Sugar Derivatives

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Glycomics for Drug Discovery: Metabolic Perturbation in Androgen-Independent Prostate Cancer Cells Induced by Unnatural Hexosamine Mimics**

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Prostate cancer is the most frequently diagnosed malignancy and the second leading cause of cancer-related deaths among men. The incidence is very high in the United States followed by Australia/New Zealand and Europe. The American Cancer Society estimated that in the United States about 240890 new cases of prostate cancer will be diagnosed and in 2011 about 33720 cases will be fatal.[1] Androgens are critical to the growth and differentiation of prostate epithelial cells. Therefore an androgen ablation therapy is the first choice for the treatment of advanced prostate cancer. However, metastatic prostate cancer eventually becomes hormone-refractory and resistant to many treatments, while it is initially responsive to androgen ablation. Although systemic chemotherapy has been evaluated in men with hormone-refractory prostate cancer for many years, there is no current standard treatment for such a metastatic androgen-independent prostate cancer.[2,3] It is clear that novel therapeutic approaches are strongly needed to improve the outlook for patients suffering from advanced prostate cancer.

Cell-surface glycans regulate many different states of cancer progression such as proliferation, invasion, angiogenesis, and metastasis.[4] Cancer cells often express many aberrant glycoforms when compared with normal cells. It is documented that the expression of highly branched N-glycans is distinctly enhanced during the proliferation of different cancer cells and metastasis.[5,6] Recently, Novotny and coworkers reported an increase of the expression of highly branched N-glycans, such as tri- and tetra-antennary structures, in the serum of human metastatic prostate cancer patients when compared with the expression level in the serum of healthy individuals.[7] We also observed similar alterations of N-glycan profiles in the human prostate cancer cell line PC-3 in comparison with those of normal human prostate epithelial cells.[8] Therefore, it is considered that inhibitors and modifiers of the biosynthesis of such highly branched glycans may become novel candidates for cancer therapy.[9–13] The Golgi N-glycan biosynthetic pathway seems to be ultrasensitive to the hexosamine flux that is required for the production of tri- and tetra-antennary N-glycans, which regulate topologies/distribution and functional roles of different cell-surface glycoprotein receptors.[14] Increasing the intracellular uridyl diphosphate *N*-acetyl-glucosamine (UDP-GlcNAc) concentration leads to increased branching of N-glycans of such glycoprotein receptors[15] and enhances the affinity with the cell-surface galectin-3, a key lattice-forming lectin.[16,17] In cancer cells, the interaction between glycoprotein receptors having multiple hyperbranched N-glycans and galectin-3 seems to be crucial for retaining growth-promoting receptors at the cell surface by forming galectin-mediated glycoprotein lattice. Reagents that can control the intracellular UDP-GlcNAc levels and N-glycan branching by perturbing a hexosamine biosynthetic pathway may become promising leads to the discovery of a new class of anti-prostate-cancer drugs. GlcNAc can normally be salvaged from glycoprotein turnover by degradation in lysosomes and be employed for further enzymatic modifications in the cytoplasm to re-enter the UDP-GlcNAc pool (Scheme 1 A). We hypothesized that GlcNAc analogues bearing a fluorine atom at the C4 position[18–20] might depress the expression levels of such hyperbranched N-glycans in human prostate cancer PC-3 cells[21] by perturbing the hexosamine biosynthetic pathway.

Herein, we communicate for the first time evidence for and the mechanism of a metabolic inhibitory effect on human prostate cancer cell proliferation by a new class of 2-acetamido-2,4-dideoxy-4-fluoro-D-glucopyranose (**1**, FGN-2Ac) derivatives, notably the known 2-acetamido-2,4-dideoxy-1,3,6-tri-*O*-acetyl-4-fluoro-D-glucopyranose (**2**, FGN-1236Ac)[18] and the novel 2-acetamido-2,4-dideoxy-3,6-di-*O*-acetyl-4-fluoro-D-glucopyranose (**3**, FGN-236Ac; Scheme 1 B, C). Synthetic 4F-GlcNAc-1-phosphate is converted into UDP-4F-GlcNAc (**5**) efficiently in vitro by the recombinant human UDP-GlcNAc pyrophophorylase in the presence of uridine triphosphate (UTP);[22] thus per-*O*-acetate **2** and its analogue may be accepted in the hexosamine biosynthetic pathway leading to compound **5**, an unnatural sugar nucleotide (Scheme 1 C). We thought that an interconversion between closed and open forms at the anomeric carbon of compound **3** (1-OH analogue) might influence a series of metabolic reactions of enzymes comprising the GlcNAc salvage pathway and result in a different biological activity against cancer cell growth in comparison with that of per-*O*-acetate **2**. To obtain a set of the required derivatives **1**–**4**, we

