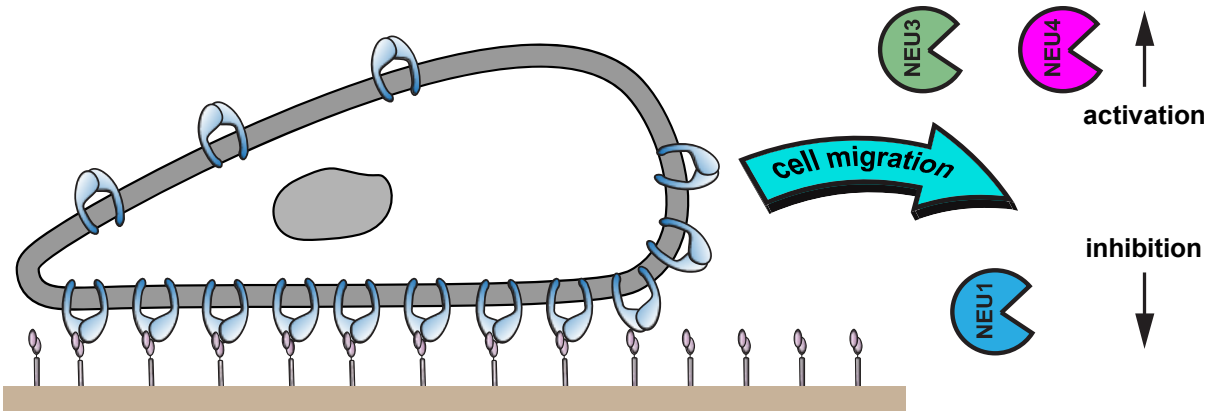
Isoenzyme-Selective Inhibitors of Human Neuraminidases Reveal Distinct Effects on Cell Migration

Md. Amran Howlader, Tianlin Guo, Radhika Chakraberty, Christopher W. Cairo*

Department of Chemistry, University of Alberta, Alberta, Canada T6G 2G2

* Corresponding author: ccairo@ualberta.ca, tel. 780-492-0377, fax 780-492-8231

Graphical Abstract



ABSTRACT

The human neuraminidase enzymes (NEU1, NEU2, NEU3, and NEU4) are a class of enzymes implicated in pathologies including cancer and diabetes. Several reports have linked neuraminidase activity to the regulation of cell migration in cancer cells. Using an in vitro cell migration assay on fibronectin (FN) coated surfaces, we have investigated the role of these enzymes in integrin-mediated cell migration. We observed that neuraminidase inhibition caused significant retardation of cell migration in breast cancer (MDA-MB-231) and prostate cancer (PC-3) cell lines when using inhibitors of NEU3 and NEU4. In contrast, inhibition of NEU1 caused a significant increase in cell migration for these same cell lines. We concluded that blockade of human neuraminidase enzymes with isoenzyme-selective inhibitors can lead to disparate results and has significant potential in the development of anti-cancer or wound healing therapeutics.

1. Introduction

Human neuraminidase enzymes (hNEU) are glycoside hydrolases which release sialic acids from glycolipid and glycoprotein substrates.[1] Currently, hNEU isoenzymes that have been identified include NEU1,[2] NEU2,[3] NEU3,[4] and NEU4.[5] Though the four human neuraminidase isoenzymes catalyze the same hydrolysis reaction of sialoglycoconjugates (SGC), their specific targets and expression allow them to play differing roles in human physiology. One process that illustrates the diversity of hNEU function is the migration of cells.[6, 7] NEU1 has been reported to downregulate cell migration in human lung cancer cells and to suppress metastasis in mice.[6, 8] NEU1 also regulates lysosomal exocytosis, which may influence the invasiveness of cancers.[9] In contrast, NEU2 has been linked to increased cell survival and motility of prostate cancer cells.[10] Increased expression of NEU3 has been reported in human colon,[11] melanoma,[12] glioblastoma,[13] and prostate cancer cells[14] where the enzyme affects cell motility[6] and tumor growth.[15] Additionally, NEU3 activity is implicated in axon regeneration.[16] NEU4 is overexpressed in human neuroblastoma resulting in increased cell proliferation,[17] but it is downregulated in human colon cancer cells where expression correlates with increased motility and metastasis.[18] These findings suggest significant, but highly diverse, roles played by hNEU enzymes in cell migration and cancer metastasis.

Integrins are glycoprotein receptors that play central roles in cellular migration, metastasis, and transmigration.[19, 20] Fibronectin (FN) is a major extracellular matrix (ECM) ligand of the α4β1 and α5β1 integrins (also called VLA-4 and VLA-5, respectively)[21] commonly expressed on many cell types.[22, 23] Integrins are regulated, in part, by dynamic shuttling of receptors between the membrane and endocytic compartments by clathrin-dependent and clathrin-independent mechanisms.[24-27] The endo- and exo-cytosis of integrins is known to feature selective uptake and spatially-organized processes;[28] furthermore, integrin cluster size and organization is critical to the strength of the resulting adhesions.[29] The glycosylation of β1 integrins plays an important role in the endocytosis and function of integrins.[30] For example, the N-glycan structure of α5β1 influences endocytosis of the receptor through interactions with Galectin-3.[31-33] The N-glycan of the α5 integrin is critical to cell migration and may affect endocytosis.[34] Integrin-galectin interactions are attenuated by increased α2,6-linked sialic acid content, which masks lectin binding sites.[35, 36] Thus, hNEU may participate in integrin regulation by altering integrin-lectin binding.[37] Indeed, NEU1 is known to modify the N-glycan of the β4 integrin.[8]

The expression of multiple hNEU isoenzymes in most cell types makes it challenging to study the role of individual enzymes in cell migration. One strategy that has emerged is the use of selective small-molecule inhibitors of individual isoenzymes. We and other groups have worked to identify inhibitors of hNEU with excellent selectivity and nanomolar potencies, a selection of these are provided in **Table 1**.[38] Commonly used inhibitors include DANA **1** (2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid), a pan-selective inhibitor of the four hNEU isoenzymes with modest preference for NEU3 and NEU4.[39] The first compound designed as a selective inhibitor of hNEU was C9-BA-DANA **2**,[40] which targets NEU1. The antiviral and bacterial NEU inhibitor, zanamivir **3**, has low micromolar activity against NEU2 and NEU3.[41, 42] The first NEU3-selective inhibitor reported was compound **4**.[39] The most potent inhibitor reported for any hNEU is the NEU4-selective inhibitor, C9-4HMT-DANA **5**.[43] We considered that the availability of this panel of inhibitors would allow us to analyze specific contributions of individual hNEU isoenzymes to cell migration.

In the current study, we examined the role of hNEU isoenzymes in the migration of four different human cancer cell lines from prostate (PC-3), breast (MDA-MB-231), lung (A549), and cervical (HeLa) tissue. We hypothesized that this panel of different cell lines could reveal the range of activity for hNEU isoenzymes found in disparate tissues. Furthermore, we wanted to use a panel of inhibitors that could interrogate multiple isoenzymes, including NEU1, NEU3, and NEU4. We observed that, not only do cell lines show different levels of response to the same inhibitor, but that targeting of specific isoenzymes can have contrary effects. These results suggest that interrogating hNEU isoenzyme activity with non-selective inhibitors could mask important activities and make a strong case for the further development and application of selective inhibitors of this family of human enzymes.

