Targeting Pro-Invasive Oncogenes with Short Chain Fatty Acid-Hexosamine Analogues Inhibits the Mobility of Metastatic MDA-MB-231 Breast Cancer Cells

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Per-butanoylated *N*-acetyl-D-mannosamine (Bu_4ManNAc), a SCFA-hexosamine cancer drug candidate with activity manifest through intact *n*-butyrate-carbohydrate linkages, reduced the invasion of metastatic MDA-MB-231 breast cancer cells unlike per-butanoylated-D-mannose (Bu_5Man), a clinically tested compound that did not alter cell mobility. To gain molecular-level insight, therapeutic targets implicated in metastasis were investigated. The active compound Bu_4ManNAc reduced both MUC1 expression and MMP-9 activity (via down-regulation of CXCR4 transcription), whereas "inactive" Bu_5Man had counterbalancing effects on these oncogenes. This divergent impact on transcription was linked to interplay between HDACi activity (held by both Bu_4ManNAc and Bu_5Man) and NF-κB activity, which was selectively down-regulated by Bu_4ManNAc. Overall, these results establish a new therapeutic end point (control of invasion) for SCFA-hexosamine hybrid molecules, define relative contributions of molecular players involved in cell mobility and demonstrate that Bu_4ManNAc breaks the confounding link between beneficial HDACi activity and the simultaneous deleterious activation of NF-κB often found in epigenetic drug candidates.

Introduction

Over the last 30 years, short chain fatty acid (SCFA[a])-monosaccharide hybrid molecules have been investigated by two groups of researchers. By far, their most successful use has been for metabolic glycoengineering of the cell surface[1,2] where SCFA have been exploited to enhance the cellular uptake of monosaccharide analogues.[3,4] Earlier, in the reverse strategy, carbohydrates were used as delivery vehicles for *n*-butyrate, a histone deacetylase inhibitor (HDACi) used for epigenetic cancer therapy.[5] In both applications, the hybrid molecules were assumed to be hydrolyzed by esterases or other lipases upon entering a cell and any ensuing biological activity was attributed to either the liberated sugar or SCFA moieties. In this study, overlaid upon, and dominating, the glycosylation-specific effects of the core sugar and the HDACi activity of the accessory SCFA, we investigated the therapeutic possibilities of a third source of biological activity derived from molecular species with intact SCFA-hexosamine ester linkages (Figure 1).

At the outset, a brief review of the first two uses of SCFA-monosaccharide hybrid molecules is useful for understanding why the therapeutic prospects of this class of compounds are worth revisiting. Hybrid molecules consisting of a SCFA ester linked to a carbohydrate were first employed by cancer biologists who sought to exploit the sugar as a delivery vehicle for *n*-butyrate. *n*-Butyrate is liberated by cytosolic esterases upon cellular uptake of the prodrug, and the resulting histone deacetylase inhibitory (HDACi) activity of this SCFA slows the growth of cancer cells by up-regulating the cell cycle checkpoint protein p21, halting cell cycle progression and contributing to apoptosis. In studies dating from the 1980s, clinical testing of Bu_5Man (compd **2** in Figure 2) and similar butanoylated carbohydrates showed moderate, but ultimately disappointing, efficacy.[5] SCFA-monosaccharides subsequently have been superseded by small molecule HDACi such as suberoylanilide hydroxamic acid (SAHA),[6] a drug that was FDA approved under the trade name Zolinza in 2006 for the treatment of cutaneous T cell lymphoma (CTCL), a form of non-Hodgkin's lymphoma.[7]

During the past decade, as the fortunes of SCFA-monosaccharides in cancer treatment waned, a subset of these molecules found new life in metabolic glycoengineering and, in the process, shed light on the shortcomings of the clinical tests. In early metabolic glycoengineering experiments, a technique that involves the introduction of non-natural monosaccharides into biosynthetic glycosylation pathways for the display of chemically modified, i.e., engineered, glycans on the cell surface,[2,8,9] acetate was linked to the sugar to increase membrane permeability.[3,4] As a consequence, monosaccharide concentrations required to elicit robust cellular responses dropped from the millimolar to the micromolar range.[4,10,11] Because of the success of this strategy with acetate, our group employed longer chain SCFA of increased lipophilicity to further increase membrane permeability and analogue uptake. Specifically, the relative abilities of acetate- (as Ac_4ManNAc), propionate- (as Pr_4ManNAc), and *n*-butyrate-modified (as Bu_4ManNAc) *N*-acetyl-D-mannosamine (ManNAc) to support flux into the sialic acid pathway progressively increased from 600- to 1800- to 2100-fold.[12]

Coincident with its favorable impact on metabolic flux into the sialic acid pathway, Bu_4ManNAc proved to be moderately toxic to cancer cells.[12] This observation, reminiscent of the use of Bu_5Man for cancer treatment,[5,13] raised the possibility that the new compound also could be exploited therapeutically. As a first step in evaluating whether Bu_4ManNAc was a superior drug candidate compared to Bu_5Man, we tested its ability to

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[a] Abbreviations: SCFA, short chain fatty acids; HDACi, histone deactylase inhibitor; MMP, matrix metalloproteinase; SAR, structure−activity relationships; MUC1, mucin 1, NF-κB, nuclear factor kappa B; CXCR4, chemokine (C-X-C motif) receptor 4.

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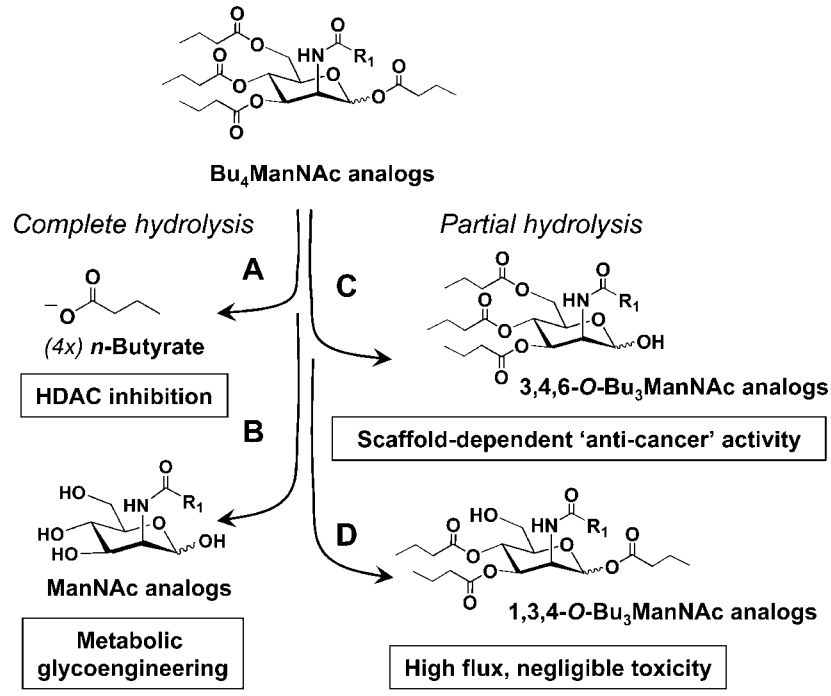

Figure 1. SCFA-ManNAc analogues have three modes of biological activity. The intracellular fate of short chain fatty acid (SCFA)-monosaccharide hybrid molecules are illustrated with *n*-butanoylated ManNAc analogues (R_1 modifications at the *N*-acyl position have been used extensively in metabolic glycoengineering; this paper focuses on the natural core sugar where R_1 is a methyl group as embodied in Bu_4ManNAc, compd **1** in Figure 2). In the past, complete hydrolysis of the analogue into the SCFA (in this case, *n*-butyrate (A)) and the carbohydrate (e.g., R_1-modified ManNAc (B)) moieties has been assumed to account for the biological activities of the parent molecule through HDACi-mediated chromatin remodeling and glycosylation, respectively. (C) Recently, we reported that partial hydrolysis products of peracylated hexosamines, exemplified by tributanoylated ManNAc analogues lacking the SCFA group at either the C1-OH or C6-OH position, have distinct biological activities that are not primarily dependent on either the HDACi activity of *n*-butyrate or on the impact of the core sugar on glycosylation.[15] In particular, C6-derivatized hexosamine analogues (such as compds **1**, **3**, and **5** in Figure 2) support unique scaffold-dependent anticancer responses. (D) By contrast, "inactive" analogues not based on the hexosamine scaffold or lacking an SCFA at the C6 position have negligible toxicity, do not suppress metastatic oncogenes and introduce high metabolic flux into glycosylation pathways.[15]

