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Metabolic Monosaccharides Altered Cell Responses to Anticancer Drugs

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Abstract

Metabolic glycoengineering has been used to manipulate the glycochemistry of cell surfaces and thus the cell/cell interaction, cell adhesion, and cell migration. However, potential application of glycoengineering in pharmaceutical sciences has not been studied until recently. Here, we reported that Ac₄ManNAc, an analog of N-acetyl-D-mannosamine (ManNAc), could affect cell responses to anticancer drugs. Although cells from different tissues and organs responded to Ac₄ManNAc treatment differently, treated cells with increased sialic acid contents showed dramatically reduced sensitivity (up to 130 times) to anti-cancer drugs as tested on various drugs with distinct chemical structures and acting mechanisms. Neither increased P-glycoprotein activity nor decreased drug uptake was observed during the course of Ac₄ManNAc treatment. However, greatly altered intracellular drug distributions were observed. Most intracellular daunorubicin were found in the perinuclear region but not the expected nuclei in the Ac₄ManNAc treated cells. Since sialoglycoproteins and gangliosides were synthesized in the Golgi, intracellular glycans affected intracellular signal transduction and drug distributions seem to be the main reason for Ac₄ManNAc affected cell sensitivity to anticancer drugs. It was interesting to find that although Ac₄ManNAc treated breast cancer cells (MDA-MB-231) maintained the same sensitivity to 5-Fluorouracil, the IC₅₀ value of 5-Fluorouracil to the same Ac₄ManNAc treated normal cells (MCF-10A) was increased by more than 20 times. Thus, this Ac₄ManNAc treatment enlarged drug response difference between normal and tumor cells provides a unique opportunity to further improve the selectivity and therapeutic efficiency of anticancer drugs.

Keywords

glycoengineering; metabolic monosaccharide; anticancer drugs; drug targeting; drug metabolism; sialic acid

INTRODUCTION

Carbohydrates contribute to the welfare of cells in several ways by supplying cells with energy, participating in synthesis of cell architectures, and providing the appropriate microenvironment (extracellular matrix, ECM) for cells residing and growing.

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Glycoproteins and glycolipids carry oligosaccharide chains that are built from nine different monosaccharides. Sialic acids (sias), a family of nine-carbon acidic sugars usually expressed at the termini of oligosaccharide chains [1, 2], are typically the outermost monosaccharide units on the glycan chains of glycolipids and glycoproteins. Most cellular glycans are located on the outer surface of the plasma membrane, and the mammalian cell surface is covered by a dense layer of glycoconjugates anchored to the plasma membrane, called glycocalyx, [3].

The biosynthesis pathway for sialic acid in mammalian cells has been unraveled in considerable detail. Sialic acid biosynthesis in mammalian cells is supported by flux through the hexosamine pathway which produces UDP-GlcNAc [4]. A relatively minor proportion of the millimolar levels of this cytosolic nucleotide sugar is converted to ManNAc, a dedicated precursor for sialic acid biosynthesis that produces Neu5Ac in the cytosol and then enters the nucleus where CMP-Neu5Ac is synthesized [5, 6]. This nucleotide sugar is then transported into lumen of the Golgi where they are used by several sialyltransferases to produce α 2,3-, α 2,6-, or α 2,8-linked sialoglycoproteins or gangliosides [7].

Metabolic glycoengineering is a recently developed technique with which monosaccharide analogs can be introduced into cell glycosylation pathways and thus biosynthetically incorporated into cellular architecture, especially glycocalyx, endowing the cell surfaces with novel chemical and biophysical properties [8–12]. ManNAc analog-based sialic acid engineering efforts have blossomed in the past several years, growing from the initial alkyl chain extensions to now include chemical functional groups normally absent from the glycocalyx [13–16]. An initial opportunity of sialic acid engineering is that the modified sugars can constitute immunogenic epitopes when introduced into the body. By adjusting the dose and timing of administration of the non-natural sugar, the targeting of an antibody specific for the altered polysialic acid was restricted to tumor cells through this innovative passive immunotherapy approach [15]. It has also been reported that *N*-phenylacetyl analogs, when incorporated into tumor-associated carbohydrate antigens (TACA) such as sTn or GM3, are sufficiently antigenic to raise antibody titers [17]. In addition, since ManNAc analogs can alter the glycochemistry of cell surface, they are becoming fairly general tools for affecting cell adhesion. By installing non-natural sialosides that interact differently with their cognate receptors, ManNAc analogs have been widely applied in altering cell/cell interaction, cell adhesion, and cell migration [12, 18]. For example, glycoengineering has been employed to fluorinate mammalian cell surfaces resulting in modestly decreased adhesion of HL-60 cells to fibronectin [19].

