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Hexosamine analogs: from metabolic glycoengineering to drug discovery

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Abstract

Metabolic glycoengineering, a technique pioneered almost two decades ago wherein monosaccharide analogs are utilized to install non-natural sugars into the glycocalyx of mammalian cells, has undergone a recent flurry of advances spurred by efforts to make the methodology more efficient. This article describes the versatility of metabolic glycoengineering, which is a prime example of ‘chemical glycobiology,’ and gives an overview of its capability to endow complex carbohydrates in living cells and animals with interesting (and useful!) functionalities. Then an overview is provided describing how acylated monosaccharides, a class of molecules originally intended to be efficiently-used, membrane-permeable metabolic intermediates, have led to the discovery that a subset of these compounds (e.g. tributanoylated hexosamines) display unanticipated ‘scaffold-dependent’ activities; this finding establishes these molecules as a versatile platform for drug discovery.

Nature's biopolymers

Carbohydrates are nature's third biopolymer

Carbohydrates have been described as ‘the third chain of living organisms’ [1]; this article begins by discussing critical similarities and differences that render these molecules, compared to the two other classes of biopolymers (nucleic acids and proteins), uniquely suited to a metabolic substrate-based approach to manipulation (an overview of this technique — referred to as ‘metabolic glycoengineering’ in this article and also commonly referred to as ‘metabolic oligosaccharide engineering’ [2*] — is provided in Figure 1). Then, a brief description of challenges and opportunities provided by this emerging technology is given followed by a discussion of another burgeoning endeavor — carbohydrate-based drug discovery — that is being propelled by modified monosaccharides used in metabolic glycoengineering.

Comparing carbohydrates with nucleic acids and proteins

There are many similarities between the three classes of mammalian biopolymers. For example, they are all assembled from basic set of ‘building blocks’ into multi-mers that vary greatly in size. Biologically active nucleic acids in humans, for example, range from 22-mer siRNAs to 245 000 000-mer chromosomes; peptides from the three amino acid glutathione to the 34 350 residue protein titin; and carbohydrates from monosaccharides and

disaccharides to hyaluronic acid, which can be assembled from as many as 50 000 monosaccharide residues. Another similarity is that individual members of each class are routinely modified during, or more often after, synthesis to increase chemical and structural diversity.

For every similarity shared by natural biopolymers, there are equally compelling differences. Some are obvious from simple inspection; for example, the ability of carbohydrates to form branched structures supports incredible diversity compared to the restricted linear conformations of nucleic acids and peptides [3]. Other differences are not immediately apparent, but are equally profound; for example the absence of a template precludes the use of PCR-like methods to study and manipulate carbohydrates leaving this field years behind other facets of cellular and molecular biology [4].

Challenges faced in studying and manipulating carbohydrates have been a blessing in disguise insofar as they have provided impetus for sustained efforts to manipulate these molecules in ways outside of the conventional methods used in genetics and molecular biology. These efforts are epitomized by metabolic glycoengineering, a field that has uncovered another major difference between carbohydrates and other biopolymers. This latent feature lies in the ability of several glycosylation pathways to biosynthetically incorporate non-natural monosaccharide building blocks into mature glycans, an ability that is in stark contrast to proteins where the molecular machinery that assembles amino acids into peptide chains is virtually refractory to chemical variants. For example, the incorporation of non-natural amino acids often requires the corequisite engineering of critical biosynthetic components (e.g. the tRNA charging system), which so far has primarily been accomplished in prokaryotic systems [5*,6] (conversely, metabolic glycoengineering has flourished in mammalian cells with substantial progress reported only very recently in bacteria [7*]). Nucleic acids are similarly finicky about substrates, and in fact have elaborate proofreading mechanisms to correct biosynthetic mistakes; this feature essentially precludes the incorporation of non-natural nucleotides. In the next section, a brief description of the out-lying biopolymer, carbohydrates, highlights chemical diversity that can be incorporated into cellular glycans through the use of monosaccharide analogs.

