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Pharmacological, Physiochemical, and Drug-Relevant Biological Properties of Short Chain Fatty Acid Hexosamine Analogs used in Metabolic Glycoengineering

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Abstract

In this study, we catalog structure activity relationships (SAR) of several short chain fatty acid (SCFA)-modified hexosamine analogs used in metabolic glycoengineering (MGE) by comparing *in silico* and experimental measurements of physiochemical properties important in drug design including of. We then describe the impact of these compounds on selected biological parameters that influence the pharmacological properties and safety of drug candidates by monitoring P-glycoprotein (Pgp) efflux, inhibition of cytochrome P450 3A4 (CYP3A4), hERG channel inhibition, and cardiomyocyte cytotoxicity. These parameters are influenced by length of the SCFA (e.g., acetate vs. n-butyrate), which are added to MGE analogs to increase the efficiency of cellular uptake, the regioisomeric arrangement of the SCFAs on the core sugar, the structure of the core sugar itself, and by the type of *N*-acyl modification (e.g. N-acetyl vs. N-azido). By cataloging the influence of these SAR on pharmacological properties of MGE analogs, this study outlines design considerations for tuning the pharmacological, physiochemical, and the toxicological parameters of this emerging class of small molecule drug candidates.

Graphical Abstract

Keywords

Metabolic Oligosaccharide Engineering; P450; hERG; Pgp Efflux; Carbohydrate Drug Design

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Introduction

Glycosylation of proteins and lipids – which endows these biomacromolecules with additional complexity in the form of complex carbohydrates (typically known as “glycans”) – is ubiquitous across all domains of life. Glycans play numerous fundamental roles that profoundly affect cellular physiology; for example, in healthy organisms they regulate embryonic development, the immune system, and ECM- and cell-cell communication.^{1–5} In disease, aberrant glycosylation is a near universal feature of cancer⁶ and plays defining roles in many other complex ailments ranging from diabetes to neurological disorders.^{7,8} The ubiquitous association between pathologies and glycosylation has spurred efforts to understand the underlying mechanisms that produce atypical glycans found in diseased cells and attempts to manipulate the affected biochemical pathways to correct the underlying biochemical defects. This report focuses on metabolic glycoengineering (MGE), a method where exogenously-supplied carbohydrate analogs intercept intracellular metabolic pathways to manipulate glycan biosynthesis. *In vivo* demonstration of this strategy -- which initially focused on sialic acid -- was reported 25 years ago by Reutter's group⁹ and since then many intriguing indications that MGE holds potential to exploit or correct disease-related glycan abnormalities to treat disease have emerged (including many additional publications from Reutter and colleagues^{10–12}). For example, based on the frequent overexpression of sialic acid in cancer, MGE has been employed to target cancer cells and tumors with toxic or imaging agents;^{13,14} in a different context, biochemical muscle defects associated with reduced flux through the sialic acid biosynthetic pathway can be rescued using MGE in rodent models of GNE myopathy.^{15–17}

Monosaccharide analogs used in MGE usually are modified with ester-linked short chain fatty acids (SCFAs) to mask their hydroxyl groups to improve cellular uptake (Fig. 1); this approach is required because no plasma membrane transporters exist for non-natural analogs used in MGE. As a result, the multiple hydroxyl groups of the monosaccharide hinders cellular uptake by impeding passage through the lipophilic plasma membrane and concentrations exceeding 50 millimolar can be required to achieve saturating levels of surface modification in cell culture experiments.^{18,19} Peracetylation of a monosaccharide's hydroxyl groups, a strategy dating back almost 35 years for ManNAc analogs,^{20,21} increases metabolic uptake by ~600 fold or more by facilitating passive membrane diffusion; efficiency is improved further in a successive manner as longer short-chain fatty acids (e.g., propionate [~1800-fold increased efficiency] and butyrate [~2100 increased efficiency]) are used in place of acetate.^{22,23} Tri-butanoylated analogs, where one hydroxyl group is left unmodified, are taken up by cells even more efficiently,²⁴ presumably because the amphipathic nature of these molecules reduces membrane sequestration of the more highly (and uniformly) lipophilic per-acylated analogs.²⁵ Unexpectedly, we found that the regioisomeric placement of the three butyrate groups on the core sugar influenced biological activity.^{23,24,26,27} In particular “1,3,4-O-Bu₃ManNAc” analogs supported robust metabolic high-flux into the sialic acid pathway with minimal side effects whereas the “3,4,6-O-Bu₃ManNAc” isomer triggered a suite of non-glycosylation related effects (in addition to supplying flux into the sialic acid pathway) including down-regulation of pro-metastatic oncogenes and inhibition of NF-κB signaling;^{23,26,28} the latter effect -- when employed with

the corresponding “3,4,6” GlcNAc and GalNAc analogs -- holds anti-inflammatory activity and the ability to reverse pathology associated with osteoarthritis.^{29–32}

Although these (and additional) studies have established that the addition of ester-linked SCFAs, especially n-butyrate, to hexosamine analogs increases cellular uptake and efficiency and can elicit beneficial bioactivity for treating disease, there has been minimal experimental characterization of the physiochemical, toxicological, and pharmacological properties required for the clinical translation and commercialization of this class of drug candidates. Based on parameters often used to predict drug-like properties (e.g., molecular mass, lipophilicity, the number of rotatable bonds), the butanoyl-modification strategy does not intuitively appear to be favorable. Accordingly, in previous efforts towards clinical translation we side-stepped pharmacological pitfalls by employing polymer-based delivery systems; examples of this approach include use of sebacic acid-PEG polymers that enable controlled release of butanoylated ManNAc analogues³³ and PLGA constructs that deliver “3,4,6”-butanoylated GlcNAc and GalNAc analogues to treat osteoarthritis.³² A biopolymer-based strategy, however, is not applicable to many disease conditions that could benefit from MGE-based therapy such as metastatic cancer, where the target cells could be anywhere in the body as compared to treating osteoarthritis, where the drug formulation can be directly injected into an already-known site of damage (e.g., a knee joint). Consequently, we reasoned that having viable options for non-encapsulated MGE analogs would considerably expand the scope of and hasten the clinical translation of this technology. Accordingly, we undertook the comparative analysis of pharmacological properties of several acetyl- and butanoyl-modified MGE analogs. Our results demonstrate that important design considerations for MGE analogs, which include the composition and regioisomeric placement of the SCFA “protecting groups” as well as the chemical structure of the N-acyl group found at the C2-position of hexosamines (which is often a non-natural functional group in MGE), not only influence biological activity (as we previously reported) but also the physiochemical and pharmacological properties of this emerging class of drugs.