Here we reported that tetra-O-acetyl-2-acetamido-2-deoxy-beta-D-mannose (Ac₄ManNAc), an analog of ManNAc, changed cell's sensitivity to the anti-cancer drugs dramatically. Cells from different tissues/organs of different species responded to Ac₄ManNAc treatment differently. Therefore, metabolic glycoengineering might provide a unique opportunity to differentiate the responses of normal and tumor cells to anticancer drugs to maximize the therapeutic efficiency.

MATERIALS AND METHODS

Materials

N-Acetylneuraminic acid (ManNAc) and Calcein-AM were purchased from Sigma-Aldrich Co (St. Louis, MO). BCA Protein Assay Kit was purchased from Thermo Scientific Inc (Barrington, IL). MTT was purchased from Invitrogen Inc (Camarillo, CA). Tetra-O-acetyl-2-acetamido-2-deoxy-beta-D-mannose (Ac₄ManNAc) was a gift from New Zealand Pharmaceuticals Ltd (Palmerston North, New Zealand).

Cell Cultures

CHO-K1 (Chinese hamster ovary), NIH/3T3 (Swiss mouse embryo tissue), A549 (Human lung adenocarcinoma epithelial cell line), MDA-MB-231 (human breast adenocarcinoma), MCF-10A (mammary epithelial cells), and SC (mouse schwann cells) were obtained from American Type Culture Collection (ATCC). A549 and CHO-K1 cells were grown in F12K, NIH/3T3 and SC cells in DMEM, MDA-MB231 in L-15, and MCF-7 in MEM supplemented with 0.01mg/l insulin. All mediums were supplemented with 10% Fetal Bovine Serum (FBS). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂.

Determination of total sialic acids in cells [20]

Cells cultured on culture flasks were scraped off, washed, and then re-suspended in PBS. Harvested cells were lysed through five freeze-thaw circles. A sample of 250 µl cell lysates was then mixed with 50 µl of 0.04 M periodic acid. The solutions were thoroughly mixed and allowed to stand in an ice bath for 20 min. After the addition of the resorcinol reagent (625 µl), the solutions were mixed, placed in an ice bath for 5 min, heated at 100 °C for 15 min, and then cooled in tap water. After adding 625 µl tert-butyl alcohol, the solutions were thoroughly mixed and placed in a 37 °C water bath for 3 min to stabilize the color. Solutions were measured for absorbance at 630 nm using microplate spectrophotometer (Biotek Instruments, Inc.) after cooling down to room temperature. Total sialic acids in cells were determined using a standard curve and normalized to total cell numbers.

Measurement of P-glycoprotein (P-gp) activity [21]

Cells were grown onto 24-well plates. At the end of cultivation, the growth medium was first aspirated off and then the cells were rinsed three times with pre-warmed (37°C) PBS. Collected cells were pre-incubated in 500 µl of serum free F12K medium at 37 °C for 1 hour, and followed by incubation with 5 µM Calcein-AM for another hour. At the end of incubation, the Calcein-AM solution was aspirated off, and collected cells were immediately rinsed three times with an ice-cold stop solution (PBS containing 0.1% w/v sodium azide). Cells were lysed (37 °C for 30 min) with 100 µl of lysis solution (0.5% v/v Triton X-100). Lysates were then analyzed for Calcein using a fluorescent spectrophotometer (Biotek Instruments, Inc.) by setting excitation and emission wavelengths at 485nm and 535 nm, respectively. The data was corrected against the auto fluorescence from control (Calcein-AM untreated) cells. Calcein amount in lysates was quantified using a standard curve and normalized to intracellular accumulation of Calcein based on the total proteins in the lysates. The protein content in cell lysates was determined using the BCA protein assay reagent kit.

Drug sensitivity assay

Sensitivity of cells to anti-cancer drugs were tested using MTT based cytotoxicity assay [22]. Briefly, cells cultured in 96-well plates were washed and fed with 100 µL complete medium containing different concentrations of anti-cancer drugs. At the end of 24 h incubation, 10 µL of MTT solution (5 mg/ml) were added into each well. After 4 h of incubation, the developed color in each well was dissolved by DMSO and quantified at 570 nm using a microplate reader. The half maximal inhibitory concentration (IC₅₀) of each drug on the specific cell was calculated.