Overview of metabolic glycoengineering

Non-natural monosaccharides are incorporated into cellular glycans

On the surface, metabolic glycoengineering is a straight-forward technique where non-natural analogs intercept the glycosylation apparatus of a cell and are bioprocessed effortlessly into surface glycans by this ‘black box’ (Figure 1). In practice, the glycosylation pathways of a mammalian cell are a highly interconnected paragon of complexity (as outlined in the Supplementary Materials, and described in more detail elsewhere, such as the KEGG Pathway database [8]) consisting of 1–3% of the human genome, or (at a minimum) 225–250 components. Overall, metabolic glycoengineering can substantially increase the chemical diversity of cellular glycans; although this methodology was pioneered with the sialic acid pathway, three additional pathways now have been successfully exploited (GalNAc, GlcNAc, and fucose). Accordingly, non-natural analogs can now gain metabolic access to 4 of the 10 monosaccharides commonly found in mammal glycans adding chemical variety beyond the natural functional groups common in sugars (e.g. phosphates, sulfates, and the ubiquitous hydroxyl groups) as well as reversible and transiently installed functionalities (such as *O*-acetates, phosphates, and sulfates, Figure 2).

Metabolic glycoengineering as a biological and chemical tool

Applications for metabolic glycoengineering can be divided into two broad categories. The first roughly mimics the consequences of natural modifications made to glycans, which

involve very subtle (or in some cases quite dramatic) changes to biological recognition events and downstream cellular behaviors. To give an illustrative example, cellular responses can be exquisitely tuned by the judicious placement — or absence — of a single hydroxyl in a large glycan structure. Remarkably, this group can make a substantial difference despite being located within a veritable sea of similar chemical functionalities. A premier example is provided by Neu5Gc, a sugar that can be installed into surface glycans enzymatically [9] or through ManNAc analogs [10] (Figure 2c). Neu5Gc is normally largely absent from human cells [11] and the difference of its single added oxygen atom compared to the predominant human form of sialic acid, Neu5Ac, has been implicated in brain evolution [12] and the function of the immune system through the regulatory effects of Siglecs [13]. Similarly, an added methyl group (e.g. $R_1 = -CH_3$ in Figure 1e) engenders biological responses that include dampened viral binding and infectivity [14], changes to integrin binding [15], perturbation of gene regulation [16], alterations to neonatal cell fate [17], and changes to the immunogenicity of the host glycan [18]. The numerous functional groups that have been installed into glycans by metabolic glycoengineering (see reviews [2*, 19*] or Supplementary Materials), often with much more flamboyant structural changes than proffered by the hydroxyl or methyl group, provide rich opportunities to further explore glycochemical space — in essence expanding the sugar code [20] — to modulate biological recognition phenomena.

From its inception metabolic glycoengineering has been a chemistry-driven enterprise. Early manifestations of a chemical emphasis included efforts to install bulky groups such as fluorophores or biotin in sialosides directly via derivatized CMP-Neu5Ac ‘building blocks’ [21]. Building on this chemical heritage, a significant advance was embodied in the introduction of bioorthogonal chemical functional groups capable of ‘chemoselective ligation’ reactions that allow organic chemistry to be done in aqueous environments selectively between a glycan-displayed functional group and a complementarily derivatized ligand [22]. This concept was first demonstrated in the form of the ketone group of levulinoyl-bearing analogs [23]; the ketone is not normally found in mammalian glycans and is absent from the cell surface allowing selective chemical reaction with exogenously delivered hydrazide or aminoxy derivatized ligands [24]. In recent developments, bioorthogonal reactions have been expanded to include the targeting of sugar-displayed thiols [25], diazirines [26], alkynes [27], and azides [28] (Figure 2d). Particularly intriguing is the recent development of cycloaddition reactions (e.g. ‘click’ reactions between glycan-displayed azides or alkynes) that avoid toxic copper catalysts [29]; these innovative approaches promise to facilitate *in vivo* imaging of the glycome [30••] now being pursued in model organisms such as the zebrafish [31].