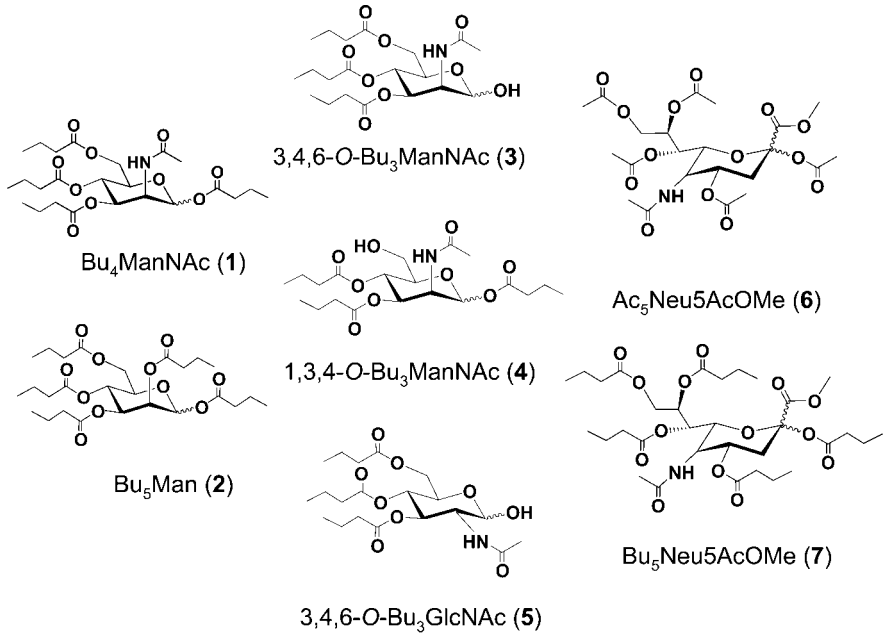

Figure 2. Chemical structures of SCFA-derivatized monosaccharide analogues used in this study.

inhibit cell growth and induce apoptosis in several cancer lines and found that it inhibited proliferation over a 3−5 day period consistent with the HDACi activity of *n*-butyrate.[14] Cells treated with Bu_5Man, by contrast, subsequently recovered and resumed robust cell growth (thereby offering an explanation for the only modest clinical efficacy of this compound), whereas cells treated with Bu_4ManNAc underwent apoptosis over a two week period.[14] The ability of Bu_4ManNAc to augment growth inhibition with apoptosis was a positive step in cancer drug design, but an even more encouraging development was the subsequent discovery that Bu_4ManNAc, unlike Bu_5Man, inhibited the pro-metastatic oncogene MUC1.[15] On the basis of its

prominent role in promoting metastatic breast cancer, MUC1 provides an attractive, but to date recalcitrant, therapeutic target. Because of the difficulty of modulating MUC1 with current small molecule drug candidates, new compounds such as Bu_4ManNAc that suppress this pro-invasive oncogene are worthy of investigation; accordingly, in the first experiments undertaken in this study, we tested whether Bu_4ManNAc could inhibit the mobility of metastatic breast cancer cells.

The favorable outcome of the initial experiments reported in this paper, where Bu_4ManNAc inhibited invasion in MDA-MB-231 cells (in contrast to Bu_5Man that had no impact on cell mobility), provided impetus to further probe the underlying mechanism for two reasons. First, mechanisms beyond MUC1 influence the invasive behavior of metastatic cells making it likely that additional factors were involved in modulating cell mobility. Second, despite the favorable features of Bu_4ManNAc (i.e., the induction of apoptosis and suppression of MUC1), its prospects in cancer drug development were clouded by potential pitfalls arising from its hydrolysis products, *n*-butyrate and ManNAc. For example, in some types of cancer, HDACi suppress proliferation while also activating NF-κB,[16] which leads cells to become drug resistant, refractory to apoptosis, or increasingly metastatic. Thus, *n*-butyrate generated by Bu_4ManNAc had the potential to negate the benefits of MUC1 suppression via NF-κB activation. Moreover, the pro-invasive predisposition of *n*-butyrate threatened to be exacerbated by ManNAc, the second hydrolysis product of Bu_4ManNAc, because ManNAc is the dedicated metabolic precursor for sialic acid biosynthesis, a sugar that alters the adhesive properties of cells and that has been associated with a highly metastatic phenotype in cancer.

Because of the potential pitfalls introduced by the hydrolysis products of Bu_4ManNAc, the anti-invasive properties of this compound, which ultimately proved to be an attribute of structural features of the molecular species with intact SCFA-hexosamine linkages (Figure 1), were evaluated throughout this study against the backdrop of cellular responses emanating from *n*-butyrate and ManNAc. The results reinforce the hypothesis that the hexosamine core structure can serve as a scaffold for pharmacophore development,[15,17] overturning the previously held premise that SCFA-carbohydrate molecules primarily function as prodrugs. Importantly, we extend the newfound "intact ester linkage" mode of activity from a single oncogene (MUC1) to additional therapeutic candidates (e.g., MMP-9, CXCR4, and NF-κB) implicated in metastasis and show that the molecular-level changes translate into altered cell level behavior with attributes that are attractive for confronting malignant disease.

Results and Discussion

Rationale for the General Experimental Design. The lead compound Bu_4ManNAc had both of the SAR features, an *N*-acyl substitution at the C2 position and an ester-linked SCFA group at the C6 position, hypothesized to provide the anticancer activities uniquely held by "active" SCFA-hexosamine analogues (Figure 1). For comparison purposes, Bu_5Man (compd **2**, Figure 2) was an attractive "negative" control for several reasons (note that other compounds shown in Figure 2 were also used as controls for specific experiments). First, being hexose-based, Bu_5Man did not have the critical *N*-acyl group at the C2 position required for critical anticancer activities, such as induction of apoptosis[14] or MUC1 suppression,[15] found in hexosamine-based analogues. Another important feature of Bu_5Man is that its core sugar mannose can be converted to 6-phosphofructose and preferentially shunted into energy production rather than used for maximizing metabolic flux into biosynthetic pathways. In this regard Bu_5Man differed significantly from Bu_4ManNAc, which generates ManNAc, a sugar dedicated to sialic acid biosynthesis. This point was important because, at the outset of these experiments, the relative contributions of the HDACi activity of *n*-butyrate, sugar-specific responses, and the scaffold-dependent activity were unclear; hence employing different core sugars helped clarify the importance of glycosylation. Finally, because Bu_5Man had already undergone clinical testing, it was an established benchmark for cancer therapy using SCFA-carbohydrate hybrid molecules. Although it is difficult to extrapolate from cell culture experiments to in vivo efficacy, the expectation was that Bu_4ManNAc needed to show clearly superior results in cell culture tests compared to Bu_5Man (which was only moderately successful in vivo) to warrant further drug development interest.

Bu_4ManNAc Selectively Inhibited Invasion. Previously, Bu_4ManNAc had been shown to suppress MUC1, whereas Bu_5Man enhanced levels of this pro-invasive oncogene in the highly metastatic MDA-MB-231 breast cancer line.[15] Because invasion is a multifactorial process, we were interested in testing whether the modulation of MUC1 by these analogues translated into corresponding changes to the invasive behavior of cells or whether the changes to this oncogene were masked by compensating or offsetting factors. In particular, because hydrolysis of Bu_4ManNAc results in the production of ManNAc that is subsequently converted to sialic acid, any benefit gained from MUC1 suppression could have been easily negated by the pro-invasive tendencies of this sugar. Therefore, the first objective of this study was to determine if Bu_4ManNAc reduced invasion more effectively than the MUC1-enhancing analogue Bu_5Man.

Accordingly, to test cell mobility, MDA-MB-231 cells that had been preincubated with Bu_4ManNAc and Bu_5Man was evaluated in modified Boyden chamber assays. Two variations of this assay were used to study either migration (measured by passage of cells through a porous membrane) or invasion (measured by penetration through Matrigel). Results obtained at the minimally cytostatic concentration of 50 μM showed a trend toward preferential inhibition of invasion compared to migration after two days of exposure to Bu_4ManNAc (Figure 3A). Increased Bu_4ManNAc concentrations almost completely inhibited invasion after two days (Figure 3B), and significant inhibition of both invasion and migration were seen after three days of exposure to 50 μM of this analogue (Figure 3C). By contrast, Bu_5Man did not change either end point at $\leq$125 μM (data are shown for 50 μM in Figure 3D).

Reduced Mobility of Bu_4ManNAc-Treated Cells Was Not Due to Cytotoxicity. Upon demonstrating the therapeutically useful end point of reduced invasion in Bu_4ManNAc-treated metastatic breast cancer cells, we next sought to understand the relative contributions of the recently discovered third mode of scaffold-dependent activity of this SCFA-hexosamine hybrid molecule (e.g., through MUC1 suppression) in comparison to the responses emanating from the hydrolysis products *n*-butyrate and ManNAc. But first we eliminated the trivial possibility that the toxicity of Bu_4ManNAc contributed substantially to reduced mobility of the analogue treated cells. To do so, we carefully avoided thresholds previously associated with cytotoxicity that included analogue concentration, cell density, and duration of analogue exposure. For example, over a 2 or 3 day period, Bu_4ManNAc approaching 500 μM is required for apoptosis;[12] therefore the current experiments were performed at less than a third of this level (i.e., at $\leq$150 μM).