Drug uptake and distribution in cells

Cells were seeded on collagen coated 8-well glass chambers (2×10^4 /well) and cultured at 37 °C for 12 hours. The formed cell monolayer was washed, fed with fresh medium supplemented with 500µM Ac₄ManNAc, and then cultured for an additional 24 h. At the end of the incubation period, daunorubicin was added into the wells of cultured cells at a

final concentration of 1.0 μM . Daunorubicin uptake and intracellular distribution was studied after 30 minutes of incubation using confocal laser scanning microscopy [23].

RESULTS

Ac₄ManNAc increased sialic acid synthesis in cells

Synthesized ManNAc derivatives usually have poor cell permeability. Ac₄ManNAc showed 600 times increased cellular uptake efficiency in comparison with ManNAc [12]. Externally supplied Ac₄ManNAc was metabolically processed by the sialic acid biosynthetic pathway and converted to the corresponding sialic acids. Increased total sialic acid was found in all treated cells from different tissue and species (Fig. 1). This Ac₄ManNAc initiated sialic acid synthesis was time and concentration dependent. Increased sialic acid contents occurred in Ac₄ManNAc treated cells after 12 h of incubation and reached a peak around 24 h (Fig. 2). If cells were supplied with low concentrations (<100 μM) of Ac₄ManNAc, we saw a gradual sialic acid content decrease after then. However, if a high concentration (500 μM) of Ac₄ManNAc was used, the sialic acid contents in cells could be maintained at the maximal level for up to 72 h. The same sialic acid synthesis kinetics was observed in other three tested cells (data not shown). There was a good correlation between increased sialic acid synthesis and supplied Ac₄ManNAc concentrations in the range of 0~500 μM . Decreased sialic acid synthesis was observed in cells treated by high concentration Ac₄ManNAc (>1,000 μM) due to dramatically increased cytotoxicity of Ac₄ManNAc at these concentrations. It is known that Ac₄ManNAc can be hydrolyzed by intracellular unspecific esterases to release acetic acids [31]. Accumulation acetic acids in cells will result in intracellular pH decrease and thus induce cytotoxicity. However, low concentration (<500 μM) of Ac₄ManNAc had limited effects on cell viability and cell growth. In the concentration range of 0~500 μM , Ac₄ManNAc treated cells maintained the same growth rates and morphologies as untreated cells (data not shown).

It should be noted that although Ac₄ManNAc concentration and exposure time affected sialic acid synthesis, Ac₄ManNAc mediated sialic acid synthesis was primarily determined by the intrinsic sialic acid synthesis ability of a cell. Cells that initially had high sialic acid contents were always associated with high sialic acid expression in the Ac₄ManNAc treatment (Fig. 1). In addition, the ability of Ac₄ManNAc in increasing sialic acid synthesis in cells seemed to be limited. The sialic acid contents in all four treated cells were only increased by 2~3 times.

Altered drug sensitivity in Ac₄ManNAc treated cells

It is known that many drugs have DNA or intracellular proteins/enzymes as their targets. This is extremely true for anticancer drugs. Almost all anticancer drugs need to cross cell membranes to enter into cytoplasm and transport into the nuclei to exert their biological functions [24]. Because of dramatically increased total sialic acid contents in treated cells, there is the possibility that Ac₄ManNAc may alter cell responses to anticancer drugs by affecting diffusion into cells and drug interaction with intracellular targets

Drug sensitivity of cells was tested on six anticancer drugs with distinct chemical structures and different acting mechanisms (Fig. 3). Interestingly, all six tested drugs had dramatically increased IC₅₀ on Ac₄ManNAc treated cells (Tab. 1). A resistance ratio (R-ratio = $\text{IC}_{50}^{\text{treated}}/\text{IC}_{50}^{\text{untreated}}$) was used to quantify developed drug resistance in Ac₄ManNAc treated cells. The average R-ratio of six drugs on all four tested cells was about 24. The greatest R-ratio (>131) was found for gemcitabine while the smallest R-ratio (1.3) was found for daunorubicin on 500 μM Ac₄ManNAc treated A549 and CHO-K1 cells, respectively. Cisplatin and daunorubicin had low average r-ratios (2.8 and 5.9 respectively),

implying that these two drugs were less sensitive to increased sialic acid synthesis in Ac₄ManNAc treated cells.

