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Metabolic Oligosaccharide Engineering with *N*-Acyl Functionalized ManNAc Analogues: Cytotoxicity, Metabolic Flux, and Glycan-display Considerations

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Abstract

Metabolic oligosaccharide engineering is a maturing technology capable of modifying cell surface sugars in living cells and animals through the biosynthetic installation of non-natural monosaccharides into the glycocalyx. A particularly robust area of investigation involves the incorporation of azide functional groups onto the cell surface, which can then be further derivatized using “click chemistry.” While considerable effort has gone into optimizing the reagents used for the azide ligation reactions, less optimization of the monosaccharide analogues used in the preceding metabolic incorporation steps has been done. This study fills this void by reporting novel butanoylated ManNAc analogues that are used by cells with greater efficiency and less cytotoxicity than the current “gold standard,” which are peracetylated compounds such as Ac₄ManNAz. In particular, tributanoylet, *N*-acetyl, *N*-azido, and *N*-levulinoyl ManNAc analogues with the high flux 1,3,4-*O*-hydroxyl pattern of butanoylation were compared with their counterparts having the pro-apoptotic 3,4,6-*O*-butanoylation pattern. The results reveal that the ketone-bearing *N*-levulinoyl analogue 3,4,6-*O*-Bu₃ManNLev is highly apoptotic, and thus is a promising anti-cancer drug candidate. By contrast, the azide-bearing analogue 1,3,4-*O*-Bu₃ManNAz effectively labeled cellular sialoglycans at concentrations ~3 to 5-fold lower (e.g., at 12.5 to 25 μM) than Ac₄ManNAz (50 to 150 μM) and exhibited no indications of apoptosis even at concentrations up to 400 μM. In summary, this work extends emerging structure activity relationships that predict the effects of short chain fatty acid modified monosaccharides on mammalian cells and also provides a tangible advance in efforts to make metabolic oligosaccharide engineering a practical technology for the medical and biotechnology communities.

Keywords

Metabolic oligosaccharide engineering; glycosylation; sialic acid biosynthesis; ManNAc analogues; apoptosis; cell surface labeling

Introduction

Metabolic oligosaccharide engineering (MOE) emerged about 20 years ago when the Reutter group demonstrated that the mammalian sialic acid biosynthetic pathway (Fig. 1)

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had sufficient substrate permissivity to accommodate non-natural analogues of *N*-acetylmannosamine (ManNAc, Cpd **1** in Fig. 2) (Kayser et al. 1992). As a result, the treated cells display non-natural sialic acids with novel physical and chemical properties on their surfaces, opening the door for a host of nascent applications (Campbell et al. 2007). For example, ManNLev (**2**), a ketone-bearing analogue that established that bio-orthogonal functional groups could be installed into the glycocalyx via MOE (Mahal et al. 1997), has been exploited for fluorescence-based quantification (Mahal et al. 1997), glycoform remodeling (Yarema et al. 1998), and delivery of various therapeutics (e.g., ricin (Mahal et al. 1997), doxorubicin (Nauman and Bertozzi 2001), and gene therapy (Lee et al. 1999)). The sub-physiological pH required for ketones to undergo bioorthogonal reactions with hydrazide binding partners (Mahal et al. 1997; Yarema 2002) spurred a search for additional chemoselectively-functionalized forms of ManNAc leading to the inclusion of thiols (Du et al. 2011; Hua et al. 2011; Sampathkumar et al. 2006c), aryl azides (Han et al. 2005), alkynes (Chang et al. 2009; Hsu et al. 2007), and diazirines (Bond et al. 2009; Tanaka and Kohler 2008) in analogue design. Of these many options, azido-derivatized analogues (e.g., ManNAz, **3**) have become the emerging favorites due to their facile incorporation into glycoconjugates and multiple chemoselective ligation reaction options that include the use of the Bertozzi modification of the Staudinger reaction (Saxon and Bertozzi 2000) and several variations of 'click chemistry' (Codelli et al. 2008; Kii et al. 2010; Kirshenbaum and Holub 2010; Kolb et al. 2001; Stöckmann et al. 2011). In fact, azido-ManNAc analogues have reached commercial fruition (Qian et al.) with many efforts underway to apply them for research (Neves et al. 2011), biomedicine (Yang et al. 2011), and materials science (Dirks et al. 2007).

In contrast to the numerous options for click chemistry that have been recently developed, less effort has been devoted towards improving the ManNAc analogues used for the preceding metabolic labeling step. In particular, a limitation of this promising technology that quickly became apparent was the low efficiency by which ManNAc analogues (e.g., ManNLev or ManNAz, Cpd **2** or **3**) were used by cells; concentrations of 30 to 50 millimolar or more were often required to maximize surface labeling (Yarema et al. 1998). These concentrations imply that to install one non-natural sialoside on the cell surface, approximately 10^6 ManNAc precursors need to be added to the culture medium (Fig. 1a,b). Such high exogenous concentrations of ManNAc analogues were required, at least in part, because there are no plasma membrane transporters for this monosaccharide, which is typically not found in the diet but is instead only produced as a transient intermediate inside a cell. To overcome this problem, the peracetylation strategy used to increase the cellular uptake of disaccharide primers of *O*-glycan biosynthesis (Sarkar et al. 1995) was applied to ManNAc analogues (Lemieux et al. 1999), with a gain of efficiency of ~ 600 fold (Fig. 1c) (Jones et al. 2004). Currently, virtually all MOE experiments using azide-modified ManNAc employ the peracetylated form of this analogue (Ac₄ManNAz, **3a**), which has the two potential drawbacks discussed below: suboptimal labeling efficiency and potential cytotoxicity