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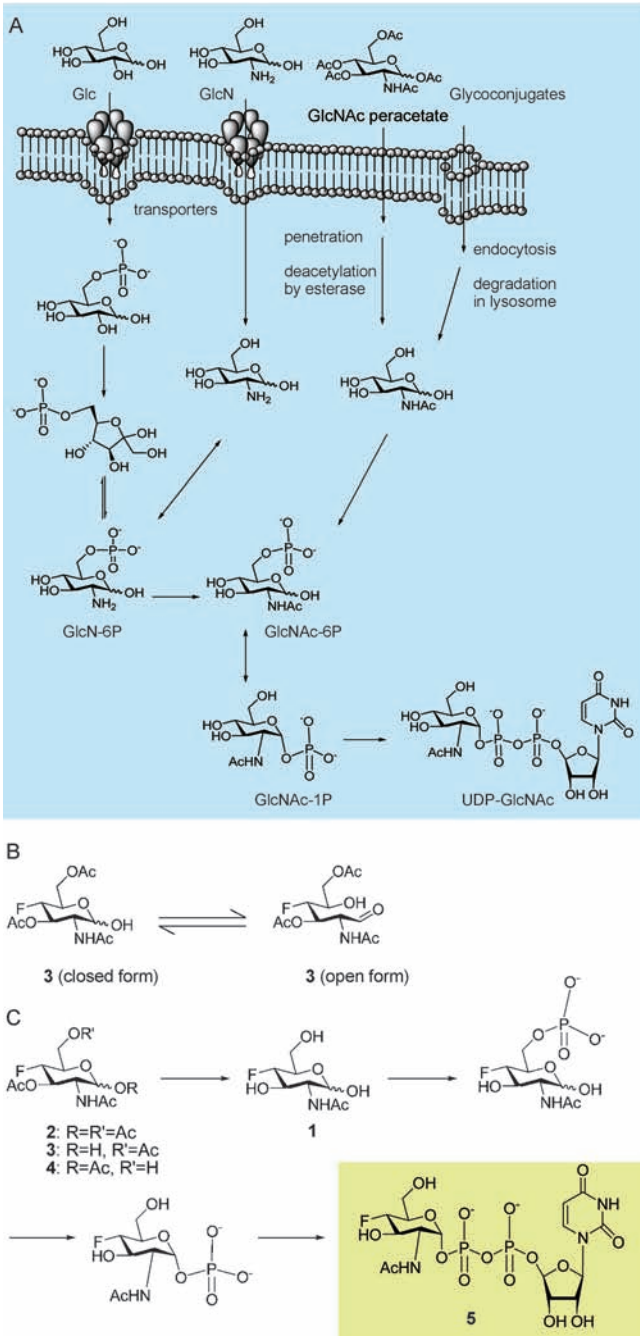
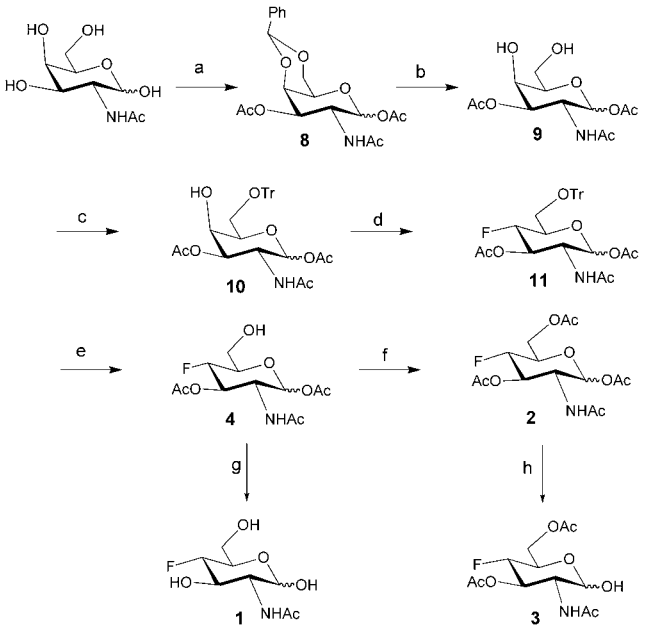