Applications — toward disease intervention

Bioorthogonal chemistry is empowering a second wave of applications for metabolic glycoengineering that range from the just-mentioned zebrafish glycomic experiments to crosslinking glycoconjugates *in situ* and isolating (and characterizing) glycoconjugates from a complex biological milieu (such as the brain [32]). As an example of *in situ* crosslinking, the study of glycoprotein interactions has been given a new dimension by arylazide-bearing or diazirine-bearing sialic acids analogs used to investigate *in situ* binding partners for CD22 [26,33]. In a tangential direction, isolation of metabolically labeled glycans is spawning new glycomics methods, exemplified by ‘tagging by substrate’ techniques used to characterize *O*-GlcNAc modified proteins [23-25]. Another promising strategy is to install functional groups not normally found in sugars as artificial receptors for the delivery of genes [34], toxins such as ricin [23], small molecule drugs such as doxorubicin [35], or imaging agents such as MRI contrast agents [36]. The delivery of imaging agents to visualize a tumor, or therapeutics designed to kill a cancer cell, by targeting analogs toward tumor-associated

carbohydrate antigens (TACA) complements efforts that exploit metabolic glycoengineering to increase the antigenicity of TACA for immunotherapy [37*,38]. Together, even this brief list of applications illustrates the versatility and potential breadth of this emerging technology.

Practical efforts have led to new horizons

Sugar-based drugs — improving their clouded prospects

As the field of metabolic glycoengineering edges toward clinical endpoints, the spotted record of carbohydrate-based drug development looms as a potential stumbling block. To underscore this point, even fairly enthusiastic discussions have included phrases such as ‘bittersweet promise’ [39] and ‘sweet and sour’ [40] in their titles. Difficulties inherent in using sugars therapeutically begin with poor pharmacological properties that include rapid serum clearance and extend issues such as low binding affinities; in general, nanomolar affinities sought in a conventional drug candidate give way to complex ‘cluster glycoside’ effects where both avidity and binding specificity are determined by multiple weak interactions [41,42,43*]. Even in the rare case when a carbohydrate-based drug has broken through and achieved clinical and commercial success such as heparin sulfate, which has become an indispensable anticoagulant, high profile (and deadly) contamination issues have clouded this success [44]. Against this inauspicious background, metabolic glycoengineering has nonetheless made slow but steady progress, mirroring the growing realization that monosaccharides are versatile scaffolds for drug discovery [45].

SCFA-hexosamine analogs: increased efficiency is a first step toward clinical translation

To be broadly useful as drugs, the longstanding problem of inefficient analog utilization (Figure 3a–d) must be overcome. Efforts to increase efficiency began by constructing precursors (aka ‘pro-drugs’) that have improved qualities at the cell level by exploiting the age old strategy utilized by aspirin [46] where ester-linked short chain fatty acid (SCFA) are employed to increase the lipophilicity of hydrophilic drug candidates. This strategy has been applied successfully to simple sugars that lack membrane transport mechanisms with ester-linked acetates, in particular, employed for improving the membrane permeability of monosaccharides and disaccharides [19*]. Our laboratory extended this concept from acetates to longer SCFA such as propionate and *n*-butyrate (as Pr₄ManNAc and Bu₄ManNAc, Figure 3e) based on the notion that further increases in lipophilicity would enhance membrane permeability [47]. Correctly, we found the expected trend where analog utilization increased with SCFA length (Figure 3f); interestingly, amphiphathic molecules (such as 1,3,4-*O*-Bu₃ManNAc (Figure 3e)) provided the largest enhancement in flux [48*].

Further investigation of tributanoylated analogs (which had attractive Lipinski Rule-of-5 properties in addition to their high-flux character [49]) led to the discovery of intriguing SAR that overturned the long held assumption that biological responses emanated from the hydrolysis products (e.g. *n*-butyrate and ManNAc, Figure 3g and h). To elaborate, *any* tributanoylated isomer of ManNAc was expected to exhibit similar bioactivity because they each provided three equivalents of *n*-butyrate for each monosaccharide upon hydrolysis. Experimental testing, however, showed that 3,4,6-*O*-Bu₃ManNAc elicited dramatically different responses than 1,3,4-*O*-Bu₃ManNAc (Figure 4a) [48*]; 1,3,4-*O*-Bu₃ManNAc lacked the cytotoxic and growth inhibitory effects of Bu₄ManNAc while 3,4,6-*O*-Bu₃ManNAc showed enhanced toxicity and also inhibited pro-invasive oncogenes [48*]. The latter responses, which are attractive for cancer therapy, are linked to the suppression of NF-κB-driven gene expression for analogs bearing a SCFA at the C6 position [50,51•]. Overall, the SAR that separate these ‘anti-cancer’ responses from ‘high-flux’ properties extends to — and in fact dominate — glycosylation and HDACi responses that emanate

from the hydrolysis products of the analogs. In particular, the presence or absence of a SCFA group at the C1 or C6 position extend to 'Lev' core sugars where the 1,3,4-*O*-isomer lacks the toxicity of Ac₄ManNLev [48*] while 3,4,6-*O*-Bu₃ManNLev shows exacerbated responses compared to perbutanoylated ManNLev (Figure 4a). This newfound ability to separate flux from toxicity by leaving the C6-OH group of ManNAc analogs unprotected promises to enhance the safety of labeling and glycomic efforts.