There was a good correlation between increased sialic acid synthesis and drug resistance development in cells. For a specific cell, high concentration Ac₄ManNAc treatment was associated with greatly increased sialic acid contents and drug resistance in cells (Tab. 2). For example, R-ratios of camptothecin and etoposide from 100 μ M Ac₄ManNAc treated A549 cells were ten (1.9 vs 11.8) and eleven (1.6 vs >17.2) times less than the R-ratios of these two drugs from 500 μ M Ac₄ManNAc treated A549 cells, respectively. Daunorubicin was an exception. It had very close R-ratios on 100 μ M and 500 μ M Ac₄ManNAc treated cells. It was interesting that we did not see a correlation between increased drug resistance and sialic acid content in cells. The greatest and smallest average R-ratio values of six anti-cancer drugs were found on A549 (R-ratio=36) and CHO-K1 (R-ratio=9.1) cells, respectively. Both of these two cells had low sialic acid contents (Fig. 1). Similarly, although cells treated by 500 μ M Ac₄ManNAc could maintain stable sialic acid contents during the time course of 24–72 h (Fig. 2), the R-ratios of anticancer drugs measured at 24 and 48 h might vary greatly (Tab. 3): camptothecin and 5-Fluorouracil had close R-ratios on 500 μ M Ac₄ManNAc treated NIH/3T3 cells at 24 and 48 h; on the contrary, the R-ratios of daunorubicin and etoposide on the same cell at 24 and 48 h were about 3~4 times in difference.

Drug resistance mechanisms

All six tested anticancer drugs have DNA and/or DNA/RNA synthesis related enzymes as targets. For example, daunorubicin causes tumor cell death by directly intercalating in DNA and by inhibiting reverse transcriptase and RNA polymerase. These drugs need to cross cell membranes and transport into nuclei in order to exert their activities. P-glycoprotein (P-gp) is a well-known cell membrane-associated efflux protein. Dramatically increased P-gp expression and activity is a primary reason of developed drug resistance in cancer cells [25]. According to a proposed model, P-glycoprotein in cell membranes continuously pumps drugs from plasma membranes out into the cytoplasm and extracellular fluids. Surprisingly, increased sialic acid synthesis and modified glycocalyx had little effects on P-gp activity/ expression in these cells. P-gp activity in all tested cells kept unchanged during the course of Ac₄ManNAc treatment (Fig. 4).

We then examined cell uptake and intracellular distribution of drugs in Ac₄ManNAc treated cells. Due to the intrinsic fluorescence, altered intracellular distribution of daunorubicin in cells was visualized under confocal laser scanning microscopy (Fig. 5). Unlike evenly distributed daunorubicin between cytoplasm and nuclei in untreated cells (Fig. 5A & 5B), less amount of daunorubicin was found in the nuclei in Ac₄ManNAc treated cells (Fig. 5C & 5D). Most intracellular daunorubicin in Ac₄ManNAc treated cells were located in the perinuclear region. Similar intracellular drug distribution changes were found in all Ac₄ManNAc treated cells.

To further reveal the acting mechanism of Ac₄ManNAc, we had examined Ac₄ManNAc affected glycocalyx in cells. In untreated cells, more than 90% of cell sialic acid existed as glycocalyx. Although Ac₄ManNAc treatment did increased the amounts of glycoconjugate-bound sialosides, about 70% of increased sialic acid contents are in the form of “free” sialic acid (Fig. 6). This result is in agreement with P-gp activity (Fig. 4) and intracellular drug distribution (Fig. 5) assays and implies that glycocalyx may not play an important role in Ac₄ManNAc induced drug resistance.

It is known that the use of short chain fatty acid hexosamine monosaccharide hybrid molecules in metabolic glycoengineering often suffer from off-target effects [33]. To

confirm the involvement of sialic acids, we had compared the effects of Ac₄ManNAc and ManNAc. The cytotoxicity of etoposide and daunorubicin were tested on two cell lines, CHO-K1 and NIH/3T3, which expressed almost the same amount of total sialic acid in response to the treatment of ManNAc and Ac₄ManNAc (Tab. 4). Both tested drugs gave very close R-ratio on ManNAc and Ac₄ManNAc treated CHO-K1 and NIH/3T3 cells, confirming the contribution of sialic acid.