2. Results and discussion

2.1 Inhibitors of NEU3 and NEU4 suppress cell migration of PC-3 and MDA-MB-231 cells

To understand the specific functions of hNEU isoenzymes on cell migration, we investigated β1 integrin-mediated cell migration of four human cell lines (PC-3, MDA-MB-231, A549, HeLa). HeLa and A549 cells express α5β1 integrin, with low levels of α4β1.[37, 44] PC3 cells are known to express β1 integrins.[45] Glycosylation of the α5β1 integrin is critical for cell migration in MBA-MB-231 cells.[46] To restrict our findings to the β1 integrin, we used a FN-coated surface for cell migration assays, following previously reported methods.[32, 47] A panel of reported hNEU inhibitors with isoenzyme selectivity were either synthesized as reported or

obtained from commercial sources (**Table 1**). To screen the activity of these compounds in each cell line, we tested their activity to alter cell migration at doses which were approximately ten times that of the lowest reported IC_{50} value of each compound. In these experiments, cytochalasin D (CytoD), an actin depolymerizing agent,[48] was used as a positive control, and showed significant reduction of migration across all cell lines (**Figure 1**, **Table S1**).

We first examined HeLa and A549 cell lines using a set of inhibitors with mixed activity as well as those that target NEU3 (compounds **1**, **3**-**5**). Several inhibitors tested in HeLa did not show significant reduction of cell migration, with only compound **5** having significant reduction (94 ± 3 %; **Figure 1A**). We previously found that both **5** and DANA **1** were active in a similar assay with HeLa cells; however, we note that the time of incubation for the current experiment is substantially longer here (3 h vs 21 h), which likely explains the discrepancy.[37] Testing in A549 cells found that DANA **1** was a moderate inhibitor of migration (90 ± 2%; **Figure 1B**), while the other compounds tested had no effect. These results suggested that cell lines show differential responses to the same compounds, and we proceeded to test our inhibitor panel on additional lines.

Testing in PC-3 cells identified multiple compounds as active inhibitors of cell migration (**Figure 1C**). The pan-selective inhibitor, DANA **1**, was active at 100 μM (82 ± 2%) and testing at a lower concentration (1.5 μM) did not show significant activity. Zanamivir **3** was an active inhibitor at 10 μM (78 ± 3%), but showed no effect at 1.5 μM. The NEU3-selective inhibitor, **4**, showed substantial inhibition at 250 μM (61 ± 2%), but had no effect at 1.5 μM. We note that although more potent NEU3 inhibitors have been identified,[49] **4** was tested here due to its excellent selectivity for NEU3.[39] The NEU4-selective inhibitor **5** was active at 1.5 μM (90 ± 3%). MDA-MB-231 cells showed some similarities in sensitivity to hNEU inhibitors observed for PC-3 cells (**Figure 1D**). DANA **1** was not active over the range of 1.5 to 100 μM; while zanamivir **3** was only active at 10 μM (63 ± 4%). The NEU3-selective inhibitor, **4**, was only active at the higher concentration tested (250 μM; 79 ± 3%); while the NEU4-selective inhibitor, **5**, was active at 1.5 μM (74 ± 2%).

These initial migration data confirmed that inhibition of individual isoenzymes gave variability in the responses of individual cell lines. Furthermore, these data suggested that compounds with activity for NEU3 and NEU4 were active as inhibitors of cell migration in PC-3 and MDA-MB-231 cells. The HeLa and A549 cells were less sensitive to all compounds

tested. We therefore focused our attention on understanding the role of hNEU isoenzymes in PC-3 and MDA-MB-231 cells in more detail.

2.2 Inhibition of NEU1 activates cell migration

After identifying PC-3 and MDA-MB-231 cells as sensitive to hNEU inhibitors, we proceeded to investigate the effects of inhibitors for NEU1 and NEU4. We used C9-BA-DANA **2**, a compound selective for the human NEU1 enzyme,[40] and C9-4HMT-DANA **5**, which was identified as a potent and selective inhibitor for NEU4.[43] These two compounds offered selectivity among the inhibitors, and we hypothesized that they would be most likely to show significant effects. We tested the dose-dependent effect of C9-BA-DANA **2** in PC-3 and MDA-MB-231 cells (**Figure 2A & 2B**). We were surprised to observe that treatment of both cell lines with compound **2** at 10-100 μM caused a significant *activation* of cell migration (up to 167 ± 9%, PC-3; 163 ± 10%, MDA-MB-231, **Table S2**). This was in contrast to the effects of other compounds tested above, which had no effect or else acted as inhibitors of migration. Testing of compound **5**, a selective inhibitor of NEU4, over a similar range found *inhibition* of cell migration between 1.5 – 100 μM (**Figure 2C & 2D**). Together, these experiments confirmed that targeted inhibition of individual hNEU isoenzymes can have opposing effects in cells.

To investigate the role of NEU1 in cell migration using a different model, we employed a previously reported human embryonic kidney (HEK-293) cell line which overexpressed NEU1.[50] We confirmed that NEU1 expression levels in these cells were elevated (approximately 5-fold relative to control cells) using western blotting (**Figure S1**). Determination of basal cell migration confirmed that increased NEU1 expression resulted in a large *decrease* in cell migration (62 ± 4 %) compared to untransfected HEK-293 cells (100 ± 2 %; **Figure S1**). This result is consistent with our finding that inhibition of NEU1 resulted in *increased* migration.

We next sought to understand if the action of hNEU inhibitors with opposing effects on migration could compete with each other. Our initial results with the pan-selective inhibitor, DANA **1** (**Figure 1**), where the inhibitor had no detectable effect in some cases, could be explained through mixed effects on NEU1 versus other isoenzymes. Thus, we tested the action of compound **2** on cell migration in the presence of NEU3 and NEU4 inhibitors (**Figure 3, Table S3**). Compound **4**, a NEU3-selective inhibitor, partially blocked the increased cell migration observed for **2** alone in both cell lines (125 ± 4%, PC-3; 131 ± 7%, MDA-MB-231). The NEU4-selective inhibitor, **5**, was able to block the activating effect of **2**, reducing the effect

in both cell lines (103 ± 6%, PC-3; 102 ± 6%, MDA-MB-231). Combination of NEU1, NEU3, and NEU4 inhibitors returned cell migration to control levels (**2**+**4**+**5**, 103 ± 5%, PC-3; 111 ± 9%, MDA-MB-231). Finally, we observed that compounds **4** and **5** together had reduced effect on cell migration relative to each compound alone (104 ± 2%, PC-3; 116 ± 5%, MDA-MB-231), perhaps as a result of different enzyme targets. These experiments confirmed that inhibitors of individual hNEU isoenzymes can have competing effects on cell migration and highlight the advantages of highly-selective inhibitors.

It is possible that reduced cell migration observed upon inhibitor treatment was due to toxicity of compounds to cells. To test this hypothesis, we evaluated the cytotoxicity of compounds **1**-**5** in PC-3 and MDA-MB-231 cells. Compounds were tested under identical conditions used in cell migration experiments, with the highest concentration used for each compound tested. We did not observe any significant cytotoxicity for most hNEU inhibitors, with the notable exception of C9-BA-DANA **2** (**Figure S2**, **Table S4**). Treatment of cells with the NEU1-selective C9-BA-DANA **2** at 100 μM resulted in significant reduction in cell viability in both cell lines (PC-3, 71 ± 3%; MDA-MB-231, 74 ± 2%). CytoD induced marginal toxicity for MDA-MB-231 cells only (90 ± 2%). Since C9-BA-DANA **2** treatment of cells resulted in increased migration, we conclude that the toxicity of the compound was moderate and was unlikely to interfere with the migration assay. Overall, these results rule out compound toxicity as an explanation for reduced cell migration.