In a complementary manner, the enhanced repertoire of anti-cancer properties now established in C6-derivatized analogs improves therapeutic prospects for this class of compounds. A particularly promising feature of the 'anti-cancer' compounds is their ability to simultaneously inhibit HDACi and NF-κB. To explain briefly, HDACi remodel chromatin to the transcriptionally active form leading to the re-expression of cell cycle checkpoint proteins such as p21 that halt the growth of cancer cells. At the same time, HDACi expose DNA binding sites for NF-κB proteins that facilitate transcription and, because NF-κB-driven genes often support the progressive development of cancer or block the action of pro-apoptotic therapeutic regimens, HDACi do not offer a general strategy for cancer drug development (although they are finding a niche for treatment of cancers that are not characterized by NF-κB activation). Consequently the combination of HDACi activity and NF-κB inhibition in a single molecule (such as Bu₄ManNAc or 3,4,6-*O*-Bu₃ManNAc [50]) represents a potentially significant advance in the types of cancer that can be safely and effectively treated.

The dual action nature of analogs alluded to in the previous paragraphs, where 'high-flux' or 'anti-cancer' properties are highlighted, is in reality a gross oversimplification. Recent evidence augments a previous survey of SAR that established that cytotoxicity was influenced by the *N*-acyl group of peracetylated ManNAc analogs [47] suggesting that *each* and *every* change made to a SCFA-hexosamine analog can tune biological activity. Although far from exhaustive, preliminary transcriptional profiling of cancer cell lines exposed to a handful of these compounds shows that even subtle structure variations can elicit substantial changes in patterns of gene expression, suggesting that copious ability to modulate biological activity is lurking within this class of prospective therapeutics [51••]. Consequently, the 'R₂' groups from Figure 1 in reality should be sub-divided into 'R₂'-'R₅' because different arrangements, or combinations, of SCFA on a single hexosamine scaffold elicit distinctive responses [48*]. Combining the two dozen or so R₁ groups already reported with the epimers and anomers of common mammalian hexosamines, close to 10⁵ structures can be assembled simply by using (or not using) 2, 3, 4, and 5 carbon straight chain SCFA (Figure 4b); numerous other substituents could be attached to a hexosamine scaffold for drug discovery purposes to increase this number by orders of magnitude.

Conclusions and future directions

A goal of this article was to introduce the reader to a fascinating group of very simple compounds, SCFA-hexosamine hybrid molecules, that grew out of the metabolic engineering field but now also exemplify the emerging concept that carbohydrates can serve as unexpectedly facile scaffolds for drug discovery. In the future, significant challenges remain for translation of these largely chemistry-driven research efforts to clinical reality. On the practical side, efficient *in vivo* delivery of sugar-based drugs remains a largely unsolved issue, although encouragingly, tributanoylated analogs (such as 3,4,6-*O*-Bu₃ManNLev) have favorable Lipinski Rule-of-5 properties for orally available drugs [49,52]. Mechanistically, the mode of NF-κB inhibition is currently unknown, but the direct binding of analogs to elements of NF-κB pathway is given plausibility by AutoDock modeling that shows physiologically relevant binding affinities of 3,4,6-*O*-Bu₃ManNAc to NFKB1 (Figure 4c, for context, natural lectin-carbon binding is summarized elsewhere and