DISCUSSION

Most cellular glycans are on the outer surface of the plasma membrane. Glycocalyx is in a position to mediate and modulate cell-matrix, cell-cell, and cell-molecule interactions which are critical to the development and function of complex multicellular organisms. Therefore, previous studies have been focused on how extracellular supplement with ManNAc analogs would affect cell adhesion, cell-surface interaction, and other cell surface sialic acid related functions [15–19]. In this study, we reported for the first time that ManNAc analogs could greatly affected cells' sensitivity to anticancer drugs (Tab. 1–3).

It is known that cancer cells frequently display glycans at different levels or with fundamentally different structures than those observed on normal cells [26, 27]. Enhanced expression of terminal α 2–6-linked sialic acid on cell surface N-linked glycans and of Sialyl-Lewis X on O-linked glycans often correlates with poor prognosis of many human malignancies [26]. In addition, α 2–6-linked sialic acid is able to affect integrin function and thus up-regulate the expression of the ST6GAL1 gene in carcinomas of the colon, breast, cervix, choriocarcinomas, acute myeloid leukemias and some malignancies of the brain [27]. However, altered glycocalyx can not be the direct reason of increased drug resistance in Ac₄ManNAc treated cells. Although there was good correlation between increased sialic acid contents and drug resistance development (Fig. 2 and Tab. 1), Ac₄ManNAc treatment had very limited effects on cell uptakes of drugs (Fig. 4). All cells expressed steady P-gp (Fig. 4) during Ac₄ManNAc treatment. On the contrary, dramatically altered intracellular drug distributions and locations were found in Ac₄ManNAc treated cells (Fig. 5). Daunorubicin in Ac₄ManNAc treated cells is primarily located in the perinuclear region where CMP-Neu5Ac is accumulated before it is utilized by several sialyltransferases to produce sialoglycoproteins or gangliosides. Therefore, it is reasonable to believe that intracellular glycans, both glycoproteins and glycolipids, affected intracellular drug distributions and possible signal transductions are responsible for Ac₄ManNAc treatment induced drug resistance in cells. Indeed, it has been found that intracellular glycans are abundant within the nucleus and cytoplasm where they can serve as regulatory switches. Corroborating evidence that gene expression is influenced by sialic acid glycoengineering has been gleaned from a decade of studies, beginning with experiments where ManNAc analogs altered the fate of embryonic neural cells [28] and culminating with recent evidence that ManNAc analogs can function as signaling molecules [29], perhaps, through the metabolic intermediate CMP-Neu5Ac (or an analog such as CMP-Sia5Pr) during the steps where the biosynthetic pathway transits the nucleus. Our finding that about 70% of Ac₄ManNAc increased sialic acid in cells exists as “free” sialic acid (Fig. 6) supports above conclusion.

It was interesting to find that that the R-ratios of the six anti-cancer drugs we tested fall into two groups, 5-Fluorouracil, etoposide, camptothecin, and gemcitabine have high resistance ratios (R-ratio>13), while the R-ratios of cisplatin and daunorubicin are lower than 6. This phenomenon may be related to the different acting mechanism of these drugs. 5-Fluorouracil, etoposide, camptothecin, and gemcitabine interrupt the DNA and RNA synthesis in cells by either binding to DNA/RNA directly or interacting with DNA/RNA synthesis related enzymes existing in nuclei. On the contrary, cisplatin and daunorubicin

have multi targets which locate in both nucleus and cytoplasm. There is a report showing that treatment with D-mannosamine would condense the nucleolus [30]. We have found condensed nucleolus in some cells treated with high concentrations Ac₄ManNAc (data not shown).

Although factors such as Ac₄ManNAc dose and exposure time may affect cells' response to drugs, Ac₄ManNAc affected cell sensitivity to anti-cancer drugs is primarily determined by the nature of cells (Tab. 1–3). Cell sensitivity to a specific drug varied greatly among cells. For example, although etoposide have close IC₅₀ values on untreated CHO-K1 (IC₅₀=38.3 μg/ml) and NIH/3T3 (IC₅₀=35.8 μg/ml) cells, the sensitivity of Ac₄ManNAc treated CHO-K1 (IC₅₀=57.1 μg/ml) and NIH/3T3 (IC₅₀=1470 μg/ml) cells to the same drug is decreased by 1.5 and 27.9 times, respectively (Tab. 1). It was exciting to find that Ac₄ManNAc treated normal and tumor cells responded to anti-cancer drug differently. 5-Fluorouracil had very close cytotoxicity to normal human breast cell MCF-10A (IC₅₀=52 μg/mL) and human breast cancer cell MDA-MB-231 (IC₅₀=87 μg/mL). Although the cytotoxicity of 5-Fluorouracil to Ac₄ManNAc treated MDA-MB-231 cells was barely changed (IC₅₀=109 μg/mL), the sensitivity of Ac₄ManNAc treated normal breast cell MCF-10A to 5-Fluorouracil was increased by more than 20 times (IC₅₀=1,119 μg/mL) (Fig. 7).