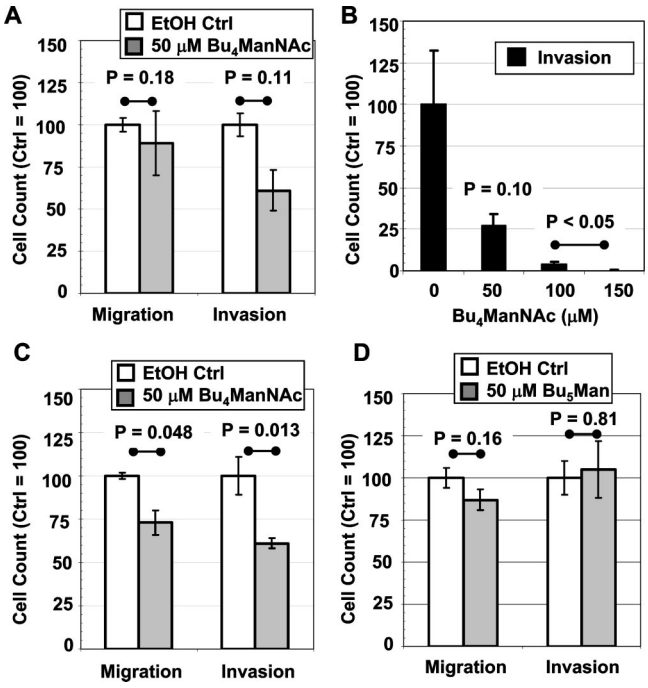

Figure 3. Effects of $Bu_4ManNAc$ and Bu_5Man on cell mobility. (A) Two day pretreatment with the minimally cytostatic concentration of 50 μM $Bu_4ManNAc$ did not have a statistically significant effect on migration or invasion in MDA-MB-231 cells. Higher concentrations (e.g., up to 150 μM (B)) or a longer incubation period (e.g., three days, (C)), however, did result in a preferential decrease in invasion compared to migration. (D) In similar experiments, cells treated with Bu_5Man did not experience a change in migration or invasion. Error bars indicate SEM for technical replicates ($n = 6$ for (A), (C), and (D) and $n = 3$ for B) using a double-sided t test.

A concentration of $\sim$ 150 μM can activate apoptosis and lead to cell death, but only on time scales approaching 2 weeks;[14] therefore in the current experiments, a maximum incubation period of 3 days was used. A third parameter, cell density, was also carefully controlled to avoid low seeding densities (e.g., 6.25×10^4 cells/ml[4]) associated with toxicity.

A series of assays verified that $Bu_4ManNAc$ had minimal cytotoxicity at the concentrations (i.e., $\leq$ 150 μM) used to evaluate mobility and associated genes. In these experiments, proliferation was slower in cells incubated with $Bu_4ManNAc$ than with Bu_5Man; however, an increase in cell number during incubation with the analogue indicated that the cells nonetheless retained viability and were actively growing (Figure 4). The small proportion of the cells that were dying, indicated with the "rounded up" morphology in Figure 4A (and evaluated more thoroughly as previously described by DNA fragmentation analysis,[12] PI/annexin staining,[18] and flow cytometry histogram analysis of DNA content and cell cycle status,[14] *data not shown*), were excluded from further analysis. As an additional safeguard to separate the effects of the analogues on cell mobility from toxicity, the WST-8 assay was employed to normalize the number of viable cells used for subsequent analysis (e.g., for loading into the migration or invasion chambers); in addition, aliquots of cells were kept separate from the invasion assays and reevaluated at end of the assay by the WST-8 method to ensure viability was maintained throughout the entire experiment. Further evidence that nonspecific toxicity did not explain the results presented in this paper came from microarray analysis previously done under similar conditions where >98% of the gene probe sets did not show changes in expression over analogue concentrations up to 125 μM.[14] Moreover, the genes that were influenced by the analogues were skewed toward up-regulation but, because these genes were not involved in the activation of apoptosis, these results were not consistent with the transcriptional programs expected during cell death. The robust sialic acid production in $Bu_4ManNAc$-treated cells (Figure 4D) was yet another indication that the cells were not dying (we have previously found that sialic acid production dramatically declines at analogue concentrations that lead to cell death[4]).

Reduced Invasion Cannot be Explained by *n*-Butyrate Generated by $Bu_4ManNAc$. Once toxicity was discounted as playing a significant role in the reduced mobility of $Bu_4ManNAc$-treated cells, we next investigated the relative impacts of respective hydrolysis products of this analogue, specifically, *n*-butyrate and ManNAc. We had previously extensively investigated *n*-butyrate in other cancer lines, both as the sodium salt and ester-linked to sugars, and found that while the expected HDACi activity was manifest in changes to histone acetylation, ancillary chromatin remodeling did not provide a convincing explanation for the divergent activities of Bu_5Man and $Bu_4ManNAc$ on apoptosis,[14] MUC1,[15] or on cell mobility (as described in this paper for the MDA-MB-231 line). This point is illustrated by the data shown in Figure 5 for MDA-MB-231 cells, where modest changes to histone H3 acetylation occurred (panels A and B) that were reflected in the expected up-regulation of cell cycle checkpoint protein p21 (panel C). Despite the "hallmark" HDACi activity exhibited by both *n*-butanoylated analogues, the overall transcriptional changes elicited by $Bu_4ManNAc$ and Bu_5Man differed dramatically. The different effects of the analogues were evident in previous microarray analysis where each analogue regulated a completely different set of genes[14] and in this study where MUC1 (e.g., see Figure 5D) was up-regulated by one analogue (i.e., by Bu_5Man) and down-regulated by the other (i.e., by $Bu_4ManNAc$). These divergent responses indicated that HDACi activity played only a partial role in regulating genes involved in cell mobility because, if *n*-butyrate generated from $Bu_4ManNAc$ and Bu_5Man hydrolysis played a substantial role, each compound should have led to similar responses.

Responses to $Bu_4ManNAc$ are Not a Result of Combined *n*-Butyrate and ManNAc Activity. Having ruled out substantial contributions from either *n*-butyrate or ManNAc in the anti-invasive activities of $Bu_4ManNAc$, the possibility remained that the divergent transcriptional activities of this compound compared to Bu_5Man resulted from a *combination* of HDACi responses and glycosylation effects derived from *n*-butyrate and the sugar acting as separate molecules. This possibility was difficult to rigorously discount because the simplest definitive experiment, the simultaneous addition of the appropriate equimolar quantities of *n*-butyrate and ManNAc to the culture medium, was not conclusive due to the poor cellular uptake of these compounds as individual molecules (as described previously[14]). Therefore a different approach was taken in this study based on the premise that, if sialic acid produced from $Bu_4ManNAc$ contributed to the anticancer activity of this compound, peracylated sialic acid analogues should be highly efficient at eliciting these responses because they directly produce sialic acid (and *n*-butyrate) upon ester hydrolysis. Accordingly, perbutanoylated Neu5Ac (as the C1-methyl ester $Bu_5Neu5AcOMe$, **7**) was synthesized and tested in comparison with peracetylated Neu5Ac (as the C1-methyl ester $Ac_5Neu5AcOMe$, **6**). The results of proliferation assays (shown in Figure 6A,B) followed the patterns associated with "inactive"

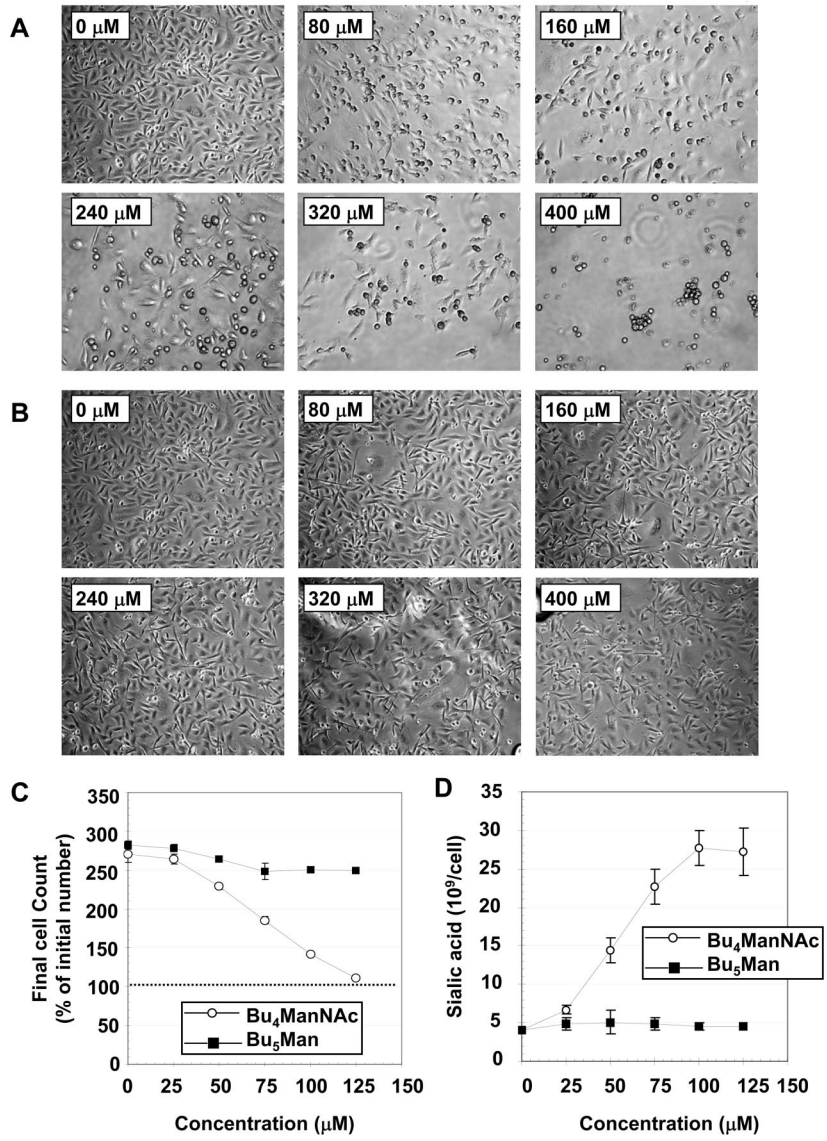

Figure 4. Effects of $Bu_4ManNAc$ and Bu_5Man on cell viability, growth inhibition, and sialic acid production in MDA-MB-231 cells. Phase contrast micrographs of cells incubated with $Bu_4ManNAc$ (A) and Bu_5Man (B) for 3 days show the growth inhibitory properties of these analogues and the "rounded-up" morphology indicative of dying cells. (C) Quantitatively, $Bu_4ManNAc$ inhibited proliferation more effectively than Bu_5Man; at concentrations used in the subsequent experiments, however, the cells remained viable and continued to proliferate (cell counts were determined by trypsinization of viable cells followed by Coulter counting analysis). (D) Sialic acid levels increased in $Bu_4ManNAc$-treated MDA-MB-231 cells in a dose-dependent manner while Bu_5Man did not change levels of this sugar. Error bars indicate standard error of the mean (SEM) and in each case $n \geq 3$.

hybrid analogues such as Bu_5Man,[14] where only HDACi behavior indicative of *n*-butyrate was manifest. Specifically, $Ac_5Neu5AcOMe$, which delivered the weakly acting SCFA acetate, had no measurable effect on proliferation (Panel A), whereas $Bu_5Neu5AcOMe$, which delivered the stronger acting SCFA *n*-butyrate, inhibited proliferation over 3−5 days, after which time the cells recovered and resumed robust growth (Panel B).