Our group reasoned that the presumed mechanism for the improved efficiency of acetylated analogues – increased lipophilicity that facilitates diffusion through the plasma membrane – could be enhanced by longer short chain fatty acid (SCFA) groups. Consistent with this hypothesis, analogues derivatized with *O*-propionyl (Pr₄ManNAc) or *O*-butanoyl (Bu₄ManNAc, **1b**) were more efficient than Ac₄ManNAc (Fig. 1d) (Jones et al. 2004; Kim et al. 2004). The butanoylated ManNAc analogues, however, had considerable toxicity that interestingly was ameliorated by certain tributanoylated analogues (e.g., 1,3,4-*O*-Bu₃ManNAc, **1c** (Aich et al. 2008)) that we initially synthesized because of their favorable Lipinski Rule of 5 properties for orally available drugs (Sampathkumar et al. 2006a). The high-flux, low toxicity properties of 1,3,4-*O*-Bu₃ManNAc (Fig. 1e) extended to ManNLev

(e.g., 1,3,4-*O*-Bu₃ManNLev, **2c**) but has not been evaluated with azido analogues. Accordingly, one objective of the current study was to evaluate the tributanoylated ManNAz analogue 1,3,4-*O*-Bu₃ManNAz (**3c**) to assess its flux and labeling characteristics based on the hypothesis that it will be superior to Ac₄ManNAz (**3a**).

A second aspect of this work was to assess the relative toxicities of butanoylated ketone- and azido-modified ManNAc analogues. This part of the study was based on the discovery that “3,4,6” tributanoylated ManNAc analogues (e.g., “3,4,6”-*O*-Bu₃ManNAc, **1d**) had dramatically different properties than their 1,3,4 counterparts (e.g., 1,3,4-*O*-Bu₃ManNAc, **1c**) (Aich et al. 2008). Instead of the “non-toxic” character of **1c**, **1d** had relatively high toxicity in cell culture and also had a suite of additional properties that foreshadowed efficacy as an anti-cancer drug (such as NF-κB inhibition and reduction in the invasive potential of metastatic breast cancer cells (Campbell et al. 2008; Elmouelhi et al. 2009; Wang et al. 2009)). These findings led us to reason that the “3,4,6” tributanoylated ManNAc analogues might be sufficiently toxic to also induce apoptosis in cancer cells *in vivo*. In particular, 3,4,6-*O*-Bu₃ManNLev, (**2d**) was postulated to have the potential to combine the apoptotic propensity of Ac₄ManNLev (Kim et al. 2004) with the added beneficial features of 3,4,6-*O*-Bu₃ManNAc (i.e., inhibition of proliferation, induction of apoptosis, and reduction of metastatic potential) to create a superior cancer drug candidate. Azido analogues (i.e., 3,4,6-*O*-Bu₃ManNAz, **3d**) will also be valuable if they maintain the anti-cancer properties of **1d** while being able to label the tumor associated carbohydrate antigens (TACA) of cancer cells efficiently. Once TACA are labeled several therapeutic options exist, which include targeting of the TACA by the host immune system with increased effectiveness (Chefalo et al. 2004; Chefalo et al. 2006) or by delivering a second exogenously supplied agent (e.g., a gene delivery vector (Lee et al. 1999), a small molecule drug such as doxorubicin (Nauman and Bertozzi 2001), or a protein toxin such as ricin (Mahal et al. 1997)).

Based on the potentially conflicting (for labeling purposes) or complementary (for cancer therapy) properties of butanoylated ManNAc analogues, the work presented here helps deconvolute structure activity relationships (SAR) that make analogues suited for metabolic labeling versus drug development. To facilitate this evaluation, we used two extremes of *N*-acyl groups – the natural *N*-acetyl group that has minimal toxicity and is well accommodated by the sialic acid pathway (i.e., it provides high flux) and the ketone-containing *N*-acyl group that is poorly processed by the pathway and has a substantial degree of toxicity. Superimposed on these extremes is the emerging favorite for MOE labeling applications; specifically, the *N*-acyl azido group that can be exploited in click chemistry (all analogues used in this study are shown in Figure 2, with the previously unreported compounds 3,4,6-*O*-Bu₃ManNLev, **2d**; Bu₄ManNAz, **3b**; 1,3,4-*O*-Bu₃ManNAz, **3c**; and 3,4,6-*O*-Bu₃ManNAz, **3d** highlighted in boxed format).

Materials and Methods

Materials

Media, fetal bovine serum (FBS), and Dulbecco's Phosphate Buffered Saline (PBS) were purchased from Invitrogen (Carlsbad, CA). Penicillin and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO). Detergents and reagents for SDS gel electrophoresis were purchased from Bio-Rad Laboratories (Hercules, CA). BCA protein assay kits and SuperSignal West Pico Chemiluminescent Substrate was obtained from Pierce Biotechnology (now Thermo Scientific, Rockford, IL). Protease inhibitor Complete (EDTA-free) was purchased from Roche (Nutley, NJ). ManNAc analogues were synthesized and characterized as previously described; compounds **1a**, **1b**, and **2a** (Kim et al. 2004); compounds **1c**, **1d**, **2b**, and **2c** (Aich et al. 2008); and compound **3a** (Saxon and Bertozzi

2000); the previously unreported compounds **2d**, **3b**, **3c**, and **3d** are described in the Supporting Information.

