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Synthesis of *N*-acetyl Glucosamine Analogs as Inhibitors for Hyaluronan Biosynthesis

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Abstract

Elevated hyaluronan expression is a hallmark of many types of cancer. Therefore, inhibition of hyaluronan biosynthesis can potentially slow the growth of tumor cells. Herein, we explore a chain termination strategy to reduce hyaluronan synthesis by tumor cells. Several analogs of glucosamine were prepared, which contained modifications at the C-3 positions. These analogs can possibly cap the nonreducing end of a growing hyaluronan chain, thus lowering the amount of hyaluronan synthesized. Upon incubation with pancreatic cancer cells, a fluorine-containing glucosamine analog was found to exhibit significant inhibitory activities of hyaluronan synthesis. Furthermore, it drastically reduced the proliferation of cancer cells.

Keywords

Biosynthesis; Chain terminator; Hyaluronan; Inhibitor; Salvage pathway

Introduction

Hyaluronan (HA), a member of the glycosaminoglycan family, is composed of repeating disaccharides of β -1,3-*N*-acetyl-glucosamine- β -1,4-glucuronic acid.^[1] HA plays important roles in tumor development, metastasis, and drug resistance.^[2–4] HA expression is elevated in a variety of tumors,^[5] with the high levels of HA shown to have prognostic values for various cancers, such as non-small cell lung, bladder, gastric, and prostate cancer.^[6–9] Therefore, inhibition of HA biosynthesis can be an interesting approach toward cancer treatment.

HA biosynthesis is mediated by HA synthases,^[10] which use UDP- α -*N*-acetyl-D-glucosamine (UDP-GlcNAc) and UDP- α -D-glucuronate (UDP-GlcA) as substrates to sequentially transfer GlcNAc and GlcA to the nonreducing end of a growing HA chain. 4-Methylumbelliferone (4-MU) was found to be an HA synthesis inhibitor,^[11] which functions by suppressing the expression of HA synthases.^[12] 4-MU can also serve as an acceptor for

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Supporting Information **Available**: Selected NMR spectra and images of the particle exclusion assay at low magnification.

UDP-GlcA, thus competing for the limited intracellular pool of UDP-GlcA and further reducing the amount of HA produced.^[13,14] 4-MU can reduce proliferation, motility, invasion, and metastatic properties of various types of cancer including prostate cancer and breast cancer.^[12,15–18] These observations have demonstrated the utility of HA biosynthesis inhibitors as potential antitumor agents.

Herein, we report an alternative approach to inhibit HA biosynthesis through potential early chain termination. We envision that if a modified GlcNAc analog such as **1–3** is incorporated into an HA chain, as the C-3 position does not bear a free hydroxyl moiety, HA synthesis will be terminated, leading to a reduction of the amount of HA synthesized. The key to the success of this strategy is the incorporation of the potential chain terminators (e.g., **1–3**) into the growing HA chain. The GlcNAc donor (i.e., UDP-GlcNAc) is synthesized inside the cells primarily from glucose through a series of enzymatic reactions.^[19] However, there exists a salvage pathway, which utilizes exogenous GlcNAc as the source for UDP-GlcNAc.^[20] The salvage pathway has a broad substrate scope and is well tolerant of GlcNAc structural modifications, which can allow the transformation of a variety of GlcNAc analogs into UDP glycosyl donors.^[21] The HA synthase can potentially utilize the UDP-GlcNAc analog as a glycosyl donor and incorporate the modified GlcNAc into the HA chain. The chain termination strategy has been applied to inhibition of biosynthesis of a variety of glycans^[22] including sialyl Lewis X, β -glucan, heparan sulfate, and chitin.^[23–28] Herein, we report the chemical synthesis of GlcNAc analogs and investigate their abilities to inhibit HA synthesis by cancer cells. A fluorine-containing GlcNAc analog showed high inhibitory activities toward HA synthesis and cancer cell proliferation.

Results and Discussion

Our synthetic targets are the 3-OMe, 3-oxo, and 3-F^[29] analogs of GlcNAc **4–6**. The hydroxyl groups of these analogs are protected as acetates to reduce their hydrophilicity and increase their potential to cross cell membranes during cellular incubation. Acetate groups have been shown to significantly enhance intracellular concentrations of exogenous carbohydrates added to mammalian cells, which can then be deacetylated in situ by cytosolic esterases.^[30]

In our initial route for the synthesis of these GlcNAc derivatives, we opted to use furanose oxazoline **8** as a key intermediate since it contains a free 3-OH moiety. Compound **8** was conveniently prepared in large quantities from commercially available GlcNAc **7** using anhydrous FeCl₃ as a catalyst in acetone.^[31] With oxazoline **8** in hand, methylation of its free 3-OH group by MeI and NaH gave compound **9** in quantitative yield. Subsequent acid-catalyzed hydrolysis of compound **9** with *p*-toluene sulfonic acid (PTSA) furnished the desired 3-*O*-methyl-*N*-acetyl- β -glucosamine **1**, which was acetylated using acetic anhydride in pyridine to give the product **4** in 50% overall yield over the two steps (Sch. 1).

Next we explored the synthesis of the 3-oxo GlcNAc derivative **5**. Swern oxidation of **8** produced the ketone **10** in 92% yield (Sch. 2a). However, acid-catalyzed deprotection of **10** in a similar manner, as in the synthesis of compound **4**, failed to provide the desired product **2**. One possible explanation was that compound **2** was unstable under the acidic reaction

condition. Based on this consideration, we designed an alternative route to avoid the acidic condition.