2.3 Inhibition of cell migration is dependent on hNEU isoenzyme expression

The extent of effects for hNEU inhibitors varied among the four cell lines tested (**Figure 1**). To better understand these differences, we investigated the native expression of NEU1 and NEU3 proteins in each cell line using western blotting (**Figure S3**). Expression levels were quantified by densitometry of western blots and normalized to PC-3 cells. The cell lines which were least sensitive to NEU3 inhibitors, HeLa and A549, had the highest expression of the enzyme (6- and 14-fold relative to PC-3). The cell lines which were most sensitive to NEU3-selective compounds (PC-3 and MDA-MB-231) had the lowest expression levels among the cells tested. We observed smaller differences in NEU1 expression between cell lines. Although NEU1 expression in PC-3 and MDA-MB-231 showed a minor decrease as compared to HeLa and A549, these differences were not significant. These findings suggest that cells with lower expression of NEU3 are more sensitive to the action of NEU3 inhibitors. This effect may be similar to that of kinase inhibitors which show increased sensitivity in cells with decreased expression of their targets.[51]

To validate the specificity of the observed effects for inhibitors of hNEU isoenzymes on cell migration, we designed an siRNA-based knockdown experiment. We used NEU1 siRNA, NEU3 siRNA, and a scrambled control (SC) to reduce expression of the enzymes in MDA-MB-231 cells and observed the effects of these changes on cell migration (**Figure 4** & **Table S5**). Reduced expression of NEU1 and NEU3 was confirmed by western blotting (**Fig S4** & **S5**). NEU1 siRNA treated cells showed significantly higher cell migration (220 ± 30%) compared to the control group (100 ± 5%), consistent with the effects of specific NEU1 inhibitor treatment resulting in increased cell migration (**Figure 2**). Reduced expression of NEU3 after siRNA treatment resulted in significant reductions of cell migration (53 ± 7 %) compared to control. Experiments with a NEU4 siRNA showed a similar decrease in cell migration; however, we were unable to confirm reduced expression of the enzyme by western blotting. We concluded that specific reduction in NEU isoenzyme expression had similar effects to pharmacological inhibition of individual isoenzymes.

2.4 Exogenous glycolipids modulate cell migration

To investigate the mechanism of action of hNEU inhibitors on cell migration, we tested the role of exogenous glycolipids on cell migration. Glycolipids such as GM3 and LacCer are known to affect integrin-mediated cell migration in HeLa and A549 cells,[37] and the concentration of these glycolipids could be altered by the activity of NEU3 or NEU4 enzymes, but should not be affected by NEU1.[4, 52] We treated cells with exogenous GM3 (50 ng mL^{-1}) and LacCer (50 ng mL^{-1}), in the presence of serum, to favor cell membrane insertion (**Figure 5**).[53] We found that the addition of GM3 caused a significant decrease in cell migration of PC-3 cells (73 ± 7 %), but there was no measurable effect on MDA-MB-231 cells. However, in both cell lines LacCer treatment increased migration (130 ± 7%, PC-3; 150 ± 11%, MDA-MB-231). LacCer is the expected hydrolysis product of GM3 from NEU3, and has previously been reported to alter β1 integrin membrane organization, endocytosis, and cell migration.[54]

2.5 Inhibitors of hNEU do not induce large changes to cellular glycolipids

The effect of hNEU inhibition is likely due to changes in specific SGC (e.g. glycolipids or glycoproteins) on cells which modulate integrin activity. NEU3 and NEU4 are implicated by our results in regulation of integrin-mediated cell migration, and these enzymes are known to modify glycolipid targets.[7, 52] We implemented an LC-MS based technique to quantify changes in glycolipids from PC-3 cells which were most sensitive to inhibitors, after treatment with hNEU inhibitors.[55-57] We quantified glycolipid concentrations for several glycolipid species on

cells treated with inhibitors (**Figure S6**). In general, the three most abundant glycolipids were GM2, GM3, and GM1. There was some variability in the levels of GM2 and GM3 detected for inhibitor-treated cells, but no statistically significant differences from control were observed. We concluded that, while glycolipid levels may be affected by inhibitor treatment, these changes were not detectable in our assay. Possible explanations for this observation are that the effects of inhibitor treatment may be below our detection limits for glycolipids, they may be only transient effects, or that glycoprotein targets of the enzymes are more critical.

2.6 Modulation of Cell surface sialoglycoconjugates by hNEU inhibitors

To explore the effects of inhibitors on cellular SGC, we examined changes to gross sialic acid content after inhibitor treatment. We quantified total sialic acid content by using fluorescent-conjugated lectins and flow cytometry. Cells were labeled with MAL (*Maackia amurensis* lectin), PNA (*Arachis hypogaea* lectin, peanut agglutinin), or SNA (*Sambucus nigra* agglutinin) (**Figure S7**). Cells were initially treated with buffer, **1** (100 μM), or NanI (0.1 RU). We observed significant changes in binding for MAL and PNA when both cell lines were treated with NanI. The binding of MAL (specific for sialic acid) was reduced, while PNA binding (specific for terminal galactose residues) was increased.[19] SNA (which prefers Neu5Ac-α2,6-Gal terminal sites) was only reduced on PC-3 cells after NanI treatment. Although these findings were consistent with removal of sialic acids from the cell surface by NanI, treatment of cells with DANA **1** at 100 μM was unable to significantly alter the binding of MAL, PNA, or SNA to cells. High doses of DANA **1** (1 mM) were able to increase MAL and PNA staining in MDA-MB-231 (**Figure S8**). Furthermore, high dose treatment of MDA-MB-231 cells with zanamivir **3** (100 μM) resulted in an increase of MAL staining. These data confirmed that both **1** and **3** were able to affect gross sialic acid content of cells. However, we concluded that these changes may be small or limited to specific targets under the conditions used for cell migration assays, as they were not detectable by lectin staining at these lower concentrations.

2.7 Effects of human neuraminidases on glycosylation and endocytosis of β1 integrin

Our results confirmed that hNEU inhibitor treatment could result in changes to cellular SGC, including glycolipids and glycoproteins, which may affect integrin activity. Thus, we next tested for direct effects of hNEU on β1 integrin glycosylation and its endocytosis. The β1 integrin heterodimer is known to be heavily N-link glycosylated, and its glycosylation state may affect activity.[30, 32, 36, 46] To obtain evidence of changes to β1 integrin glycosylation, we

performed western blotting of the β1 chain of the integrin heterodimer after treatment with NanI, NEU3, and NEU4 in MDA-MB-231 cells, as this line was sensitive to inhibitors (**Figure S9**). We observed that the β1 integrin had a large decrease in molecular weight when treated with NanI, and a second smaller band appeared after treatment with NEU3 or NEU4. These data are consistent with a change in the integrin glycoform due to hNEU enzymes, most likely a loss of sialic acid consistent with previous experiments using β2 integrins.[57] To test whether NEU activity altered endocytosis of the integrin, we measured changes in the relative β1-integrin endocytosis in PC-3 and MDA-MB-231 cells. Endocytosis was determined through biotinylation of cell surface proteins with a GSH-sensitive label, followed by treatment with GSH after an incubation period. Internalized proteins retained the biotin label, and were captured on immobilized streptavidin beads, followed by a western blot for the β1 integrin to provide insight into changes in the distribution of the protein.[58, 59] Conditions included the addition of exogenous enzymes, glycolipids, and hNEU inhibitors (**Figure 6**). Although we performed multiple replicates, these data were variable and only a few conditions showed significant changes. Treatment with GM3 resulted in a small increase in endocytosis for PC-3 cells. LacCer treatment elevated endocytosis in both cell lines, but this change was not significant. Exogenous NEU3 and NanI had no significant effects in either cell line. The NEU3 inhibitor **4** and NEU1 inhibitor **2** both had activity in migration assays for MDA-MB-231 and PC-3 cells (**Figure 1**). In testing treatment with **2** or **4** for changes in endocytosis of the integrin, we found that **2** showed significant reduction of endocytosis in both cell lines. Compound **4** only showed significant reduction of endocytosis in PC-3 cells. Inspection of the integrin blots from these experiments provides further confirmation of changes to the glycoform of the integrin, as lower molecular weight bands can be observed in several conditions. We caution that additional studies are needed to test if there are differences in the time course of endocytosis as well as the effects of other inhibitors not tested here.