Materials and Methods

Materials

All compounds and HPLC grade solvents were purchased from Sigma Aldrich. SCFA-modified hexosamines were synthesized and characterized as previously described.^{24,27}

In-silico ADME property predictions

Physiochemical characterization of absorption, distribution, metabolism, and excretion (ADME) properties were predicted *in silico* using SwissADME (<http://www.swissadme.ch/>).³⁴ Results as well as the SMILES formulas for each tested analog were tabulated and listed in Table S1 in the **Supporting Information**.

HPLC characterization (determination of lipophilicity)

One milligram of each analog was dissolved in acetonitrile and analyzed by HPLC using published methods³³ to determine comparative retention times. Measurements were performed by injecting 20 µL of sample into the HPLC system (Waters Delta 600 pump with

2996 photo diode array) using a 4.6×250 mm C18 reverse phase column (Agilent HC-C18(2), 5 μ m). Each injection was performed with a flow rate of 0.8 mL/min consisting of an isocratic mobile phase of a 3:1 mixture of acetonitrile:water. Retention time differences were calculated for pairs of analogs and k-means clustering was performed using R Studio (<https://www.rstudio.com/>), setting the number of clusters to five.

Chromatographic hydrophobicity indexing

An established method was used for chromatographic hydrophobicity indexing (CHI) of SCFA-hexosamine analogs.³⁵ Briefly, theophylline, phenyltetrazole, benzimidazole, colchicine, phenyltheophylline, acetophenone, indole, propiophenone, butyrophenone, and valerophenone were used to calibrate elution from our HPLC system with the C18 column used for profiling the HPLC retention times of the tested analogues. SCFA-hexosamine analogs were injected in triplicate and CHI values were calculated for each analogue using the obtained average of the retention times.

Aqueous solubility

A measured amount of hexosamine analog (~2 mg of each compound, with exact masses recorded) was placed into 1.5 mL microcentrifuge tubes and MilliQ water was added to dissolve or suspend each analog to a concentration of 2 mg/mL with three replicates performed in each case. (While we are aware that physiological pH is 7.4 and many water solubility experiments are performed in pH-buffered solutions, none of the compounds we tested contain amine or carboxylic acid functionalities that alter the water solubility of our analogs as a function of pH; accordingly, the solubility of our compounds in purified water accurately represents physiological conditions.) Each sample was vortexed vigorously and the compounds were allowed to dissolve for 24 h at room temperature (RT) at which point each tube was centrifuged at $21,130 \times g$ for 5 min. Empty centrifuge tubes were weighed and the mass of each was recorded. An aliquot of each sample (450 μ L) was transferred to the empty pre-weighed tubes, which were dried via lyophilization. Each analog-containing tube was weighed and the amount of compound was determined by the difference in mass with the pre-weighed empty tubes; the concentrations of the dissolved analogs were then calculated based on the volume of water initially added to each sample.

P-glycoprotein (Pgp) efflux characterization

SCFA-modified hexosamine analogs were assayed to evaluate their impact on Pgp efflux using Promega's Pgp-Glo™ Assay System (catalog #V3601) following the manufacturer's protocol #TB341; the volume of the solvent vehicle (ethanol) was limited to <1% in all cases, which we previously showed had no impact on the biological parameters under investigation.^{18,22} Readings were performed using the Synergy™2 Multi-Mode Microplate Reader (Biotek). The concentrations of analogs tested were 100 μ M (tri-butanoylated, azido, and per-butanoylated analogues), 200 μ M (per-acetylated analogs), or 400 μ M (parent hexosamines). A heatmap was generated using R Studio.

Cytochrome P450 3A4 (CYP3A4) inhibition

Several SCFA-modified hexosamine analogs were assayed for their potential as CYP3A4 inhibitors by using Promega's P450-Glo™ CYP3A4 (Luciferin-PPXE) DMSO-Tolerant Assay (catalog #V8912) according to the manufacturer's protocol #TB325. The volume of ethanol, the solvent vehicle, was <1% in all cases. Readings were performed using Synergy™2 Multi-Mode Microplate Reader (Biotek). The concentrations of analogs chosen were identical to the Pgp assay. A heatmap was generated using R Studio.

hERG inhibition

Potential cardio liability was assessed using Life Technologies™ Predictor™ hERG Fluorescence Polarization Assay Kit (catalog # PV5365) according to the manufacturer's protocol. Analogs were tested in doses ranging from 69.7 pM to 1 mM (in 3-fold serial dilutions) and fluorescence polarization measurements were performed using a Tecan Safire2™ Microplate Reader. Inhibition curves were fit using GraphPad Prism and IC₅₀ values were extrapolated.

MTT analysis and LIVE/DEAD assays of neonatal rat ventricular cardiomyocytes

Isolation and growth of neonatal rat ventricular cardiomyocytes (NRVCMs) was performed using established methods³⁶ in accordance with the Johns Hopkins Committee on Animal Care and Use along with all state and federal regulations. In 96-well plates, 5×10^3 NRVCMs were plated into each well and were allowed to adhere for 48 h while grown in DMEM (Corning 10–13-CV) supplemented with 10% fetal bovine serum (Corning 35–016-CV) and the specified concentration of antibiotics (ThermoFisher 15140122). After adherence for an initial 48 h, the cells were treated with 3,4,6-O-Bu₃ManNAc or 3,4,6-O-Bu₃GlcNAc at 0, 50, 100 and 200 μM for 24 and 48 h post-adherence after which the cells were tested for viability using the MTT assay following the manufacturers protocol (ThermoFisher M6494). To confirm the observed toxicity related to the 3,4,6-tributanoylated analogs tested, tissue culture plates (12-well) were seeded with NRVCMs at a density of $2.5 \times 10^5/\text{cm}^2$. NRVCMs were treated with 3,4,6-O-Bu₃ManNAc or 3,4,6-O-Bu₃GlcNAc for 12, 24, or 48 h by directly adding each analog (100 mM stocks in ethanol) to the media after seeding. NRVCMs were also treated with an equivalent of volume of ethanol as a vehicle control in parallel with the SCFA-hexosamine analogue treatments. LIVE/DEAD assays (ThermoFisher L3224) were performed on each well at each time point following the manufacturer's protocols. Cells were imaged at four locations in each well using a Zeiss Axio Observer inverted fluorescence microscope. Each experiment was repeated in triplicate.