Scheme 1. Mammalian hexosamine biosynthetic pathway and compounds used in this study. A) Possible pathway using Glc, GlcN, or GlcNAc salvaged from endocytic glycoconjugates. Per-*O*-acetate of GlcNAc exogenously added can penetrate into cells across the plasma membrane owing to enhanced hydrophobicity. B) Equilibrium between the open and closed forms of **3**. C) Proposed synthesis of the unnatural sugar nucleotide **5** from compounds **2**–**4** by hijacking a biosynthetic pathway.

designed a facile and efficient synthetic route from *N*-acetylgalactosamine (GalNAc) as a starting material that might be feasible for large-scale synthesis as indicated in Scheme 2. The effect of compounds **1**–**4** on PC-3 cell viability after 48 h treatment is shown in Figure 1 A. As anticipated, FGN-1236Ac (**2**) showed a significant inhibitory effect on PC-3 cell proliferation ($IC_{50} = 277\ \mu M$; IC_{50} = concentration required for 50% inhibition of target cells), whereas the parent non-*O*-acetylated FGN-2Ac (**1**) did not have any influence owing to the poor cell permeability. Surprisingly, FGN-236Ac (**3**) exhibited the highest growth inhibition against PC-3 cells ($IC_{50} = 61\ \mu M$) among all 4F-GlcNAc analogues tested herein. In contrast, FGN-123Ac (**4**) having a 6-OH group exhibited little inhibitory effect, indicating that the position of *O*-acetyl groups influences the metabolic acceptability of these molecules on the cytoplasmic side. Strikingly, a partially *O*-acetylated GlcNAc analogue having a 1-OH group also showed a similar type of activity to that of FGN-236Ac (**3**; Figure S1 in the Supporting Information), thus suggesting that the free hemiacetal group liberated predominantly during de-*O*-acetylation within cells may specifically perturb further enzymatic modification processes. Because similar results were obtained in a common human lung cancer cell line, A549 (Figure S2 in the Supporting Information), we may conclude that per-*O*-acetylated derivative FGN-1236Ac (**2**) is a precursor of the metabolic inhibitor of human cancer cell proliferation, and spontaneous liberation of the hemiacetal group during de-*O*-acetylation within cells induces perturbation in the GlcNAc salvage pathway. The inhibitory effect on PC-3 cell growth of FGN-236Ac (**3**) seemed to be higher than that of 5-fluorouracil (5-FU) or cisplatin (Figure 1 B).

The significant inhibitory effects of FGN-236Ac (**3**) on PC-3 cell growth prompted us to elicit the available UDP-



Scheme 2. A synthetic route for the synthesis of 4F-GlcNAc analogues **1**–**4** from GalNAc. Reagents and conditions: a) 1. $PhCH(OMe)_2$, CSA, CH_3CN, RT, 8 h. 2. Ac_2O, pyridine, RT, 12 h, 98%. b) H_2, Pd/C, MeOH/dioxane, RT, 2 h, 99%. c) Triphenylmethyl chloride, Et_3N, DMAP, CH_2Cl_2, RT, 13 h, 83%. d) DAST, pyridine, CH_2Cl_2, RT, 8 h, 85%. e) 80% AcOH, 40 °C, 5 h, 67%. f) Ac_2O, pyridine, RT, 23 h, 100%. g) K_2CO_3, MeOH, −20 °C, 4 h, 94%. h) Benzylamine, THF, RT, 3 h, 68%. CSA = camphorsulfonic acid, DMAP = 4-(dimethylamino)pyridine, DAST = *N*,*N*-(diethylamino)sulfur trifluoride, Tr = triphenylmethyl.