3. Conclusion

Using migration assays and a unique panel of specific hNEU inhibitors, we have investigated the involvement of hNEU in regulation of integrin-mediated cell migration. Testing for general responses to inhibitors found different magnitudes of response for the same inhibitors among a sample of four human cell lines (PC-3, MDA-MD-231, A549, and HeLa). These differences were attributable in part to differences in hNEU expression levels. Inhibitors of NEU3 and NEU4 blocked cell migration to various degrees. In contrast, treatment of cells with the NEU1 inhibitor, C9-BA-DANA **2**, had an activating effect on cell migration; while

overexpression of the NEU1 enzyme in cells resulted in a corresponding decrease in basal cell migration. The effects of isoenzyme-selective inhibitors were competitive with each other, as co-administration of NEU3 or NEU4 inhibitors with NEU1 inhibitors produced no net effect on cell migration. Experiments with exogenous glycolipids suggested a role for GSL in regulation of β1 integrin-mediated migration on FN. These observations did not exclude a role for changes to other SGC, such as glycoproteins or the integrin itself. Lectin-staining of cells did not show significant changes in cellular SGC after hNEU inhibitor treatment, which likely include both glycolipids and glycoproteins. However, western blotting of the β1 integrin after exogenous NEU treatment confirmed that NEU3 and NEU4 induced changes in the molecular weight of the protein, consistent with desialylation. These two results may suggest that specific glycoprotein targets are being modified by NEU, rather than gross changes to cellular SGC. Finally, we evaluated changes to β1 integrin endocytosis on cells treated with exogenous NEU enzymes and hNEU inhibitors. These experiments revealed that NEU inhibitor treatment generally decreased endocytosis of the integrin.

The modification of cellular SGC, including glycolipids and glycoproteins, may contribute to the regulation of β1 integrin-mediated cell migration. We observed that exogenous GM3 inhibited migration of PC-3 cells consistent with previous findings.[37, 60] Treatment of cells with LacCer increased cell migration in both PC-3 and MDA-MB-231 cells. LacCer is known to activate β1 integrin clustering through activation of Src,[54] and to enhance endocytosis of β1 integrin[54] by a clathrin-independent mechanism.[61] Notably, NEU3 has been found to be associated with caveolin-1 and is found within membrane ruffles.[62, 63] Disruption of NEU3 function is expected to result in changes to GSL levels in cells. NEU3 knock-outs show increased levels of LacCer, and, when combined with a HexA knock-out, result in large increases in GM2 levels.[64] Treatment with NEU3 shRNA resulted in increased GM2 levels in neuronal cells.[65] This may be the result of increased levels of GM3 due to the loss of NEU3 activity; as knock-outs of GM3 synthase lead to reduced GM2 levels.[66] GM2 has direct molecular interactions with the β1 integrin,[67] and likely regulates integrin function in tumor cells through CD82.[68] Colon cancer cells show increased expression of NEU3, and a concomitant increase in LacCer concentrations.[11] LacCer and other GSL are internalized via caveolin-related mechanisms.[61] We were unable to observe significant changes in cellular GSL after inhibitor treatment. We note that our findings do not exclude a role for changes to integrin glycosylation in regulating cell migration. We find evidence of desialylation of β1 integrin after NEU treatment, consistent with previous studies.[8, 30, 57]

Our finding that selective inhibitors of hNEU can have disparate cellular effects provides an important caveat for future studies.[38] First, we note that testing of non-selective inhibitors, such as DANA **1**, can provide misleading results. In our case, testing with only DANA **1** would have led us to erroneously conclude that hNEU inhibitors only block cell migration. The same may also be true for commercially available viral NEU inhibitors such as zanamivir **3**, which had mixed effects on NEU2 and NEU3.[41, 42] Studies that exploit isoenzyme-selective hNEU inhibitors are becoming more common;[37, 69-71] however, identifying new, more selective, and more potent inhibitors remains an active area of investigation.[38, 39, 43, 49, 72] Such compounds may provide new avenues for therapeutics.[38]

The regulation of cell migration by hNEU, and the availability of small molecule inhibitors for these enzymes, suggest potential therapeutic applications. Blocking of integrin-mediated cell migration has been explored in the development of anti-adhesive strategies in cancer.[73, 74] Thus, understanding the specific roles of hNEU activity in regulating cell migration will be essential to testing the feasibility of inhibitors as therapeutics. Increased NEU1 expression has been shown to block endothelial cell migration, but NEU3 had only a minor effect on this process.[75] In endothelial cells increased NEU1 activity leads to desialylation of CD31, and reduced cellular adhesion.[76] Increased NEU1 expression has also been shown to decrease epithelial cell migration.[77] Sialidase activity accelerates fibrosis, and can be activated by cytokines in A549 and PBMC.[78] Our observation that NEU1 overexpression reduced integrin-mediated cell migration on FN is consistent with previous findings that NEU1 overexpression reduced invasiveness of tumor cells.[9]

The contrasting roles of NEU1 and NEU3/NEU4 in the regulation of cell migration suggest that selective targeting could allow for applications in wound healing.[79, 80] While anti-adhesive strategies are generally concerned with blocking cell migration, wound healing applications may benefit from strategies to activate cell migration. The regulation of β1 integrin is critical for wound healing.[80] Our findings suggest that NEU3/NEU4 activates cell migration, while NEU1 inhibits cell migration. Correspondingly, inhibitors of NEU3/NEU4 will block cell migration and inhibitors of NEU1 will activate cell migration.[78] Therefore, strategies to enhance NEU3/NEU4 activity or to block NEU1 should lead to increased β1 integrin-mediated cell migration.

In this study, we have shown that β1 integrin-mediated cell migration is differentially regulated by individual hNEU isoenzymes. Selective inhibitors for hNEU isoenzymes revealed that NEU1 inhibition was activating, while NEU3 and NEU4 inhibition attenuated cell

migration. These effects were competitive, and we confirmed that inhibitors with mixed activity, or mixtures of selective inhibitors, could mask effects on cell migration. These results highlight the need for selective tools to target hNEU, and the fact that isoenzymes can play distinct roles in regulating cellular processes. Finally, the importance of integrin receptors to cancer metastasis and wound healing suggest that hNEU inhibitors may provide a new target for pharmaceutical strategies.

4. Methods

4.1 Cell culture

All cell lines were cultured according to ATCC guidelines. Briefly, HeLa and MDA-MB-231 cell line were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in 5% CO_2 and 37 °C in a humidified incubator. PC-3 and A549 were cultured in Ham's F-12k medium supplemented by 10% FBS and 1% penicillin-streptomycin in 5% CO_2 at 37 °C incubator. All experiments were performed with cell of passage 5 to passage 15.