The impact of $Bu_5Neu5AcOMe$ on proliferation closely mimicked that of Bu_5Man (as previously reported[14]). In more detailed analysis, this compound also strongly increased MUC1 mRNA levels (Figure 6C), a distinctive feature of Bu_5Man that was consistent with reports that *n*-butyrate can up-regulate mucin transcription.[19,20] Finally, cells treated with $Bu_5Neu5AcOMe$ experienced moderate enhancement of NFKB1 (NF-κB p105) and down-regulation of CXCR-4 (Figure 6C), which are two additional distinguishing features of "inactive" analogues (the significance of which is discussed in more detail later in this report). These results established that sialic acid did not synergistically combine with *n*-butyrate to modulate the pro-invasive oncogenes under investigation. They also indicated that anticancer responses held by $Bu_4ManNAc$ cannot be reproduced when *n*-butyrate is appended to sialic acid, thereby strengthening the unique ability of the hexosamine core structure to support scaffold-dependent responses by eliminating another monosaccharide (i.e., sialic acid in addition to hexoses and glycerol that were previously discounted[5,14]) from having the critical SAR required for this new mode of bioactivity.

In a further experiment that conclusively ruled out a major role for hydrolysis products in mediating the anti-invasive effects of $Bu_4ManNAc$, the impact of 3,4,6-*O*-$Bu_3ManNAc$, 1,3,4-*O*-$Bu_3ManNAc$, and 3,4,6-*O*-$Bu_3GlcNAc$, (compounds **3**, **4**, and **5** in Figure 2) on MUC1 expression were compared (Figure 7A). These three analogues generate similar molar equivalents of *n*-butyrate, but 3,4,6-*O*-$Bu_3ManNAc$ and 1,3,4-*O*-$Bu_3ManNAc$, two compounds that support similar levels of sialic

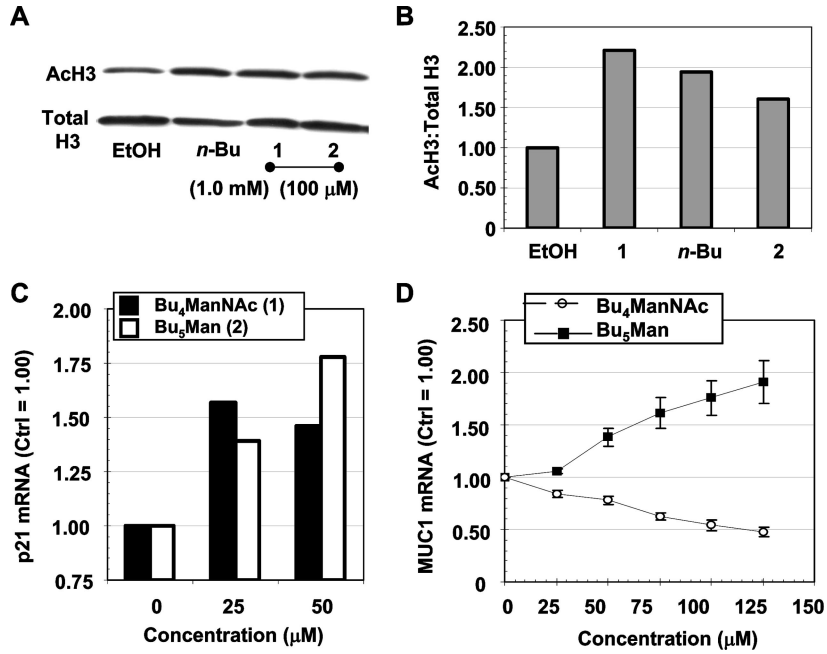

Figure 5. $Bu_4ManNAc$ and Bu_5Man have characteristic HDACi activity. (A) Western blots showed that the acetylation of histone H3 increased in MDA-MB-231 cells exposed to either $Bu_4ManNAc$, Bu_5Man, or to *n*-butryate for 3 days, as quantified in (B) by densitometry. (C) Measurement of mRNA levels by qRT-PCR showed that p21 was up-regulated by both analogues. (D) $Bu_4ManNAc$ decreased MUC1 mRNA levels while Bu_5Man had the opposite effect on this gene. In (A−B), representative data from one of three independent experiments are shown. In (C), p21 data consistent with previous testing[14] are shown, while errors bars represent SEM and $n \geq 3$ for (D).

acid production (Figure 7B), had opposing effects on MUC1 transcription, thereby unambiguously establishing that a simple combination of *n*-butyrate and ManNAc does not suppress this oncogene. By contrast, 3,4,6-*O*-$Bu_3GlcNAc$, a compound that does not generate ManNAc or by extension sialic acid, down-regulated MUC1 as effectively as 3,4,6-*O*-$Bu_3ManNAc$; together these results eliminated the possibility that the core sugar as a separate molecule (i.e., hydrolysis product) tunes the HDACi activity of *n*-butyrate in a manner that could account for the impact of $Bu_4ManNAc$ on MUC1 or invasion.

Having concluded that the distinctive transcriptional responses to $Bu_4ManNAc$ and Bu_5Man could not be explained by the HDACi activity of *n*-butyrate, either alone or in combination with the core sugar, the formal possibility remained that the core sugar contributed to the divergent invasive behavior of $Bu_4ManNAc$ and Bu_5Man strictly through changes to the cell surface glycosylation patterns. More specifically, the altered mobility of $Bu_4ManNAc$-treated cells could have been a consequence of robust sialic acid production (Figure 4D) that changed their adhesive properties. Consequently, to test whether ManNAc contributed to the reduced mobility of $Bu_4ManNAc$-treated cells in the absence of the HDACi influence of *n*-butyrate, cells were incubated with sufficient ManNAc (up to 50 mM) needed to replicate the levels of sialic acid (Figure 8A) produced in cells treated with the *n*-butanoylated analogues. Subsequent testing of ManNAc-treated cells in mobility assays revealed that there was a negligible impact on migration (data not shown), while there was a biphasic response in the invasion assays with a spike in activity at ∼10 mM that diminished at higher levels (Figure 8B). In light of this pro-invasive response to ManNAc, the ability of $Bu_4ManNAc$ to inhibit cell mobility in the face of similar levels of sialic acid production (as shown in Figure 4) was all the more remarkable and highlighted the need for broadening the scope of the investigation to fully appreciate the anti-invasive activity of this compound.

$Bu_4ManNAc$ and Bu_5Man Both Reduced MMP-9 Activity in Zymogram Assays. The molecular basis for the reduced invasion observed in $Bu_4ManNAc$ treated cells was investigated in more detail by using various assays (described in more detail below) that also provided insight into the lack of a functional response seen in Bu_5Man-treated cells. First, the activities of matrix metalloproteinases (MMPs), important players in extracellular matrix degradation during metastasis,[21] were tested at both the functional and gene expression levels. In the initial experiments, MMP activity was monitored in conditioned medium and the involvement of the majority of MMP family members was ruled out by a lack of bands observed on casein gels (data not shown). By contrast, by monitoring gelatinase activity, which is only characteristic of two MMPs (MMP-2 and MMP-9), a band was identified that was ∼92 kDa (Figure 9A) that corresponded to the mass of MMP-9 (MMP-2, which is ∼72 kDa, was not observed). Interestingly, unlike for MUC1 where the analogues had divergent effects, both $Bu_4ManNAc$ and Bu_5Man reduced the activity of MMP-9 harvested from conditioned medium (Figure 9A,B). Taken together, the results for MUC1 and MMP-9 provide a plausible explanation for the impact of these analogues on invasion at the cell level. Specifically, $Bu_4ManNAc$ suppressed both pro-invasive proteins, which was consistent with the decreased invasion observed at the cell level. By contrast, Bu_5Man up-regulated MUC1 while down-regulating MMP-9 activity, making it plausible that the two responses offset each other accounting for the unchanged cell mobility.