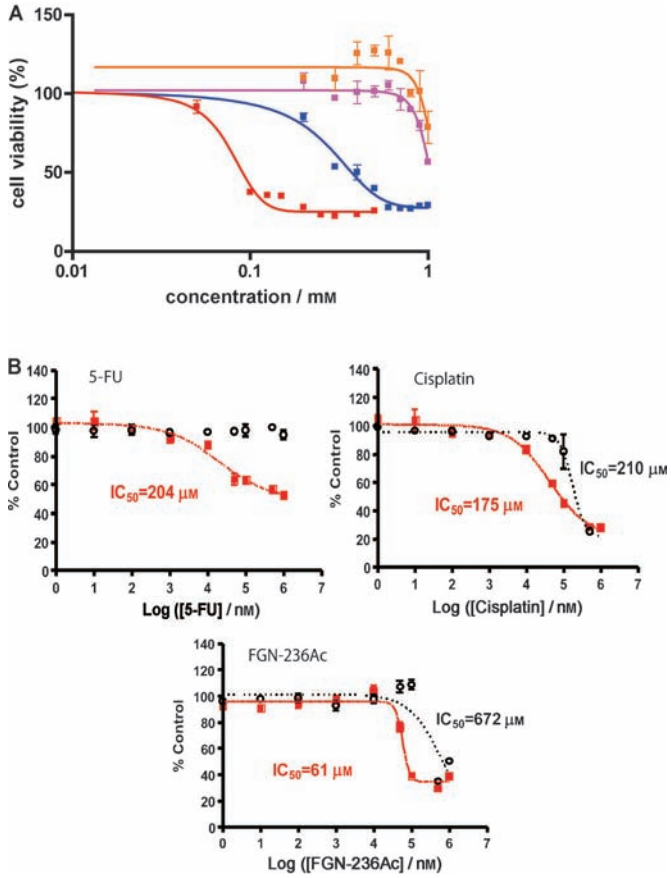


Figure 1. Inhibition of PC-3 cell growth by exogenous addition of unnatural GlcNAc mimics. A) Cell viability after 48 h incubation with 4-fluorine-containing compounds FGN-2Ac (**1**, pink trace), FGN-1236Ac (**2**, blue trace), FGN-236Ac (**3**, red trace), and FGN-123Ac (**4**, orange trace) was determined by the method described in the Supporting Information. B) Cell-growth arrest of PC-3 cells by FGN-236Ac (**3**) in comparison with two representative chemotherapy agents, 5-FU and cisplatin. Red squares correspond to measurements with PC-3 cells and white circles to those with normal prostate epithelial cells. % Control = the percentage of living cells.

GlcNAc concentration in the intracellular sugar-nucleotide pools and the alteration of cell-surface whole N-glycan profiles during cell cultivation in the presence of this class of unusual GlcNAc mimics. To verify that UDP-4F-GlcNAc (**5**) can be synthesized within PC-3 cells from fluorine-containing unusual sugars, the relative levels of intracellular UDP sugars were evaluated by using cells cultured in the presence of compounds **2**–**4** (50 μM). Considering that FGN-236Ac (**3**) completely arrested PC-3 cell growth at a dose of about 100 μM (Figure 1A), administration of 50 μM of compounds is an appropriate condition to test the effect on the GlcNAc salvage biosynthetic pathway independent from cell-growth inhibition. The HPLC and MS analysis using authentic sugar nucleotides[23] clearly revealed that all three compounds used herein can be efficiently converted within PC-3 cells into UDP-4F-GlcNAc (**5**), and in contrast, the UDP-GlcNAc level seemed to be dramatically decreased (Figure 2, and Figures S4 and S5 in the Supporting Information). The intracellular accumulation of UDP-4F-GlcNAc (**5**) in the presence of FGN-236Ac (**3**) appears to be at a lower level than the UDP-4F-GlcNAc levels observed in the presence of two other analogues, FGN-1236Ac (**2**) and FGN-123Ac (**4**).

It is likely that the decrease of the UDP-GlcNAc level and the accumulation of UDP-4F-GlcNAc (**5**) directly affect the total N-glycan profiles of PC-3 cells either through changes in the availability of UDP-GlcNAc or through an inhibitory effect of UDP-4F-GlcNAc (**5**) against different GlcNAc transferases.