4.2 Sources of reagents

DANA **1**, **2**, **4**, and **5** were prepared as previously reported.[39, 43, 49, 72] Zanamivir **3** (Sigma-Aldrich, USA) and Cytochalasin D (Sigma-Aldrich, USA) were obtained from commercial sources and used as provided. Stock solutions for all the compounds, except cytochalasin D, were made using ultra-pure water (18.2 MΩ). Stock solutions of cytoD were made in DMSO, and the final concentration of DMSO in all samples was less than 0.1%. The FITC-conjugated *Maackia amurensis* lectin (Bioworld, USA), FITC-conjugated *Sambucas nigra* agglutinin (EY Labs, USA), and AF647-conjugated PNA (Thermo Fischer, USA) were obtained from commercial sources.

4.3 Neuraminidase enzymes

Recombinant NEU3 and NEU4 were prepared and purified from *E. coli* following an established procedure.[81] *Clostridium perfringens* neuraminidase was purchased (Sigma-Aldrich) and was found to consist predominantly of the NanI isoenzyme based on molecular weight determined from SDS-PAGE.[37] NEU activity was determined using a standard 4MU-NANA assay, where 1 relative unit (RU) was defined as 1 μmol 4MU-NANA substrate cleavage per min.

4.4 Migration Studies

Cell migration studies were performed using an Oris 96 well plate assay kit (Platypus Technologies, USA). Each well of the plate was coated using 100 µL of fibronectin (10 µg/mL in PBS, Calbiochem, USA) for 2 h and 100 µL of IgG-free BSA (200 µg/mL in PBS) for 1 h. After removal of BSA, stoppers were placed in each well. Cells were placed into each well (100 µL of 50 x 10^4 cells/mL) and incubated for 18 h to achieve 80%-90% confluence. The stoppers were removed, cells were gently washed with PBS, and medium supplemented with treatment conditions or medium alone was reintroduced in each well. Wells were imaged under bright field illumination using a 5x objective with a Nikon T1 Eclipse inverted microscope. The plate was incubated for 21 h at 37 °C in a humidified incubator, and wells were imaged again and analyzed with Image J software. Each condition was repeated on different days to account for intra- and inter-day variability, and buffer and cytoD controls were used on every plate. Migration rates were normalized to the intra-day buffer control. Normalized replicates were then pooled together for statistical analysis. Migration data were normalized using the following equation: Normalized migration, $r = \left(\frac{\mathrm{A0} - \mathrm{A21}}{M_B}\right) \times 100$. Where A_0 was the cell-free zone at zero hours (µm^2), A_{21} was the area covered during 21 h incubation (µm^2), and M_B was the mean area covered by cells treated with only medium (µm^2).

4.5 Western blotting

Cells lysates were prepared in TBST buffer (1% Tween 20, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, and protease and phosphatase inhibitors), clarified by centrifugation, and boiled in Laemmli sample buffer. Lysates were separated by 10% SDS–PAGE. Lysate protein concentration was determined by bicinchoninic acid assay (BCA assay kit, Thermofisher, USA). Gels were transferred to nitrocellulose, blocked for 1 h at room temperature in TBS containing 5% milk (Biorad, USA), and then incubated in 1% TBST overnight at 4 °C with antibodies against beta-actin (anti-β actin antibody; 1:5,000; Abcam, ab8227), β1 integrin (Anti-integrin beta-1 antibody; 1: 5,000; clone EP1041Y, Abcam), α5 integrin (Anti-Integrin alpha-5 antibody; 1:1,000; clone EPR7854, Abcam), NEU1 (mouse monoclonal to human anti-neuraminidase 1; 1:1,000; Santa Cruz, clone F-8), and NEU3 antibody (mouse monoclonal to human anti-neuraminidase 3, clone 11b; 1:3,000; MBL), followed by the appropriate HRP conjugated secondary goat anti-rabbit antibody (1:5,000; Abcam) or goat anti-mouse antibody (1:3000; Biorad). Skim milk was used as blocking agent for actin blots. Western blots were developed by ECL (BioRad, USA) according to the manufacturer's instructions.

4.6 Transfection of MDA-MB-231 cells with siRNA for NEU1 and NEU3

The NEU1 siRNA, NEU3 siRNA, and scrambled control siRNA (SC siRNA) were procured from Dharmacon, USA. The sequences are presented in Table S6. To avoid contamination bands appearing in the blots from exogenously added NEU3, MDA-MB-231 cells were alternatively treated with NEU1 siRNA and NEU3 siRNA and the lysate was collected and blotted to determine knock down efficiency. Briefly, of siRNA (20 µL) and of lipofectamine 2000 (10 µL) was mixed in serum-free DMEM medium. Then, 1 x 10^7 cells (MDA-MB-231) were treated with the indicated siRNA mixtures for 72 hours following the manufacturer's protocol (Thermofisher, USA) without antibiotics. After the incubation time, cells were collected and lysed using RIPA buffer for blotting or used for cell migration experiments.

4.7 Analysis of gangliosides in PC-3 cells treated with inhibitors using LC-MS

Expression and purification of EGCase was performed based on the reports of Albrecht et al.,[56] using a gene encoding recombinant EGCase I with a His-tag in a pET30 vector. The protein was purified on a Ni-NTA superflow column, concentrated, and stored at 4 °C. The activity of EGCase I was tested using ganglioside substrate GM3. One unit of EGCase I was defined as the amount of enzyme that hydrolyzes 1 µmol of GM3 per minute at 30 °C. Ganglioside extraction and purification was performed based on previous reports.[82] Briefly, 1 x 10^6 cells were centrifuged to a pellet and homogenized in ice cold water (4 mL/g). Methanol and chloroform were added after vigorous mixing to a final ratio of 4:8:3 chloroform:methanol:water (v/v/v). This mixture was centrifuged (1500 RPM for 15 mins), and the supernatant was recovered and diluted with 0.173 volumes of water. After mixing, the suspension was centrifuged again, the upper phase was recovered, and transferred to a fresh tube. The sample was then purified on a SepPak C18 cartridge and evaporated to dryness under a stream of nitrogen. The final extract was re-suspended in sodium acetate buffer (50 mM, pH 5.2) containing 1 mg mL^{-1} sodium cholate. Gangliosides were then incubated for 18 hours at 30 °C with 0.092U EGCase to release the corresponding glycans. EGCase released glycans were labeled with anthranilic acid (30 mg anthranilic acid, 20 mg boric acid, 40 mg sodium acetate, and 45 mg sodium cyanoborohydride) at 80 °C for 45 mins, with an internal standard (maltose) added. The sample was purified on a discovery DPA-6S amide-HILIC column as described,[55] and reduced under vacuum. Three replicates from each condition were analyzed as labelled glycans by LC-MS using an Agilent 1200 SL HPLC system and a normal-phase column (Accucore-150-Amide-HILIC, 2.6 µm, 2.1 x 150 mm, Thermo Fisher). The fluorescence detector was set to monitor at excitation 320 nm, emission 420 nm and all

chromatography was performed at 40 °C. The binary solvent system followed a linear gradient with a flow rate of 0.4 mL min^{-1} (Solvent A: 100 mM ammonium formate, pH 4.45; Solvent B: acetonitrile). Quantification of glycolipid concentrations was calculated by reference to an internal standard. Data analysis was performed using the Agilent MassHunter Qualitative Analysis software package version B.07.01.