MMP-9 Activity was Correlated with CXCR-4 Transcription Rather Than with Pro-MMP-9 mRNA Levels. Because of enticing therapeutic possibilities inherent in the ability of $Bu_4ManNAc$ and Bu_5Man to reduce MMP-9 activity, we sought to understand the molecular basis of this finding more thoroughly. One explanation was that the HDACi activity of these analogues (see Figure 5) reduced pro-MMP-9 transcription (although HDACi usually activate gene expression,

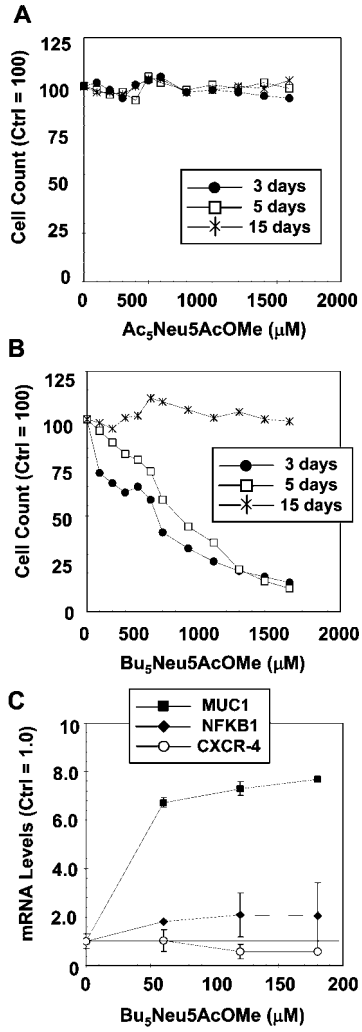

Figure 6. SCFA-sialic acid analogues follow the pattern set by "inactive" hexosamine analogues. (A) MDA-MB-231 cells exposed to Ac_5Neu5AcOMe did not experience growth inhibition at any time point, whereas (B) the proliferation of cells incubated with Bu_5Neu5AcOMe was inhibited over the first 3−5 days. The cells subsequently recovered and resumed robust growth by the 15th day. (C) Transcription patterns of MUC1, CXCR-4, and NFKB1 in Bu_5Neu5AcOMe treated MDA-MB-231 cells follow the pattern set by the "inactive" compound Bu_5Man. The data shown in (A) and (B) are averaged from triplicate experiments and in (C) from two independent experiments, each measured by qRT-PCR in quadruplicate. Error bars were omitted for clarity, in all cases SEM was ≤5%.

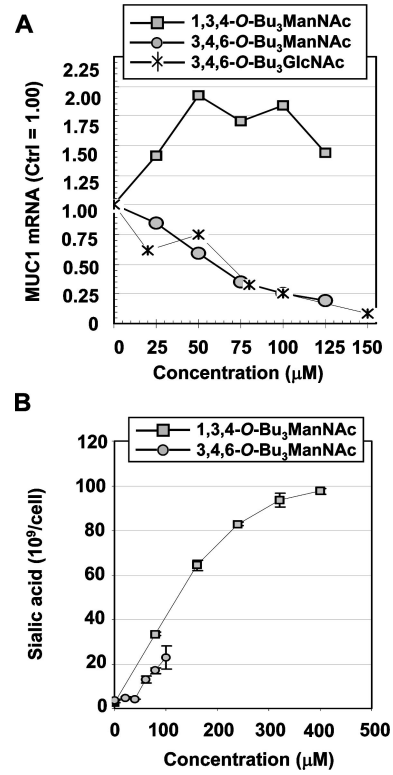

Figure 7. SAR implicated analogues with intact SCFA-hexosamine structures as prerequisite for "active" responses. (A) The C6-OH ManNAc analogue 1,3,4-*O*-Bu_3ManNAc failed to suppress MUC1 transcription unlike the two C6-SCFA derivatized analogues 3,4,6-*O*-Bu_3ManNAc and 3,4,6-*O*-Bu_3GlcNAc. Representative data from one of three independent experiments are shown; error based on the coefficient of variance (CV) for cycle number of quadruplicate replicates was ≤2% (error bars were obscured by the symbols and therefore omitted from the graph). (B) Both 1,3,4-*O*-Bu_3ManNAc and 3,4,6-*O*-Bu_3ManNAc supported robust sialic acid production at low concentrations (production from 3,4,6-*O*-Bu_3ManNAc was inhibited by the toxicity of this analogue at ≥150 μM), establishing further that the hydrolyzed core sugar was not responsible for MUC1 suppression. Error bars represent SEM, $n = 3$.

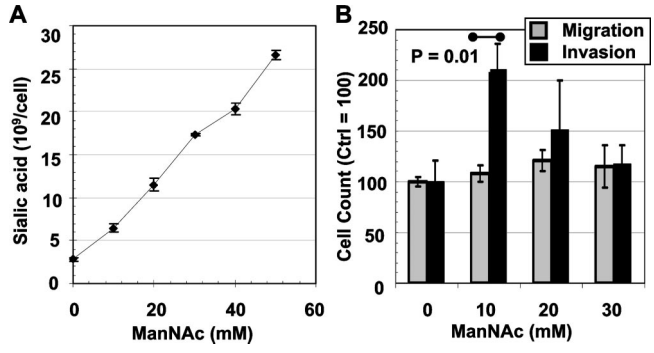

Figure 8. The suppression of invasion by Bu_4ManNAc cannot be explained by sialic acid production. (A) Millimolar concentrations of ManNAc increased intracellular sialic acid in MDA-MB-231 cells to levels comparable to cells treated with ≤125 μM of Bu_4ManNAc (see Figure 4D or 1,3,4-*O*-Bu_3ManNAc and 3,4,6-*O*-Bu_3ManNAc, in Figure 7B). (B) ManNAc increased invasion in MDA-MB-231 cells incubated with low millimolar concentrations (e.g., at 10 mM shown) of this sugar, but this potentiation was lost at higher concentrations. Errors bars represent SEM and $n \geq 3$ for each data set shown.

they are also capable of down-regulating transcription[22,23]). Measuring mRNA levels by qRT-PCR, however, revealed that Bu_4ManNAc had no impact on pro-MMP-9 transcription while Bu_5Man actually increased mRNA levels (Figure 9C). Thus, needing to probe deeper to explain the reduced MMP-9 activity observed in the zymograms, we tested the expression of CXCR4. This protein, an established therapeutic target for HIV as well as cancer,[24] regulates the secretion and activation of pro-MMP-9.[25] qRT-PCR revealed that CXCR4 mRNA levels were down-regulated by both Bu_4ManNAc and Bu_5Man (Figure 9D). Because CXCR4 plays a critical role in the secretion and activation of pro-MMP-9,[26] its down-regulation by both analogues provided a compelling mechanism to explain the reduced MMP-9 activity observed in culture medium of cells incubated with either compound.

Transcriptional Control of Genes Regulated by Bu_4ManNAc Involves NF-κB. The evaluation of MMP-9 and CXCR4, important therapeutic targets implicated in invasion, complemented the MUC1 data and helped explain the anti-invasive attributes of Bu_4ManNAc and the lack of a cell level response to Bu_5Man. These findings, however, left the important issue of how the analogues engaged the underling mechanisms that

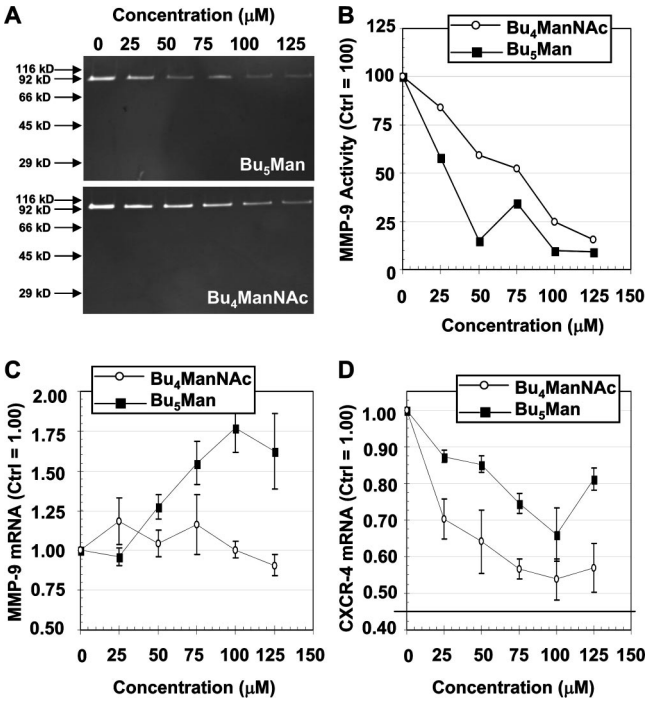

Figure 9. Control of MMP-9 activity in MDA-MB-231 cells by $Bu_4ManNAc$ and Bu_5Man. (A) Zymographic analysis of medium conditioned by MDA-MB-231 cells incubated with $Bu_4ManNAc$ and Bu_5Man for 3 days showed a dose-dependent decrease in MMP-9 gelatinase activity for both compounds (bands from a representative experiment were quantified by densitometry and shown in (B). Similar results were obtained in two additional independent experiments. qRT-PCR analysis of pro-MMP-9 (C) and CXCR4 (D) mRNA levels in cells exposed to $Bu_4ManNAc$ and Bu_5Man for 3 days. Error bars indicate SEM for independent experiments done in triplicate.