GlcNAc **7** was selectively benzylated at the anomeric position using concentrated HCl leading to compound **11**,^[28] which was followed by benzylidenation of the 4,6-hydroxyl groups generating compound **12**^[32] in an excellent 95% yield (Sch. 2b). Subsequent Swern oxidation of **12** produced 3-uloside derivative **13** in 90% yield. However, when subjected to the mild catalytic hydrogenation condition to remove the benzyl and benzylidene groups, compound **13** was transformed to multiple species. One of the major side products isolated was ketone **14**, which was presumably formed due to a retro-Claisen reaction through nucleophilic attack of methanol on **2** (Sch. 2c). Therefore, compound **2** would most likely be unstable in a nucleophilic solvent such as water and was not pursued further.

We shifted our attention to the preparation of the 3F-GlcNAc derivative **6**. The first route explored was the substitution of the free 3-OH in oxazoline **8** by a fluorine atom. However, treatment of **8** with diethylaminosulfur trifluoride (DAST) gave a complex product mixture. Exchanging **8** in this reaction with compound **12** or the corresponding epimers containing axial 3-OH did not yield any desired fluorine substitution product. This was consistent with a literature report where the yield for fluorine substitution onto C3 of a GlcNAc analog was only 10%.^[29]

Kajihara and coworkers described that 6-OH of a glucosamine analog could be substituted with fluorine with a modest yield (~38%) when phthalimide (Phth) was used as the protective group for the nitrogen.^[33] To test the effect of Phth on our fluorination reaction, we started from the Phth-containing glucosamine tetraacetate **15**,^[34,35] which was efficiently converted to β -benzyl glycoside **16**^[36] using $\text{BF}_3 \cdot \text{Et}_2\text{O}$ in CH_2Cl_2 (Sch. 3). Subsequent acetate removal with 0.3 M NaOMe in a mixture of $\text{MeOH}-\text{CH}_2\text{Cl}_2$ at -10°C to 0°C produced triol **17** in 77% yield. The low reaction temperature was important to ensure the phthalimide ring was not opened by NaOMe. The *O*-4 and *O*-6 free hydroxyl groups in **17** were protected as a benzylidene acetal by treatment with benzaldehyde dimethyl acetal in the presence of a catalytic amount of camphorsulfonic acid to yield the benzylidene glycoside **18**.^[36] To install the fluorine atom in the equatorial orientation, it was necessary to invert the configuration of the 3-OH group. This was accomplished through a four-step double-inversion protocol. Compound **18** was treated with Tf_2O in CH_2Cl_2 in the presence of pyridine to convert the free hydroxyl group into a good leaving group (i.e., triflate). Displacement of the triflate by acetate via an $\text{S}_{\text{N}}2$ manner yielded the 3-OAc glycoside, which was subsequently deprotected with NaOMe at -10°C to produce the corresponding allosamine glycoside **19**. Fluorination of compound **19** was accomplished using DAST, resulting in the inversion of configuration at *C*-3 to access the fluorinated analog mixed with a side product. Upon removal of the benzylidene-protecting group, we were able to purify the desired product **20** and identify the side product as **21**; **21** is presumably formed due to elimination of hydrogen fluoride followed by tautomerization and opening of the phthalimide ring. The overall yield of **20** from benzylidene glycoside **18** was 28%.

To remove the Phth group in **20**, it was initially treated with methylamine, but this attempt resulted in multiple degradation products. In contrast, heating **20** with excess ethylene

diamine in n-butanol at 90°C allowed the access to the free amine derivative, which on subsequent acetylation provided the acetamide **22** in 80% yield (Sch. 3). Pd-catalyzed hydrogenolysis of **22** followed by acetylation led to the desired product **6** with the α -anomer as the major product in 60% yield.

With compounds **4** and **6** in hand, we evaluated their abilities to inhibit HA biosynthesis in human pancreatic cancer cell line KP1-NL cells. 4-MU was used as a positive control. The amount of HA secreted into the culture medium as well as that residing on the cell surface was quantified by an ELISA-like assay using a Hyaluronan Assay Kit and reported on a per cell basis (Table 1). While compound **4** was not very effective in inhibiting HA biosynthesis, compound **6** significantly reduced the amount of HA formed, with an inhibition potency comparable to that of 4-MU.

As HA present in the pericellular matrix can create a barrier to the cell membrane, its presence can be visualized by the steric exclusion of fixed red blood cells to form a clear, halo-like area surrounding the KP1-NL cells (Fig. 3 and Fig. S1 in supporting information). Treatment of the cells with hyaluronidase, an enzyme capable of HA degradation, reduced the clear area around the cells to 48.2% (Fig. 3b; for quantification of the size of clear area see Fig. 3f). Compound **4** was much less potent, only leading to a small reduction to 93.1% of the vehicle control (Fig. 3d,f). In comparison, compound **6** (100 μ M) reduced the area to 40.9% (Fig. 3e,f), which was consistent with the ELISA analysis results supporting the idea that **6** could significantly inhibit HA synthesis. 4-MU (100 μ M) generated the largest change in the area under the same assay condition (Fig. 3c,f).

Next we analyzed the effects of compounds **4** and **6** on cell proliferation using an Alamar blue assay (Fig. 4). At the concentration of 100 μ M, 4-MU did not show much inhibitory activities against the pancreatic cancer cell line KP1-NL cells. 3-Methoxy analog **4** (100 μ M) reduced the proliferation of the cancer cells to 60.5% of the DMSO control, while the 3-fluorine-containing analog **6** exhibited the highest antiproliferative activities, leading to a 91% reduction in cell numbers at 100 μ M. The IC₅₀ value of **6** was determined to be 30 μ M.