4.8 Endocytosis of β1 integrin

Biotin-based assays were performed as previously described[59] with slight modifications. MDA-MB-231 or PC-3 cells were grown in 10% FBS-containing medium on 6 cm dishes to 80% confluence. The cells were placed on ice and washed once with cold PBS. Cell surface proteins were labeled with 0.8 mg mL^{-1} of EZ-link cleavable sulfo-NHS-SS-biotin (Thermo Scientific, USA) in for 60 min at 4 °C. Unbound biotin was washed away with cold medium, and pre-warmed 10% serum-containing medium with or without treatments were added to cells. Conditions included incubation with NanI (pH 7.2, 2 h, 0.1 RU), NEU3 (pH 7.2, 3 h, 500 μRU), GM3 (50 ng/mL), LacCer (50 ng/mL), compound **4** (250 μM, 1 h), and compound **2** (100 μM, 1 h). The biotin-labelled surface proteins were allowed to internalize at 37 °C for 30 min, after which the cells were placed quickly back on the ice with cold medium. The remaining biotin at the cell surface after internalization was removed with GSH buffer (75 mM sodium chloride, 1 mM magnesium chloride, and 0.1 mM calcium chloride, 50 mM GSH, 80 mM sodium hydroxide, and 10% FBS) for 30 min at 4 °C, followed by repetitive washes with cold PBS. The cells were lysed by scraping in lysis buffer (150 mM sodium chloride 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, phosphatase and protease inhibitor cocktail) and incubation at 4 °C for 30 min. The lysate was clarified by ultra-centrifugation at 18,000 x g for 10 min. Supernatants were collected and biotinylated proteins were captured using immobilized streptavidin and analyzed by western blot for changes in integrin endocytosis.

4.9 Cell viability

Toxicity of compounds was assayed under similar conditions that were used in migration studies. Briefly, wells in a 96 well plate were charged with 100 μL of 50 x 10^4 cells mL^{-1} cells and incubated for 18 h in 5% CO_2 at 37 °C in a humidified incubator. Cells were then treated with the indicated conditions for 21 h. The final concentration of the compounds was the same as that used for migration studies. After incubation, 20 μL of MTS solution (Promega, USA) was added to each well and incubated for 2 h. The absorbance of soluble formazan produced

by viable cells from MTS was measured at 490 nm using SpectraMax M2 (Molecular Devices, USA) plate reader. For each condition, the experiment was conducted with replicates on different days to consider the intra- and inter-day variabilities. Absorbance for each replicate of conditions was normalized to that of intra-day buffer control.

4.10 Measurement of cell surface sialic acid

Changes in cellular SGC on MDA-MB-231 and PC-3 cell lines after treatment with neuraminidase inhibitors and bacterial neuraminidase enzymes was assessed using flow cytometry. Cells were treated with 10 mM EDTA in PBS for 1.5 h to detach cells from culture plates and incubated for 18 h in fresh growth media. Cells were then treated with hNEU inhibitors in growth medium and incubated for 21 h. For NanI-treated cells, after harvesting cells, 0.06 mg mL^{-1} NanI was used to treat cells for 2 h. Cells (1 mL, 10 x 10^4 cells mL^{-1}) were labeled by incubation with AF 647-PNA (20 μg mL^{-1}), FITC-SNA (20 μg mL^{-1}), and FITC-MAL (5 μg mL^{-1}) for 30 mins separately. Cells were analyzed using a BD Accuri C6 flow cytometer. Doublet discrimination and gating was used to select monodisperse cells. Experiments were conducted with 4-6 replicates on different days to consider the intra- and inter-day variabilities. Fluorescence of each replicate conditions was normalized to that of the intra-day buffer control.

5. Acknowledgments

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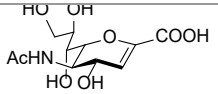
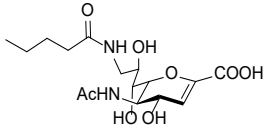
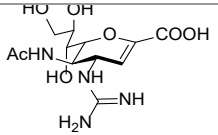
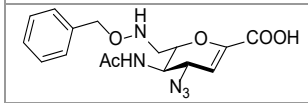
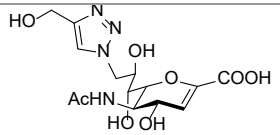
Supporting information available. This material is available free of charge via the internet. Numerical data for cell migration and cytotoxicity, siRNA knockdown and expression levels, migration after glycolipid treatment, HEK293 NEU1 overexpression, lectin staining, and integrin blots.

Table 1: Structures and activity of hNEU inhibitors used in this study.

Structure	Identifiers	IC_{50} [μM][d]				Selectivity[e]
		NEU1	NEU2	NEU3	NEU4	
	DANA[39] **1**	80	90	6	13	NEU3/4 (6X)
	C9-BA-DANA[a, 40, 72] **2**	3	>500	110	220	NEU1 (35X)
	zanamivir[42] **3**	>500	8	4	48	NEU2/3 (6X)
	**4**[b, 39]	>1000	920	24	>1000	NEU3 (39X)
	C9-4HMT-DANA[c, 43] **5**	>500	>500	80	0.16	NEU4 (500X)

a, Referred to as compound "10h" in original citation.[40] This compound has been named as a C9-butyl-*N*-amide derivative of DANA (C9-BA-DANA).[69] This could more appropriately be named as a C9-pentylamide, but we maintain this nomenclature for consistency with the literature.

b, Referred to as compound 5c in original citation.[39]

c, Referred to as compound 6 in original citation.[43] Named here as the C9-(4-hydroxymethyltriazolyl)-DANA.

d, IC_{50} values from previous studies against a 4MU-NANA substrate are provided with the indicated citation.

e, Selectivity based on IC_{50} values cited (and may be an upper limit), with fold-selectivity between the best and next-best target shown in parenthesis. For inhibitors with similar activity against more than one target, both targets are listed, and the selectivity given is based on the lowest potency.