As we know, drug resistance poses a significant problem and is responsible for the failure of chemotherapy [34]. Now nearly 50% of cancer patients either are completely resistant to chemotherapy or respond only transiently for the first treatment, after which they are no longer affected by commonly used anticancer drugs. To overcome this problem, a substantially larger dose of the drug is often administered in order to maintain therapeutic level. However, most anticancer drugs are associated with considerable toxicity, and the inadvertent exposure of normal cell types to these compounds due to higher doses administered frequently lead to increases in drug toxicity or other undesired side effects. Currently, chemotherapy has reached limits of improvement achievable by increasing the dose to the maximum tolerable (MTD) levels or raising the MTD through improved host rescue methods [35]. This metabolic monosaccharides enlarged drug sensitivity difference among cells as demonstrated in Fig. 7 may provide a unique opportunity to tune the drug targeting ability (e.g. differentiate normal and tumor cells) to maximize the therapeutic efficiency of drugs.

An issue faced by a practical-minded metabolic glycoengineering is the generally modest efficiency at which analogs enter a cell. Although Ac₄ManNAc at these concentrations had little effects on cell viability, slow growth was found in all Ac₄ManNAc treated cells (data not shown). In addition, Ac₄ManNAc concentrations of more than 100 μM were needed to maximize cellular responses (Tab. 2). It has proven that more lipophilic ManNAc analogs such as propionyl-(Pr₄ManNAc) and n-butanoyl- (Bu₄ManNAc) are associated with increased efficiency in comparison with Ac₄ManNAc [32]. Therefore, Pr₄ManNAc and n-butanoyl- (Bu₄ManNAc) may be better candidates than Ac₄ManNAc for real applications. In addition, other peracetylated N-acyl analogs of ManNAc including N-propionyl- (ManNPr), N-butanoyl- (ManNBu), N-iso-butanoyl- (ManNiBu), and N-phenylacetyl-D-mannosamine (ManNPac) are also of great interest. Besides increased lipophilicity and thus cell uptakes, because these peracetylated analogs can bypass N-acetylneuraminic acid synthase (NANS) bottleneck in two important ways, they may prove to be more effective and efficient than Ac₄ManNAc for potential clinical applications. Studies on these ManNAc analogs are undergoing.

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Fig. 1.

Ac₄ManNAc increased sialic acid synthesis in cells. Cells were cultured in the absence (untreated) and presence (treated) of 500 μM Ac₄ManNAc for 48 hr. Total sialic acid contents in cells were measured at the end of 48 h incubation. Data represents the mean and SD of three independent tests

Fig. 2.

A typical sialic acid synthesis kinetics in Ac_4ManNAc treated cells. NIH/3T3 cells were cultured with 100 μM and 500 μM Ac_4ManNAc for 72 h. Total sialic acid contents in cells were measured at different incubation time. Data represents the mean and SD of three independent tests

Fig. 3.
Chemical structures of anticancer drugs

Fig. 4.

Effects of Ac₄ManNAc on P-glycoprotein activity. A549 cells were cultured in the presence of 500 µM Ac₄ManNAc. Changes of calcein accumulation in Ac₄ManNAc treated cells were measured at different time points. Data represents the mean and SD of three independent tests

Fig. 5.

Intracellular distributions of daunorubicin. NIH/3T3 cells were cultured in the absence (untreated) and presence (treated) of 500 μM Ac₄ManNAc for 24 h. At the end of incubation, daunorubicin was added (final concentration = 1.0 μM) and incubated with cells for 30 min. Daunorubicin distributions in untreated (**A**) and Ac₄ManNAc treated (**C**) cells were acquired using confocal microscopy. The fluorescence intensity change across an untreated (**B**) or Ac₄ManNAc treated (**D**) cell was measured using NIH image software ImageJ. Positions of nuclei in ImageJ graphics were indicated.