Statistical Analysis

Quantitative data are reported as the mean $\pm$ SEM. One-way ANOVA with Dunnett's *posthoc* test or Tukey's *posthoc* test was performed to determine statistical difference between testing groups as appropriate using GraphPad Prism. Differences were considered statistically significant at $*p < 0.05$.

Results

In silico characterization

Physiochemical descriptors and predictions for absorption, distribution metabolism, and excretion (ADME) parameters, pharmacokinetic properties, and the overall druglike nature of hexosamines GlcNAc, GalNAc, ManNAc along with SCFA-modified derivatives used in MGE (Table S1) were calculated using SwissADME. Radar plots with a shaded area representing an optimal zone of physiochemical characteristics for oral bioavailability were generated for each analog (Fig. 2A) representing a typical profile for each class of analog as shown). Many (or most) of the *in silico* predictions were expected. For example, modification of an analog with increasingly long SCFAs (e.g., Bu vs. Ac) increased overall flexibility, size, lipophilicity, and insolubility. Other *in silico* predictions were less obvious, for example, the regioisomeric arrangement of SCFAs (e.g., 3,4,6-O-Bu₃ vs. 1,3,4-O-Bu₃ modification) led to interesting differences between the two isomers, where overall polarity was predicted to be greater for the 3,4,6-O-Bu₃-modified analogs with other subtle differences predicted for lipophilicity, size, polarity and insolubility (regioisomeric differences were predicted for analogs having both the natural core hexosamine and the non-natural N-acyl azido-modification).

In silico predictions for gastrointestinal absorption and potential for brain penetration were also calculated (Fig. 2B). None of the typical molecules used in MGE were predicted to penetrate the blood brain barrier (BBB, shaded yellow area), which is expected due to their high polar surfaces areas and molecular weights compared to most small molecule drugs that achieve BBB penetration. Analogs with tri-butanoylation patterns (either 3,4,6-O-Bu₃ 1,3,4-O-Bu₃ regioisomers) on any hexosamine scaffold met predicted cutoffs for passive gastrointestinal adsorption (white area). Additional quantitative *in silico* predicted values for physiochemical parameters, lipophilicity, water solubility, and drug-likeness as well as for pharmacokinetically-important characteristics were calculated and tabulated (Table S1); several aspects of these results (e.g., log p values) are discussed in more detail below.

Lipophilicity (HPLC characterization)

To quantify the impact of SCFA and N-acyl SAR on analogue lipophilicity, we considered measuring the partition coefficients (log P) using the established shake flask method that partitions a test compound between water and octanol, followed by quantification of material dissolved in the aqueous layer. This method, however, lacked sensitivity and reproducibility, leading us to pursue reverse phase C18 HPLC analyses where retention times can be correlated with log P values to reflect compound's lipophilicity. The retention time data (Table S2 and Fig. 3) showed several expected and also some unanticipated results. For example, in some cases different per-acetylated hexosamine (e.g., Ac₄GalNAc and Ac₄ManNAc) had almost identical RT (Fig. 3A), which might be expected because these molecules are isomers; similarly, it was expected that the increased lipophilicity gained from substitution of acetate with butyrate groups would increase RT as verified by comparing Ac₄ManNAc with Bu₄ManNAc (Fig. 3B) or Ac₄GlcNAc with Bu₄GlcNAc (Fig. 3C).

Unanticipated and non-obvious results also emerged; for example, we found that the identity of the core hexosamine could tune RT. This was illustrated by the difference in RT between peracetylated ManNAc and perbutanoylated ManNAc of ~3.6 min (Fig. 3B) whereas the corresponding increase for the respective GlcNAc analogs was < 3 min (Fig. 3C). In another example, the comparison of perbutanoylated GalNAc, GlcNAc, and ManNAc showed distinct RTs for each isomer (Fig. 3D); the ability of the core sugar to influence lipophilicity was also observed (albeit to a lesser extent) by comparing 1,3,4-O-Bu₃GlcNAc with 1,3,4-O-Bu₃ManNAc (Fig. 3E). The presence of an N-azido functional group consistently increased RT, as seen by comparing 1,3,4-O-Bu₃GlcNAc with 1,3,4-O-Bu₃GlcNAz (Fig. 3F) or Bu₄GalNAc with Bu₄GalNAz (Fig. 3G); interestingly, the effect was much greater (~2.5 min vs. ~0.7 min) when the core sugar was GalNAc (vs. GlcNAc). Finally, the regioisomeric arrangement of butyrate groups on the core molecule measurably (albeit mildly) influenced RT, as seen by the comparison of 1,3,4-O-Bu₃GlcNAc with 3,4,6-O-Bu₃GlcNAc (Fig. 3H).

k-Cluster analysis

The data presented in Figure 3 represents only a small fraction of pair-wise comparisons that can be made between different combinations of analogs for which we measured RT (Table S2 lists retention times for all of the tested analogs). To perform a more comprehensive analysis of our data we performed k-cluster analysis where analogs were grouped by holding all SAR variables constant except for one. To briefly explain this approach, k-cluster analysis groups sets of objects so that objects in the same group (i.e., a cluster) are more similar to each other than to objects in other clusters; k-cluster analysis is a common approach for exploratory data mining that -- although not (necessarily) holding practical experimental value -- can often be used to generate new hypotheses and design follow-up experiments. To conduct k-cluster analysis, we generated differences in retention times (RT) for all theoretically possible pairs of analogs (as outlined in Fig. 4A/B); this set of RT values represent distinct chemical transformations (examples of a single variable “chemical transformations” include (i) the presence or absence of an N-acyl azido group [e.g., Bu₄GlcNAc vs. Bu₄GlcNAz]; (ii) a conformational difference at a single stereocenter [e.g., Bu₄GalNAz vs. Bu₄GlcNAz]; or (iii) the presence or absence of a SCFA [e.g., 1,3,4-O-Bu₃GlcNAz vs. Bu₄GlcNAz; the former analog lacks an ester-linked butyrate group at the C6 position] between each pair of analogs; the entire compilation of such pairs is provided in Table S2).