We employed glycoblotting-based enrichment analysis to characterize the whole *N*-glycome of PC-3 cells.[24,25] Advantageous in this method is that the sensitivity of the Golgi N-glycan biosynthetic pathway to the hexosamine flux can be directly monitored as the up-regulation of hyperbranched *N*-glycoforms of PC-3 cells that were treated with high concentrations (10–100 mM) of free GlcNAc (Figure S5 in the Supporting Information). In other words, direct monitoring of the total cellular glycome[8,26] is a facile and efficient assay to estimate the available GlcNAc concentration within cells during dynamic cancer progression. Surprisingly, treatment of PC-3 cells with three fluorine-containing GlcNAc mimics (**2**–**4**, 50 μM) down-regulated remarkably the expression levels of most hyperbranched N-glycans such as tri- and tetra-antennary glycoforms, which were profiled as characteristic cellular and serum biomarkers for human prostate cancer[7,8] (Figure 3). As anticipated, treatment with 5-FU, cisplatin, or UV light did not induce any notable changes in the N-glycan profiles. Moreover, we could not see any significant ion peaks originating from 4F-GlcNAc-terminated N-glycans in the lower-molecular-weight region of MALDI-TOF-MS (Figure S6 in the Supporting Information), thus indicating that

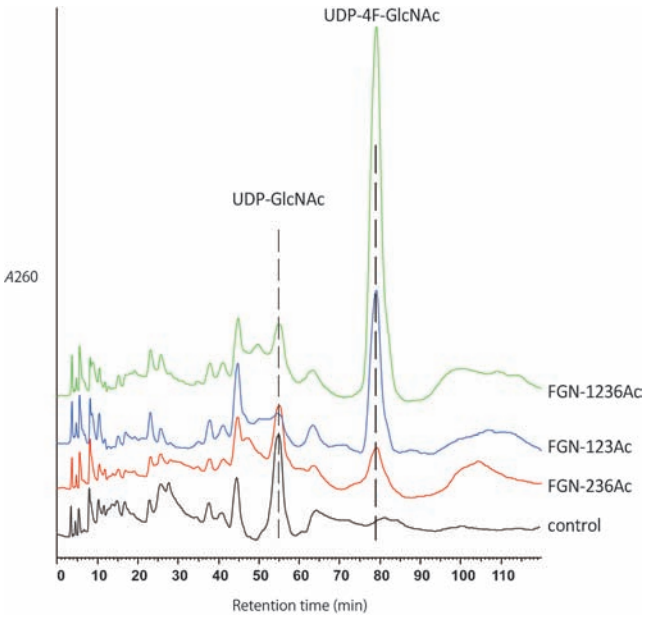


Figure 2. Accumulation of UDP-4F-GlcNAc in sugar-nucleotide pools of PC-3 cells. HPLC analysis of sugar nucleotides of PC-3 cells treated with 50 μM of 4F-GlcNAc mimics **2**–**4** and 0.5% DMSO as a control for 48 h. Sugar nucleotides were separated on a Develosil RPAQUEOUS column (250×4.6 mm) and detected by measuring the absorbance at 260 nm (*A*260).

non-natural UDP-4F-GlcNAc (**5**) cannot be incorporated into the Golgi *N*-glycosylation pathways instead of the native UDP-GlcNAc. Interestingly, it was reported that 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-5-thio-α-D-glucopyranose (Ac-5S-GlcNAc) can be salvaged within COS-7 cells and be converted into the unnatural UDP-5S-GlcNAc, and disturb the synthesis of *O*-linked GlcNAc residues (*O*-GlcNAc), whereas 5S-GlcNAc was not incorporated.[27] Furthermore, no significant changes in the expression levels of major bi-antennary N-glycans of PC-3 cells were observed in the presence of **2** and **3** at a concentration of 50 μM (Figure S7 in the Supporting Information). This finding suggests that an intracellular UDP-GlcNAc level of at least approximately 1 mM is retained, which is needed for the *N*-acetylglucosaminyltranferases (GnTs), GnT-I and GnT-II, because the values of the Michaelis constant K_m obtained in vitro of GnT-I and GnT-II for UDP-GlcNAc are 0.04 and 0.96 mM, respectively.[15,16] These results imply that, within PC-3 cells, UDP-4F-GlcNAc (**5**) is a highly specific and efficient metabolic inhibitor of GlcNAc transferases that are responsible for the synthesis of tri- and tetra-antennary N-glycans, notably GnT-IV and GnT-V (K_m values of GnT-IV and GnT-V are 5.0 and 11.0 mM, respectively).[15,16] Most importantly, the loss of the hyperbranched N-glycans caused by a 50 μM dose of **2**–**4** did not correlate with the results of cell-growth inhibition as shown in Figure 1 A. It means that 6-*O*-acetylated 4F-GlcNAc analogues bearing a free hemiacetal group might efficiently inhibit other enzymes and result in the perturbation of the total hexosamine biosynthetic pathway. This conclusion was supported by the lower accumulation of UDP-4F-GlcNAc (**5**) in PC-3 cells after treatment with **3** when compared with the