Cell Growth Conditions

All cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Jurkat wild-type cells were cultured in RPMI 1640 medium (Mediatech; Manassas, VA) supplemented with 5.0% fetal bovine serum (FBS, Atlanta Biologicals; Lawrenceville, GA) and antibiotic solution containing 1.0% of a 100× stock solution of penicillin and streptomycin (P/S; Invitrogen, Carlsbad, CA). Human pancreatic adenocarcinoma cell lines, SW1990 and PANC-1, were grown in 1:1 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and P/S. Chinese hamster ovary (CHO) cells were cultured in DMEM supplemented with 10% FBS, antibiotic solution and 2.0 mM L-glutamine. MDA-MB-231 breast cancer cells were cultivated in RPMI 1640 supplemented with 10% FBS and P/S. All cells were maintained at 37 °C in a humidified atmosphere containing 5.0% CO₂. Cell Dissociation Buffer, enzyme free, PBS based was purchased from Millipore (Billerica, MA). TrypLE™ Express, a stable trypsin replacement enzyme, was from Invitrogen. As a technical note, all assays reported in this study were done in the presence of FBS, which comprises a rich source of sialoglycoconjugates that can be scavenged by the sialic acid biosynthetic pathway and compete with uptake and incorporation of the exogenously supplied ManNAc analogues (Mantey et al. 2001; Tangvoranuntakul et al. 2003). This method, however, was used to reflect widely used cell culture conditions that employ FBS and also to be relevant to in vivo testing, where competition from serum glycoconjugates is unavoidable.

Cell Counting Assays

For a typical growth inhibition assay, analogue (from a 10 mM stock solution in EtOH) was added to 24-well tissue culture (T. C.) plates at a range of concentrations between 0 to 400 μM depending on the analogue (for example, highly toxic analogues were tested between 0 and 100 μM) and the EtOH was allowed to evaporate. Jurkat cells (10⁵) in 0.5 ml of medium were added to each well on Day 0. On Day 3 100 μl of cell suspension was counted in triplicate by using a Beckman Coulter Z2 Coulter Particle Count and Size Analyzer. Alternately, on Day 3 fresh medium (1.0 ml) was added to each well, cell cultures were mixed by gentle pipetting and cells were maintained until Day 5 when counting was done (for one experiment, shown in Figure 4, cells were evaluated after 15 days, as previously described (Sampathkumar et al. 2006b)). Results were typically compared to a control sample that did not contain analogue; the number of cells in this sample was arbitrarily set at 100.

Caspase Activity Assays

Jurkat cells were plated in 6-well T. C. plates at a density of 10⁶ cells per well in 3.0 mL of cell culture medium containing the appropriate amount of pre-aliquoted and evaporated analogue (from a 50 mM stock solution in EtOH) to give a final concentration of 20, 50, 100 or 200 μM. The cells were incubated for either 5.0 or 24 h. After incubation, cells in each well were thoroughly mixed, 1.0 ml was transferred to a 1.5 ml Eppendorf tube, and two 100 μl cell counts were done. Equivalent numbers of cells in 50 μl aliquots were placed separately in fresh 1.5 ml Eppendorf tubes for the Caspase 3/7 assay and for the Caspase 9 assay. An equivalent volume (50 μl) of Caspase-Glo 3/7 Assay Reagent or Caspase-Glo 9 Reagent (Promega; Madison, WI) was added to the appropriate tube, mixed, and gently rocked on a mechanical shaker until measured in a luminometer after 30, 60, and 90 min.

Cell Cycle Arrest Analysis

Cell cycle arrest analysis followed published methods (Sampathkumar et al. 2006b). Briefly, Jurkat cells were incubated with analogue in 6-well T. C. plates as described above for three days, counted, and separated into samples each containing 10^6 cells, and washed twice with PBS. The cells were then centrifuged and resuspended in 500 μ l PBS and added to 4.5 ml of 70% aqueous ethanol and refrigerated at 4°C for ≥ 24 h. After the cells were fixed, they were centrifuged twice to completely remove the ethanol. The cells were then resuspended in 500 μ l propidium iodide (PI) / ribonuclease A (RNase A) staining solution (BD Pharmingen, Catalog No. 550825; San Diego, CA). The samples were incubated in the dark at room temperature for 30 min and then analyzed by flow cytometry (FACSCalibur flow cytometer; Becton Dickinson, San Jose, CA, USA). An area-width plot of FL2 (PI fluorescence) was used to exclude cell aggregates and only include single cell populations for cell cycle determination. At least 10,000 gated events were recorded for each sample.

Determination of Sialic Acid Levels in Analogue-treated Cells

Jurkat cells were plated in 6-well T. C. plates at a density of 10^6 cells per well in 2.0 ml of culture medium. The appropriate volume of analogue (or ethanol, for control samples) was added to each well from a 50 mM stock solution. The cells were incubated for 48 or 72 h and then lysed by three freeze-thaw cycles. Lysates were analyzed using an adaptation of the periodate-resorcinol assay (Jourdain et al. 1971; Yarema et al. 2001) to quantify total sialic acid (i.e., the oxidation step was performed for 15 min on ice, which allows both free monosaccharides and glycoconjugate-bound sialic acid to be measured). For each experiment, test samples were compared to a standard curve created using known concentrations of *N*-acetylneuraminic acid (Pfanstiehl; Waukegan, IL).

Glycoconjugate or Cell surface Labeling Assays

For glycoconjugate labeling or cell surface quantification or visualization assays, the appropriate volume of analogue (typically experiments compared Ac₄ManNAz (**3a**) and 1,3,4-*O*-Bu₃ManNAz (**3c**) in parallel under identical conditions) was added to each well of a 6-well T. C. plate from a 50 mM stock solution to give final concentrations in the range of 0 to 150 μ M and the ethanol was allowed to evaporate prior seeding the cells. Cells were seeded into analogue-containing wells in their respective growth medium at less than 25% confluency to ensure that they would be able to maintain robust growth over the course of the ensuing two day experiment. An equivalent number of cells, from the same initial batch (e.g., harvested from a 10 cm T.C. plate or T75 flask, as needed), was added to each well and the plate was incubated for two days. After incubation, the cells were used for bioorthogonal glycan blot analysis, flow cytometry, or live cell imaging, respectively, as described below.