Ultimately, the compiled RT values reflect changes in an analog’s lipophilicity expected after any transformation, including theoretical substitution of hexosamine cores between cores. To visualize this data, changes in retention time values (RT) for each pair of analogs was plotted against the retention time for each initial analogue before chemical transformation and k-cluster analysis was used to produce clusters of analogs (Fig. 4C and Table S2) that retain comparable lipophilicity after a given chemical transformation. The k-cluster analysis was arbitrarily tasked to produce five clusters, two of which (green and red) have distinctly higher lipophilicity than the other three (light blue, blue, and black).

Although many transformations in the k-cluster analysis resulted in expected outcomes (e.g., the “red” cluster entirely included highly lipophilic, per-butanoylated analogs), in other

cases the chemical transformations were less intuitive. For example, the “green” cluster also was based on per-butanoylated analogs (e.g., Bu₄GalNAz) but mapped these highly lipophilic compounds to a subset of the less lipophilic per-acetylated (e.g., Ac₄GalNAc) or tri-butanoylated (e.g., 1,3,4-OBu₃GlcNAc) analogs; the “blue” cluster was essentially the reverse of this where per-acetylated and tri-butanoylated analogs were mapped entirely to per-butanoylated analogs). Additional non-obvious analog k-clustering included the transformation of 1,3,4-O-Bu₃ManNAc to perbutanoylated ManNAc or GlcNAc, which was clustered in the “black” group, whereas comparable transformation to perbutanoylated GalNAc was grouped in the “green” cluster. Finally, clustering patterns were not universal, as transformation of 3,4,6-O-Bu₃ManNAc and GlcNAc to per butanoylated hexosamines remained in the same cluster (green). The complex effect of the hexosamine scaffold on the physiochemical characteristics of MGE analog SAR we uncovered with routine RP-HPLC profiling provided motivation to quantitatively assess lipophilicity using HPLC.

Characterization of lipophilicity using Chromatographic Hydrophobicity Indexing (CHI)

The molecules (theophylline, phenyltetrazole, benzimidazole, colchicine, phenyltheophylline, acetophenone, indole, propiophenone, butyrophenone, and valerophenone) depicted in Fig. 5A (and listed in Table S3), were used to generate a standard curve to relate CHI values to retention times; this information allowed us to assign CHI values to our test compounds. After calibration of our HPLC system and determination of analog retention times under the same conditions, we scored the CHI of each MGE analog and presented this information in Fig. 5B.

The complex nature of MGE physicochemical properties of SCFA- and N-acyl modified hexosamines -- already deduced from the preceding k-cluster analysis -- was further evident from the CHI measurements. For example, there were small differences in hydrophobicities between peracetylated GlcNAc and peracetylated GalNAc (GlcNAc < GalNAc) as well as between 3,4,6-O-Bu₃GalNAc and 3,4,6-O-Bu₃GlcNAc (GalNAc < GlcNAc); the only structural difference between these sets of isomers was axial versus equatorial orientation of the hexosamine's C4-OH group. Interestingly, “Ac₄” substitution reversed the order of hydrophobicity of the two core hexosamines (GlcNAc vs. GalNAc) compared to the corresponding “Bu₃” analogs. The CHI data showed that in addition to the ability of an appended SCFA to influence a core sugar's hydrophobicity, the core sugar also affected hydrophobicity when the SCFA was held constant. The latter effect was illustrated by perbutanoylated hexosamines where hydrophobicity increased from Bu₄GalNAc < Bu₄ManNAc < Bu₄GlcNAc. The complexity of MGE analog chemical space extended further, with the regioisomeric placement of SCFA groups on the core hexosamine also having an impact on hydrophobicity; this effect was illustrated by differences in values observed between “1,3,4” and “3,4,6” tributanoylated GlcNAc (1,3,4-O-Bu₃GlcNAc < 3,4,6-O-Bu₃GlcNAc) and ManNAz (1,3,4-O-Bu₃ManNAz > 3,4,6-O-Bu₃ManNAz). Finally, the presence of an N-acyl azide group had an impact on lipophilicity (e.g., 1,3,4-O-Bu₃GlcNAc < 1,3,4-O-Bu₃GlcNAz), with the azido group increasing CHI values.

Because log P values are often regarded as the gold standard for measuring lipophilicity during drug development (for context, the log P of 1791 approved drugs developed over the

past 60 years is ~ 2.5 ³⁷), we correlated our experimentally obtained CHI values with consensus log P values calculated using SwissADME (Table S1). To do so, we plotted the CHI values against the calculated consensus log P values (Fig. 5C) and found that there was relatively poor correlation between the *in silico* predictions and experimentally measured values reflected in CHI scores ($r^2=0.684$). To further demonstrate these differences, analogs were ranked (Fig. 5D) by the CHI score (along with their consensus log P ranking in parentheses that was calculated using SwissADME) to illustrate the disparate rank orderings generated using *in silico* methods compared to experimental data. Overall these comparisons showed that this *in silico* algorithm was relatively accurate in predicting analogs with the natural core hexosamines (e.g., although slight discrepancies arose in precise rank ordering, structurally similar analogues [e.g., Bu₄GalNAc, Bu₄GlcNAc, and Bu₄ManNAc] were assigned fairly consistent values). The *in silico* tools, however, struggled to assign accurate values to azido-modified hexosamine analogs (e.g., 1,3,4-O-Bu₃ManNAz was predicted to be one of the least lipophilic analogs but experiments results showed that it had the second highest lipophilicity of the test compounds). Based on these results, combined with the growing importance of non-natural hexosamine analogues in MGE, it was evident that *in silico* prediction tools are not capable of fully predicting the impact of SAR of the lipophilicity of these compounds; this parameter, therefore must be measured experimentally for highly accurate determination.

