrinsic differences between both sugars that should be taken into account. ManNAc and sialic acid analogues can show significant differences with respect to their incorporation efficiency into sialoglycans, selectivity for the sialic acid pathway and effects on biological processes and thus can

lead to different results in glycoengineering experiments. ManNAc and sialic acid analogues should therefore be compared using parameters such as summarized in **Table II** to be able to select the best analogue depending on the type of application. Such comparative studies are especially needed, regarding the growing number of ManNAc and sialic acid analogues with one or more functionalities that is becoming available to probe and alter the biological functions of sialic acids in single cells and whole organisms. Comparative studies can be difficult to pursue for instance, because of limited availability of analogues or restricted access to technology to assess their metabolism and to quantify incorporation. These hurdles will be overcome with an increasing availability of ManNAc and sialic acid analogues, rapid progress in the development of robust readouts e.g. click chemistry, advances in the mass spectrometry of glycans and their metabolites and growing multidisciplinary research efforts. Eventually, these studies will enable researchers in the field of glycobiology and beyond to explore the numerous applications of sialic acid glycoengineering using ManNAc and sialic acid analogues.

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Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CMAH	Cytidine-monophosphate-N-acetylneuraminic acid hydroxylase
CMAS	CMP sialic acid synthase
CMP	Cytidine monophosphate
GalNAc	N-Acetylgalactosamine
GlcNAc	N-acetylglucosamine
GNE	UDP-GlcNAc 2-epimerase/ManNAc kinase
KDN	2-keto-3-deoxy-D-glycero-D-galacto-nononic acid
ManNAc	N-Acetylmannosamine
MNK	ManNAc kinase
NAL	N-acetylneuraminic acid lyase
NANP	Neu5Ac-9-P-phosphatase
NANS	N-acetylneuraminate synthase
NCAM	Neural cell adhesion molecule
Neu5Ac	N-Acetylneuraminic acid
Neu5Gc	N-Glycolylneuraminic acid
NPL	N-acetylneuraminate lyase
Pep	Phosphoenolpyruvate
PI	Inorganic phosphate
RenBP	Renin-binding protein
Siglec	Sialic acid-binding immunoglobulin-like lectin
UDP	Uridine diphosphate

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Figure Legends

Figure 1 Chemical structure of N-Acetylmannosamine (ManNAc) and N-Acetylneuraminic acid (Neu5Ac).

Figure 2 Sialic acid biosynthesis pathway and entry points of ManNAc and sialic acid analogues. The *de novo* biosynthesis of sialic acid is derived from the metabolism of glucose. Three enzymatic steps convert glucose into uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), the biochemical precursor for the biosynthesis of Neu5Ac as well as other carbohydrates. The bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE) converts UDP-GlcNAc into N-Acetylmannosamine (ManNAc) via its epimerase domain (GNE) and subsequently produces ManNAc-6-phosphate using its kinase activity (MNK). ManNAc-6-phosphate is converted into Neu5Ac-9-phosphate by the N-acetylneuraminate synthase (NANS) and dephosphorylated by Neu5Ac-9-P-phosphatase (NANP) to yield Neu5Ac. In the nucleus, Neu5Ac is conjugated with cytidine monophosphate (CMP) by the CMP sialic acid synthase (CMAS) and transported into the Golgi system via the CMP-sialic acid transporter SLC35A1.Cytosolic CMP-sialic acid levels regulate the *de novo* synthesis of sialic acids by feedback inhibition of GNE. In the Golgi, 20 sialyltransferase isoenzymes link sialic acid to glycans. Sialoglycans present at the cell surface or in intracellular compartments like lysosomes can be cleaved by sialidases to release free sialic acids. Free lysosomal sialic acids can be transported into the cytosol via the sialin (SLC17A5) transporter for recycling in the sialic acid biosynthesis pathway or degradation into ManNAc and pyruvate by N-acetylneuraminate pyruvate lyase (NPL) (Freeze, H.H., Hart, G.W., et al. 2015, Varki, A., Schnaar, R.L., et al. 2015b).