Bioorthogonal Glycan Blot Analysis

Bioorthogonal glycan blot analysis was performed to analyze azido-modified glycoconjugates from analogue-treated cells. After metabolically labeling with Ac₄ManNAz (**3a**) or 1,3,4-*O*-Bu₃ManNAz (**3c**), cells were detached with dissociation buffer and transferred to 1.5 ml Eppendorf tubes, in which they were washed with 1.0 ml PBS. Cells were then resuspended in 200 μ l of Click-iT reaction mixture containing 1.0 mM biotin-alkyne (Qingdao Vochem, Qingdao, China) and incubated for 45 min at room temperature. Cells were then washed and lysed using RIPA buffer (Sigma-Aldrich) supplemented with protease inhibitor (Fermentas, Glen Burnie, MD) and total protein quantified with BCA protein assay kit (Thermo Scientific Pierce). Equal quantities of protein samples in Laemmli sample buffer with 5% β -mercaptoethanol were run on SDS polyacrylamide gel (12%) for 2.0 h at 140 V and transferred to nitrocellulose membrane for 1.0 h in Tris/Glycine transfer

buffer at 100 V. The membranes were then blocked with 5.0% (w/v) BSA in TBS with Tween®20 (0.1% v/v) (TBS-T) for 1.0 h and probed with streptavidin-horseradish peroxidase (HRP) (Cell Signaling Technology, Beverly, MA) for 1.0 h. The membranes were washed in TBS-T and biotin-tagged cell surface azido glycoconjugates recognized by streptavidin-HRP were visualized using the Colorimetric HRP substrate reagent kit (Bio-Rad) and by the Supersignal West Pico chemiluminescent reagents (Pierce Biotechnology, Inc., Rockford, IL). The membranes were then scanned with NIH ImageJ software to obtain a semi-quantitative measurement of cell surface azido glycoconjugate expression.

Cell Surface Azide Quantification by Flow Cytometry

To label cells for quantification of cell surface azide groups, a fresh Click-iT reaction cocktail (Invitrogen) was prepared for each experiment by combining and mixing 880 μ L of Click-iT buffer reaction, 100 μ L of Click-iT additive C, 50 μ L of a 50mM CuSO₄ solution, 30 μ L of 5.0 M NaCl, and immediately prior to labeling, adding 10 μ L of a 100 mM Alexa Fluor 488 Alkyne (Invitrogen) in the dark. Adherent cells were detached by dissociation buffer (Millipore, Billerica, MA) and transferred to 1.5 ml Eppendorf tubes, in which they were washed with 1.0 ml PBS. The cells were then resuspended in 200 μ L Click Reaction Mixture and incubated at room temperature for 45 min in the dark and gently flipped every 15 min to ensure the cells interacted evenly with the labeling mixture. The samples were then washed twice with PBS with 5.0% BSA. Flow cytometry (FACSCalibur flow cytometer; Becton Dickinson, San Jose, CA, USA) was performed for each sample and the azide expression levels were quantified by geometric mean of FL1 fluorescence using FlowJo Software (Tree Star, Ashland, OR).

Cell Surface Azide Visualization via Fluorescence Microscopy

Visualization of azido-tagged glycoconjugates on three adherent cell lines (PANC-1, SW1990, and CHO) was analyzed by fluorescent microscopy after incubation for two days with Ac₄ManNAz or 1,3,4-*O*-Bu₃ManNAz as described above. After two days, cells were washed with PBS, taking care not to detach them. To each well, 200 μ L of Click-iT reaction mixture containing Alexa Fluor 488 Alkyne was added. Also, 10 μ L of a 1.0 mM 4,6-diamidino-2-phenylindole (DAPI) solution was added to each well to stain the nuclei of the cells. After incubation with these reagents for 45 min at room temperature, the cells were rinsed three times with PBS containing 5.0% BSA in PBS. Images were taken using a Nikon eclipse Ti live-cell microscope with a 50 $\times$ objective (Nikon Instruments Inc., Melville, NY). Fluorescence pictures of Alexa488 and DAPI labeled cells were recorded for the same exposure time and overlay with the Nikon Imaging System.

Results and Discussion

Structure-activity Relationships of Azide and Ketone-modified ManNAc Analogues that Contribute to Growth Inhibition and Cytotoxicity

An important consideration when planning a MOE experiment is to avoid analogue-induced cytotoxicity. For *in vitro* cell culture experiments, this endpoint can be rapidly evaluated by monitoring cell counts (we have previously shown that this data, when used for evaluating ManNAc analogues, closely mirrors more sophisticated approaches such as MTT assays (Campbell et al. 2008; Wang et al. 2006)); such data is shown in Figure 3a for ManNAc (**1**), ManNLev (**2**), and the peracetylated versions of these sugars (**1a** and **2a**, which are 2-3 orders of magnitude more cytotoxic). When moving from acetylation to butanoylation (a strategy that increases the efficiency of analogue utilization several fold, Fig. 1c,d), the SAR become more complex. For example, a comparison of Ac₄ManNAc (**1a**) with its perbutanoylated Bu₄ManNAc (**1b**) shows that the latter compound is considerably more cytotoxic, an effect that is exacerbated slightly by 3,4,6-*O*-Bu₃ManNAc (**1d**) and

ameliorated by 1,3,4-*O*-Bu₃ManNAc (**1c**) (Fig. 3b). A similar comparison of the “Lev” compounds shows that all of these analogues were more cytotoxic than their “NAc” counterparts with the exception of 1,3,4-*O*-Bu₃ManNLev (**2c**), which did not show a significant reduction in cell growth over the concentration range tested (Fig. 3c). The “NAz” derivatives showed an unexpected response insofar as the previously regarded “non-toxic” 1,3,4-butanoylated pattern, exemplified by 1,3,4-*O*-Bu₃ManNAz (**3c**), showed a greater reduction in cell number than Ac₄ManNAz (**3a**).