In conclusion, we have developed synthetic routes to GlcNAc analogs bearing structural modifications at C-3 positions as potential inhibitors of HA biosynthesis. While the 3-OMe compound **4** was readily prepared, the intermediate to 3-oxo analog was found to be unstable. To synthesize the 3-F-containing analog **6**, fluorination of the C-3 turned out to be highly challenging. The Phth group used for protection of the nitrogen was crucial, which gave better yield for the fluorination step compared to the previous report.^[29] Upon incubation of human pancreatic cell line with compounds **4** and **6**, the 3-F analog **6** significantly reduced the amount of HA both in secreted form and on the cancer cell surface. The inhibitory activity of **6** was further confirmed by a particle exclusion assay and **6** exhibited significant antiproliferative activities against pancreatic cancer cells. It is possible that besides the potential for serving as a chain terminator, 3-F GlcNAc analog **6** can reduce HA biosynthesis by interfering with other cellular processes. The exact mechanism will require further investigation.^[37]

Experimental Section

General Experimental Procedures

All reactions were carried out under nitrogen with anhydrous solvents in flame-dried glassware, unless otherwise noted. Chemicals used were reagent grade as supplied except where noted. Compounds were visualized by UV light (254 nm) and by staining with a yellow solution containing $\text{Ce}(\text{NH}_4)_2(\text{NO}_3)_6$ (0.5 g) and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (24.0 g) in 6% H_2SO_4 (500 mL). Flash column chromatography was performed on silica gel 60 (230–400 mesh). NMR spectra were referenced using residual CHCl_3 ($\delta^1\text{H}$ NMR 7.26 ppm) and CDCl_3 ($\delta^{13}\text{C}$ NMR 77.0 ppm). Peak and coupling constant assignments are based on ^1H NMR, ^1H - ^1H gCOSY, and/or ^1H - ^{13}C gHMQC and ^1H - ^{13}C gHMBC experiments.

2-Methyl-(1,2-dideoxy-5,6-O-isopropylidene- α -D-glucofurano)-[2,1-d]-2-oxazoline (8)^[38]

Anhydrous FeCl_3 (15 g, 0.092 mol) was added to a suspension of 2-acetamido-2-deoxy-D-glucopyranose **7** (10 g, 0.068 mol) in dry acetone (200 mL), and the mixture was stirred and heated to reflux for 20 min with exclusion of moisture. The solution was cooled to 0°C, followed by addition of triethylamine (35 mL), acetone (135 mL), and a solution of sodium bicarbonate (21.3 g) in water (135 mL) with continuous stirring. Acetone, triethylamine, and some water were then removed in vacuo at <30°C. The mixture was extracted with diethyl ether (5 × 200 mL) and the combined extracts dried over MgSO_4 and concentrated at rt to give compound **8** as brownish syrup (7.15 g, 65%). The ^1H NMR spectrum is identical to what is reported in the literature.^[38] ^1H NMR (600 MHz, CDCl_3) δ 1.33 (s, 3H, CH_3), 1.39 (s, 3H, CH_3), 2.00 (s, 3H, N = COCH_3), 2.92 (s, 1H, OH-3), 3.72 (dd, 1H, $J = 3.6, 9.6$ Hz, H-4), 3.97 (dd, 1H, $J = 4.8, 8.4$ Hz, H-6), 4.11 (dd, 1H, $J = 5.4, 7.8$ Hz, H-6'), 4.28–4.32 (m, 1H, H-5), 4.39 (d, 1H, $J = 3$ Hz, H-3), 4.44 (dd, 1H, $J = 1.2, 4.8$ Hz, H-2), 6.14 (d, $J = 4.8$ Hz, H-1).

2-Methyl-(1,2-di-deoxy-3-methoxy-5,6-O-isopropylidene- α -D-glucofurano)-[2,1-d]-2-oxazoline (9)

Compound **8** (0.4 g, 1.64 mmol) was dissolved in DMF (15 mL) and cooled to 0°C. NaH (0.08 g, 3.29 mmol) was added in three portions with vigorous stirring followed by dropwise addition of MeI (0.15 mL, 2.47 mmol). The reaction mixture was allowed to warm up to rt. After 3 h, DMF was evaporated in vacuo and the residue coevaporated twice with toluene. The resultant residue was dissolved in CH_2Cl_2 (20 mL) and washed with ice cold water and the aqueous phase extracted twice with CH_2Cl_2 (2 × 20 mL). The organic phases were combined, washed with brine (30 mL), and dried with Na_2SO_4 . Upon filtration and concentration in vacuo, compound **9** was obtained in 96% yield (0.4 g). $[\alpha]_{\text{D}}^{20} - 15.5$ ($c = 1$, CH_2Cl_2); ^1H NMR (600 MHz, CDCl_3) δ 1.27 (s, 3H, CH_3), 1.33 (s, 3H, CH_3), 1.94 (s, 3H, N = CCH_3), 3.40 (s, 3H, OCH_3), 3.71 (dd, 1H, $J = 3.6, 9$ Hz, H-4), 3.77 (d, 1H, $J = 4.2$ Hz, H-3), 3.91–3.94 (m, 1H, H-6), 3.97–3.99 (m, 1H, H-6'), 4.21 (m, 1H, H-5), 4.42 (dd, 1H, $J = 1.8, 6$ Hz, H-4), 6.01 (d, 1H, $J = 6.6$ Hz, H-1); ^{13}C NMR (150 Hz, CDCl_3) δ 14.3, 25.5, 26.99, 57.98, 67.0, 72.7, 74.8, 81.5, 83.7, 107.1, 109.2, 167.2. HRMS $\text{C}_{12}\text{H}_{20}\text{NO}_5$ [$\text{M} + \text{H}^+$] calc. 258.1341 found 258.1333.