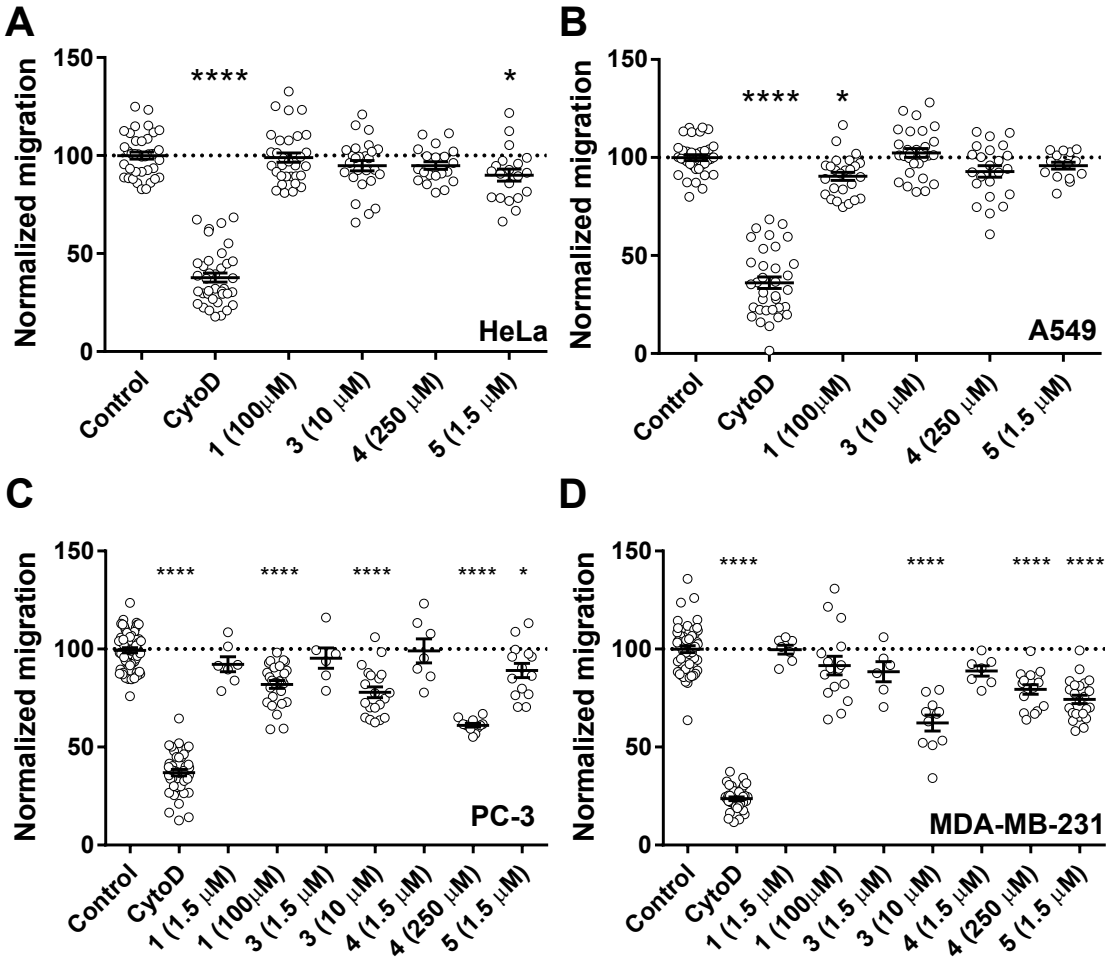


Figure 1. Inhibition of cell migration using inhibitors of neuraminidase enzymes. Normalized cell migration of four different cells treated with specific isoform-selective or non-selective inhibitors of human neuraminidase enzymes. A) HeLa, B) A549, C) PC-3, and D) MDA-MB-231. The data is presented as mean ± SEM, and conditions were compared to control using one-way ANOVA and Dunnett's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

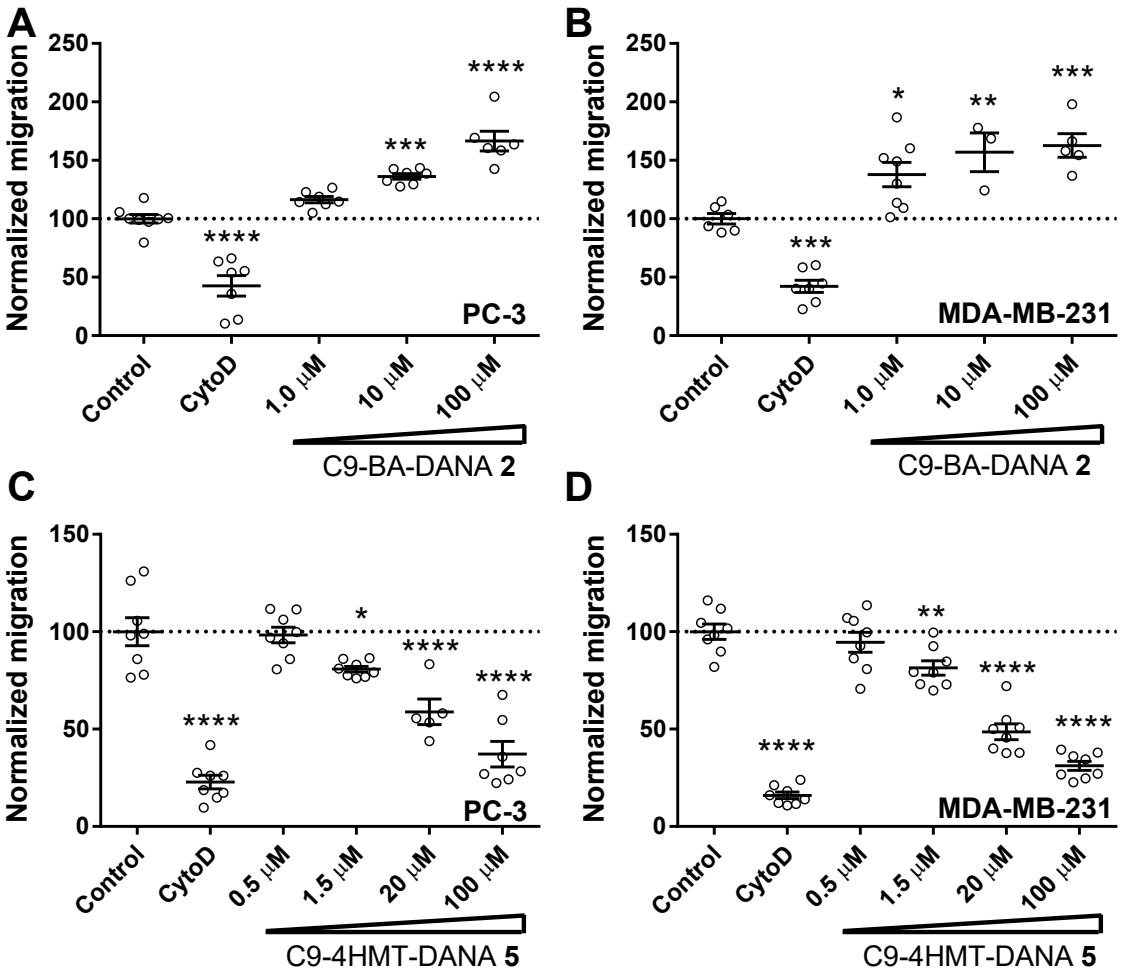


Figure 2. Dose-dependent cell migration modulation by hNEU inhibitors. Cell lines A) PC-3 and B) MDA-MB-231 were treated with 1.0, 10, and 100 μM C9-BA-DANA (**2**) and cell migration was determined. Cell lines C) PC-3 and D) MDA-MB-231 were treated with 0.5, 1.5, 20, and 100 μM C9-4HMT-DANA (**5**) and cell migration was determined. Cytochalasin D (197 nM) was used as a positive control. The data is presented as mean ± SEM, and conditions were compared to control using one-way ANOVA and Dunnett's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

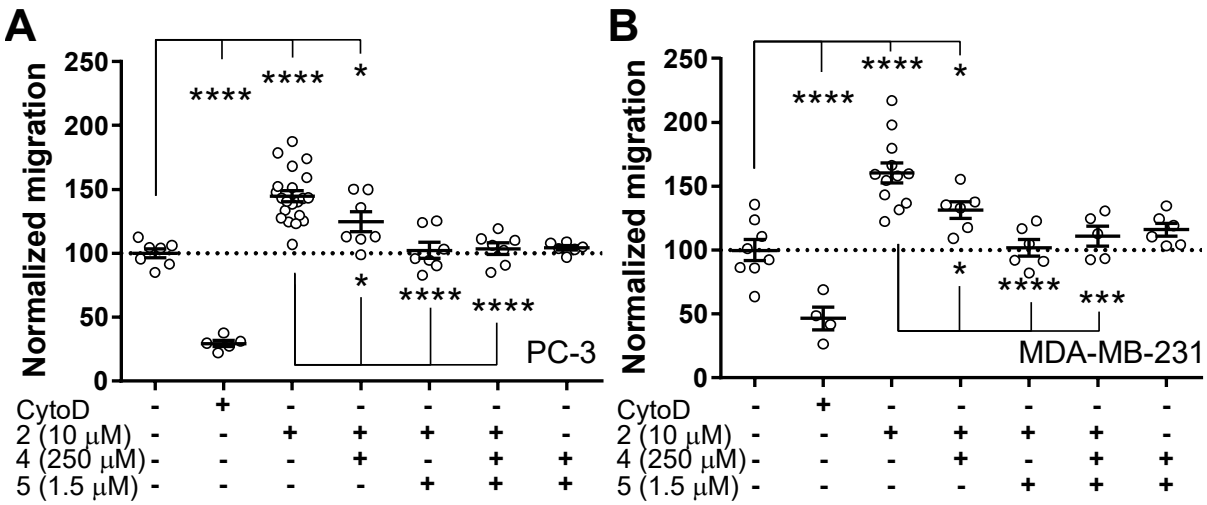


Figure 3. Combined effects of NEU inhibition. Normalized cell migration of four different cell lines treated specific NEU1 inhibitors alone or in combination with specific inhibitors of NEU3 and NEU4 enzymes in A) PC-3, and B) MDA-MB-231 cell lines. The data is presented as mean ± SEM, and conditions were compared to control using one-way ANOVA and Dunnett's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