Aqueous solubility

Solubility in water is an important practical endpoint in drug design that is based on the lipophilicity of a molecule. Briefly, when a lipophilic molecule is placed in an aqueous solution, the surrounding water molecules are forced to organize into an “ice-like” structure generating an entropic penalty that progressively reduces solubility as lipophilicity increases. For example, the acetamido motif can exquisitely and differentially regulate the preferred choice of hydration sites and subsequent conformational populations of GlcNAc and GalNAc.³⁸ Similarly the impact of carbohydrate configuration on hydration can alter the recrystallization-inhibition properties of teleost fish antifreeze glycoproteins³⁹ and mono- and disaccharides have anti-freezing properties due to the ability of carbohydrates to disrupt the preordering of water at the interfaces between bulk water and the quasi-liquid layer.⁴⁰ Based on this precedent, which involves complex molecular behavior that remains difficult to predict using *in silico* tools or even reliably extrapolate from lipophilicity data, we experimentally tested aqueous solubility. We measured solubility up to 2 mg/mL, which is twice the 1 mg/mL benchmark that represents the ‘slightly soluble’ solubility definition according to the United States Pharmacopeia (USP).⁴¹ Overall, 35–40% of drugs have a solubility of less than 5 mg/mL;⁴¹ accordingly, compounds with solubilities of ≥ 2 mg/mL represent the lower end of this range. As shown in Figure 6 (and Table S4) the aqueous solubility data revealed both expected and unexpected results that are a function of SAR. As expected, the peracetylated (“Ac₄”) analogs had greater solubility than their perbutanoylated (“Bu₄”) counterparts (Fig. 6A). Removal of a butyrate group to produce either “1,3,4” or “3,4,6” tri-butanoylated analogs partially restored the water solubility of the peracetylated compounds (Fig. 6B). As an aside, we did not evaluate di-butanoylated or tri-acetylated analogs in this study because we have previously shown that the loss of either a butyrate group from a tri-butanoylated analog or the loss of a single acetate from a peracetylated

hexosamine prevents membrane diffusion and subsequent cell uptake,^{26,28} thereby rendering these compounds ineffective for MGE experiments.

Azido modification of the N-acyl position of a hexosamine^{19,42,43} creates attractive MGE analogs because of this functional group's well known role as a reaction partner in "click chemistry" but the azide functional group also imparts added lipophilicity. This effect was observed to extend to water solubility when 1,3,4-O-Bu₃ManNAc was compared with 1,3,4-O-Bu₃ManNAz but was less pronounced for 1,3,4-O-Bu₃GlcNAz (Fig. 6C(i)); this result demonstrated that analog solubility can be influenced by the structure of the core hexosamine as observed when comparing the azido-modified, tri-butanoylated hexosamines 1,3,4-O-Bu₃GlcNAz and 1,3,4-O-Bu₃ManNAz. Finally, in apparent contrast to the "1,3,4" analogues, the azido group appeared to increase the solubility of perbutanoylated analogues (Fig. 6C(ii); a caveat to this comparison is that the core sugar was a second variable that may have influenced solubility).

P-Glycoprotein (Pgp) efflux

We next determined P-glycoprotein (Pgp) efflux, which is important in the success of drug delivery because this membrane transporter, is widely distributed throughout the body and influences the cellular uptake of hundreds of structurally diverse therapeutic agents and often jeopardizes the success of drug delivery.⁴⁴ In our experiments, Pgp efflux of SCFA hexosamine analogs was minimally (if at all) influenced by SAR in several cases (Fig. 7A and Table S5). For example, no significant difference was observed when comparing 3,4,6- or 1,3,4,-tributanoylated compounds with each other or when comparing peracetylated analogues with solvent vehicle controls. However some distinct differences were noted; for example Pgp efflux was positively correlated with the number of SCFA groups ester linked to the core hexosamine with perbutanoylated SCFA analogs exhibiting increased Pgp substrate activity. Finally, there were measurable differences that depended on the core sugar; for example perbutanoylated GlcNAc had lower activity than either perbutanoylated ManNAc or GalNAc.

CYP3A4 inhibition

Many drugs are deactivated by cytochrome P450 3A4 (CYP3A4) while others affect the activity of this oxidizing enzyme (generally, through inhibition); overall CYP3A4 is a major source of variability in drug pharmacokinetics and response⁴⁵ and is thus an important parameter to consider in early stage development. In these experiments we tested the impact of selected compounds that represented extremes of lipophilicity ranging from highly hydrophilic unprotected (i.e., the natural, unmodified "perhydroxyl" hexosamines) to highly lipophilic perbutanoylated analogs on CYP3A4 inhibition (Fig. 7B and Table S6). Unprotected ManNAc had negligible effect on CYP3A4, while tri-butanoylated GlcNAc (1,3,4- and 3,4,6-) substituted analogs showed a similar lack of CYP3A4 inhibition. When "3,4,6" tributanoyl-substituted hexosamines were compared, differences were observed between GlcNAc, GalNAc, and ManNAc. Differences in CYP3A4 inhibition also depended on the pattern or extent of butanoyl-ester substitution with perbutanoylated ManNAc, GlcNAc, and GalNAc differing from corresponding 3,4,6-O-Bu₃-modified ManNAc, GlcNAc and GalNAc. Next, we observed positive correlation between overall molecule

lipophilicity and CYP3A4 inhibition; in particular more inhibition was observed when the core sugar was constant and the appended SCFA groups were increased in size, (e.g., Ac₄ManNAc < Bu₄ManNAc). Finally, azido incorporation influenced CYP3A4 inhibitory activity (e.g., 3,4,6-Bu₃ManNAc > 3,4,6-Bu₃ManNAz); interestingly, the added lipophilicity imparted by the azido group did not increase CYP3A4 inhibition, which is inconsistent with the effects observed with SCFA substitutions where increasing lipophilicity was positively correlated with CYP3A4 inhibition.