1,4,6-Tri-O-acetyl-2-acetamido-2-deoxy-3-methoxy-- β -glucopyranoside (4)

Compound **9** (0.204 g, 0.793 mmol) was dissolved in 1:2 H₂O/THF mixture (10 mL:20 mL) and *p*-toluenesulfonic acid (0.06 g, 0.264 mmol) was added to the reaction mixture then stirred overnight. The reaction was quenched with Et₃N to a neutral pH and the solvents were evaporated in vacuo. The residue was washed several times with CH₂Cl₂ and the resultant white powder dried under vacuum. The residue was then dissolved in pyridine (8 mL). Ac₂O (0.5 mL) was added and the reaction mixture was stirred at rt overnight. The reaction mixture was concentrated. The residue was dissolved in CH₂Cl₂, washed with H₂O and brine, dried over Na₂SO₄, and purified by column chromatography (CH₂Cl₂/MeOH, 8:1) to give compound **4** in 50% (0.143 g) yield over two steps. $[\alpha]_D^{20} + 10.5$ (*c* = 1, CH₂Cl₂); ¹H NMR (600 MHz, CDCl₃) δ 1.99 (s, 3H, NHCOCH₃), 2.07 (s, 3H, COCH₃), 2.10 (s, 3H, COCH₃), 2.11 (s, 3H, COCH₃), 3.40 (s, 3H, OCH₃), 3.75–3.84 (m, 3H, H-2, H-4, H-5), 4.09 (dd, 1H, J = 2.4, 10.8 Hz, H-6), 4.23 (dd, 1H, J = 4.5, 12 Hz, H-6'), 5.02 (t, 1H, J = 8.4 Hz, H-3), 5.70 (d, 1H, J = 7.2 Hz, H-1), 5.89 (d, 1H, J = 6.6 Hz, CONH); ¹³C NMR (125 Hz, CDCl₃) 20.8, 20.84, 20.9, 23.4, 53.8, 58.0, 62.0, 68.5, 72.9, 79.2, 91.97, 169.4, 169.41, 170.2, 170.7. HRMS C₁₅H₂₃NO₉ Na[M + Na⁺] calc. 384.1271 found 384.1259.

Benzyl-2-acetamido-2-deoxy- α -D-glucopyranoside (11)

N-Acetyl glucosamine **7** (6.00 g, 27.1 mmol) was dissolved in benzyl alcohol (50 mL) and concentrated HCl (2.9 mL) was added. The mixture was heated to 90°C for 3 h, cooled to rt, and then poured onto 500 mL Et₂O and stored overnight at –20° C. The resulting precipitate was recovered by filtration and rinsed with Et₂O and hexanes to yield 17.64 g of crude material, which was purified by silica gel chromatography (8% to 15% MeOH/CH₂Cl₂) to provide **11** (5.98 g, 71%) as white foam. Comparison of ¹H NMR with literature values confirmed the identity of compound **11**.^[28] ¹H NMR (500 MHz, CD₃OD), δ 1.95 (s, 3H, COCH₃), 3.37–3.39 (m, 1H), 3.69–3.73 (m, 3H), 3.82 (d, 1H, J = 9.5 Hz), 3.89 (dd, 1H, J = 3.5, 11 Hz), 4.49 (d, 1H, J = 12 Hz), 4.74 (d, 1H, J = 11.5 Hz), 7.28–7.40 (m, 5H, aromatic).

Benzyl-2-acetamido-2-deoxy-4,6-O-benzylidene- α -D-glucopyranoside (12)

Compound **11** (0.412 g, 1.32 mmol) was dissolved in DMF (20 mL) followed by the addition of benzaldehyde dimethyl acetal (0.6 mL, 3.97 mmol) and a catalytic amount of *p*-toluenesulfonic acid (0.123 g, 0.53 mmol). The reaction mixture was stirred for 3 h. DMF was removed in vacuo and the resultant white residue was suspended in saturated sodium bicarbonate solution. Upon filtration, the residue was washed several times with hexanes/EtOAc/CH₂Cl₂ (4/1/0.5) solvent system then dried under vacuum to afford compound **12** in 95% yield (0.5 g). Comparison of ¹H NMR with reported literature values confirmed the identity of compound **12**.^[32] ¹H NMR (600 MHz, CDCl₃) δ 1.82 (s, 3H, CH₃), 3.59 (t, 1H, J = 9 Hz, H-6), 3.75 (t, 1H, J = 10.2 Hz, H-6'), 3.84–3.91 (m, 1H, H-5), 3.92–3.95 (m, 1H, H-4), 4.23–4.26 (m, 2H, H-2, H-3), 4.93 (d, 1H, J = 12 Hz, CH₂Ph), 4.74 (d, 1H, J = 12 Hz, CH₂Ph), 4.93 (d, 1H, J = 3.5 Hz, H-1), 5.57 (s, 1H, CHPh), 5.82 (d, 1H, J = 8.4 Hz, CONH), 7.33–7.51 (m, 10H, aromatic).