Fig. 6. Comparison of glycoconjugate-bound sialic acid in untreated and Ac_4ManNAc treated CHO-K1 and NIH/3T3 cells. Cells were cultured in the absence (untreated) and presence (treated) of $500 \mu\text{M}$ Ac_4ManNAc for 48 hr. Total sialic acid (TSA) and glycoconjugate-bound sialic acid (bound SA) contents in cells were measured at the end of 48 h incubation. Data represents the mean and SD of three independent tests

Fig. 7. Ac₄ManNAc affected responses of normal (MCF-10A) and tumor (MDA-MB-231) breast cells to 5-Fluorouracil. Cells were treated with 500 μM Ac₄ManNAc for 48 h before 5-Fluorouracil was added. The IC₅₀ values were determined using MTT assay. Data represents the mean and SD of three independent tests

Table 1

Cytotoxicity (IC_{50} , $\mu\text{g/ml}$) of anticancer drugs measured on 500 μM Ac₄ManNAc treated cells after 48 hr incubation.

Cells	5-Fu	CDDP	DNR	VP-16	CPT	Gem	Average R-ratio
CHO-K1							
Untreated	42.9	84.3	1.06	38.3	4.5	>1000	
Treated	>1000	331	1.41	57.1	70.5	>1000	
R-ratio	>23.3	3.9	1.3	1.5	15.7	NA	9.1
NIH/3T3							
Untreated	51.5	246	2.30	35.8	38.5	59.0	
Treated	>1000	>1000	6.29	>1000	72	>1000	
R-ratio	>19.4	>4.1	2.7	>27.9	1.9	>16.9	12.2
A549							
Untreated	22.6	53.0	0.13	29.0	4.4	7.6	
Treated	>1000	76.1	1.34	>500	51.7	>1000	
R-ratio	>44.2	1.4	10.3	>17.2	11.8	>131	36.0
SC							
Untreated	19.4	200	0.45	42.0	4.7	68.6	
Treated	>1000	319	4.1	362	119	368	
R-ratio	>51.5	1.6	9.1	8.6	25.3	5.4	16.9
Average R-ratio	34.6	2.8	5.9	13.8	13.7	51.1	

Table 2

Cytotoxicity (IC₅₀, µg/ml) of anticancer drugs measured on 100 µM Ac₄ManNAc treated cells after 48 hr incubation

Cells	5-Fu	DNR	VP-16	CPT
CHO-K1				
Untreated	43.5	1.1	41.5	4.9
Treated	>1000	1.4	68.9	6.9
R-ratio	>23	1.3	1.7	1.4
NIH/3T3				
Untreated	198	1.85	36.8	24.2
Treated	>1000	7.28	186	>1000
R-ratio	>5.1	3.9	5.1	>41
A549				
Untreated	23.3	0.11	24.4	4.2
Treated	93	1.27	38.0	7.8
R-ratio	4	12.7	1.6	1.9
SC				
Untreated	21.1	0.94	45.5	5.7
Treated	>1000	3.69	361	11.8
R-ratio	>47.4	3.9	7.9	2.1

Table 3

Cytotoxicity (IC₅₀, µg/ml) of anticancer drugs measured on 500µM Ac₄ManNAc treated NIH/3T3 cells after 24 and 48 hr incubation

	5-Fu	DNR	VP-16	CPT	Average *R-ratio
24h					
Untreated	53.8	2.35	31.9	33.8	
Treated	>1000	20	234	48	
R-ratio	>18.6	8.5	7.3	1.4	9.0
48h					
Untreated	51.5	2.30	35.8	38.5	
Treated	>1000	6.29	>1000	72	
R-ratio	>19.4	2.7	>27.9	1.9	13.0

Table 4

Cytotoxicity (IC₅₀, µg/ml) of anticancer drugs measured on ManNAc treated cells after 48 hr incubation.

	CHO-K1		NIH/3T3	
	Ac ₄ ManNAc (500 μM), 48 h incubation			
Total sialic acid (μM/10 ⁷ cells)	0.383 ± 0.04		1.695 ± 0.064	
	ManNAc (500 μM), 48 h incubation			
Total sialic acid (μM/10 ⁷ cells)	0.367 ± 0.03		1.696 ± 0.082	
	IC ₅₀ (μg/ml)			
	DNR	VP-16	DNR	VP-16
Untreated	1.3	32.7	2.5	38.8
Treated with ManNAc	2.3	67.6	4.0	768.0
R-ratio	1.8	2.1	1.6	19.8