To facilitate comparisons of the effect of the *N*-acyl group with each pattern of tributanoylation, the “3,4,6” trio of *N*-acyl modified analogues are directly compared in Figure 3e and the “1,3,4” analogues in Figure 3f. A comparison of the three “3,4,6” derivatized tributanoylated ManNAc analogues (Fig. 3e) verified that the *N*-levulinoyl side chain can be combined with this pattern of butanoylation to give a highly toxic phenotype; this result was predicted from previous SAR that separately established the cytotoxicity of the *N*-acyl levulinoyl group (Kim et al. 2004) and “3,4,6” *O*-acylation pattern (Aich et al. 2008). The resulting analogue 3,4,6-*O*-Bu₃ManNLev (**2d**) was the most cytotoxic ManNAc analogue yet evaluated in our laboratory, with an IC₅₀ lower than 20 μM not only for Jurkat cells but for a panel of other human cancer cells (data not shown). An unexpected trend, however, emerged when comparing the “1,3,4” analogues (Fig. 3f). Although all of these compounds were much less toxic than their 3,4,6 counterparts (note the different scale used for the x-axis), the “NAz” analogue (1,3,4-*O*-Bu₃ManNAz, **3c**) most potently reduced the cell count. This result was surprising because in many previous experiments in our laboratory and elsewhere, the *N*-acyl azido modification and “1,3,4” pattern of SCFA derivatization – when used separately – were virtually non-toxic.

Time Dependence of Cytotoxicity

A minor difference between the data shown in panels (a) through (d) of Figure 3 and panels (e) and (f) were that the former data illustrates cell counts five days after the cells were given a one-time dose of analogue while the latter were evaluated after three days. By directly comparing cell counts (Fig. 4a), there was little difference between either of these “short” time periods for 3,4,6-*O*-Bu₃ManNAc (**1d**). Figure 4a also shows that a one-time dosage above a certain threshold (e.g., ~ 100 μM) results in complete cell death after about two weeks while certain previously growth inhibited cells (e.g., those at ~75 μM) below this threshold recover and resume normal growth (repeated dosing exacerbates analogue toxicity, as we discuss in more detail elsewhere (Aich et al. 2010)). A similar recovery is seen for azido analogues with an especially pronounced effect for 1,3,4-*O*-Bu₃ManNAz (**3c**); as shown in Figure 3, this analogue induced substantial growth inhibition at 3 or 5 days but had no observable impact on cell viability at 15 days (Fig. 4). The fact that cells treated with certain analogues can recover and return to robust growth two weeks later results indicate that short term cytotoxicity could be due to transient growth inhibition rather than to cell death. This view is confirmed by plotting the data as the total number of cells compared to the number seeded, as shown in Figure 4c for azido analogues. When depicted in this manner, cell death only necessarily occurs for cell counts below 100 (which only happens for the “toxic” 3,4,6 analogue).

Deconvoluting Cell Death (apoptosis) From Transient Growth Inhibition – Part 1. Caspase Activity

To help delineate the boundaries between apoptosis and transient growth inhibition, caspase activity was analyzed in analogue treated cells. None of the “1,3,4” analogues – even 1,3,4-*O*-Bu₃ManNAz (**3c**) that elicited substantial (Fig. 3d) albeit transient (Fig. 4b) growth inhibition measurably increased caspase activity at concentrations of ≤ 200 μM (data not shown). By contrast, analysis of caspase 3/7 and caspase 9 activity for the “Bu₄” and “3,4,6”

analogues for the “Lev” core (shown in Fig. 5a & b, respectively in comparison with the toxic “3,4,6” form of “NAc”) and for the “NAz” core (panels c and d) reveals several time-dependent increases in caspase activity. At short time points (e.g., at 5 h) it is striking that only two conditions substantially increased caspase activity over background levels; one of these was for caspase 3/7 for Bu₄ManNLev (**2b**) at the highest test concentration of 200 μ M. Interestingly, the other was for 3,4,6-*O*-Bu₃ManNLev (**2d**), where an increase in caspase 3/7 activity was observed only at the lowest analogue concentration tested of 20 μ M. Presumably at higher concentrations, the cells had already progressed sufficiently into apoptosis that caspase activity was no longer required; a similar response has been described previously for apoptosis-inducing cancer drugs such as etoposide (Benjamin et al. 1998) and paclitaxel (Au and Wientjes 1999). At the 24 h time points, many significant increases in caspase activity (for both 3/7 and 9) were seen; these changes were roughly dose dependent although a drop off in activity was sometimes observed at higher concentrations (e.g., for 3,4,6-*O*-Bu₃ManNAz, **3d**) presumably because – similar to **2d** at 5 h – apoptosis had already been initiated and the dying cells no longer needed to maintain caspase activity.