Benzyl-2-acetamido-2-deoxy-4,6-O-benzylidene-3-oxo- α -D-glucopyranoside (13)

To a solution of COCl_2 (0.11 mL, 1.25 mmol) and DMSO (0.16 mL, 2.23 mmol) in CH_2Cl_2 (30 mL) cooled to -78°C and stirred for 20 min was added compound **12** (0.36 g, 0.894 mmol) dissolved in CH_2Cl_2 (20 mL). After 20 min, Et_3N (0.63 mL, 4.47 mmol) was added and the reaction mixture allowed to warm to rt then quenched with H_2O (100 mL). The aqueous layer was extracted with CH_2Cl_2 (50 mL). The organic layers were combined and washed with brine and dried over MgSO_4 . Compound **13** was obtained in 90% yield (0.32 g)

by recrystallization from hexanes/ $\text{EtOAc}/\text{CH}_2\text{Cl}_2$ (5/1/0.5). $[\alpha]_{\text{D}}^{20} + 38.8$ ($c = 0.1$, CH_2Cl_2); ^1H NMR (500 MHz, CDCl_3) δ 2.00 (s, 3H, CH_3), 3.90 (t, 1H, $J = 10.5$ Hz, H-6), 4.11–4.16 (m, 1H), 4.29 (dd, 1H, $J = 5, 10.5$ Hz, H-6'), 4.37 (d, 1H, $J = 10.5$ Hz, H-4), 4.50 (d, 1H, $J = 12$ Hz, CH_2Ph), 4.69 (d, 1H, $J = 12$ Hz, CH_2Ph), 4.95–4.97 (m, 1H, H-2), 5.41 (d, 1H, $J = 4.5$ Hz, H-1), 5.56 (s, 1H, CHPh), 6.22 (d, 1H, $J = 8.0$ Hz, CONH), 7.25–7.49 (m, 10H, aromatic); ^{13}C NMR (125 Hz, CDCl_3) δ 23.2, 59.1, 66.6, 69.6, 70.8, 77.0, 77.2, 77.5, 82.9, 100.8, 102.2, 126.6, 128.2, 128.6, 128.9, 129.6, 136.5, 170.1, 195.2. HRMS $\text{C}_{22}\text{H}_{24}\text{NO}_6$ $[\text{M} + \text{H}^+]$ calc. 398.1580 found 398.1577.

Benzyl-3,4,6-tri-O-acetyl-2-deoxy 2-phthalamido- β -D-glucopyranoside (16)

Compound **15** (2.2 g, 4.61 mmol) was dissolved in CH_2Cl_2 (30 mL), followed by addition of BnOH (1.1 mL, 9.22 mmol). BF_3OEt_2 (1.75 mL, 11.4 mmol) was added dropwise and the reaction mixture was stirred at rt for 24 h. The mixture was diluted with CH_2Cl_2 (20 mL) and washed with saturated NaHCO_3 (3×20 mL). The aqueous layer was extracted with CH_2Cl_2 (60 mL) and the combined organic phases were dried over Na_2SO_4 , filtered, concentrated, and purified by column purification (hexanes/ EtOAc 3:2) to yield compound **16** (1.92 g, 79%). Comparison of ^1H NMR with reported literature values confirmed the identity of compound **16**.^[36] ^1H NMR (600 MHz, CDCl_3) δ 1.86 (s, 3H, COCH_3), 2.02 (s, 3H, COCH_3), 2.13 (s, 3H, COCH_3), 3.85–3.88 (m, 1H, H-5), 4.19 (dd, 1H, $J = 2.4, 12$ Hz, H-6), 4.33–4.39 (m, 2H, H-2, H-6'), 4.53 (d, 1H, $J = 12$ Hz, CHPh), 4.84 (d, 1H, $J = 12.6$ Hz, CHPh), 5.17 (t, 1H, $J = 9.6$ Hz, H-4), 5.37 (d, 1H, $J = 8.4$ Hz, H-1), 5.77 (dd, 1H, $J = 9.0, 10.2$ Hz, H-3), 7.08–7.79 (m, 9H, aromatic).

Benzyl-4,6-O-benzylidene-2-deoxy-2-phthalamido- β -D-glucopyranoside (18)

Compound **16** (1.88 g, 3.79 mmol) was dissolved in $\text{MeOH}/\text{CH}_2\text{Cl}_2$ solvent mixture (3:2, 20 mL) and cooled to -10°C followed by dropwise addition of 0.3M NaOMe in MeOH (6 mL). Upon completion of the reaction as established by TLC in 2 h, the reaction mixture was neutralized with Amberlite ion exchange resin to pH 7. The crude triol **17** was concentrated and the residue was coevaporated with toluene twice followed by drying under vacuum overnight. The residue was then suspended in CH_3CN (15 mL) and $\text{PhCH}(\text{OCH}_3)_2$ (0.7 mL, 4.55 mmol) was added, followed by addition of camphorsulfonic acid (0.2 g, 0.87 mmol). The mixture was stirred for 3 h then quenched with Et_3N upon completion of reaction as confirmed by TLC. Solvents were evaporated and the residue was purified by column chromatography (hexanes/ EtOAc 3:1) to yield compound **18** (1.42 g, 77%). The identity of compound **18** was confirmed by comparison with reported literature values.^[36] ^1H NMR (600 MHz, CDCl_3) 3.62–3.67 (m, 2H, H-4, H-6), 3.85–3.88 (m, 1H, H-6), 4.29–4.32 (m, 1H, H-2), 4.42 (dd, 1H, $J = 4.8, 10.8$ Hz, H-5), 4.52 (d, 1H, $J = 12.6$ Hz, CHPh), 4.63–

4.67 (m, 1H, H-3), 4.84 (d, 1H, $J = 12$ Hz, CHPh), 5.28 (d, 1H, $J = 8.4$ Hz, H-1), 5.58 (s, 1H CHPh) 7.03–7.79 (m, 14H, aromatic).