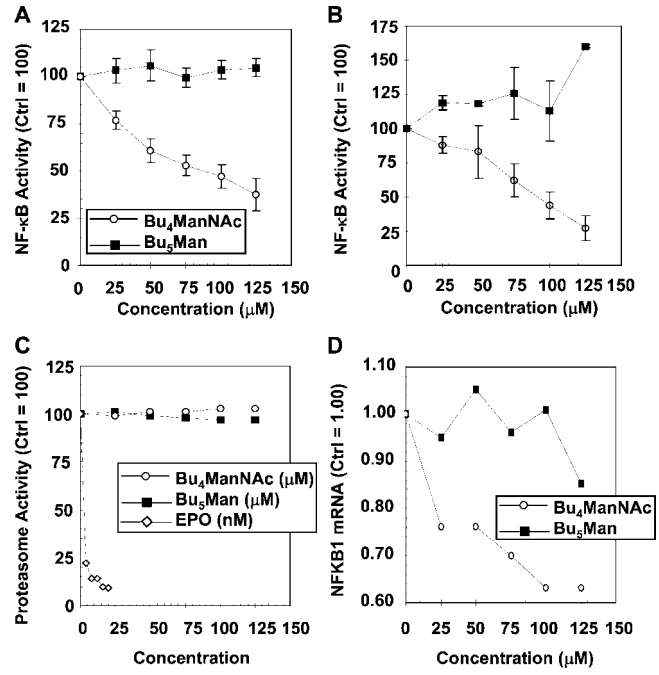

Figure 10. Analysis of NF-κB in HEK AD293 and MDA-MB-231 cells treated with $Bu_4ManNAc$, Bu_5Man, and controls. (A) HEK AD293 cells that were transfected with reporter gene plasmids and then incubated with either $Bu_4ManNAc$ or Bu_5Man; Bu_5Man had no effect on NF-κB activity in the reporter gene assay, while $Bu_4ManNAc$ provided substantial inhibition in this cell line. (B) Similar results were observed for $Bu_4ManNAc$ and Bu_5Man in the reporter gene assay in electroporated MDA-MB-231 cells. Error bars for the transcription factor reporter assays in (A) and (B) represent SEM for independent experiments done in triplicate. (C) Incubation with either analogue for 3 days did not alter proteasome activity in MDA-MB-231 cells, whereas a 6 h treatment with epoxomicin produced potent inhibition; SEM was $\leq$4% for all data points (and error bars were not included because they were generally smaller than the data symbols). (D) Endogenous NFKB1 (p105) mRNA levels were reduced by $Bu_4ManNAc$ but not by Bu_5Man in MDA-MB-231 cells; error calculated based on the coefficient of variance (CV) for cycle number of quadruplicate replicates was $\leq$2% (not shown).

controlled gene expression largely unresolved. Certain genes (e.g., p21 involved in cell cycle arrest and CXCR4 implicated in the control of MMP-9 gelatinase activity) were regulated in a manner consistent with HDACi activity of *n*-butyrate generated from the hydrolysis of the parent compound. By contrast, HDACi could not explain the impact of these compounds on other genes, exemplified by MUC1, that were regulated in a dramatically different fashion by each compound. Clearly HDACi, or most likely *any* single regulatory mechanism by itself, did not adequately explain the impact of $Bu_4ManNAc$ on gene expression.

Accordingly, we considered alternative possibilities to account for the discordant effects of these analogues on gene regulation and the NF-κB family of transcription factors that mediate inflammation in normal cells and, among other functions in cancer, help orchestrate invasion garnered interest for two reasons. First, depending on the type of malignancy, HDACi can activate NF-κB and offset anticancer benefits;[16] at a minimum, we wanted to eliminate this possibility for our analogues. Second, and of specific relevance to this study, MUC1 contains an NF-κB binding domain in its promoter region[27,28] and conversely, MUC1 can modulate constitutive NF-κB signaling found in cancer cells.[27] This crosstalk led us to speculate that the link between reduced invasion (Figure 3B,C) and the transcription of MUC1 (Figure 5D) in cells incubated with $Bu_4ManNAc$ plausibly involved NF-κB.

NF-κB activity in cells exposed to $Bu_4ManNAc$ or Bu_5Man was evaluated in two ways. First, a reporter gene assay was used to measure transcriptional activity driven by an NF-κB consensus sequence upstream of the luciferase gene. In this assay, a luciferase reporter plasmid was cotransfected with a second plasmid encoding β-galactosidase driven by an SV promoter and relative differences in luciferase to β-galactosidase signaled changes in NF-κB transcriptional activity. The MDA-MB-231 line used throughout this study proved difficult to transfect by standard lipofectamine protocols; consequently, these assays were first performed in the human HEK AD293 line where transfection efficiencies of >90% could be obtained routinely. In these cells, the NF-κB activator TPA and inhibitor EPO had the expected effects (see Figure S1 in the Supporting Information), thereby validating the assay in our hands; it is noteworthy that a relatively high level of NF-κB transcriptional activity was found in unstimulated cells, likely reflecting high basal levels of NF-κB activity associated with the cancerous phenotype.[27] In subsequent analogue testing, $Bu_4ManNAc$ decreased NF-κB transcriptional activity in a dose-dependent manner, while Bu_5Man had negligible effect (Figure 10A).

Having found that NF-κB activity in analogue-treated HEK cells reflected the impact of $Bu_4ManNAc$ and Bu_5Man on invasion in MDA-MB-231 cells (i.e., $Bu_4ManNAc$ was inhibitory and Bu_5Man hade no effect), we next tested NF-κB activity in the breast cancer line by using electroporation (which was more effective than lipid-based transfection in MDA-MB-231 cells). The reporter assay showed similar results in the MDA-MB-231 cells (Figure 10B) as obtained in the HEK cells (Figure

10A) with the exception that Bu_5Man showed a trend toward being slightly activating (instead of having an negligible impact on activity). In both lines, however, it was unambiguous that $Bu_4ManNAc$ dramatically inhibited NF-κB in a dose-dependent manner, resulting in a ~75% reduction at 125 μM. It is worth noting that because NF-κB activity was normalized to an internal control (a cotransfected plasmid with the β-galactosidase gene driven by an SV promoter), the observed decrease in pathway activity was not an artifact of nonspecific suppression of transcription or translation due to nonspecific cytotoxicity.

$Bu_4ManNAc$ Inhibits the Endogeneous NF-κB Pathway. Having demonstrated that $Bu_4ManNAc$ inhibited NF-κB activity in reporter gene assays, we next verified that this compound had a similar impact on the endogenous NF-κB pathway. Reduction of proteasome activity, a common but by no means ubiquitous characteristic of small molecule NF-κb inhibitors, was tested as a potential mechanism underlying the suppressive effects of $Bu_4ManNAc$ on NF-κB. The proteasome inhibitor epoxomicin (EPO), which blocks degradation of ubiquinated IκB by sequestering NF-κB in the cytoplasm, was used as a positive control in this assay; unlike this compound, however, neither $Bu_4ManNAc$ nor Bu_5Man altered the degradation of a luminescent proteasomal substrate (Figure 10C). Reduced NF-κB signaling in the context of normal proteasome function suggested $Bu_4ManNAc$ inhibited this pathway by regulating the transcription of NF-κB proteins themselves. Therefore, as a second method to assess the impact of the analogues on NF-κB activity, we used qRT-PCR to monitor p105 (NFKB1) mRNA levels. This gene encodes a subunit of the NF-κB transcription complex (cleavage of p105 yields p50, one of five subunits that assemble to form NF-κB) and is itself an NF-κB target gene.[29]

Consistent with the reporter plasmid assays, $Bu_4ManNAc$ suppressed NFKB1 transcription while Bu_5Man did not significantly alter its expression (Figure 10D), indicating that this analogue directly altered, or interacted with, NF-κB proteins rather than affecting this signaling pathway by a peripheral mechanism such as proteasome activity. It is noteworthy that current NF-κB inhibitors, while often proficient at knocking down transiently elevated levels of NF-κB resulting from pathway activating factors such TNFα, fail to reduce the elevated basal NF-κB activity characteristic of certain types of cancer. In one study, even small inhibitory RNAs targeted at NF-κB proteins did not substantially inhibit the elevated basal activity of NF-κB in MDA-MD-231 cells;[30] consequently, the discovery that $Bu_4ManNAc$ inhibited endogenous NF-κB represents a potentially important therapeutic advance.