Figure 3 Reported ManNAc (blue) and sialic acid analogues (C-5 in red; C-9 in gray) developed for sialic acid glycoengineering. 1. (Collins, B.E., Fralich, T.J., et al. 2000); 2. (Chefalo, P., Pan, Y., et al. 2006); 3. (Lieke, T., Gröbe, D., et al. 2011); 4. (Jacobs, C.L., Goon, S., et al. 2001, Kayser, H., Zeitler, R., et al. 1992, Keppler, O.T., Stehling, P., et al. 1995); 5, 26. (Dafik, L., d'Alarcao, M., et al. 2008, Hadfield, A.F., Mella, S.L., et al. 1983); 6, 10. (Stairs, S., Neves, A.A., et al. 2013); 7. (Cole, C.M., Yang, J., et al. 2013); 8. (Saxon, E. and Bertozzi, C.R. 2000); 9. (Hsu, T.L., Hanson, S.R., et al. 2007); 11. (Xiong, D.-C., Zhu, J., et al. 2015); 12. (Andersen, K.A., Aronoff, M.R., et al. 2015); 13. (Bateman, L.A., Zaro, B.W., et al. 2013); 14, 19. (Hong, S., Lin, L., et al. 2015); 15. (Patterson, D.M., Jones, K.A., et al. 2014); 16, 21. (Dold, J.E., Pfotzer, J., et al. 2017); 17, 21. (Niederwieser, A., Späte, A.K., et al. 2013, Späte, A.K., Schart, V.F., et al. 2014); 18. (Tanaka, Y. and Kohler, J.J. 2008); 20. (Mahal, L.K., Yarema, K.J., et al. 1997); 22. (Sampathkumar, S.-G., Li, A.V., et al. 2006); 23-25, 33-36, 38-41, 44. (Oetke, C., Brossmer, R., et al. 2002); 27, 28. (Luchansky, S.J., Goon, S., et al. 2004); 29. (Büll, C., Heise, T., et al. 2017); 30. (Feng, L., Hong, S., et al. 2013); 31. (Homann, A., Qamar, R.-u., et al. 2010); 32. (Dafik, L., d'Alarcao, M., et al. 2010); 37. (Xie, R., Hong, S., et al. 2012); 42. (Zeng, Y., Ramya, T., et al. 2009); 43. (Patterson, D.M., Nazarova, L.A., et al. 2012); 45. (Han, S., Collins, B.E., et al. 2005).

Table I General considerations for ManNAc and sialic acid analogues.

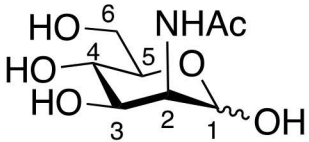
	ManNAc analogues	**Sialic acid analogues**
Chemical modification potential	– Simple (1 step) modification – Only C-2 and C-4 modifications are tolerated by GNE, NANS and NANP – Size of modification is mainly limited by GNE – Does not allow conversion into corresponding sialic acid analogues with C-3 or C-4 modifications – Suitable for peracetylation or other prodrug modifications	– Modification more challenging – Small modifications from C-3 to C-9 are generally tolerated by enzymes involved in sialylation – Chemo-enzymatic synthesis from ManNAc possible – C-3 to C-9 modification feasible (C-3, C-5, C-7, C-9 achieved; C-4 and C-8 possible) – Suitable for peracetylation or other prodrug modifications
Metabolism	– Uptake of unprotected ManNAc analogues is poor (1-10 mM) and potentially mediated via a so far unknown transporter – Peracetylation improves membrane permeability (100-1000 times higher uptake) – Conversion into sialic acids via three rate-limiting enzymes – Utilization in hexosamine pathway possible through conversion into GlcNAc by GlcNAc 2-epimerase	– Uptake of unprotected sialic acid is poor (1-10 mM) and potentially mediated by pinocytosis into lysosomes and export into cytosol via SLC17A5 – Peracetylation improves membrane permeability (100-1000 times higher uptake) – Directly available for CMP activation by CMAS after deacetylation – Utilization in hexosamine pathway possible through degradation into ManNAc by NPL
Applications	– Restricted to cells/species expressing GNE, NANS and NANP – Sialic acid glycoengineering – Metabolic flux analysis – Detection of genetic effects upstream of GNE (MNK domain), NANS or NANP	– Incorporation independent from GNE, NANS and NANP – Sialic acid glycoengineering – Metabolic flux analysis – Detection of genetic effects downstream of NANP and in the lysosomal transporter SLC17A5

Table II Criteria for the evaluation of ManNAc and sialic acid analogues using the example of peracetylated ManNAc and sialic acid and their frequently used C-5 alkyne and azido variants.