hERG inhibition and cardiomyocyte toxicity

Fluorescence polarization assays were performed to monitor inhibition of the human Ether-a-go-go-Related Gene (hERG) potassium channel, which comprises another liability in drug development because off target inhibition of this channel can prolong the QT interval and cause cardiac arrhythmia.⁴⁶ In these assays, the first notable result was that the “1,3,4” analogs 1,3,4-O-Bu₃GlcNAc and 1,3,4-O-Bu₃ManNAc showed no detectable inhibition up to 1 mM (Fig. 8 and Table S7), which was the highest concentration tested because this level exceeds the ~100 to 150 μ M^{27,47} concentrations typically required to achieve maximal MGE responses. By contrast, the “3,4,6” analogs showed inhibition in the ~1 to 100 micromolar range for 3,4,6-O-Bu₃GlcNAc and 3,4,6-O-Bu₃ManNAc (Fig. 8B & C) with the ManNAc analog showing ~13-fold greater potency. Interestingly, a comparison of 1,3,4-O-Bu₃GlcNAc (for which we observed no inhibition) to 1,3,4-O-Bu₃GlcNAz revealed that the azido modification increased hERG inhibition from undetectable levels to an IC₅₀ of 2.85 μ M for 1,3,4-O-Bu₃GlcNAz.

The hERG inhibition results, which were particularly striking for the “3,4,6” analogs, led us to test whether these potent analogs would exhibit corresponding cardiomyocyte cytotoxicity. Accordingly we tested tributanoylated 3,4,6 analogs for dose- and time-dependent cytotoxicity using the MTT assay and found that the 3,4,6 analogs significantly adversely affected cell viability;. In most cases, however, the hERG and MTT assay provided consistent results, for example 3,4,6-O-Bu₃GlcNAc and 3,4,6-O-Bu₃ManNAc, both showed inhibition in the 10 $\pm$ 5 μ M range in the hERG assays (after 12, 24, or 48 h, Fig. 9A, B respectively) and significantly reduced NRVCN cell viability when evaluated using the MTT assay after 24 or 48 h of analog exposure.

Discussion

It has become increasingly clear that a paradigm shift is needed for small molecule drug discovery efforts because current efforts -- which are focused on generating highly potent molecules against specified targets -- are becoming increasingly unproductive suffering from declining rates of clinical advancement and stagnation of the number of FDA-approved small molecule drugs. Based on the failure of many otherwise promising drug candidates during clinical testing, the idea that a compound's overall physiochemical properties can have a significant impact on successful clinical translation has regained importance as a parameter in drug design. A conundrum exists, however, insofar that although the potency and selectivity of many small molecule drug candidates can be enhanced through the incorporation of lipophilic moieties (for example, the introduction of n-butyrate groups

increases potency the of our MGE analogs) this approach often results in poorer physiochemical properties that ultimately hinder translation in a tradeoff that has been dubbed “molecular obesity”.³⁷ Interestingly, many longstanding cancer therapeutics such as alkylating agents and hormonal agents do not even have nanomolar potencies but instead are active in the micromolar to even millimolar range in assay-based screening.⁴⁸ The success of some of these modestly potent molecules can be attributed to favorable physiochemical properties that contribute to efficacy; in retrospect, these properties often are more important than very high (e.g., nanomolar or better) target-based potency *per se*.⁴⁸

Against this backdrop, we characterized the physicochemical properties of a panel of SCFA-derivatized carbohydrate analogs used in MGE and also evaluated these drug candidates for potential liabilities by measuring P-glycoprotein efflux, CYP3A4 inhibition, and hERG inhibition. To begin, we conducted *in silico* analysis of ADME properties of MGE analogs (as compiled in Table S1 and summarized in graphical form in Fig. 2) and found that the parent hexosamines have good “drug-like” properties from an ADME perspective but -- as discussed earlier -- suffer from extremely inefficient cell uptake that makes clinical translation impractical. This problem is (largely) solved by peracetylation, which results in only slight deviation from acceptable *in silico* ADME properties (Fig. 2A, second graphic); this prediction is consistent with the demonstrated *in vivo* efficacy of orally-delivered Ac₄ManNAc in treating hereditary inclusion body myopathy (now referred to as “GNE” myopathy⁴⁹).¹⁷ Based on these considerations, one conclusion is that peracetylation provides an avenue for clinical translation of MGE analogs. However, based the many intriguing (and often superior) biological activities of butanoyl-modified hexosamine MGE analogs that we have discovered,^{23,24,26,27,29–32,47,50} we sought to clarify whether these compounds also had -- or could be endowed with -- similarly suitable ADME properties for *in vivo* use.

Because lipophilicity and molecular flexibility are key molecular properties that (usually negatively) influence ADME, it was not surprising that *in silico* prediction of butanoyl-modified MGE analogs often showed parameters outside of accepted values for orally-available drugs (Fig. 2A; a complete tabulation of the number of rotatable bonds, hydrogen bond donors, and hydrogen bond acceptors of our molecules, which are widely used metrics for oral bioavailability of small molecule drugs⁵¹ is given in Table S1). By comparing butyrate-modified analogs we observed that numerous ADME parameters were tuned by the number and regioisomeric placement of this SCFA on the core sugar as well as the chemical composition of the core sugar itself; based on this information we reasoned that it should be possible to semi-rationally design analogs with improved ADME properties.

To test whether the *in silico* predictions held “real world” value, we experimentally tested several ADME endpoints. Of these, the first physiochemical property we measured was lipophilicity; this parameter is often measured as a log P value from octanol partition coefficient experiments but in this project we used reverse phase HPLC to rank order the lipophilicity of the analogs (we found HPLC analysis to be more sensitive and reproducible than conventional solvent extraction assays). Based on our experimental results, the lipophilic properties of the MGE analogs we tested depended on the core sugar, the composition of the N-acyl amide functional group, the length of the SCFA used a protecting

group, and the regioisomeric arrangement of the SCFAs in ways that were not always reliably predicted by the *in silico* tools. In particular, our experimental data contrasted with calculated log P values; for example, *in silico* calculations were poor at predicting rank order differences we observed in the lipophilicity of regioisomers and were off by as much as an order of magnitude for N-azido-modified analogs. These results serve as a reminder that log P values calculated through computational means can vary considerably from actual values.