generally falls in the -4 to -11 kcal/mol range [53]). Confirming or debunking this hypothesis, plus meshing NF- κ B-driven responses with those emanating from HDACi and glycosylation effects of the analogs, is sure to provide ample fodder for the third decade of metabolic glycoengineering, and take this field in interesting new directions beyond original (and still vibrant!) efforts to re-engineer complex carbohydrates with novel chemical and structural properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Overview of metabolic glycoengineering. **(a)** Monosaccharides taken in by cells intercept glycosylation pathways (see the Supplementary Materials for more detail) that **(b)** remodel and assemble simple sugars into complex surface-displayed glycans and extracellular matrix polysaccharides. **(c)** In metabolic glycoengineering, monosaccharide analogs, such as 'R₁'-modified ManNAc analogs (two specific examples, ManNPhAc and ManNLev are shown in Figure 3), intercept the relevant biosynthetic pathway and **(d)** are incorporated into surface and secreted glycans thereby increasing the chemical diversity of glycocalyx (Figure 2). **(e)** Efforts to increase the efficiency of analog uptake and utilization through 'R₂' groups (Figure 3) led to the discovery of SAR that have established 'scaffold-dependent' activities for this class of molecules that hold promise for — among other things (Figure 4) — selectively killing cancer cells **(f)**.

Figure 2.

Natural and glycoengineered chemical diversity in carbohydrates. **(a)** In addition to the ubiquitous hydroxyl groups associated with carbohydrates, carboxylic acids and *N*-acetyl groups are found in many monosaccharides used for glycan biosynthesis. **(b)** After glycans are assembled, specific hydroxyl groups can be modified with sulfates, phosphates, or acetates. **(c)** Metabolic glycoengineering can be used to install additional chemical diversity, including hydroxyls in subcellular contexts important for modulating biological recognition, exemplified by the conversion of Neu5Ac to Neu5Gc through the use of ManNGc. **(d)** Groups not naturally found in sugars such as thiols, diazines, alkynes, and azides can be installed via metabolic glycoengineering; these groups (as well as others) can be exploited for bioorthogonal chemoselective ligation reactions to append various ligands to the cell surface.

Figure 3.

Strategies to increase flux of non-natural analogs into glycosylation pathways. **(a)** ManNAc has no effective transport mechanism through the plasma membrane, a problem compounded by non-natural variants such as ManNLev; **(b)** 30–75 mM of these analogs are required to maximize flux into the sialic acid pathway and presentation of non-natural sialosides such as Sia5Lev on the cell surface **(c)**. **(d)** ManNPhAc is used much more efficiently, presumably because of the hydrophobic phenyl ring that facilitates membrane diffusion, as strategy that is extended in the use of SCFA-derivatized ‘pro-drugs’ **(e)** that have ester-linked acetate, propionate, or *n*-butyrate groups that increase cellular uptake by three or more orders of magnitude. **(f)** The SCFA groups are removed by nonspecific esterases upon uptake by a cell **(g)** and (at least in the case of *n*-butyrate), support HDACi activity **(h)** while the core sugar is freed to impinge upon glycosylation pathways. **(i)** Thus, acylated analogs support very high levels of flux into the sialic acid pathway, with intracellular levels of sialic acid increasing from the low micromolar to as high as millimolar levels. The downstream effects of increased flux on glycosylation are not always clearcut; for example the impact of ‘high-flux’ analogs such as 1,3,4-*O*-Bu₃ManNAc on global patterns of glycosylation can be relatively minor for natural core sugars (such as ManNAc), which can be explained by constriction points that prevent highly elevated levels of intracellular sialic acid from being reflected on the cell surface such as **(j)** limited CTP supplies for creating CMP-sialic acids and **(k)** lack of sialyltransferases or acceptor sites needed for the ultimate installation of sialic acid on the cell surface. Conversely, acylation is critical for increasing the replacement of natural sialosides with non-natural variants (e.g. ManNLev) that have reduced metabolic processing.

Figure 4.

The hexosamine as a scaffold for drug discovery. **(a)** Bu₄ManNAc, a lead drug candidate, has a complex mixture of biological activities emanating from glycosylation, SCFA responses, and newfound 'scaffold-dependent' effects as evidenced by the dramatically different biological responses of the two tributanoylet isomers 1,3,4-*O*-Bu₃ManNAc and 3,4,6-*O*-Bu₃ManNAc. **(b)** Revisiting SAR, close to 10⁵ hexosamine-based structures can be assembled from the current repertoire of components. **(c)** The NF-κB-suppressive analog is modeled to bind to NFκB1 with moderate, but physiologically relevant, affinity.