	Ac_4ManNAc/Az/Al	**Ac_5SiaNAc/Az/Al**	**References**
Synthesis	– 2 steps	– 8 steps	(Saxon, E., Luchansky, S.J., et al. 2002)
Uptake	– Passive diffusion and deacetylation by esterases	– Passive diffusion and deacetylation by esterases	(Wang, Z., Du, J., et al. 2009)
Effects on sialic acid biosynthesis enyzmes	– Not reported	– Not reported	
Incorporation efficiency *in vitro*	– 2-4 times lower compared to sialic acid analogues in THP-1, Jurkat, HEK293 and HL-60 cells at 100 µM – Low/no incorporation into secreted transferrin	– 2-4 times higher compared to ManNAc analogues in THP-1, Jurkat, HEK293 and HL-60 cells at 100 µM – Incorporation into secreted transferrin	(Büll, C., Heise, T., et al. 2015)
Selectivity for sialoglycan synthesis	– Incorporation detectable in SLC35A1 knockout cells – Sialyltransferase inhibitor reduces labeling by 50% – Incorporation into sialidase-resistant cell surface glycans – Indications for metabolism in hexosamine pathway	– No incorporation into SLC35A1 knockout cells – Sialyltransferase inhibitor reduces labeling by 80% – Selective utilization in the sialic acid biosynthesis pathway	(Büll, C., Heise, T., et al. 2015, Dube, D.H., Prescher, J.A., et al. 2006, Luchansky, S.J., Yarema, K.J., et al. 2003, Neves, A.A., Stockmann, H., et al. 2011)
Off-target effects	– Acetyl groups can induce cysteine *S*-glycosylation (2 mM) – Inhibition of proliferation and cytotoxicity >50 µM – Induction of differential gene expression – Reduction of cellular respiration rate and enhanced ROS production >20 µM – Inhibition of neurite outgrowth and capillary sprouting >50 µM	– Acetyl groups can induce cysteine *S*-glycosylation (2 mM) – No cytotoxic effects found at concentration up to 2 mM – No effects on cellular function reported	(Bayer, N.B., Schubert, U., et al. 2013, Han, S.S., Lee, D.E., et al. 2017, Han, S.S., Shim, H.E., et al. 2018, Jones, M.B., Teng, H., et al. 2004, Kang, K., Joo, S., et al. 2015, Qin, W., Qin, K., et al. 2018, van den Bijgaart, R.J.E., Kroesen, M., et al. 2019)
***In vivo* incorporation efficiency and safety**	– 2 times lower systemic incorporation compared to sialic acid analogue – No labeling of brain sialoglycans (liposome formulation) – No toxicity reported	– 2 times higher systemic incorporation compared to ManNAc analogue – Effective labeling of brain sialoglycans (liposome formulation) – No toxicity reported	(Büll, C., Heise, T., et al. 2015, Xie, R., Dong, L., et al. 2016)
Diagnostic Use	– Detection of genetic defects in NANS in patient-derived fibroblasts	– Detection of genetic defects in NANS in patient-derived fibroblasts	(van Karnebeek, C.D., Bonafe, L., et al. 2016)
Therapeutic Use	– Oral administration improves muscle phenotype in GNE myopathy mouse model	– Not evaluated	(Malicdan, M.C., Noguchi, S., et al. 2012)