Deconvoluting Cell Death (apoptosis) From Transient Growth Inhibition – Part 2. DNA Analysis

As outlined above, certain analogues activated caspases; moreover, this activation was roughly consistent with analogues that most effectively reduced cell counts (as shown in Fig. 3). However, not all cells died even for “toxic” analogues, while caspase activity was not observed at all for the “1,3,4,” analogues. These data suggested that another mechanism was involved in the reduced cell counts; one possibility was that transient growth inhibition resulted from the histone deacetylase inhibitory (HDACi) properties of hydrolyzed *n*-butyrate (Sampathkumar et al. 2006b). Therefore a more sophisticated level of analysis was conducted to test if the analogues increased the proportion of cells at the G2/M cell cycle checkpoint, indicative of HDACi mediated growth arrest. In these tests, the DNA content of cells treated with analogues at the IC₅₀ (as well as above and below, as determined in Fig. 3) was measured by flow cytometry. At concentrations slightly less than the IC₅₀, e.g., 10 μ M for 3,4,6-*O*-Bu₃ManNLev **2d** (Fig. 6a), 20 μ M for 3,4,6-*O*-Bu₃ManNAz **3d** (panel b), or 40 μ M for Bu₄ManNAz **3b** (panel c), cells accumulated at in G2/M with a small but noticeable increase in the sub-G₀ apoptotic population and with a concomitant decrease in cells in the G0/G1 phase. As the concentrations exceeded the IC₅₀ of each of these compounds, the cells became more dramatically apoptotic as shown by DNA degradation and fragmentation (i.e., the sub-G₀ population increased dramatically). Of note, an increase of apoptotic cells occurred at lower concentrations for **2d** than for **3d**, confirming the potentially toxic phenotype of the *N*-levulinoyl group. By contrast, comparing **3d** (panel b) with 1,3,4-*O*-Bu₃ManNAz (**3c**, panel d) shows the latter analogue produces virtually no sub-G₀ apoptotic cells even at the relatively high concentration of 200 μ M, consistent with an inability to kill cells (Fig. 4b) despite relatively potent growth inhibition at early time points (Fig. 3d).

Characterization of Azido Analogue-Driven Metabolic Flux in Jurkat Cell

All four SCFA-derivatized ManNAz analogues tested in this study increased sialic acid production in Jurkat cells (Fig. 7a). The largest increase resulted from 1,3,4-*O*-Bu₃ManNAz (**3c**) while sialic acid production from Ac₄ManNAz (**3a**) was also quite robust (although **3a** lagged **3c** at all concentrations tested). By contrast, Bu₄ManNAz (**3b**) and 3,4,6-*O*-Bu₃ManNAz (**3d**) only increased sialic acid production up to ~75 μ M, after which production declined most likely due to the cytotoxicity of these analogues. For comparison purposes, the increase in sialic acid observed in cells treated with the azido analogues was ~3-fold lower than seen for the equivalent series of ManNAc analogues (i.e., **1a**, **1b**, **1c** & **1d**; Fig. 7b). This diminished flux was expected based on the lower sialic acid production observed for *N*-acyl modified analogues (Jacobs et al. 2001); it is noteworthy, however, that

flux from the azido analogues was still relatively high compared to alkyl chains of a similar length (e.g., ManNPent or ManNHex (Kim et al. 2004)). Testing of the *N*-levulinoyl analogues is not reported here because ManNLev does not increase cellular levels of sialic acids (Kim et al. 2004) even though it is capable of intersecting the sialic acid pathway and replacing cell surface sialosides with Sia5Lev (Mahal et al. 1997). ManNLev illustrates how the ultimate goal of MOE – incorporation of monosaccharide analogues into glycoconjugates – is not adequately addressed by measuring total cellular levels of sialic acid because much of the sialic acid produced in ManNAc-treated cells remains in the free monosaccharide form (Jones et al. 2004).

ManNAz Incorporation into Glycoconjugates in Jurkat and Various Cell Types

Having confirmed that both Ac₄ManNAz (**3a**) and 1,3,4-*O*-Bu₃ManNAz (**3c**) increase sialic acid levels in analogue treated Jurkat cells (Fig. 7a) at levels that are not cytotoxic (e.g., at ≤ 300 μM, Fig. 4b), we measured Sia5Az incorporation into glycoproteins by using bioorthogonal glycan blots (which are a variation of “Eastern” blots used to detect glycosylated molecules; in our case we specifically probe for azido-modified sialosides). In these experiments, five concentrations (12.5, 25, 50, 100, and 150 μM) of **3a** and **3c** were tested under identical conditions. The initial testing in Jurkat cells showed a dose dependent increase in signal for **3a** over the concentration range that was tested while incorporation for **3c** was essentially maximized at 12.5 μM (Fig. 8a, with quantification in panel f). This result is consistent with the data shown in Fig. 7a where the **3c** supports almost as much sialic acid production at 12.5 μM as **3a** does at 100 μM. Interestingly, a comparison with several other cell lines showed that incorporation of either analogue into Jurkat cells was rather modest, as shown by panels (b) through (e) of Figure 8 for SW1990, MDA-MB-231, CHO, and PANC-1 cell lines, respectively (the exposures are conducted under identical conditions, with quantification for each set of data in (f)). These results suggest that when working with a previously untested cell type, optimization of analogue incorporation is more important for **3a**, which shows a dose dependent increase for almost all cell lines over 0-150 μM (a commonly used concentration range in MOE experiments) while incorporation of **3c** is already robust at 12.5 μM (a very low concentration for most MOE experiments).