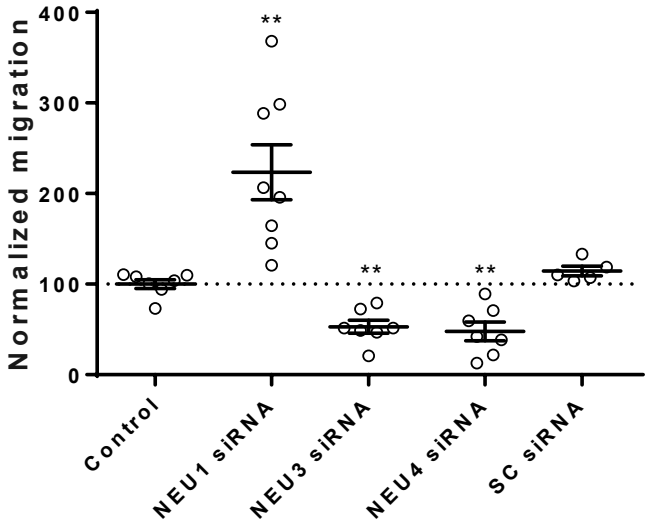


Figure 4. Effect of siRNA knockdown of NEU1, NEU3, and NEU4 on cell migration. Normalized cell migration of MDA-MB-231 cells treated NEU1 siRNA, NEU3 siRNA, NEU4 siRNA, and a scrambled control (SC siRNA) are shown. Changes in expression levels of NEU isoenzymes were confirmed by western blotting (see Supporting information). The data are presented as mean ± SEM, and conditions were compared to control using one-way ANOVA and Dunnett's t-test (**, $p \leq 0.01$).

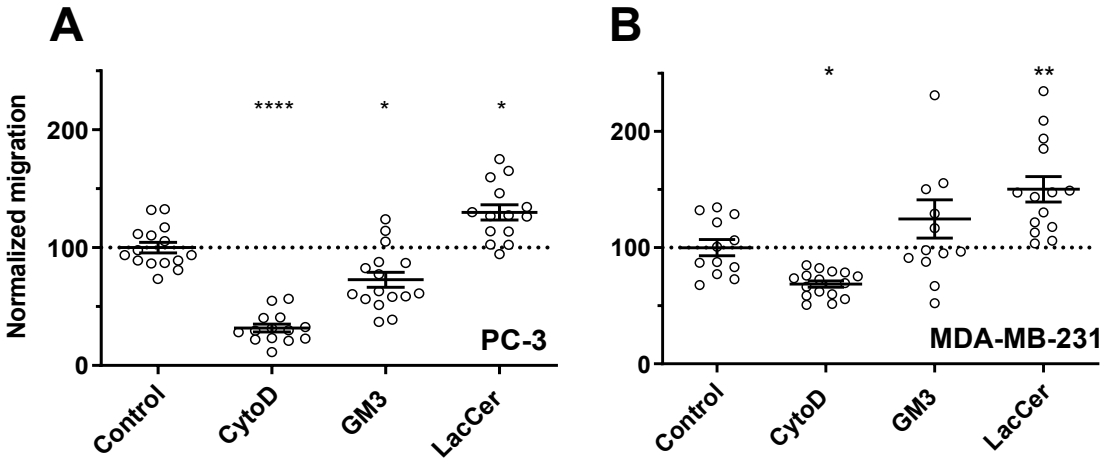


Figure 5. Effect of glycolipids on cell migration. Normalized migration of cells treated with exogenous glycolipids GM3 (50 ng/mL) and lactosylceramide (50 ng/mL) in A) PC-3 and B) MDA-MB-231 are shown. The data is presented as mean ± SEM, and conditions were compared to control using one-way ANOVA and Dunnett's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

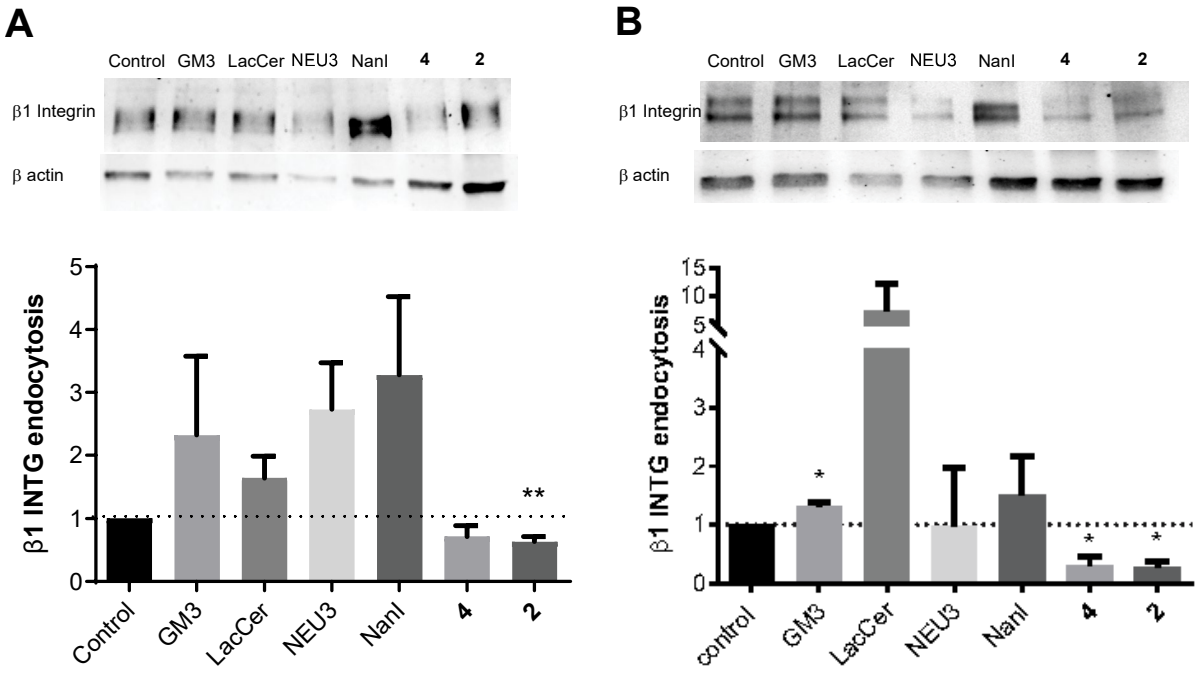


Figure 6. Neuraminidase enzymes affect the endocytosis of β1 integrin. The endocytosis of the β1 integrin was determined using biotinylation and western blotting. Blots are shown in the top row for the β1 integrin, and a β actin loading control is shown in the bottom row. Cell lines A) MDA-MB-231 and B) PC-3 were analyzed after treatment with glycolipids, enzymes (NanI or NEU3), or hNEU inhibitors (**4** and **2**). A representative blot from at least two experiments are shown. Densitometry was used to compare changes in the integrin band to the loading control, and values are plotted as the mean ± SEM and conditions were compared to control using student's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$).

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