Anticancer SAR of SCFA-hexosamine Analogues Extend to NF-κB. The close correlation between reduced invasion and NF-κB in $Bu_4ManNAc$-treated cells led us to investigate whether the anticancer SAR previously observed for apoptosis[14] or MUC1[15] extended to NF-κB. Specifically, we tested whether NF-κB was governed by the same controlling mechanism as the other attractive anticancer attributes of $Bu_4ManNAc$ (i.e., MUC1 suppression and apoptosis), where both an *N*-acyl group at the C2 position and a SCFA substituent at the C6 position were critical for activity (see Figures 11 and 12), by comparing 3,4,6-*O*-$Bu_3ManNAc$, 1,3,4-*O*-$Bu_3ManNAc$, and 3,4,6-*O*-$Bu_3GlcNAc$, (compds **3**, **4**, and **5** in Figure 2). This experiment verified the close link between the SAR responsible for suppression of MUC1 and reduction of NF-κB activity as the two C1-OH analogues (**3** and **5**) once again showed almost identical inhibitory responses (Figure 11) despite having core sugars that, as hydrolysis products, engaged different glycosylation pathways. The fact that the C6-OH analogue **4** showed

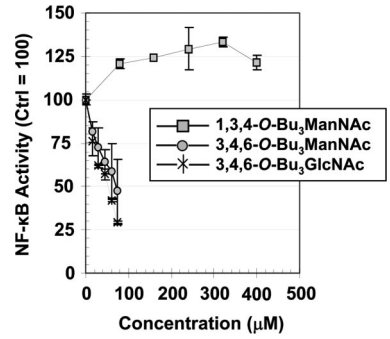

Figure 11. SAR of tributanoylated hexosamine analogues extend to NF-κB. The effects of three tributanoylated hexosamine derivatives 1,3,4-*O*-$Bu_3ManNAc$ (**3**), 3,4,6-*O*-$Bu_3ManNAc$ (**4**), and 3,4,6-*O*-$Bu_3GlcNAc$ (**5**) on NF-κB activity was measured by using the luciferase reporter assay in HEK AD293 cells exposed to each of the analogues. Error bars indicate SEM of independent experiments measured in triplicate.

no inhibition of NF-κB (slight activation, in fact) while based on a ManNAc core identical to **3** emphasized that the SAR of the intact pharmacophore dominated any latent effects emanating from the hydrolysis products. Another significant ramification of these SAR is that simultaneous suppression of MUC1 and NF-κB can be obtained with the GlcNAc scaffold, thereby avoiding the potentially deleterious impact of sialic acid derived from a ManNAc core on cancer progression. In other cases, such as in the use of non-natural ManNAc analogues (i.e., with R_1 in Figure 1 not a methyl group) to install immunogenic sialic acids in tumor-associated carbohydrate antigens for cancer vaccine development,[31,32] a ManNAc core may ultimately be advantageous for cancer therapy. Either way, the results described in this paper lay the foundation for a highly versatile platform that exploits the emergence of carbohydrates as scaffolds for drug discovery.[33]

Conclusions

Revisiting the therapeutic prospects of SCFA-monosaccharide hybrid molecules was spurred by the discovery of therapeutically relevant activities not derived from, as had previously been presumed, the hydrolysis products of these compounds but instead from structural features of the intact prodrug (see Figure 1 and references[15,17]). In particular, three specific factors provided renewed interest in this class of drug candidates. First, there is a growing appreciation that therapeutic compounds that contain ester linkages, long exemplified by aspirin, are not exclusively prodrugs; instead, the parent molecule can have important biologic activity.[17] Second, recent SAR have revealed critical differences between the SCFA-carbohydrates previously evaluated in clinical trials with only moderate success and the current hexosamine-based compounds. Specifically, an *N*-acyl group at the C2 position of a 6-carbon sugar is critical for anticancer activity, and it is this C2 *N*-acyl group, found in hexosamines but not in carbohydrates previously used for *n*-butyrate delivery such as glycerol, glucose, or mannose, that distinguishes the current report from earlier efforts. Finally, the identification of important therapeutic targets involved in invasion, including MUC1, MMP-9, CXCR4, and NF-κB, suppressed by the lead compound $Bu_4ManNAc$, provides a new direction for drug discovery by expanding efforts previously directed at killing cancer cells to now also reducing their metastatic potential.

The first important experimental result described in this paper, verification that the lead compound $Bu_4ManNAc$ inhibited the

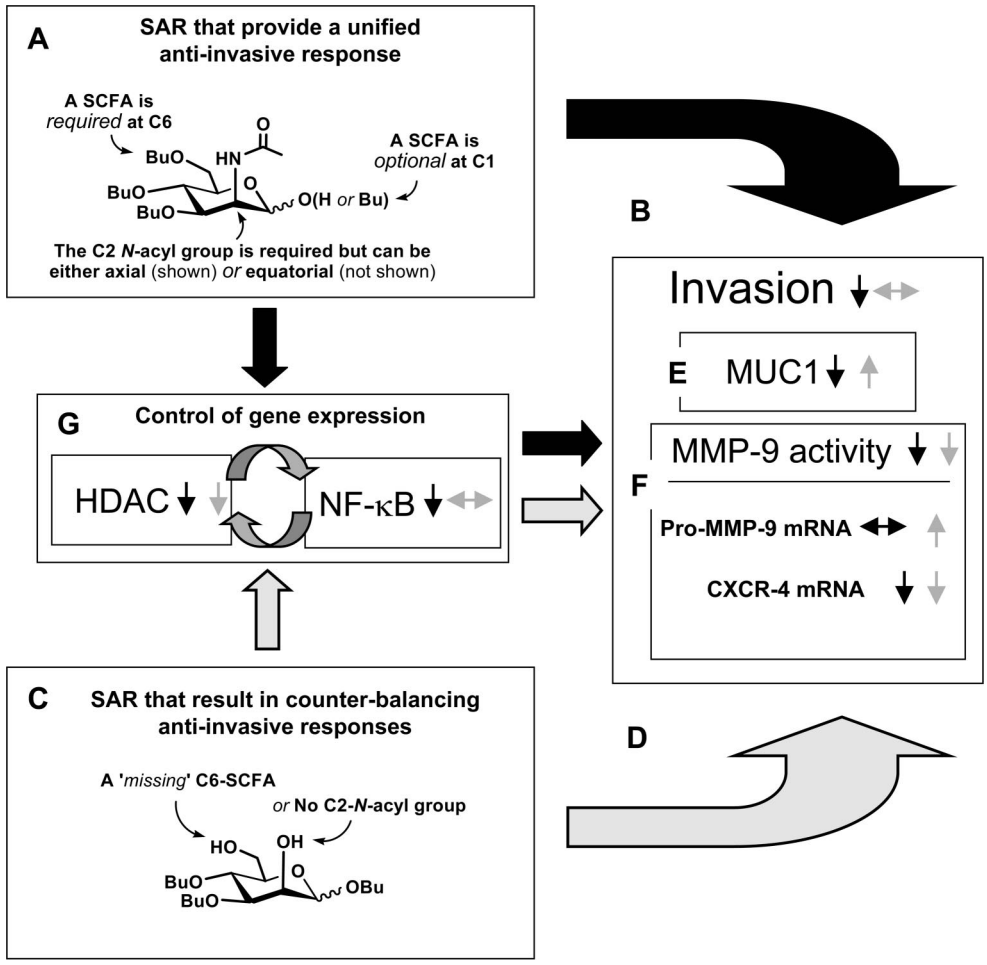

Figure 12. Summary of the anti-invasive effects of MDA-MB-231 cells to Bu_4ManNAc and the muted response to Bu_5Man. (A) Structure−activity relationships (SAR) are summarized that are required for a unified molecular level response that reduces invasion through suppression of MUC1 and MMP-9 activity ((B), and dark arrows in (E) and (F)). By contrast, the SAR shown in (C) (bottom) resulted in offsetting molecular responses ((D), and gray arrows in (E) and (F)) that did not change the invasive behavior of cells. (E) The ability of Bu_4ManNAc to inhibit invasion was a consequence of coordinated down-regulation of MUC1 and (F) MMP-9 activity; in turn, MMP-9 activity was the aggregate result of the dominance of CXCR-4 transcription over pro-MMP-9 mRNA expression. (G) A working hypothesis is that influence of Bu_4ManNAc and Bu_5Man on invasion ultimately is a matter of balance between the differing impacts of these analogues on HDAC and NF-κB.

invasive behavior of metastatic breast cancer cells, established a new therapeutic end point, control of pro-invasive oncogenes, for SCFA-hexosamines. In addition, data presented in this paper strongly confirmed the hypothesis that the unique biological activity of this class of molecules is derived from intact ester-linkages found in the parent molecules instead of merely resulting from the hydrolysis products of the prodrug. Importantly for the control of pro-invasive oncogenes, this third mode of scaffold-dependent activity dominated the potentially pro-invasive attributes of the hydrolysis products *n*-butyrate and ManNAc as well as illuminated shortcomings in previous strategies that used broadly comparable molecules (i.e., SCFA-carbohydrates in general) in cancer therapy: namely that the wrong core sugars were utilized as delivery vehicles. Overall, combined with the specific biological attributes discussed below and the growing interest in carbohydate-based drug candidates,[33] this work established SCFA-hexosamines as an attractive class of molecules for the continuing development of antimetastatic agents.

Although the impetus for this study originated in the ability of Bu_4ManNAc to suppress MUC1, we found that this compound's capacity to influence invasive behavior at the cell level was critically dependent on suppression of additional therapeutic targets including MMP-9 (via indirect regulation through CXCR4) and NF-κB. Significantly, neither of two important molecular players that regulate invasive behavior, MUC1 and MMP-9, could by themselves explain the cell-level effects of Bu_4ManNAc and Bu_5Man on mobility. Instead, the active compound Bu_4ManNAc evoked a unified molecular-level response that led to reduced invasion whereas the "inactive" analogue Bu_5Man elicited counteracting molecular level responses that resulted in no change in cellular behavior (as outlined in Figure 12).