Benzyl-2-deoxy-3-fluoro-2-phthalamido- β -D-glucopyranoside (20)

To a solution of compound **18** (0.833 g, 1.71 mmol) dissolved in CH_2Cl_2 (30 mL), pyridine (0.7 mL, 8.54 mmol) was added and the mixture was cooled to -20°C . TiF_4 (0.6 mL, 3.42 mmol) dissolved in CH_2Cl_2 (1 mL) was added drop-wise and the reaction mixture was allowed to warm up to rt within 1.5 h. The reaction mixture was diluted with CH_2Cl_2 (20 mL) and then quenched with saturated NaHCO_3 , washed with water and brine, dried over Na_2SO_4 , and concentrated under reduced pressure at ambient temperature (25 – 30°C). The resultant yellow syrup was dissolved in toluene, TBAOAc (3.09 g, 10.26 mmol) added, and the mixture stirred at 65°C overnight. The mixture was allowed to cool to rt then diluted with EtOAc. Solvents were evaporated and the resultant residue was purified by column chromatography (hexanes/EtOAc 3.5:1) to yield benzyl-3-*O*-acetyl-2-deoxy-4,6-*O*-benzylidene-2-phthalamido- β -D-allopyranoside. $[\alpha]_{\text{D}}^{20} -47.0$ ($c = 0.5$, CH_2Cl_2); ^1H NMR (600 MHz, CDCl_3) δ 2.07 (s, 3H, COCH_3), 3.86–3.93 (m, 2H, H-4, H-6), 4.18–4.22 (m, 1H, H-6), 4.44–4.49 (m, 2H, H-2, H-4), 4.64 (d, 1H, $J = 10.8$ Hz, CHPh), 4.93 (d, CHPh $J = 12$ Hz, CHPh), 5.61 (s, 1H, CHPh), 5.77–5.78 (m, 1H, H-3), 6.15 (d, 1H, $J = 8.4$, H-1), 7.21–7.84 (m, 14H, aromatic). HRMS $\text{C}_{30}\text{H}_{27}\text{NO}_8\text{Na}$ $[\text{M} + \text{Na}^+]$ calc. 552.1634 found 552.1595. Benzyl-3-*O*-acetyl-2-deoxy-4,6-*O*-benzylidene-2-phthalamido- β -D-allopyranoside (0.6 g, 1.13 mmol) was dissolved in $\text{MeOH}/\text{CH}_2\text{Cl}_2$ (3:2, 10 mL), cooled to -10°C , and treated with 0.3M NaOMe solution in methanol (3 mL). After 1 h, the reaction was complete as confirmed by TLC and subsequently quenched with Amberlite ion exchange resin (IR 120) to pH 7. Solvents were evaporated and the resultant white solid was coevaporated with toluene (3×10 mL) then dried under vacuum for 2 h. The allosamine analog **19** was dissolved in CH_2Cl_2 (10 mL) in a 50-mL falcon tube and cooled to -5°C . This was followed by dropwise addition of DAST (0.27 mL, 6.78 mmol) and the reaction mixture was allowed to warm to rt over 3 h. The reaction mixture was cooled to -5°C followed by dropwise addition of MeOH to destroy the excess DAST, concentrated, and passed through a short silica column. The resultant mixture of compound was dissolved in $\text{MeOH}/\text{CH}_2\text{Cl}_2$ (3:2, 10 mL) followed by addition of AcCl (0.16 mL, 2.26 mmol), then stirred overnight at rt. The reaction mixture was quenched with Et_3N to pH 7. Solvents were evaporated in vacuo and the resultant residue was purified by column chromatography (hexanes/EtOAc/MeOH 3:1:0.1) to acquire the desired compound **20** (0.17 g, 28% yield from **18**) and elimination product **21** (0.07 g, 14.5%). **20**: $[\alpha]_{\text{D}}^{20} -64.0$ ($c = 0.5$, CH_2Cl_2); ^1H NMR (600 MHz, CDCl_3) δ .29 (s, 1H, OH), 3.11 (s, 1H, OH), 3.52–3.54 (m, 1H, H-5), 3.89–3.99 (m, 3H, H-4, H-6, H-6'), 4.34–4.39 (m, 1H, H-2), 4.53 (d, 1H, $J = 12$ Hz, CHPh), 4.79 (d, 1H, $J = 12$ Hz, CHPh), 5.13–5.25 (m, 3H), 7.05–7.26 (m, 5H, aromatic), 7.22–7.80 (m, 4H, aromatic); ^{19}F NMR (282 MHz, CDCl_3) -194.44 (dt, 1F, $J = 51.9, 13.8$ Hz). HRMS $\text{C}_{21}\text{H}_{20}\text{NaO}_6\text{NF}$ $[\text{M} + \text{Na}^+]$ calc. 424.1167 found 424.1172.

Benzyl-3-deoxy-2-(methylcarbamoyl)benzoate-6-methoxy-4-oxo- β -D-glucopyranoside (21)

$[\alpha]_{\text{D}}^{20} -51.4$ ($c = 0.5$, CH_2Cl_2); ^1H NMR (600 MHz, CDCl_3) 2.24 (dd, 1H, $J = 4.2, 13.2$ Hz, H-3), 2.48 (t, 1H, $J = 13.2$ Hz, H-3), 3.32 (s, 3H, OCH_3), 3.36 (s, 3H, OCH_3), 3.73–3.74

(m, 1H, H-5), 3.88–3.96 (m, 2H, H-6, H-6'), 4.43–4.48 (m, 1H, H-2), 4.58 (d, 1H, $J = 12$ Hz, CHPh), 4.79 (d, 1H, $J = 12$ Hz, CHPh), 5.33 (d, 1H, $J = 8.4$ Hz, H-1), 7.08–7.16 (m, 5H, aromatic), 7.70–7.84 (m, 4H, aromatic); ^{13}C NMR (150 Hz, CDCl_3) 32.6, 49.4, 49.95, 50.6, 60.9, 71.2, 80.1, 98.7, 99.4, 123.3, 123.6, 127.6, 127.8, 127.9, 128.1, 128.3, 128.5, 131.4, 131.5, 131.7, 134.0, 167.6, 167.8, 204.4, 204.7. HRMS $\text{C}_{23}\text{H}_{29}\text{N}_2\text{O}_7$ $[\text{M} + \text{NH}_4^+]$ calc. 445.1975 found 445.1994.