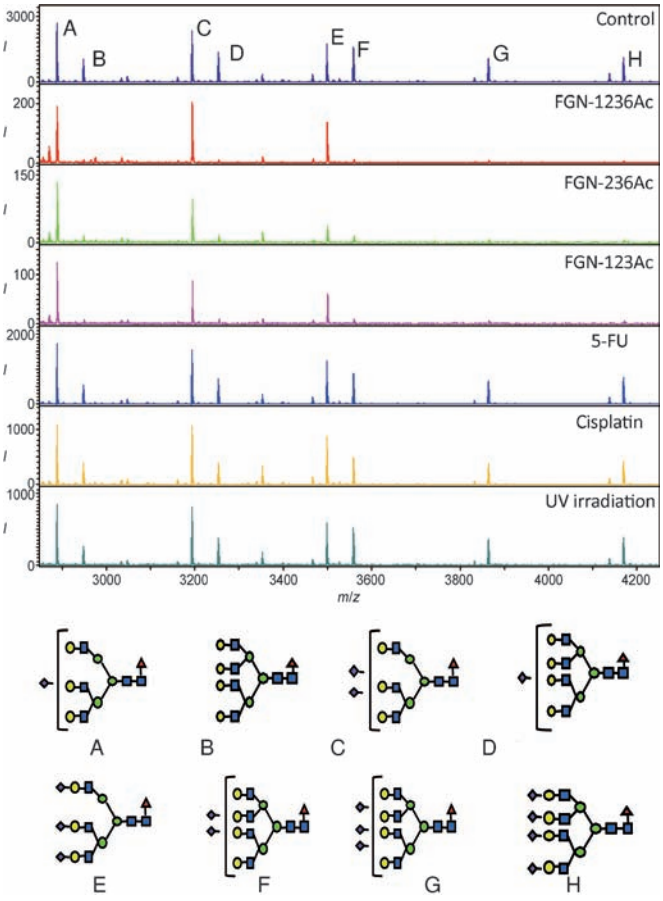


Figure 3. Perturbation of the Golgi *N*-glycosylation biosynthetic pathway by 4F-GlcNAc analogues. Hyperbranched N-glycans of PC-3 cells cultured in the presence of different GlcNAc mimics, 5-FU, cisplatin, and UV light. MALDI-TOF-MS focused on the high-molecular-weight region involving major tri-antennary and tetra-antennary N-glycans. Structures were identified by using tandem MS and the GlycoMod Tool and the GlycoSuite website. Yellow circle: galactose, blue square: *N*-acetylglucosamine, green circle: mannose, red triangle: fucose, purple diamond: sialic acid.