The finding that Bu_4ManNAc did not trigger the counterproductive relationship between HDACi and NF-κB found in some cancer drug candidates invokes the principle described by Minucci and Pelicci[34] that epigenetic factors maintain a "matter of balance" between genes that simultaneously promote malignancy and those that suppress cancer progression. When this balance is thrown out of kilter, as manifest in the highly invasive phenotype of metastatic MDA-MB-231 cells, it can be extremely difficult to remedy this aberrant behavior with a single therapeutic agent. This point is illustrated by unchanged mobility of cells treated with Bu_5Man, or other "inactive" analogues, that modulate chromatin structure via the HDACi activity of their *n*-butyrate groups. In essence, cancers cells exhibit an altered, but multifaceted, equilibrium that is difficult to get "back in balance" and was a feat that could only be achieved through

the combined HDACi activity and NF-κB suppression held by active analogues such as Bu_4ManNAc. Ultimately, the emerging strategy of coupling HDACi activity with NF-κB inhibition in a single molecule, described in this paper for Bu_4ManNAc (and other analogues with the appropriate SAR shown in Figure 12A), promises to be an important step in intensifying efforts to develop epigenetic therapies for invasive breast cancer.

Experimental Procedures

Synthesis of SCFA-monosaccharide Hybrid Molecules. The structures of the *n*-butyrate monosaccharide hybrid molecules used in this study are shown in Figure 2. These compounds, 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-butanoyl-α,β-D-mannopyranose (Bu_4ManNAc, **1**[12]), 1,2,3,4,6-penta-*O*-butanoyl-α,β-D-mannopyranose (Bu_5Man, **2**[14]), 2-acetamido-3,4,6-tri-*O*-butanoyl-2-deoxy-α,β-D-mannopyranose (3,4,6-*O*-Bu_3ManNAc, **3**[15]), 2-acetamido-1,3,4-tri-*O*-butanoyl-2-deoxy-α,β-D-mannopyranose (1,3,4-*O*-Bu_3ManNAc, **4**[15]), 2-acetamido-3,4,6-tri-*O*-butanoyl-2-deoxy-α,β-D-glucopyranose (3,4,6-*O*-Bu_3GlcNAc, **5**[15]), and 5-actetamido-2,4,7,8,9-penta-*O*-acetyl-3,5-dideoxy-1-methyl ester-D-glycero-D-galacto-2-nonulopyranosonic acid (Ac_5Neu5AcOMe, **6**[35]) were synthesized, purified, and characterized as described in the references provided. The previously unreported compound 5-actetamido-2,4,7,8,9-penta-*O*-butanoyl-3,5-dideoxy-1-methyl ester-D-glycero-D-galacto-2-nonulopyranosonic acid (Bu_5Neu5AcOMe, **7**) was synthesized and characterized as described in the Supporting Information. For long-term storage, compounds were maintained at −20 °C after lyophilization and stock solutions used for cell culture assays were made periodically as needed by dissolving the analogues in ethyl alcohol (EtOH) and storing at 4 °C; in both cases, compounds were stable over several months of storage.

Cell Culture. Cell lines were obtained from the ATCC (Manassas, VA) and stored in aliquots in liquid nitrogen. MDA-MB-231 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). HEK AD293 cells were maintained in DMEM supplemented with 10% FBS. Unless noted otherwise, cells were plated at a density of 27000 cells/cm^2 in 6-well plates for analogue testing and, at the time of plating, stock solutions of analogues in EtOH, or *n*-butyrate in PBS, were added to cell culture medium such that the final EtOH concentration was 0.4% (v/v) in all samples (control experiments established that this level of ethanol had no effect on the biological parameters tested in this report). Cells were cultured at 37 °C in a water saturated, 5.0% CO_2 incubator.

In Vitro Invasion and Migration Assays. Modified Boyden chambers (BD Biosciences, San Jose, CA) were used to measure the migration and invasion of MDA-MB-231 cells as described by Albini and co-workers.[36] Briefly, migration chambers tested chemotaxis through 8.0 μm pores in response to a serum gradient. Invasion chambers contained a layer of Matrigel on top of the porous membrane. After pretreatment with analogue for two or three days, cells were trypsinized, resuspended in serum-free RPMI-1640 medium, counted, tested for viability, and seeded at a density of 50000 viable cells per chamber (equal seeding of viable cells was confirmed using the WST-8 assay (Dojindo, Gaithersburg, MD). Following a 24 h incubation period at 37 °C, migrating or invading cells were stained with Diff-Quik (Andwin Scientific, Addison, IL) and counted using MetaMorph software (Molecular Devices Corporation, Downingtown, PA); aliquots of cells were also reevaluated at this 24 h time point by the WST assay to ensure continued viability.

Determination of the Total Sialic Acid Content of Cells. A modification of the periodate-resorcinol assay originally described by Jourdian and co-workers,[37] adapted for smaller volumes,[4,38] was used to measure sialic acid levels in analogue-treated cells. Briefly, trypsinized cells were washed twice and resuspended in PBS, counted using a Coulter model Z2 cell counter, and lysed by three freeze−thaw cycles. Lysates (300 μL) were oxidized by the addition of 5.0 μL of 0.4 M periodic acid, cooled on ice for 10 min, reacted with 500 μL of freshly prepared resorcinol mixture (10% 0.5 M resorcinol, 10% 2.5 mM CuSO4, 44% concentrated HCl, 36% dd H_20), and heated at 100 °C for 15 min. After cooling, 500 μL of *tert*-butyl alcohol was added and optical density readings at 630 nm were promptly recorded (the signal fades by ∼25% over the course of an hour; typically readings were completed within five minutes, a time frame where no measurable changes occur). The cellular content of sialic acid, typically expressed as the number of molecules of sialic acid per cell, was calculated based on the number of cells harvested before lysis and on a calibration curve derived from standards of sialic acid (Pfanstiehl, Waukegan, IL) simultaneously assayed alongside the samples derived from the cells.

Analysis of Histone Acetylation by Immunoblotting. MDA-MB-231 cells (5.0×10^5) were plated into 100 mm dishes containing complete medium and allowed to form monolayers for 24 h prior to treatment with the specified compounds. After incubation for two days with analogue or sodium *n*-butyrate, cells were detached by scraping and were collected together with floating cells and medium, pelleted by centrifugation, and washed twice with PBS. Histones were extracted from nuclei following a published procedure.[39] Following the determination of protein concentration by the micro BCA assay (Pierce, Rockford, IL), histone extracts (4.2 μg protein/well) were separated by SDS-PAGE using 18% polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA). Samples then were blotted onto nitrocellulose and immunostained with antibodies against histone H3 (total) and acetyl-histone H3 (Upstate, Lake Placid, NY). Densitometry was performed using NIH ImageJ software.

Analysis of Gene Expression. mRNA for gene expression analysis was extracted from cells using Trizol (Invitrogen, Carlsbad, CA), treated with DNase (Ambion, Austin, TX) to remove contaminating genomic DNA, and purified using RNeasy spin columns (Qiagen, Valencia, CA). RNA quality was assessed with UV spectroscopy and agarose gel electrophoresis. For quantitative real-time PCR (qRT-PCR), cDNA was obtained using the SuperScript III First Stand synthesis kit (Invitrogen) and amplified in an ABI 7700 thermocycler using a SYBR green master mix (Applied Biosystems, Foster City, CA). Primer sequences are given in Table S2 in the Supporting Information. All Ct values were measured in quadruplicate (typically the SEM was smaller than the symbols used in the figures and has not been shown). Relative gene expression was calculated using average Ct values according to the $2^{-\Delta\Delta Ct}$ method.[40,41] Correct amplification was verified by agarose gel electrophoresis of the PCR products.

Zymogram Analysis of MMP Activity. To measure the activity of secreted matrix metalloproteinases (MMPs), MDA-MB-231 cells were stimulated with 100 ng/mL TPA (Sigma, St. Louis, MO), incubated for three days, washed twice with serum-free medium to remove serum proteases, and incubated with serum-free medium for an additional day. The culture medium was harvested, and the volumes of conditioned media were adjusted according to cell number before measuring MMP activity using SDS-PAGE gels containing either gelatin or casein (Biorad, Hercules, CA). Following electrophoresis to separate the MMPs according to molecular weight, the zymograms were incubated in renaturation and development buffers according to the manufacturer's protocol (Biorad, Hercules, CA). Counterstaining with Coomassie Blue identified protease activity as regions of degraded substrate (either gelatin or casein). Densitometry was performed using the NIH ImageJ software.

Reporter Gene Assays for Measurement of NF-κB. Transactivation activity of NF-κB was assessed with luciferase reporter plasmids (Stratagene, La Jolla, CA). To normalize for transfection efficiency, reporter plasmids were cotransfected with an equal mass of plasmid encoding β-galactosidase under the control of a SV promoter (Promega, Madison, WI). MDA-MB-231 and HEK 293 cells were transfected using a BTX electroporator and Lipofectamine 2000 (Invitrogen, Carlsbad, CA), respectively, according to the manufacturer's directions. Following a 24 h recovery period after transfection, the cells were replated and

treated with the specified compounds as described. Activities of luciferase and β-galactosidase were measured via chemiluminescent and colorimetric assays (Promega, Madison, WI), respectively. Normalized transactivation activity of NF-κB was calculated from ratios of luciferase and β-galactosidase activities.

Proteasome Activity. Proteasome activity was assayed using a succinyl luminogenic proteasome substrate (Promega, Madison, WI). Viable MDA-MB-231 cells (100000 cells in 100 μL of RPMI) were incubated with the luminogenic substrate according to the manufacturer's instructions. Proteasome activity was calculated from luminescent activity normalized to that of untreated controls. The proteasome inhibitor epoxomicin (Sigma, St. Louis, MO) provided a positive control.

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Supporting Information Available: Synthesis and characterization of analogues; supporting information for cell-based assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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