Measurement and Imaging of Glycoconjugate Labeling in Live Cells

An attractive feature of MOE experiments, including those exploiting glycan-targeted click chemistry, is their ability to measure glycan expression on the surfaces of living cells (Hong et al. 2010) or organisms (Laughlin et al. 2008). Measuring cell surface levels of Sia5Az by flow cytometry for the lines that robustly incorporated Ac₄ManNAz (**3a**) and 1,3,4-*O*-Bu₃ManNAz (**3c**) (i.e., the PANC-1, SW1990, and CHO lines, Fig. 8) showed that **3c** was more efficient of a labeling reagent than **3a** and these cell lines again displayed more surface labeling than the Jurkat line (Fig. 9a, b). The increased efficiency of **3c** over **3a** was particularly pronounced at lower concentrations (e.g., 12.5 and 25 μM), where **3c** was 4 to 5 fold more effective for the CHO and SW1990 cell lines (Fig. 9c).

In a complementary method to flow cytometry, which just provides a single averaged value for each cell, fluorescent microscopy imaging shows that the Neu5Az labeling is fairly evenly, but not entirely uniformly dispersed across the cell surface (Fig 10). Moreover, there are distinctive distributions observed in the various cell lines, with a distinctive staining of cell-cell boundaries in SW1990 and CHO cells compared to the PANC-1 line. Part of this unevenness is likely due to different proteins expressed in each cell type (represented by the data shown in Fig. 8) but another source of variability might arise from the repertoire of sialyltransferases expressed in each cell type (human cells, for example, express a subset of 20 different sialyltransferases encoded by the human genome). It is intriguing to speculate that individual sialyltransferases process CMP-Neu5Az with various levels of efficiency,

thus skewing the distribution of Neu5Az-bearing glycoconjugates (we are testing this possibility in work beyond the scope of the current publication).

Concluding Comments

In this paper, we characterize mainly expected, and a few unexpected, properties of previously unreported ManNAc analogues. In particular, the SAR that specify toxicity (the “3,4,6” tributanoylation pattern, Fig. 1f) or non-toxicity (the “1,3,4” tributanoylation pattern, Fig. 1e) were demonstrated to be robust to modifications at the *N*-acyl position of ManNAc. For example, the combination of 3,4,6-*O*-tributanoylation with the *N*-levulinoyl group (in the form of 3,4,6-*O*-Bu₃ManNLev, **2d**) resulted in the most apoptotic ManNAc analogue yet tested, which is a valuable lead in cancer drug development of this class of compounds. Even for the MOE researcher not interested in cancer drug development, the results presented in this paper outline conditions where transient growth inhibition can be differentiated from the onset of apoptosis, to design “safe” concentrations of analogues to use in an experiment. Moreover, considering the growing use of analogues other than those based on ManNAc in MOE experiments (Du et al. 2009; Wu and Wong 2011), the extension of the butanoylation patterns to the SAR of GalNAc and GlcNAc (Elmouelhi et al. 2009; Wang et al. 2009) indicate the analogue design principles outlined in this paper will have farther reaching consequences.

In a second aspect of this report, a detailed comparison of azido analogues Ac₄ManNAz (a commercially available compound increasingly used for the many burgeoning applications of click chemistry) and 1,3,4-*O*-Bu₃ManNAz revealed that the latter compound had several advantages. For one, it exhibited no long term toxicity (although, for many routine applications, the concentrations of Ac₄ManNAz needed for labeling are not overtly toxic, either (Soriano del Amo et al. 2010)). More importantly, in no case was the tributyrated analogue *less* efficient than Ac₄ManNAc; rather, in several cell lines, 1,3,4-*O*-Bu₃ManNAz was several fold (e.g., up to 5-fold, Fig. 9) more efficient. For *in vitro* cell culture assays, or even rodent testing, this difference may not be highly significant, but as MOE moves into larger scale applications (e.g., recombinant protein production (Luchansky et al. 2004; Möller et al. 2011) or large animals that require gram quantities of analogue that would cost in the tens of thousands of dollars) the more efficient analogue could provide substantial financial benefits. Finally, it is intriguing to note that recombinant protein production has sought to inhibit cell proliferation in favor of production of the recombinant protein (Arden et al. 2004), 1,3,4-*O*-Bu₃ManNAz may have the ability to perform this task as an unintended but welcome side effect.

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Figure 1. Use of SCFA-derivatized ManNAc analogues to increase flux into the sialic acid pathway

(a) Under normal cell culture conditions with most mammalian cells type, approximately one million (10^6) molecules of ManNAc analogue must be added to the culture media to install one analogue-derived sialoside on the cell surface (b). (c) Peracetylation of ManNAc increases the efficiency ~ 600 fold, such that only $\sim 1,600$ molecules of analogue needs to be added to the media to install one surface sialoside; this gain in efficiency is presumably due to the enhanced membrane permeability of the acylated analog, which was consistent with the even greater efficiency of perbutanoylated analogue (e.g., Bu₄ManNAc, where ~ 500 molecules were required (d)). (e) As a final step in increasing efficiency, omission of one ester-linked butyrate increased efficiency yet another $\sim 25\%$ with "1,3,4" analogues being high flux and "safe" (e.g., with negligible side effects such as the cytotoxicity seen for peracetylated analogues). (f) By contrast, tributanoylated analogues with a "3,4,6" pattern of substitution showed enhanced cytotoxicity and the ability to modulate biological responses (such as signaling pathways) via "whole molecule" mechanisms (as discussed in detail elsewhere (Elmouelhi et al. 2009; Wang et al. 2009)). Examples of "R" groups are shown in Figure 2.

"Natural" ManNAc analogues:

Ketone-derivatized levulinoyl analogues:

Azido-derivatized analogues:

In all cases, Ac = -COCH₃ and Bu = -COCH₂CH₂CH₃

Figure 2. Structures of the ManNAc analogues used in this study

Top row: acetyl- and *n*-butanoyl-derivatized analogues with the natural ManNAc "core" structure; middle row: analogues with a ketone group (highlighted in blue); bottom row: analogues with an azide group (highlighted in red). In all cases the OH group resulting from the "missing" *n*-butanoyl of "1,3,4" and "3,4,6" analogues is highlighted in yellow and the four previously unreported compounds are indicated by the boxes.