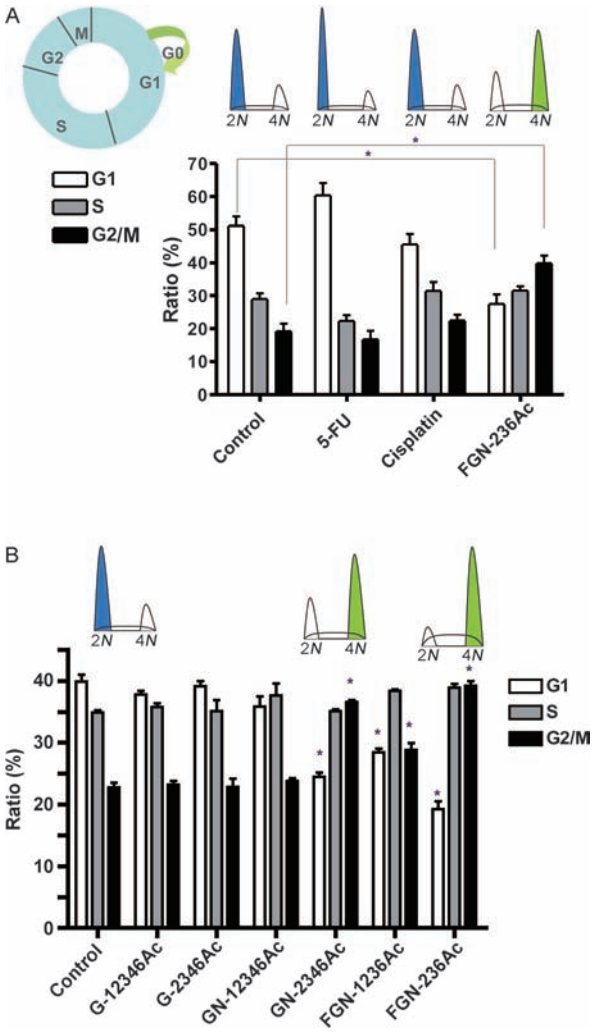


Figure 4. G2/M cell-cycle arrest and apoptosis analyzed by flow cytometry. A) A schematic presentation of the cell cycle is shown on the left, with the mitotic phase (M) and the interphase, which consists of G1, G0, S, and G2. Cell-cycle distribution in PC-3 cells treated with DMSO (0.5%) as control, 5-FU (50 μM), cisplatin (50 μM), and FGN-236Ac (50 μM) for 48 h (*$P < 0.01$ compared with control). B) Cell-cycle distribution in PC-3 cells treated with the shown related sugar derivatives (G: Glc, GN: GlcNAc, numbers 1–6 indicate the positions of acetylation). Above the histograms in (A) and (B), an indication of the ploidy (2*N*, 4*N*) of the cells is given. During the G2/M phases, cells are usually tetraploid (4*N*), that is, they have double the number of chromosomes.

UDP-4F-GlcNAc levels observed after treatment with **2** and **4** (Figure 2). However, notably the hyperbranched *N*-glycoforms are essential for the retention of cancer cell surface glycoprotein receptors through the formation of galectin-mediated lattice that greatly influences cancer malignancy phenomena, such as invasion and metastasis.[5,6] It should be emphasized that 4F-GlcNAc derivatives are potential reagents that reduce expression levels of hyperbranched N-glycans during cancer-cell progression, although confirmation by using appropriate aggressive cancer cells or such disease animal models remains to be carried out.

Cell flow cytometry of PC-3 cells treated with **3** (50 μM) for 48 h showed a marked increase in the cell population in the G2/M phases from 19.2% (control) to 39.9% with a concomitant decrease of the cells in the G1 phase from 51.2% (control) to 27.5% (Figure 4A). In contrast, treatment with 50 μM 5-FU or cisplatin for 48 h showed no significant differences in the populations of phases G1 (60.4% and 45.5%) and G2/M (16.8% and 22.5%), because 5-FU is known to be one of the S-phase-specific anticancer agents. The induction of G2/M cell-cycle arrest was most efficient after treatment with **3** (50 μM) compared with related sugar compounds (Figure 4B). This finding clearly indicates that the hemiacetal group of GlcNAc is essential for the specific cell-cycle arrest, when other hydroxy groups involving the C-6 position are acetylated. The estrogen metabolite 2-methoxyestradiol[28] also induces G2/M cell-cycle arrest and apoptosis in LNCaP, DU145, ALVA-31, and PC-3 cells in vitro, and inhibits the growth of androgen-independent prostate cancer in a transgenic mouse model in vivo.[29] To assess the role of **3** in the apoptosis, double staining with annexin V-FITC (FITC = fluorescein isothiocyanate) and propidium iodide (PI) was employed for PC-3 cells treated with **3** for 48 h at concentrations of 50, 100, 200 μM to differentiate early apoptotic cells and late apoptotic/necrotic cells, respectively (Figure S8 in the Supporting Information). 66.6% of PC-3 cells treated with **3** (200 μM) underwent apoptosis (9.7% early apoptosis and 56.9% late apoptosis/necrosis), whereas no significant changes were observed at concentrations of **3** below 100 μM. These results may motivate us to target the late cell-cycle stage for therapeutic intervention in androgen-insensitive diseases,[29] and the novel class of non-natural GlcNAc derivatives would allow for expanding the available chemical space to discover promising anti-prostate cancer drugs.

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