Figure 1

N-Acetylmannosamine



N-Acetylneuraminic acid

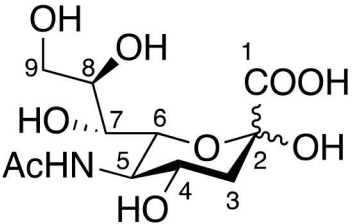


Figure 2

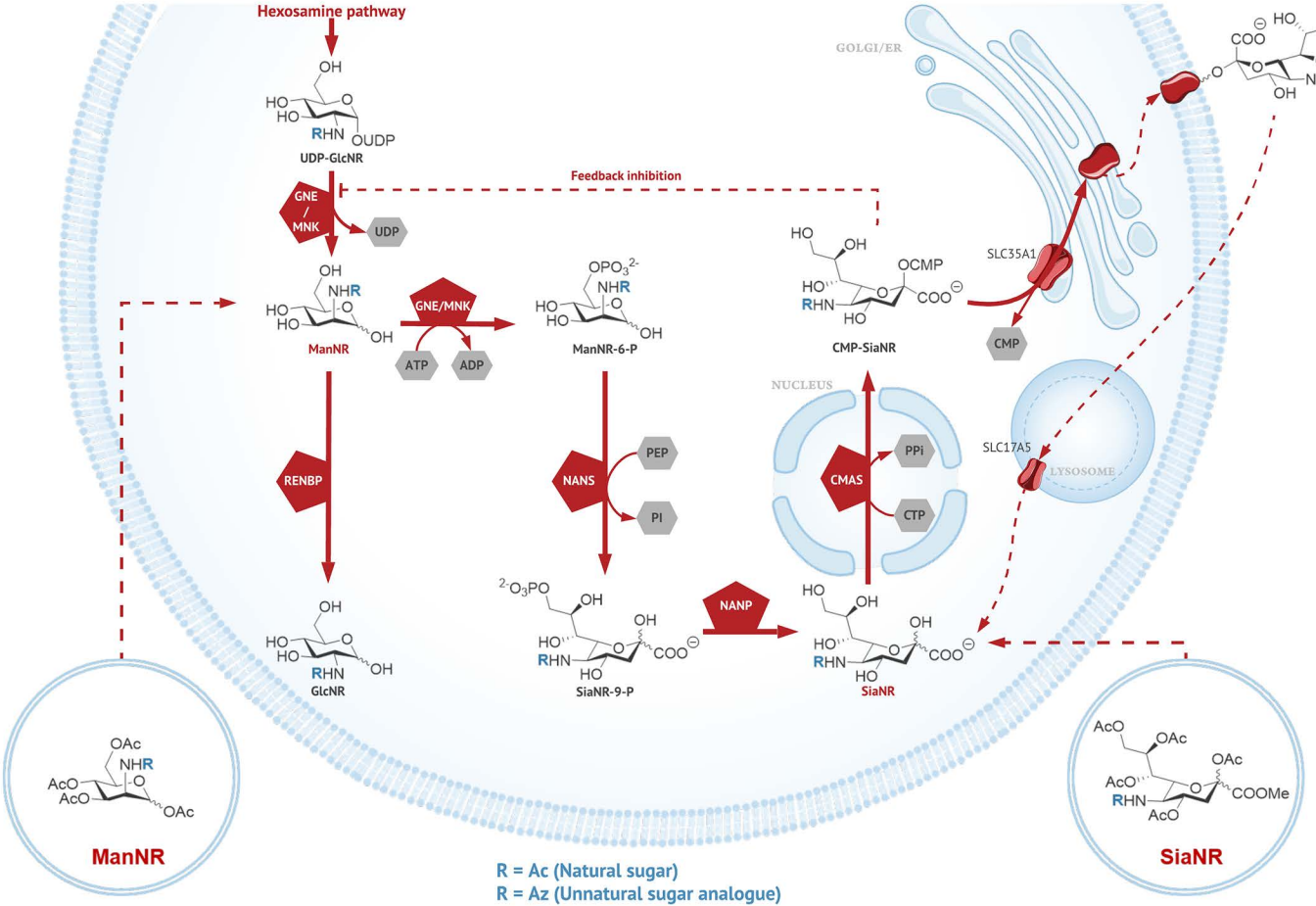


Figure 3