Benzyl-4,6-tri-O-acetyl-2-acetamido-2-deoxy-3-fluoro- β -D-glucopyranoside (22)

Compound **20** (0.34 g, 0.847 mmol) was dissolved in n -butanol (10 mL) and treated with ethylene diamine (6 mL). The reaction mixture was heated at 90°C for 23 h. Solvents were then removed under reduced pressure and the resultant residue was coevaporated with toluene (3×10 mL), which was followed by drying in vacuo for 3 h. The intermediate free amine was dissolved in pyridine (10 mL), followed by addition of Ac_2O (6 mL), and was stirred overnight at rt. The mixture was treated with EtOH to react with excess Ac_2O , concentrated, dissolved in CH_2Cl_2 , washed with water and brine, and dried over Na_2SO_4 , followed by purification by column chromatography (hexanes/EtOAc/ CH_2Cl_2 /MeOH, 2:1:1:0.2) to afford compound **22** (0.27 g, 80%). ^1H NMR (600 MHz, CDCl_3) δ 1.94 (s, 3H, NHCOCH_3), 2.09 (s, 3H, COCH_3), 2.10 (s, 3H, COCH_3), 3.33–3.38 (m, 1H, H-2), 3.66–3.67 (m, 1H, H-5), 4.14–4.17 (m, 1H, H-6), 4.27 (dd, 1H, $J = 5, 12.5$ Hz, H-6') 4.57 (d, 1H, $J = 12.5$ Hz, CHPh), 4.88 (d, 1H, $J = 12$ Hz, CHPh), 5.10–5.24 (m, 3H, H-1, H-3, H-4), 5.78 (d, 1H, $J = 7$ Hz, CONH); ^{19}F NMR (282.2 MHz, CDCl_3) –193.26 (dt, 1F, $J = 53.3, 12.4$ Hz). HRMS $\text{C}_{19}\text{H}_{25}\text{FNO}_7$ $[\text{M} + \text{H}^+]$ calc. 398.1615 found 398.1595.

1,4,6-Tri-O-acetyl-2-acetamido-2-deoxy-3-fluoro- α -D-glucopyranoside (6)

Compound **22** (20 mg, 0.05 mmol) was dissolved in MeOH (6 mL) and treated with Pd/C (30 mg). The reaction mixture was stirred under hydrogen for 36 h and the reaction confirmed as complete by TLC. The mixture was filtered and concentrated and the residue coevaporated with toluene (3×10 mL) followed by drying under vacuum. The resultant residue was dissolved in pyridine (3 mL), treated with Ac_2O (0.5 mL), and stirred overnight at rt. The mixture was concentrated; washed with 1M HCl, saturated NaHCO_3 , and brine; and dried over Na_2SO_4 . Column purification (hexanes/EtOAc, 1.5:1) afforded compound **6** (15 mg, 76% yield over two steps). $[\alpha]_{\text{D}}^{20} +10.1$ ($c = 1$, CH_2Cl_2); ^1H NMR (600 MHz, CDCl_3) δ 2.03 (s, 3H, NHCOCH_3), 2.09 (s, 3H, COCH_3), 2.11 (s, 3H, COCH_3), 2.16 (s, 3H, COCH_3), 3.93–3.96 (m, 1H, H-5), 4.06–4.09 (m, 1H, H-6), 4.22 (d, 1H, $J = 4.2, 12.6$ Hz, H-6'), 4.58–4.69 (m, 2H, H-2, H-3), 5.25–5.30 (m, 1H, H-4), 5.52 (d, 1H, $J = 7.8$ Hz, NH), 6.20 (t, 1H, $J = 3$ Hz, H-1); ^{13}C NMR (150 Hz, CDCl_3) 20.6, 20.7, 20.8, 23.2, 50.9, 50.96, 61.3, 68.1, 68.2, 69.5, 69.6, 88.97, 90.2, 90.9, 91.0, 168.4, 169.0, 170.0, 170.7; ^{19}F NMR (282.2 MHz, CDCl_3) –194.24 (dt, 1F, $J = 50.8, 13.5$ Hz). HRMS $\text{C}_{14}\text{H}_{24}\text{N}_2\text{O}_8\text{F}$ $[\text{M} + \text{NH}_4^+]$ calc. 367.1517 found 367.1512.

Determination of HA Concentration by an ELISA-Like Assay

KP1-NL cells (passage 3) were seeded in 35-mm culture dishes (6×10^5 cells/dish) and cultured for 24 h. Cells were further cultured in fresh medium containing each compound for 48 h. The culture supernatants were collected and the cells were lysed by 1% Nonidet P-40,

140 mM NaCl, 10 mM EDTA, and 20 mM Tris-HCl (pH 7.4). HA released into the culture medium and accumulated in cell layers was quantified by an ELISA-like assay using HA-binding protein (HABP) according to the manufacturer's instructions for the Hyaluronan Assay Kit (Seikagaku Co., Tokyo, Japan). The absorbance at 490 nm (control wavelength, 630 nm) of each well was measured by a microplate spectrophotometer xMark (Bio-Rad, Tokyo, Japan). The quantity of HA was expressed per live cell number. The viability of cells was assessed by trypan blue staining.