Figure 3. Cytotoxic properties of SCFA-derivatized *N*-acyl, *N*-levulinoyl, and *N*-azido ManNAc analogues

(a) Comparison of the cytotoxicity of ManNAc and ManLev when incubated with Jurkat cells for five days in the free monosaccharide and peracetylated forms. (b) Comparison of acylation patterns (e.g., peracetylation compared to perbutanoylation or the “1,3,4” vs. the “3,4,6” tributanoylated analogues) of the natural ManNAc “core” with the same information shown for the respective ManLev analogues in (c) and for ManNAz in (d). Panels (e) and (f) show a direct comparison of the three JV-acyl varieties of the “3,4,6” vs. the “1,3,4” tributanoylated analogues, respectively. Error bars shown in (e) and (f) represent SEM from $n \geq 3$ replicates (note that in (a)-(d), error bars were omitted for clarity).

Figure 4. Comparison of the *N*-acyl group and time dependence of ManNAc analogue cytotoxicity

(a) The relative number of Jurkat cells incubated with 3,4,6-*O*-Bu₃ManNAc for various lengths of time is shown. (b) The long term (15 day) impact of SCFA-derivatized ManNAc analogue treatment (comparison with short term effects can be made with the data shown in Fig. 3d or panel (c)). (c) Cell counts based on the starting, rather than the final, number of cells.

Figure 5. Caspase activation – a surrogate measure for apoptosis

Caspase activity displayed for analogues at 20 μM , 50 μM , 100 μM , and 200 μM showing time and dose-dependent onset of apoptosis. Panels (a) and (c) represent ManNLev-based analogues and panels (b) and (d) shows the equivalent data for ManNAz-based compounds; also, panels (a) and (b) depict caspase 3/7 activity and panels (c) and (d) show caspase 9 activity. In all cases, data for 5 and 24 hour time points are shown. Error bars represent the SEM for $n \geq 3$ replicates.

(a) 3,4,6-*O*-Bu₃ManNLev (IC₅₀ ~ 15 μM) (b) 3,4,6-*O*-Bu₃ManNAz (IC₅₀ ~ 30 μM) (c) Bu₄ManNAz (IC₅₀ ~ 75 μM) (d) 1,3,4-*O*-Bu₃ManNAz (IC₅₀ ~ 100 μM) **Figure 6. Cell cycle arrest and apoptosis analysis of analogue-treated Jurkat cells**

Cells were incubated for 3 days with (a) 3,4,6-*O*-Bu₃ManNLev, (b) 3,4,6-*O*-Bu₃ManNAz, (c) Bu₄ManNAz, and (d) 1,3,4-*O*-Bu₃ManNAz after which the DNA content was measured by PI/RNase A staining.

Figure 7. Comparison of Azido-ManNAc flux in Jurkat cells

Intracellular sialic acid levels in cells incubated with peracetylated and three varieties of butanoylated ManNAz analogues (panel a) or ManNAc analogues (panel b). Error bars represent the SEM for $n \geq 3$ replicates.

Figure 8. Comparison of glycoconjugate labeling with Ac₄ManNAz and 1,3,4-*O*-Bu₃ManNAz by bioorthogonal glycan blots

(a) Jurkat, (b) SW 1990, (c) MDA-MB-231, (d) CHO, or (e) PANC-1 cells were metabolically labeled with either analogue for 48 h and cell surface azido groups were conjugated with biotin-alkyne, lysed, and equal quantities of protein were loaded for each sample, resolved by SDS-PAGE, and visualized by incubation with streptavidin-HRP. In all cases, samples were treated with (from left to right) 12.5, 25, 50, 100, 150 μ M Ac₄ManNAz or 1,3,4-*o*-Bu₃ManNAz. Lanes indicated with (-) represent control samples from cells treated with neither analogue. (f) The intensity of the labeled glycoconjugates in each lane was measured using NIH ImageJ software and samples from a particular cell line were compared to the signal from cells incubated with 12.5 μ M Ac₄ManNAz. Error bars represent the SEM for $n \geq 3$ replicates.

Figure 9. Comparison of surface labeling with 1,3,4-*O*-Bu₃ManNAz and Ac₄ManNAz by flow cytometry

(a) PANC-1, SW1990, Jurkat and CHO cells treated with (or without) 1,3,4-*O*-Bu₃ManNAz (black bars) or Ac₄ManNAz (outline bars) for two days, detached and incubated for 1.0 h with and click-labeled with Alexa Fluor®488 alkyne as described in experimental procedures; these data are obtained from $n \geq 3$ flow cytometry replicates, for which representative histograms are shown in panel (b) for PANC-1 and SW1990 cells. (c) Summary of the geometric means of samples treated with 1,3,4-*O*-Bu₃ManNAz divided by the geometric means of their Ac₄ManNAz counterparts. Error bars represent the SEM for $n \geq 3$ replicates.

Figure 10. Comparison of surface labeling with Ac₄ManNAz and 1,3,4-O-Bu₃ManNAz by fluorescence microscopy

Representative images of PANC-1, SW1990, and CHO cells after treatment with 100 μ M Ac₄ManNAz or 1,3,4-O-Bu₃ManNAz followed by labeling with Alexa488-Alkyne to detect surface azide groups and DAPI to stain nuclei (the procedure for labeling of cells is described in detail in the text). The fluorescence intensity landscape is shown for dotted the line indicated on each image. Scale bar = 25 μ m. Representative images of analogue-treated cells are given in the Supporting Information.