We next cataloged aqueous solubility -- a property inherently linked to lipophilicity -- which is important in drug design because it influences oral bioavailability. The butanoylated analogs we tested had solubility values that the USP characterizes as “practically insoluble” to “very slightly soluble” (i.e., 1 mg/mL to almost an order of magnitude lower). The peracetylated analogs had improved water solubility of > 1 mg/mL as expected because of their lower lipophilicity. These findings provide guidelines for implementing animal studies as well as for clinical translation in humans; for example, the marginal solubility of the perbutanoylated analogs predicts that they will have poor oral availability and may require special formulation strategies;⁵² as an aside, we are already formulating butanoylated analogs for controlled-release delivery.^{32,33} Alternatively, the k-cluster analysis (Fig. 4) presents the intriguing possibility (although mostly theoretical at this point) that some butyrate-modified analogs (in particular tri-butanoylated compounds) have “cross-talk” with peracetylated compounds in a manner where a single variable chemical transformation holds the potential to considerably improve the ADME properties of an MGE drug candidate

To complement our analyses of physicochemical properties, we conducted a set of functional ADME-relevant assays designed to uncover biological liabilities for clinical translation. We first tested the impact of the analogs on P-glycoprotein efflux, a parameter that we expected to be influenced by analog SAR because physicochemical properties have previously been implicated in Pgp efflux liability.⁵³ These assays showed that, in general, our analogs elicited modest or even negligible P-glycoprotein efflux; for example, tri-butanoylated compounds were not significantly different than vehicle-treated controls. Because MGE analogs have undergone extensive evaluation for the study and treatment of cancer,^{13,14,28,50,54–59} this finding potentially advances the design of more efficacious SCFA-modified MGE analogs by avoiding SAR that contribute to or exacerbate Pgp efflux, which is a common mechanism by which cancer cells become drug-resistant.^{60–62}

To further characterize the safety profile of our MGE analogs, which is a key aspect of early-stage drug design; we evaluated cytochrome P450 activity because FDA guidelines since the early 1990s have required new molecular entities to be evaluated for inhibition of key metabolizing enzymes⁶³). Specifically, we tested several analogs against CYP3A4 -- a member of the family of cytochrome P450 oxidizing enzymes -- that metabolizes more than 50% of all drugs.⁶⁴ Considering that our analogs are designed for cancer treatment (amongst other applications), understanding how these drug candidates interact with a key metabolizing enzyme such as CYP3A4 is critical. By cataloging CYP3A4 inhibition (Fig. 7B), we demonstrated that SAR again tuned this important ADME property and although -- based on the relatively small sample set tested to date -- no clear design principles have emerged to avoid this liability; experimental testing using straightforward assays is advised to accurately evaluate this potential liability. Importantly, our results show that by altering

SAR, potentially deleterious effects on cytochrome P450 activity can be minimized, thereby increasing the safety of an MGE drug candidate.

Finally, we screened the impact of selected analogs on hERG channels to provide additional perspective on the safety of these compounds; specifically, hERG inhibition has been associated with cardiotoxicity due to QT interval prolongation which increases the risk of fatal cardiac arrhythmias⁶⁵. As was common in this study, hERG channel inhibition depended on molecular flexibility and lipophilicity (i.e., the extent and patterns of butanoylation), which are key molecular features that influence hERG channel inhibition in other contexts.⁶⁶ Less expectedly, we observed distinct differences for hERG inhibition between the 1,3,4-tri-butanoylated and the 3,4,6-tri-butanoylated analogs that potentially shed light on the “whole molecule” activity of the “3,4,6”-modified analogs that distinguish this set of regioisomers from their “1,3,4” counterparts.^{23,24,26} Originally we hypothesized that many of the “whole molecule” effects of the “C6”-modified analogs -- including increased apoptosis in cancer cells -- were due to inhibition of NF- κ B.²⁸ Interestingly, and providing another mechanism to explain these results, hERG channels are aberrantly-expressed on tumor cells where they regulate apoptosis and proliferation; furthermore, hERG expression in tumor cells is correlated with increased NF- κ B activity.⁶⁷ The SAR from our current study suggests that 3,4,6-tributanoylated analogs may have the ability to induce apoptosis by inhibiting hERG channels that are abundantly expressed on cancer cells, possibly in combination with NF- κ B inhibition. Although these results provide “added value” for cancer treatment, the possibility of analog-provoked hERG inhibition raises a cautionary note with respect to cardiotoxicity potential; more positively, the set of analogs we tested suggested that hERG and cardiotoxicity can be avoided through selection of appropriate SAR features (e.g., use of the safe “1,3,4” tributanoylated analogs when possible).

Summary and Conclusions

This report describes, for the first time to our knowledge, experiments that characterize the physiochemical properties and FDA-relevant safety parameters for representative SCFA-modified monosaccharide analogs used in MGE applications. As evidence mounts from cell-based experiments that MGE has many potential biomedical and clinical applications, one stumbling block to actual clinical translation lies in the perceived high concentrations required for biological activity, which are often in the micromolar and sometimes even in the millimolar range. But, as discussed above, the “dogma” that high potency in the nanomolar range is required in drug design and development is countered by conventional small molecule therapeutics that have modest activity when tested using *in vitro* screening assays but have become successful drugs because of their attractive pharmacological properties and high safety margins.³⁷ Based on these ideas, we reasoned that more importance should be given to the physiochemical properties of small molecules and the associated biological properties of Pgp efflux, CYP3A4, and hERG channels.