Particle Exclusion Assay

KP1-NL cells (passage 5) plated at 2.5×10^4 cells in a 35-mm dish were cultured for 24 h and then further cultured for 48 h with or without 100 μ M of the compound of interest in fresh medium containing 0.1% DMSO. An aliquot of glutaraldehyde-stabilized sheep erythrocytes (5×10^8) in 0.75 mL of PBS was then added to the culture medium. After 15 min, the culture was observed using an inverted phase-contrast microscope (Olympus IMT-2).^[39] The sizes of the clear areas were quantified using ImageJ and the standard deviations of at least five areas in each image were reported.

Estimation of Cell Proliferation

Cell numbers were estimated using the Alamar Blue assay, which is based on the oxidation–reduction reaction of cells.^[40] Briefly, KP1-NL cells (passage 3, 625 cells/well) were cultured overnight in 90 μ L of medium in 96-well flat-bottomed microplates (Iwaki Glass Co., Chiba, Japan) in triplicate and were further cultured for 72 h with or without 0.1 mM of the test compound in freshly served medium containing 0.1% DMSO. Alamar Blue working solution (10 μ L, BioSource, Camarillo, CA) was then added to each well and the plate was further incubated for 3 h at 37°C in a CO₂ incubator. The fluorescence of each well was measured at ex 544 nm and em 590 nm using a Fluoroskan II (Japan Flow Laboratories, Tokyo, Japan). Reported values represented the means of replicate wells and the standard deviations for each are reported. The reaction was linear in the range of 300–3500 fluorescence units, corresponding to 300–20,000 KP1-NL cells/well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures of compounds 1–3 (color figure available online).

Figure 2.
Structures of compounds 4–6 (color figure available online).

Figure 3.

Effect of various compounds on the formation of a pericellular HA matrix by KP1-NL cells as assessed by a particle exclusion assay. Representative pictures of KP1-NL cells following incubation with (a) DMSO control; (b) *Streptomyces* hyaluronidase (1.0 U/mL); (c) 4-MU (100 μ M); (d) GlcNAc analog **4** (100 μ M); and (e) GlcNAc analog **6** (100 μ M). Fixed sheep erythrocytes (the small cells in the pictures) were then added to the cells to help visualize the pericellular matrix around the cancer cells. The pictures were taken at identical magnification (each picture represents an area of 100 μ m in width). (f) Quantification of the relative sizes of areas in particle exclusion assay assessed using Image J.

Figure 4.

Number of KP1-NL cells upon incubation with 4-MU, compound **4**, and compound **6** respectively at 100 μ M for 72 hours as determined by Alamar blue assay. The number of cells treated with DMSO was set as 100% (color figure available online).

Scheme 1.

Reagents and Conditions: i) Acetone/2 eq FeCl₃, reflux, 60°C, 65%; ii) 2 eq NaH, 1.5 eq MeI, DMF, 0°C to rt, 96%; iii) 0.4 eq PTSA, THF/H₂O 2:1; iv) Ac₂O/Pyridine, rt, 50%.

Scheme 2.

Reagents and Conditions: i) 1.4 eq COCl_2 , 2.5 eq DMSO, 5 eq Et_3N , -75°C to rt, 92%; ii) 0.4 eq PTSA, $\text{THF}/\text{H}_2\text{O}$ 2:1; iii) BnOH , HCl , 90°C , 71%, iv) 5 eq $\text{PhCH}(\text{OMe})_2$, 0.6 eq camphorsulfonic acid, DMF, rt, 95%, v) 1.4 eq COCl_2 , 2.5 eq DMSO, 5 eq Et_3N , -75°C to rt, 90%; vi) H_2 , Pd/C, MeOH.

Scheme 3.

Reagents and Conditions: i) 2 eq BnOH, 3 eq BF_3OEt_2 , CH_2Cl_2 , 79%; ii) 0.3 M NaOMe, MeOH/ CH_2Cl_2 , -10°C ; iii) $\text{PhCH}(\text{OMe})_2$, 0.08 eq camphorsulfonic acid, CH_3CN , 77%; iv) 2 eq Tf_2O , 5 eq pyridine, -20°C to 10°C ; 6 eq tetrabutylammonium acetate, toluene, 60°C %; v) 6 eq DAST, CH_2Cl_2 , -5°C to rt; vi) 6 eq AcCl, MeOH/ CH_2Cl_2 , 28% yield from **18**; vii) $\text{H}_2\text{N}(\text{CH}_2)_2\text{NH}_2$, nBuOH, 90°C ; acetic anhydride, pyridine rt, 80%; viii) H_2 , Pd/C, MeOH; acetic anhydride, pyridine, rt, 60%.

Table 1
Amount of HA secreted into culture medium and on surface of human pancreatic cancer cell KP1-NL upon incubation with various compounds

		Culture medium		Cell surface	
		Amount of HA ($\mu\text{g}/10^6$ cells)	% of control	Amount of HA ($\mu\text{g}/10^6$ cells)	% of control
DMSO			100		100
4-MU	10 μM	3.02 $\pm$ 0.03	99.2	0.17 $\pm$ 0.01	100.2
	100 μM	1.66 $\pm$ 0.04	54.9	0.12 $\pm$ 0.01	68.4
4	10 μM	2.95 $\pm$ 0.02	97.5	0.15 $\pm$ 0.02	89.1
	100 μM	2.65 $\pm$ 0.01	87.7	0.14 $\pm$ 0.02	81.8
6	10 μM	2.40 $\pm$ 0.02	79.6	0.14 $\pm$ 0.01	82.9
	100 μM	1.89 $\pm$ 0.06	62.7	0.11 $\pm$ 0.01	64.9