Related to MGE, therefore we believe that increased focus should be placed on optimizing physiochemical properties, ADME, toxicity, and improving the pharmacodynamic profiles for existing (e.g., peracetylated) and emerging (e.g., butanoylated) analogs. As the data

presented in this paper shows, the core sugar, the SCFA protecting groups, their regioisomeric arrangement on the core sugar, and the N-acyl group all combine to influence pharmacological endpoints that are important for clinical translation. Throughout this study we have used experimentally tractable, rapid screening assays to begin assembling a scientific foundation of ADME-relevant properties for MGE analogs; in the future as clinical translation proceeds, we anticipate that these parameters will be studied using increasingly rigorous methodology. Looking forward based on these results, we speculate that analogs can be designed with further improved properties that will help advance MGE-based therapies into the clinic. In conclusion, this study establishes that ADME, toxicity, pharmacodynamic, and physiochemical properties can be tuned based on composition of the core hexosamine, the identity and regioisomeric placement of the SCFA protecting groups, and the chemical nature of the N-acyl functional group and confirms that the hexosamine template ²⁶ is a versatile scaffold for the development of new drugs with many options for tuning biological activity and pharmacological properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Overview of SCFA hexosamine analog "druggability."

(A) The hexosamine scaffold has over 100,000 theoretical permutations²⁶, in which the R_1 , R_3 , R_4 , and R_6 naturally-occurring or synthetic ester-linked SCFA groups can be mix-and-matched to improve the efficiency of analog uptake into cells and tune biological activity while the R' group is either the natural acetate or non-natural "NAz" moiety in this study (overall, several dozen R' -modified analogs have been reported^{19,68}). (B) The removal of the ester-linked protecting groups by non-specific intracellular esterases "activates" the core sugar for incorporation into glycosylation pathways^{69,70}. In the current work, we expand the evaluation of these compounds to include (C) physicochemical properties relevant to pharmacokinetics and (D) biological endpoints related to ADME.

Figure 2. *In silico* prediction of ADME properties.

(A) Representative physicochemical profiles of SCFA-hexosamine analogs were calculated using SwissADME. The shaded red area indicates the regions optimal physicochemical parameters for drug-likeness for oral administration. The calculated parameters for the six physicochemical properties (lipophilicity, size, polarity, solubility, flexibility and saturation) for each type of analog shown are outlined in red. (B) BOILED-Egg plot⁷¹ showing calculated $\log P$ (WLOGP) versus total polar surface area (TPSA) illustrating the predicted abilities of MGE analogs to penetrate the BBB (yellow) or undergo passive human intestinal absorption (HIA) through the gastrointestinal tract (white area). The blue dots indicate that all analogs evaluated were expected to be effluxed from the central nervous system via P-glycoprotein transporters (Pgp).

Figure 3. HPLC analysis.

Retention times were determined for each analog using RP-HPLC; selected example sets of retention times differences between analogs are shown in panels (A-I) as discussed in more detail in the main text.

(A) Representative chromatogram

(B) Method for calculating ΔRT

$$\mathbf{A} = \{RT_1, RT_2, \dots, RT_n\}$$

$$\mathbf{A} \times \mathbf{A} = \{(RT_1, RT_2), (RT_1, RT_3), \dots, (RT_n, RT_1), \dots, (RT_n, RT_{n-1})\}$$

$$\Delta RT = \{x - y \mid (x, y) \in \mathbf{A} \times \mathbf{A}\}$$

(C) Cluster analysis

Figure 4. k-Cluster analysis of RT data.

(A) Retention times were cataloged for each analog using RP-HPLC, representative data is shown. (B) Ordered pairs of retention times for all possible combinations of analogs were generated and the difference in retention times between each analog was calculated (ΔRT). (C) Using the differences between retention times for all pairs of analogs that were calculated (ΔRT), a k-cluster analysis was performed in order to group analog pairs that have similar changes in retention time after a specific change(s) in chemical structure. Each colored group represents a set of chemical transformations that produces a similar net

change in retention time for each ordered pair of analogs. See the **Supporting Information** (Table S2) for a specific list of chemical transformations in each cluster.

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Figure 5. Chromatographic hydrophobicity index (CHI) analyses

(A) CHI values for MGE analogs were determined using set of molecules for calibrating RP-HPLC. (B) The calculated CHI scores for the analogs shown are listed, with each color representing a similar group of chemical transformations for the given hexosamine cores. (C) Experimental CHI scores correlate poorly with *in silico* modeling predictions using Swiss-ADME for consensus log P values. (D) The analogs tested for their CHI were ranked according to their experimentally determined values with the *in silico* lipophilicity rankings listed in parentheses. No analogs were calculated to have the same CHI score.

Figure 6. Aqueous solubility map.

Colored squares indicate the type of hexosamine core following the conventions provided by Glycopedia (http://www.glycopedia.eu/IMG/pdf/the_symbolic_representation_of_monosaccharides_2014.pdf). The area of each square is proportional to the experimentally measured quantitative value for water solubility (exact values are provided in the **Supporting Information** (Table S4)). Each node of the map is connected to another node that is differentiated by the listed modification(s) and **A**, **B**, **C(i)** and **C(ii)** denote relationships discussed in the main text.

(A) Pgp efflux **(B) CYP3A4 inhibition** **Figure 7. Pgp efflux and CYP3A4 inhibition.**

The indicated analogs were screened for (A) Pgp efflux (B) and CYP3A4 inhibition. Heat maps were generated to depict the combinatorial effects of the core hexosamine, SCFA modification, and composition of the N-acyl group. Gray areas denote analogs that do not exist or were not tested.

Figure 8. HERG channel activity inhibition.

(A) E-1041, a positive control and (B-H) MGE analogs were screened for HERG channel inhibition. (I) Non-linear regression was used to fit the IC_{50} curves to the inhibition data and IC_{50} values were approximated (see Supplementary Table S7).

Figure 9.

Cardiomyocytes were incubated for 12, 24, and 48 h with (A) 3,4,6-O-Bu₃GlcNAc and (B) 3,4,6-O-Bu₃ManNAc at 50 μ M each. Significantly higher cell death was apparent beginning 12 to 24 h after commencing incubation with each analog. (C). MTT assays conducted after 24 and 48 h confirm the dramatic decline in cell viability upon treatment with these “3,4,6” tri-butanylated hexosamine analogs.